Prevention of Ocular Inflammation in Endotoxin-Induced Uveitis with Resveratrol by Inhibiting Oxidative Damage and Nuclear Factor–κB Activation

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PURPOSE. Resveratrol is known as one of the antioxidant polyphenols contained in red wine and grape skin. The purpose of the present study was to investigate the role of resveratrol in ocular inflammation in endotoxin-induced uveitis (EIU).

METHODS. EIU was induced in male C57/B6 mice at the age of 6 weeks by a single intraperitoneal injection of lipopolysaccharide (LPS). Animals had received oral supplementation of resveratrol at the doses of 5, 50, 100, or 200 mg/kg for 5 days until LPS injection. Twenty-four hours after LPS administration, leukocyte adhesion to the retinal vasculature was examined with a concanavalin A lectin perfusion-labeling technique. Retinal and retinal pigment epithelium (RPE)-choroidal levels of intercellular adhesion molecule (ICAM)-1, monocyte chemotactic protein (MCP)-1, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nuclear translocation of nuclear factor (NF)-κB p65 were evaluated by enzyme-linked immunosorbent assay. Retinal and RPE-choroidal activities of silent information regulator two ortholog (SIRT) 1 were measured by deacetylase fluorometric assay.

RESULTS. Resveratrol pretreatment led to significant and dose-dependent suppression of leukocyte adhesion to retinal vessels of EIU mice compared with vehicle application. Protein levels of MCP-1 and ICAM-1 in the retina and the RPE-choroid of EIU animals were significantly reduced by resveratrol administration. Importantly, resveratrol-treated animals showed significant decline of retinal 8-OHdG generation and nuclear NF-κB P65 translocation, both of which were upregulated after EIU induction. RPE-choroidal SIRT1 activity, reduced in EIU animals, was significantly augmented by treatment with resveratrol.

CONCLUSIONS. Resveratrol prevented EIU-associated cellular and molecular inflammatory responses by inhibiting oxidative damage and redox-sensitive NF-κB activation. (*Invest Ophthalmol Vis Sci.* 2009;50:3512–3519) DOI:10.1167/iovs.08-2666

R esveratrol (3,5,4-trihydroxystilbene), one of dietary polyphenols found in red wine and grape skin, is known to have an antioxidant effect[1] for reducing cardiovascular events. This mechanism may contribute to the so-called French paradox, which refers to the phenomenon that the French experience a relatively low incidence of cardiovascular disease despite a high-calorie and high-fat diet.[2] Resveratrol was reported to exhibit various bioactivities, including antitumorigenic,[3] antiangiogenic,[4] and neuroprotective[5] effects. Recently, silent information regulator two ortholog (SIRT) 1, a known regulator of aging,[6] has been shown to be activated by resveratrol.[7] Indeed, administration of resveratrol extended the life spans of yeast,[7] *Caenorhabditis elegans,*[8] *Drosophila melanogaster,*[8,9] short-lived fish *Nothobranchius furzeri,*[10] and mice fed a high-fat diet.[11] However, little is known about the effect of resveratrol on ocular disorders.

Recently, it has become widely recognized that inflammatory processes are involved in pathogenic mechanisms in vision-threatening retinal diseases such as diabetic retinopathy[12] and age-related macular degeneration.[13] A potential common therapeutic strategy against those retinal diseases is likely to consist in regulating inflammation. Various chemical mediators are shown to play key roles in ocular inflammation, together with retinal leukocyte adhesion and vascular leakage. These inflammation-related molecules include proinflammatory cytokines interleukin (IL)-6[14] and tumor necrosis factor (TNF)-α,[15] the arachidonic acid cascade enzyme cyclooxygenase-2[16] and its major metabolite prostaglandin E2,[17] monocyte chemotactic protein (MCP)-1,[18] angiotensin II type 1 receptor,[19,20] intracellular signaling pathways JAK (Janus kinase)/STAT (signal transducer and activator of transcription) 3[14,19] and IκB kinase/ nuclear factor (NF)-κB,[21] and leukocyte adhesion molecules such as intercellular adhesion molecule (ICAM)-1[22] and vascular adhesion protein-1.[23] Oxidative stress is also suggested to be pathogenic in inducing inflammation in the eye.[24,25] Endotoxin-induced uveitis (EIU) is an established animal model for ocular inflammation.[26] Oxidative biomarkers are shown to be elevated in EIU,[27] suggesting that inflammation and oxidative stress cooperatively contribute to its pathogenesis. Herein, we report the preventive effect of the antioxidant resveratrol on EIU-associated inflammation and oxidative stress in the eye.

METHODS

Animals and Induction of EIU

Male C57BL/6 mice (CLEA, Tokyo, Japan) at the age of 6 weeks were used. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ethics committee of our institution approved all surgical interventions and animal care procedures, which were in accordance with the Guidelines and Policies for Animal Surgery provided by the Animal Study Committees of the Central Institute for Experimental Animals of Keio University. Each animal received a single intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli* (Sig-
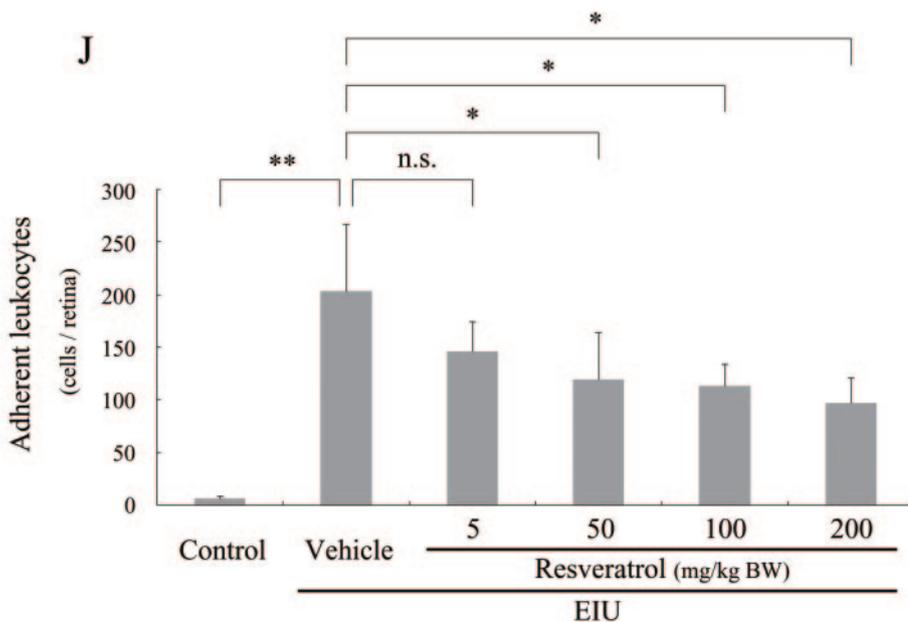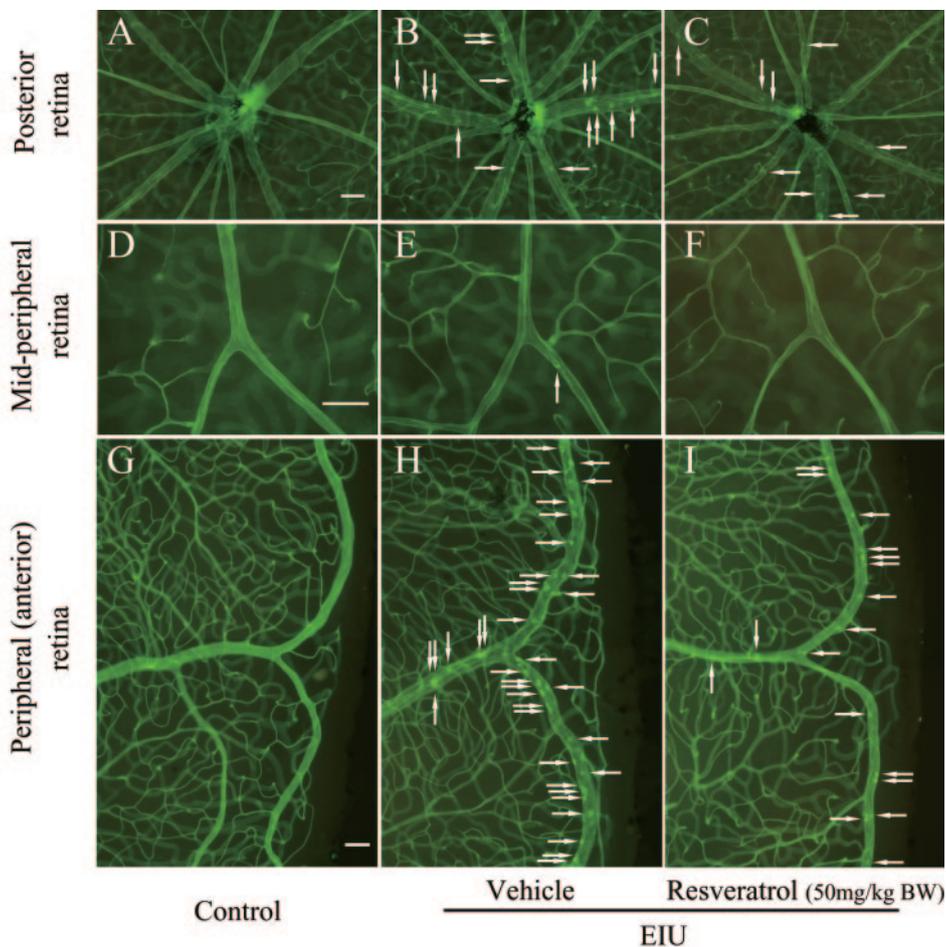
FIGURE 1. Suppression of retinal leukocyte adhesion with resveratrol. Flatmounted retinas from vehicle-treated control mice (**A**, **D**, **G**), vehicle-treated EIU mice (**B**, **E**, **H**), and resveratrol-treated (50 mg/kg BW) EIU mice (**C**, **F**, **I**). Vehicle-treated EIU mice showing increased adherent leukocytes (*arrows*) compared with controls. (**J**) Quantification of adherent retinal leukocytes. Resveratrol-treated (50, 100, or 200 mg/kg BW) EIU mice showed significantly fewer adherent leukocytes in a dose-dependent fashion than vehicle-treated EIU mice. Scale bar, 100 μm. $n = 6$ to 17. **$P < 0.01$; *$P < 0.05$.

ma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) at the dose of 9 mg/kg body weight (BW).

Treatment with Resveratrol

Mice were orally administered vehicle (6.67% dimethyl sulfoxide [DMSO] in PBS) or resveratrol (Sigma-Aldrich, St. Louis, MO) at the dose of 5, 50, 100, or 200 mg/kg BW by using gastric intubation daily for 5 days until the injection of LPS. Preliminary experiments confirmed that the vehicle DMSO solution did not affect EIU-related parameters, including cellular (Fig. 1) and molecular (Fig. 2) inflammatory responses, oxidative damage to DNA (Fig. 3), and NF-κB activation (Fig. 4) in murine eyes (data not shown). Thus, vehicle-treated age-
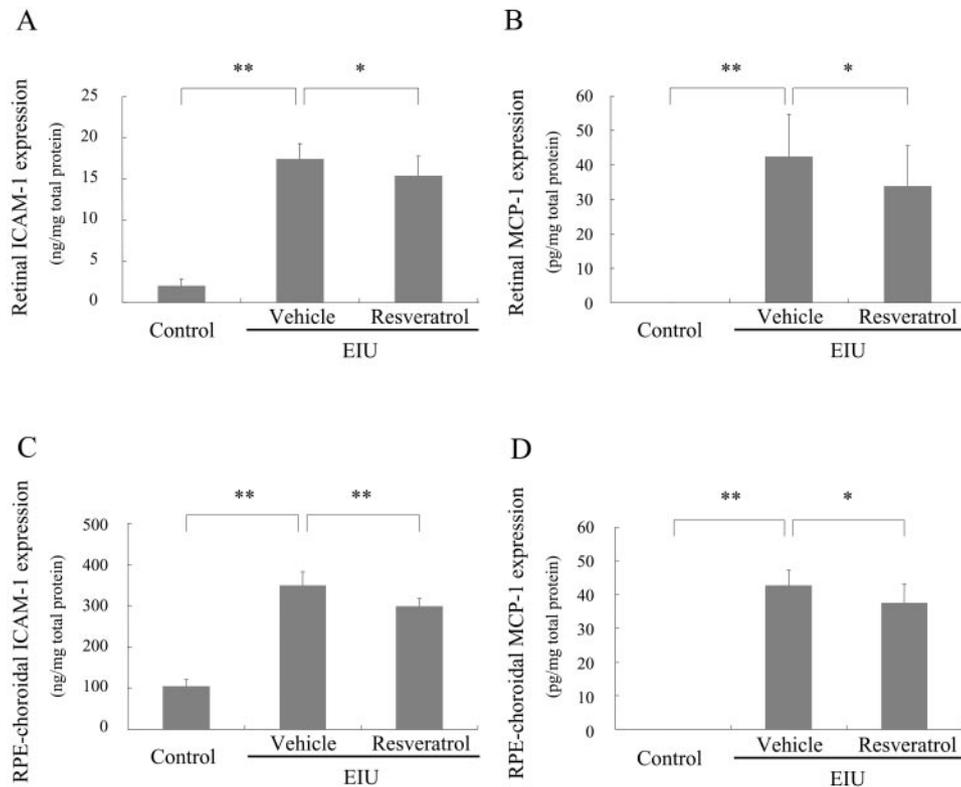
FIGURE 2. Suppression of retinal and RPE-choroidal expression of ICAM-1 and MCP-1 with resveratrol. Retinal ICAM-1 (**A**) and MCP-1 (**B**) protein levels in vehicle-treated EIU mice, significantly elevated compared with those in vehicle-treated normal controls, were significantly suppressed by treatment with resveratrol (50 mg/kg BW). Similarly, RPE-choroidal ICAM-1 (**C**) and MCP-1 (**D**) protein levels in vehicle-treated EIU mice, significantly elevated compared with those in vehicle-treated normal controls, were significantly suppressed by treatment with resveratrol (50 mg/kg BW). $n = 6$ to 17 and 6 to 30 for ICAM-1 and MCP-1, respectively. $**P < 0.01$; $*P < 0.05$.

matched normal mice served as controls throughout the study (Figs. 1–4). The dose of 50 mg/kg BW was equivalent to that applied to protect against neuronal injury associated with cerebral ischemia-reperfusion in mice.[28]

Quantification of Retinal Adherent Leukocytes

The retinal vasculature and adherent leukocytes were imaged by perfusion-labeling with fluorescein-isothiocyanate (FITC)-coupled concanavalin A lectin (ConA; Vector Laboratories, Burlingame, CA), as described previously.[20,21,29] Twenty-four hours after LPS injection, the chest cavity was opened and a 24-gauge cannula was introduced into the left ventricle under deep anesthesia. After injection of 2 mL PBS to remove erythrocytes and nonadherent leukocytes, 2 mL FITC-conjugated ConA was perfused. After the eyes were enucleated, the retinas were flatmounted. The flatmounts were imaged with an epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and the total number of ConA-stained adherent leukocytes per retina was counted in a masked fashion.

Enzyme-Linked Immunosorbent Assay for ICAM-1 and MCP-1

Twenty-four hours after LPS injection, the animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina and the retinal pigment epithelium (RPE)-choroid complex were carefully isolated and placed into 100 μL lysis buffer (0.02 M HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], 10% glycerol, 10 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 1% Triton, 100 mM NaF, 4 mM EDTA [ethylenediaminetetraacetic acid], pH 8.0) supplemented with protease inhibitors (2 mg/L aprotinin, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2.5 μM pepstatin A) and then were sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. ICAM-1 and MCP-1 levels in the supernatant were determined with the mouse ICAM-1 and MCP-1 ELISA kits (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's protocols. The tissue sample concentration was calculated from a standard curve and cor-

rected for protein concentration evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA).

Quantification of Oxidative Damage to DNA

Oxidative stress to the retina and choroid was evaluated by quantifying the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) as oxidatively modified DNA. Three hours after EIU induction, mice were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina and the RPE-choroid complex were carefully isolated, and total DNA was purified (DNeasy Blood & Tissue kit; Qiagen, Valencia, CA) according to the manufacturer's instruction. The 8-OHdG levels were measured with a DNA oxidation kit (Highly Sensitive 8-OHdG Check ELISA; Japan Institute for the Control of Aging, Fukuroi, Japan) according to the manufacturer's protocol. The 8-OHdG standard (0.5–40 ng/mL) or 15 to 20 μg DNA purified from the retina was incubated for 1 hour with a monoclonal antibody against 8-OHdG in a microtiter plate precoated with 8-OHdG. After the final color was developed with the addition of 3,3′,5,5′-tetramethylbenzidine, absorbance was then measured at 450 nm. Tissue sample concentration was calculated from a standard curve and corrected for DNA concentration.

ELISA for NF-κB p65 Subunit after Nuclear Extraction

Three hours after EIU induction, mice were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina and the RPE-choroid complex were carefully isolated and homogenized in 50 μL hypotonic buffer (10 mM HEPES-KCl, 1 mM β-mercaptoethanol, 1 mM dithiothreitol). After incubation on ice for 10 minutes, the homogenate was vortexed for 10 seconds and centrifuged. The supernatant was discarded, and the pellet was resuspended in 100 μL lysis buffer in the presence of protease inhibitors and was incubated on ice for 10 minutes. Cellular debris was removed by centrifugation at 15,000 rpm for 15 minutes at 4°C, and 10 μg protein was subjected to ELISA for the p65 subunit of NF-κB. Activation of NF-κB was determined by measuring the p65 protein level in the nuclear extracts with
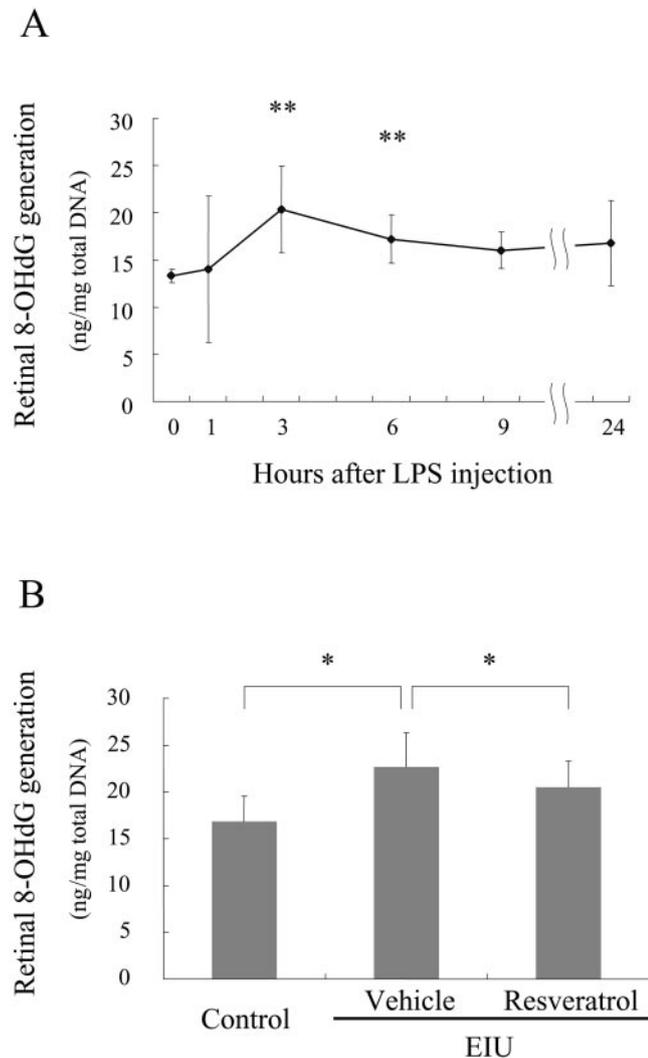
FIGURE 3. Suppression of retinal 8-OHdG generation with resveratrol. (A) After LPS injection, retinal 8-OHdG levels significantly increased, peaked at 3 hours, and reached a plateau from 6 to 24 hours. $n = 4$ to 6. **$P < 0.01$. (B) Retinal 8-OHdG levels in vehicle-treated EIU mice, significantly elevated compared with those in vehicle-treated controls, were significantly suppressed by treatment with resveratrol (50 mg/kg BW). $n = 8$ to 17. *$P < 0.05$.

a NF-κB detection kit (p65 Transcription Factor Assay kit; Active Motif, Carlsbad, CA) according to the manufacturer's instruction. Tissue sample concentration was calculated from a standard curve and corrected for protein concentration and evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific).

Western Blot Analyses for IκB-α

Protein extracts were obtained from the homogenized RPE-choroid complex 3 hours after EIU induction. Each sample containing 30 μg total protein was separated by SDS-PAGE and electroblotted to polyvinylidene fluoride membrane (ATTO, Tokyo, Japan). After blocking nonspecific binding with 5% skim milk, the membranes were incubated with a rabbit monoclonal antibody against IκB-α (Cell Signaling Technology, Beverly, MA) or an anti–α-tubulin antibody (1:2000; Sigma) at 4°C overnight. Membranes were then incubated with biotin-conjugated secondary antibodies followed by avidin-biotin complex (1:2000; Vectastain ABC Elite Kit; Vector Laboratories) or a horseradish peroxidase–conjugated goat antibody against rabbit IgG (1:5000; Bio-Source, Camarillo, CA). Signals were visualized with an enhanced

chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol.

Deacetylase Fluorometric Assay for SIRT1

Twenty-four hours after LPS injection, the animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina and the RPE-choroid complex were carefully isolated and were placed in 100 μL lysis buffer and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. SIRT1 activity in the supernatant was determined (SIRT1/Sir2 Deacetylase Fluorometric Assay kits; CycLex, Ina, Japan) according to the manufacturer's protocols.

Statistical Analysis

All results were expressed as mean ± SD. Values were processed for statistical analyses (Mann-Whitney U test), and differences were considered statistically significant at $P < 0.05$.

RESULTS

Suppression of Retinal Leukocyte Adhesion with Resveratrol

To evaluate inflammatory cell responses in EIU, leukocytes adherent to the retinal vasculature were imaged by perfusion labeling with FITC-coupled ConA. Leukocyte counts were evaluated in the posterior retina around the optic disc (Figs. 1A–C), the midperipheral retina near the equator of the globe (Figs. 1D–F), and the peripheral (anterior) retina next to the ora serrata (Figs. 1G–I). The total number of adherent leukocytes per retina was significantly ($P < 0.01$, Fig. 1J) higher in vehicle-treated EIU mice (Figs. 1B, 1E, 1H; 203.5 ± 63.4 cells) than in vehicle-treated controls (Figs. 1A, 1D, 1G; 6.3 ± 2.2 cells). Resveratrol treatment (50, 100, or 200 mg/kg BW) to EIU mice led to significant ($P < 0.05$ for each; Fig. 1J) suppression of leukocyte adhesion in a dose-dependent manner (119.2 ± 44.5 cells, 112.8 ± 21.0 cells, 96.5 ± 24.0 cells, respectively), compared with vehicle-treated EIU animals (203.5 ± 63.4 cells).

Suppression of Retinal and RPE-Choroidal Expression of ICAM-1 and MCP-1 with Resveratrol

To investigate molecular mechanisms underlying retinal leukocyte adhesion (Fig. 1), protein levels of ICAM-1 (Figs. 2A, 2C) and MCP-1 (Figs. 2B, 2D) in the retina (Figs. 2A, 2B) and the RPE-choroid (Figs. 2C, 2D) were evaluated by ELISA. Retinal ICAM-1 and MCP-1 protein levels were significantly ($P < 0.01$ for both) higher in vehicle-treated EIU mice (17.5 ± 1.82 ng/mg and 42.5 ± 12.2 pg/mg, respectively) than in vehicle-treated controls (1.97 ± 0.91 ng/mg and nondetectable, respectively). Systemic administration of resveratrol (50 mg/kg BW) to EIU animals significantly ($P < 0.05$ for both) reduced ICAM-1 and MCP-1 protein levels (15.4 ± 2.43 ng/mg and 33.8 ± 11.7 pg/mg, respectively). RPE-choroidal ICAM-1 and MCP-1 protein levels were significantly ($P < 0.01$ for both) higher in vehicle-treated EIU mice (349.0 ± 33.8 ng/mg and 42.6 ± 4.60 pg/mg, respectively) than in vehicle-treated controls (104.9 ± 15.7 ng/mg and nondetectable, respectively). ICAM-1 and MCP-1 protein levels (299.3 ± 18.4 ng/mg and 37.6 ± 5.64 pg/mg, respectively) in the RPE-choroid were significantly ($P < 0.01$ and $P < 0.05$, respectively) reduced by systemic administration with resveratrol at the dose of 50 mg/kg BW to EIU animals.

Suppression of Retinal 8-OHdG Generation with Resveratrol

To examine oxidative stress as a potential stimulus for EIU-related cellular (Fig. 1) and molecular (Fig. 2) inflammatory
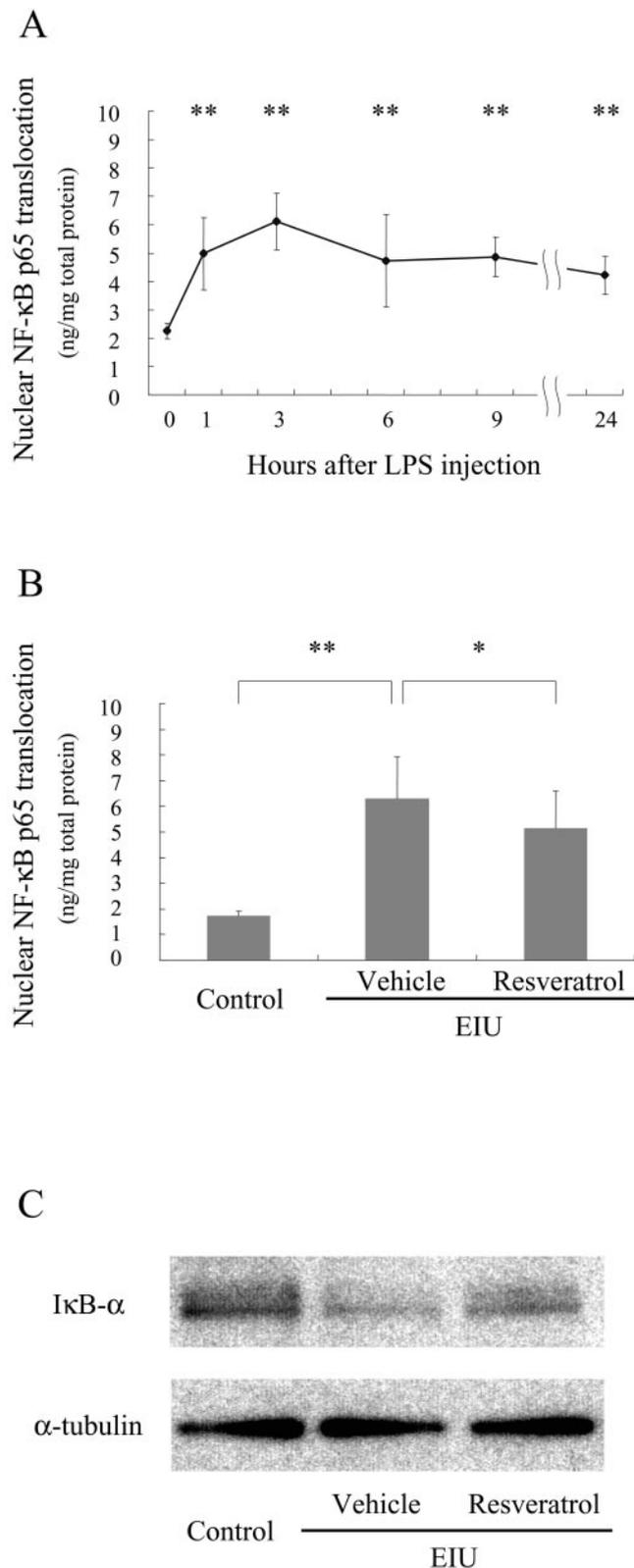
events, retinal generation of 8-OHdG, a marker of oxidative damage to DNA, was analyzed by ELISA (Fig. 3). After EIU induction, retinal 8-OHdG levels significantly ($P < 0.01$) increased, peaked at 3 hours, and reached a plateau from 6 to 24 hours (Fig. 3A). Thus, the time point to evaluate resveratrol-induced suppression of 8-OHdG generation was determined to be 3 hours after LPS injection. Similarly, RPE-choroidal 8-OHdG generation tended to peak at 30 minutes after EIU induction, but the difference was not statistically significant (data not shown). Retinal 8-OHdG levels 3 hours after EIU induction were significantly ($P < 0.05$, Fig. 3B) higher in vehicle-treated EIU mice (22.7 ± 3.61 ng/mg) than in vehicle-treated controls (16.9 ± 2.72 ng/mg). Systemic administration of resveratrol (50 mg/kg BW) to EIU animals significantly ($P < 0.05$, Fig. 3B) reduced 8-OHdG generation (20.5 ± 2.81 ng/mg).

Suppression of Nuclear NF-κB Translocation and IκB-α Degradation with Resveratrol

To investigate proinflammatory signal transduction in the downstream of oxidative stress (Fig. 3), redox-sensitive NF-κB activation was examined with ELISA by measuring the p65 protein level in the nuclear extracts from the RPE-choroid complex (Fig. 4). Retinal levels of NF-κB p65 translocated in the nucleus measured by the present method were undetectable. After EIU induction, nuclear NF-κB p65 levels significantly ($P < 0.01$) increased, peaked at 3 hours, and reached a plateau from 6 to 24 hours (Fig. 4A), which was temporally correlated with the time course of LPS-induced oxidative damage (Fig. 3A). Thus, the time point to evaluate resveratrol-induced suppression of NF-κB activation was determined to be 3 hours after LPS injection. Nuclear NF-κB p65 levels at 3 hours after EIU induction were significantly ($P < 0.01$; Fig. 4B) higher in vehicle-treated EIU mice (6.31 ± 1.61 ng/mg) than in vehicle-treated controls (1.73 ± 0.18 ng/mg). Systemic administration of resveratrol (50 mg/kg BW) to EIU animals significantly ($P < 0.05$; Fig. 4B) reduced NF-κB p65 levels (5.15 ± 1.43 ng/mg). To further confirm NF-κB involvement in the treatment with resveratrol, we used Western blot analysis to examine the protein levels of IκB-α that had to be degraded for nuclear NF-κB translocation. RPE-choroidal IκB-α protein levels, substantially reduced in EIU mice 3 hours after induction, were partially reversed by treatment with resveratrol at the dose of 50 mg/kg BW (Fig. 4C), supporting the data on nuclear NF-κB translocation (Fig. 4B). Western blot analysis results were comparable in three independent experiments.

## Activation of SIRT1 in the RPE-Choroid with Resveratrol

To examine a possible role played by SIRT1 in EIU-related inflammatory events, retinal (Fig. 5A) and RPE-choroidal (Fig. 5B) SIRT1 activities were analyzed by deacetylase fluorometric assay. Retinal ($91\% \pm 5\%$ of control) and RPE-choroidal ($92\% \pm 7\%$ of control) SIRT1 activities were significantly ($P < 0.01$ for both) lower in vehicle-treated EIU mice than in vehicle-treated controls. Systemic administration of resveratrol (50 mg/kg BW) to EIU animals led to a significant ($P < 0.05$) increase in SIRT1 activity in the RPE-choroid ($99\% \pm 7\%$ of control), but not in the retina ($91\% \pm 6\%$ of control).

DISCUSSION

The present study reveals, for the first time to our knowledge, the inhibitory effect of resveratrol on ocular inflammation,

FIGURE 4. Suppression of nuclear NF-κB translocation and IκB-α degradation with resveratrol. (**A**) After LPS injection, nuclear NF-κB p65 levels significantly increased, peaked at 3 hours, and reached a plateau from 6 to 24 hours. $n = 6$ to 8. **$P < 0.01$. (**B**) Nuclear NF-κB p65 levels in vehicle-treated EIU mice, significantly elevated than those in vehicle-treated controls, were significantly suppressed by treatment with resveratrol (50 mg/kg BW). $n = 6$ to 21. **$P < 0.01$; *$P < 0.05$. (**C**) Protein levels of IκB-α were substantially reduced in vehicle-treated EIU mice compared with vehicle-treated controls. Systemic administration of resveratrol (50 mg/kg BW) to EIU animals partially inhibited IκB-α degradation. $n = 3$.

FIGURE 5. Activation of SIRT1 in the RPE-choroid with resveratrol. Retinal (A) and RPE-choroidal (B) SIRT1 activities were significantly ($P < 0.01$ for both) lower in vehicle-treated EIU mice than in vehicle-treated controls. Systemic administration of resveratrol (50 mg/kg BW) to EIU animals led to a significant ($P < 0.05$) increase in SIRT1 activity in the RPE-choroid (B) but not in the retina (A). $n = 11$ to 12. **$P < 0.01$; *$P < 0.05$.
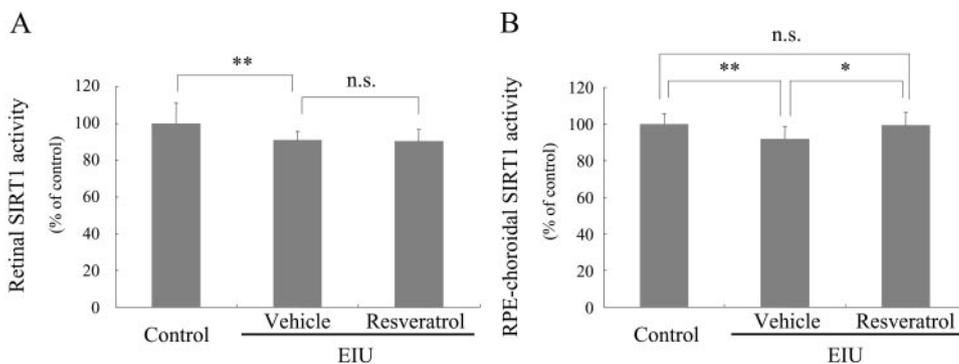
together with underlying molecular mechanisms. Resveratrol ameliorated EIU-related parameters including cellular (Fig. 1) and molecular (Fig. 2) inflammatory responses, LPS-induced oxidative damage (Fig. 3), redox-sensitive NF-κB activation (Fig. 4), and SIRT1 deactivation (Fig. 5).

Resveratrol is one of the polyphenols contained abundantly in red wine, grape skin, and peanut skin.[2] The term polyphenol refers to a complex group of compounds that include in their structure a benzene ring bearing one or more hydroxyl groups (i.e., phenolic hydroxyl groups). Phenolic hydroxyl groups are subject to oxidation because of their low oxidoreduction potential, accounting for the antioxidative effect of polyphenols.[30] Resveratrol has been shown to have various bioactivities, including antitumorigenic,[3] antiangiogenic,[4] neuroprotective,[5] vasodilative,[31] and antioxidative[1,32] effects. Application of resveratrol is shown to prevent tumorigenesis by inhibiting cyclooxygenase activation,[3] one of the key steps in the arachidonic acid cascade-induced inflammation. Consistent with recent studies showing the anti-inflammatory property of resveratrol on various disease models,[33,34] the present data are the first to demonstrate its inhibitory effect on the pathogenesis of ocular inflammation in EIU.

Leukocyte adhesion, one of the most prominent pathologic events in the inflamed retina of EIU, was successfully inhibited by oral administration of resveratrol (Fig. 1). In addition to the investigation into cellular responses, parallel experiments revealed that resveratrol application led to significant suppression of the inflammation-related molecules MCP-1 and ICAM-1 (Fig. 2), both of which play pivotal roles in leukocyte recruitment[35] and adhesion[22] during inflammatory status. Recently, resveratrol has been shown to attenuate TNF-α–induced MCP-1 expression in adipocytes[36] and cigarette smoke-induced upregulation of ICAM-1 in rat arteries.[34] In accordance with these in vitro[36] and in vivo[34] data, the present study unraveled molecular mechanisms underlying resveratrol-induced inhibition of inflammation in the eye.

Increasing evidence has suggested that oxidative stress contributes to the pathogenesis of LPS-induced inflammation in various organs, including the brain,[37] lung,[38] liver[39] and joint.[40] In addition, in EIU eyes, oxidative stress, evaluated by malondialdehyde, was significantly augmented after LPS injection.[27] Because oxidative stress is likely to be a potential stimulus for inducing cellular (Fig. 1) and molecular (Fig. 2) inflammatory events, we investigated the time course of 8-OHdG generation, the predominant marker of oxidative damage to DNA (Fig. 3A). Reasonably, 8-OHdG generation peaked at 3 hours after EIU induction, which preceded the prominent phase of cellular and molecular responses (24 hours, the time point for EIU-related inflammatory parameters). Importantly, resveratrol pretreatment led to significant suppression of 8-OHdG generation at 3 hours after EIU induction (Fig. 3B). This is explained, at least in part, by the chemical structure of resveratrol, which exerts antioxidative action attributed to

multiple phenolic hydroxyl groups characteristic of polyphenols.

Recently, it has been widely recognized that NF-κB, the p65/p50 heterodimer, enters the nucleus to bind the κB sequence after IκB phosphorylation and degradation caused by various stimuli and promotes the transcription of target genes, including ICAM-1 and MCP-1.[41] Given that NF-κB is a known redox-sensitive transcription factor functioning as proinflammatory signaling in the downstream of oxidative stress,[42] we investigated the role of NF-κB in resveratrol-induced suppression of EIU-related inflammation (Fig. 4). Previous studies on EIU[43] showed that immunohistochemistry for the p65 subunit of NF-κB detected a large number of nuclei positive for p65 in EIU tissue specimens, indicative of NF-κB activation in EIU. In our present study, to examine the time course of NF-κB activation in EIU, we used ELISA for p65 after nuclear extraction (Fig. 4A) as a more accurate technique to quantify the levels of NF-κB activation. Interestingly, nuclear p65 levels peaked at 3 hours and reached a plateau from 6 to 24 hours after LPS injection (Fig. 4A), which was temporally correlated with the time course of oxidative damage to DNA (Fig. 3A), suggesting a close link between LPS-induced oxidative stress and redox-sensitive NF-κB activation in EIU. Antioxidant resveratrol pretreatment led to significant suppression of NF-κB activation at 3 hours after LPS injection (Fig. 4B). This finding is compatible with those of several recent in vitro studies[37,44] showing that resveratrol inhibited LPS- or TNF-α–induced NF-κB activation in vascular endothelial cells and adipocytes. The anti-inflammatory effects of resveratrol, shown in the present study, were thought to be sequential events whereby its anti-oxidant action led to the inhibition of NF-κB–induced expression of inflammatory molecules.

In addition to its antioxidant property, resveratrol is known to function as a potent activator of the histone deacetylase SIRT1, a molecule responsible for regulating life span.[7,11] Interestingly, resveratrol has been shown to attenuate NF-κB activation by enhancing SIRT1 activity, leading to significant suppression of TNF-α–induced cell death[45] and amyloid β–induced neuronal injury.[46] In accordance with these in vitro studies, the present in vivo data (Fig. 5) show that oral application of resveratrol prevented LPS-induced deactivation of SIRT1 in the RPE-choroid. These findings on SIRT1 are consistent with resveratrol-induced suppression of nuclear NF-κB p65 translocation and IκB-α degradation in the RPE-choroid (Fig. 4). In concert with the antioxidant effect of resveratrol on the retina (Fig. 3), the present data suggest dual mechanisms of resveratrol as an antioxidant agent and a SIRT1 activator for suppressing NF-κB–mediated inflammation in the eye (Fig. 6).

Although resveratrol has not been applied in clinical practice until recently, it has received orphan-drug designation by the US Food and Drug Administration for MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes) syndrome. Clinically, ocular inflammation such as
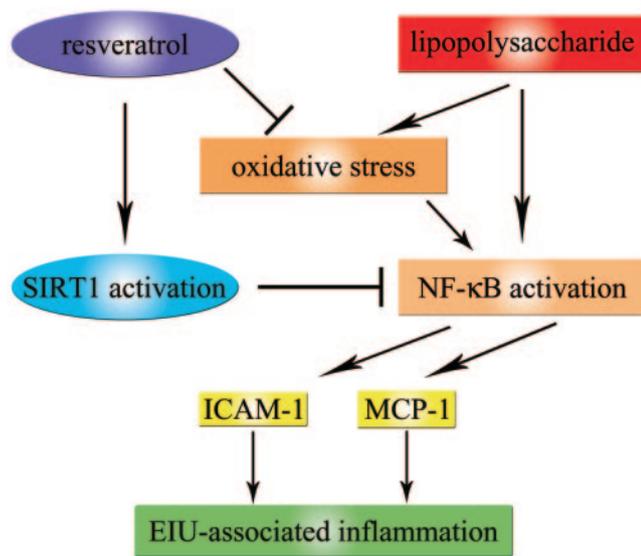
FIGURE 6. Schematic showing possible mechanisms in resveratrol-induced suppression of EIU-associated ocular inflammation. Note the dual pathways mediated by resveratrol as an antioxidant agent and SIRT1 activator to suppress LPS-induced NF-κB activation and subsequent expression of inflammation-related molecules ICAM-1 and MCP-1.

chronic endogenous uveitis is treated primarily with topical or systemic application (or both) of corticosteroids. During long-term treatment with corticosteroids, however, care must be taken to guard against ocular and systemic complications, including cataract, glaucoma, diabetes, hypertension, and osteoporosis. Accordingly, the establishment of additive and preventive anti-inflammatory approaches is desirable to decrease the rate and degree of corticosteroid-induced complications. Our present data provide molecular evidence of the potential validity of resveratrol supplementation as a therapeutic strategy to suppress ocular inflammation.

References

1. Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. *Lancet.* 1993;341:1103–1104.
2. Siemann EH, Creasy LL. Concentration of the phytoalexin resveratrol in wine. *Am J Enology Viticulture.* 1992;43:49–52.
3. Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science.* 1997;275:218–220.
4. Brakenhielm E, Cao R, Cao Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. *FASEB J.* 2001;15:1798–1800.
5. Wang Q, Xu J, Rottinghaus GE, et al. Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain Res.* 2002;958:439–447.
6. Bordone L, Guarente L. Calorie restriction, SIRT1 and metabolism: understanding longevity. *Nat Rev Mol Cell Biol.* 2005;6:298–305.
7. Howitz KT, Bitterman KJ, Cohen HY, et al. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature.* 2003;425:191–196.
8. Wood JG, Rogina B, Lavu S, et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature.* 2004;430:686–689.
9. Bauer JH, Goupil S, Garber GB, Helfand SL. An accelerated assay for the identification of lifespan-extending interventions in *Drosophila melanogaster. Proc Natl Acad Sci U S A.* 2004;101:12980–12985.
10. Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Curr Biol.* 2006;16:296–300.
11. Milne JC, Lambert PD, Schenk S, et al. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature.* 2007;450:712–716.
12. Joussen AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J.* 2004;18:1450–1452.
13. Ambati J, Anand A, Fernandez S, et al. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. *Nat Med.* 2003;9:1390–1397.
14. Izumi-Nagai K, Nagai N, Ozawa Y, et al. Interleukin-6 receptor-mediated activation of signal transducer and activator of transcription-3 (STAT3) promotes choroidal neovascularization. *Am J Pathol.* 2007;170:2149–2158.
15. Koizumi K, Poulaki V, Doehmen S, et al. Contribution of TNF-alpha to leukocyte adhesion, vascular leakage, and apoptotic cell death in endotoxin-induced uveitis in vivo. *Invest Ophthalmol Vis Sci.* 2003;44:2184–2191.
16. Shiratori K, Ohgami K, Ilieva IB, Koyama Y, Yoshida K, Ohno S. Inhibition of endotoxin-induced uveitis and potentiation of cyclo-oxygenase-2 protein expression by alpha-melanocyte-stimulating hormone. *Invest Ophthalmol Vis Sci.* 2004;45:159–164.
17. Bellot JL, Palmero M, Garcia-Cabanes C, Espi R, Hariton C, Orts A. Additive effect of nitric oxide and prostaglandin-E2 synthesis inhibitors in endotoxin-induced uveitis in the rabbit. *Inflamm Res.* 1996;45:203–208.
18. Mo JS, Matsukawa A, Ohkawara S, Yoshinaga M. Role and regulation of IL-8 and MCP-1 in LPS-induced uveitis in rabbits. *Exp Eye Res.* 1999;68:333–340.
19. Kurihara T, Ozawa Y, Shinoda K, et al. Neuroprotective effects of angiotensin II type 1 receptor (AT1R) blocker, telmisartan, via modulating AT1R and AT2R signaling in retinal inflammation. *Invest Ophthalmol Vis Sci.* 2006;47:5545–5552.
20. Nagai N, Oike Y, Noda K, et al. Suppression of ocular inflammation in endotoxin-induced uveitis by blocking the angiotensin II type 1 receptor. *Invest Ophthalmol Vis Sci.* 2005;46:2925–2931.
21. Nagai N, Izumi-Nagai K, Oike Y, et al. Suppression of diabetes-induced retinal inflammation by blocking the angiotensin II type 1 receptor or its downstream nuclear factor-kappaB pathway. *Invest Ophthalmol Vis Sci.* 2007;48:4342–4350.
22. Becker MD, Garman K, Whitcup SM, Planck SR, Rosenbaum JT. Inhibition of leukocyte sticking and infiltration, but not rolling, by antibodies to ICAM-1 and LFA-1 in murine endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 2001;42:2563–2566.
23. Noda K, Miyahara S, Nakazawa T, et al. Inhibition of vascular adhesion protein-1 suppresses endotoxin-induced uveitis. *FASEB J.* 2008;22:1094–1103.
24. Hollyfield JG, Bonilha VL, Rayborn ME, et al. Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat Med.* 2008;14:194–198.
25. Sellak H, Franzini E, Hakim J, Pasquier C. Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood.* 1994;83:2669–2677.
26. Rosenbaum JT, McDevitt HO, Guss RB, Egbert PR. Endotoxin-induced uveitis in rats as a model for human disease. *Nature.* 1980;286:611–613.
27. Satici A, Guzey M, Gurler B, Vural H, Gurkan T. Malondialdehyde and antioxidant enzyme levels in the aqueous humor of rabbits in endotoxin-induced uveitis. *Eur J Ophthalmol.* 2003;13:779–783.
28. Gao D, Zhang X, Jiang X, et al. Resveratrol reduces the elevated level of MMP-9 induced by cerebral ischemia-reperfusion in mice. *Life Sci.* 2006;78:2564–2570.
29. Ishida S, Usui T, Yamashiro K, et al. VEGF164 is proinflammatory in the diabetic retina. *Invest Ophthalmol Vis Sci.* 2003;44:2155–2162.
30. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell.* 2008;132:344–362.
31. Nagaoka T, Hein TW, Yoshida A, Kuo L. Resveratrol, a component of red wine, elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and potassium channels. *Invest Ophthalmol Vis Sci.* 2007;48:4232–4239.

32. Mizutani K, Ikeda K, Kawai Y, Yamori Y. Protective effect of resveratrol on oxidative damage in male and female stroke-prone spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol.* 2001;28:55–59.

33. Birrell MA, McCluskie K, Wong S, Donnelly LE, Barnes PJ, Belvisi MG. Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-kappaB-independent mechanism. *FASEB J.* 2005; 19:840–841.

34. Csiszar A, Labinskyy N, Podlutsky A, et al. Vasoprotective effects of resveratrol and SIRT1: attenuation of cigarette smoke-induced oxidative stress and proinflammatory phenotypic alterations. *Am J Physiol Heart Circ Physiol.* 2008;294:H2721–H2735.

35. Fuentes ME, Durham SK, Swerdel MR, et al. Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1. *J Immunol.* 1995;155:5769–5776.

36. Zhu J, Yong W, Wu X, et al. Anti-inflammatory effect of resveratrol on TNF-alpha-induced MCP-1 expression in adipocytes. *Biochem Biophys Res Commun.* 2008;369:471–477.

37. Choi HB, Ryu JK, Kim SU, McLarnon JG. Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. *J Neurosci.* 2007;27:4957–4968.

38. Li L, Shoji W, Takano H, et al. Increased susceptibility of MER5 (peroxiredoxin III) knockout mice to LPS-induced oxidative stress. *Biochem Biophys Res Commun.* 2007;355:715–721.

39. Watanabe N, Miura S, Zeki S, Ishii H. Hepatocellular oxidative DNA injury induced by macrophage-derived nitric oxide. *Free Radic Biol Med.* 2001;30:1019–1028.

40. Tsuji G, Koshiba M, Nakamura H, et al. Thioredoxin protects against joint destruction in a murine arthritis model. *Free Radic Biol Med.* 2006;40:1721–1731.

41. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol.* 1996;14:649–683.

42. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature.* 2000;408:239–247.

43. Suzuki Y, Ohgami K, Shiratori K, et al. Suppressive effects of astaxanthin against rat endotoxin-induced uveitis by inhibiting the NF-kappaB signaling pathway. *Exp Eye Res.* 2006;82:275–281.

44. Carluccio MA, Siculella L, Ancora MA, et al. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler Thromb Vasc Biol.* 2003;23:622–629.

45. Yeung F, Hoberg JE, Ramsey CS, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.* 2004;23:2369–2380.

46. Chen J, Zhou Y, Mueller-Steiner S, et al. SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. *J Biol Chem.* 2005;280:40364–40374.