CHOP Deletion and Anti-Neuroinflammation Treatment With Hesperidin Synergistically Attenuate NMDA Retinal Injury in Mice

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Abstract

Background: Glaucoma is a leading cause of blindness worldwide and is characterized by degeneration associated with the death of retinal ganglion cells (RGCs). It is believed that glaucoma is a group of heterogeneous diseases with multifactorial pathomechanisms. Here, we investigate whether anti-inflammation treatment with an ER stress blockade can selectively promote neuroprotection against NMDA injury in the RGCs.

Methods: Retinal excitotoxicity was induced with an intravitreal NMDA injection. Microglial activation and neuroinflammation were evaluated with Iba1 immunostaining and cytokine gene expression. A stable HT22 cell line transfected with an NF-kB reporter was used to assess NF-kB activity after hesperidin treatment. CHOP-deficient mice were used as a model of ER stress blockade. Retinal cell death was evaluated with a TUNEL assay.

Results: In the NMDA injury group, Iba1-positive microglia increased 6 h after NMDA injection. Also at 6 h, pro-inflammatory cytokines and chemokines increased, including TNFα, IL-1b, IL-6 and MCP-1. In addition, the MCP-1 promoter-driven EGFP signal, which we previously identified as a stress signal in injured RGCs, also increased; hesperidin treatment suppressed this inflammatory response and reduced stressed RGCs. In CHOP-deficient mice that received an NMDA injection, the gene expression of pro-inflammatory cytokines, chemokines, markers of active microglia, and inflammatory regulators was greater than in WT mice. In WT mice, hesperidin treatment partially prevented retinal cell death after NMDA injury; this neuroprotective effect was enhanced in CHOP-deficient mice.

Conclusions: These findings demonstrate that ER stress blockade is not enough by itself to prevent RGC loss due to neuroinflammation in the retina, but it has a synergistic neuroprotective effect after NMDA injury when combined with an anti-inflammatory treatment based on hesperidin.

Introduction

Glaucoma has become the most frequent cause of irreversible blindness worldwide. It is characterized by a progressive loss of retinal ganglion cells (RGCs) that results in characteristic visual field defects[1]. One of the most important risk factors is elevated intraocular pressure (IOP), which promotes mechanical stress in RGCs and leads to the development of glaucoma. However, many patients, especially in Asia, experience a continued progression of visual field defects despite normal IOP, suggesting that IOP-independent factors also influence glaucoma progression[2]; thus, glaucoma is a multifactorial disease. Studies of in vitro human samples and in vivo animal models have provided some evidence of the triggers that cause RGC loss. These include many well-known contributors to the pathogenesis of RGC death, and include such events and molecules as oxidative stress[3][4], a low level of ocular blood flow[5], genetics[6], excessive calpain activation[7], glutamate excitotoxicity[8], and neuroinflammation[9].

In animal models, N-methyl- D-aspartate (NMDA), an agonist of the glutamate receptors, is commonly used to induce excitotoxicity and RGC death. NDMA was chosen after research revealed that glutamate

levels were elevated in the vitreous body of humans and monkeys with glaucoma [10]. Moreover, mice deficient in GLAST, a glutamate transporter, have become established as a model of normal tension glaucoma (NTG); furthermore, an NMDA receptor antagonist (memantine) prevents RGC degeneration[11], suggesting that glutamate receptor-mediated neurotoxicity has an important role in RGC loss in glaucoma. Finally, memantine has been found to prevent RGC loss in a high-IOP model of glaucoma [12]. Together, these findings indicate that NMDA receptors are involved in RGC loss in glaucoma even when IOP is not elevated, making it important to clarify the pathomechanism of excitotoxicity to prevent RGC death.

After NMDA injury is induced, the first event is a calcium influx into the cells via the NMDA receptors. This calcium overload has been shown to promote excessive calpain activation, oxidative stress and ER stress in the retina[13][14]. These findings reveal that multiple cell death signaling pathways are activated, and that therapeutic approaches with a single target may be not sufficient to prevent RGC death caused by NMDA injury. On the other hand, approaches with multiple targets may be effective. Our previous work showed that hesperidin, a plant-derived bioflavonoid, was a noteworthy candidate for a multifunctional treatment. We found that hesperidin countered oxidative stress, suppressed calpain activation, and was an anti-inflammatory, with the result that it could prevent RGC death in mice after NMDA injury[14]. This led us to hypothesize that combining hesperidin treatment with an ER stress blockade might be even more effective, and have the potential to rescue RGCs more effectively than a single-target therapeutic approach. To test this hypothesis, we examined the proposed combined treatment in C/EBP homologous protein (CHOP)-deficient mice, which we chose because CHOP is well-known as a key molecule for the promotion of RGC death under ER stress[15][16]. Thus, the present study investigated whether anti-inflammatory treatment with hesperidin had a synergistic effect in mice with genetic CHOP deletion, and attempted to determine whether this was a practical approach to preventing RGC death in mice after NMDA injury.

Material And Methods

Animals

Eight-to-twelve-week-old male C57BL/6 mice were purchased from SLC). CHOP knockout mice (C57BL/6 background) were used in this study, and their littermates were used as controls [17]. The animals were maintained at Tohoku University Graduate School of Medicine under a cycle of 12 h of light and 12 h of dark. The animal experiments in this study adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement on the use of animals in ophthalmic and vision research, and were approved by the institutional animal care and use committee of the Guidelines for Animals in Research.

NMDA-induced retinal injury and hesperidin treatment

NMDA injury was induced in the animals as previously described[14]. In brief, 15 μM of NMDA (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) was injected intravitreally (2 μl/eye) under anesthesia, which was administered with sodium pentobarbital diluted with PBS (78 mg/kg). The

hesperidin treatment comprised an injection of 15 μM of NMDA and 17% hesperidin (αG hesperidin PAT-T; Glico, Tokyo) in PBS[14]. PBS vehicle was also injected by itself as a control.

RGC stress reporter assay using an AAV2/2 vector in vivo

To detect RGC damage, we used an AAV2/2-EGFP vector driven with an Mcp-1 promoter, as in our previous report[18]. This virus (1×10^{12} gc/ml) was injected (2 μ l/injection) into the vitreous cavity of the mice, under anesthesia. After four weeks, NMDA with or without hesperidin was injected into the vitreous. Six hours later, whole retinal mounts were prepared and fluorescence images were captured. EGFP-positive cells were counted as previously described[14].

Immunohistochemistry

Retinal cryosections were prepared as previously described[19]. The cryosections were blocked with blocking buffer (10% donkey serum in Tw-PBS) at room temperature for 1 h and then incubated overnight with rabbit anti-Iba1 (1:200, #019-19741, Wako Pure Chemical Industries, Osaka, Japan) and rabbit anti-CHOP (GADD153) (1:200, Santa Cruz, sc-575) as the primary antibodies at 4° C. After washing with Tw-PBS, the sections were incubated in Alexa Fluor 488 conjugated with goat anti-rabbit IgG antibody (1:500, Invitrogen) or in blocking buffer at room temperature for 1 h. To detect CHOP antibodies, the immunoreaction signal was amplified with TSA plus (PerkinElmer, Boston, MA, USA) as previously described [20]. The sections were mounted on Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA, USA). Whole retinal sections were scanned and immunoreactive-positive cells were counted with a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan). Representative fluorescence images were captured with an Axiovert 200 (Carl Zeiss AG, Feldbach, Switzerland).

Quantitative RT-PCR

Total RNA extraction, cDNA synthesis and qPCR were performed as previously described[14]. Predesigned primers and probes, purchased from Life Tec, were used as follows: *TNF1a* (Mm00441883_g1), *IL-1b* (Mm00434228_m1), *IL-6* (Mm00446100_m1), *MCP-1* (Mm00441242_m1), *Ddit3* (Mm00492097_m1) and *Gapdh* (Mm01256744_m1). The data were analyzed using the comparative Ct method (2-ΔΔCT), normalized to an endogenous control (GAPDH mRNA).

Cell culture and transfection

An HT22 cell line, comprising mouse hippocampal neuronal cells, was kindly gifted by Prof. Yoko Hirata (Gifu University, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° C with 5% CO_2. To establish HT22 cells stably expressing the NF-κB reporter gene (HT22/NF-κB/luc), the cells were transfected with pGL4.32 [luc2P/NF-κB-RE/Hygro] (Promega) vector using lipofectamine 2000 transfection reagent (Thermo Fisher). The stably transfected cells were selected with hygromycin B (400 μg/mL, Nacalai Tesque) for two weeks.

NF-κB reporter assay

To determine the effect of hesperidin on inflammatory cytokine-mediated activation of NF-κB, the HT22/NF-κB/luc cells were seeded at a density of 5,640 cells/well and were incubated overnight in 96-well plates. The cells were pretreated with hesperidin for 2 h, and then treated with TNFα (20 ng/mL) or vehicle control for 4 h. Luciferase activity was measured using the ONE-Glo luciferase assay system kit (Promega) according to manufacturer's instructions. Luminescence was measured with a PHERAstar plate reader. The relative luciferase activity of each group was compared to the control group.

Cell viability assay

To determine the effect of hesperidin on cell viability, the HT22/NF-κB/luc cells were seeded at a density of 5,640 cells/well and were incubated overnight in 96-well plates. The cells were pretreated with hesperidin for 2 h, and then treated with TNFα (50 ng/mL) for 4 h. Alamar blue reagent (Invitrogen) was added and the fluorescence intensity was measured (560 nm excitation and 590 nm emission) with an absorption spectrometer (Vmax; Molecular Devices, Sunnyvale, CA, USA) as previously described[14].

Measurement of MCP-1 mRNA after TNFα treatment

HT22 cells were seeded at a density of 5,640 cells/well and were incubated overnight in 96-well plates. The cells were pretreated with hesperidin for 2 h, and then treated with TNFα (50 ng/mL) or vehicle control for 8 h. Extraction of total RNA and reverse transcription were conducted with the SuperPrep cell lysis and RT kit for qPCR (Toyobo) according to the manufacturer's instructions. The cDNA was mixed with TaqMan Fast Universal PCR Master mix (Life Technologies) and TaqMan probes (Life Technologies). Quantitative RT-PCR was performed with an initial denaturation step at 95° C for 20 seconds, followed by 40 cycles at 95° C for 3 seconds and 60° C for 30 seconds with a 7500 Fast Real-Time PCR System. For a relative comparison of gene expression, we analyzed the results of the qRT-PCR data with the comparative Ct method (2-ΔΔCT), normalized to Gapdh, an endogenous control. the following TaqMan probes were used: MCP-1 (Mm00441242; Life Technologies) and Gapdh (Mm99999915; Life Technologies).

Immunoblot analysis

Protein separation and transfer and antibody reaction and detection were performed as previously reported. Briefly, RIP3 antibody (Sigma, #R4277, 1:4000 dilution) and b-actin antibody (Sigma, #A5316, 1:5000 dilution) were used as the primary antibodies. For the HT22 cells, the nuclear fraction and cytosol fraction were separated using a nuclear/cytosolic fractionation kit (Cell Biolabs, #AKR-171). Anti-NF-kB antibody (Cell Signaling, #8242, 1:5000 dilution) and anti-IkB antibody (Cell Signaling, #4814, 1:5000 dilution) were used as primary antibodies. Anti-lamin B (Santa Cruz, #sc-6217, 1:5000 dilution) was used as a loading control for each fraction.

Cryosections and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL).

Apoptotic cells were detected with an ApopTag Red In Situ Apoptosis Detection Kit (Chemicon International, Inc., Temecula, CA, USA) according to the manufacturer's instructions[21]. The nuclei were stained with 4' -6' -diamidino-2-phenylindole (DAPI) in Vectashield (H-1200; Vector, Burlingame, CA, USA). Fluorescence images of whole retinal sections of mice were captured and TUNEL-positive cells were counted with a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan), as previously described[21].

Statistical analysis

Statistical comparisons used a one-way ANOVA followed by Dunnett's test or the Tukey-Kramer test to compare the mean in multiple groups and an unpaired t-test to compare pairs of samples. The statistical comparisons in figure 4 were performed with a two-way ANOVA followed by Holm-Sidak's multiple comparison test to assess the synergistic effect of CHOP deletion and hesperidin treatment. The significance level was set at $p < 0.05$.

Results

Hesperidin suppressed the inflammatory response to NMDA-induced excitotoxicity in the mouse retina

We previously found that NMDA-induced excitotoxicity increased pro-inflammatory cytokines in the retina[8]. Therefore, in this study, we evaluated microglial activation and the expression level of inflammatory regulators. Retinal sections taken from mice showed that the number of Iba1-positive microglia increased starting 6 h after NMDA injection (Fig. 1A-B). Our previous study showed that intravitreous injection of hesperidin suppressed TNFα expression[14], so we set out to determine whether hesperidin treatment ameliorated the pro-inflammatory response and inflammation-caused RGC stress in mice. We performed a qRT-PCR analysis and found that the transcription of inflammatory cytokines, such as TNFα, IL-1b, and IL-6, and ccl2 mRNA, were upregulated in the NMDA-injured retinas, and that this upregulation was significantly suppressed in the hesperidin-treated mice (Fig. 1D-F). In a previous study, we found that MCP1 promoters increased in the RGCs after optic nerve crush, and that AAV2-Mcp1-promoter-driven enhanced green fluorescence protein (EGFP) was useful as an early stress response reporter[18]. To test whether this stress promoter was also effective in an NMDA injury model, we counted the number of EGFP-positive RGCs after NMDA injection with or without hesperidin treatment in AAV2-Mcp1-promoter EGFP-injected mice. In PBS-treated retinas, there were few EGFP-positive RGCs in the retina. Retinal NMDA injury strongly increased the number of EGFP-positive RGCs, and this increase was totally suppressed by hesperidin treatment 6 h after NMDA injection (Fig. 1G-H). These results suggest that an Mcp1 promoter drives RGC damage under neuroinflammation, similar to the effects of optic nerve crush in the retina, and that hesperidin reduced RGC damage by suppressing neuroinflammation.

Hesperidin suppressed TNFα-induced NF-kB activation in vitro

The above results are consistent with our previous work showing that hesperidin has anti-inflammatory effects in the NMDA-injured retina [14]. To identify the mechanisms underlying the anti-inflammatory effect of hesperidin in the retina, we evaluated the activation of NF-kB, a transcriptional factor known as a

key regulator of cytokine expression. We first prepared stable transfected HT22 cells with a NF-kB-luciferase reporter signal plasmid. After TNFα stimulation, an NF-kB reporter gene assay showed that NF-kB activity was strongly increased and that 0.01%, 0.1% and 1% hesperidin treatment significantly suppressed this increase (Fig. 2A). The transcriptional expression of MCP-1 was also suppressed by pre-treatment with 1% hesperidin before TNFα stimulation (Fig. 2B). To determine whether hesperidin had a cytotoxic effect, we performed a calcein assay of cells that received hesperidin and TNFα stimulation. Even with 1% hesperidin treatment, cell viability was not significantly changed (Fig. 2C), suggesting that the reduction of NF-kB activity and MCP-1 expression were not due to cell death or reduced cell numbers. The transcriptional activity of NF-kB is revealed by nuclear translocation and regulated by IkB; thus, we set out to determine whether hesperidin could suppress NF-kB translocation and IkB degradation. Immunocytochemistry analysis showed that the NF-kB immunofluorescence signal was translocated into the nuclei in TNFα-treated HT22 cells, and revealed that pre-treatment with hesperidin suppressed NF-kB immunoreactivity in the nuclei (Fig. 2D). Similarly, an immunoblot analysis showed that NF-kB protein signal intensity increased in the nuclear fraction after TNFα stimulation and was reduced after pre-treatment with hesperidin (Fig. 2E). In contrast, the IkB content of the cytosolic fraction was reduced after TNFα stimulation, and this reduction was reversed after hesperidin treatment (Fig. 2F). The results described above suggest that the anti-inflammatory effect of hesperidin is due to the inhibition of NF-kB nuclear translocation in NMDA-injured retinas.

Hesperidin treatment synergistically reduces apoptotic cell death in the retinas of CHOP-deficient mice

A previous study showed that ER stress involves NMDA-induced retinal damage. Furthermore, the intravitreal injection of NMDA induces CHOP expression in the RGCs and RGC death in mice. Interestingly, CHOP-deficient mice are resistant to this effect with low doses of NMDA (2-5 nmol), but not when the dose is high (10 nmol) [13]. We hypothesized that CHOP-deficient mice that receive a high dose of NMDA undergo activation of augmented cell death pathways, such as neuroinflammation, in addition to the ER stress cell death pathway. To test this hypothesis, we compared the expression levels of inflammatory response signals in CHOP-deficient mice and control mice. In the retinas of wild-type (WT) mice, inflammatory cytokines and chemokines, such as TNFα, IL-1β, IL-6 and MCP-1, significantly increased 24 h after NMDA injection (Fig. 3A-D). Quantitative PCR also showed that transcriptional levels of TSPO, known as an activated microglia marker[22], increased 24 h after NMDA injection (Fig. 3E). A recent study reported that the RIP3 signaling cascade mediates the pro-inflammatory response[23]. The gene expression of RIP3 and TNF-receptor 1 (TNF-R1), upstream of RIP3, were also significantly elevated 24 h after NMDA injection (Fig. 3F and H). Immunoblot analysis also showed that the content of RIP3 in the NMDA-injured retinas was higher than in PBS-injected retinas (Fig. 3G). Interestingly, these inflammatory molecules were more highly expressed after NMDA injection in the retinas of CHOP-deficient mice than WT mice (Fig. 3A-H).

If increased inflammatory molecules contribute to retinal cell death in NMDA-injured CHOP-deficient mice, we speculated that anti-inflammatory treatment should be partially preventative. To test this, we treated CHOP-deficient and WT mice with intravitreal hesperidin in addition to NMDA injection (Fig. 4A). After 24

h, TUNEL-positive apoptotic cells in GCL increased in the retinas of NMDA-treated WT mice, while they tended to decrease (p=0.08) in the WT mice that also received hesperidin (Fig. 4B). Moreover, consistent with previous reports[13], the CHOP-deficient mice showed no ability to resist RGC death after NMDA injury compared with WT mice. However, apoptotic cell death in the GCL was clearly reduced in the hesperidin-treated CHOP-deficient mice compared with the WT mice (Fig. 4B). On the other hand, these synergistic rescue in combination with hesperidin treatment and CHOP deletion could not reveal in INL (Fig. 4C). This result described above suggest synergistic neuroprotective effect when combined with ER stress blockade and an anti-inflammatory treatment based on hesperidin was revealed to RGC, but not inner retinal cells such as bipolar cells or horizontal cells.

Discussion

This study focused on the synergistic neuroprotective effect of anti-inflammatory treatment and ER stress blockade in RGCs. We found that inflammatory molecules in the retinas of CHOP-deficient mice were more numerous than in WT mice after NMDA injury. Hesperidin treatment suppressed the inflammatory response in NMDA-injured retinas and clearly ameliorated apoptotic cell death in the retinas of CHOP-deficient mice. These results demonstrate that neuroinflammation may be a redundant contributor to retinal cell death in CHOP-deficient mice after NMDA injury, and that simultaneous inhibition of ER stress and neuroinflammation is an effective neuroprotective strategy for the treatment of excitotoxic retinal disorders.

Previously, we revealed that hesperidin could not only suppress neuroinflammation, but also oxidative stress and the over-activation of calpain after NMDA injury[14]. On the other hand, hesperidin could not attenuate ER stress pathways, such as CHOP, GRP78 or HSP60 (Supp Fig. 1). Thus, we considered that a method combining hesperidin treatment and ER stress blockade would be a reasonable approach to modify multiple cell death signaling pathways in NMDA-injured RGCs. After retinal detachment, photoreceptor cell death is caused mainly by apoptosis. However, caspase inhibition decreases apoptosis and increases necroptosis, and it has been reported that inhibiting both apoptosis and necroptosis, with Z-VAD and Nec-1, respectively, effectively prevents photoreceptor cell death[24]. Thus, the inhibition of specific signaling pathways is not enough for effective neuroprotection, due to the augmentation of other cell death pathways, and inhibiting multiple signal cascades will be necessary to provide significant neuroprotection against retinal cell death.

Previous work has demonstrated that hesperidin inhibits cell migration and proliferation in vitro[25][26], and our results clearly show that hesperidin treatment prevents neuroinflammation in the retina. To test whether this anti-inflammatory effect was due to suppression of microglial infiltration, we performed immunostaining. Retinal Iba1 immunostaining showed that the number of retinal microglia did not significantly change in mice after NMDA injury (data not shown). This finding suggests that the anti-inflammatory effect of hesperidin is directly due to the suppression of NF-kB translocation and activity. Moreover, TNFα has recently been identified as a necessary and sufficient factor to induce RGC death, and it has been shown that it acts by promoting neurotoxic astrocyte states[27]. In a mouse model of

glaucoma, TNFα levels increased after elevating intraocular pressure, and the retinal microglia mediated the cytotoxic effects of TNFα[28]. In addition, hesperetin has been shown to inhibit microglia-mediated neuroinflammation by suppressing cytokines and the iNOS and MAPK pathways[29]. Thus, hesperidin may contribute to neuroprotection of the RGCs by suppressing microglial activity and the inflammatory response, but it does not prevent cell infiltration into the retina.

In CHOP-deficient mice, we found that hesperidin did not prevent retinal cell death after NMDA damage, despite the elevation of CHOP after NMDA injection[13]. We hypothesized that this may have been due to other cell death pathways being augmented in CHOP-deficient mice. In the current study, we found that an excessive inflammatory response was promoted in CHOP-deficient mice after NMDA injury.

Proinflammatory cytokines are important contributors to retinal cell death. The augmentation of the inflammatory response in CHOP-deficient mice may be due to the involvement of necroptosis. Necroptosis is known as “programmed necrosis” and is activated by the TNF/RIP kinase pathway under conditions of apoptosis inhibition[30]. Necrosis involves membrane rupture and the induction of an inflammatory response, such as macrophage infiltration and proinflammatory cytokine elevation. Necroptosis promotes the activation of the RIP1/RIP3 pathway and the suppression of caspase-8 activation[31]. ER stress promotes caspase-8 activation[32], suggesting that caspase-8 activation may be inhibited in CHOP-deficient mice after NMDA injury. Consistent with the above, we observed that RIP3 was elevated in the NMDA-injured retinas of CHOP-deficient mice. The RIP3 molecule also regulates inflammation and has a role as a key mediator of inflammation[23][33]. In addition, inflammation augments microglial activation. We showed that TSPO, a marker of microglial activation[22], was also elevated after NMDA injection in CHOP-deficient mice. This suggests that the microglia in CHOP-deficient mouse retinas may be highly activated, resulting from the inflammatory response after NMDA injury. In summary, the retina in CHOP-deficient mice may be susceptible to an excessive inflammatory response and microglial activation, resulting from the induction of necroptotic cell death in association with the activation of the RIP1/RIP3 pathway after NMDA injury.

Declarations

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Authors’ contributions

KS, TS, MOO, MO, SM, YS, TY, and KF performed the experimental work. KS, MOO, MY, NH, KO, and KMN conceived and designed the experiments, analyzed the data and advised on data interpretation. KS and TN composed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animal experiments were approved by Tohoku University, following the Guidelines for Animals in Research. The protocol number is 2017-229.

Consent for publication

Not applicable.

Competing interests

None of the authors have any competing interests in the manuscript

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Figures

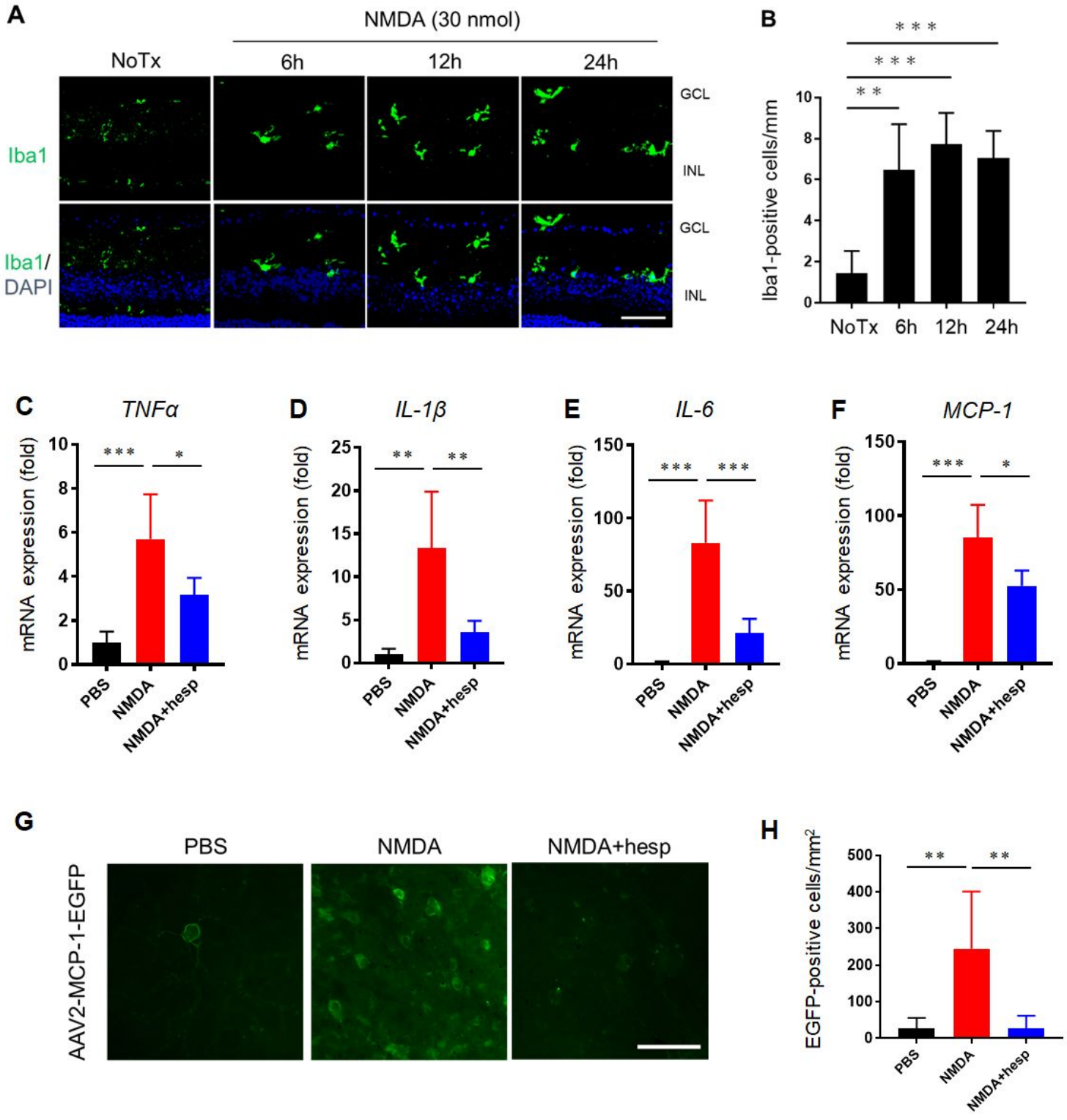

Figure 1

The effects of hesperidin as an anti-inflammatory and attenuator of the RGC stress reporter response in NMDA-injured retinas. (A) Representative immunohistochemistry images showing Iba1-positive microglia (shown in green) in the retina after NMDA injection. Scale bar: 50 μm. (B) Histogram showing the number of Iba1-positive cells in whole retinal sections (N=4-5). This statistical analysis used Dunnett's test. Transcriptional levels of TNFα (C), IL-1b (D), IL-6 (E) and MCP-1 (F) in the retinas of mice treated with

hesperidin 6 h after NMDA injury (N=6). This statistical analysis used the Tukey-Kramer test. (G) The MCP-1 promoter-driven EGFP signal (AAV2-Mcp1-EGFP) in RGCs 6 h after NMDA injury with or without hesperidin. (H) Histogram showing the average number of EGFP-positive cells in the samples from (G) (N=4-6). Error bars denote SD. ▯P < 0.05, ▯▯P < 0.01, ▯▯▯P < 0.001.

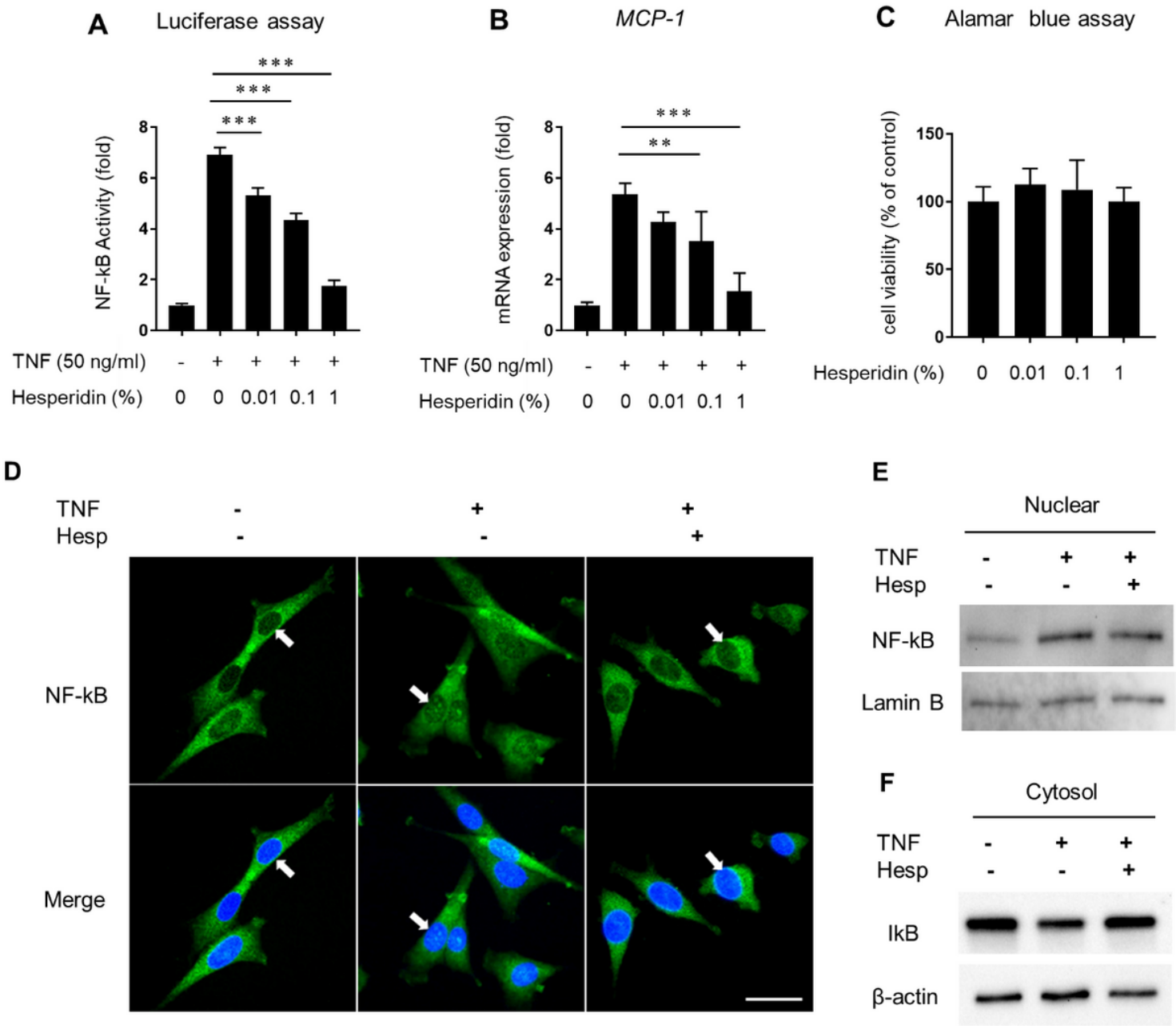

Figure 2

Suppression of NF-kB activity by hesperidin in vitro. (A) Cells were pre-treated with hesperidin for 2 h and stimulated with TNFα for 4 h. Then, NF-kB activity was measured with a luciferase assay. This statistical analysis used Dunnett's test. (B) Relative expression levels of MCP-1 mRNA in HT22 cells after hesperidin treatment, 8 h after TNFα stimulation. This statistical analysis used Dunnett's test. (C) Histogram showing cell viability, assessed after treatment with various concentrations of hesperidin for 2 h and treatment with TNFα for 4 h, represented as quantitative data from an Alamar blue assay. The data are

shown as the percentage of viable cells compared to cells treated with vehicle without hesperidin under TNFα stimulation. (D) Representative immunofluorescence images showing the distribution of NF-kB after treatment with TNFα and/or 1% hesperidin. Immunoblot images for NF-kB in the nucleus (E) and IkB in the cytosol (F) after treatment with TNFα and/or hesperidin. Error bars denote SD. ▯P < 0.05,▯▯P < 0.01,▯▯▯P < 0.001.

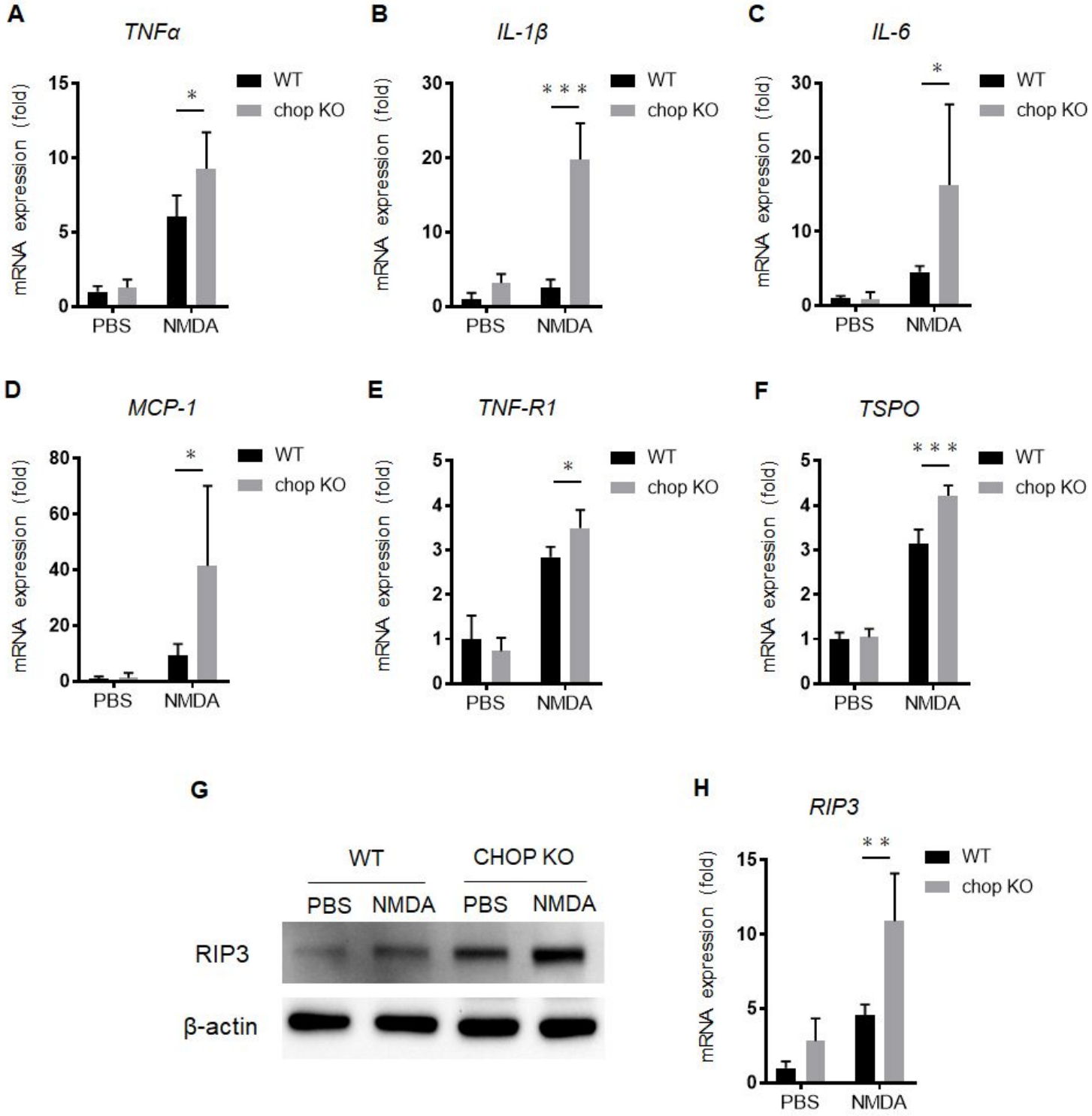

Figure 3

CHOP deficiency promoted an inflammatory response in NMDA-treated retinas. The relative mRNA levels of inflammatory molecules such as TNFα (A), IL-1b (B), IL-6 (C), MCP-1 (D), TNF-R1 (E) and TSPO (F) were compared in the eyes of WT mice and CHOP-deficient mice 24 h after treatment with NMDA. This statistical analysis used the Student t-test. RIP3 protein levels (G) and mRNA levels (H) were measured with Western blotting and qPCR. This statistical analysis used the Student t-test. Error bars denote SD (N=4-6).□P < 0.05,□□P < 0.01,□□□P < 0.001.

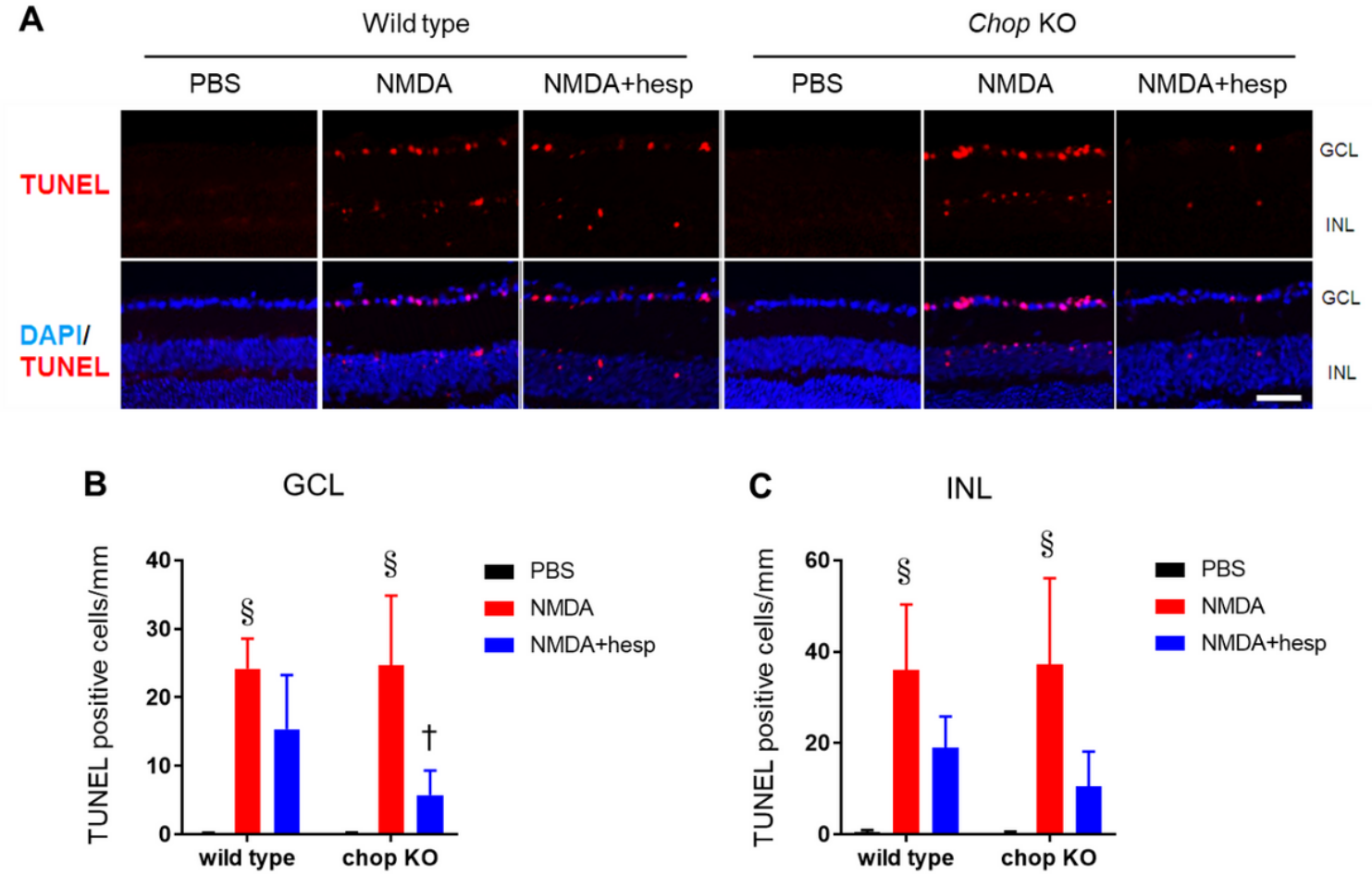

Figure 4

Hesperidin augmented protection against apoptotic cell death in the retinas of CHOP-deficient mice. (A) Retinal sections from WT or CHOP-deficient mice were analyzed with a TUNEL assay to detect apoptotic cell death in the retina 24 h after treatment with hesperidin and NMDA injury. (B) Histogram showing the average number of TUNEL-positive cells in whole retinal sections of the GCL (B) and INL (C). Scale bar: 50 μm. Error bars denote SD (N=6-9). This statistical analysis used the Tukey-Kramer test (§: p<0.05 vs. PBS-injected wild-type or CHOP-deficient mice). This statistical analysis used a two-way ANOVA followed by the Holm-Sidak multiple comparison test (□: p<0.05 vs. NMDA-injured wild-type mice with hesperidin treatment).

Supplementary Files

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- FigureS1.docx