Article

Discovery Medicine 2025; 37(194): 525–534
https://doi.org/10.24976/Discov.Med.202537194.44

Anti-Inflammatory Effect of Calycosin on Hypoxia-Induced Retinal Pigment Epithelium Cells

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Published: 20 March 2025

Background: Calycosin is thought to have anti-cancer and anti-inflammatory characteristics; however, more research is needed to determine how it impacts retinal pigment epithelium (RPE) cells. This study aims to explore the effects of calycosin on RPE cells under hypoxia.

Methods: Experimental hypoxia was induced by treating RPE cells with cobalt chloride for 2, 4, and 6 h. To investigate the effect of calycosin on RPE cells under hypoxia, RPE cells were treated with calycosin and cobalt chloride ($CoCl_2$). Cells were assessed for viability (Cell Counting Kit-8 assay) and apoptosis (flow cytometry). Inflammatory cytokines (enzyme-linked immunosorbent assay) and genes or proteins related to apoptosis and the hypoxia-inducible factor-1α (*HIF-1*α)/nuclear factor-κB (*NF-*κ*B*) axis (quantitative real-time polymerase chain reaction and western blot) were measured.

Results: Under hypoxic conditions, RPE cells showed reduced viability but increased levels of inflammation and apoptosis. The *NF-*κ*B* pathway was activated, and *HIF-1*α, apoptosis/*NF-*κ*B* pathway-related proteins (cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP); phosphorylated-p65 (p-p65), p-p65/p65), and inflammatory cytokines (interleukin-6 (IL-6) and interleukin-8 (IL-8)) were upregulated ($p < 0.001$). Calycosin weakened the effects of hypoxia on RPE cells ($p < 0.05$).

Conclusion: Calycosin inhibits the *HIF-1*α/*NF-*κ*B* axis and protects RPE cells from hypoxia-induced inflammation and apoptosis.

Keywords: calycosin; retinal pigment epithelium cells; hypoxia-inducible factor-1α/nuclear factor-κB axis

Introduction

Retinopathy of prematurity (ROP) has been reported to cause visual abnormalities and disabilities in preterm infants, along with retinal ischemia, which can cause injury and blindness in those with ROP [1,2]. Interruption of the retinal blood supply induces hypoxia and retinal cell damage [3]. Retinal pigment epithelium (RPE) cells are located near choroidal capillaries where they are vulnerable to ischemia or hypoxia and where the movement of nutrients, metabolites, and fluid between the outer retina and the choroidal blood supply occurs [4]. It has been reported that oxidative damage to the RPE is one of the pathogenesis of ROP [4,5]. Furthermore, hypoxia promotes the secretion of basic fibroblast growth factor (bFGF), which plays an essential role in the onset of ROP [6]. The incidence of ROP-associated blindness in high-income nations has been reported to be less than 10% of total number of pre-term born infants. Conversely, in low- and middle-income countries, due to an increase in the survival of pre-term born infants and limitations in fundoscopic follow-up, the incidence is higher than 40% [7]. In a systematic study that reviewed the prevalence of ROP between 1985 and 2021, the total prevalence of ROP was 31.9%, and that of severe ROP was 7.5%. The highest ROP prevalence was found in low-middle-income countries with high mortality rates [8]. While adequate screening programs can prevent ROP-induced blindness, in some middle-income Asian nations, the number of tertiary eye care centers that can perform this type of surgery is limited. Furthermore, poor prognosis, retinal detachment, and shortage of experienced surgeons and anesthetists make management of ROP and its associated blindness difficult [9]. Therefore, new guidelines and protocols are urgently required.

As an isoflavonoid phytoestrogen with both anti-inflammatory and anti-tumor properties, calycosin is a component of *Astragali radix* [10,11]. Shi *et al.* [12] reported that calycosin mitigated chondrocyte inflammation by regulating phosphatidylinositol 3-kinase/protein kinase B (*PI3K*/*AKT*) and nuclear factor-κB (*NF-*κ*B*) pathways. In the mice with renal ischemia-reperfusion injury (IRI), calycosin reduced early growth response 1 (*EGR1*) expression and attenuated *NF-*κ*B*-mediated inflammation to produce a protective effect on renal IRI [13]. Inflammation has been shown to play an important role in both physiological and pathological retinal angiogenesis. There is growing evidence that perinatal inflammation or infection contributes to the pathogenesis of ROP [7,14,15]. Neonatal inflammation is a key regulator of ROP development and progression

[7], and systemic inflammation in neonates can disrupt retinal vessel development and induce pathological features of ROP in animal models [16,17]. Nevertheless, it is unclear whether calycosin plays an anti-inflammatory role in ROP.

The impact of hypoxia-inducible factor-1α (*HIF-1*α) on hypoxia has been reported [18,19]. The function of *HIF-1*α is mediated through the regulation of the *NF-*κ*B* pathway [20–22]. However, it is unclear whether *HIF-1*α and the *NF-*κ*B* axis are also implicated in the effect of calycosin on the ROP. Therefore, we explored the effects of calycosin on RPE cells and its possible interaction with *HIF-1*α and the *NF-*κ*B* axis *in vitro* in an attempt to figure out a clinical strategy for ROP prevention and treatment in the future. Herein, RPE cells were treated with cobalt chloride ($CoCl_2$) and then calycosin, and the effect of calycosin on the viability, apoptosis, inflammation, and *HIF-1*α/*NF-*κ*B* axis was examined.

Materials and Methods

Cell Culture and Treatment

Dulbecco's modified Eagle's medium/F12 medium (PM150312, Procell, Wuhan, China) containing 10% fetal bovine serum (164210, Procell, Wuhan, China) and 50 U/mL penicillin–50 µg/mL streptomycin (PB180120, Procell, Wuhan, China) was prepared for culture (37 °C, 5% CO_2) of the human RPE cell line ARPE-19 (BNCC337713, BeiNa Bio, Beijing, China). ARPE-19 cells were identified by short tandem repeat (STR) and tested for mycoplasma, and they were found to be free of mycoplasma contamination.

Cobalt chloride ($CoCl_2$, #60818) and calycosin (B9938) were purchased from Sigma-Aldrich (St. Louis, MO, USA). To establish the hypoxia model, ARPE-19 cells were treated with 600 µmol/L $CoCl_2$ for 2, 4, and 6 h as previously described [2]. To determine the effects of calycosin on hypoxia-treated ARPE-19 cells, calycosin (1, 5, 10, 20, 50, and 100 µmol/L) was added for 48 h, and cells were subsequently transferred to $CoCl_2$ (600 µmol/L) for 6 h. The concentration of calycosin was based on an earlier study [23].

Cell Counting Kit-8 (CCK-8) Assay

APRE-19 cells (5×10^3/well) were seeded in 96-well plates (37 °C, 5% CO_2) and subjected to hypoxia for 2, 4, and 6 h, and the viability was examined. Subsequently, cells were treated with calycosin (1, 5, 10, 20, 50, and 100 µM) for 48 h, and the viability was re-examined. The cells in the control group were cultured normally without any treatment. The cells were cultured with 10 µL of CCK-8 reagent (CA1210, Solarbio, Beijing, China) for 4 h at 37 °C, followed by absorbance measurement at 450 nm by using a Microplate Reader (iMark, Bio-Rad, Hercules, CA, USA).

Table 1. Primers used for qRT-PCR.

Gene	Primers (5′-3′)
HIF-1α	
F	CAGCAGACTCAAATACAAGA
R	TCTATATGGTGATGATGTGG
GAPDH	
F	GAGAAGGCTGGGGCTCATTT
R	AGTGATGGCATGGACTGTGG

Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction; *HIF-1*α, hypoxia-inducible factor-1α; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, Forward; R, Reverse.

Cell viability (%) =[(OD test – OD blank)/(OD control – OD blank)] × 100%.

Flow Cytometry

The Annexin V-FITC Cell Apoptosis Kit (130-092-052, Miltenyi Biotech, Bergisch Gladbach, Germany) was used to assess apoptosis. APRE-19 cells (1×10^5/well) seeded in 96-well plates were treated with annexin V and propidium iodide (5 µL, for both) and incubated at room temperature in darkness for 15 min. Apoptosis was assessed with the Guava easyCyte Benchtop Flow Cytometer (BR168323; Luminex, Austin, TX, USA). Data were analyzed with Kaluza C Analysis Software (ver. 2.1, Beckman Coulter, Indianapolis, IN, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ARPE-19 cells (1×10^5/well) were cultured in 96-well plates (37 °C, 5% CO_2), and the cell culture supernatant was collected for the measurement of inflammatory cytokines interleukin-6 (IL-6) and IL-8 (KAC1261; BMS204-3, Invitrogen, Carlsbad, CA, USA) using corresponding ELISA kits, followed by absorbance measurement at 450 nm by using a Microplate Reader (iMark, Bio-Rad, Hercules, CA, USA). A standard curve was drawn with optical density (OD) values as ordinate and the standard substance concentration as abscissa. Specimen OD values were used to identify the cytokine level of samples to be tested.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from ARPE-19 cells using TRIzol (15596-018, Invitrogen, Carlsbad, CA, USA), followed by determination of concentration using a NanoDrop Lite Spectrophotometer (ND-LITE, Thermo Fisher Scientific, Waltham, MA, USA). The First Strand cDNA Synthesis Kit (K1651, Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis. The qRT-PCR Kit (600182, Agilent, Santa Clara, CA, USA) was used for amplification of target genes in a Touch real-time PCR system (CFX384, Bio-Rad, Hercules, CA, USA) at 98

Table 2. Antibodies used for western blot.

Antibody	Host	Dilution ratio	Catalog no.
HIF-1α	mouse	1:1000	ab1
Cleaved caspase-3	rabbit	1:500	ab32042
Cleaved PARP	rabbit	1:5000	ab32064
p65	rabbit	1:2000	ab16502
p-p65	rabbit	1:5000	ab86299
GAPDH	rabbit	1:10,000	ab181602
Goat anti-rabbit IgG H&L	goat	1:2000	ab205718
Goat anti-mouse IgG H&L	goat	1:2000	ab205719

All primary and secondary antibodies were purchased from Abcam (Cambridge, UK). Abbreviations: HIF-1α, hypoxia-inducible factor-1α; PARP, poly (ADP-ribose) polymerase; p-p65, phosphorylated-p65; IgG, Immunoglobulin G.

°C (2 min), followed by 40 cycles at 95 °C (15 s) and 60 °C (30 s) [24]. Relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method, and the internal reference was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [25]. Table 1 lists the primer sequences.

Western Blot

Total proteins were extracted from cells using radio immunoprecipitation assay (RIPA) lysis buffer (R0010, Solarbio, Beijing, China), and protein levels were measured using the Bicinchoninic Acid Protein Kit (PC0020, Solarbio, Beijing, China) as previously described [26]. In brief, 20 µg protein lysate from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (P1200, Solarbio, Beijing, China), transferred onto polyvinylidene fluoride membranes (YA1701, Solarbio, Beijing, China), blocked (5% fat-free milk, 2 h), and incubated with primary antibodies (4 °C, overnight, GAPDH as the internal reference). Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (RT, 1 h), followed by washing in Tris-buffered saline containing Tween-20 (TBST, T1085, Solarbio, Beijing, China) three times. Target proteins were visualized with an enhanced chemiluminescence kit (PC0018S, Solarbio, Beijing, China), followed by image acquisition (iBright CL750 Imaging System, A44116, Thermo Fisher Scientific, Waltham, MA, USA) and densitometry (ImageJ 5.0, National Institutes of Health, Bethesda, MD, USA). Table 2 lists the antibodies used.

Statistical Analysis

Data from independently repeated (thrice) experiments are represented as mean ± standard deviation (SD). Data were analyzed by GraphPad Prism 8.0 (GraphPad Inc., La Jolla, CA, USA) and SPSS 20.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA), followed by the Tukey post hoc test, was used for statistical significance determination. $p < 0.05$ was deemed statistically significant.

Results

Hypoxia Suppressed Cell Viability Yet Promoted HIF-1α Expression and Apoptosis in RPE Cells

The chemical structure of calycosin is shown in Fig. 1A. To understand how hypoxia influences RPE cells, viability was evaluated after cells were exposed to $CoCl_2$ (2/4/6 h). We found that hypoxia caused a decrease in viability at 4 and 6 h (Fig. 1B, $p < 0.05$), suggesting hypoxia suppresses RPE cell viability.

Given that *HIF-1α* is involved in hypoxia [27], we measured its expression in RPE cells after hypoxia and found that its expression increased during hypoxia at 4 and 6 h (Fig. 1C–E, $p < 0.001$), showing *HIF-1α* is upregulated by hypoxia.

Compared to the control group (without hypoxia treatment), the apoptosis rate was higher after hypoxia (Fig. 1F,G, $p < 0.001$), indicating hypoxia promotes apoptosis in RPE cells. Furthermore, the expression levels of cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP), two apoptosis-related factors [28], increased during hypoxia at 4 and 6 h (Fig. 1H,I, $p < 0.001$).

Hypoxia Caused Inflammation and NF-κB Pathway Activation in RPE Cells

Cytokine levels were measured by ELISA, and inflammatory cytokines (IL-6 and IL-8) in RPE cells were upregulated during hypoxia at 4 and 6 h (Fig. 2A,B, $p < 0.001$), indicating hypoxia aggravates inflammation in RPE cells.

Next, we measured the levels of *NF-κB* pathway-related factors in RPE cells and found that both p-p65 expression and the p-p65/p65 ratio were increased during hypoxia at 4 and 6 h (Fig. 2C–E, $p < 0.001$), revealing hypoxia activates the *NF-κB* pathway in RPE cells.

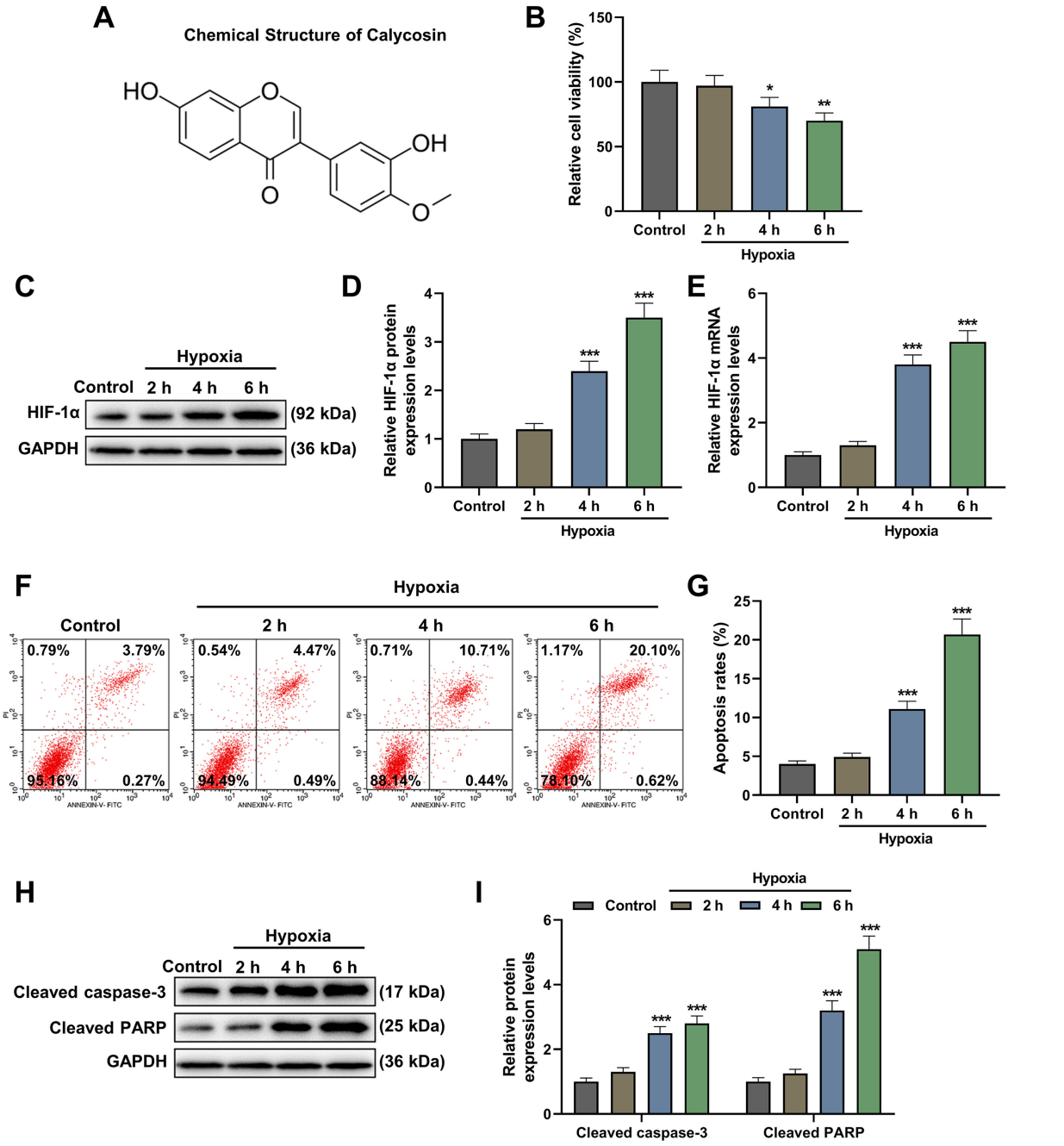

Fig. 1. Hypoxia suppressed cell viability yet promoted *HIF-1α* expression and apoptosis in RPE cells. (A) Chemical structure of calycosin. In RPE cells, after 2/4/6-h hypoxia treatment, the detection was performed on (B) the viability (CCK-8 assay), (C,D) relative *HIF-1α* protein expression (western blot, GAPDH as internal control), (E) relative *HIF-1α* mRNA expression (qRT-PCR, GAPDH as internal control), (F,G) the apoptosis (flow cytometry), and (H,I) relative protein expressions of cleaved caspase-3 and cleaved PARP (western blot, GAPDH as internal control). All experiments were repeated triplicate and data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. Control. RPE, retinal pigment epithelium; *HIF-1α*, hypoxia-inducible factor-1α; qRT-PCR, quantitative real-time polymerase chain reaction; PARP, poly (ADP-ribose) polymerase; CCK-8, Cell Counting Kit-8.

Calycosin Attenuated the Influences of Hypoxia on Viability, HIF-1α Expression and Apoptosis in RPE Cells

Based on the CCK-8 assay, we found that calycosin significantly affected cell viability when cells were treated with concentrations of calycosin above 10 μmol/L (Fig. 3A, $p < 0.05$). Therefore, calycosin at 1, 5, and 10 μmol/L was used for subsequent studies and designated as calycosin-low (L), calycosin-medium (M), and calycosin-high (H).

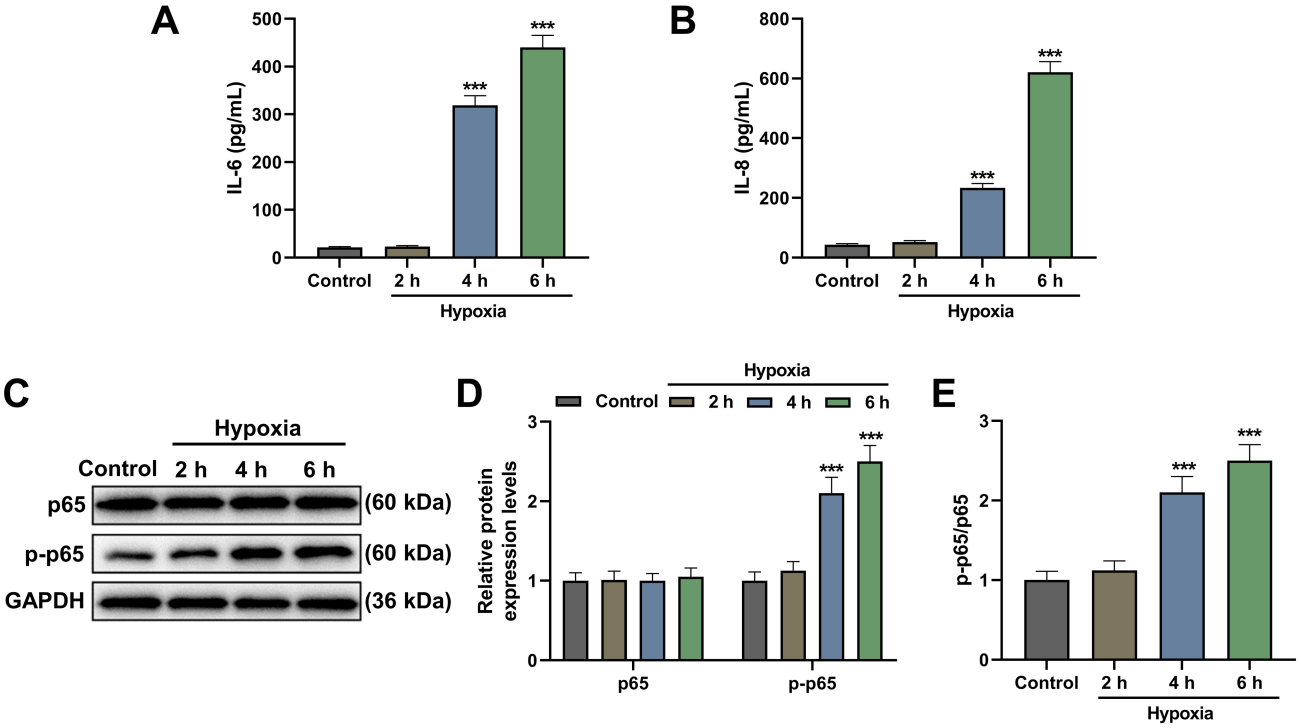

Fig. 2. Hypoxia caused inflammation and *NF-κB* pathway activation in RPE cells. In RPE cells after 2/4/6-h hypoxia intervention, (A,B) levels of inflammatory cytokines IL-6 (A) and IL-8 (B) (ELISA); (C,D) relative protein expression of p-p65 and p65 (western blot, GAPDH as the internal control); and (E) ratio of p-p65/p65 were assessed. All experiments were independently performed in triplicate, with data represented as mean ± standard deviation (SD). $^{***}p < 0.001$, vs. Control. *NF-κB*, nuclear factor-κB; IL-6, interleukin-6; IL-8, interleukin-8; ELISA, enzyme-linked immunosorbent assay; p-p65, phosphorylayted-p65.

We treated RPE cells with 1, 5, and 10 µmol/L calycosin for 48 h and then exposed the cells to hypoxia for 6 h. Using the CCK-8 assay, we found that hypoxia decreased the cell viability, whereas calycosin enhanced the cell viability of hypoxia-treated RPE cells (Fig. 3B, $p < 0.05$).

We also measured *HIF-1α* expression after hypoxia and calycosin treatment and observed that *HIF-1α* expression was upregulated following hypoxia, whereas calycosin decreased *HIF-1α* expression in hypoxia-treated RPE cells (Fig. 3C–E, $p < 0.001$).

Finally, we examined RPE cell apoptosis by measuring apoptosis-related factor expression. We found that after hypoxia, both apoptosis rates and apoptosis-related factors (cleaved caspase-3 and cleaved PARP) expression increased (Fig. 3F–I, $p < 0.001$) but decreased by calycosin treatment (Fig. 3F–I, $p < 0.05$), suggesting calycosin attenuates the impact of hypoxia on viability, *HIF-1α* expression, and apoptosis in RPE cells.

Calycosin Alleviated the Role of Hypoxia in Inflammation and NF-κB Pathway Activation in RPE Cells

Subsequently, we determined the effects of hypoxia and calycosin treatment on inflammation and *NF-κB* pathway activation in RPE cells. We found that the levels of IL-6 and IL-8 decreased by calycosin treatment in hypoxia-treated RPE cells (Fig. 4A,B, $p < 0.001$). Additionally, hypoxia increased the levels of p-p65 and p-p65/p65, whereas the levels were decreased by calycosin in hypoxia-treated RPE cells (Fig. 4C–E, $p < 0.001$).

Discussion

ROP, a devastating neurovascular disease of the retina in newborn infants, is one of the leading causes of preventable blindness in children [29,30]. RPE cells have been suggested to contribute to the retinal secretome, and their role in the pathophysiology of those most neovascular pathologies within the retina, for instance, age-associated macular degeneration, diabetic retinopathy, and ROP, has been reported [31]. RPE cells are also determinants of retinal disease, and their survival is critical in ocular health [32]. As a state that occurs both systemically and locally within the human body, hypoxia has been indicated to impact several organs and tissues such as the retina [33]. The response to hypoxia, which is involved in retinal development, physiology, and pathophysiology, is considered a fundamental phenomenon that is under the regulation of HIFs [34]. As a heterodimeric transcription factor, HIF-1 comprises two subunits, *HIF-1α* and *HIF-1β* [35]. *HIF-1α* is regulated by both oxygen levels and oxygen-sensitive post-translation modifications, while *HIF-1β* is constitu-

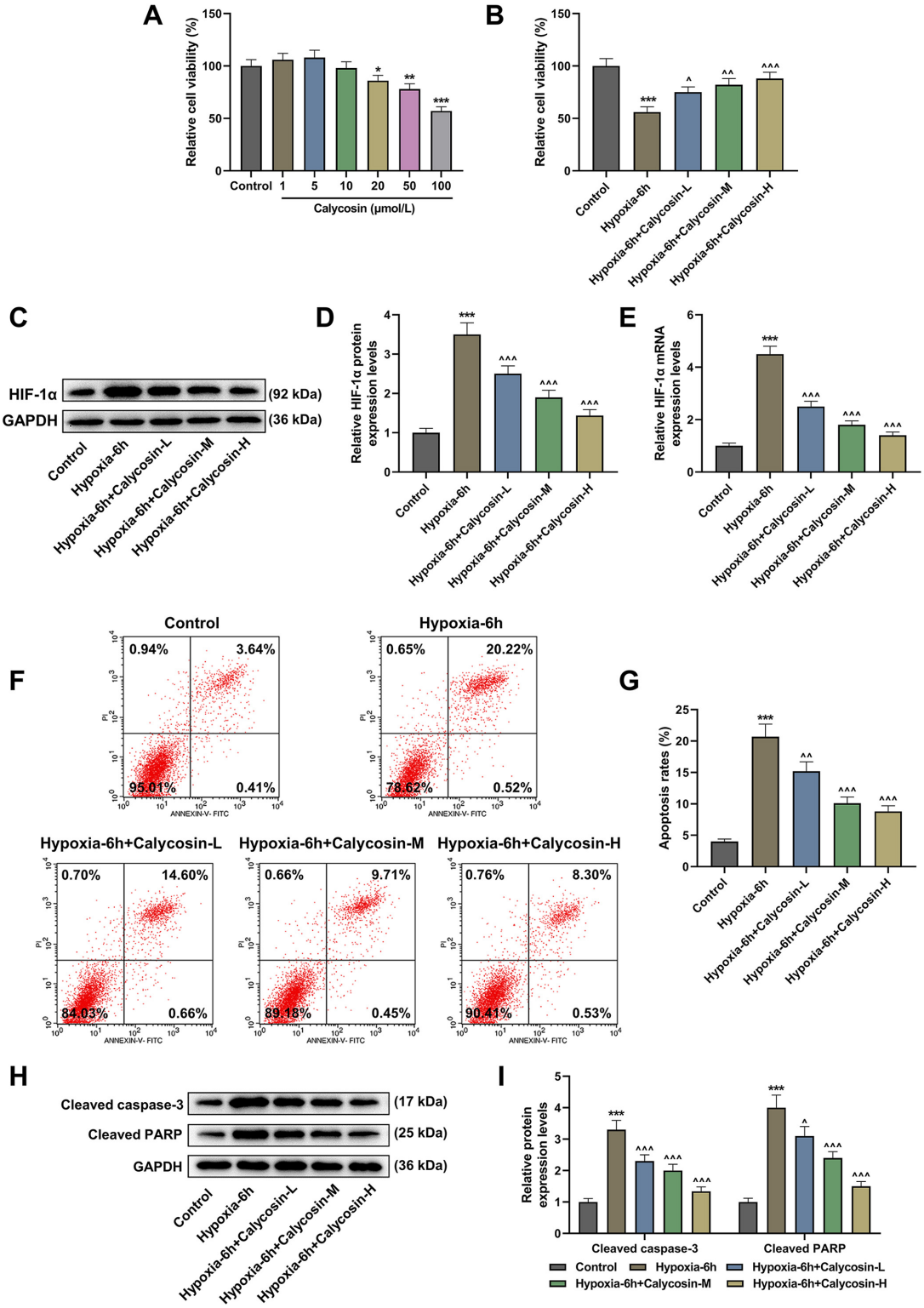

Fig. 3. Calcysosin attenuated the influences of hypoxia on viability, *HIF-1α* expression, and apoptosis in RPE cells. (A) Effect of calcysosin on cell viability (CCK-8 assay). (B) Viability of hypoxia- and calcysosin-treated RPE cells (CCK-8 assay). (C,D) Relative *HIF-1α* protein expression in hypoxia- and calcysosin-treated RPE cells (western blot, GAPDH as the internal control). (E) Relative *HIF-1α* mRNA expression in hypoxia- and calcysosin-treated RPE cells (qRT-PCR, GAPDH as the internal control). (F,G) Apoptosis of hypoxia- and calcysosin-treated RPE cells (flow cytometry). (H,I) Relative protein expression of cleaved caspase-3 and cleaved PARP in hypoxia- and calcysosin-treated RPE cells (western blot, GAPDH as the internal control). All experiments were independently performed in triplicate, with data represented as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. Control; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, vs. hypoxia-6 h.

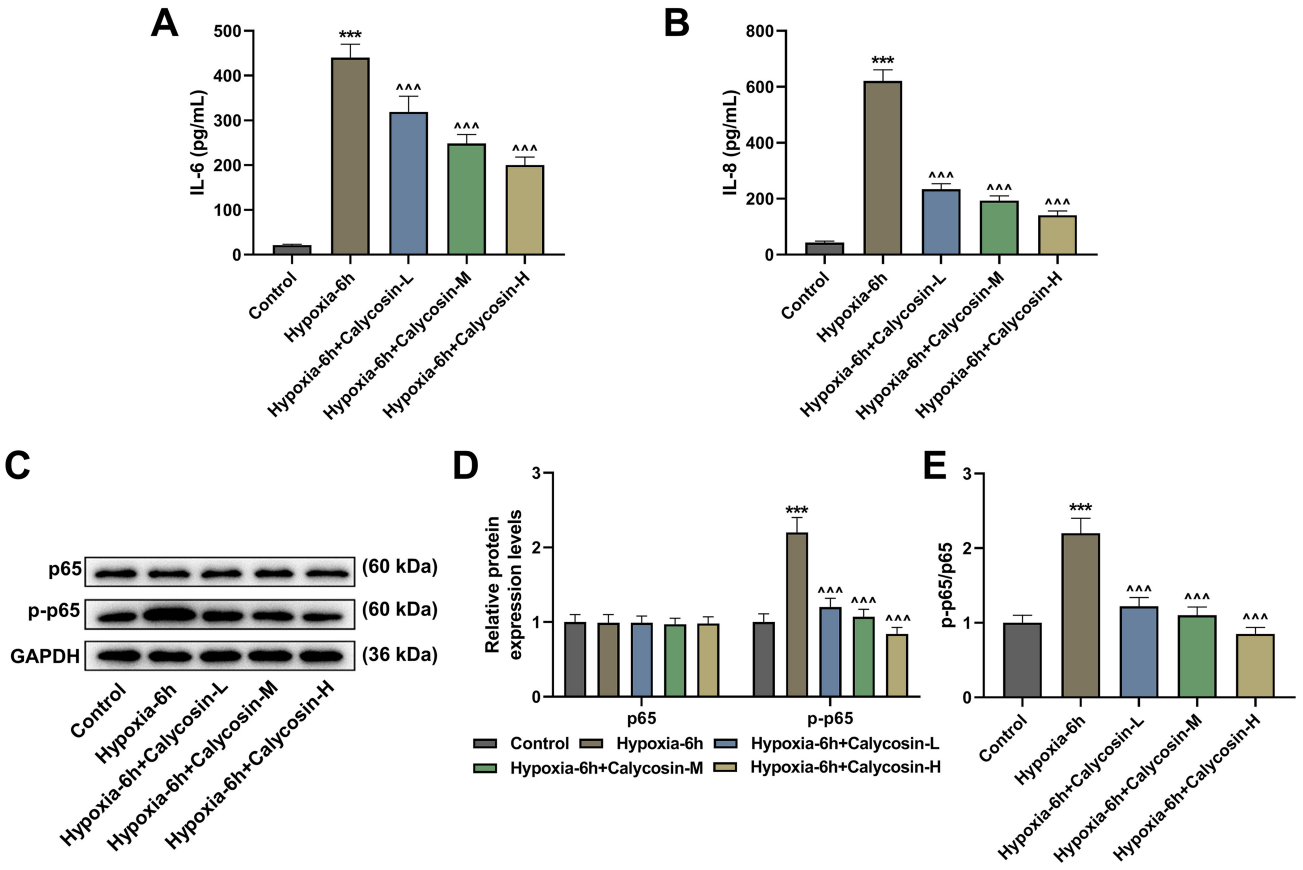

Fig. 4. Calycosin alleviated the roles of hypoxia in inflammation and *NF-κB* pathway activation in RPE cells. (A,B) Levels of inflammatory cytokines IL-6 (A) and IL-8 (B) in hypoxia- and calycosin-treated RPE cells (ELISA). (C,D) Relative protein expression of p-p65 and p65 in hypoxia- and calycosin-treated RPE cells (western blot, GAPDH as the internal control). (E) The ratio of p-p65/p65 in hypoxia- and calycosin-treated RPE cells. All experiments were independently repeated in triplicate, with data represented as mean ± standard deviation (SD). $^{***}p < 0.001$, vs. Control; $^{\wedge\wedge\wedge}p < 0.001$, vs. hypoxia-6 h.

tively expressed within the nucleus [36]. *HIF-1α* can impact the pathology of retinal diseases [37–39]. In addition, the balance between RPE cell proliferation and apoptosis is responsible for retinal diseases, and several factors, including cleaved caspase-3 and cleaved PARP, facilitate RPE cell apoptosis [28,40]. Casapse-3, a member of the caspase family, is activated or cleaved under the stimulation of the N-methyl-D-aspartate (NMDA) receptor, which promotes apoptosis [41]. The PARP family plays roles in DNA repair and cell apoptosis, and cleaved PARP is an apoptotic gene as well [42]. In our study, we used $CoCl_2$ to induce hypoxia in RPE cells *in vitro* and found that both HIF-1α and apoptosis-related genes (cleaved caspase-3 and cleaved PARP) were increased, although cell viability was decreased. These data imply that hypoxia negatively impacts viability and drives apoptosis and *HIF-1α* expression in RPE cells *in vitro*.

During inflammation, retinal cell hypoxia may be caused by an increase in oxygen consumption to support the increased metabolic activity of retinal cells. Early changes in blood flow have also been reported to contribute to acute and chronic inflammation of the retinal vasculature, which causes capillary dropout and progressive and irreversible ischemia and hypoxia [43,44]. Two inflammatory cytokines, IL-6 and IL-8, play roles in inflammation of hypoxia-treated RPE cells [33]. IL-6 is an acute inflammatory cytokine, whereas IL-8 is a granulocyte-attracting chemokine, and their levels are upregulated upon hypoxia induction [33]. In our study, their levels in RPE cells were upregulated upon hypoxia induction, indicating hypoxia can provoke inflammation in RPE cells *in vitro*.

As the main bioactive compound of *Astragali radix*, calycosin has been used in traditional Chinese medicine as an herbal medicine, with several anti-inflammatory and anti-tumor effects [45]. The pharmacological effects of calycosin and its associated compounds on hypoxia and ischemia have been elucidated [46–48]. Given that there is insufficient information on the efficacy of calycosin in hypoxia-induced RPE cells, in this study, we applied calycosin to hypoxia-induced RPE cells and found that hypoxia-associated factor expression, apoptosis, and inflammation were alleviated, while cell viability was promoted and apoptosis-related factors expressions and inflammatory cytokines were downregulated. These results show that caly-

cosin protects RPE cells from hypoxia-induced inflammation and apoptosis. However, the mechanism remains to be elucidated.

Canonical and non-canonical signaling pathway activation is required for transcription factors within the *NF-κB* pathway to mediate biological functions [49]. Canonical activation of the *NF-κB* pathway produces inflammation-related mediators and hypoxia [50,51]. P65, a dimeric transcription factor, and phosphorylated P65 are components of the *NF-κB* pathway [52,53]. In addition, the role of the *HIF-1α*/*NF-κB* axis has been discovered in lymphangiogenesis, shikonin inhibits lymphangiogenesis *in vitro* by interfering with the *NF-κB*/*HIF-1α* pathway [54]. However, it is unclear whether it is also implicated in hypoxia. In our study, we observed that hypoxia promoted both p-p65 and the p-p65/p-65 ratio in RPE cells, whereas calycosin weakened these effects in hypoxia-treated RPE cells, implying the impact of calycosin in protecting RPE cells from hypoxia-induced inflammation and apoptosis may be achieved by suppressing *HIF-1α* and the *NF-κB* axis.

This study has several limitations. We investigated the effects of calycosin and its interaction with the *HIF-1α*/*NF-κB* axis *in vitro*, but the effects need to be examined *in vivo*. Whether calycosin protects hypoxia-induced RPE cells by regulating *HIF-1α* and the *NF-κB* axis also remains to be investigated.

Conclusion

Taken together, we report that hypoxia results in decreased cell viability and increased inflammation and apoptosis of RPE cells. It also activates *HIF-1α* and the *NF-κB* pathway in RPE cells *in vitro*, whereas calycosin exerts ameliorative effects. Our study provides new evidence on the effects of calycosin in hypoxia-treated RPE cells, and our findings help provide a research basis for future clinical prevention and treatment of ROP.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Author Contributions

TZ designed the research study; BW, WH and HZ performed the research; RC collected and analyzed the data. All authors have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors have given the final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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