Doctor of Philosophy

Statins inhibit gliosis of MIO-M1, a Müller glial cell line, induced by TRPV4 activation

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Department of Medicine

Youn Hye Jo

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Supervisor: Kyung Rim Sung

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Youn Hye Jo

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This certifies that the dissertation
of Youn hye Jo is approved

Committee Chair Dr._____Jae Yong Kim_____

Committee Member Dr.___Kyung Rim Sung_____

Committee Member Dr.___Joo Yong Lee_____

Committee Member Dr.___Ho-Seok Sa_____

Committee Member Dr.___Hyewon Chung_____

Department of Medicine
University of Ulsan, Korea
August 2022

Abstract

Purpose: We characterized Müller cell gliosis induced by activation of transient receptor potential vanilloid-type 4 (TRPV4) and assessed whether statins could modulate the gliosis.

Methods: The human Müller cell line, used to analyze gliosis caused by glaucomatous stimulation. To induce Müller gliosis in MIO-M1 cells, GSK101 was used to activate TRPV4, and Müller gliosis was evaluated by analyzing vimentin, nestin, and glial fibrillary acidic protein (GFAP) expression. The expression level of TNF-α was determined by ELISA. To evaluate GSK101 activation of the NF-κB pathway, p65 phosphorylation was measured by western blotting, and nuclear translocation of p65 and IκBα phosphorylation were assessed by immunostaining. To assess the effect of statins on MIO-M1 gliosis and its signaling pathway, cells were pretreated for 24 h with simvastatin, atorvastatin, and lovastatin before GSK101 treatment.

Results: Vimentin, nestin, and GFAP expression was upregulated by GSK101 treatment of MIO-M1 cells, confirming gliosis induction. Statins effectively inhibited gliosis. The expression of the cytokine TNF-α, which can induce RGC apoptosis, was increased by GSK101. To investigate the signaling pathway leading to TNF-α expression, NF-κB activation was determined. Phosphorylation and nuclear translocation of p65 and IκBα phosphorylation, which occurs prior to p65 activation, were induced. Statins suppressed GSK101-mediated phosphorylation of IκBα and p65 and p65 translocation.

Conclusions: Statins can mitigate gliosis human Müller cell line. Because TRPV4 activation in Müller cells reflects glaucoma pathophysiology, statins are potential therapeutic agents to prevent RGC death.

Contents

Introduction

Glaucoma is a progressive neurodegenerative eye disease characterized by irreversible structural damage to retinal ganglion cells (RGCs).[1,2] RGCs play critical roles in the transfer of visual information from the retina to the brain via their axons, i.e., retinal nerve fiber layer (RNFL). In glaucoma, axonal degeneration and apoptotic cell death of RGCs ultimately lead to irreversible vision loss. While lowering intraocular pressure (IOP) indirectly protects RGCs, there is no proven glaucoma treatment that directly targets RGCs. Therefore, understanding the mechanism of RGC dysfunction is important for the development of new glaucoma treatment strategies.

RGCs receive physiological and pathological support from glial cells, such as astrocytes and Müller cells. Healthy Müller cells are specialized radial glial cells that span the entire retina and provide metabolic support. However, changes in Müller cells are involved in almost all types of retinal degeneration, such as age-related macular degeneration, diabetic retinopathy, and glaucoma, and normal retinal function.[3,4] When surrounding tissues are damaged by increased IOP or inflammation, Müller cells induce neuronal degeneration and edema by gliotic alteration and may form glial scars, which interfere with repair and regeneration of retinal tissues.[3,5] In addition, Müller gliosis is induced by glaucomatous stimulation and implicated in RGC apoptosis. Tumor necrosis factor-alpha (TNF-α), a detrimental cytokine, is mainly synthesized and released by activated Müller glial cells and induces RGC apoptosis.[6-11] Thus, it can be postulated that suppression of Müller gliosis is neuroprotective. Studies of Müller cell function *in vitro* are challenging because the cells tend to rapidly differentiate in culture, making it difficult to isolate pure cell populations. In 2002, MIO-M1 was reported as the first spontaneously immortalized human Müller cell line.[12] Thereafter, MIO-M1 cell lines were used in several *in vivo* studies[13,14] for evaluating Müller cells. We also used MIO-M1 cells in this study.

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Because the transient receptor potential vanilloid-type 4 (TRPV4) channel contributed to the resting membrane potential of Müller cells, its specific agonist, GSK1016790A (GSK101), was used to activate gliosis.[15,16] Then vimentin,[17] nestin,[18] and glial fibrillary acidic protein (GFAP)[19,20] were used to verify induction of Müller gliosis. Because GSK101 is a candidate for inducing gliosis through TNF-α, we assayed TNF-α levels in response to GSK101 and monitored how they changed after statin treatment.

NF-κB (p50/p65), an inducible nuclear transcription factor, regulates inflammatory factors, including TNF-α.[21,22] NF-κB is inactive when bound to IκB (inhibitor κB). When NF-κB is released from IκB, it is free to translocate to the nucleus, where it acts as a transcription factor. Thus, p65 and phospho-p65 were assayed in the nucleus and cytosol, respectively, after GSK101 and statin treatments. IκB kinase (IKK) complex phosphorylation occurs in response to upstream signals after activation of the IκB kinase complex (IκBα/IκBβ/IκBγ). Active IKK induces IκB degradation, and NF-κB is released. Thus, we analyzed the concentration of phospho-IκBα by immunostaining after GSK101 and statin pretreatments.

Statins, widely used as hyperlipidemia treatments, not only regulate cholesterol biosynthesis but also exert pleiotropic effects, such as fibrosis, inflammation, and immune response control.[23-25] Statins' neuroprotective effects have been reported in many central nervous system (CNS) diseases, such as ischemic stroke, Alzheimer's disease, and multiple sclerosis.[26] Therefore, we expected statins would have beneficial effects on the optic nerve and RNFL. In fact, several previous studies have indicated an association between glaucoma and statins.[27-34] As clinical evidence about the protective effect of statins on glaucoma development and progression has increased, *in vitro* studies have also been published. The secreted protein, acidic and rich in cysteine (SPARC) and extracellular matrix (ECM) protein have emerged as major modulators of IOP through changing the ouflow facility, and lovastatin inhibits the expression of TGF-β2-mediated SPARC in human trabecular meshwork cells.[35] In addition, we reported

that statins exert antifibrotic effects by inhibiting the expression of TGF-β2-mediated ECM molecules in astrocytes of the human optic nerve head (ONH).[36,37] However, no research about the direct effects of statins on Müller glial cells as protection for dysfunctional RGCs has been conducted.

In this paper, we assessed whether Müller gliosis could be induced by activation of TRPV4, a mechanosensor that may response to increased IOP. To do this, we evaluated the expression of gliosis markers, TNF-α expression, and activation of the NF-κB pathway. Then we determined whether statins could modulate TRPV4-mediated Müller gliosis.

Methods

Cell culture

  MIO-M1, a human Müller glial cell line, was grown in GlutaMAX^{TM} DMEM medium supplemented with 50 U/mL penicillin, 50 g/mL streptomycin (Invitrogen-Gibco-Life Science Technology, Karlsruhe, Germany) and 10% fetal bovine serum (FBS; Invitrogen-Gibco-Life Science Technology).[38] MIO-M1 cells were maintained at 37 , 5% CO_2 in a humidified incubator until the cells reached 80% confluency. The cells were detached by treatment with TrypLE (Invitrogen-Gibco-Life Science Technology), and the resulting cell suspension was protected with a neutral trypsin solution (Invitrogen-Gibco-Life Science Technology).[39]

Reagents

  For investigation of the NF-κB pathway, IκBα, p65, and phospho-p65 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Vimentin, nestin, and GAPDH antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The Human Quantikine TNF-α ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA). The GFAP antibody and RIPA Cell Lysis buffer were purchased from ThermoFisher Scientific (Fair Lawn, NJ, USA). GSK1016790A (GSK101) was purchased form Sigma–Aldrich (St. Louis, MO, USA).

Immunofluorescent staining of human Müller glial (MIO-M1) cells

MIO-M1 cells were seeded on 12-mm glass coverslips placed in 24-well culture plates in growth medium. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After three washes with phosphate-buffered saline (PBS), the cells were incubated for 1 hour (h) at 37 with primary antibodies (1:50) in PBS with 1% bovine serum albumin (BSA). After washing three times in PBS for 5 min each, the cells were incubated for 1 h at room temperature with secondary antibody (Cy-3, Alexa594) (1:100) in PBS. Gliosis of MIO-M1 cells was confirmed through positive staining for vimentin, nestin, and GFAP. Cells were incubated with 1 µg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen-Gibco-Life Science Technology) to stain the nuclei. The coverslips were mounted with FluorSave (Calbiochem, San Diego, CA, USA), and fluorescent images were captured using an inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and analyzed with Zeiss ZEN imaging software (Carl Zeiss Microscopy GmbH).

Separation of nuclei and cytosol

MIO-M1 cells were seeded in 6-well culture plates. Cells were pretreated with statins followed by 25 nM of GSK101, a TRPV4 agonist. Nuclei and cytosols were separated using the Pierce™ NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, cell lysis was demonstrated using the CER-I and CER-II lysis buffers of the NE-PER kit. The tube containing CER-I was centrifuged for 5 min at ~16,000 x g in a microcentrifuge and the supernatant (cytosol) transferred to a new Eppendorf tube. After suspending the insoluble fraction, ice-cold NER was added, and the cells were incubated for 40 min. After adding

CER-II lysis buffer, centrifugation for 10 min at 16,000 x g, the supernatant (nuclei) was transferred to a new Eppendorf tube, and each sample was analyzed by western blotting.

Western blot analysis

Each cytosolic and nuclear sample was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Cytiva, MA, USA), which were blocked in BSA for 1 h. The blots were incubated with antibodies against phospho-IκBα (p-IκBα), GFAP, and GAPDH (Santa Cruz Biotechnology, Inc.); p65 (Cell Signaling Technology); and phospho-p65 (p-p65; Invitrogen) in blocking solution overnight at 4℃. The membranes were washed with PBST and then incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The membranes were developed using the Enhanced Chemiluminescence (ECL) detection system (Santa Cruz Biotechnology, Inc.). Band densities were quantified using FUJIFILM Science Lab Image Gauge Ver. 4.0 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Quantification of TNF-α

MIO-M1 cells were cultured in 6-well plates and then treated with GSK101 for 24 h or statins for 1 h followed by GSK101. The MIO-M1-cell culture medium was analyzed for TNF-α using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.[40] The absorbance at 450 nm was determined by an automated microplate reader (Vmax; Molecular Devices, Palo Alto, CA, USA), and the results were analyzed using Prism 6.05 (GraphPad Software, Inc., San

Diego, CA, USA).

Statistical analysis

   All values are expressed as means ± standard deviations (SD). The results represent three separate

experiments conducted under the same conditions. Unpaired t tests and one-way ANOVAs were used

for statistical comparison. All statistical analyses were performed using an ANOVA test with Prism 6.05

(GraphPad Software, Inc.). *P<0.05 and **P<0.001 were considered significant.

Results

Evaluation of Müller gliosis in MIO-M1 cells and the effect of statins

  To evaluate Müller gliosis by TRPV4 activation, the expression of the Müller gliosis indicators vimentin, nestin, and GFAP was analyzed by immunostaining. Treatment with the TRPV4 agonist GSK101 upregulated vimentin, nestin, and GFAP expression (Fig. 1A, B, C). Pretreatment with simvastatin, atorvastatin, and lovastatin effectively suppressed the induction of gliosis marker proteins.

Figure 1. GSK treatment induces reactivation of MIO-M1 cells and gliosis, and statins suppress these events. MIO-M1 cells were treated with GSK101 alone for 24 h or pretreated with each statin for 6 h and then followed by GSK101 treatment for 24 h. Then cells were stained for the expression of (A) vimentin, (B) nestin, and (C) GFAP, which are the indicators of gliosis. The nucleus was labeled with DAPI. GSK101 at 25 nM increased vimentin, nestin, and GFAP expression. Statins pretreatment at 10 nM attenuated these expressions. n = 4 for each figure.

A

B

9

C
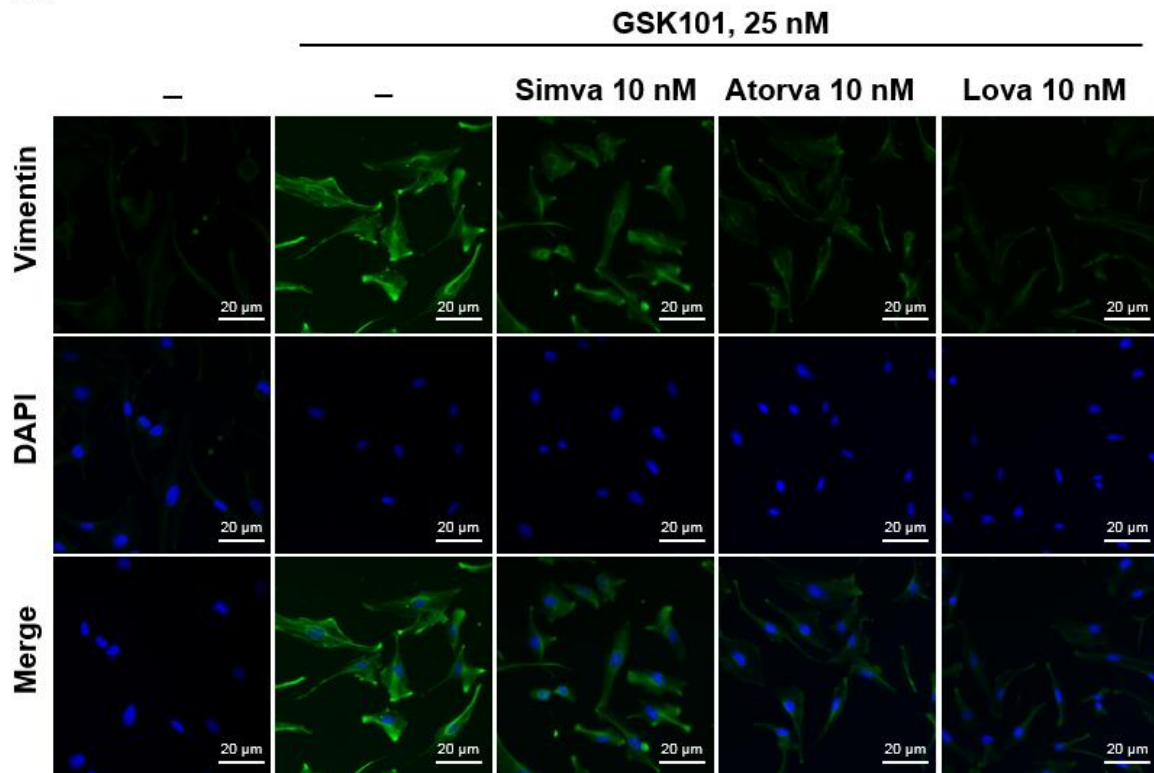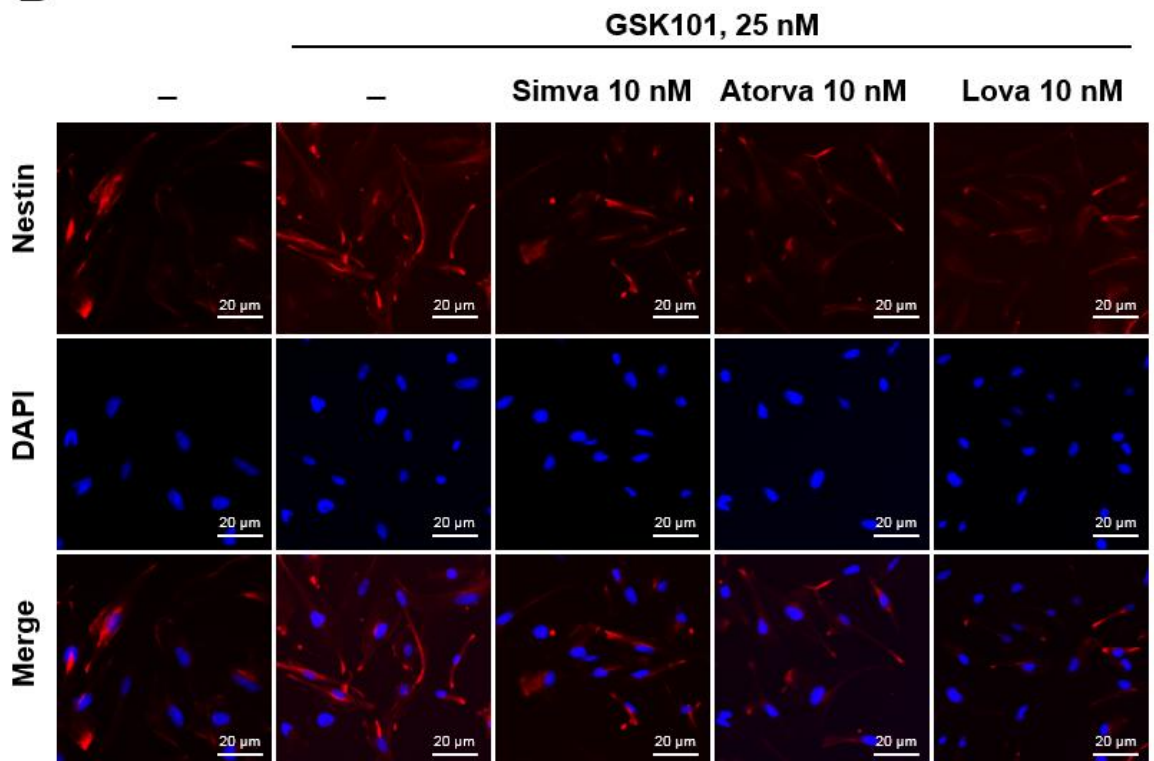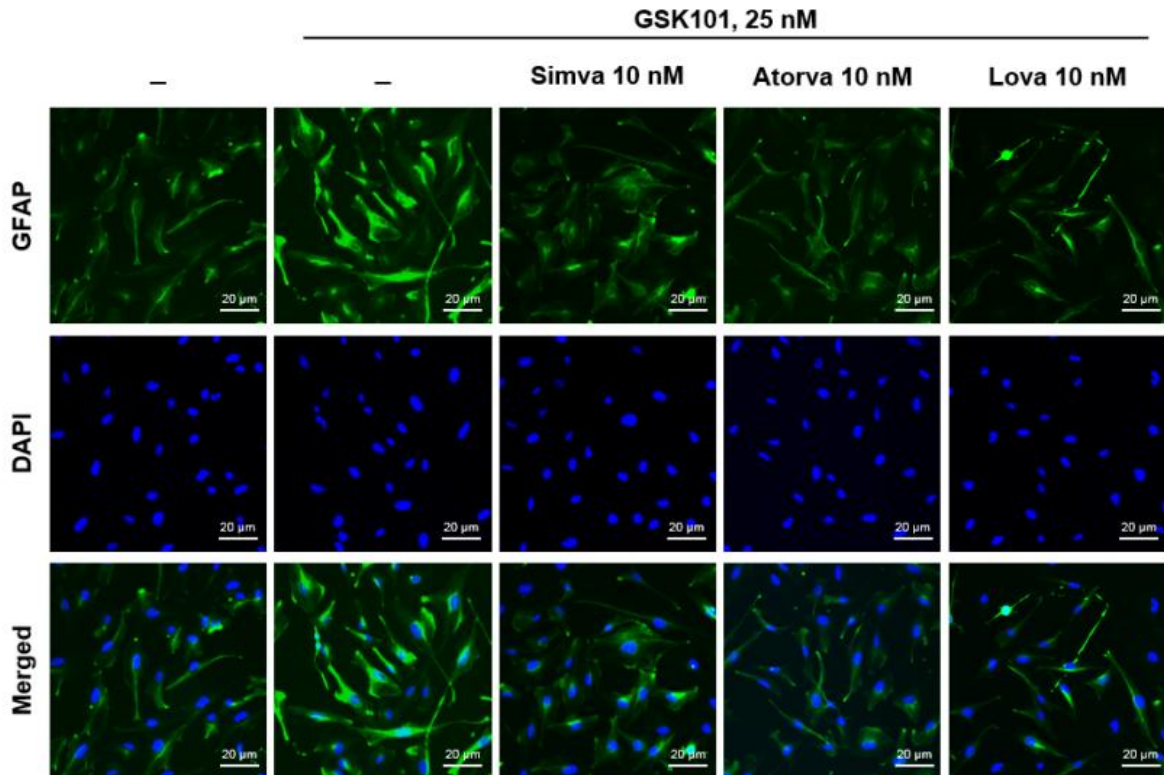
TNF-α as a candidate to induce gliosis and its reduction by statins

  Then we asked how the response of Müller gliosis to TRPV4 activation leads to detrimental effects

on RGC. The most plausible candidate is TNF-α because it can induce RGC apoptosis.[6-11,41]

Therefore, we asked whether TNF-α production increases in GSK101-treated MIO-M1 cells. The

cells were treated with GSK101 concentrations ranging from 1 to 50 nM. TNF-α expression was

significantly induced at GSK101 concentrations ≥10 nM (Fig. 2A). We also tested whether statins

could suppress GSK101-induced TNF-α expression. With 50 nM GSK101, TNF-α production was

induced 14-fold more than with 1 nM GSK101. Pretreatment with statins suppressed TNF-α

production, even at concentrations as low as 1 nM (Fig. 2B, C, D).

Figure 2. GSK101 mediated induction of TNF-α levels were suppressed by pretreatment of statins. MIO-M1 cells were treated either with GSK101 alone for 24 h or pretreated with (B) simvastatin, (C) atorvastatin, and (D) lovastatin for 1 h and then followed by GSK101 treatment for 24 h With 50 nM GSK101, TNF-α production was induced 14-fold than at a 1-nM concentration. Pretreatment with statins suppressed TNF-α production from 1-nM concentrations. Asterisks indicate statistical significance. *P < 0.05, **P < 0.01. n = 4 for each figure.
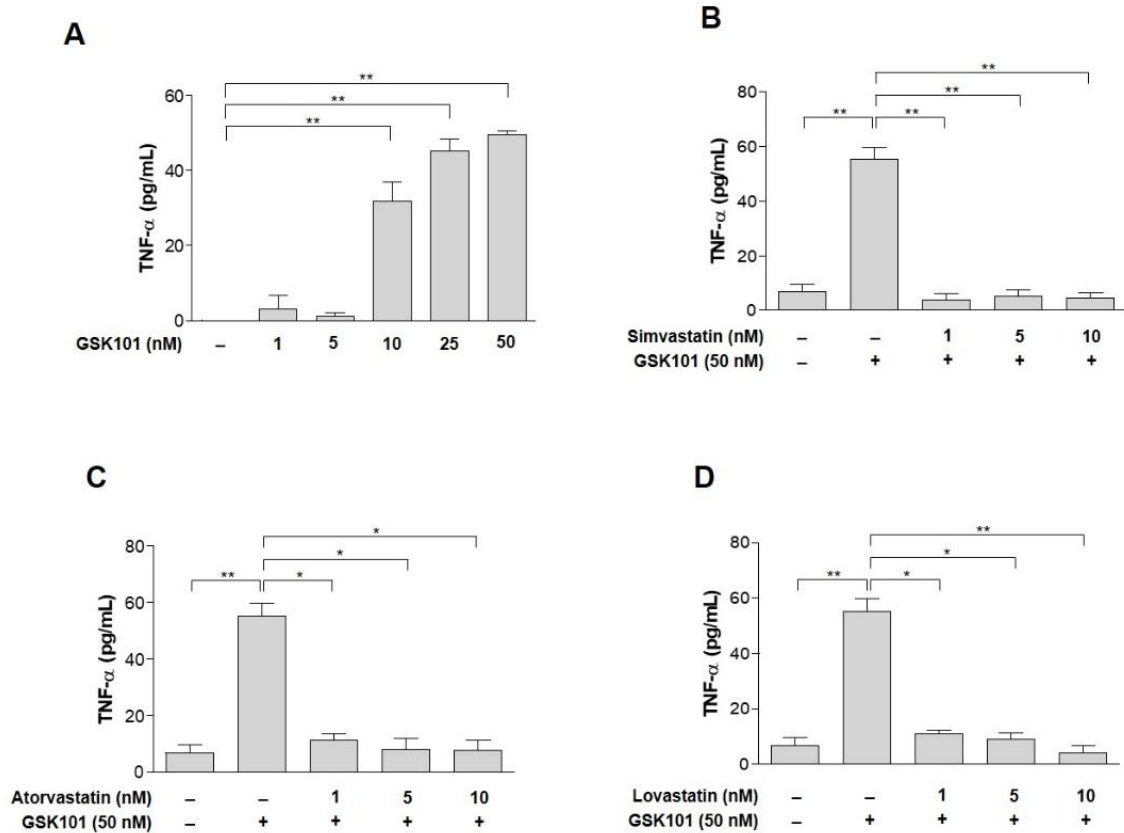
NF-κB pathway in MIO-M1 cells

  Because we confirmed that TRPV4 activation induces NF-κB activation and TNF-α secretion, and statins inhibit these events, we determined which step of the NF-κB pathway was inhibited to suppress TNF-α expression and consequently protect RGCs. NF-κB forms homo- or hetero-dimers with DNA-binding proteins. p65 is a major component of these dimers; when NF-κB is activated, p65 is phosphorylated, and the complex translocates to the nucleus. Therefore, we assayed p65 phosphorylation in cytosolic and nuclear fractions with GAPDH and lamin B1 as respective internal controls. We observed robust induction of p65 phosphorylation in both the cytosolic and nuclear fractions by treatment with GSK101 in a dose-dependent manner (Fig. 3). These data suggest that TNF-α induction by GSK101 is mediated by the NF-κB pathway in MIO-M1 cells.

Figure 3. Phospho-p65 levels were induced by GSK101 in primary Müller cells. MIO-M1 cells were treated with increasing does of GSK101 for 24 h. Cell lysates were fractionated into nucleus and cytosol fractions and then subjected to western blot. From 6 nM GSK101, p65 phosphorylation was significantly induced in both the cytosolic and nuclear fractions in a dose-dependent manner. GAPDH and Lamin B1 were served as the internal controls for cytosol and nucleus fractions, respectively. Asterisks indicate statistical significance. *P < 0.05, **P < 0.01. n = 4.

Cytosol   Nucleus

GSK101 (nM)   −   3   6   12   25   50   −   3   6   12   25   50

p-p65

p65

GAPDH

Lamin B1
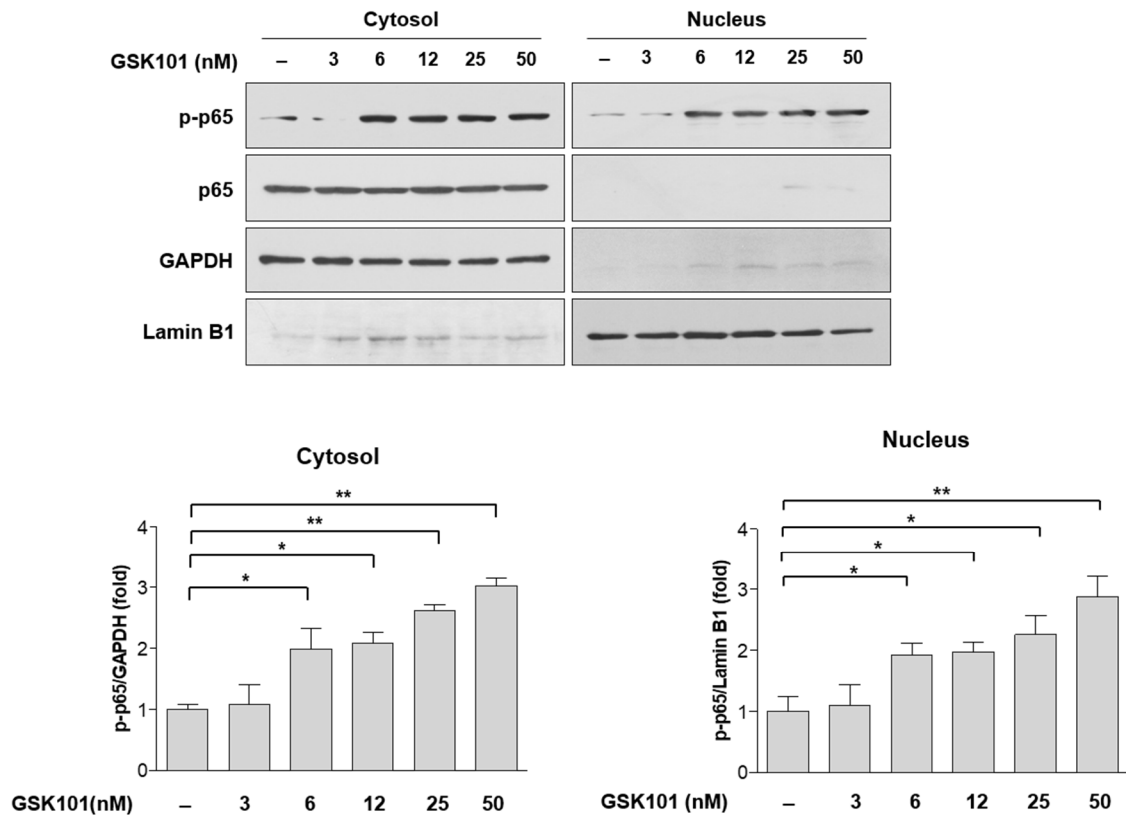
Statins inhibit the NF-κB pathway

Because statins can suppress TNF-α induction, we tested whether they could suppress GSK101-
mediated p65 phosphorylation. Treatment with 5 nM GSK101 consistently induced p65
phosphorylation in cytosolic and nuclear fractions (Fig. 4A–C). Pretreatment with 0.2 nM of any of
the statins significantly decreased p65 phosphorylation in the nucleus, and pretreatment with 0.2 nM
simvastatin, 1 nM atorvastatin, and 10 nM lovastatin decreased p65 phosphorylation in the cytosol
(Figs. 4A–C).

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Figure 4. GKS101 mediated phosphorylation of p65 was attenuated by statins. MIO-M1 cells were treated either with GSK101 alone for 24 h or pretreated with (B) simvastatin, (C) atorvastatin, and (D) lovastatin for 6 h and then followed by GSK101 treatment for 24. The cytosol and nucleus fractions were subjected to western blot. P65 phosphorylation significantly decreased in the nucleus after pretreatment with 0.2 nM of all three statins and decreased in the cytosol after pretreatment with 0.2 nM simvastatin, 1 nM atorvastatin, and 10 nM lovastatin. GAPDH and Lamin B1 were served as the internal controls for cytosol and nucleus fractions, respectively. n = 4 for each figure.

B

	Cytosol						Nucleus					
		GSK101 25 nM							GSK101 25 nM			
Atorvastatin (nM)	–	–	0.2	1	5	10	–	–	0.2	1	5	10

p-p65 (S311)

p65

GAPDH

Lamin B1

15
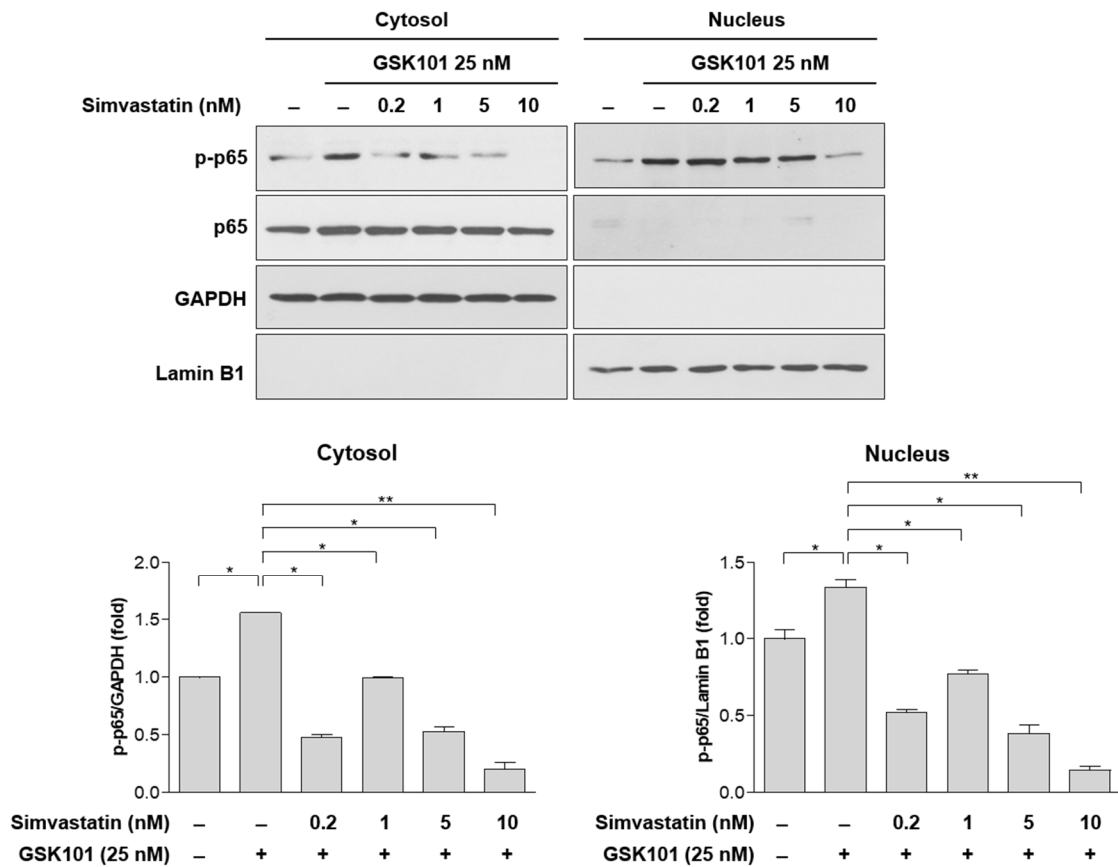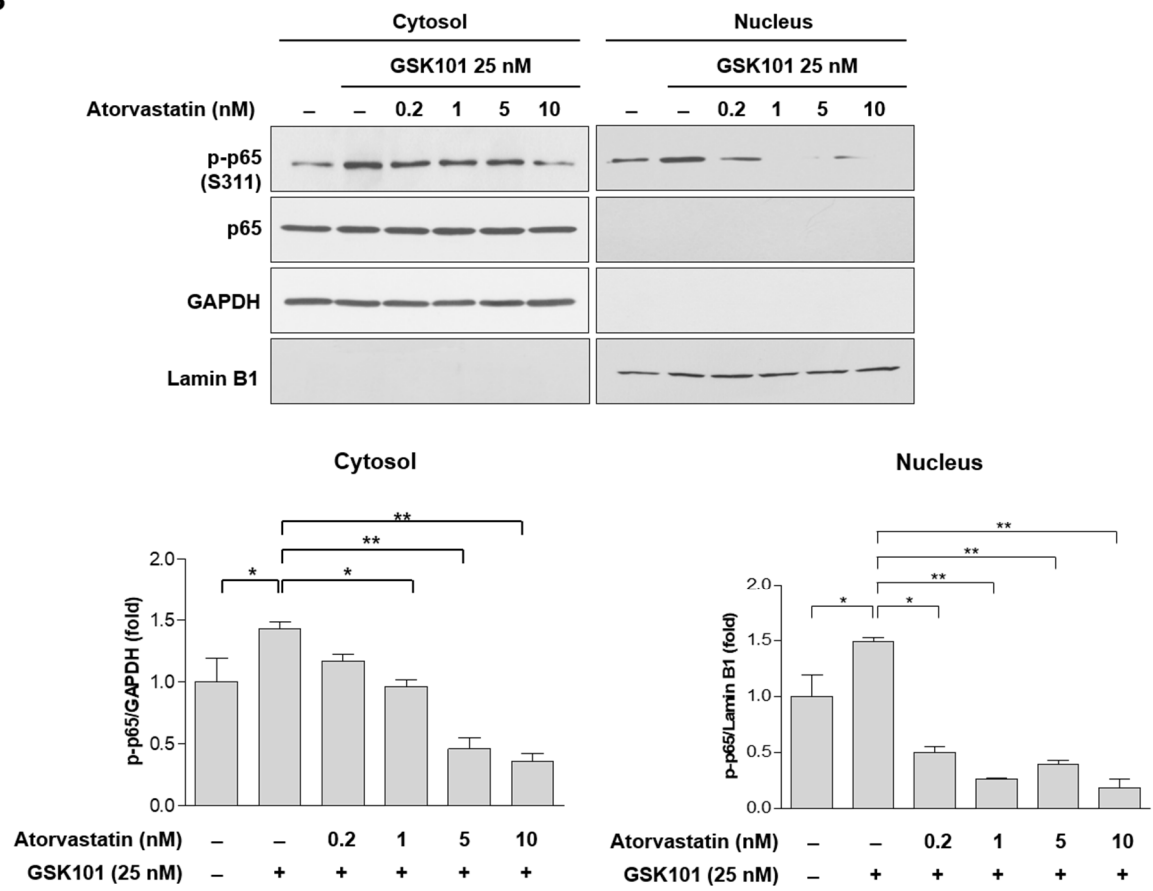
C
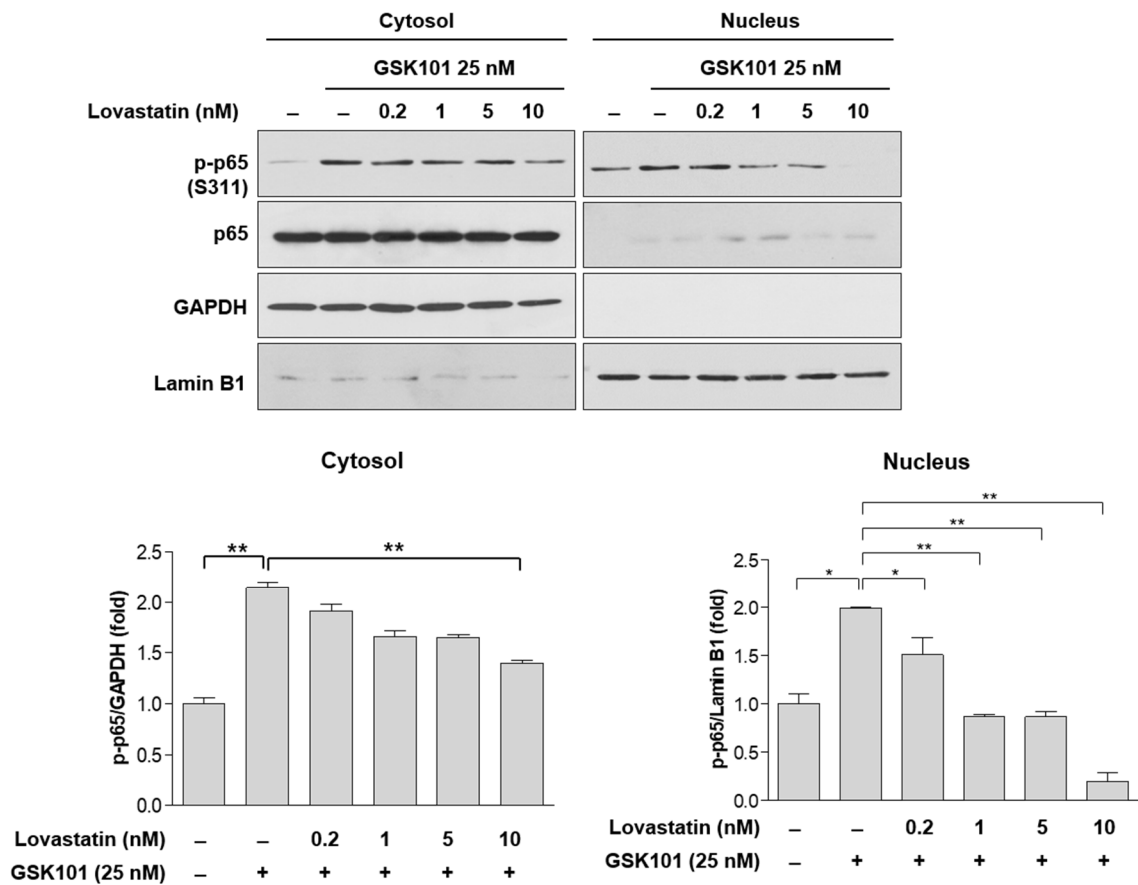
Statins reduce IκBα phosphorylation

To confirm that statins suppressed the NF-κB pathway, we assessed the phosphorylation of the inhibitory protein IκBα by immunocytochemistry. IκBα functions as an inhibitor through interaction with the NF-κB complex. However, IκBα phosphorylation by stimulatory signals promotes IκBα ubiquitination and degradation, which induces NF-κB complex activation. Consistent with Figs. 3 and 4, GSK101 treatment increased the immunoreactivity for phospho-IκBα in the cytoplasm and nucleus. Treatment with statins prior to GSK101 treatment suppressed IκBα phosphorylation. All three statins were potent attenuators of GSK-mediated IκBα phosphorylation upstream of p65 activation in MIO-

M1 cells (Fig. 5). These results, that is, on which part of the NF-κB pathway the statin acts to suppress

the expression of TNF-α is summarized in figure 6.

Figure 5. Statins attenuate GSK-mediated IκBα phosphorylation, which occurs upstream of p65

activation. MIO-M1 cells were treated either with GSK101 alone for 24 h or pretreated with

simvastatin, atorvastatin, or lovastatin for 6 h and then followed by GSK101 treatment for 24 h. Then

cells were fixed and stained for pIκBα. Pretreatment with 10 nM statins prior to GSK101 treatment

suppressed IκBα phosphorylation. All three statins were potent attenuators of GSK-mediated IκBα

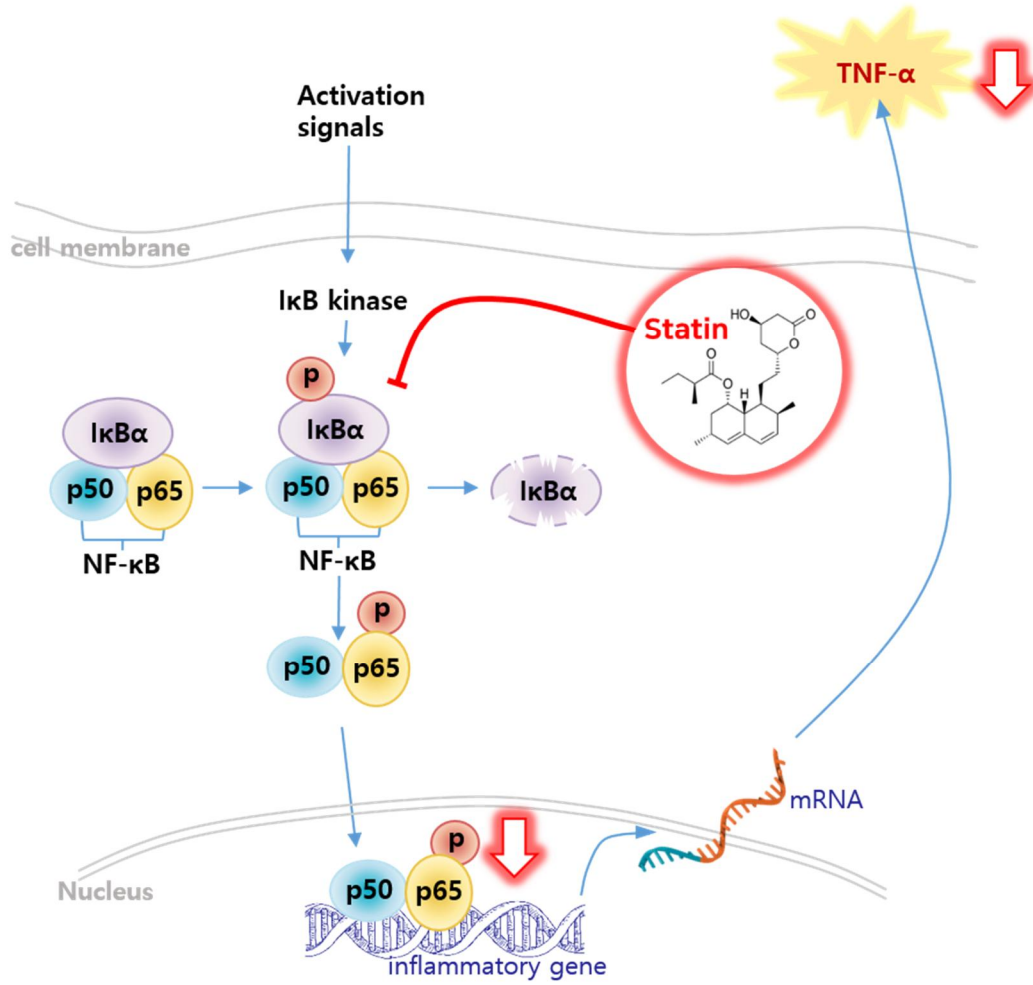phosphorylation upstream of p65 activation in Müller glial MIO-M1 cells. n = 4
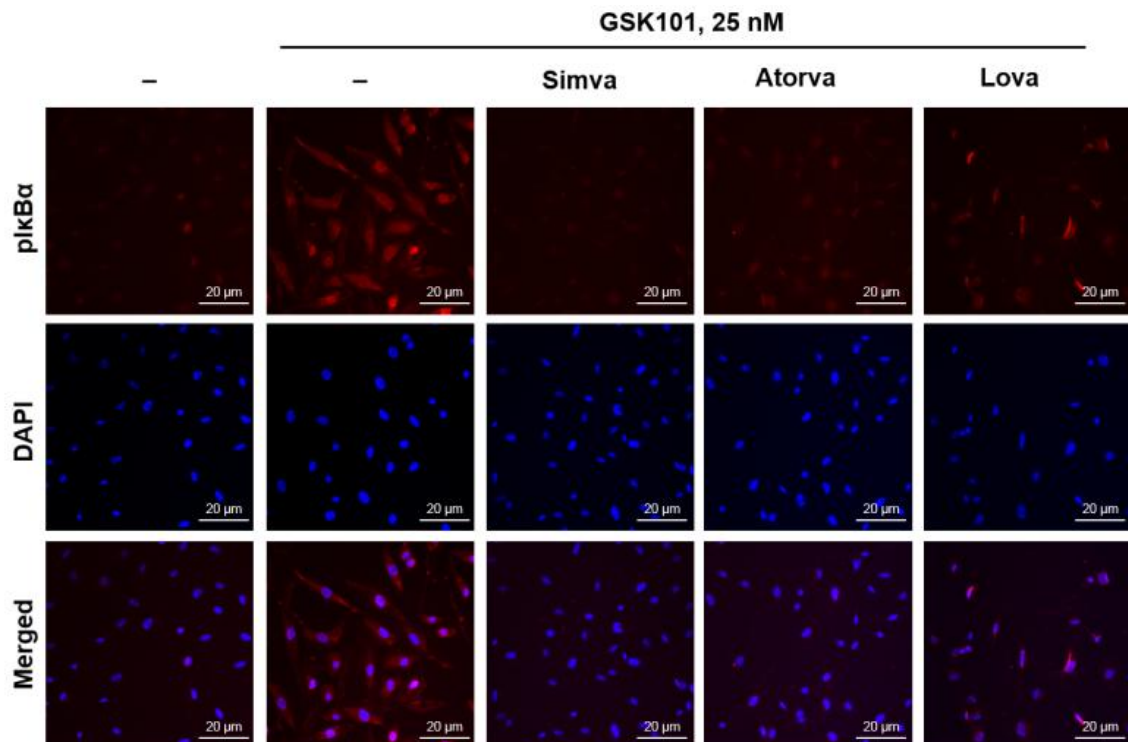
Figure 6. Statins attenuate IκBα phosphorylation, which occurs upstream of p65 activation, and consequently suppresses the expression of TNF-α, which induces RGC apoptosis.

Discussion

The present study aimed to determine whether Muller gliosis is induced by activation of TRPV4, a mechanosensor responding to increased IOP, and whether statins attenuate it. Our study showed that GSK treatment, the agonist of TRPV4, induced reactivation of MIO-M1 cells and gliosis, and statins suppressed these events. GSK101 also induced TNF-α expression, which was suppressed by pretreatment of statins. This study suggests an important aspect of statins' protective effects in reactive Müller gliosis and it is noteworthy in that it revealed that statins have potential as novel glaucoma treatment agents with neuroprotective effects.

Statins are prescribed for hyperlipidemia treatment; they lower blood cholesterol by competitively inhibiting HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway. Since statin is structurally similar to HMG-CoA, the active site of enzyme competes with the native substrate. This competition reduces the rate at which mevalonate is produced, the next molecule to synthesize cholesterol. In addition, inhibition of HMG-CoA by statins hampers downstream synthesis of isoprenoids, which are crucial lipid attachments for intracellular signaling molecules, such as Rho, Rac1, and CDC42.[42,43] Suppression of protein prenylation is thought to be involved in the modulation of immune function, improvement of endothelial function, and other pleiotropic cardiovascular benefits.[42,44,45]

Considerable clinical evidence indicates that statins have beneficial effects on glaucoma patients. De Castro et al.[29] showed that statins may slow the progression of suspected glaucoma as measured by confocal scanning laser ophthalmoscopy. McGwin et al.[28] demonstrated that the prevalence of open angle glaucoma (OAG) was lower in patients who had taken statins for more than 24 months. Also, Leung et al.[46] reported that simvastatin may stabilize visual field progression. In a retrospective

longitudinal cohort analysis, statin use was associated with a significant reduction in OAG risk.[27] A systemic review and meta-analysis indicated that short-term statin use was associated with reduced glaucoma incidence, but the effect of statins on glaucoma progression and IOP was unclear.[47] Furthermore, a large cohort study suggested that statin use significantly reduced OAG risk in hyperlipidemic patients.[27] Our recent study also showed that statins aided RGC survival by inhibiting ECM remodeling of the ONH.[37] As clinical evidence for the protective effect of statins on OAG development and progression has accumulated, *in vitro* studies on astrocytes and trabecular meshwork cells have also been published.[35,37,48] However, this study is the first to elucidate the protective effect of statins against glaucomatous stimuli in Müller glial cells.

Müller glia play crucial roles in RGC survival, and Müller gliosis contributes to glaucoma pathogenesis.[49,50] Müller glia span the entire retina and have neuroprotective, as well as detrimental, effects in response to glaucoma-related neuronal injury via signaling cascades.[4,5,51-55] After retinal insults, Müller glia undergo reactivation, so-called Müller gliosis, characterized by the increased expression of intermediate filaments, such as vimentin, nestin, and GFAP.[51] TRPV4 is a membrane calcium channel activated by osmotic or mechanical pressure-related insults, causing calcium flow into the cell, increasing the intracellular calcium ion concentration.[56,57] TRPV4 induces gliosis and neurodegeneration in the brain[58,59] and is expressed in the retina, specifically in Müller glia. TRPV4 activation appears to be involved in RGC apoptosis. In a study using dissociated RGC cultures, RGC apoptosis and Müller gliosis increased due to TRPV4-agonist induced TRPV4 activation;[60,61] in a study using porcine retinal explants treated with the TRPV4-specific antagonist RN-1743, RGC survival increased.[62] Therefore, the mechanism of inflammation induction by TRPV4 has received attention. We used MIO-M1 cells with GSK101 as a TRPV4 channel agonist: expression of each intermediate filament, vimentin, nestin, and GFAP, increased in MIO-M1 cells treated with 25 nM GSK101 and

decreased by treatment with 10 nM statins (Fig. 1). That is, we found that TRPV4 and statins respectively induces and reduce Müller gliosis of MIO-M1 cells caused by any mechanism.

We hypothesized that TNF-α production in Müller glia is involved in this mechanism because TRPV4 activation increases the secretion of proinflammatory cytokines, including TNF-α, from glia in other cells.[63] Although some studies have shown that increased IOP activates TRPV4,[64,65] it is not well-established that TRPV4 activation in Müller cells is linked with TNF-α production and glaucoma pathology. We found that TNF-α was induced by GSK101 in MIO-M1 cells (Fig. 2). Therefore, this study provides evidence that TRPV4 is closely related to glaucoma pathophysiology through Müller gliosis and TNF-α production.

TNF-α is a well-known proinflammatory cytokine with multiple functions in the immune response, and its expression is induced by transcription factors in the NF-κB pathway (p50–p65 complex).[66-68] It is mainly synthesized and released by activated glial cells and is involved in glaucomatous neurodegeneration; thus, it is considered a therapeutic target for improving neuroprotection.[2,6,8-11,69-71] In animal models, increased IOP, increased TNF-α in Müller glia,[72,73] and Müller gliosis resulted in increased TNF-α expression.[74] TNF-α was also increased in the retinas of glaucoma patients.[75-77] Lebrun-Julien et al.[73,78] reported that intravitreal injection of TNF-α reduces the RGC numbers and causes optic nerve degeneration. Furthermore, TNF-α inhibitors prevent Müller gliosis and retinal ganglion cell loss.[79,80] We found that TNF-α production was increased by TRPV4 activation in MIO-M1 cells and decreased by statins, implying that statins could be therapeutic agents with neuroprotective effects for glaucoma patients, if proven by animal models, which will be our next research project.

We also confirmed that TNF-α induction by GSK101 in MIO-M1 cells was mediated by the NF-κB pathway, which statins inhibited by attenuating IκBα phosphorylation. These findings agree with

previous studies.[71,81,82] Shi et al.[71] demonstrated that excitotoxic damage leads to selective NF-κB activation, which induces TNF-α production in Müller glial cells. Ahn et al.[81] showed that statins had a role in overcoming chemoresistance through modulation of NF-κB in human myeloid leukemia KBM-5 cells. In addition, Tu et al.[82] demonstrated that simvastatins inhibited the NF-κB pathway by suppressing IκBα phosphorylation and degradation in the nuclei of pulposus cells. However, this is the first study to reveal the activation of TNF-α through activated TRPV4 in Müller glia, its inhibition by statins, and the effects statins exert on the NF-κB pathway.

This study has some limitations. First, we used a Müller cell line, MIO-M1, rather than primary Müller cells. Therefore, in our next study, we will check whether the same action occurs in primary Müller cells through primary Müller cell culture or *in vivo* and furthermore in animal experiments. Numerous studies in the field of neuroinflammation have shown the potential of statins,[83,84] but there are also some reports concluded that statins are ineffective.[85,86] Second, although TNF-α induces RGC apoptosis as a proinflammatory cytokine, the present study did not show that RGC apoptosis occurs when TRPV4 is activated. In future experiments, we will need to examine RGC apoptosis *in vivo* or in a Müller–RGC co-culture model. Third, we only examined Müller gliosis caused by TRPV4 activation, but TRPV4 is also expressed in RGCs and bipolar cells and can change with increased IOP.[56] Therefore, the effect of statins on TRPV4 activation in RGCs and bipolar cells should be investigated. Finally, NF-κB pathway activation is one way that TRPV4 signaling occurs. TRPV4 signaling increases intracellular Ca^{2+} concentration, which increases calpain.[87] There is a report that statins inhibit calpain activation;[87] therefore, the effects of TRPV4 activation in Muller or RGCs cannot be limited to the NF-κB pathway. It will be interesting to examine calpain activity on statins.

In conclusion, TRPV4 activation reiterates glaucomatous stimulation by GSK101 in human MIO-

M1 cells, increasing TNF-α expression, and statins inhibit this expression by interfering with IκBα phosphorylation. Numerous studies have shown that TNF-α signaling is involved in glaucomatous neurodegeneration and is thus a possible treatment target to improve neuroprotection.[2,69-71] This understanding of the TNF-α expression pathway caused by glaucoma stimulation in Müller cells, and the reduction of TNF-α expression by statins, suggest statins as potential therapeutic agents for glaucoma treatment. This study has revealed an important aspect of statins' protective effects in reactive Müller gliosis.

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요약

목적: Transient receptor potential vanilloid-type 4 (TRPV4) 활성화로 Müller cell gliosis가 유도되는지 확인하고 statin이 이에 영향을 미치는지 확인하고자 한다.

방법: 녹내장 자극에 의해 유발된 gliosis를 분석하는데 human Müller cell line인, MIO-M1 cell를 사용하였다. Müller gliosis를 유도하기 위해 GSK101을 사용하여 TRPV4를 활성화하였고, Müller gliosis는 vimentin, nestin 및 glial fibrillary acidic protein(GFAP) 발현을 통해 확인하였다. TNF-α의 발현 수준은 ELISA로 측정하였다. NF-κB pathway에서 GSK101 활성화를 평가하기 위해, Western blotting으로 p65의 인산화를 확인하였고, 면역염색으로 p65의 핵 전위 및 IκBα 인산화를 평가하였다. MIO-M1 gliosis 과정에서 Statin의 영향을 평가하기 위해 GSK101 처리 전 simvastatin, atorvastatin, 그리고 lovastatin으로 24시간 동안 전처리하여 실험을 반복하였다.

결과: Vimentin, nestin 및 GFAP 발현은 MIO-M1 cell의 GSK101 처리에 의해 발현이 증가하는 것을 확인하였고, Statin은 이를 효과적으로 억제하였다. Retinal ganglion cell (RGC)의 세포사멸을 유도할 수 있는 사이토카인 TNF-α의 발현은 GSK101에 의해 증가되었다. TNF-α 발현으로 이어지는 신호 전달 경로를 확인하기 위해 NF-κB 활성화를 확인하였고, p65의 인산화, 핵 전위 및 IκBα 인산화가 유도되는 것을 확인하였다. Statin은 GSK101을 매개로 IκBα와 p65의 인산화, p65 핵 전위를 억제하는것을 확인하였다..

결론: 본 연구에서는 MIO-M1 cell에서 TRPV4 활성화로 Müller cell gliosis가 유도되고, Statin이 이를 억제한다는 것을 확인하였다. Müller cell gliosis는 녹내장자극에 의해 유도되고 RGC의 세포사멸을 야기하므로, 이를 억제하는 Statin은 녹내장에서 잠재적 치료제로 사용될 수 있을 것이다.