Inflamm. Res.
DOI 10.1007/s00011-015-0894-x

Inflammation Research

ORIGINAL RESEARCH PAPER

Anti-angiogenic and anti-inflammatory effect of Magnolol in the oxygen-induced retinopathy model

Boyu Yang[1] · Yue Xu[1] · Shanshan Yu[1] · Yongsheng Huang[1] · Lin Lu[1] · Xiaoling Liang[1]

Received: 15 September 2015 / Accepted: 27 October 2015

Abstract
Objective In the present study, we investigated the effects of Magnolol on the retinal neovascularization (RNV) and local glial cells in an oxygen-induced retinopathy (OIR) model and explored their molecular mechanisms.
Materials and methods Neonatal C57BL/6J mice were subjected to 75 % O_2 ± 5 % from postnatal day (P) 7 to P12 and subsequently returned to room air. Mice were injected with 25 mg/kg Magnolol intraperitoneally once a day from P12 to P17, then retinas were harvested and flat-mounted to assess the retinal vessels, astrocytes and microglia. To clarify the molecular mechanisms of Magnolol, we observed the level of inflammatory cytokines such as interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1, tumor necrosis factor-α, and analyzed the hypoxia-inducible factor (HIF)-1α/vascular endothelial growth factor (VEGF) pathway in OIR mice.
Results Intraperitoneal administration of Magnolol resulted in significant reduction of RNV without retinal toxicity or perturbation of developmental retinal angiogenesis. In addition, Magnolol preserved the astrocyte morphology and diminished the activation of microglia. Moreover, Magnolol down regulated the expression of inflammatory cytokines and inactivated the HIF-1α/VEGF pathway.
Conclusions These results indicated that Magnolol might have potential for the treatment of pathological retinal angiogenesis and glial dysfunctions via anti-inflammation and inhibition of HIF-1α/VEGF pathway.

Keywords Magnolol · Oxygen-induced retinopathy · Retinal neovascularization · Astrocytes · Microglia · Inflammation · HIF-1α/VEGF

Introduction

Ischemic retinopathy (IR), mainly includes retinopathy of prematurity (ROP), vascular occlusions, diabetic retinopathy and age-related macular degeneration, is the cause of blindness in all ages. It is characterized by retinal neovascularization (RNV) and glial dysfunctions [1]. As the most potent angiogenic factor, it is undoubted that vascular endothelial growth factor (VEGF) plays a pivotal role in the development of IR and has become an ideal target for regression of the RNV [2, 3]. However, intravitreal injection of anti-VEGF agents may cause several significant concerns such as endophthalmitis, inflammation, retinal fibrosis, potential neuronal and glial toxicity, off-target effect and short-lived effect [4, 5].

Recent studies have proved that retinal glial cells including macroglia and microglia are likely to be altered in the IR [1, 6]. Astrocytes and Müller cells belong to the retinal macroglia and play an assistant role in retinal development, metabolism and function maintenance. Astrocytes and Müller cells can provide support for retinal neurons [7], secrete VEGF for angiogenic sprouts [8], preserve the blood–retinal barrier [9] and act as a template to guide the development of retinal vasculature [10, 11]. Another retinal glial cell type is microglia, which is considered as the resident macrophages of retina. Microglia are

Responsible Editor: John Di Battista.

Boyu Yang and Yue Xu have contributed equally to this work.

✉ Xiaoling Liang
liangxlsums@qq.com

[1] State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510000, Guangdong Province, People's Republic of China

Published online: 07 November 2015

quiescent in the postnatal period but turn into activated state in response to hypoxia or inflammation. It has been found that microglia release both neurotoxic and pro-angiogenic factors in mice oxygen-induced retinopathy (OIR) model. In hypoxia, activated microglia produce excess amounts of inflammatory cytokines such as cytokines interleukin (IL)-1β [12], monocyte chemoattractant protein-l (MCP-1) [13] and tumor necrosis factor-α (TNF-α) [14] and then may contribute to OIR development. Thus, an ideal anti-neovascularization agent to against IR should focus on not only the prevention of pathologic RNV, but also the protection of retinal glial cells, as well as the inhibition of microglia-mediated inflammatory responses.

Magnolol, a traditional Chinese herbal medicine, is a polyphenolic binaphthalene compound extracted from the stem bark of Magnolia officinalis. Recent evidences revealed that Magnolol exerts beneficial effects in various diseases because of its anti-inflammatory, anti-oxidative, antineoplastic, anti-angiogenic and neuroprotective effects [15–19]. Magnolol has been reported to exhibit the anticancer activity via inducing apoptosis and suppressing hypoxia-induced VEGF expression and angiogenesis [20–23]. Moreover, Magnolol is able to protect neurons against ischemia injury [24] and suppress oxidative and inflammatory responses induced by interferon-γ and lipopolysaccharide in microglial cells [25]. Taken together, these observations imply that Magnolol is likely to be an effective alternative treatment in IR. However, the therapeutic effects of Magnolol in OIR model have not been documented.

In the present study, we investigated the effects of Magnolol on the development of RNV, local glial cells in an OIR model and explored the molecular mechanism. Our results revealed that Magnolol partially suppressed the RNV, prevented glial degeneration and inhibited microglia-mediated inflammatory responses via hypoxia-inducible factor (HIF)-1α/VEGF pathway.

Materials and Methods

OIR model

All animal experiments in this study were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center (Guangzhou, China). C57BL/6J mice from the Animal Laboratory of Zhongshan Ophthalmic Center were used. The OIR model was induced as described by Smith et al. [26]. Briefly, neonatal C57BL/6J mice with their nursing mothers were exposed to 75 % O_2 ± 5 % for five consecutive days from postnatal day (P) 7 to P12 and subsequently returned to room air (RA).

Magnolol administration

Magnolol (Selleckchem, Houston) solution was freshly prepared as 100 mM in vehicle dimethylsulfoxide (DMSO). OIR mice were randomly assigned into three groups (10, 25 and 50 mg/kg Magnolol) to choose the best concentration. Age-matched mice maintained in RA from birth to P17 served as normal group. After choosing the best concentration, the pups form normal group and OIR group were injected with 25 mg/kg Magnolol intraperitoneally once a day from P12 to P17. That is, there were four groups in our next experiments: (1) normal group, (2) normal + 25 mg/kg Magnolol group, (3) OIR group and (4) OIR + 25 mg/kg Magnolol group. After 5 days of intraperitoneal injection, mice were euthanized and their retinas were prepared for morphology or molecular biology studies.

Immunostaining on whole-mount retinas

Mice at P17 were euthanized, and eyes were enucleated and fixed with freshly prepared 4 % paraformaldehyde for 2 h. Retinas were dissected from the choroid and sclera, blocked and permeabilized in PBS containing 5 % BSA and 0.5 % Triton X-100 overnight at 4 °C, and then incubated overnight at 4 °C with red-light-absorbing dye labeled (Alexa Fluor 568; Invitrogen, Carlsbad, CA) Griffonia simplicifolia isolectin B4 (IB4, 1:50, a marker for vessels; Invitrogen). To assess the astrocyte and microglia, retinas were incubated with glial fibrillary acidic protein (GFAP, a marker for astrocyte) rabbit mAb (1:50; Millipore, Bedford, MA) or ionized calcium-binding adaptor molecule 1 (IBA-1, a marker for microglia) rabbit mAb (1:50; Wako, Osaka,Japan) overnight at 4 °C, which was followed by the incubation with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:100; Cell Signaling Technology, Beverly, MA) for 2 h at room temperature (RT). Next, retinas were washed with PBS and mounted on microscope slides in mounting medium (Aqua-Polymount; Polysciences, Warrington, PA). Retinas were examined by fluorescence microscopy (AxioCam MRC; Carl Zeiss, Thornwood, NY) and by confocal microscopy (Zeiss 510; Carl Zeiss). Areas of vaso-obliteration and vitreoretinal neovascular tufts were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Semiquantification of astrocyte rescue in the RNV areas

To assess the astrocytic morphology, mice retinas from normal group, normal + Magnolol (25 mg/kg) group, OIR group, and OIR + Magnolol (25 mg/kg) group were dissected, flat-mounted and stained by GFAP at P17. We scored the extent of astrocyte persistence as the previous

scoring criteria we have documented [27]. Specifically, a score of 1–2 indicated retinas with a great number of astrocytes in the RNV areas that were observed to form a completely or fairly normal astrocytic template. A score of 3–4 indicated retinas with fewer astrocytes remaining in the RNV areas than normal retinas; these astrocytes lacked the normal cellular processes, and the standard network observed in a normal astrocytic template was not present. A score of 5–6 indicated very few astrocytes remaining in the RNV areas. The astrocytes from the six fields (the RNV areas) of each retina were graded. Then, the mean and standard error of these grades from twelve retinas per group were statistically analyzed.

Quantification of activated microglia in the RNV areas

To assess the microglia activation, mice retinas from the four groups were dissected, flat-mounted, and stained by IBA-1 at P17. Microglial cells from normal mice retinas had a round, small flattened cell body and large ramified processes that extended radially when viewed in retinal flat mounts; this morphology was typical for resting microglia. Activated microglial cells were recognized by their enlarged cell body and retracted processes. The activated and resting microglial cells from the six fields (the RNV areas) of each retina were calculated. Then, the mean and standard error of these cell numbers from twelve retinas per group were statistically analyzed.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated using Trizol reagent (Invitrogen). Reverse transcriptase reaction was performed using reverse transcriptase reagent kit with gDNA Eraser (Takara, Tokyo). qRT-PCR was performed using the SYBR Green qRT-PCR Master mix (Biotool, Houston). Mouse β-actin served as an internal standard of mRNA expression. Primers for mice IL-1β, IL-6, MCP-1, TNF-α and β-actin were used: IL-1β Forward 5′-GAA ATG CCA CCT TTT GAC AGT G-3′, IL-1β Reverse 5′-TGG ATG CTC TCA TCA GGA CAG-3′; IL-6 Forward 5′-AGT TGT GCA ATG GCA ATT CTG A-3′, IL-6 Reverse 5′-AGG ACT CTG GCT TTG TCT TTC T-3′; MCP-1 Forward 5′-CCC CAC TCA CCT GCT GCT ACT-3′, MCP-1 Reverse 5′-GGC ATC ACA GTC CGA GTC ACA-3′; TNF-α Forward 5′-AAG CCT GTA GCC CAC GTC GTA-3′, TNF-α Reverse 5′-GGC ACC ACT AGT TGG TTG TCT TTG-3′; β-actin forward 5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′, β-actin reverse 5′-ATG GAG CCA CCG ATC CAC A-3′. Samples were compared using the relative threshold cycle, and the relative fold difference in expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. All reactions were run in triplicate.

Western blot

For Western blot experiments, retinas at P17 were harvested and lysed using RIPA (Beyotime, Shanghai) buffer containing a protease inhibitor PMSF (1:100; Beyotime). Total protein was sized fractionized by electrophoresis in 10 % SDS-polyacrylamide gels at 120 V for 1.5 h at RT and transferred to polyvinylidene difluoride filter membrane (Millipore, Bedford, MA) at 100 V for 1.5 h at 4 °C. The membranes were blocked with 5 % skim milk for 2 h at RT and incubated with primary antibody against HIF-1α (anti-rabbit, 1:500; Novus Biologicals, Littleton), VEGF (anti-mouse, 1:500; Santa Cruz, Dallas, Texas) and GAPDH (anti-rabbit, 1:1000; Santa Cruz) at 4 °C overnight. The membranes were then washed and incubated with second antibody goat anti-rabbit or goat anti-mouse conjugated with horseradish peroxidase (1:2000; Southern-Biotech, Birmingham, Alabama) for 2 h and visualized using an enhanced chemiluminescence system (Millipore).

Immunofluorescent staining

After fixation in 4 % paraformaldehyde, eyes were equilibrated in 30 % sucrose and embedded in OCT (Sakura Finetek, Torrance, California). They were then fast frozen in liquid nitrogen and cut into 6-μm-thick sections. Retinal sections were placed on a glass slide, washed with PBS, and permeabilized with 1 % Triton X-100 for 20 min at RT before blocking with 10 % normal goat serum for 2 h. Then, sections were incubated overnight at 4 °C with rabbit monoclonal primary antibodies for GFAP (1:200; Millipore) and mouse monoclonal primary antibodies for VEGF (1:200; Santa Cruz). After washing in PBS, sections were incubated for 2 h at RT with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody and Alexa Fluor 555-labeled goat anti-mouse secondary antibody (Cell Signaling Technology) at 1:500 dilution and were counterstained with DAPI for 10 min. Retinas were examined by confocal microscopy (Zeiss 510; Carl Zeiss).

Statistical analysis

All data were presented as mean ± SEM. Statistical analyses were performed by independent Student's *t* test, or one-way ANOVA followed by Bonferroni post hoc test with SPSS software version 20.0 (SPSS Inc, Chicago, IL). *P* values <0.05 were considered to be statistically significant. Each experiment consisted of at least three replicates per condition.

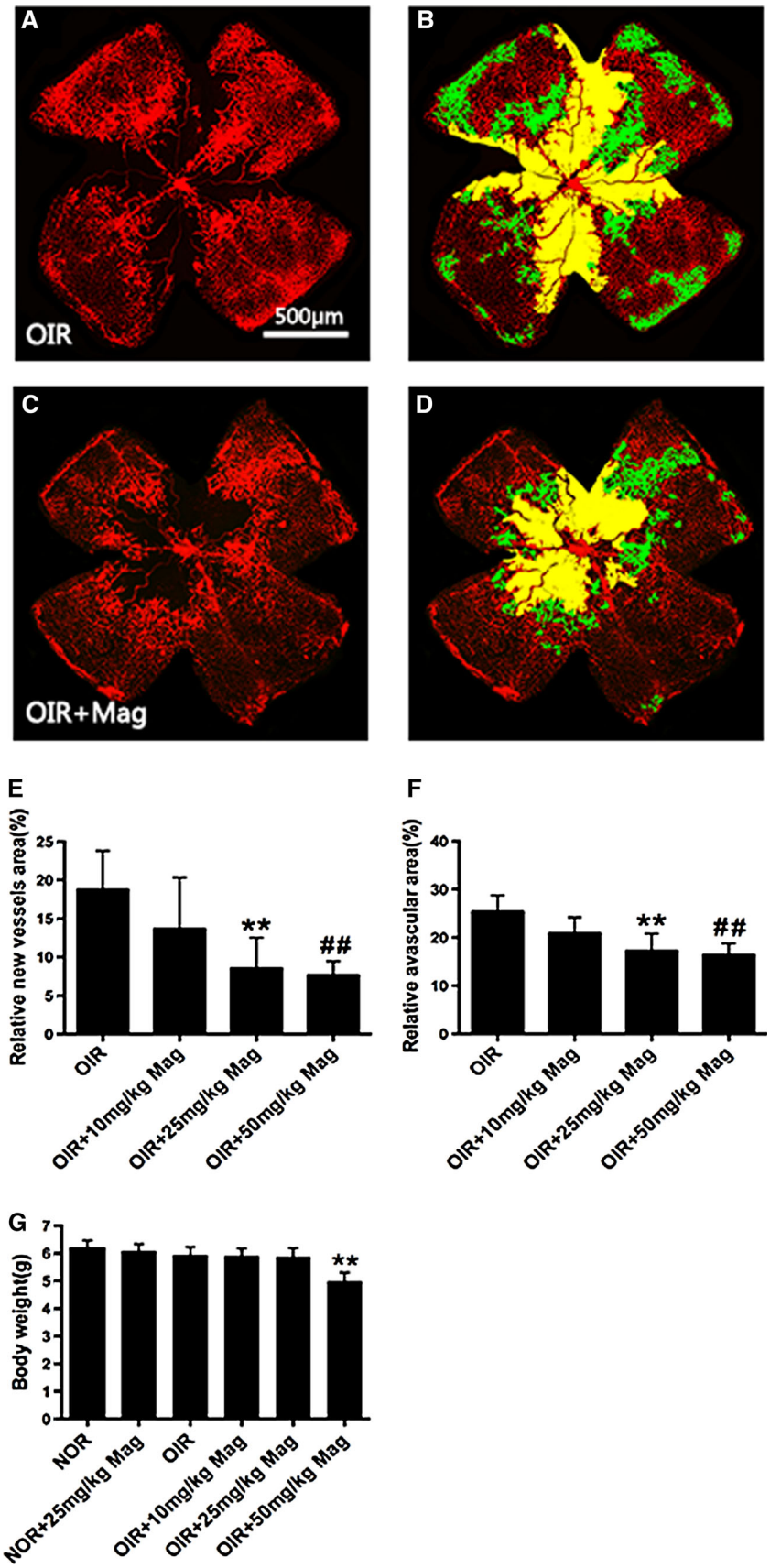


Fig. 1 Magnolol reduces RNV areas and avascular areas in OIR mice. Mice retinas from OIR group (**a**, **b**) and OIR + Magnolol (25 mg/kg) group (**c**, **d**) were harvested at P17 and subjected to whole-mount immunostaining with IB4 (*red*), showing neovascular areas (*green*) and avascular areas (*yellow*). **e** The RNV areas were measured after Magnolol injection at different concentrations (10, 25 and 50 mg/kg) in OIR mice. **f** The retinal avascular areas were measured after Magnolol injection at different concentrations (10, 25 and 50 mg/kg) in OIR mice. **g** The body weight was measured at P17 after Magnolol injection at different concentrations (10, 25 and 50 mg/kg) in normal and OIR mice. Data were presented as mean ± SEM. $n = 12$ retinas from 12 mice for whole-mount immunostaining. $^{**}P < 0.01$ and $^{\#\#}P < 0.01$, comparing Magnolol versus OIR groups. *Scale bar* indicates 500 μm. *NOR* normal, *Mag* Magnolol (color figure online)

Results

Magnolol attenuates retinal vaso-obliteration and neovascularization in OIR mice

To observe the retinal vessels, OIR mice retinas were harvested at P17, flat-mounted and stained with IB4 (Fig. 1a–d). Five days after intraperitoneal injection of Magnolol, there were dose-dependently fewer neovascular areas (Fig. 1e) and avascular areas (Fig. 1f) in the Magnolol-injected eyes compared to the OIR group and reached its lowest level at the concentrations of 25 and 50 mg/kg. But after 50 mg/kg injection, the weight of OIR mice markedly lost; however, 25 mg/kg Magnolol did not influence the body weight of both normal and OIR mice (Fig. 1g). Therefore, 25 mg/kg might be the best concentration that we could use in the next experiments.

Next, we evaluated whether Magnolol affected the physiological retinal angiogenesis in normal mice and reduced the neovascular tuft in OIR mice. As normal mice retinas at P17 (Fig. 2a, e), the physiological retinal angiogenesis was not affected after Magnolol injection (Fig. 2b, f). The retinas of OIR mice showed a multiple of neovascular tufts covered at the leading edge of retinal vessels (Fig. 2c, g); however, the areas of neovascular tufts were significantly reduced after the treatment with 25 mg/kg Magnolol (Fig. 2d, h). These results indicated that Magnolol did not mitigate physiological retinal angiogenesis in the retinal development and could dramatic inhibition of OIR pathologic RNV.

Magnolol preserves the astrocytic morphology and inhibits microglia activation in OIR mice retinas

Compared with normal C57BL/6J mice retinas (Fig. 3a–d), a large amount of neovascular tufts and damaged astrocytes in RNV areas could be observed in OIR retinas at P17 (Fig. 3i–l), and most of the astrocytes demonstrated a score of 3–6 (Fig. 3q). After intraperitoneal injection of Magnolol in OIR mice, astrocytes formed a better network and retained a relative normal stellate/dendritic morphology accompanied by a decreased neovascular tufts (Fig. 3m–p), and most of the astrocytes demonstrated a score of 1–4 (Fig. 3q). In addition, Magnolol had no effect on the astrocytic morphology in normal mice retinas (Fig. 3e–h).

In response to injury or inflammation, microglia became activated in OIR mice retinas at P17, presented as enlarged cell body and retracted processes (Fig. 4i–l, q). After treatment with Magnolol, OIR mice retinas showed decreased activation of microglial cells (Fig. 4s) and more microglial cells had ramified processes that extended radially (Fig. 4m–p, r). Similarly, Magnolol had no effect on the microglial morphology in normal mice retinas (Fig. 4e–h). These results indicated that the vascular rescue

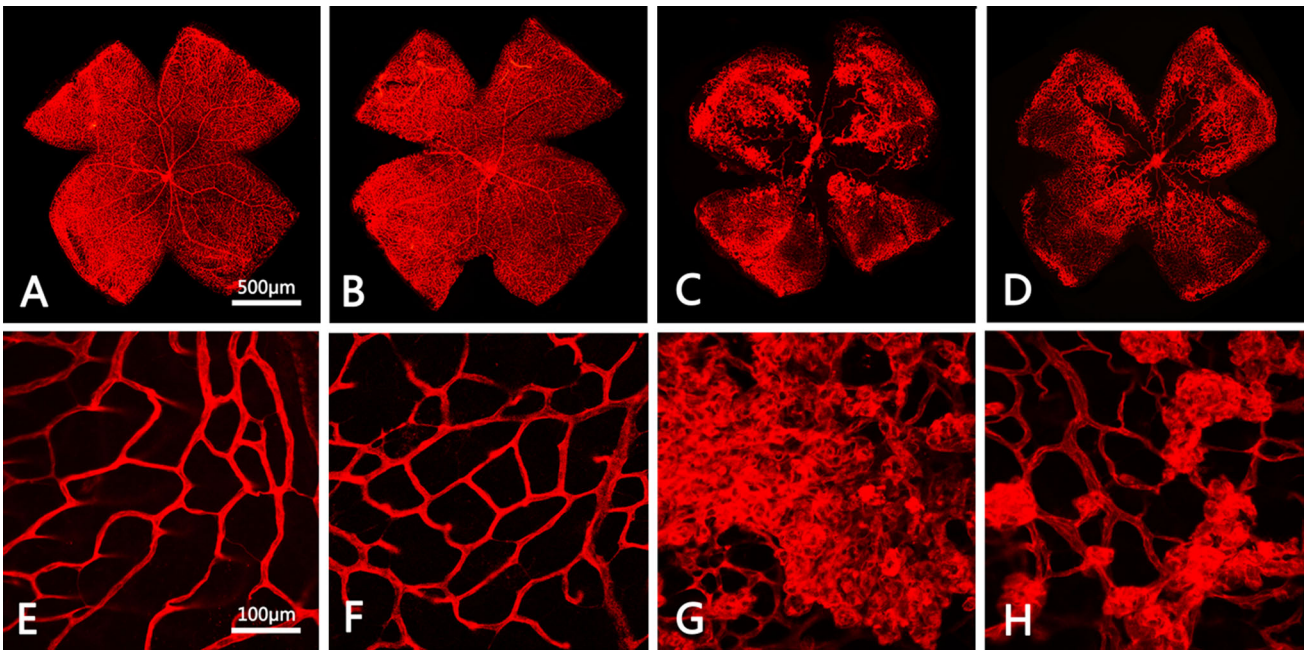
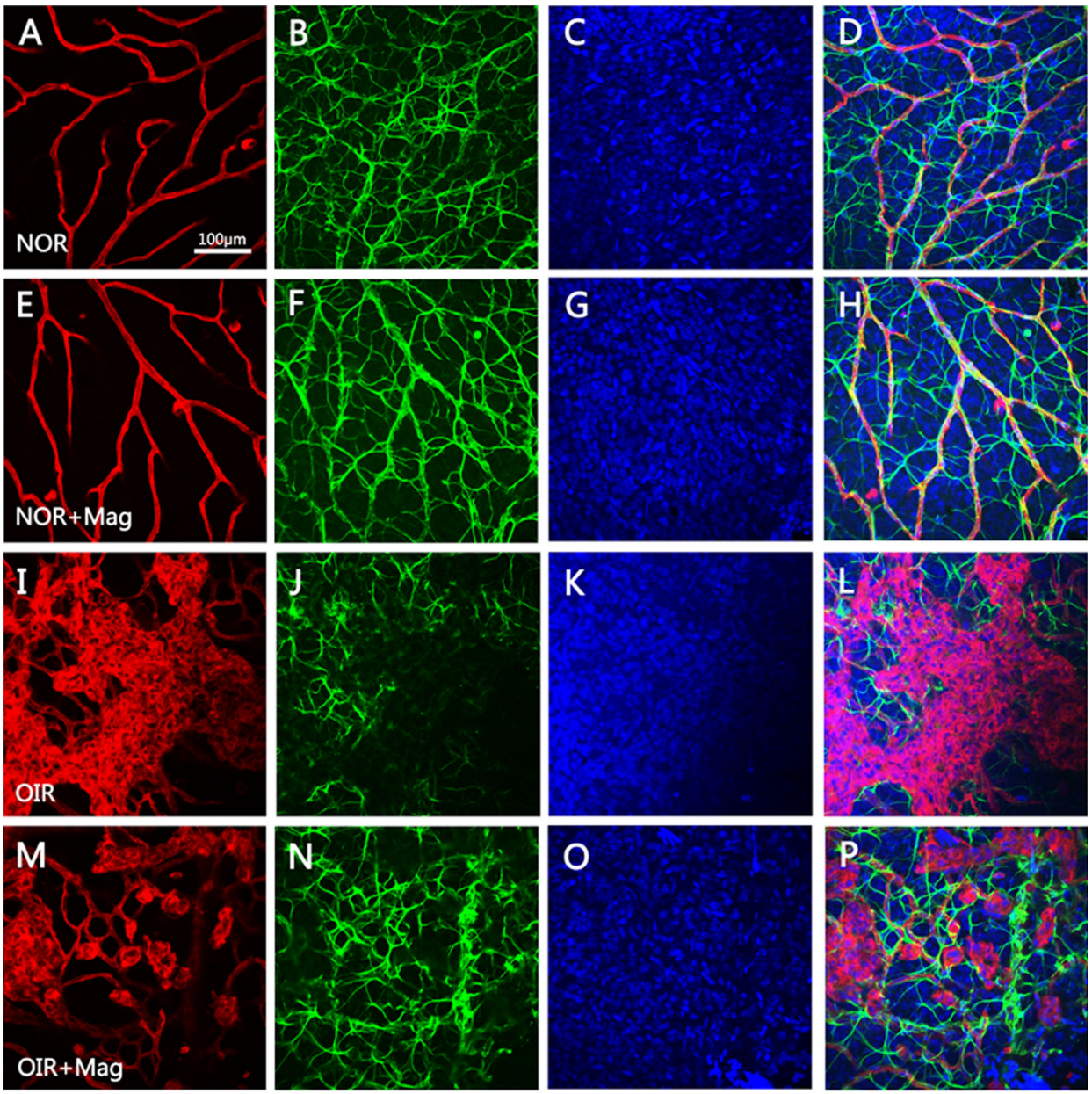


Fig. 2 Magnolol attenuates retinal vaso-obliteration and neovascularization in OIR, but has no effect on the retinal vasculature of normal mice. Mice retinas from normal group (**a**, **e**), normal + Magnolol (25 mg/kg) group (**b**, **f**), OIR group (**c**, **g**) and OIR + Magnolol (25 mg/kg) group (**d**, **h**) were harvested at P17 and subjected to whole-mount immunostaining with IB4 (*red*). *Scale bar* indicates 500 μm (**a**–**d**) and 100 μm (**e**–**h**) (color figure online)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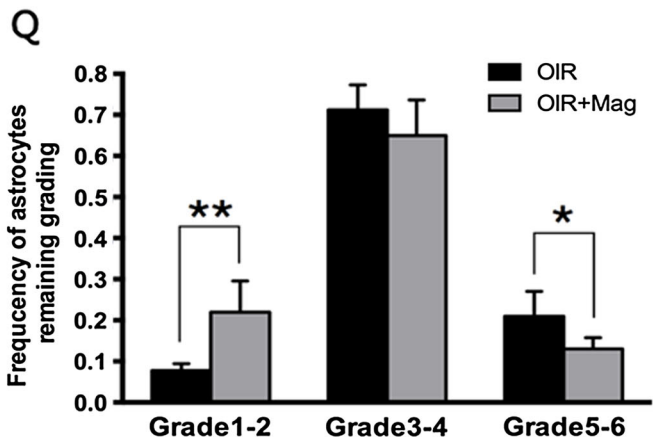
Q
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Frequency of astrocytes remaining grading
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
*
Grade1-2
Grade3-4
Grade5-6

◀**Fig. 3** Magnolol preserves the astrocytic morphology in OIR, but has no effect on the astrocytic morphology in normal mice retinas. Mice retinas from normal group (**a**–**d**), normal + Magnolol (25 mg/kg) group (**e**–**h**), OIR group (**i**–**l**) and OIR + Magnolol (25 mg/kg) group (**m**–**p**) were harvested at P17 and subjected to whole-mount immunostaining with anti-GFAP (*green*) and anti-IB4 (*red*). GFAP co-localized with IB4 in the merged image (**d**, **h**, **l**, **p**; *yellow*). *Scale bar* indicates 100 μm. **q** The relative frequency of each grade was used for statistical analyses of OIR and OIR + Magnolol (25 mg/kg) retinas. Data were presented as mean ± SEM. $n = 12$ retinas from 12 mice. $^{*}P < 0.05$ and $^{**}P < 0.01$. All four images are of the same region. *NOR* normal, *Mag* Magnolol (color figure online)

was associated with protection of the endogenous astrocytes and microglial cells in Magnolol-injected OIR retinas.

Magnolol decreases mRNA expression of inflammatory cytokines in OIR mice retinas

To determine whether the anti-angiogenic and neuroprotective effects of Magnolol on OIR retina may be due to reduced inflammatory cytokines, we examined the mRNA expression of IL-1β (Fig. 5a), IL-6 (Fig. 5b), MCP-1 (Fig. 5c) and TNF-α (Fig. 5d) by qRT-PCR. As shown in Fig. 5, a prominent increase of IL-1β, IL-6, MCP-1 and TNF-α mRNA expression could be observed in OIR retinas at P13, P14 and P17. Magnolol significantly reduced retinal IL-1β, IL-6, MCP-1 and TNF-α mRNA expression in OIR mice. However, the injection of Magnolol to normal mice did not alter the mRNA levels of these inflammatory cytokines, compared to normal controls.

Magnolol reduces GFAP expression in Müller cells and down-regulates HIF-1α and VEGF expression in OIR mice retinas

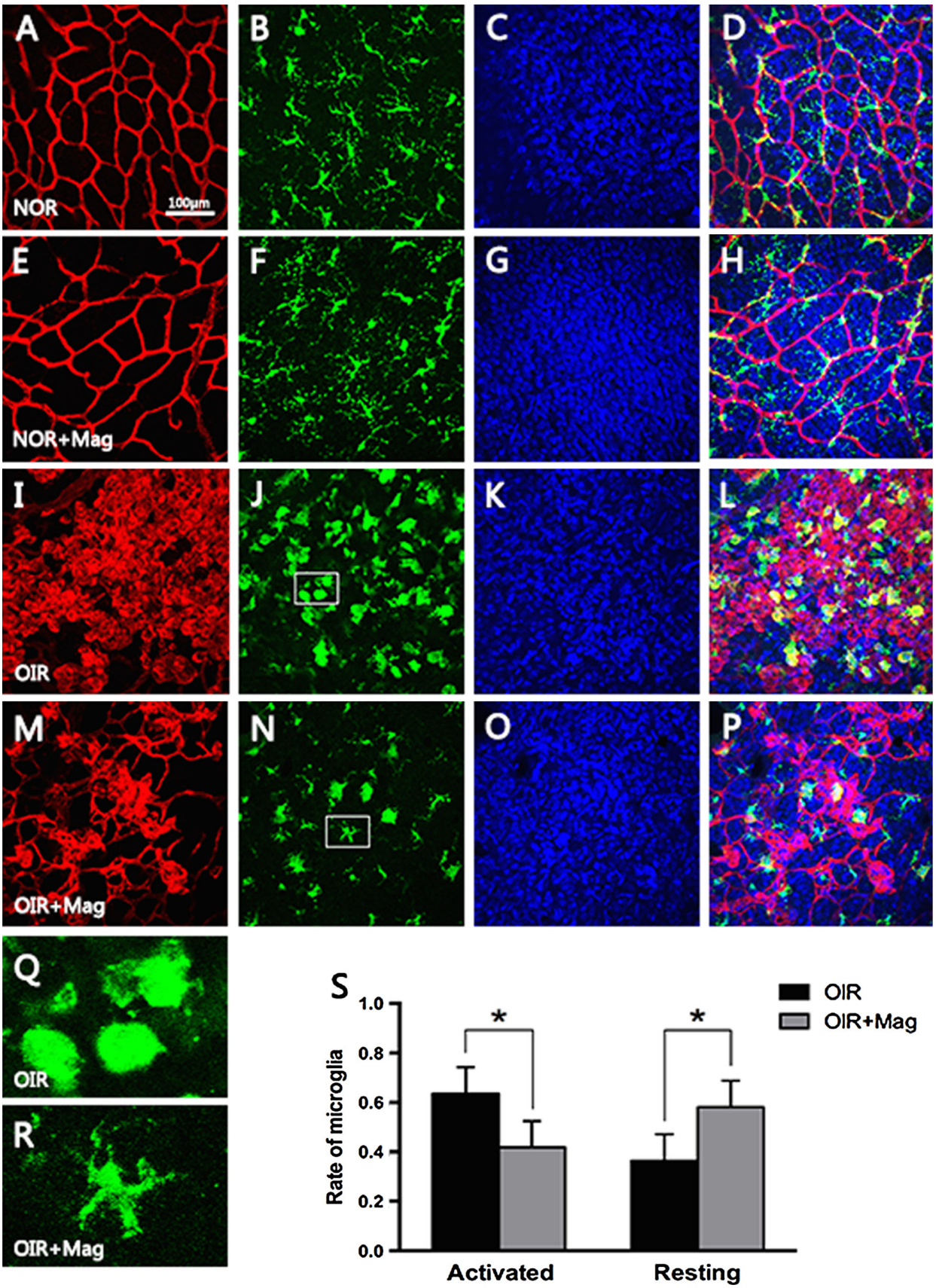
To identify the molecular mechanism of Magnolol involved in protection of the glial cells and its anti-angiogenic properties, we performed Western blot to examine the expressions of HIF-1α (Fig. 6a) and VEGF (Fig. 6b) in the retinas of normal and OIR mice at P17. We found that the levels of HIF-1α and VEGF expression were increased significantly in the retinas of OIR group compared with the normal group. Decreased levels of HIF-1α and VEGF were detected in Magnolol injection group in OIR mice retinas, and no difference was observed in normal mice retinas between administrated group and non-administered group.

To further determine the staining changes of VEGF and GFAP in the OIR mice retinas after Magnolol treatment, we performed immunohistochemistry staining on transverse cryosections. Our results showed that immunostaining of VEGF was obviously increased in OIR group (Fig. 7i) and remarkably decreased in OIR retinas after Magnolol treatment (Fig. 7m). In OIR group, activated Müller glia were observed as GFAP-positive cells with processes spanning the entire retina (Fig. 7j). Magnolol had no effect on GFAP immunolabeling (Fig. 7f) in normal mice retinas, but could reduce the reactive expression of GFAP in the Müller glia (Fig. 7n) in retina of OIR mice.

Discussion

IR is a major cause of blindness in developed and developing countries. RNV occurs after a period of ischemia caused by the retinal vessel regression, occlusion or cessation of vascular development. Respond to hypoxia, RNV is the pathologic process with resident retinal vascular endothelial cell proliferation combined with the development of new vessels from preexisting vasculature. These new vessels grow within retina and then grow toward the retinal surface or into the vitreous [28]. It can result in epiretinal fibrosis, vitreous hemorrhage and tractional retina detachment, leading to irreversible vision loss. OIR is a useful and intensively investigated model for the research on molecular mechanisms of IR. Abnormal vasculature identified by central vaso-obliteration and peripheral pathological RNV is the most well-recognized characteristic in OIR model. In this study, the reduction in pathological RNV could be observed in OIR retinas after Magnolol treatment and was associated with a significant improvement of the physiological revascularization in hypoxic areas. In addition, the avascular areas and RNV areas in normal mice retinas showed no significant difference between the Magnolol injection group and non-injection group. These results supported the concept that Magnolol could effectively prevent pathologic RNV in OIR mice and does not have an adverse effect on growth of normal retinal vasculature.

As the two major systems in retina, vascular and nervous networks show a high degree of anatomical parallelism and functional cross talk. It is important to understand the interplay among angiogenesis and the glial response in IR. Retinal astrocytes and Müller cells can provide support for neurons and are necessary for maintaining the normal function of retina [7]. During early retinal vascular development, astrocytes provide the template on which the endothelial cells migrate to form the vascular network. Astrocytes also express VEGF to stimulate the endothelia filopodia growth and extension [10, 29]. Numerous studies indicated decreased cell number and degeneration of astrocytes in OIR model [27, 30], and these changes were occurred between P13 and P18 [31]. After intraperitoneal injection of Magnolol in OIR mice, the density of astrocytes remained high and the cells formed a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O
P
Q
OIR
R
OIR+Mag
S
Rate of microglia
1.0
0.8
0.6
0.4
0.2
0.0
*
*
OIR
OIR+Mag
Activated
Resting

◀**Fig. 4** Magnolol inhibits microglia activation in OIR, but has no effect on the microglia morphology and activity in normal mice retinas. Mice retinas from normal group (**a**–**d**), normal + Magnolol (25 mg/kg) group (**e**–**h**), OIR group (**i**–**l**) and OIR + Magnolol (25 mg/kg) group (**m**–**p**) were harvested at P17 and subjected to whole-mount immunostaining with anti-IBA1 (*green*) and anti-IB4 (*red*). IBA1 co-localized with IB4 in the merged image (**d**, **h**, **l**, **p**; *yellow*). *Scale bar* indicates 100 μm. **q**, **r** A highly magnified image of selected areas from **j** and **n** illustrates the morphology of microglia in OIR group and Magnolol treatment group. **s** The rates of activated and resting microglia were used for statistical analyses of OIR and OIR + Magnolol (25 mg/kg) retinas. Data were presented as mean ± SEM. $n = 12$ retinas from 12 mice. $^{*}P < 0.05$. All four images are of the same region. *NOR* normal, *Mag* Magnolol (color figure online)

better network, which contributed to accelerate physiological revascularization and correspondingly reduced pathologic RNV.

Müller cells, another specialized macroglial cell, span the transverse retina from the inner limiting membrane to the outer limiting membrane. As above mentioned, Müller cells can bring support for retinal neurons, maintain the blood–retinal barrier and express angiogenic growth factors that guide blood vessels growth. Like astrocytes, Müller cells can stimulate retinal vascular endothelial cell proliferation during hypoxia [32]. In normal mice retinas, GFAP is restricted to astrocytes and is expressed very limited in Müller cells. However, GFAP is expressed in Müller cells when they are under pathological condition, including hypoxia [26], neural degeneration [33] or trauma [34]. In accordance with previous studies, we observed the loss of astrocytes was accompanied by an increased number of GFAP-active Müller cells in the retina of OIR mice. In contrast, Magnolol markedly reduced the activation of Müller cells and preserved the astrocytes network.

Resident microglia play an important role in promoting and maintaining normal retinal vascularization during early development [35]. During retinal vascular development, microglial cells have a close spatial relationship with angiogenic tip cells at its turning and branching point. Considered as the retinal immune cells, microglia keep quiescent in the postnatal period but can rapidly respond to damage in the activated process, proliferating and migrating to the injury site [36]. In OIR, microglia have the potential to contribute to RNV and relate to the intravitreal blood vessels [35, 37]. In addition, greatly elevated microglial cell numbers have been shown in neovascular zones in OIR mice retinas [38, 39]. Our data also suggested increased microglial cells in activated state in RNV areas of OIR retina. In contrast, Magnolol diminished the

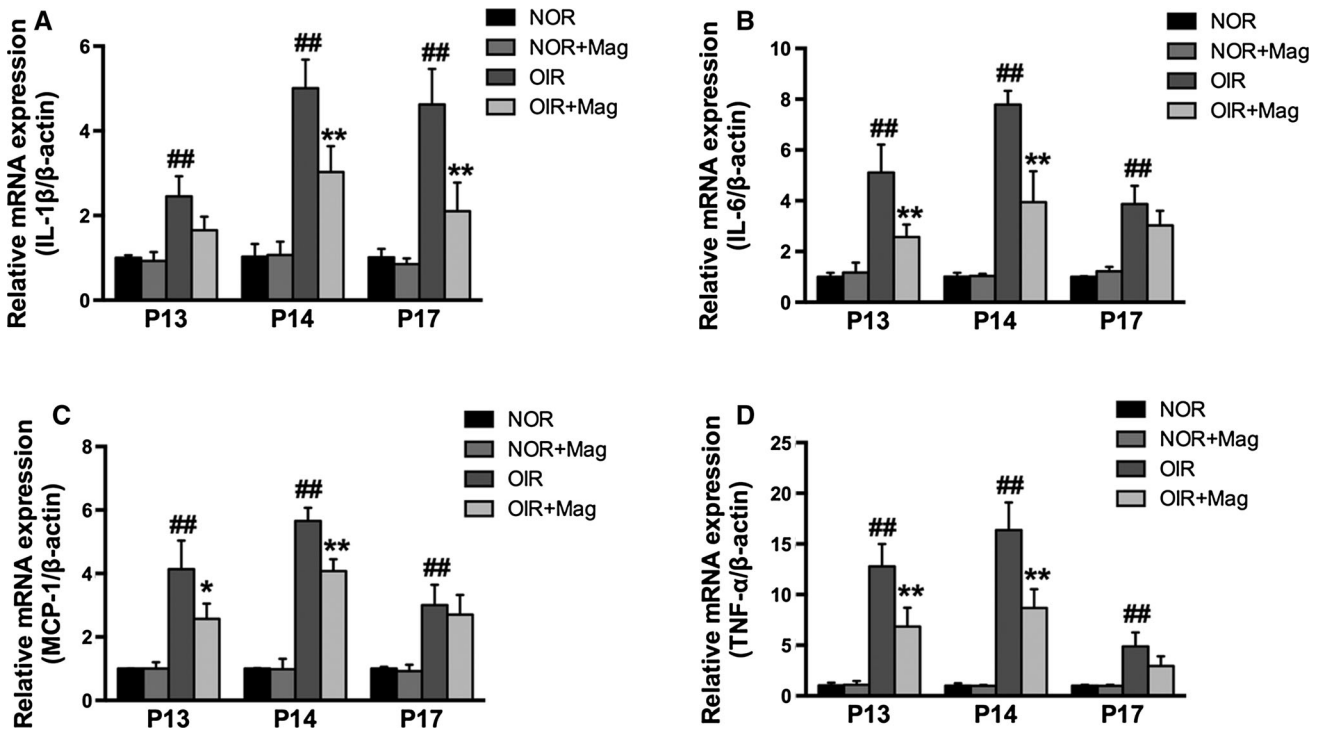

Fig. 5 Magnolol decreases mRNA expression of inflammatory cytokines in OIR mice retinas. Mice retinas from each group were harvested at P13, P14, P17 and detected by qRT-PCR. mRNA levels of IL-1β (**a**), IL-6 (**b**), MCP-1 (**c**) and TNF-α (**d**) were significantly increased in OIR groups and reduced with Magnolol (25 mg/kg) treatment. Data were presented as mean ± SEM. $n = 3$, with triplicates per experiment. $^{*}P < 0.05$, $^{**}P < 0.01$ comparing Magnolol versus OIR groups; $^{\#\#}P < 0.01$ comparing OIR versus normal groups. *NOR* normal, Mag Magnolol

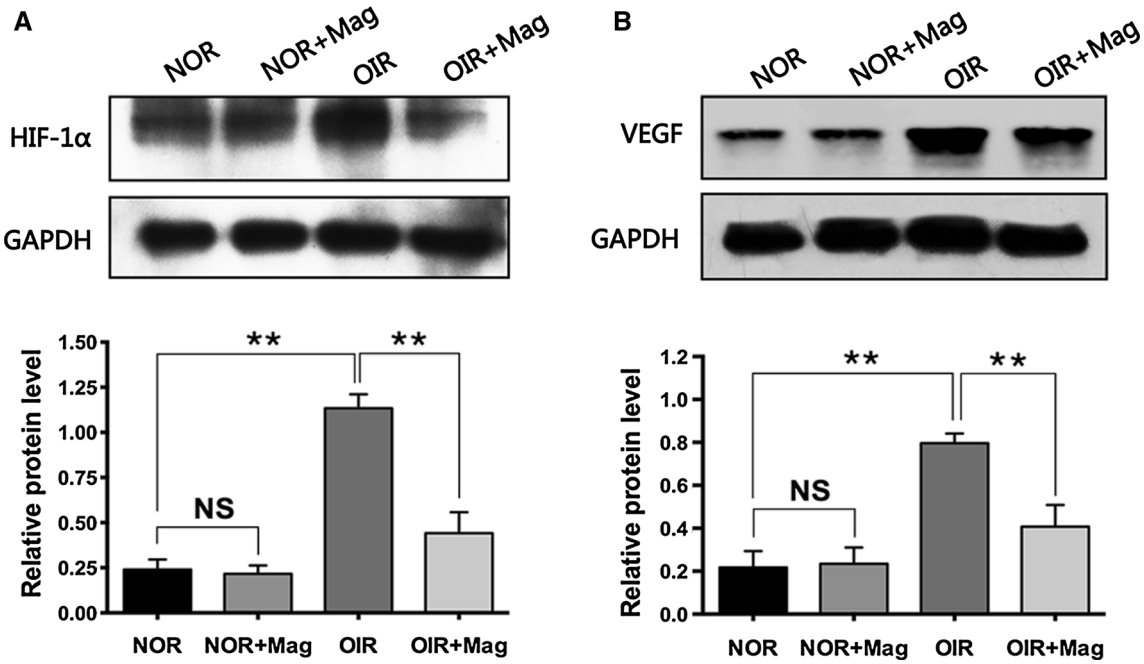

Fig. 6 Magnolol down-regulates HIF-1α and VEGF expression in OIR mice retinas and has no effect on normal mice retinas. Mice retinas from each group were harvested at P17. The protein expression of HIF-1α (**a**) and VEGF (**b**) at each group was measured by Western blot. The *bar chart* shows the relative ratio of HIF-1α (**a**) and VEGF (**b**) to GAPDH at each group. Data were presented as mean ± SEM. $n = 3$, with triplicates per experiment. $^{**}P < 0.01$. NS not statistically significant, *NOR* normal, *Mag* Magnolol

activation of microglia concomitant with decreased pathologic RNV.

Inflammation has been considered as the major pathogenic factor in OIR; the microglial cells play a pivotal role in the process. It has been reported that inflammatory cytokines such as IL-1β, IL-6, MCP-1 and TNF-α can be released by microglia following hypoxic–ischemic injury and are markedly enhanced when compared to normal conditions [12–14, 40]. In early stages of IR, excessive IL-1β released by activated microglia can induce retinal microvascular injury. Meanwhile, IL-1β also sustains microglial activation [12]. In retinal ischemia injury model, IL-6 is up-regulated and mainly expressed by microglial/phagocytic cells [40]. Apart from the microglial cells, injured Müller cells also release IL-6 in reaction to stimuli [41, 42]. MCP-1, another inflammatory cytokine expressed by microglia in hypoxia, is also looked as the chemokine interacting between activated microglia and vascular elements in OIR. MCP-1 has the potential angiogenic effect and may work as a direct angiogenic factor, or relate to the recruitment of pro-angiogenic macrophages and interaction with other cytokines [38]. The fourth inflammatory factor we investigated in this study is TNF-α, one of the best known mediators released by microglial cells. Prior research provided evidence that the inhibition of TNF-α significantly improves physiological angiogenesis and reduces pathological neovascularization in OIR model [43]. As a conclusion, retinal hypoxia may initiate inflammation by activation of microglia; in turn, enhanced inflammation factors further promote the activation of microglial cells, resulting in the pathological angiogenesis [44]. Our study suggested that Magnolol with the anti-inflammatory effect might reduce the accumulation and activation of microglia; hence, it could play a pivotal role in attenuating RNV and decreasing glial degeneration.

HIF-1 is well known for its key role in hypoxia-induced responses. It is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β, the inducible oxygen-sensitive subunit and constitutive oxygen-insensitive subunit, respectively. The protein level of HIF-1α is tightly regulated by cellular oxygen concentration, while HIF-1β is unaffected [45]. In normoxic condition, HIF-1α is continuously synthesized and its prolyl residues are hydroxylated by prolyl hydroxylase (PHD). Then, hydroxylated HIF-1α is captured by the von Hippel–lindau (VHL) tumor suppressor protein, leading to ubiquitination and degradation by ubiquitin–proteasome. In contrast, the degradation processes are impaired under hypoxia and thus enhance the stability of HIF-1α [46]. Subsequently, more stabilized HIF-1α entered into nucleus, resulting in the formation of HIF-1 complex that binds to DNA hypoxia response element to induce the transcription of hypoxically regulated genes [47], such as VEGF. Numerous studies have confirmed that the HIF-1α/VEGF pathway is closely

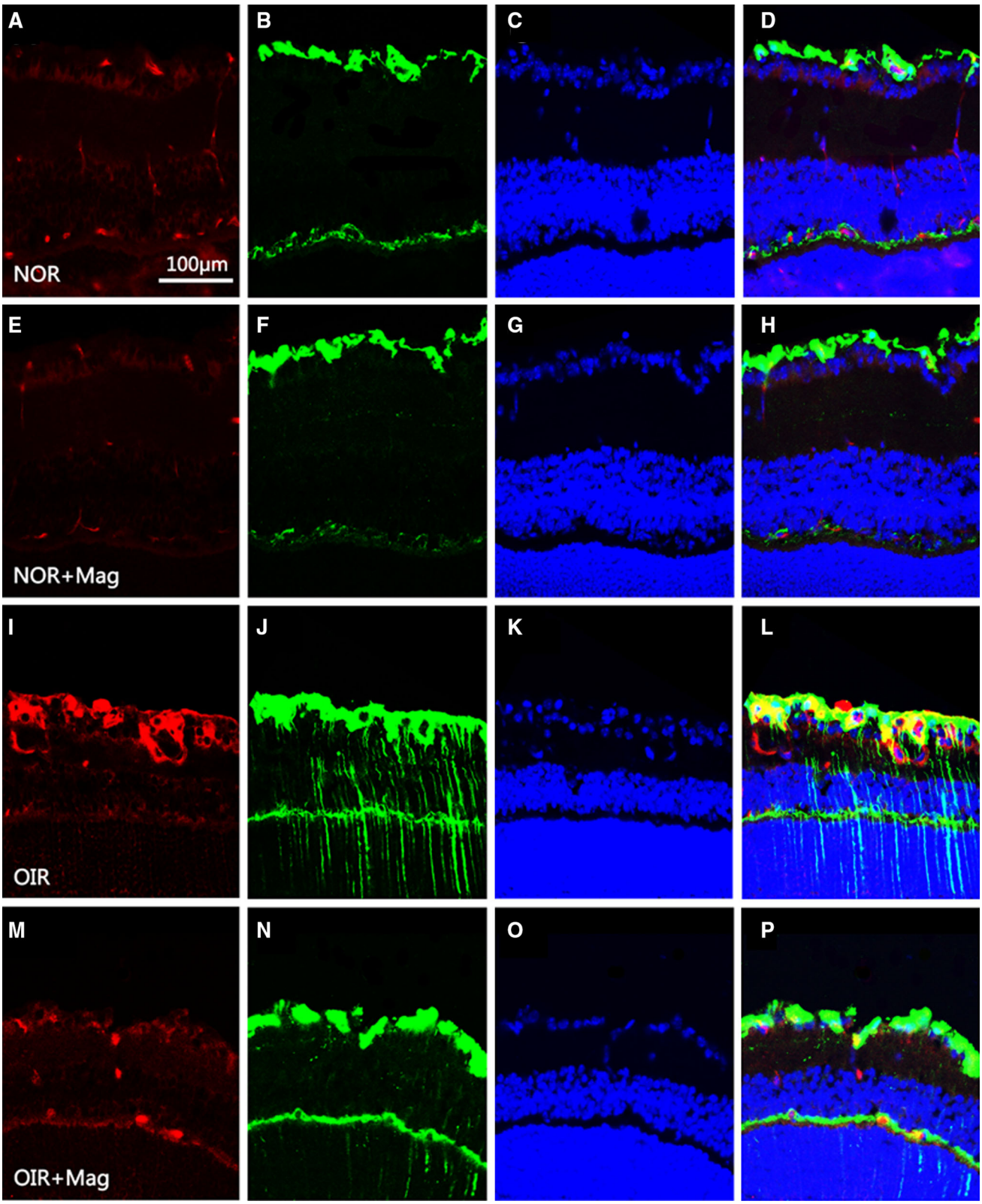

Fig. 7 Magnolol reduces GFAP expression in Müller cells and down-regulates VEGF expression in OIR retinas. Mice retinal frozen sections at P17 from normal group (**a**–**d**), normal + Magnolol (25 mg/kg) group (**e**–**h**), OIR group (**i**–**l**) and OIR + Magnolol (25 mg/kg) group (**m**–**p**) were immunostained with VEGF (**a**, **e**, **i**, **m**; *red*), GFAP (**b**, **f**, **j**, **n**; *green*), DAPI (**c**, **g**, **k**, **o**; *blue*) and Merge (**d**, **h**, **l**, **p**; *yellow*). *Scale bar* indicates 100 μm. $n = 12$ retinas from 12 mice for immunofluorescent staining. *NOR* normal, *Mag* Magnolol (color figure online)

associated with pathogenesis of ROP [27, 30]. Additionally, it confirmed that regulate HIF-1α can inhibit pathological RNV without affecting physiological angiogenesis [48]. Therefore, HIF-1α has been viewed as a promising target for anti-angiogenesis therapy in IR. Chen et al. have found that Magnolol suppressed hypoxia-induced angiogenesis through inhibition of HIF-1α/VEGF pathway in human bladder cancer cells. Except the effect of decreased HIF-1α protein accumulation, Magnolol also enhances HIF-1α protein degradation and inhibits its protein synthesis [20]. In our study, the anti-angiogenic effect of Magnolol was related to HIF-1α degradation and subsequent attenuation of VEGF expression. Importantly, Magnolol-targeting HIF-1α resulted in significant reduction in pathological neovascularization without disturbing physiological angiogenesis during the retinal vascular development.

In summary, we concluded that Magnolol protects against hypoxia-induced injury of retinal glial cells and lessens RNV via anti-inflammatory and inhibition of HIF-1α/VEGF pathway. Importantly, Magnolol could not mitigate physiological retinal angiogenesis in the retinal development. Therefore, Magnolol is a potential candidate to prevent the RNV in IR. Further studies are necessary to assess the cytoprotective effects and mechanisms of Magnolol on the angiogenesis and glial rescue from the cellular/microenvironmental level.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No. 81470030).

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