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Vision preservation during retinal inflammation by anthocyanin-rich bilberry extract: cellular and molecular mechanism

Seiji Miyake[1,2,3], Noriko Takahashi[1,2], Mariko Sasaki[1,2], Saori Kobayashi[3], Kazuo Tsubota[1] and Yoko Ozawa[1,2]

Anthocyanin-rich bilberry extract, a plant-derived antioxidant, has been utilized as a popular supplement for ocular health worldwide. However, it is unclear whether this extract has any biological effect on visual function, and the mechanism for such an effect is completely unknown. In this study, we generated a mouse model of endotoxin-induced uveitis (EIU) that shows retinal inflammation, as well as uveitis, by injecting lipopolysaccharide. We pretreated the mice with anthocyanin-rich bilberry extract and analyzed the effect on the retina. Anthocyanin-rich bilberry extract prevented the impairment of photoreceptor cell function, as measured by electroretinogram. At the cellular level, we found that the EIU-associated rhodopsin decreased and the shortening of outer segments in photoreceptor cells were suppressed in the bilberry-extract-treated animals. Moreover, the extract prevented both STAT3 activation, which induces inflammation-related rhodopsin decrease, and the increase in *interleukin-6* expression, which activates STAT3. In addition to its anti-inflammatory effect, the anthocyanin-rich bilberry extract ameliorated the intracellular elevation of reactive oxygen species and activated NF-κB, a redox-sensitive transcription factor, in the inflamed retina. Our findings indicate that anthocyanin-rich bilberry extract has a protective effect on visual function during retinal inflammation.

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Recent technological progress has led to the synthesis of medically useful chemical compounds. On the other hand, there is a long historical precedent for developing medical drugs from living things, often in the form of plant extracts. For example, digitalis, a cardiac drug, was first discovered in the leaves of the digitalis flower (*Digitalis purpurea* L.),[1] and morphine, an analgesic drug, was found in the opium poppy (*Papaver somniferum* L.).[2]

Here, we focus on an edible berry, the bilberry (*Vaccinium myrtillus* L.), which contains abundant anthocyanins. Anthocyanins are the water-soluble glycosides of anthocyanidins, which are flavylium cation derivatives. Fifteen different anthocyanins are found in bilberry.[3] Previous reports proposed that the anthocyanins are the responsible molecules for the bilberry's main pharmaceutical effects, which include antioxidant activity,[4–6] and its free-radical scavenging property.[7] The anthocyanin-rich bilberry extract has long been a popular treatment for various eye conditions. For example, it is believed to improve night vision, for which clinical trials have been performed.[8] Cataract and glaucoma are also thought to be prevented by this extract.[9] However, the extent of its effects on visual function and the underlying biological mechanisms have not been elucidated.

Excessive oxidative stress destroys tissue in various diseases, including neurodegenerative diseases such as amyotrophic lateral sclerosis,[10] cardiovascular diseases,[11] and cancers.[12] Ocular disease, including age-related macular degeneration,[13] is also a result of oxidative stress caused by the overproduction of reactive oxygen species (ROS). As the antioxidant, anthocyanin-rich bilberry extract, might have a tissue-protecting effect, it may have pharmaceutical application in human preventive therapy.

We previously proved that some plant-derived molecules can prevent or attenuate retinal impairment: lutein prevents neural dysfunction and resveratrol prevents vascular dysfunction in the retina in a mouse model of

[1]Laboratory of Retinal Cell Biology, Keio University School of Medicine, Tokyo, Japan; [2]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan and [3]Wakasa Seikatsu, Kyoto, Japan
Correspondence: Dr Y Ozawa, MD, PhD, Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: ozawa@a5.keio.jp (new) or yoko-o@sc.itc.keio.ac.jp (available until March 2012)

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endotoxin-induced uveitis (EIU).[14,15] In this model, innate immunity causes an impairment in visual function, which can be recorded by electroretinogram (ERG),[15–17] and abnormal leukocyte adhesion, which is related to abnormal circulation caused by inflammatory cytokines produced in the retina.[18] The ERG of these mice shows impaired photoreceptor cell function that is associated with the excessive STAT3-dependent degradation of rhodopsin, a visual substance.[17,19]

In this study, we revealed the biological effects of anthocyanin-rich bilberry extract on visual function in the EIU mouse as well as its molecular mechanism. Our findings support the potential future application of this extract as a preventive therapy.

MATERIALS AND METHODS

Preparation of Bilberry Extract Suspension and Endotoxin

Bilberry extract (containing about 39% anthocyanins) provided by Wakasa Seikatsu (Kyoto, Japan) was suspended in phosphate-buffered saline (PBS) at a concentration of 50 mg/ml. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma-Aldrich, St Louis, MO, USA) was dissolved in PBS at a concentration of 1 mg/ml.

Animals and Treatments

C57BL/6 mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and housed in an air-conditioned room ($23 \pm 1\,^{\circ}C$) under 12 h dark/12 h light cycles (light on from 8:00 to 20:00), with free access to standard diet (CLEA Japan) and tap water. The mice were randomly divided into three groups. Anthocyanin-rich bilberry extract (500 mg/kg body weight) for the bilberry group or PBS for the control and vehicle groups was administered orally to the mice for 4 days. At 3 h after the last administration, the vehicle and bilberry groups received a single intraperitoneal injection of LPS (6.0 mg/kg body weight) to generate the EIU model, whereas PBS was injected into the control group. The mice were evaluated 1.5 (for measurement of phosphorylated NF-κB) and 24 (for others) h after the LPS or PBS injection, at which times the pathological changes in the retina are obvious.[15,20] All the animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Electroretinogram

Mice were dark adapted for at least 12 h, prepared under dim red illumination, and anesthetized with 70 mg/kg body weight of pentobarbital sodium (Dainippon Sumitomo Pharmaceutical, Osaka, Japan). They were placed on a heating pad throughout the experiment. The pupils were dilated with one drop of a mixture of 0.5% tropicamide and 0.5% phenylephrine (Santen Pharmaceutical, Osaka, Japan). The ground electrode was a needle placed subcutaneously in the tail, and the reference electrode was placed in the mouth. Active electrodes were gold wires placed on the cornea. Recordings were made (PowerLab System 2/25; AD Instruments, NSW, Australia), and the responses were differentially amplified and filtered through a digital band-pass filter ranging from 0.313 to 1000 Hz to yield a- and b-waves. Light pulses of $800\,cd \cdot s/m^2$ and 4 ms duration were delivered through a commercial stimulator (Ganzfeld System SG-2002; LKC Technologies, Gaithersburg, MD, USA). Electrode impedance was checked before and after each measurement in all the animals, using a built-in feature of the instrument. The implicit times of the a- and b-waves were measured from the onset of the stimulus to the peak of each wave. The amplitude of the a-wave was measured from the baseline to the trough of the a-wave, and the amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave.

Immunoblot Analyses

The retina was isolated and placed in lysis buffer, including protease inhibitor cocktail (Complete, EDTA-free; Roche, Mannheim, Germany) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich), and exposed to 10 periods of sonication of 1 sec each on ice using a Handy Sonic UR-20 (Tomy Seiko, Tokyo, Japan) at a power control dial setting of 3. The sonicated suspension was left on ice for 30 min, centrifuged at 20 400 *g* for 15 min at $4\,^{\circ}C$, and the supernatant was transferred to a new 1.5-ml tube. The lysate was treated with Laemmli's sample buffer and separated by 12.5% SDS-polyacrylamide gel electrophoresis, as described previously.[21] The proteins were electrically transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) in a Trans-Blot SD Cell (Bio-Rad Laboratories, Hercules, CA, USA) at 19 V for 90 min. After the transfer, the membrane was blocked with 5% skim milk in TBS-T or TNB blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% TSA Blocking Reagent (PerkinElmer Life Sciences, Waltham, MA, USA)), then incubated overnight at $4\,^{\circ}C$ with a rabbit anti-rhodopsin antibody (1:100 000; LSL, Osaka, Japan) or rabbit anti-phospho-STAT3 antibody (1:1000; Cell Signaling Technology, Beverly MA, USA) or rabbit anti-phospho-NF-kappaB p65 (Ser536) (93H1) antibody (1:2000; Cell signaling Technology), and mouse anti-α-tubulin (1:5000; Sigma-Aldrich). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody. Finally, the signals were detected using the enhanced chemiluminescence system (ECL Blotting Analysis System; Amersham, Arlington Heights, IL, USA), measured with the ImageJ program (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), and normalized to α-tubulin.

Measurement of Outer Segment Length

Retinal sections (10 μm) were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (HE), and the outer segment (OS) length was measured in the posterior retina at four points, two on either side of the optic nerve that were

200 and 500 μm apart, using the ImageJ program, and averaged. All the sections were examined under a microscope equipped with a digital camera (BIOREVO BZ-9000, Keyence, Osaka, Japan).

Quantitative Real-Time RT-PCR

The retina was placed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract the total RNA. Complementary DNA (cDNA) was made by adding 1μg of the total RNA to the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA), and reverse-transcribed according to the manufacturer's instruction. The mRNA levels of rhodopsin, interleukin-6 (IL-6), and TNF-α were normalized to that of the GAPDH mRNA. PCR was performed using the StepOnePlus Real Time PCR system (Applied Biosystems), and the delta delta CT method was used to quantify gene expression. The following gene-specific primers were used: *rhodopsin* forward: 5′-GCTTCCCTACGC CAGTGTG-3′ and reverse: 5′-CAGTGGATTCTTGCCGCAG-3′; *il-6* forward: 5′-GAGGATACCACTCCCAACAGACC-3′ and reverse: 5′-AAGTGCATCATCGTTGTTCATACA-3′; *tnf-α* forward: 5′-ACGTGGAACTGGCAGAAGAG-3′ and reverse: 5′-GGTCTGGGCCATAGAACTGA-3′; and *gapdh* forward: 5′-TGTCTTCACCACCATGGAGA-3′ and reverse: 5′-AGTCT TCTGGGTGGCAGTGA-3′.

Measurement of ROS

The protocol for measuring ROS was based on a published method[22] with some modifications.[14] Eyes were enucleated and immediately frozen in OCT compound (Sakura Finetek, Torrance, CA, USA). Unfixed cryosections (10 μm) were incubated with 5 μM dihydroethidium (DHE; Invitrogen-Molecular Probes, Eugene, OR, USA) for 10 min at room temperature. DHE reacts with intracellular superoxide anion and is converted to the red fluorescent compound ethidium in nuclei. The sections were examined using a microscope equipped with a digital camera (BIOREVO BZ-9000, Keyence) under the same exposure conditions, and the intensity of staining in the inner plexiform layer (INL) was measured in the posterior retina at four points, two on either side of the optic nerve that were 200 and 500 μm apart, using the ImageJ program, and averaged.

Statistical Analyses

Values were expressed as the mean ± s.d. An unpaired Student's *t*-test was used to assess the statistical significance of differences. $P < 0.05$ was regarded as significant.

RESULTS

Protective Effect of Anthocyanin-Rich Bilberry Extract on Visual Function During EIU

To examine the effect of the anthocyanin-rich bilberry extract on visual function during EIU, we measured the ERG response. Compared with the control, the amplitudes of the a- and b-waves, which reflect the function of photoreceptor cells and secondary or later neurons, respectively, were decreased, and the implicit time of the b-wave was prolonged in the EIU model mice (Figures 1a–c and e). However, these changes were all prevented by the treatment with bilberry extract. No change was observed in the implicit time of the a-wave among the three groups (Figure 1d).

Preservation of the Rhodopsin Level by Anthocyanin-Rich Bilberry Extract During EIU

Because the a-wave change on the ERG reflects photoreceptor cell abnormality, we examined the rhodopsin protein level by immunoblot analysis (Figures 2a and b). The rhodopsin protein was significantly reduced in the retina of the EIU models, and treatment with the anthocyanin-rich bilberry extract prevented this reduction.

The rod OSs, where rhodopsin is concentrated, were measured in the retinal sections by HE staining. The OS length was significantly shortened during EIU, and this shortening was prevented by the treatment with anthocyanin-rich bilberry extract (Figures 2c and d). No changes were found in the other retinal layers in the retinal sections, consistent with a previous report.[14]

Inhibitory Effect of Anthocyanin-Rich Bilberry Extract on the STAT3 Activation Induced in the Retina with EIU

In the retina with inflammation, the decrease in rhodopsin level is correlated with STAT3 activation.[16,17] Immunoblot analysis showed that activated STAT3 was upregulated during EIU, and this increase was suppressed by the treatment with anthocyanin-rich bilberry extract (Figure 3). Thus, STAT3 activation was negatively correlated with the rhodopsin level.

Suppression of a Proinflammatory Cytokine by Anthocyanin-Rich Bilberry Extract in the Retina with EIU

One of the proinflammatory cytokines that activates STAT3 is IL-6. The expression level of *IL-6* mRNA measured by real-time PCR was significantly upregulated during EIU (Figure 4), and this increase was suppressed by anthocyanin-rich bilberry extract treatment.

Reduction of Oxidative Stress by Anthocyanin-Rich Bilberry Extract in the Retina with EIU

To evaluate the involvement of oxidative stress, we used DHE, which is converted to a red fluorescent compound in nuclei when it reacts with ROS.[23] The red fluorescence, representing ROS, was upregulated in the GCL, INL, and ONL of the retina in EIU models; however, this change was prevented by the treatment with anthocyanin-rich bilberry extract (Figure 5).

Suppression of a NF-κB by Anthocyanin-Rich Bilberry Extract in the Retina with EIU

To further explore the relationship between ROS and *IL-6* induction, we focused on a redox-sensitive transcription factor, NF-κB,[24] which can be in the upstream of

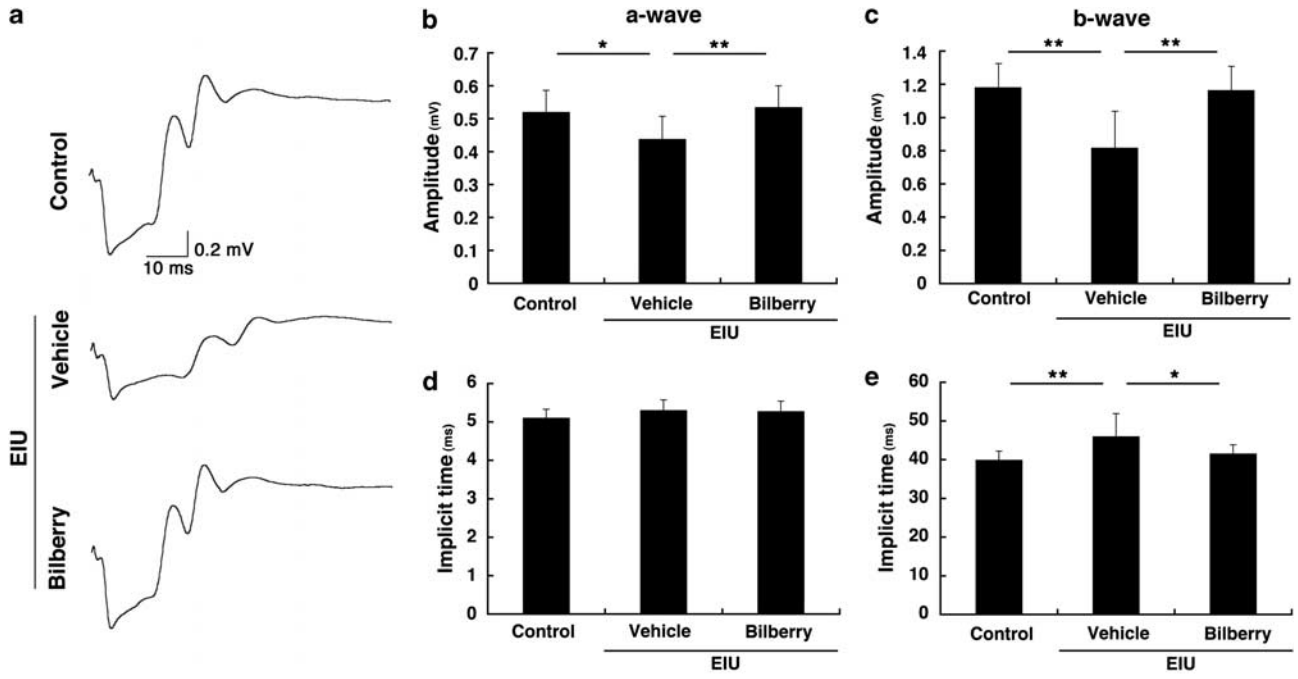

Figure 1 Protective effect of anthocyanin-rich bilberry extract on visual function during EIU. Representative responses from an individual mouse in each group (**a**). The decreases in a-wave amplitude (**b**) and b-wave amplitude (**c**) during EIU were significantly suppressed by treatment with anthocyanin-rich bilberry extract. The implicit time of the a-wave was not changed (**d**). The prolonged implicit time of the b-wave during EIU was prevented by treatment with anthocyanin-rich bilberry extract (**e**). $^{*}P<0.05$ and $^{**}P<0.01$; $n=5$ or 6.

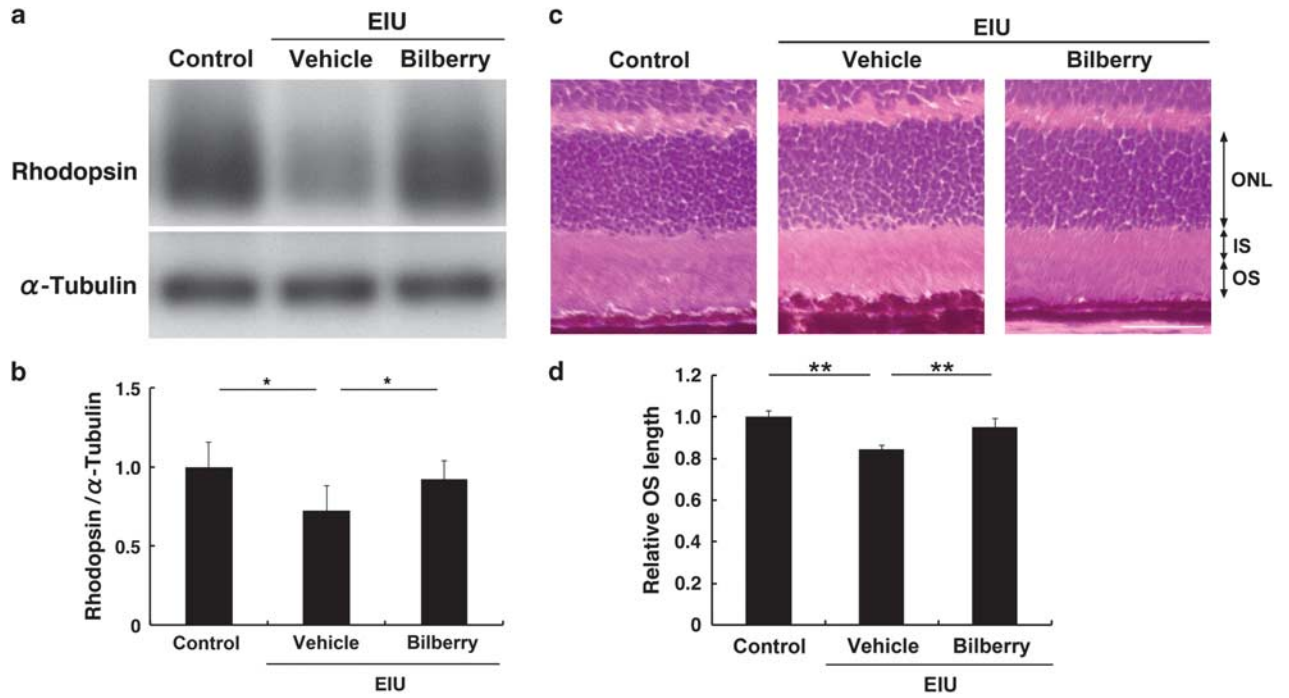

Figure 2 Preservation of the rhodopsin level by anthocyanin-rich bilberry extract during EIU. Immunoblot analysis (**a**, **b**). A decrease in the rhodopsin protein during EIU was prevented by treatment with anthocyanin-rich bilberry extract. $^{*}P<0.05$; $n=5$. Retinal sections stained with hematoxylin and eosin (**c**, **d**). A decrease in the length of the photoreceptor OS during EIU was prevented by treatment with anthocyanin-rich bilberry extract. $^{**}P<0.01$; $n=4$. Scale bar, 50 μm. IS, inner segment; ONL, outer nuclear layer; OS, outer segment.

IL-6. We measured phosphorylated p65, a component of activated NF-κB, in the retina after EIU induction using immunoblot analysis. The level of activated NF-κB was elevated in the retina during EIU; however, it was significantly suppressed by the treatment with anthocyanin-rich bilberry extract (Figure 6).

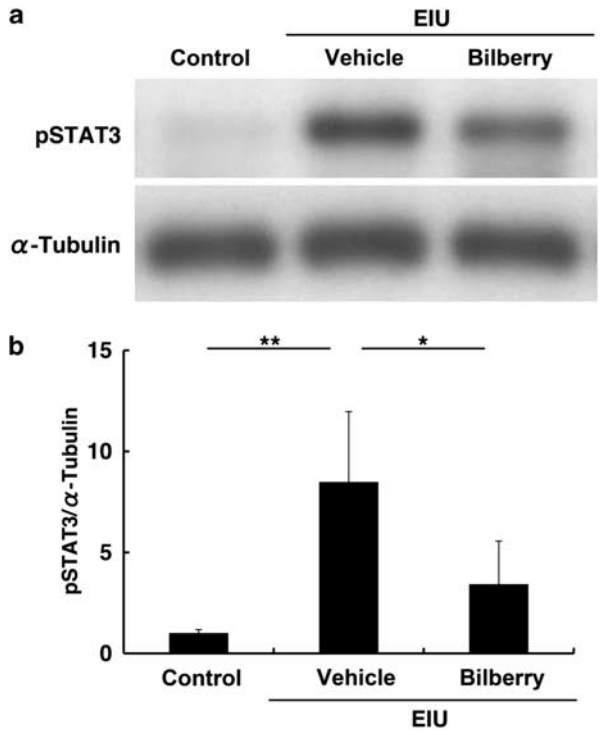

Figure 3 Inhibitory effect of anthocyanin-rich bilberry extract on STAT3 activation induced in the retina with EIU. Immunoblot analysis (**a**, **b**). Activated STAT3 was evaluated by measuring pSTAT3. The increase in pSTAT3 during EIU was significantly prevented by treatment with anthocyanin-rich bilberry extract. $^{*}P<0.05$ and $^{**}P<0.01$; $n=5$. pSTAT3, phosphorylated STAT3.

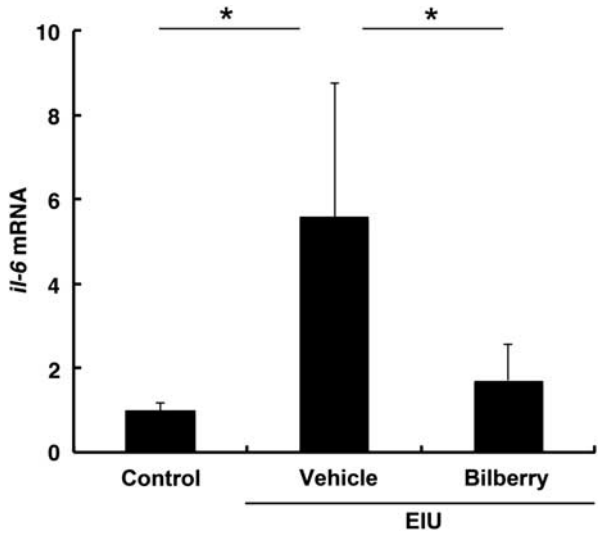

Figure 4 Suppression of proinflammatory cytokine by anthocyanin-rich bilberry extract in the retina with EIU. Real-time PCR. The mRNA level of *IL-6* was upregulated in the retina of the EIU model, and was suppressed by anthocyanin-rich bilberry extract. $^{*}P<0.05$; $n=5$, 6, and 6. *IL-6*, *interleukin-6*.

DISCUSSION

Here, we demonstrated that anthocyanin-rich bilberry extract has antiinflammatory and antioxidative tissue-protective effects, and elucidated the molecular mechanism for these effects, in EIU model mice. Visual function, including photoreceptor cell function, was well preserved by treatment with anthocyanin-rich bilberry extract (Figure 1). The reduction in rhodopsin protein and shortening of OS that accompany retinal inflammation were prevented by treatment with anthocyanin-rich bilberry extract (Figure 2). These effects correlated with the suppression of the proinflammatory molecules, activated STAT3 (Figure 3) and IL-6 (Figure 4), and the suppression of ROS production (Figure 5) and NF-κB activation (Figure 6), all of which are induced locally in the retina during inflammation.

Visual stimuli are received by photoreceptor cells, propagate through the neural network of the retina, and are finally passed to the optic nerve. The ERG response reflects the function of each retinal cell: the a-wave response reflects photoreceptor cell function, whereas the b-wave reflects the inner retinal cells, such as second-order neurons or interneurons, and may be influenced by a-wave changes. Our findings indicated that inflammation impaired photoreceptor cell function, but anthocyanin-rich bilberry extract protected the photoreceptor cells from this impairment.

The rhodopsin protein level was correlated with the OS length in this study. Rhodopsin knockout mice that lack both opsin alleles fail to form rod OSs, although the retina develops normally.[25,26] In hemizygous knockout mice, the rhodopsin protein level in the whole retina is approximately half that in wild-type mice, and OS length and light sensitivity are reduced to about half than their wild-type values.[26] In addition, in *Drosophila*, rhodopsin has an essential role in the development of the photosensitive membrane, the rhabdomere.[27] The absence of rhodopsin influences cytoskeletal molecules, such as F-actin and unconventional myosin, which are associated with the photosensitive membrane. Therefore, rhodopsin is indispensable for photoreceptor cell morphogenesis, including of the OS, as well as for visual signal transduction. These findings suggest a causal relationship between the changes in rhodopsin level and OS length, and are consistent with the idea that the anthocyanin-rich bilberry extract protected the OS length by preventing the reduction of the rhodopsin level in the retina. In addition, Sasaki *et al*[14] confirmed by TUNEL assay that apoptosis is not induced in the retina during inflammation, suggesting that the decrease in the rhodopsin protein level is not dependent on the number of the photoreceptor cells but on the protein level in each cell.

In both homozygous and hemizygous rhodopsin knockout mice, photoreceptor cells are lost in adulthood. Thus, a persistent lack of rhodopsin due to prolonged inflammation may subsequently cause photoreceptor cells to degenerate. Therefore, the anthocyanin-rich bilberry extract may have a role in preventing this late-occurring neurodegeneration. As a matter of practicality, the inflammation in the EIU model does not last more than 10 days,[17,28] which may be why apoptosis is not observed.

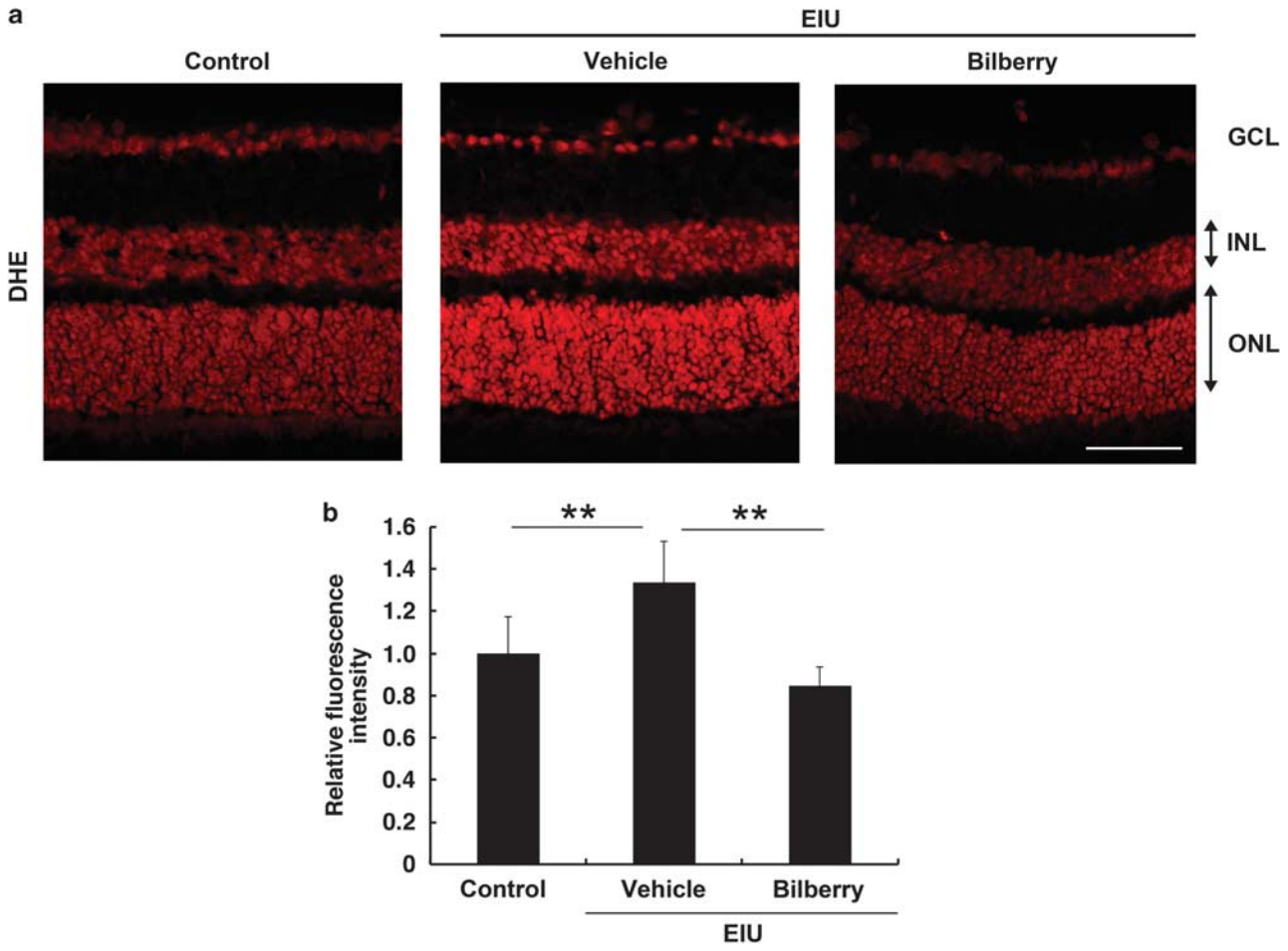

Figure 5 Reduction of oxidative stress by anthocyanin-rich bilberry extract in the retina with EIU. Representative digital images of retinal sections reacted with DHE in each group (**a**). Fluorescence intensity was measured using ImageJ and averaged (**b**). The increase in DHE fluorescence during EIU was suppressed by treatment with anthocyanin-rich bilberry extract. $^{**}P<0.01$; $n=4$. Scale bar, 50 μm. DHE, dihydroethidium.

The decrease in rhodopsin was correlated with STAT3 activation and IL-6 expression. Our previous study showed that the STAT3-related decrease in rhodopsin during inflammation is caused by a posttranscriptional mechanism, which we found to be excessive degradation through the ubiquitin–proteasome system.[17,19] Activated STAT3 induces an E3-ubiquitin ligase, Ubr1, which enhances *rhodopsin* degradation during inflammation, and ROS may modify rhodopsin protein, which accelerates ubiquitination and subsequent degradation. We also observed that the rhodopsin mRNA level was not changed under the control condition or during inflammation, regardless of treatment with anthocyanin-rich bilberry extract (data not shown), consistent with the involvement of a posttranscriptional modification. Therefore, the same mechanism of enhanced degradation was likely to account for the reduced rhodopsin level in the current study. The anthocyanin-rich bilberry extract suppressed both the STAT3 activation and ROS production, which may have prevented the UPS-mediated degradation of rhodopsin.

Although STAT3 can be activated by various kinds of ligands, this study showed that one of the candidates for its activation in the retina with inflammation was IL-6. In fact, IL-6 is known to be expressed in Müller glial cells,[29] which induces the downstream activation of STAT3. Anthocyanin-rich bilberry extract may have worked by blocking this vicious cycle of local IL-6–STAT3 signaling in the retina. In contrast, the level of TNF-α, another proinflammatory cytokine, did not change in this study (data not shown).

Oxidative stress is a major triggering factor for local inflammation and tissue damage during inflammatory processes.[19] DHE has been used to detect superoxide anion produced in a variety of biological systems *in vivo* and *in vitro*. It was recently found that DHE can also react with other oxidants, such as H_2O_2 and $ONOO^-$.[30] Thus, DHE's red fluorescence during retinal inflammation may have indicated several kinds of ROS accumulated in the retina. Most of the 15 anthocyanins contained in bilberry[3] are delivered intact to the eye or retina of living animals.[31,32] These previous findings support the idea that ingested anthocyanins were delivered to the retina of the EIU mice in this study, where they directly scavenged ROS.

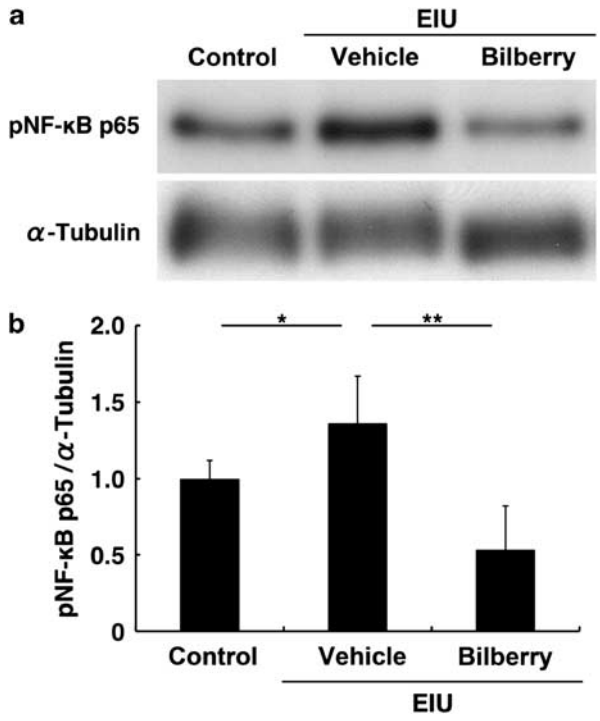

Figure 6 Suppression of a NF-κB by anthocyanin-rich bilberry extract in the retina with EIU. Immunoblot analysis (**a**, **b**). Activated NF-κB was evaluated by measuring phosphorylated p65. The increase in activated NF-κB after EIU induction was significantly suppressed by treatment with anthocyanin-rich bilberry extract. $^{*}P<0.05$ and $^{**}P<0.01$; $n=5$, 6, and 7.

Activated NF-κB was increased in the retina where ROS accumulated because of EIU, and was suppressed after ROS decreased by anthocyanin-rich bilberry extract, suggesting that the extract's effect on this redox-sensitive transcription factor was by modulating ROS as an antioxidant. The target gene of NF-κB involves IL-6, which supports the idea that the effect of anthocyanin-rich bilberry extract on STAT3 activation was by suppressing ROS-induced NF-κB–IL-6 axis.

Interestingly, anthocyanin-rich bilberry extract inhibits angiotensin-converting enzyme (ACE) activity in vascular endothelial cells, by its ability to bind and chelate the Zn at ACE's active site.[33] Angiotensin II is highly upregulated during retinal inflammation, and the angiotensin II type 1 receptor–STAT3 activation pathway also contributes to the decrease in rhodopsin during inflammation.[16] Thus, the anthocyanin-rich bilberry extract may suppress the rhodopsin reduction by modifying this signaling through angiotensin II. Alternatively, angiotensin II is known to stimulate the local gene expression of proteins related to iron metabolism,[34] which may upregulate ion transporters and facilitate iron uptake. Iron overload leads to oxidative damage,[35] thus the anthocyanin-rich bilberry extract might inhibit this pathway to protect photoreceptor cells from oxidative stress. Therefore, anthocyanin-rich bilberry extract may inhibit multiple pathways in the retina to protect visual function.

During the past four decades, basic and clinical studies of anthocyanin-rich bilberry extract have intensified because of its potential beneficial health effects.[8,36] However, the detailed molecular mechanism(s) of this extract's effects require further investigation *in vivo* and *in vitro*. In this study, we demonstrated that anthocyanin-rich bilberry extract has anti-inflammatory and antioxidative effects *in vivo*. Our findings may contribute to the future development of new therapeutic approaches for protecting visual function during retinal inflammation.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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