

Apolipoprotein E-Containing Lipoproteins and LRP1 Protect From NMDA-Induced Excitotoxicity Associated With Reducing α 2-Macroglobulin in Müller Glia

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PURPOSE. Optic nerve damage leads to impairment of visual functions. We previously demonstrated that apolipoprotein E-containing lipoproteins (E-LPs) protect retinal ganglion cells (RGCs) from degeneration in a glaucoma model of glutamate/aspartate transporter-deficient mice. This study aimed to determine whether E-LPs protect RGCs from *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity, and to investigate the details of an indirect neuroprotective mechanism of E-LPs by reducing α 2-macroglobulin, which interferes with the neuroprotective effect of E-LPs, in Müller glia.

METHODS. Excitotoxicity was caused by intravitreal injection of NMDA, and then retinae were subjected to immunoblotting or quantitative reverse transcription-PCR. Primary cultures of mouse mixed retinal cells and mouse Müller glia were used for evaluating the effects of E-LPs on the expression of α 2-macroglobulin.

RESULTS. Intravitreal injection of E-LPs protected the optic nerve from degeneration and attenuated the increase in α 2-macroglobulin in aqueous humor and retina of rats. E-LPs directly decreased the expression and secretion of α 2-macroglobulin in primary cultures of Müller glia; this decrease in production of α 2-macroglobulin was blocked by knock-down of the low-density lipoprotein receptor-related protein 1 (LRP1) with small interfering RNA. E-LPs promoted the phosphorylation of STAT3, whereas Stattic, an inhibitor of STAT3, restored the expression of α 2-macroglobulin decreased by E-LPs.

CONCLUSIONS. In addition to our previous findings of the protection of RGCs by E-LPs, the new observations in Müller glia indicate that a reduction of the intraocular α 2-macroglobulin, regulated by the E-LP-LRP1-STAT3 pathway, might be an additional protective mechanism against excitotoxicity in the retina.

Keywords: low density lipoprotein receptor-related protein 1, α 2-macroglobulin, apolipoprotein E-containing lipoprotein, neuroprotection, glaucoma

Degeneration of retinal ganglion cells (RGCs), followed by optic nerve damage, is the major characteristics of glaucoma. Loss of RGCs directly leads to deficits of visual functions. Elevation of intraocular pressure is a modifiable primary risk factor for development of primary open-angle glaucoma.¹ However, a subset of patients with glaucoma suffers from normal tension glaucoma, a type of primary open-angle glaucoma with normal intraocular pressure (10–21 mm Hg).² Although the underlying cause of normal tension glaucoma has not been clarified, multiple factors, such as genetic variation, reduction of blood flow, deficits of growth factor supply, and oxidative stress, have been suggested to be associated with development of this disorder.^{2–4} A clinical treatment for normal tension glaucoma is commonly controlling intraocular pressure, although the efficacy of this treatment is often limited and unsatisfac-

tory.^{5,6} Thus, an improved therapeutic treatment for normal tension glaucoma by neuroprotection is desired.

An increase in the expression and secretion of apolipoprotein (apo) E-containing lipoproteins (E-LPs) in glia has been reported in response to nerve damage⁷ resulting from cerebral ischemia, traumatic brain injury, neurodegenerative disorders,⁸ and optic nerve injury.⁹ Apo E deficiency in mice increases neurodegeneration in the brain during aging¹⁰ and exacerbates ischemic insult in stroke models.^{11,12} Based on these findings, E-LPs secreted from glia have been proposed to support and protect neurons from damage.^{13,14} We previously demonstrated that E-LPs protect RGCs from neurodegeneration induced by trophic factor withdrawal in vitro,^{15,16} and from glutamate excitotoxicity in vitro and in vivo.¹⁷ The protective signals initiated by E-LPs in RGCs were mediated via the low density lipoprotein

receptor-related protein 1 (LRP1), a member of the low density lipoprotein receptor family. However, a neuroprotective role for E-LPs associated with retinal glia has not been demonstrated.

α 2-Macroglobulin, a major plasma glycoprotein, is known to inhibit a broad-spectrum of proteases and also to be one of ligands for LRP1.¹⁸ For example, the amount of α 2-macroglobulin in aqueous humor and retinae is upregulated in patients with glaucoma and in animal models of glaucoma.^{19,20} In addition, exogenous administration of α 2-macroglobulin to healthy eyes of rats induces RGC death.¹⁹ Although, in our previous study, recombinant α 2-macroglobulin itself did not induce RGC death, this glycoprotein impaired the neuroprotective effect of E-LPs in primary cultured RGCs.¹⁷ In combination, these observations support the idea that α 2-macroglobulin is associated with degeneration of RGCs, but the mechanism by which expression of α 2-macroglobulin is regulated in retinal glia has not been elucidated.

We now demonstrate that intravitreal administration of E-LPs protects RGCs from *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity. Our data also show that this excitotoxicity increases the levels of α 2-macroglobulin in aqueous humor of this excitotoxicity model, and that E-LP injection into vitreous humor attenuates the increase in α 2-macroglobulin. Furthermore, E-LPs decreased the expression and secretion of α 2-macroglobulin in primary mixed cultures of retinal cells and in Müller glia via LRP1. Moreover, STAT3 was shown to play a key role in the regulation of α 2-macroglobulin expression downstream of LRP1 in primary cultured Müller glia. Thus, in addition to the direct protection mechanisms in RGCs by E-LPs that were shown in our previous studies, data from the current studies provide the basis of an indirect neuroprotective mechanism in which α 2-macroglobulin expressed from Müller glia is reduced by E-LPs.

METHODS

Animals

Male Sprague Dawley rats (Japan SLC, Shizuoka, Japan), 7 weeks old weighing between 200 and 220 g, were used as an animal model of retinal excitotoxicity. In addition, C57BL/6J mice (2–4 days old) were used as a source of primary cultures of mixed retinal cells and Müller glia. The animals were maintained under controlled conditions at $23 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with a light cycle of 12 hours light/12 hours darkness. All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental procedures were approved by the Animal Care Committee of Tokyo University of Pharmacy and Life Sciences (approval numbers are P19-23 and P20-05).

Primary Culture of Mouse Mixed Retinal Cells

Retinal cells were isolated from retinae of male and female C57BL/6J mice (2–4 days old). Retinal tissues were collected and incubated with 0.25% trypsin (FujiFilm Wako, Osaka, Japan), in phosphate buffered saline (PBS) for 45 minutes at 37°C . The tissues were triturated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum. Retinal cells were plated in 96-well plates (Falcon, Corning, NY, USA) at a density of

5×10^5 cells/well. The mixed retinal cells were cultured for 7 to 10 days prior to use. These cells were washed twice with 100 μl /well Hank's balanced salt solution (HBSS; Invitrogen) containing 2.4 mM CaCl_2 and 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) without magnesium (because Mg^{++} blocks the activity of the NMDA receptor). Cells were incubated for 15 minutes at 37°C between each wash. The cells were then incubated with or without 300 μM NMDA and 10 μM glycine (a co-activator of the NMDA receptor) in HBSS for 15 minutes at 37°C . After the treatment, the mixed retinal cells were cultured in DMEM with or without E-LPs for 24 hours at 37°C .

Primary Culture of Mouse Müller Glia

Müller glia were isolated from retinae of male and female C57BL/6J mice (2 to 4 days old). Retinae were digested with 0.25% trypsin in PBS for 45 minutes at 37°C , then triturated in DMEM containing 10% fetal bovine serum. Cells were cultured in 6-well plates (Falcon) at a density of 1×10^7 cells/well in DMEM containing 10% fetal bovine serum. After 7 to 9 days, cells were harvested from the 6-well plates by treatment with 0.25% trypsin and replated to 96-well plates (Falcon) at a density of 2×10^4 cells/well. These cells were highly enriched in Müller glia according to immunocytochemical staining with antibodies raised against markers of Müller glia: glutamine synthetase (MAB302; Millipore, Burlington, MA, USA) and vimentin (5741; Cell Signaling Technology, Danvers, MA, USA). Primary Müller glia were cultured for at least 7 days before the experiments. For administration of E-LPs and/or Stattic, an inhibitor of STAT3 (Cayman Chemical, Ann Arbor, MI, USA), Müller glia were washed 3 times (5-minute incubation for each wash) with 50 μl /well DMEM, then incubated in DMEM with or without E-LPs and/or Stattic as indicated.

Immunoblotting

Immunoblotting was performed as described previously.^{17,21} Briefly, primary mixed retinal cells or Müller glia were harvested on ice in 30 μl /well sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β -mercaptoethanol. Aqueous humor (5 μl /eye) or conditioned medium (30 μl /well) was mixed with an equal volume of twice-concentrated sample buffer. For retinal samples, rats were euthanized by suffocation with carbon dioxide (CO_2), and then the eyes were immediately enucleated. Each retina was removed from the sclerae, added to 250 μl lysis buffer (1% Triton X-100; [MP Biomedicals, Santa Ana, CA, USA] containing 0.1% sodium deoxycholate [Fuji-Film Wako, Osaka, Japan], 1% EDTA [Dojindo, Kumamoto, Japan], and complete protease inhibitor cocktail [Roche, Basel, Switzerland] PhosStop phosphatase inhibitor cocktail [Roche], in 50 mM Tris-buffered saline). The sample was then sonicated by Ultrasonic Liquid Processor Q125 (QSON-ICA) for 20 seconds (2-second sonication \times 10 times) at 4°C . The protein concentration of retinal sample was measured by a BCA protein assay kit (Thermo Fisher Scientific). The retinal sample adjusted for protein concentration was mixed with an equal volume of twice-concentrated sample buffer. Protein samples were heated at 95°C for 5 minutes and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with primary antibodies, and then peroxidase-conjugated secondary antibodies.

Immunoreactive proteins were visualized with ImmunoStar Basic or Zeta (FujiFilm Wako). The following primary antibodies were used: mouse anti- β -actin (a5441; Sigma), goat anti-albumin (A90-134A; BETHYL Laboratories, Montgomery, TX, USA), rabbit anti- α 2-macroglobulin (sc-8517; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human apo E (k74190g; Biodesign, Saco, ME, USA), goat anti-Brn-3a (sc-31984; Santa Cruz), rabbit anti-LRP1 (2703-1; Epitomics, Burlingame, CA, USA), goat anti-apo J (600-101-198, Rockland Antibodies & Assays, Gilbertsville, PA, USA), rabbit anti-cleaved caspase 3 (9661; Cell Signaling Technology), anti-phospho-ERK/ERK (4370/4696; Cell Signaling Technology), anti-phospho-Akt/Akt, rabbit anti-phospho-GSK3 β /GSK3 β (5558/9832; Cell Signaling Technology), anti-phospho-CREB/CREB (9198/9104; Cell Signaling Technology), anti-phospho-JNK/JNK (9255/9252; Cell Signaling Technology), anti-phospho-p38/p38 (4511/8690; Cell Signaling Technology), and anti-phospho-STAT3/STAT3 (9145/9139; Cell Signaling Technology). Quantification was performed by Chemiluminescence Imaging System (Lumigraph II; ATTO Co., Tokyo, Japan) and an image analyzer (CS Analyzer; ATTO Co.).

Preparation of Reconstituted Apolipoprotein E-Containing Lipoproteins

Reconstituted E-LPs that contained 1-palmitoyl-2-oleoyl-glycerophosphocholine (POPC; Sigma) and recombinant human apo E3 (FujiFilm Wako) at a molar ratio of 100:1 were prepared as described previously.^{15,17} Briefly, 2.71 mg POPC was dissolved in chloroform then the chloroform was evaporated under nitrogen gas for 30 minutes. Tris-buffered saline (pH 7.4, 400 μ l) was added and the mixture was incubated for 1 hour on ice with mixing every 15 minutes, after which 100 μ l of 15 mg/mL sodium cholate was added to the POPC suspension. The mixture was incubated for 2 hours on ice with mixing every 15 minutes, after which 1 mg recombinant apo E3 was added and the samples were incubated for 1 hour on ice. Bio-Beads (100 mg; Bio-Rad, Hercules, CA, USA) for removal of detergent were added to the mixture, which was then rotated for 3 hours at 4°C and filtered through a 0.45 μ m filter (Millipore) to remove the beads. The reconstituted lipoproteins were subjected to ultracentrifugation in a P40ST rotor (Hitachi, Tokyo, Japan) at 160,000 \times g for 48 hours at 4°C on a discontinuous sucrose gradient consisting of: 2 mL of density 1.30 g/mL, 3 mL of density 1.2 g/mL, 3 mL of density 1.1 g/mL, and 3 mL of density 1.006 g/mL. Fractions were collected from the top of the gradient and immunoblotted for apo E, as described below. The apo E-containing fractions were concentrated using an Amicon Ultra filter, 50 kDa molecular weight cutoff (UFC905008; Millipore). The protein concentration of the lipoproteins was measured by a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Immunocytochemistry

Cells isolated from mouse retina were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 minutes, then permeabilized with 0.2% Triton X-100 in PBS for 15 minutes. The cells were then incubated with 10% goat serum, 1% BSA, and 0.2% Triton X-100 in PBS for 1 hour. Subsequently, the cells were incubated for 1 h at room temperature with mouse anti-glutamine synthetase (dilution 1:100, MAB302;

Millipore), rabbit anti-vimentin (dilution 1:100, 5741; Cell Signaling Technology), or mouse anti- β III tubulin (dilution 1:500, ab78078; Abcam, Cambridge, UK) in PBS containing 10% goat serum, 1% BSA, and 0.2% Triton X-100. The cells were washed 3 times with PBS, then incubated with Alexa Fluor 488- or 594-conjugated goat anti-rabbit IgG (dilution 1:500; Invitrogen) and Alexa Fluor 594- or 488-conjugated goat anti-mouse IgG (dilution 1:500; Invitrogen) for 1 hour at room temperature. Subsequently, the Müller glia or retinal cells were incubated with Hoechst 33342 (1 μ g/mL, Dojindo, Kumamoto, Japan) for 15 minutes at room temperature, washed 3 times with PBS, then mounted with Fluoromount/plus (Diagnostic BioSystems, Pleasanton, CA, USA). Fluorescence images were acquired by an Olympus IX71 microscope (Tokyo, Japan). The types of cells in the samples were assessed with the Multi-Wavelength Cell Scoring Module of MetaMorph software (Molecular Devices) by quantification of the number of stained cells as a percentage of the total number of cells.

RNA Silencing

Müller glia were washed twice with 200 μ l DMEM. Negative control small interfering RNA (siRNA) (1 μ M, AM4611, Silencer Select; Ambion) or siRNA specific for LRP1 (part number 4390771, Silencer Select; Ambion) was added to DMEM, as directed by the manufacturer, then electroporated into Müller glia using a NEPA21 electroporator (Nepagene, Chiba, Japan) with a CUY900-5-2-3 cell-culture-plate electrode. Electric pulse parameters were setup at 200 V, 2.5 msec pulse length, 50 msec interval for poring pulse and 30 V, 50 msec pulse length, and 50 msec interval for transfer pulse. The extent of knockdown by negative control or LRP1 siRNA was evaluated by immunoblotting. The electroporated cells were used for experiments 3 days after electroporation.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from retinae and primary cultured Müller glia by Isogen II (Nippon Gene, Tokyo, Japan), as described previously,²² then quantified by BioSpec-nano (Shimazu, Kyoto, Japan). Complementary DNAs were synthesized from 500 ng RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan). Quantitative RT-PCR was performed by the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with THUNDERBIRD SYBR qPCR Mix (TOYOBO). Data were normalized to mRNA expression of β -actin and analyzed by the $2^{-\Delta\Delta Ct}$ method. The primers used were: rat α 2-macroglobulin-forward, 5'-GTAATCCTTCTAACTGCTTCGGCG-3'; reverse, 5'-CCAATGAAGATCGTTTCATACGGA-3'; rat β -actin-forward, 5'-TGCTATGTTGCCCTAGACTTCG-3'; reverse, 5'-GTTGGCATAGAGGTCTTTACGG-3'; mouse α 2-macroglobulin-forward, 5'-TGCATCCGGGTTGGTGAC-3'; reverse, 5'-TTCATACAGATGCAGTGAGACTTTTG-3'; mouse β -actin-forward, 5'-ACGTTGACATCCGTAAAGACCT-3'; reverse, 5'-GGTGTAAAACGCAGCTCAGTAA-3'.

Generation of NMDA-Induced Excitotoxicity in Rats

Intravitreal injection of NMDA (Sigma) was performed as described previously²³ with minor modifications. Briefly,

7-week-old rats were anesthetized with 5% isoflurane, then maintained with 2.5% isoflurane. The pupil was dilated with phenylephrine hydrochloride and tropicamide eye-drops (Wakamoto Pharmaceuticals Co., Ltd., Tokyo, Japan). Subsequently, PBS (vehicle control) or 20 nmol NMDA/eye with or without 5 ng/eye E-LP in a total volume of 4 μ l was injected into the vitreous cavity. Injections were performed under a microscope using a 34-gauge needle (Nanopass, Terumo, Tokyo, Japan) connected to a micro syringe (80008; Hamilton, Reno, NV, USA) with an infusion pump (Fusion 200; Chemyx Inc., Stafford, TX, USA). The needle was inserted approximately 1.0 mm behind the corneal limbus. The eyes were enucleated 3 days after NMDA injection, and retinæ were collected for immunoblotting and quantitative RT-PCR.

Collection of Aqueous Humor From Rats

Rats were anesthetized with 5% isoflurane and then maintained with 2.5% isoflurane. Aqueous humor (5 μ l/eye) was collected at a rate of 6 μ l/min using a 34-gauge needle connected to a micro syringe with an infusion pump (Fusion 200) 3 days after intravitreal injection of NMDA. Because blood contains a large amount of α 2-macroglobulin,²⁴ contamination of the aqueous humor with blood was carefully avoided. Collected samples were immediately mixed with sample buffer for immunoblotting.

Retrograde Labeling for Measurement of RGC Survival

To evaluate whether E-LPs affected RGC survival, retrograde labeling of RGCs with a retrograde fluorescent tracer Fluoro-gold (FG) was performed in a manner similar to that described previously.^{23,25} Briefly, rats were anesthetized with 5% isoflurane, then maintained in 2.5% isoflurane. Skin of the head was incised in the midline to expose the skull and sutured (sagittal, coronal, and lambdoid sutures). The incisions were subjected to bilateral 2 mm diameter craniotomies at 0.5 mm posterolateral to the sagittal and lambdoid sutures. Superior colliculi were carefully exposed by removal of the cerebral content. A small piece of sterile sponge (gelform; Pfizer, Pearl River, NY, USA), presoaked in 10 μ l of 4% FG solution, was left on the surface of the superior colliculus. After the surgery, the rats were kept warm and allowed to recover. Seven days after the surgery, the rats were euthanized and the eyes were enucleated and fixed with SuperFix (Kurabo, Osaka, Japan) for 2 hours at 4°C. Retinæ were removed from the sclerae and divided into four quadrants (superior, inferior, nasal, and temporal) and mounted on slides. Fluorescence images of each quadrant at 2.0 mm from the optic nerve head were acquired and analyzed by counting the number of FG-labeled RGCs using the Multi Wavelength Cell Scoring Module of MetaMorph software (Molecular Devices) to avoid measurement bias.

Statistical Analysis

Statistical analyses were performed using Kaleida Graph 4.5. Differences between the two groups were determined using the unpaired Student's *t*-test. Differences among multiple groups were analyzed using 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison as a post

hoc test. A confidence level of >95 % was considered significant ($P < 0.05$).

RESULTS

Intravitreal Injection of E-LPs Protects RGCs from NMDA-induced Retinal Damage In Vivo

Intravitreal injection of NMDA induces excitotoxicity of RGCs in rats. Thus, this animal model provides a pharmacological model of retinal degeneration.^{26,27} We detected Brn-3a protein, a marker of RGCs, in the retinæ of rats 3 days after NMDA treatment. Injection of 20 nmol NMDA/eye caused a significant (approximately 72.3%) loss of Brn-3a, and intravitreal administration of 5 ng E-LPs/eye markedly attenuated the loss of Brn-3a in this excitotoxicity model (see Fig. 1A). Increased caspase 3 cleavage (an indicator of caspase 3 activation and cell death), was induced by NMDA but was significantly inhibited by the intravitreal injection of E-LPs (see Fig. 1B). These data indicate that E-LPs protect retina from NMDA-induced retinal damage in rats. We also assessed the neuroprotective effect of E-LPs against NMDA-induced optic nerve damage by examination of the retrograde labeling of RGCs with fluorescent FG (see Figs. 1C, 1D). Three days after intravitreal injection of NMDA into the rats, the number of labeled RGCs was reduced by 43.3%, whereas the administration of E-LPs largely prevented this loss (see Figs. 1C, 1D). These data indicate that the intravitreal administration of E-LPs protects RGCs from NMDA-induced excitotoxicity.

The Increase of α 2-Macroglobulin Upon NMDA Injection is Prevented by E-LPs in Aqueous Humor of an NMDA-Induced Excitotoxicity Model

The amount α 2-macroglobulin in aqueous humor of eyes has been reported to be significantly increased in patients with glaucoma.^{20,28} Thus, we examined the levels of α 2-macroglobulin in aqueous humor and the retinæ from the NMDA-induced excitotoxicity model 3 days after NMDA treatment. The intravitreal injection of NMDA significantly increased (by approximately 5.72-fold in aqueous humor and approximately 2.15-fold in the retinæ) the amounts of α 2-macroglobulin and these increases were prevented by intravitreal injection of E-LPs (see Figs. 2A, 2B). We also measured the mRNA level of α 2-macroglobulin in the retinæ. Consistent with the results from immunoblotting of α 2-macroglobulin, shown in Figures 2A and 2B, NMDA injection markedly increased the level of α 2-macroglobulin mRNA. Moreover, E-LP administration significantly prevented this increase (see Fig. 2C).

The NMDA-Induced Increase in Expression of α 2-Macroglobulin is Attenuated by E-LPs in Cultured Mixed Retinal Cells

Primary cultures of mixed retinal cells were established to determine if NMDA treatment with or without E-LPs affects the levels of α 2-macroglobulin expression in the retinal cells. The mixed retinal cells contained vimentin (a marker of Müller glia)-positive cells at $71.3 \pm 4.8\%$ of total cells and β III tubulin (a marker of neurons)-positive cells at $20.4 \pm 7.5\%$ of total cells (counted 1255 cells in 6 images) in the cultures, as revealed by immunocytochemical analysis

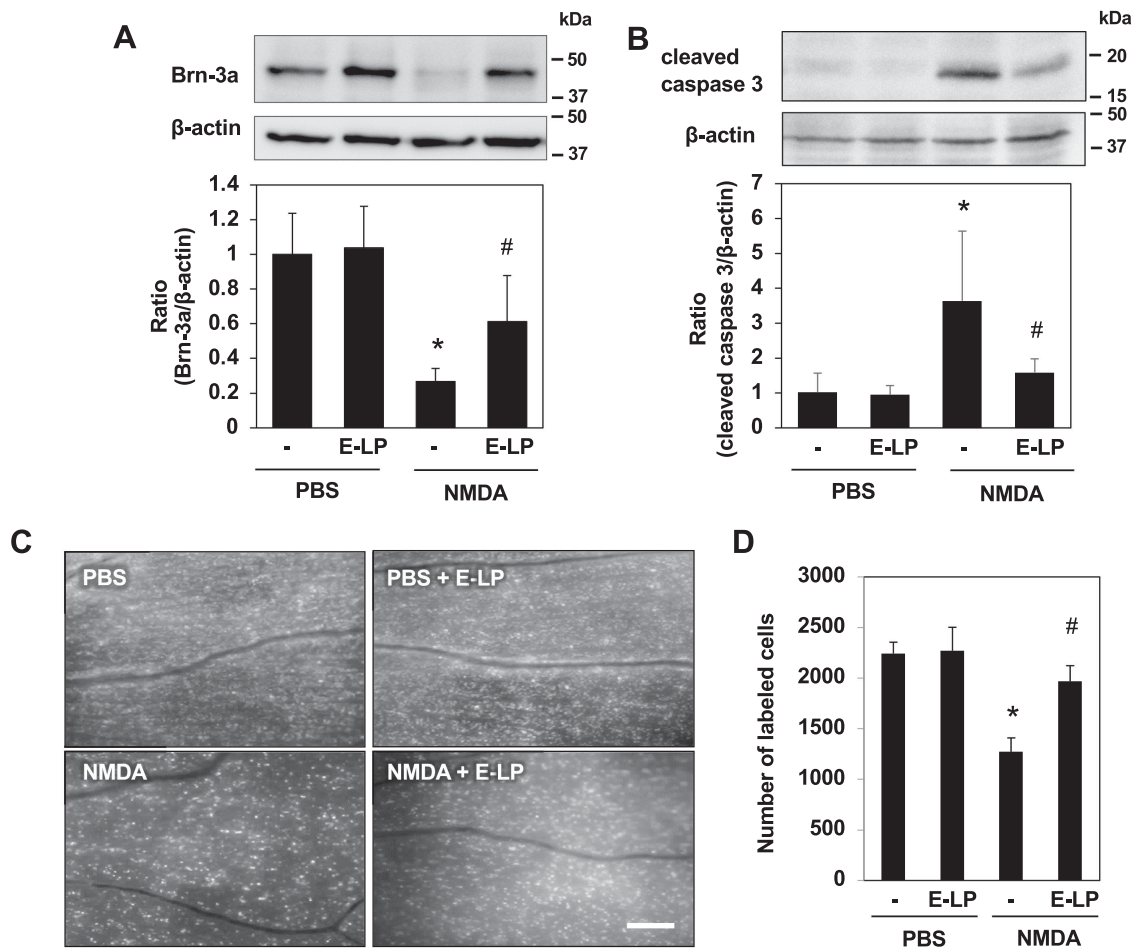


FIGURE 1. E-LPs protect RGCs from NMDA-induced retinal damage in rats. PBS (4 μ l) or NMDA (20 nmol) with or without 5 ng E-LP, was injected into the vitreous humor of 7-week-old rats. **(A)** Each retina was collected 3 days after injection, immediately prepared for immunoblot sample and subjected to immunoblotting with antibody directed against Brn3a (PBS, $n = 7$ retinae; PBS + E-LP, $n = 6$ retinae; NMDA, $n = 8$ retinae; and NMDA + E-LP, $n = 8$ retinae) or β -actin. The protein levels were normalized by the corresponding β -actin levels. Data are means \pm S.D. *, $P < 0.0001$ for PBS versus NMDA. #, $P = 0.032$ for NMDA versus NMDA + E-LP. **(B)** Each retina was collected 3 days after injection, immediately prepared for immunoblot sample and subjected to immunoblotting with antibody directed against cleaved caspase 3 (PBS, $n = 5$ retinae; PBS + E-LP, $n = 5$ retinae; NMDA, $n = 6$ retinae; and NMDA + E-LP, $n = 6$ retinae) or β -actin. The protein levels were normalized by the corresponding β -actin levels. Data are means \pm S.D. *, $P = 0.0113$ for PBS versus NMDA. #, $P = 0.0417$ for NMDA versus NMDA + E-LP. **(C)** Fluorescence images of retinal flat-mounts showing retrograde labeling of RGCs with FG. Scale bar, 100 μ m. **(D)** The number of FG-labeled cells in retinal flat-mounts was quantified ($n = 5$ independent retinae). Data are means \pm S.D. *, $P < 0.0001$ for PBS versus NMDA. #, $P = 0.0001$ for NMDA versus NMDA + E-LP. Data were analyzed with the 1-way ANOVA, Tukey's multiple comparisons test.

(see Fig. 3A). The addition of 300 μ M NMDA showed a trend to elevate the expression of α 2-macroglobulin and a significant increase of α 2-macroglobulin secretion from the mixed retinal cell cultures. Moreover, in NMDA-treated cells, E-LPs (300 ng/mL), but not the HBSS alone, reduced the expression and secretion of α 2-macroglobulin (see Figs. 3B, 3C). These results exhibit that the NMDA-induced increase of α 2-macroglobulin expression was decreased by E-LPs in the mixed retinal cell cultures. However, it was unidentified what kind of cells responded to this reduction of α 2-macroglobulin expression by E-LPs in the retinal cells. In addition, some types of cells potentially weakened and/or diluted the decreasing effect on α 2-macroglobulin expression. Thus, we further examined whether E-LPs have direct effect on α 2-macroglobulin expression in the Müller glia.

E-LPs Decrease the Expression and Secretion of α 2-Macroglobulin in Primary Cultured Müller Glia

The effects of E-LPs on α 2-macroglobulin in Müller glia were also investigated. Primary enriched cultures of Müller glia were established. Glutamine synthetase and vimentin were used as Müller glia markers (see Fig. 4A). Immunocytochemical analyses showed that glutamine synthetase- and vimentin-positive cells comprised $90.8 \pm 1.7\%$ of total cells (counted 763 cells in 12 images), whereas β III tubulin-positive cells were $2.0 \pm 0.8\%$ (counted 410 cells in 6 images) of total cells in the cultures. E-LPs (at 100 and 300 ng/mL) dose-dependently decreased the level of α 2-macroglobulin mRNA in the Müller glia (see Fig. 4B). In addition, the levels of the expression and secretion of α 2-macroglobulin were reduced (Figs. 4C, 4D) in contrast to the increase of added

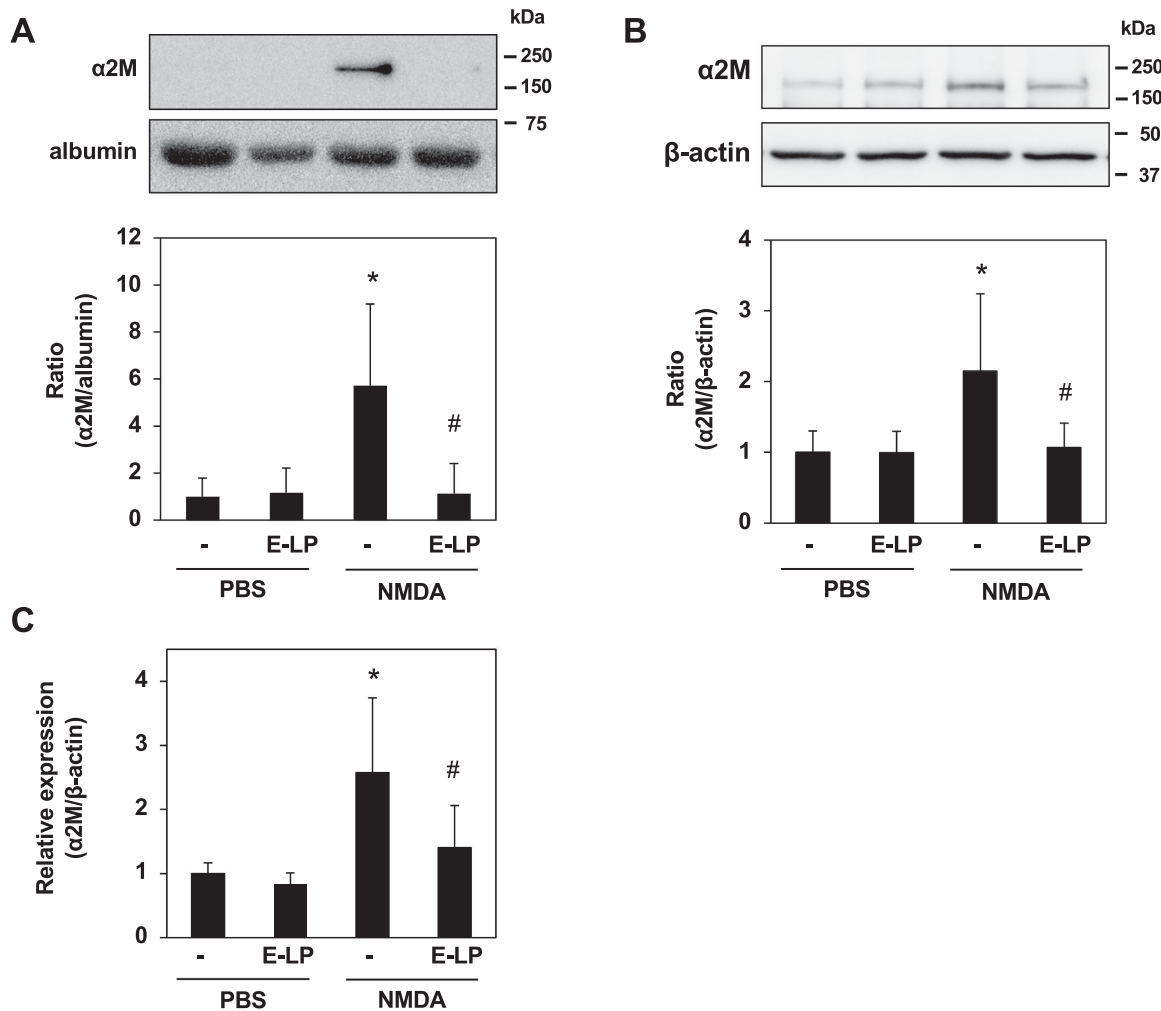


FIGURE 2. The increases in α 2-macroglobulin protein and mRNA in aqueous humor and retina, respectively, induced by NMDA injection, were reduced by intravitreal injection of E-LPs in rats. Aqueous humor and retinae of rats were collected 3 days after injection of 4 μ l of PBS or NMDA (20 nmol), with or without 5 ng E-LP. (A) Aqueous humor was subjected to immunoblotting with anti- α 2-macroglobulin ($n = 7$ independent eyes) or anti-albumin antibodies. The protein levels were normalized by the corresponding albumin levels. Data are means \pm SD. *, $P = 0.0018$ for PBS versus NMDA. #, $P = 0.0024$ for NMDA versus NMDA + E-LP. (B) Each retina was collected 3 days after injection, immediately prepared for immunoblot sample and subjected to immunoblotting with antibody directed against α 2-macroglobulin ($n = 6$ independent retinae) or β -actin. The protein levels were normalized by the corresponding β -actin levels. Data are means \pm SD. *, $P = 0.0191$ for PBS versus NMDA. #, $P = 0.0297$ for NMDA versus NMDA + E-LP. (C) α 2-Macroglobulin mRNA in retinae ($n = 8$ independent retinae) was extracted and quantified, relative to β -actin mRNA, by qPCR. Data are means \pm SD. *, $P = 0.0009$ for PBS versus NMDA. #, $P = 0.0161$ for NMDA versus NMDA + E-LP. Data were analyzed with the 1-way ANOVA, Tukey's multiple comparisons test.

E-LP (see a blot of apo E in Fig. 4C). These results show that E-LPs directly reduce α 2-macroglobulin expression in Müller glia.

Reduction of α 2-Macroglobulin Expression by E-LP is Mediated by LRP1

The expression of α 2-macroglobulin in Müller glia was significantly reduced by 300 ng/mL E-LPs (see Fig. 4). However, the reduction of α 2-macroglobulin was largely prevented by the addition of 300 nM receptor-associated protein (see Fig. 5A), a protein that inhibits the binding of E-LPs to receptors of the low-density lipoprotein receptor family. Furthermore, knockdown of LRP1 expression by siRNA (see Fig. 5B) significantly prevented the reduction in α 2-macroglobulin caused by E-LP treatment

(see Fig. 5C). These data indicate that LRP1 mediates the decrease in α 2-macroglobulin induced by E-LPs in Müller glia.

STAT3 Regulates α 2-Macroglobulin Expression in Müller Glia in Response to E-LPs

Next, we assessed intracellular signaling pathways stimulated by E-LPs in primary cultured Müller glia. Phosphorylation states of several signaling proteins were analyzed by immunoblotting 4 hours after E-LP treatment. No significant changes in phosphorylation levels of Akt, Erk, GSK3 β , CREB, JNK, and p38 were detected in response to increasing amounts (from 0 to 300 ng/mL) of E-LPs (see Figs. 6A–6F). In contrast, after 4 hours, the phosphorylation level of STAT3 was increased by E-LPs relative to PBS treatment

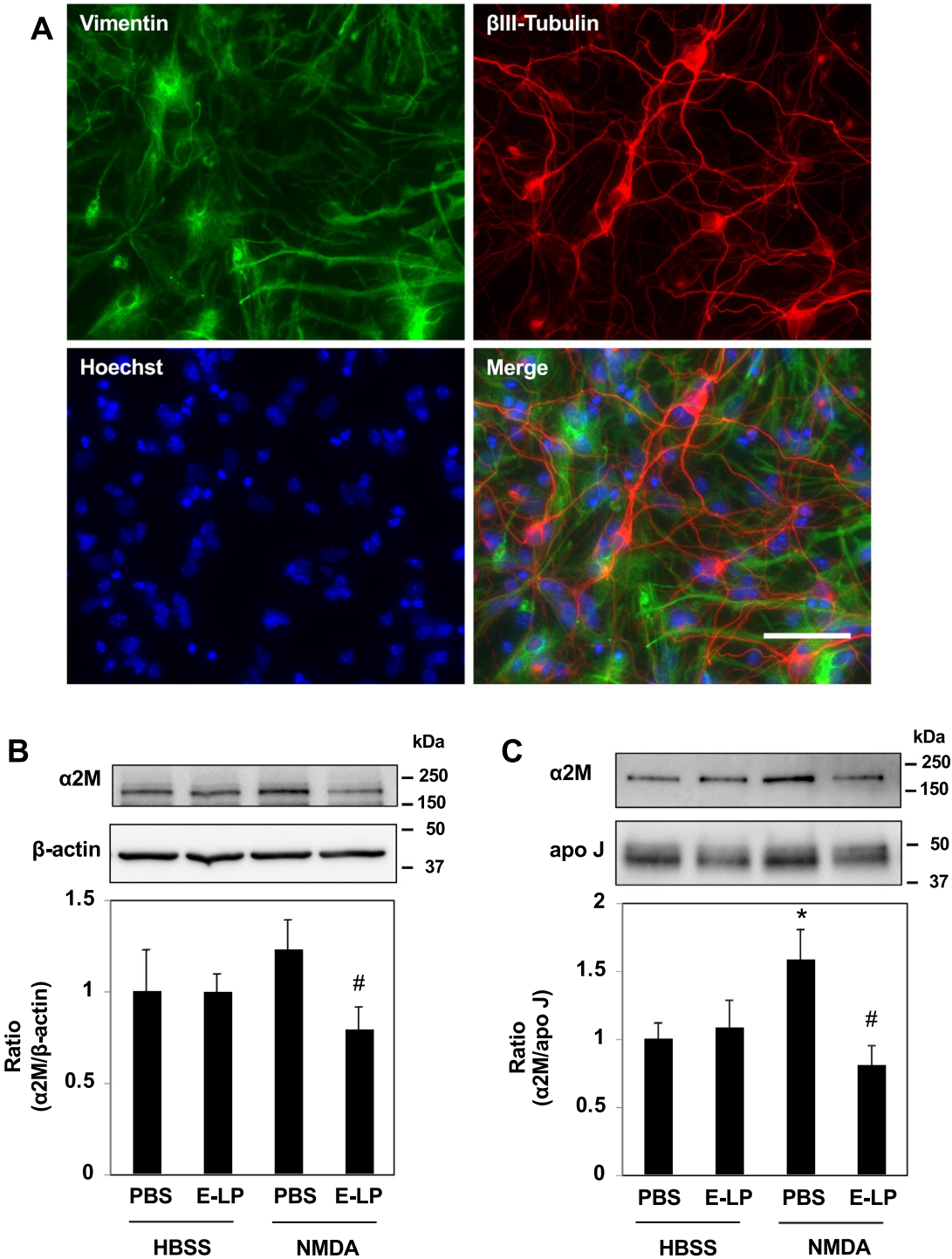


FIGURE 3. E-LPs attenuate the increased secretion of α 2-macroglobulin induced by NMDA in primary cultures of mixed retinal cells. (A) Immunocytochemistry of primary cultures of mixed retinal cells with anti-vimentin (green) and anti- β III tubulin (red) antibodies. Nuclei (blue) were stained by Hoechst 33342. Images show primary cultures of mixed retinal cells from one experiment of three independent experiments with similar results. Scale bar represents 50 μ m. (B) Cells (HBSS + PBS, $n = 7$ cell cultures; HBSS + E-LP, $n = 7$ cell cultures; NMDA + PBS, $n = 8$ cell cultures; and NMDA + E-LP, $n = 8$ cell cultures) of the mixed retinal cells were collected 24 hours after treatment with HBSS or 300 μ M NMDA treatment with PBS or 300 ng/mL E-LP, and subjected to immunoblotting with antibodies directed against α 2-macroglobulin or β -actin. The protein levels were normalized by the corresponding β -actin levels. Data are means \pm SD. #, $P = 0.0001$ for NMDA + PBS versus NMDA + 300 ng/mL E-LP. (C) Conditioned media (HBSS + PBS, $n = 5$ cell cultures; HBSS + E-LP, $n = 5$ cell cultures; NMDA + PBS, $n = 6$ cell cultures; and NMDA + E-LP, $n = 6$ cell cultures) of the mixed retinal cells were collected 24 hours after treatment with HBSS or 300 μ M NMDA treatment with PBS or 300 ng/mL E-LP, and subjected to immunoblotting with antibodies directed against α 2-macroglobulin or apo J. The protein levels were normalized by the corresponding apo J levels. Data are means \pm SD. *, $P = 0.0007$ for HBSS + PBS versus NMDA + PBS. #, $P < 0.0001$ for NMDA + PBS versus NMDA + 300 ng/mL E-LP. Data were analyzed with the 1-way ANOVA, Tukey's multiple comparisons test.

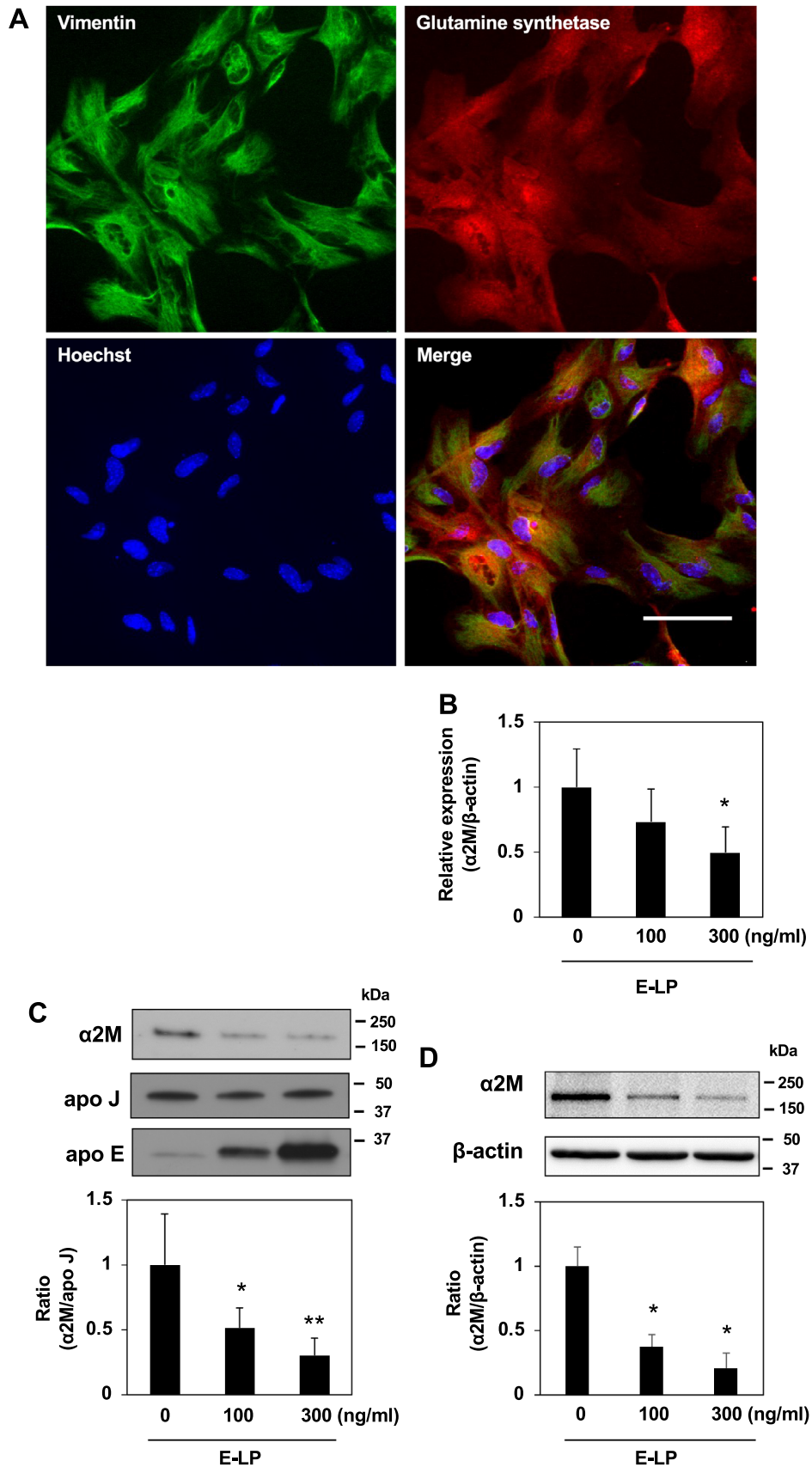


FIGURE 4. E-LPs reduce the expression and secretion of α 2-macroglobulin in primary cultured Müller glia. **(A)** Immunocytochemistry of Müller glia using anti-vimentin (green) and anti-glutamine synthetase (red) antibodies. Nuclei (blue) were stained by Hoechst 33342. Image of Müller glia is from one experiment of four independent experiments with similar results. Scale bar represents 50 μ m. **(B)** Ratio of mRNA

expression of α 2-macroglobulin or β -actin was analyzed by qPCR 24 hours after 0, 100, or 300 ng/mL treatment with E-LPs ($n = 7$ cell cultures). The mRNA expression levels were normalized by the corresponding β -actin mRNA levels. Data are means \pm SD. *, $P = 0.0072$ for 0 ng/mL E-LP versus 300 ng/mL E-LP. (C, D) Conditioned media C and cells D of Müller glia were collected 24 hours after 0, 100, or 300 ng/mL E-LP treatment ($n = 5$ cell cultures), and subjected to immunoblotting with antibodies directed against α 2-macroglobulin, apolipoprotein J, apolipoprotein E, or β -actin. The protein levels were normalized by the corresponding apo J C or β -actin D levels. Data are means \pm SD. C *, $P = 0.0483$ or **, $P = 0.0059$ for 0 ng/mL E-LP versus 100 or 300 ng/mL E-LP, respectively. D *, $P < 0.0001$ for 0 ng/mL E-LP versus 100 or 300 ng/mL E-LP. Data were analyzed with the 1-way ANOVA, Tukey's multiple comparisons test.

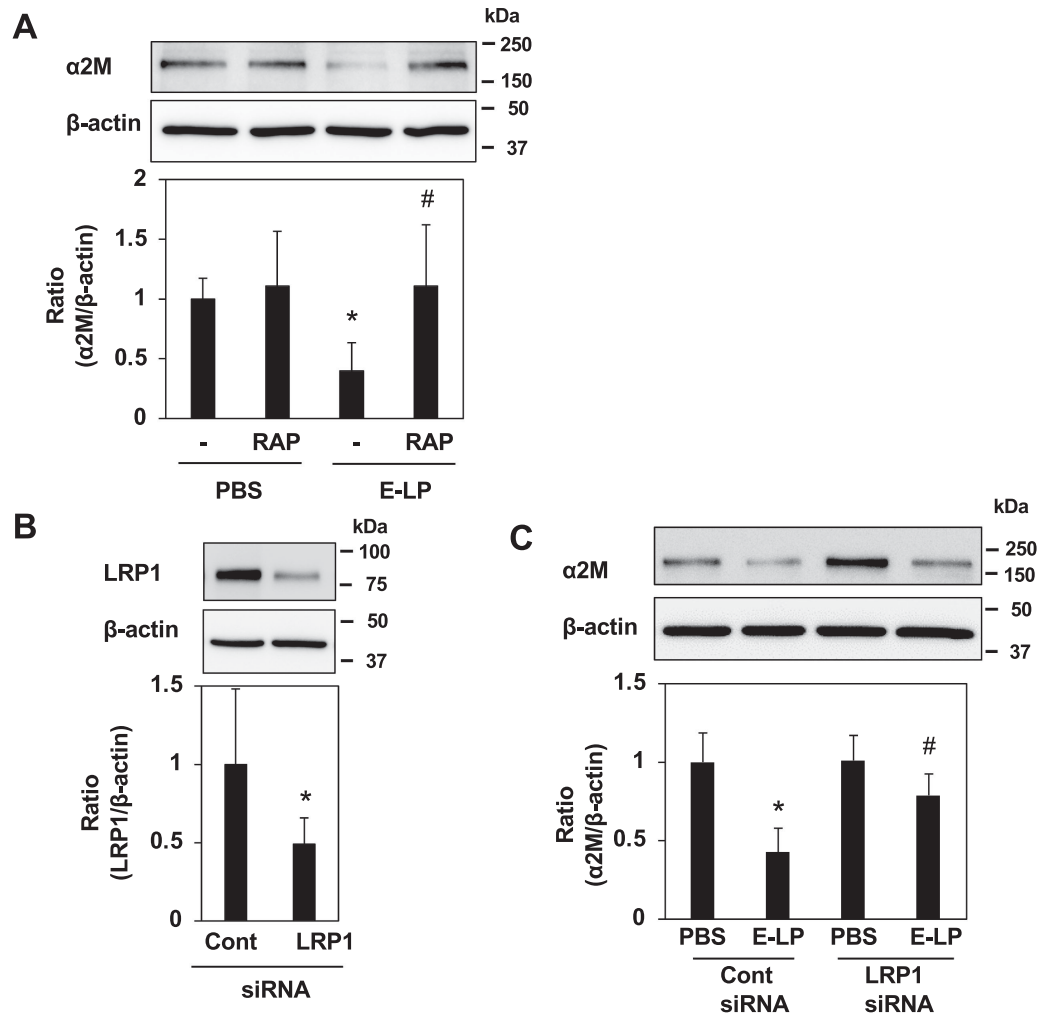


FIGURE 5. LRP1 mediates the reduction of α 2-macroglobulin by E-LPs in primary cultured Müller glia. (A) Müller glia were collected 24 hours after treatment with PBS, PBS + 300 nM receptor-associated protein (RAP), 300 ng/mL E-LP, or 300 ng/mL E-LP + 300 nM RAP ($n = 7$ cell cultures). The samples were subjected to immunoblotting with anti- α 2-macroglobulin or anti- β -actin antibodies. Data are means \pm SD. *, $P = 0.0463$ for PBS versus 300 ng/mL E-LP. #, $P = 0.0149$ for 300 ng/mL E-LP versus 300 ng/mL E-LP + 300 nM RAP. (B) knockdown of LRP1 in Müller glia was induced by LRP1 siRNA. Müller glia were collected 3 days after transfection ($n = 6$ cell cultures) and subjected to immunoblotting with anti-LRP1 or anti- β -actin antibodies. Data are means \pm SD. *, $P = 0.04922$ for control siRNA versus LRP1 siRNA. (C) Müller glia were collected 24 hours after treatment with PBS or 300 ng/mL E-LP, with knockdown by control or LRP1 siRNA ($n = 5$ cell cultures). Cell proteins were subjected to immunoblotting with anti- α 2-macroglobulin or anti- β -actin antibodies. Data are means \pm SD. *, $P = 0.0006$ for control siRNA + PBS versus control siRNA + 300 ng/mL E-LP. #, $P = 0.0261$ for control siRNA + 300 ng/mL E-LP versus LRP1 siRNA + 300 ng/mL E-LP. (A–C) The protein levels were normalized by the corresponding β -actin levels. A and C Data were analyzed with the 1-way ANOVA, Tukey's multiple comparisons test. B Data were analyzed with unpaired Student's *t*-test.

(see Fig. 6G). Because E-LPs increased the phosphorylation level of STAT3 and reduced α 2-macroglobulin expression in Müller glia, we examined whether or not a STAT3 inhibitor affected α 2-macroglobulin expression in the presence of E-LPs. Figure 7A shows that the phosphorylation level of STAT3 was significantly attenuated after 4 hours by 3 μ M Stattic (a STAT3 inhibitor). Moreover, this reduction in

phosphorylation by Stattic was restored by E-LP treatment (see Fig. 7A). Furthermore, 24 hours after E-LP treatment of Müller glia Stattic largely prevented the reduction of α 2-macroglobulin expression induced by E-LPs (see Fig. 7B). These data demonstrate that STAT3 is a key intracellular molecule that regulates the expression of α 2-macroglobulin downstream of the binding to E-LPs to LRP1 in Müller glia.

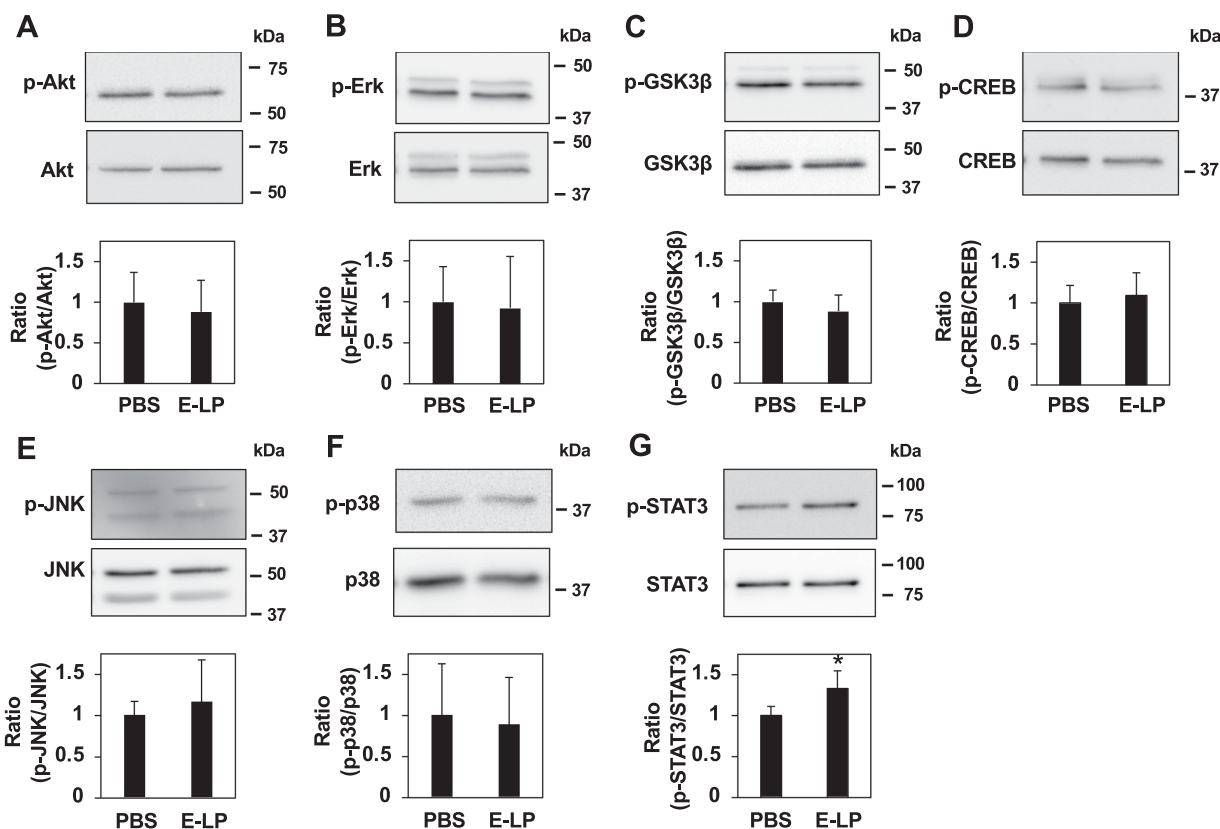


FIGURE 6. Phosphorylation of signaling molecules by E-LP treatment of primary cultured Müller glia. Müller glia were collected 4 hours after PBS or 300 ng/mL E-LP treatment ($n = 6$ cell cultures), and subjected to immunoblotting with antibodies raised against phospho-Akt or Akt (A), phospho-Erk or Erk (B), phospho-GSK3 β or GSK3 β (C), phospho-CREB or CREB (D), phospho-JNK or JNK (E), phospho-p38 or p38 (F), or phospho-STAT3 or STAT3 (G). The phosphorylated protein levels were normalized by the corresponding total protein levels. Data are means \pm SD. *, $P = 0.02737$ for PBS versus 300 ng/mL E-LP. Data were analyzed with unpaired Student's t -test.

DISCUSSION

The present study demonstrates that E-LPs act as a neuroprotectant against excitotoxicity induced by intravitreal injection of NMDA in rats. E-LPs not only directly protect RGCs via an intracellular signaling pathway through LRP1, as we previously demonstrated,^{15–17} but also attenuate the expression and secretion of α 2-macroglobulin from Müller glia via interaction of the E-LPs with LRP1. Because α 2-macroglobulin reduces the neuroprotection elicited by E-LPs in primary cultured RGCs,¹⁷ we propose that in an *in vivo* environment α 2-macroglobulin, produced by Müller glia, contributes to optic nerve survival associated with E-LPs.

In our previous study¹⁷ recombinant α 2-macroglobulin alone did not cause RGC degeneration and also did not affect the degree of glutamate-induced RGC degeneration, yet it exhibited the protective effect against trophic factor withdrawal-induced RGC death *in vitro*.¹⁵ However, α 2-macroglobulin did impair the neuroprotective effect of E-LPs in primary cultured RGCs.¹⁷ These findings support the idea that α 2-macroglobulin in the retina might act as an exacerbating factor in RGC degeneration induced by excitotoxicity *in vivo*. Thus, α 2-macroglobulin might be a potential biomarker for glaucoma in aqueous humor of animal models and human patients.^{19,20,28} We have previously reported that the level of α 2-macroglobulin in vitreous humor of *Glaxt*^{-/-} mice is increased after retinal damage.¹⁷ Although E-LPs protected RGCs from NMDA-induced retinal damage

in vivo, we had not determined whether the E-LP-induced decrease in α 2-macroglobulin in aqueous humor could be attributed to the protection of RGCs, the direct reduction of α 2-macroglobulin in glia or other mechanisms.

We have, therefore, now investigated whether expression and secretion of α 2-macroglobulin in Müller glia are promoted by NMDA-induced neuron damage and also attenuated by E-LP treatment using primary cultures of mixed retinal cells. E-LPs alone did not reduce the expression or secretion of α 2-macroglobulin in cells and media of primary cultures of mixed retinal cells (see Figs. 3B, 3C) similar to *in vivo* results (see Fig. 2), but the increase in α 2-macroglobulin levels in cells and media upon NMDA treatment were attenuated upon the addition of E-LPs. It has been reported that α 2-macroglobulin levels in glia are increased when the retina is damaged, for example, during diabetic retinopathy and glaucoma.^{19,29} Because apo E has been reported to be a neuroprotectant,^{30,31} it is possible that the prevention of the NMDA-induced upregulation of α 2-macroglobulin in primary cultures of mixed retinal cells might be due to neuroprotection by E-LPs.

We also performed experiments in primary cultured Müller glia to determine if E-LPs directly reduced the expression of α 2-macroglobulin. Addition of E-LPs to the culture medium of these cells dose-dependently decreased both the protein levels and the mRNA levels of α 2-macroglobulin in Müller glia (see Fig. 4). Furthermore, the reduction in α 2-macroglobulin expression promoted

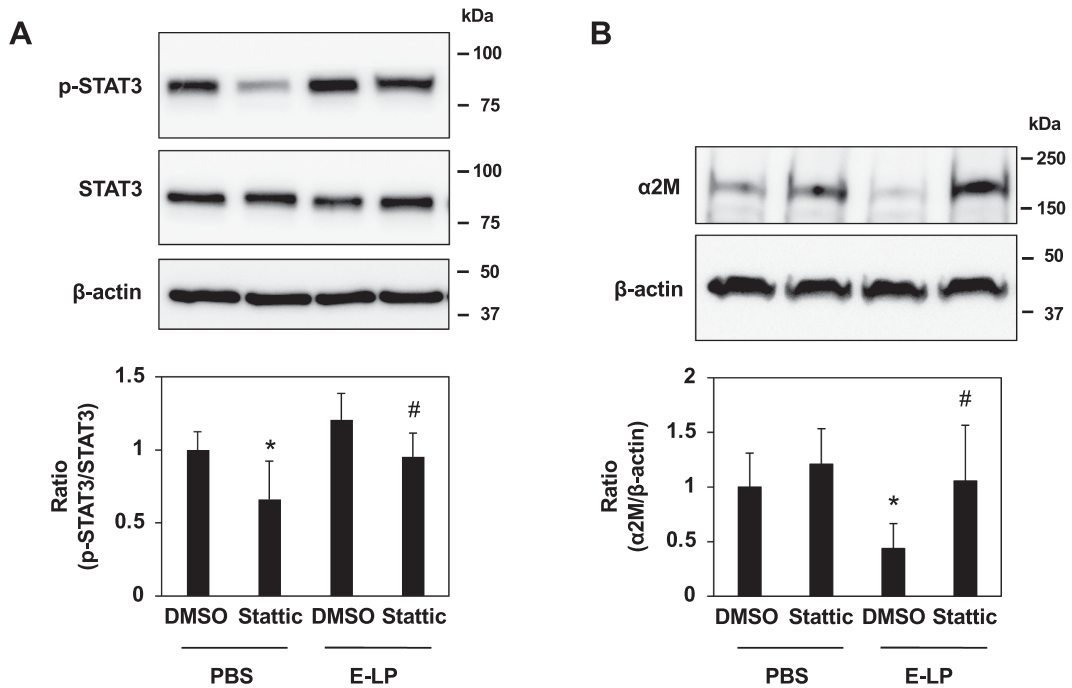


FIGURE 7. STAT3 inhibitor blocks the reduction in $\alpha 2$ -macroglobulin levels induced by E-LPs in primary cultured Müller glia. **(A)** Müller glia were treated with 3 μ M Static, a STAT3 inhibitor, or dimethyl sulfoxide (DMSO) as vehicle, in the presence of PBS or E-LPs for 4 hours ($n = 8$ cell cultures). Cells were collected for immunoblotting with anti-phospho-STAT3, anti-STAT3, or anti- β -actin antibodies. The phospho-STAT3 levels were normalized by the corresponding STAT3 levels. Data are means \pm SD. *, $P = 0.0116$ for DMSO + PBS versus 3 μ M Static + PBS. #, $P = 0.0355$ for 3 μ M Static + PBS versus 3 μ M Static + 300 ng/mL E-LP. **(B)** Müller glia were treated with 3 μ M Static or DMSO in the presence of PBS or E-LPs for 24 hours ($n = 8$ cell cultures). Cells were collected for immunoblotting with anti- $\alpha 2$ -macroglobulin or anti- β -actin antibodies. Data are means \pm SD. *, $P = 0.0317$ for DMSO + PBS versus DMSO + 300 ng/mL E-LP. #, $P = 0.0163$ for DMSO + 300 ng/mL E-LP versus 3 μ M Static + 300 ng/mL E-LP. **A and B** The protein levels were normalized by the corresponding β -actin levels. Data were analyzed with the 1-way ANOVA, Tukey's multiple comparisons test.

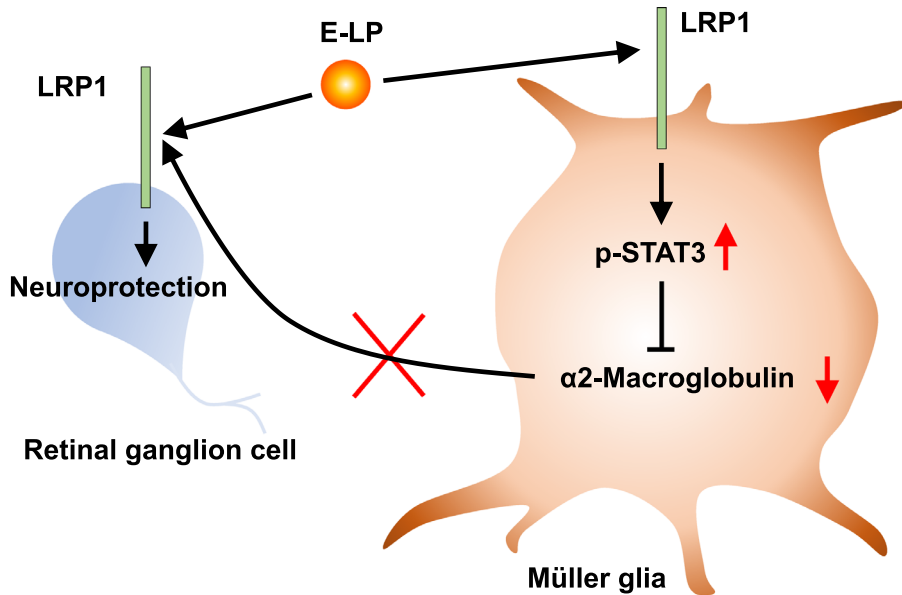


FIGURE 8. Proposed neuroprotective mechanisms by E-LPs in the retina. E-LPs protect retinal ganglion cells in a rat glaucoma model. In addition to the direct protective mechanisms in RGCs by E-LPs that were demonstrated in our previous studies, the current study indicates the basis of an indirect protective mechanism that E-LPs attenuate $\alpha 2$ -macroglobulin which interferes with neuroprotective effect of E-LPs. STAT3 regulates the expression of $\alpha 2$ -macroglobulin downstream of LRP1 in Müller glia.

by E-LPs was mediated through LRP1 (see Fig. 5). It has been reported that the increase in LRP1 expression in response to α 2-macroglobulin regulates matrix metalloproteinase activity and promotes migration of Müller glia.³² Moreover, GFAP expression in Müller glia is enhanced by α 2-macroglobulin through LRP1.³³ Furthermore, the amount of α 2-macroglobulin was shown to increase LRP1 levels at the plasma membrane via the PI3K/Akt pathway in Müller glia.³⁴ Thus, α 2-macroglobulin seems to have multiple important roles in Müller glia of the retina under physiological and pathological conditions. In this study, we demonstrate that E-LPs directly decrease the expression of α 2-macroglobulin through LRP1 in primary cultured Müller glia. The α 2-macroglobulin secretion treated with E-LPs seems to exhibit more significant changes than its mRNA or protein expression in Müller glia (see Figs. 4, 5). These observations provide us an idea that there is an additional important mechanism regulating α 2-macroglobulin secretion by E-LPs in Müller glia. In addition, glial cells other than Müller glia in the retina may also play a crucial role in glutamate recycling through NMDA receptor. Thus, a cell-specific experiment with Müller glia, other glial cells, and/or RGCs is needed to conclude a role of α 2-macroglobulin in neurodegeneration and therapeutic efficacy of E-LPs. However, further experiments are required to address these ideas.

The intracellular mechanism(s) by which α 2-macroglobulin is reduced by E-LPs via LRP1 was examined in primary cultures of Müller glia. Upon the binding of E-LPs to LRP1, STAT3 was shown to perform a key role in modulating the expression of α 2-macroglobulin downstream of LRP1. It has been reported that Wnt/ β -catenin signaling in Müller glia protects photoreceptor neurons in association with an increased level of STAT3 in the rd10 mouse model of inherited retinal degeneration.³⁵ In addition, a signal of leukemia inhibitory factor through gp130/JAK/STAT3 pathway activates Müller glia after optic nerve injury.³⁶ Moreover, exogenous ciliary neurotrophic factor initiates cytokine action mediated by STAT3 and Erk signaling in Müller glia, subsequently protecting photoreceptor neurons from degeneration in a mouse model of retinitis pigmentosa.³⁷ Thus, the presence of STAT3 in Müller glia might play a significant role in neuron survival in the retina. Consistent with these findings, our studies suggest a novel mechanism for modulating the expression of α 2-macroglobulin, which interferes with the neuroprotection provided by E-LPs. The expression and secretion of α 2-macroglobulin are reduced in a process mediated by STAT3 downstream of LRP1 in Müller glia (see Fig. 8).

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