Roles of AMP-Activated Protein Kinase in Diabetes-Induced Retinal Inflammation

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PURPOSE. AMP-activated protein kinase (AMPK) is a sensor of cellular energy status. The purpose of the present study was to elucidate the roles of AMPK in the pathogenesis of diabetic retinopathy using the known AMPK activators resveratrol and AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) in a mouse model.

METHODS. C57BL/6 mice with streptozotocin-induced diabetes were treated with resveratrol orally at 50 mg/kg for 7 days or with AICAR intraperitoneally at 100 mg/kg 24 hours before death. Retinal protein levels of phosphorylated and total AMPK, phosphorylated nuclear factor (NF)-κB p65, intercellular adhesion molecule (ICAM)-1, and vascular endothelial growth factor (VEGF) were evaluated by Western blot analysis or enzyme-linked immunosorbent assay. Retinal activity of sir- tuin (SIRT)1 was measured by deacetylase fluorometric assay. Leukocyte adhesion to the retinal vasculature was examined with a concanavalin A lectin perfusion-labeling technique.

RESULTS. Induction of diabetes in mice led to retinal AMPK dephosphorylation, which was significantly reversed by either resveratrol or AICAR. Either resveratrol or AICAR significantly reversed SIRT1 deactivation and NF-κB phosphorylation, both of which were induced in the diabetic retina. Administration of resveratrol to diabetic mice significantly reduced diabetes-induced retinal leukocyte adhesion, together with retinal expression of ICAM-1 and VEGF.

CONCLUSIONS. The present findings reveal that diabetes-induced retinal inflammation stems from downregulation of the AMPK pathway, leading subsequently to SIRT1 deactivation and NF-κB activation. The data also suggest the potential use of the AMPK activator resveratrol as a therapeutic agent for diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2011;52:9142–9148) DOI:10.1167/iovs.11-8041

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Diabetic retinopathy is a significant cause of severe vision loss and blindness in working-age adults.1 Increasing evidence suggests that the pathogenesis of diabetic retinopathy is mediated by inflammatory processes, including leukocyte adhesion and the cytokine network, causing diabetic retinopathy, which is regarded as an inflammatory disease.2–7 Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion.5,6,7 which triggers vascular hyperpermeability5,6,7 and pathologic retinal neovascularization.2 Clinically, elevated levels of vascular endothelial growth factor (VEGF) were detected in the vitreous fluid of eyes with proliferative diabetic retinopathy.9 Enhanced immunoreactivity of intercellular adhesion molecule (ICAM)-1 in retinal vessels together with leukocyte infiltration was observed in postmortem eyes with diabetic retinopathy.10 As upstream stimuli for these inflammatory molecules, recent reports, including ours, have demonstrated that oxidative stress and redox-sensitive transcription factor nuclear factor (NF)-κB signaling contribute to the pathogenesis of diabetic retinopathy.11–13

Resveratrol (3,5,4′-trihydroxystilbene), one of polyphenolic phytoalexins found in red wine and grape skin, has been reported to exhibit various bioactivities,14 including antioxidative,15 antimutagenic16,17 and neuroprotective18 effects. Recently, sirtuin (SIRT)1, a known regulator of aging,19 has been shown to be activated by resveratrol.20 As concerns its effects on the eye, we have recently revealed that resveratrol suppresses ocular inflammation in endothoxin-induced uveitis through its antioxidative property and SIRT1-activating action, both of which lead to inhibition of the NF-κB pathway.21 We have also demonstrated the neuroprotective effect of resveratrol on light-induced retinal degeneration that was mediated by the redox-sensitive transcription factor activator protein (AP)-1.22 Moreover, resveratrol has been shown to prevent neuronal damage in the diabetic retina.23 However, little is known about the effect of resveratrol on diabetes-induced retinal inflammation and microvascular complication.

Resveratrol is also known to increase the activity of AMP-activated protein kinase (AMPK).24 AMPK is a fuel-sensing kinase that ameliorates the pathogenesis of metabolic disorders, including diabetes, by controlling the expression and activation of various downstream molecules.24 The activity of AMPK is enhanced by ATP-depleting conditions such as calorie restriction and physical exercise.25 Although either resveratrol application or calorie restriction leads to SIRT1-mediated lifespan extension, the relationship between SIRT1 and AMPK remains unclear and controversial.25–28 Interestingly, AMPK-deficient mice fed with a high-fat diet failed to exhibit resveratrol-induced amelioration of diabetic parameters, suggesting the significance of AMPK as a central target for the metabolic effect of resveratrol.26 Although the AMPK pathway has been shown to be protective against and downregulated in diabetes-related pathologies in the kidney29 and skeletal muscle,30 the role of AMPK in the retina remains to be elucidated. Some in vitro data on vascular endothelial cells31,32 and leukocytes33–34
have shown that AMPK activation leads to significant suppression of NF-κB, which regulates gene expression of various proinflammatory molecules responsible for the pathogenesis of diabetic retinopathy. Therefore, we hypothesize that NF-κB-mediated retinal inflammation in diabetes results from AMPK deactivation. Herein, we report the first evidence that AMPK-activating action of resveratrol is anti-inflammatory in the pathogenesis of diabetic retinopathy.

**Methods**

**Induction of Diabetes**

C57BL/6 mice (Clea, Tokyo, Japan) at the age of 6 weeks were used for induced diabetes. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals received intraperitoneal injections of streptozotocin (STZ; Sigma, St. Louis, MO) at a dose of 60 mg/kg body weight (BW) for 5 days. Blood glucose concentrations were measured from the tail vein (Medisafe mini-GR-102; Terumo, Tokyo, Japan). Development of diabetes was defined by blood glucose more than 250 mg/dL 7 days after the first injection of STZ.

**Treatment with Resveratrol**

Eight weeks after the first injection of STZ, mice were administered vehicle (6.67% DMSO [dimethyl sulfoxide] in PBS [phosphate-buffered saline]) or resveratrol (Sigma-Aldrich, St. Louis, MO) at 50 mg/kg BW by gastric intubation daily for 7 days. As preliminary experiments confirmed that the vehicle DMSO solution did not affect diabetes-related parameters including cellular and molecular inflammatory responses in murine eyes (data not shown), vehicle-treated, age-matched, normal mice served as controls throughout the study. The dose of 50 mg/kg BW is equivalent to that applied to protect against endotoxin-induced retinal inflammation22 or light-induced retinal degeneration22 in mice.

**Treatment with AICAR**

Twenty-four hours before euthanization, mice were intraperitoneally injected with the specific AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; Santa Cruz Biotechnology, Santa Cruz, CA) at 100 mg/kg body weight (BW). A dose of 100 mg/kg BW is equivalent to that applied to protect against endotoxin-induced retinal inflammation22 or light-induced retinal degeneration22 in mice.

**Immunoblot Analyses for Phosphorylated and Total AMPK**

Eight weeks after diabetes induction, mice were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retinas were carefully isolated and placed into 100 μL of lysis buffer supplemented with protease inhibitors and then sonicated. Each sample containing 30 μg total protein was separated by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) and electroblotted to polyvinylidene fluoride (PVDF) membranes (ATTO, Tokyo, Japan). After nonspecific binding was blocked with TNP blocking buffer (PerkinElmer Life Science, Boston, MA), the membranes were incubated with a rabbit monoclonal antibody against phosphorylated AMPK (1:1000; Cell Signaling Technology, Beverly, MA) or a rabbit polyclonal antibody against total AMPK (1:1000; Cell Signaling Technology) at 4°C overnight. The membranes were then incubated with secondary antibodies followed by a horseradish peroxidase-conjugated goat antibody against rabbit IgG (1:5000; BioSource, Camarillo, CA). The signals were visualized with an ECL kit (GE Health care, Buckinghamshire, UK) according to the manufacturer’s protocol, and measured by ImageJ software (http://rsb.info.nih.gov/ij/index.html; National Institutes of Health, Bethesda, MD).

**ELISA for ICAM-1, VEGF, and Phosphorylated NF-κB p65 Subunit**

Eight weeks after the first STZ injection, animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retinas were carefully isolated and placed into 100 μL of lysis buffer (0.02 M HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], 10% glycerol, 10 mM Na3P2O7, 100 μM NaCl, 1% Triton, 100 mM NaF, and 4 mM EDTA [pH 8.0]) supplemented with protease inhibitors (2 mg/L aprotinin, 100 μM PMSF [phenylmethylsulfon fluoride], 10 μM leupeptin, and 2.5 μM pepstatin A) and then sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The levels of ICAM-1 and VEGF in the supernatant were determined with mouse ICAM-1 and VEGF enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer’s protocols. Activation of NF-κB was determined by measuring the phosphorylated NF-κB p65 levels with the phosphorylated NF-κB p65 ELISA kit (Cell Signaling Technology), according to the manufacturer’s instructions. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration evaluated by a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA).

**Deacetylase Fluorometric Assay for SIRT1**

Eight weeks after diabetes induction, animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retinas were carefully isolated, placed in 100 μL of lysis buffer and then sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The activity of SIRT1 in the supernatant was determined with an SIRT1 assay kit (SIRT1/Sir2 Deacetylase Fluorometric Assay kit; CycLex, Ima, Japan), according to the manufacturer’s protocols.

**Quantification of Retinal Adherent Leukocytes**

The retinal vasculature and adherent leukocytes were imaged by perfusion labeling with fluorescein-isothiocyanate (FITC)–coupled concavalin A lectin (Con A; Vector, Burlingame, CA), as described previously. Eight weeks after the first injection of STZ, the chest cavity was opened in animals under deep anesthesia, and a 24-gauge cannula was introduced into the left ventricle. After injection of 2 mL of PBS to remove erythrocytes and nonadherent leukocytes, 2 mL FITC-conjugated Con A was perfused. After the eyes were enucleated, the retinas were flat mounted. The flat mounts were imaged with an epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and the total number of Con A-stained adherent leukocytes per retina was counted in a masked fashion.

**Statistical Analyses**

All results are expressed as the mean ± SD. The values were processed for statistical analyses (ANOVA and post-hoc Tukey-Kramer test), and differences were considered statistically significant at P < 0.05.

**Results**

**Metabolic Parameters**

Mice with STZ-induced diabetes showed a significant decrease in body weight and a significant increase in blood glucose, compared with age-matched nondiabetic controls. Treatment with resveratrol at 7 weeks of diabetes did not significantly change these metabolic parameters (Table 1).

**Resveratrol and AICAR Reversed Diabetes-Induced Retinal AMPK Deactivation**

The roles of AMPK in the pathogenesis of diabetic retinopathy remain uncertain, although only nephropathy, out of several diabetic organ complications, has recently been associated...
Blood glucose, mg/dL 157/H11006
Body weight, g 26.6/H11006

which the AMPK pathway was downregulated. activating effect as potent as AICAR in the diabetic retina in results indicate the capability of resveratrol to exert an AMPK-

29.2% of control), compared with the vehicle treatment. These (activator as a positive control in the diabetic mice significantly (71.2% /H11006 (100% /H11006 (100%
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Systemic administration of resveratrol to diabetic animals led to a significant (P < 0.05) increase in p-AMPK/t-AMPK ratios (100.7% /H11006 (100%
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activator as a positive control in the diabetic mice significantly (71.2% /H11006 (100% /H11006 (100%
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reactive) increase in SIRT1 activity (107% ± 11.1% or 97.9% ± 3.5% of control, respectively), compared with vehicle treatment of diabetes. Consequently, the data confirmed the regulation of SIRT1 downstream of the AMPK pathway in the diabetic retina, consistent with recent data26 showing that AMPK is indispensable for the ameliorating effect of resveratrol on diabetes-related systemic parameters.

**Resveratrol and AICAR Suppressed Diabetes-Induced Retinal NF-κB Activation**

NF-κB, the major transcription factor that transduces proinflammatory signaling in the diabetic retina,12,13 has recently been shown to be regulated by AMPK31–34 and SIRT133,36 in vitro. To investigate the signal transduction downstream of AMPK activation in the diabetic retina (Fig. 1), we examined NF-κB activities with ELISA by measuring the protein levels of phosphorylated (p-) NF-κB p65 (Fig. 3). Retinal p-NF-κB p65 levels were significantly (P < 0.01) higher in the vehicle-treated diabetic mice (205% ± 65.8% of control) than in the vehicle-treated controls (100% ± 32.5% of control). Systemic administration of resveratrol to the diabetic animals significantly (P < 0.05) reduced p-NF-κB p65 levels (149% ± 55.9% of control), compared with vehicle treatment (205% ± 65.8% of control). Similarly, systemic application of AICAR to the diabetic animals led to a significant (P < 0.05) decrease in p-NF-κB p65 levels (134% ± 10.7% of control), compared with the vehicle treatment. These in vivo findings demonstrate that the AMPK-activating action of resveratrol (Fig. 1) leads to significant suppression of NF-κB activation in the diabetic retina.

**Resveratrol and AICAR Suppressed Diabetes-Induced Retinal Leukocyte Adhesion**

With a future view to clinical application, we then examined whether the AMPK activator resveratrol as well as AICAR suppresses NF-κB-induced inflammatory events in diabetic retinopathy.

with AMPK deactivation.29 To investigate the involvement of AMPK with diabetic retinopathy, we analyzed the ratios of retinal phosphorylated (p-) to total (t-) AMPK levels (Fig. 1) by immunoblot analyses. Retinal p-AMPK/t-AMPK ratios were significantly (P < 0.05) lower in the vehicle-treated diabetic mice (71.2% ± 16.3% of control) than in the vehicle-treated controls (100% ± 22.0% of control). Systemic administration of resveratrol to diabetic animals led to a significant (P < 0.05) increase in p-AMPK/t-AMPK ratios (111.5% ± 29.2% of control), compared with the vehicle treatment. These in vivo findings demonstrate that resveratrol and AICAR reversed diabetes-induced retinal AMPK deactivation. **Resveratrol and AICAR Reversed Diabetes-Induced Retinal SIRT1 Deactivation**

The involvement of SIRT1, as well as the relationship between SIRT1 and AMPK in the pathogenesis of diabetic retinopathy, remains to be elucidated. To examine a possible association of SIRT1 with diabetic retinopathy, we analyzed retinal SIRT1 activities (Fig. 2) by deacetylase fluorometric assay. Retinal SIRT1 activities (87.4% ± 6.2% of control) were significantly (P < 0.01) lower in the vehicle-treated diabetic mice than in the vehicle-treated controls (100% ± 9.7% of control). Systemic administration of the AMPK activator resveratrol or AICAR to the diabetic animals led to a significant (P < 0.01 or P < 0.05, respectively) increase in SIRT1 activity (107% ± 11.1% or 97.9% ± 3.5% of control, respectively), compared with vehicle treatment of diabetes. Consequently, the data confirmed the regulation of SIRT1 downstream of the AMPK pathway in the diabetic retina, consistent with recent data26 showing that AMPK is indispensable for the ameliorating effect of resveratrol on diabetes-related systemic parameters.
Resveratrol and AICAR suppressed diabetes-induced retinal NF-κB activation. Retinal p-NF-κB p65 levels in vehicle-treated diabetic mice, significantly elevated compared to those in vehicle-treated controls, were significantly suppressed by treatment with either resveratrol or AICAR. n = 6–16. **P < 0.01; * P < 0.05.

Resveratrol Suppressed Diabetes-Induced Retinal Expression of ICAM-1 and VEGF

To investigate molecular mechanisms underlying diabetes-induced retinal leukocyte adhesion (Fig. 4), we evaluated the protein levels of ICAM-1 (Fig. 5A) and VEGF (Fig. 5B) in the retina by ELISA. Retinal ICAM-1 and VEGF protein levels were significantly (P < 0.05 and P < 0.01, respectively) higher in the vehicle-treated diabetic mice (9.6 ± 1.9 ng/mg and 20.8 ± 2.5 pg/mg, respectively) than in the vehicle-treated controls (7.1 ± 1.0 ng/mg and 17.9 ± 1.6 pg/mg, respectively). Systemic administration of resveratrol to the diabetic animals significantly (P < 0.05 and P < 0.01, respectively) reduced ICAM-1 and VEGF protein levels (8.4 ± 1.0 ng/mg and 17.6 ± 2.1 pg/mg, respectively). These data clarify the protective effect of resveratrol on the diabetic retina through suppression of cellular (Fig. 4) and molecular (Fig. 5) responses.

Discussion

The present study reveals, for the first time to our knowledge, the protective roles of the AMPK pathway in the pathogenesis of diabetic retinopathy. Induction of diabetes in mice led to retinal AMPK deactivation, which was normalized by an AMPK-activating action exerted by resveratrol that was as potent as that induced by AICAR (Fig. 1). Resveratrol-induced recovery of SIRT1 activity, which was downregulated in the diabetic retina, depended on its AMPK-activating effect (Fig. 2). Resveratrol suppressed diabetes-induced upregulation of retinal NF-κB signaling by activating the AMPK pathway (Fig. 3). Indeed, the AMPK activator resveratrol inhibited cellular (Fig. 4) and molecular (Fig. 5) mechanisms in diabetes-induced retinal inflammation, which is known to result from NF-κB activation.

AMPK is a heterotrimeric protein comprising a catalytic α subunit and regulatory β and γ subunits. AMPK exists in the cytoplasm without activation as a sensor of cellular energy status, enters the nucleus after phosphorylation of α subunit induced by metabolic stresses, such as glucose deprivation, and regulates the expression of target enzymes related to catabolic pathways that generate ATP.59 Our present finding (Fig. 1) is the first to show significant decline of AMPK activity in the diabetic retina compared with the normal retina. This is comparable with recent data on diabetic nephropathy60 and glucose-stimulated skeletal muscle,61 both of which were associated with AMPK deactivation. Resveratrol application to diabetic mice reversed retinal AMPK deactivation (Fig. 1), NF-κB activation (Fig. 3), and leukocyte adhesion to the retinal vasculature (Fig. 4), whereas AMPK activation with AICAR mimicked the effects of resveratrol on these in vivo parameters (Figs. 1, 3, 4). These findings are explained by and compatible with recent in vitro data showing that AMPK activation led to NF-κB suppression in vascular endothelial cells31,52 and inflammatory leukocytes,53–54 both of which are the key cellular components responsible for the pathogenesis of diabetic retinopathy.

NF-κB is a transcription factor that binds to IκB in the cytoplasm without activation, enters the nucleus after IκB phosphorylation and degradation induced by various stimuli, and promotes the transcription of target genes including ICAM-1 and VEGF.59 Recently, proinflammatory stimuli have been shown to promote the phosphorylation of not only IκB but also NF-κB p65 subunit, per se, and thus the p65 phosphorylation is regarded as a marker of NF-κB activation.57–58 The current finding on AICAR-induced suppression of NF-κB activation (Fig. 3) suggests that anti-inflammatory effects of resveratrol on NF-κB-dependent cellular (Fig. 4) and molecular (Fig. 5) events is mediated, at least in part, by the recovery from diabetes-induced deactivation of the AMPK pathway (Fig. 1).

Besides AMPK activation, resveratrol is known to stimulate SIRT1 activity. SIRT1 has been suggested to be a key modulator for extending the lifespan of various species after calorie restriction.19 Interestingly, SIRT1 overexpression is shown to protect pancreatic β-cells by inhibiting the NF-κB signaling pathway through deacetylation of p65.60 In accordance with the in vitro data, the induction of diabetes in mice led to retinal SIRT1 deactivation (Fig. 2) together with NF-κB activation (Fig. 3), both of which were significantly reversed by application of resveratrol (Figs. 2, 3), suggesting a close link between SIRT1 deactivation and NF-κB activation in the diabetic retina. Interestingly, recent in vitro data have shown that AMPK activation inhibits NF-κB-induced inflammatory responses in macrophages through the effect of SIRT1 on deacetylating NF-κB.55 All evidence taken together, the molecular pathway by which resveratrol inhibits NF-κB signaling (Fig. 3) appears to involve multiple steps through the upregulation of SIRT1 enzymatic activity (Fig. 2) together with the AMPK activation (Fig. 1) as the most upstream cascade reaction.

Treatment with resveratrol to diabetic mice suppressed leukocyte adhesion to the retinal vasculature (Fig. 4), which plays a pivotal role in the development of endothelial injury, capillary loss, and retinal ischemia.4,5 As concerns the underlying molecular mechanisms, resveratrol inhibited diabetes-induced expression of ICAM-1 and VEGF (Fig. 5), both of which are known proinflammatory molecules responsible for the pathogenesis of diabetic retinopathy.5,5,6,9,59 Clinically,
FIGURE 4. Resveratrol and AICAR suppressed diabetes-induced retinal leukocyte adhesion. Flat-mounted retinas from vehicle-treated control mice (A, E, I), vehicle-treated diabetic mice (B, F, J), resveratrol-treated diabetic mice (C, G, K), and AICAR-treated diabetic mice (D, H, L). Vehicle-treated diabetic mice showing increased adherent leukocytes (arrows) compared with vehicle-treated controls. (M) Quantification of adherent retinal leukocytes. The resveratrol- or AICAR-treated diabetic mice showed significantly fewer adherent leukocytes than did the vehicle-treated diabetic mice. Scale bar, 100 μm. n = 10–18. **P < 0.01.
resveratrol is now in phase II investigations for cancer and diabetes. Preclinical data revealed that resveratrol application in mice with type 2 diabetes led to significant amelioration of insulin sensitivity and blood glucose levels. Importantly, our systemic data (Table 1) showed that the current dose and duration of resveratrol did not alter blood glucose, suggesting that the present findings (Figs. 1–5) resulted from blood glucose-independent mechanisms for inhibiting diabetes-induced retinal inflammation. Resveratrol application to diabetic patients in clinical practice may therefore be expected to protect the retina from diabetic insults through its systemic pathways for lowering blood glucose as well as its anti-inflammatory effect exerted directly on the retina via AMPK activation. Future and further studies are awaited to verify the potential use of resveratrol for the treatment of diabetic retinopathy.

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References


