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Resveratrol prevents the development of choroidal neovascularization by modulating AMP-activated protein kinase in macrophages and other cell types

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Abstract

The development of choroidal neovascularization (CNV) is a critical step in the pathogenesis of age-related macular degeneration (AMD), a vision-threatening disease. In this study, we used a mouse model of AMD to study the protective effects of resveratrol (RSV) supplementation against CNV as well as the underlying molecular mechanisms. Mice were orally pretreated with RSV daily for 5 days. On the fifth day, the mice underwent laser photocoagulation to induce CNV. One week after laser treatment, CNV volume was significantly lower in the RSV-treated mice compared with vehicle-treated animals. In addition, RSV treatment significantly inhibited macrophage infiltration into the retinal pigment epithelium (RPE)-choroid and suppressed the expression of inflammatory and angiogenic molecules, including vascular endothelial growth factor, monocyte chemotactic protein-1 and intercellular adhesion molecule-1. Importantly, RSV prevented the CNV-induced decrease in activated AMP-activated protein kinase and increase in activated nuclear factor-κB in the RPE-choroid complex. The regulatory effects of RSV on these molecules were confirmed in RPE, microvascular endothelial and macrophage cell lines. Inhibition of macrophage infiltration by RSV was confirmed by *in vitro* scratch and migration assays. RSV suppressed CNV development, reducing the levels of multiple cytokines secreted from several cell types and inhibiting macrophage migration. The direct effects of RSV on each cell type were confirmed *in vitro*. Although further studies are needed, RSV could potentially be applied in the clinic to prevent CNV development in AMD.

Keywords: Retina; AMPK; Inflammation; Macrophage; Neovascularization; Resveratrol

1. Introduction

Choroidal neovascularization (CNV) is the main pathogenesis of age-related macular degeneration (AMD), the most common cause of blindness in developed countries [1]. Anti-vascular endothelial growth factor (VEGF) therapy is used worldwide to suppress CNV [2]. The positive effects of anti-VEGF drugs are well documented [3,4], while non-responsive cases are also reported [5]. Moreover, once CNV has initiated, even when treated, it can breach the barrier consisting of Bruch's membrane and retinal pigment epithelium (RPE) cells, resulting in irreversible changes in retinal neurons. Thus, strategies to prevent CNV development are now a major focus of AMD research [6]. A large-scale clinical study, the Age-Related Eye Disease Study 2, has revealed that micronutrient supplementation with lutein and zeaxanthin together with multi-vitamins and zinc can suppress the rate of AMD progression in subjects who were in the lowest quintile of dietary lutein and zeaxanthin intake [7]. However, there also were some cases in which less effect was observed in micronutrient supplementation of lutein and zeaxanthin. Thus, the alternative approaches for prevention are still anticipated.

The molecular and cellular mechanisms underlying the development of CNV have long been studied. The role of macrophages [8,9], which are a rich source of VEGF and are recruited by the actions of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1), is well documented, as is the involvement of inflammatory mechanisms such as the renin–angiotensin system [10,11] and interleukin (IL)-6 receptor signaling [12]. Thus, inflammation and the associated oxidative stress are logical targets for preventive therapies [13]. However, practical preventive therapies have not been fully developed.

Here, we focus on resveratrol (3,5,4-trihydroxystilbene; RSV), an antioxidant and dietary polyphenol found in red wine and grape skin. A number of bioactive functions have been attributed to RSV, including anti-tumorigenic [14], anti-angiogenic [15], anti-atherogenic [16] and neuroprotective [17,18] functions. RSV can also protect the cardiovascular system from oxidative stress [19,20]. RSV has been a focus of research on aging because RSV administration extends the lifespan of yeast, *Caenorhabditis elegans* and *Drosophila melanogaster* by activating Sir2. Moreover, RSV improves the survival and health of mice fed with a high-fat diet [21]. RSV increases the activity of AMP-activated protein kinase (AMPK), a fuel-sensing kinase that ameliorates the pathogenesis of metabolic disorders such as diabetes [22,23].

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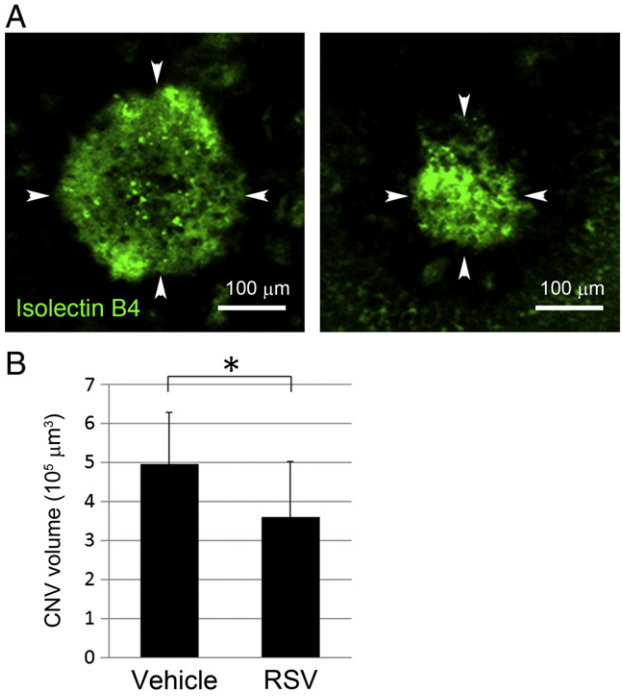

Fig. 1. RSV treatment suppressed CNV development. (A) Flat-mounted RPE-choroid complexes from vehicle- and RSV-treated mice 7 days after laser PC for CNV induction. CNV tissues were stained with lectin. (B) Graph showing the CNV volume index. RSV suppressed the CNV volume in the mouse model of AMD. $n=16$. *$P<.01$.

In addition, RSV's activation of AMPK is also involved in reverse remodeling of cardiomyocytes in the post-infarction heart through promoting autophagy [24], inducing vasodilation in the hypertensive model through increasing NO synthesis [25] and inhibiting cell proliferation and protein translation in the tumor cells interacting with estrogen receptor [26]. Thus, RSV-induced AMPK may contribute to organ health in aged mice stressed by metabolic disorders [21].

Because AMD is an aging-related disease that is often induced in cases of metabolic syndrome, RSV may represent a promising candidate for a preventive therapeutic approach. However, neither the involvement of the AMPK pathway in the mechanisms underlying CNV development nor the effects of RSV on these mechanisms are fully understood.

In this study, we pretreated mice with oral RSV and then analyzed its preventive effects on CNV development together with the underlying molecular mechanisms, focusing on AMPK activation. The direct effects of RSV on different cell types were also investigated.

2. Methods and materials

2.1. Animals

Six-week-old male C57BL/6J mice (CLEA, Tokyo, Japan) were used. All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. RSV treatment

Animals were pretreated with RSV at a daily dose of 50 mg/kg body weight (Sigma, St. Louis, MO, USA) or with phosphate-buffered saline (PBS) containing 1% dimethyl sulfoxide as the vehicle control for 5 days, starting 4 days before and ending on the day of laser photocoagulation (PC).

2.3. Induction of CNV

Laser-induced CNV is widely used as an animal model of neovascular AMD and reflects the pathogenesis of the inflammation-related CNV observed in AMD. In this model, new vessels from the choroid invade the subretinal space after PC. Laser PC was performed at 5 spots per eye around the optic disc using a slit-lamp delivery system (Novus Spectra; Lumenis, Tokyo, Japan) as previously described [9–12,27–30].

2.4. Immunohistochemistry and quantification of laser-induced CNV

One week after laser injury, the mice were sacrificed, and the eyecups were removed and incubated with 0.5% fluorescein-isothiocyanate-conjugated isolectin B4 (Vector, Burlingame, CA, USA). CNV was visualized using a scanning laser confocal microscope (FV1000; Olympus, Tokyo, Japan). The area of CNV-related fluorescence was measured using NIH (National Institutes of Health) ImageJ software. Horizontal optical sections of CNV were obtained at 1-μm intervals from the surface to the deepest focal plane. The summation of the whole fluorescent area was used as the index for CNV volume as previously described [10,11,27–30].

2.5. Immunohistochemistry for infiltrating macrophages

Whole-mount RPE-choroid complexes obtained 3 days after PC were incubated with a rat polyclonal antibody against macrophage marker F4/80 (Abcam, Raleigh, NC, USA). Avidin-Alexa 546-tagged secondary antibody (Invitrogen, Carlsbad, CA, USA) was then applied as previously described [12].

2.6. Real-time reverse-transcription (RT) polymerase chain reaction (PCR)

Total RNA was isolated from the RPE-choroid complex 1–3 days after PC and reverse-transcribed. Quantitative PCR analyses for F4/80, VEGF, ICAM-1, MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in combination with TaqMan probes as previously described [27]. The level of each mRNA was normalized to that of GAPDH.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Protein extracts were obtained from homogenized RPE-choroid complex 4 h or 3 days after CNV induction by laser PC. In the samples taken 3 days after the PC, VEGF, MCP-1 and ICAM-1 levels were determined with mouse VEGF, MCP-1 and ICAM-1 ELISA kits (R&D Systems, Minneapolis, MN, USA) as previously described [10]. The VEGF ELISA kit recognizes all isoforms of VEGF. In the samples obtained 4 h after PC, the levels of phosphorylated nuclear factor-κB (NF-κB) p65 were analyzed with a mouse phosphorylated NF-κB p65 ELISA kit (Cell Signaling Technology, Beverly, MA, USA). For the ELISA assays performed on the *in vitro* samples, the details of the sampling are described below.

2.8. Immunoblot analyses

Protein extracts were obtained from homogenized RPE-choroid complexes prepared 4 h after PC. Immunoblot analyses were performed as described previously [12]. A rabbit polyclonal antibody against phosphorylated AMPK (Cell Signaling Technology) and a mouse monoclonal antibody against α-tubulin (1:1000; T9026, Sigma-Aldrich, Tokyo, Japan) were used, and the protein level of pAMPK was normalized to that of α-tubulin.

2.9. Cell lines

Three cell lines were prepared: RPE cells (the human cell line ARPE-19), microvascular endothelial cells (the murine cell line b-End3) and macrophages (the murine cell line RAW264.7). The cells were treated with RSV (10 μM) or vehicle in serum-free DMEM/F12 (Sigma) for the ARPE-19 cells or in serum-free DMEM for the b-End3 and RAW264.7 cells. RSV was applied from 1.5 h before and through the course of stimulation with tumor necrosis factor-α (TNF-α; Sigma; 10 ng/ml for ARPE-19 and b-End3 cells; 20 ng/ml for RAW264.7 cells). To inhibit AMPK activation, the cells were pretreated with an AMPK inhibitor, compound C (10 μM; Calbiochem, La Jolla, CA, USA), under TNF-α stimulation. After a 20-min incubation, the cell lysates were subjected to ELISA for phosphorylated NF-κB p65. After a 6-h incubation, the cell lysates or culture supernatants were subjected to ELISA for ICAM-1, MCP-1 or VEGF.

2.10. Scratch assay

RAW264.7 cells were grown to confluence on 24-well tissue dishes, and a single scratch was made using a sterile 1000-μl pipette tip. The cells were washed with PBS and treated with RSV (10 μM) with or without compound C (10 μM) in serum-free DMEM containing MCP-1 (50 ng/ml; R&D Systems). Photographs were taken after 24 h, and the cell-covered area was measured by NIH Image.

2.11. Transmigration assay

The effect of RSV on macrophage migration was assessed in a 96-well microchamber (Trevigen Inc., Gaithersburg, MD, USA). RAW264.7 macrophages were added to the upper

wells, which contained RSV (10 μM) with or without compound C (10 μM). The upper wells were separated from the lower chamber by an 8-μm-pore collagen-coated polyethylene terephthalate membrane. The lower chamber contained MCP-1 (200 ng/ml; R&D Systems) in serum-free DMEM. After 24 h, the transmigrated cells were analyzed by a calcein acetomethylester system (Trevigen Inc.).

2.12. Statistical analyses

All results are expressed as the mean±S.D. The values were subjected to statistical analyses (one-way analysis of variance with Tukey's *post hoc* test). The differences were considered statistically significant at $P<.05$.

3. Results

3.1. RSV treatment suppressed CNV development

We first calculated the CNV volumes 7 days after induction in mice pretreated with control vehicle or RSV. The CNV volume was significantly suppressed by RSV treatment ($P<.01$, Fig. 1).

3.2. RSV treatment inhibited macrophage infiltration

To examine how RSV suppresses CNV development, we analyzed macrophage infiltration in the RPE-choroid complex 3 days after CNV induction, when the infiltration is at its peak [31], by immunohistochemistry for the macrophage marker F4/80 (Fig. 2A). The RSV-treated mice showed less immunoreactivity for F4/80 in the RPE-choroid complex compared with vehicle-treated animals. We further measured the mRNA level of F4/80 in the RPE-choroid by quantitative RT PCR (Fig. 2B) and found that there was a trend of suppression at day 1 and a significant suppression at day 2 in the RSV-treated mice ($P<.01$, Fig. 2B). The suppressive effect was also observed at day 3 ($P<.05$, Fig. 2B). Taken together, the results suggested that the macrophage infiltration during the process of CNV development was suppressed by RSV.

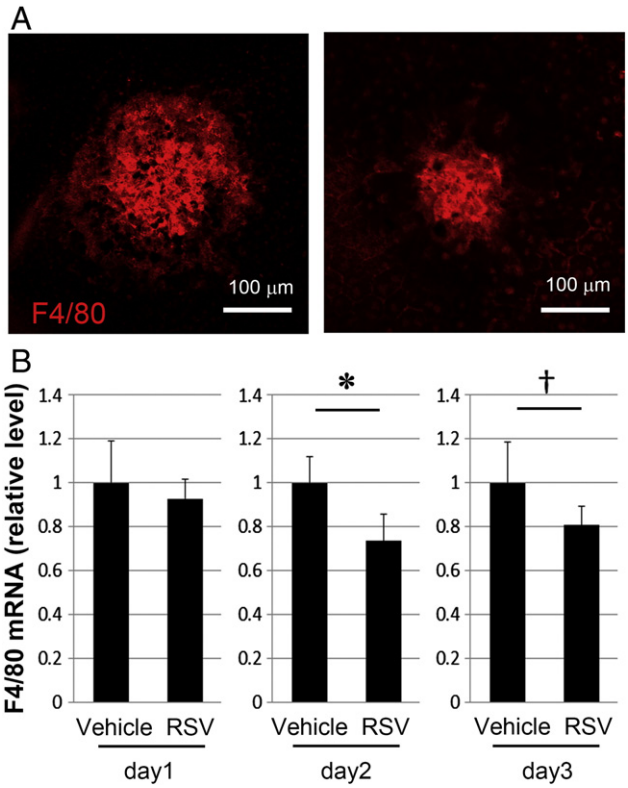

Fig. 2. RSV treatment inhibited macrophage infiltration. (A) The presence of macrophages in the RPE-choroid complexes of mice treated with vehicle or RSV was evaluated by immunohistochemistry for F4/80 3 days after CNV induction. (B) RSV suppressed the mRNA levels of F4/80 in the RPE-choroid at days 2 and 3, while it tended to suppress at day 1. $n=8–10$. *$P<.01$, †$P<.05$.

3.3. RSV treatment reduced the levels of inflammatory and angiogenic molecules

We next measured the protein levels of VEGF, ICAM-1 and MCP-1 in the RPE-choroid complex 3 days after CNV induction by ELISA. The protein levels of all of these inflammatory and angiogenic molecules [32,33] were higher in the RPE-choroid complexes of CNV-induced mice compared with age-matched normal controls. These increases were significantly suppressed by RSV treatment ($P<.01$ for VEGF and $P<.05$ for MCP-1 and ICAM-1, Fig. 3A–C).

3.4. RSV treatment activated AMPK and suppressed NF-κB in the RPE-choroid complex

Because AMPK activation is a known effect of RSV [34], we measured the levels of phosphorylated (activated) AMPK by immunoblot analysis. We found that the levels of activated AMPK were decreased in the RPE-choroid 4 h after CNV induction ($P=.005$). This reduction was prevented by RSV pretreatment ($P=.02$, Fig. 4A).

Knowing that activated AMPK can suppress NF-κB [35], a common upstream modulator of inflammatory and angiogenic molecules, we next measured levels of activated NF-κB p65 in the RPE-choroid by ELISA. The levels of phosphorylated (activated) NF-κB p65 were increased 4 h after CNV induction (Fig. 4B). RSV pretreatment significantly suppressed this change ($P=.003$), indicating that RSV may have suppressed NF-κB activation by increasing the levels of activated AMPK.

3.5. RSV inhibited NF-κB and inflammatory and angiogenic molecules in vitro

To analyze the direct effects of RSV on various cell types that may be involved in the pathogenesis of CNV, we prepared 3 cell lines, ARPE-19 cells (RPE cells), b-End3 cells (microvascular endothelial cells) and RAW264.7 cells (macrophages) (Fig. 5). ELISA analyses revealed that the protein levels of phosphorylated (activated) NF-κB were increased by TNF-α stimulation in all 3 cell lines. However, importantly, RSV treatment significantly decreased the levels of activated NF-κB p65 in all 3 cell lines ($P<.05$, Fig. 5A, D and G). Moreover, these effects were attenuated by an AMPK inhibitor, compound C ($P<.05$, Fig. 5A, D and G), indicating that RSV decreased NF-κB activation via AMPK activation in these 3 cell lines.

Next, the protein levels of inflammatory and angiogenic molecules were measured by ELISA. In the ARPE-19 RPE cell line, TNF-α induced VEGF (Fig. 5B) and MCP-1 (Fig. 5C). Both increases were significantly suppressed by RSV treatment ($P<.001$ for VEGF; $P<.05$ for MCP-1). In the b-End3 microvascular endothelial cell line, RSV application significantly reversed the TNF-α-induced increases in ICAM-1 ($P<.001$, Fig. 5E) and MCP-1 protein levels ($P<.001$, Fig. 5F). Similarly, in RAW264.7 macrophages, RSV treatment significantly ($P<.01$) blocked the TNF-α-induced increase in VEGF protein levels (Fig. 5H). Notably, all of these inhibitory effects of RSV were significantly attenuated by treatment with the AMPK inhibitor compound C ($P<.05$ for all, Fig. 5B, C, E, F and H), indicating that RSV's effects on inflammatory and angiogenic molecules were mediated by AMPK activation and the subsequent decrease in activated NF-κB.

3.6. RSV suppressed macrophage migration in vitro

To analyze the direct effects of RSV on macrophage infiltration, scratch and transmigration assays were performed. RSV significantly

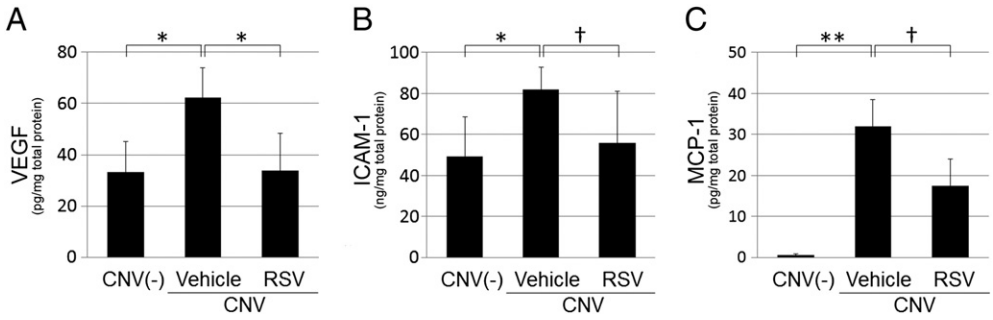

Fig. 3. RSV treatment reduced the levels of inflammatory and angiogenic molecules.Protein levels of VEGF (A), ICAM-1 (B) and MCP-1 (C) were increased 3 days after CNV induction. RSV pretreatment significantly suppressed these increases. $n=8$. $^{**}P<.001$, $^{*}P<.01$, $\dagger P<.05$.

inhibited both the invasion (Fig. 6A and B) and transmigration (Fig. 6C) of RAW264.7 macrophages. However, these effects were significantly suppressed by treatment with the AMPK inhibitor compound C. Thus, RSV suppressed macrophage migration through activation of AMPK.

4. Discussion

In the present work, we show that RSV has anti-pathogenic effects on CNV development (Fig. 1), reducing macrophage infiltration (Fig. 2) and inflammatory and angiogenic cytokines in the RPE-choroid complex (Fig. 3). RSV suppressed the decrease in activated AMPK and the increase in NF-κB in the RPE-choroid after laser PC for CNV induction (Fig. 4). *In vitro* assays confirmed that RSV suppressed NF-κB activation; reduced the levels of inflammatory and angiogenic molecules in RPE cells, vascular endothelial cells and macrophages; and inhibited the migration of macrophages via AMPK activation (Figs. 5 and 6). RSV acted directly on the cells. These data suggest that, under pathological conditions, RSV has multiple effects on 3 different types of cells in the RPE-choroid, i.e., RPE cells, vascular endothelial cells and macrophages, which may coordinately contribute to CNV development (Fig. 7).

The role of VEGF in CNV progression is well accepted [36,37]. Current standard therapy targets VEGF to suppress the progression of CNV that has already developed. However, a previous study that used pharmacological macrophage depletion with liposomal clodronate demonstrated the contribution of macrophages to CNV development: phagocytosis of liposomal clodronate by macrophages rapidly induced apoptosis without proinflammatory cytokine secretion and resulted in CNV suppression [31]. Macrophages can also be anti-angiogenic depending on their condition; the intensity of the CNV development may be relied on a balance between M1 and M2 macrophages [38–40]. The macrophages that we observed during CNV development might have included both types. Whether RSV can regulate the balance may be an interesting research topic in the future. The importance of MCP-1, which recruits macrophages, was demonstrated using MCP-1 knockout mice [8,41]. Another group showed that atorvastatin, an HMG CoA reductase inhibitor that is widely used in patients with atherosclerotic disease and hyperlipidemia, reduces macrophage infiltration and CNV size in a dose-dependent manner, in accord with decreased MCP-1 expression [42]. Moreover, ICAM-1, an adhesion factor for leukocytes that is expressed in vascular endothelial cells and precedes CNV development [10,27,43], also plays a role in CNV development: ICAM-1-deficient mice developed smaller size of CNVs in the laser-induced CNV model [9].

Given these findings, as well as the fact that macrophages are found at the damaged area of Bruch's membrane where CNV invasion can be facilitated [44,45], macrophage targeting therapy might be effective at preventing AMD progression if applied during the very

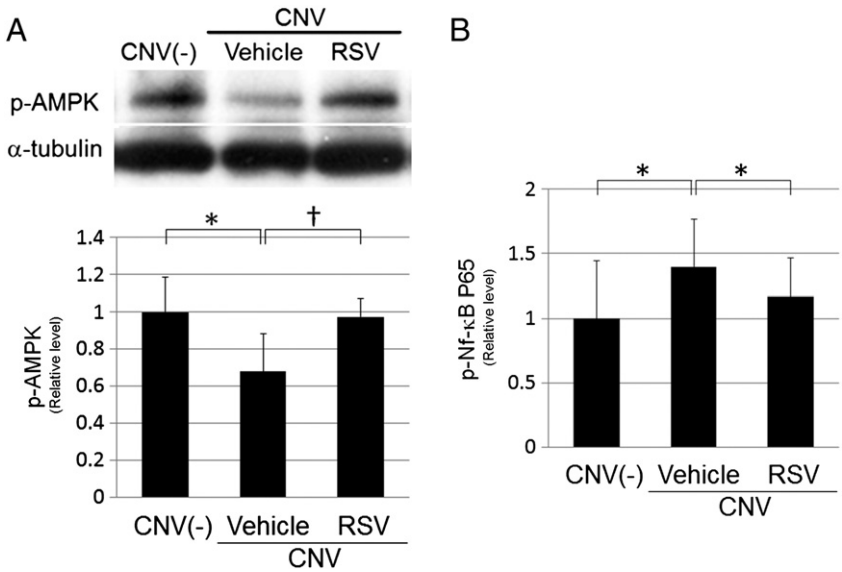

Fig. 4. RSV treatment activated AMPK and suppressed NF-κB in the RPE-choroid complex.Phosphorylated (activated) AMPK was reduced (A), and phosphorylated (activated) NF-κB p65 was induced (B) in the RPE-choroid 4 h after CNV induction. These effects were blocked by RSV. $^{*}P<.01$, $\dagger P<.05$.

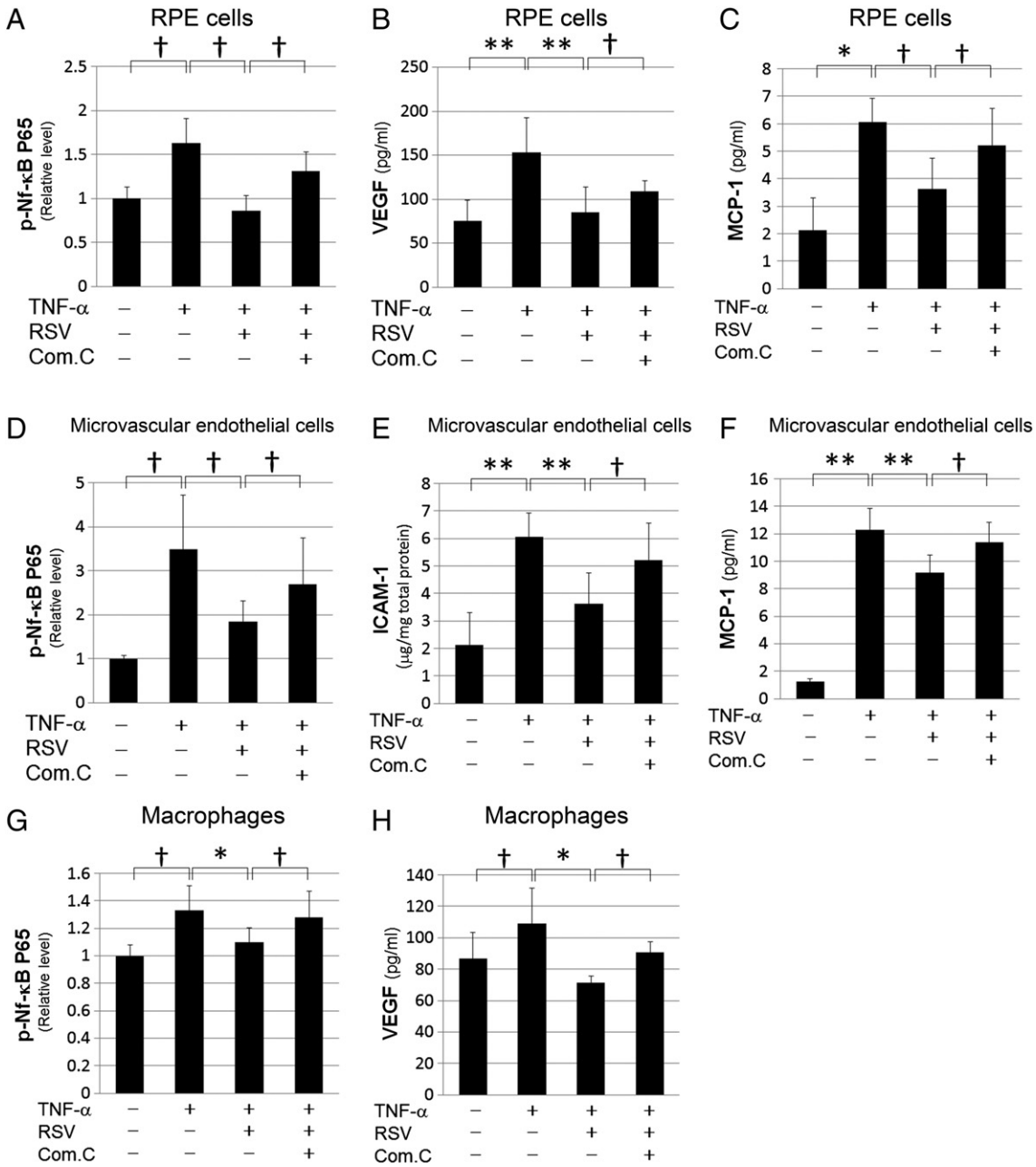

Fig. 5. RSV inhibited NF-κB and inflammatory and angiogenic molecules *in vitro*. ARPE-19 cells (A–C), b-End3 microvascular endothelial cells (D–F) and RAW264.7 macrophages (G and H) were stimulated with TNF-α. RSV significantly suppressed the phosphorylation (activation) of NF-κB by TNF-α (A, D and G) and the subsequent induction of VEGF (B and H), MCP-1 (C and F) and ICAM-1 (E). All of the effects of RSV were attenuated by an AMPK inhibitor, compound C. $n=8$. $^{**}P<.001$, $^{*}P<.01$, $\dagger P<.05$.

early stages of AMD or prior to CNV onset. Our study, showing the inhibitory effects of RSV on macrophage infiltration and cytokine expression will provide a basic therapeutic rationale for the use of RSV in AMD prevention. Both mRNAs of MCP-1 and ICAM-1 were already suppressed by RSV 1 day after CNV induction (Supplementary Fig. 1) and mRNA of F4/80 was also suppressed at day 2, while a significant reduction of VEGF mRNA was observed at day 3 in the whole RPE-choroid. These results suggested that the macrophage inducing activity that subsequently promotes CNV development can be suppressed by RSV from the early phase. Moreover, RSV also acted on RPE and vascular endothelial cells and reduced the production of cytokines related to vascular proliferation and/or macrophage infiltration (VEGF, ICAM-1 and MCP-1), suggesting that RSV might offer a multistep targeting therapy. The facts that RSV can suppress IL-6 [46,47] and IL-1b [48,49] both *in vivo* and *in vitro* further suggest the RSV's multistep targeting in terms of suppressing multiple kinds of inflammatory cytokines. Moreover, RSV could also improve the systemic condition improving insulin resistance [50] and hypertension [25] that could reduce the risk of AMD in human [51], although further studies are required.

We also found that the effects of RSV observed in this study were mediated by AMPK activation. *In vitro* data showed that the actions of RSV on RPE cells, vascular endothelial cells and macrophages were all mediated by AMPK activation. AMPK, in turn, suppressed NF-κB,

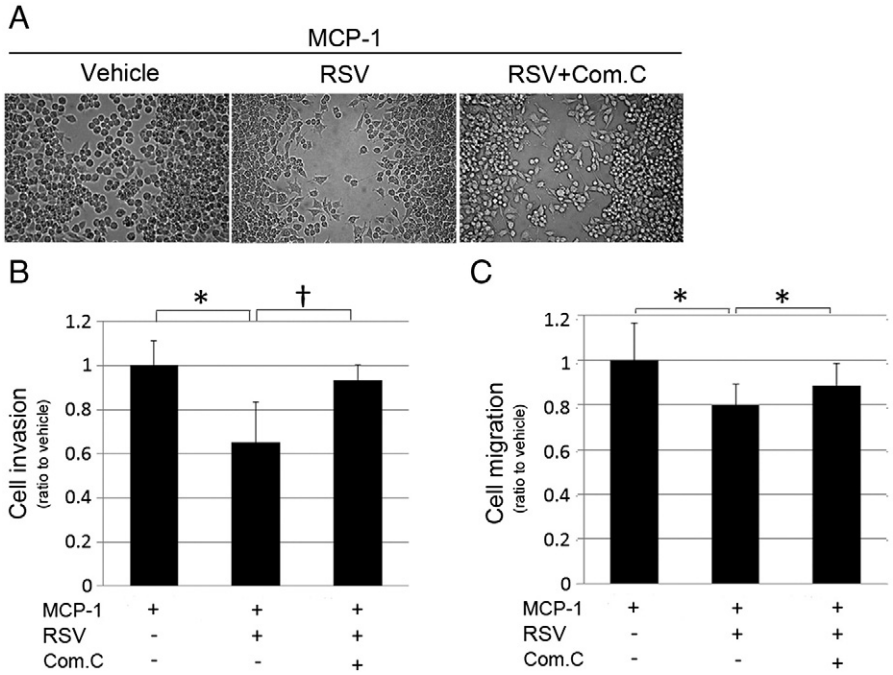

Fig. 6. RSV suppressed macrophage migration *in vitro*.Scratch (A and B) and transmigration assays (C). The effects of RSV were attenuated by an AMPK inhibitor, compound C. $n=8$. *$P<.01$, †$P<.05$.

an upstream regulator of inflammatory and angiogenic cytokines; therefore, the series of events was theoretically consistent. However, there may well be other pathways besides NF-κB that act downstream of AMPK.

A previous study reported an inhibitory effect of RSV on CNV development, specifically related to the proliferation of vascular endothelial cells [52]. RSV-induced activation of AMPK results in the phosphorylation of a serine residue of eukaryotic elongation factor 2 (eEF2) kinase, which inactivates eEF2 through threonine phosphorylation. It was previously proposed that, because eEF2 promotes protein synthesis, its inactivation might inhibit cell division. Another group reported RSV's inhibitory effect on CNV and RSV's *in vitro* effect on cell survival; RSV inhibited acrolein-induced ARPE-19 cell death by increasing the mitochondria biogenesis [53].

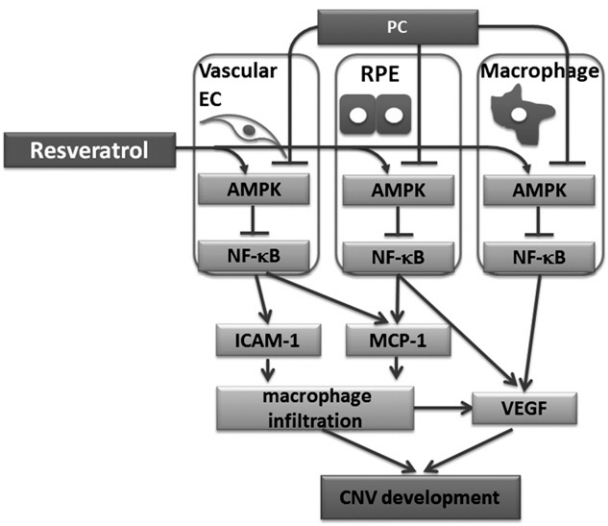

Fig. 7. A model for the preventive effects of RSV on CNV.RSV treatment significantly suppresses CNV development by preserving AMPK activation, which inhibits macrophage recruitment as well as the induction of inflammatory processes, including NF-κB activation and the subsequent up-regulation of inflammatory and angiogenic molecules such as ICAM-1, MCP-1 and VEGF in multiple cell types. PC, laser PC for CNV induction; Vascular EC, vascular endothelial cells.

Whereas the previous studies showed a specific effect of RSV on vascular endothelial cells, or suggested the effect on RPE, the present work expands on this finding to show that RSV inhibits CNV development through multiple additional mechanisms affecting macrophages as well as endothelial cells and RPE.

An anti-inflammatory function of AMPK has also been documented in other tissues and organs. In experimental autoimmune encephalomyelitis [54], acute and chronic colitis [55] and lipopolysaccharide-induced lung injury in mice [56], the induction of inflammatory proteins, such as IL-6 and TNF-α, is attenuated by the artificial activation of AMPK by 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside. In LPS-induced inflammation in rat skeletal muscle and adipose tissue, AMPK inhibits inducible nitric-oxide synthase (iNOS), a proinflammatory mediator of endotoxic shock and chronic inflammatory states, including obesity-linked diabetes [57]; this action occurs through post-transcriptional regulation of the iNOS protein. Therefore, AMPK inhibits inflammation through multiple pathways in several different cell types.

Most current therapies for AMD target VEGF. However, the continuous inhibition of VEGF to suppress CNV may be harmful for the surrounding tissues, as VEGF has important physiological roles in maintaining the choroidal vessels that feed the retina [58] and in supporting the survival of retinal neural cells under pathogenic conditions [59,60]. Moreover, once CNV has developed, the neural retina may be permanently damaged. Thus, a preventive therapy is required. The current study explored the molecular basis of CNV development. Our findings indicate that RSV exerts a therapeutic effect by preserving the level of activated AMPK under pathogenic conditions. Notably, RSV suppressed CNV development by targeting not only VEGF but also multiple other cytokines secreted from several cell types, as well as by inhibiting macrophage migration. Although

further studies are needed, RSV could be potentially applied clinically to prevent CNV development in AMD.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2014.05.015.

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