The Role of Lysophosphatidic Acid Receptor (LPA_1) in the Oxygen-Induced Retinal Ganglion Cell Degeneration

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Purpose. Although previous studies have demonstrated that hypoxia induces retinal ganglion cell (RGC) apoptosis and that transient retinal ischemia upregulates the expression of lysophosphatidic acid (LPA) receptors, it remains to be determined whether LPA_1 receptor mediates RGC degeneration during retinopathy of prematurity (ROP). By using an immortalized RGC line (RGC-5), primary neonatal RGC cultures, and oxygen-induced retinopathy (OIR) to model ROP, the authors explored whether LPA_1 receptor induces RGC degeneration and the potential mechanisms thereof.

Methods. OIR was induced by exposing rat pups to alternating cycles of hyperoxia/hypoxia from postnatal day (P) 0 to P14. RGC viability was evaluated by Fluorogold labeling. Effects of hyperoxia or hypoxia on LPA_1 expression were determined in the RGC-5 line by Western blot. Roles of hypoxia, LPA_1 receptor (with agonist, stearoyl-LPA; antagonist, THG1603; LPA_1 knock-down, shRNA-LPA_1), and Rho kinase (with inhibitor Y-27632) in mediating RGC survival and neurite outgrowth were assessed by MTT assay and phase-contrast microscopy, respectively. Expression of GFP-LPA_1 in RGC-5 under hypoxia was examined by confocal microscopy.

Results. OIR caused pronounced RGC loss in the retina. LPA_1 receptor was expressed by RGCs in retinal tissue, whereas oxygen stress induced its expression in RGC-5. Exposure to stearoyl-LPA or hypoxia substantially reduced the viability of RGCs; this was abrogated by THG1603 and shRNA-LPA_1. THG1603 and Y-27632 treatment also attenuated the adverse effects of hypoxia on RGC-5 neurite outgrowth, and their intravitreal administration prevented OIR-induced RGC loss. Interestingly, overexpression of LPA_1 increased RGC-5 susceptibility to hypoxia-induced cell loss.

Conclusions. Current data strongly support a critical role for LPA_1 receptor in mediating RGC degeneration during OIR. (*Invest Ophthalmol Vis Sci.* 2009;50:1290–1298) DOI:10.1167/iovs.08-1920

Retinal ganglion cells (RGCs) constitute the innermost neuronal layer of the retina and play a critical role in transmitting light signals to visual processing centers in the brain. Studies have revealed that RGCs are particularly sensitive to transient, mild, systemic hypoxia[1] and consequent apoptosis.[2] Nonetheless, the mediators of oxygen-induced RGC degeneration are complex and not fully known.

Exposure to variable oxygen tension predisposes the preterm retina to retinopathy of prematurity (ROP), a sight-threatening disease associated with low-birth-weight infants.[3] During ROP, fluctuations in the oxygen partial pressure of the arterial blood can lead to alternating episodes of severe and extended hyperoxemia and hypoxemia.[4] Although poorly studied, these conditions may have detrimental consequences on RGC survival.

Lysophosphatidic acid (LPA) is a small, bioactive phospholipid implicated in a wide spectrum of biological activities. LPA exerts its effects through interaction with four G protein-coupled receptors termed LPA_1, LPA_2, LPA_3, and LPA_4. The LPA_1 receptor is ubiquitously expressed in the central nervous system[5] and conducts essential functions.[6–8] With respect to cell survival, LPA_1 receptor has been shown to exhibit dual effects, exerting proliferative or cytotoxic responses in a variety of cell types.[5,6] In the normal retina, LPA_1 receptor expression has been detected with pronounced upregulation in the inner layers after ischemia.[9] Nonetheless, the role of LPA_1 in mediating ischemia-induced RGC degeneration remains obscure.

In the present study, we hypothesized that the LPA_1 receptor contributes significantly to RGC degeneration triggered by ROP. With the use of a rat model of oxygen-induced retinopathy (OIR), an established model of ROP,[10] primary RGC cultures, and an immortalized rat RGC line (RGC-5), we report that OIR elicits RGC degeneration whereas hypoxia diminishes RGC survival and neurite outgrowth in an LPA_1 receptor-dependent manner. Collectively, our findings reveal that LPA_1 receptor is a potent mediator of RGC degeneration.

Materials and Methods

Materials

Materials used in this study were as follows: N-acetylcysteine (NAC); Y-27632 (Sigma-Aldrich, St. Louis, MO); THG1603 (PCT WO 00/17348, a gift from Theratechnologies Inc., St. Laurent, QC, Canada); staurosporine (Alexis Biochemicals, San Diego, CA); hydroxystilbamidine methanesulfonate (Fluorogold; Molecular Probes, Eugene, OR); rabbit anti-LPA_1 receptor antibody (Exalpha Biologicals Inc., Maynard, MA); stearoyl lysophosphatidic acid (s-LPA; Avanti Polar Lipids Inc, Alabaster, AL); oxygen sensor (Teledyne Analytical Instruments, City of Industry, CA); and N-(2-quinolyl)valyl-aspartyl-(2, 6-difluoro-

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phenoxy) methyl ketone (Q-VD-OPh; Calbiochem, San Diego, CA).

Animals

Newborn and adult Long-Evans rats were purchased from Charles River (St. Constant, QC, Canada). All animal experiments were performed according to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of CHU Sainte-Justine (Montreal, QC, Canada).

Oxygen-Induced Retinopathy and Retrograde Labeling of Retinal Ganglion Cells

The OIR model was generated as previously described.[10] Briefly, rat mothers and pups (13–16 pups/litter) were housed from postnatal day (P) 0 to P14 in an oxygen (O_2) chamber. O_2 levels were adjusted every 24 hours between 45% and 12% O_2 (OxyCycler software; BioSpherix Ltd., Redfield, NY). Newborn litters in the control group were maintained in room air (21% oxygen). At P7, rat pups were anesthetized with isoflurane and bilaterally injected with 1 μL Fluorogold into the superior colliculus, as previously described.[11]

Intraocular Injections, Retinal Ganglion Cell Quantification, and Immunohistochemistry

Rat pups were anesthetized and injected intravitreally at P0, P3, P6, P9, and P12 with glass capillaries (approximately 60 gauge) and a microinjector (FemtoJet; Eppendorf AG, Hamburg, Germany). One eye received 2 μL vehicle (0.9% saline), and the contralateral eye was injected with an LPA_1 antagonist (THG1603; 20 mM) or Rho kinase inhibitor (Y-27632, 2 mM).

At P14, pups were killed and intracardially perfused with 4% paraformaldehyde (PFA). A suture was placed on top of the superior quadrant to facilitate orientation before eyes were fixed in 4% PFA and transferred to 30% sucrose. Enucleated eyes were frozen in optimum cutting temperature compound, and transverse sections (16 μm) were made with a cryostat. The resultant sections were stained with primary antibodies against LPA_1 receptor (1:400) followed by AlexaFluor 594 goat anti-rabbit IgG secondary antibody (1:300; Molecular Probes). Thereafter, the sections were counterstained with the nuclei marker 4′-6-Diamidino-2-phenylindole (DAPI). To quantify RGCs, only sections with the optic nerve were analyzed under a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) and were photographed with a digital camera (DMX1200; Nikon). RGC density was quantified in a masked manner by counting the number of nuclei in a defined length of retinal section and was expressed as RGCs per 500 μm.[12]

Primary RGC Cultures

Primary cultures of RGCs were derived from P7 to P8 rat pups using a two-step panning procedure, as previously described.[13,14] Briefly, retinas were dissected and dissociated enzymatically in a papain solution (Worthington Biochemicals, Lakewood, NJ). The RGCs were purified with anti–Thy-1.1 monoclonal antibodies (Cedarlane Laboratories Ltd., Burlington, ON, Canada) and were indentified by double immunolabeling.[15] First the cells were incubated with an antibody against LPA_1 (1:100) and AlexaFluor 488 secondary antibody (1:200; Molecular Probes). Then the cells were incubated with a primary antibody against β-III-tubulin (1:100; Sigma-Aldrich) and AlexaFluor 594 secondary antibody (1:200; Molecular Probes). After that, the cultures were examined under a fluorescence microscope. Negative controls were performed by replacing the primary antibody with nonimmune serum. A cell death detection kit (Boehringer Mannheim, Mannheim, Germany) was also used to identify apoptotic RGCs, according to the manufacturer's instructions. TUNEL-positive cells were counted in triplicate wells under a fluorescence microscope, and the percentage of apoptosis was calculated by using the total number of cells in these wells. After 24 hours of treatment with exposure to hypoxia, the percentage of apoptotic RGCs was not significantly increased compared with exposure to normoxia ($P > 0.05$; data not shown)

RGC-5 Cell Culture and Cell Viability Assay

The immortalized rat retinal ganglion cell line RGC-5 was kindly provided by Neeraj Agarwal (University of North Texas Health Science Center, Fort Worth, TX) and was cultured as described.[16] RGC-5 cells were induced to differentiate in serum-free medium with 1.0 μM staurosporine. In separate experiments, cells were exposed to hypoxia (2% O_2/5% CO_2) or hyperoxia (90% O_2/10% air) in air-tight chambers (Billups-Rothenberg Inc., Del Mar, CA) maintained in a tissue culture incubator. Cell viability was assessed by MTT, as previously described.[17]

Constructs and Transfection

The green fluorescent protein (GFP)-conjugated LPA_1 receptor expression plasmid (GFP-LPA_1) was generated by inserting LPA_1 cDNA (NM_053936) downstream of the cytomegalovirus promoter and GFP coding sequence in pEGFP-C1 vector (GFP-CTL) at *Xho*I and *Hin*dIII restriction sites. The construct was verified by sequencing. The shRNA-LPA_1 vector, which expresses shRNA directed against the rat LPA_1 receptor, was purchased from Open Biosystems (Huntsville, AL). The hairpin sequence of the shRNA in retroviral vector pSM2c (V2MM_65185) was sense (5′-ACGATGTCCTGGCCTATGAGAA-3′) and loop (5′-*TAGTGAAG CCACAGATGTA*-3′).

Transfections were performed with transfection reagent (FuGene HD; Roche Diagnostics, QC, Canada) according to the manufacturer's protocol to obtain greater than 80% transfection efficiency. Transfection efficiency of GFP-LPA_1 and GFP-CTL plasmid was measured by counting the percentage of the GFP-positive cells in each transfected well and was used to readjust the final data from these two groups. RGC-5 cells were seeded on sterile coverslips and transiently transfected with LPA_1-GFP or GFP-CTL expression plasmids for 36 hours. Cells were then exposed to hypoxia for different time periods and were fixed in 4% PFA. DAPI was used to stain nuclei. Confocal microscopy was performed on a laser scanning microscope (LSM-510; Zeiss, Thornwood, NY) with an oil-immersion lens (1003; Zeiss).

Western Blot Analysis and Real-Time Quantitative PCR

RGC-5 cells were seeded at a density of 1×10^6 cells per 100-mm plate and were exposed to normoxia (21% O_2), hypoxia (2% O_2), or hyperoxia (90% O_2) for 24 hours. Proteins were extracted for Western blot analysis as described.[17] Anti-LPA_1 receptor polyclonal antibody (1:2000) was used. β-Actin (1:10,000; Novus Biological) served as a loading control.

mRNA from retinal tissue and RGC-5 cells was extracted using an RNA extraction kit (Qiagen, Mississauga, ON, Canada). The following primers were used for PCR: LPA_1 receptor sense, 5′-AACCGG AGTGGAAAGTATCTAGC-3′; LPA_1 receptor antisense, 5′-AATGGCCCAGAAGACT AAGTAGG-3′. 18S PCR primers were purchased from Ambion (Austin, TX). Quantitative PCR was performed with a real-time PCR/thermal cycler system (SmartCycler; Cepheid, Sunnyvale, CA). For each sample, reactions were performed in duplicate, and threshold cycle numbers were averaged. LPA_1 receptor expression was

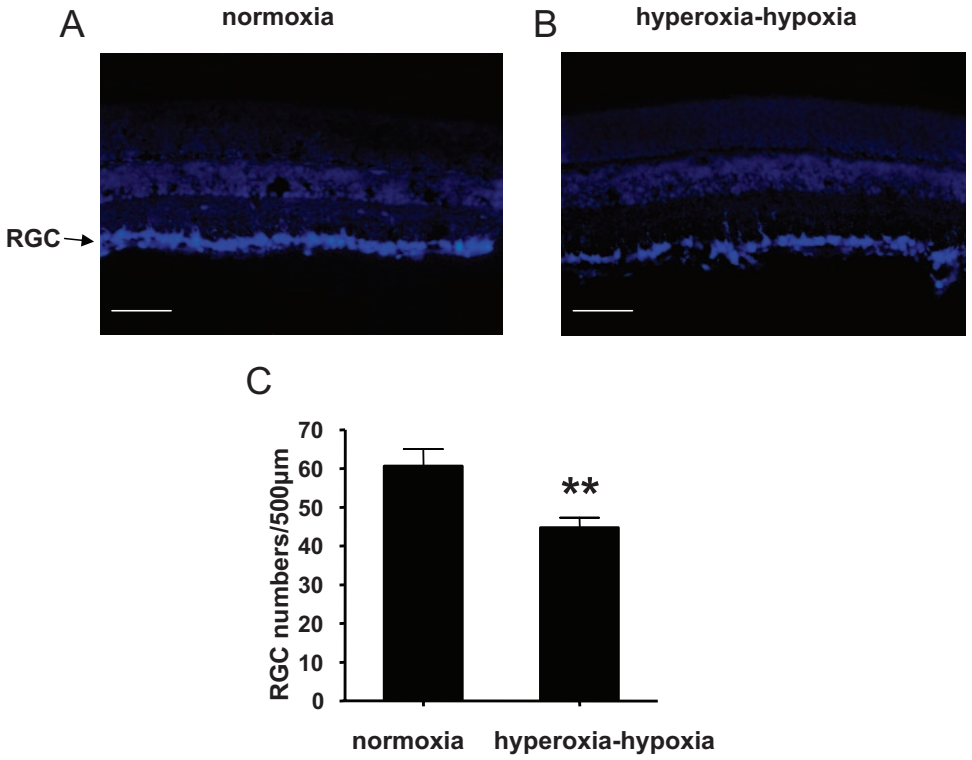

FIGURE 1. Effect of oxygen-induced retinopathy on survival of RGCs. Representative photographs of Fluorogold-labeled RGCs in the central retinas of (**A**) room air-raised pups (normoxia; $n = 10$ retinas) and (**B**) hyperoxia/hypoxia-exposed pups ($n = 10$ retinas) with (**C**) corresponding quantifications. $^{**}P < 0.01$ vs. normoxia. Scale bar, 60 μm.

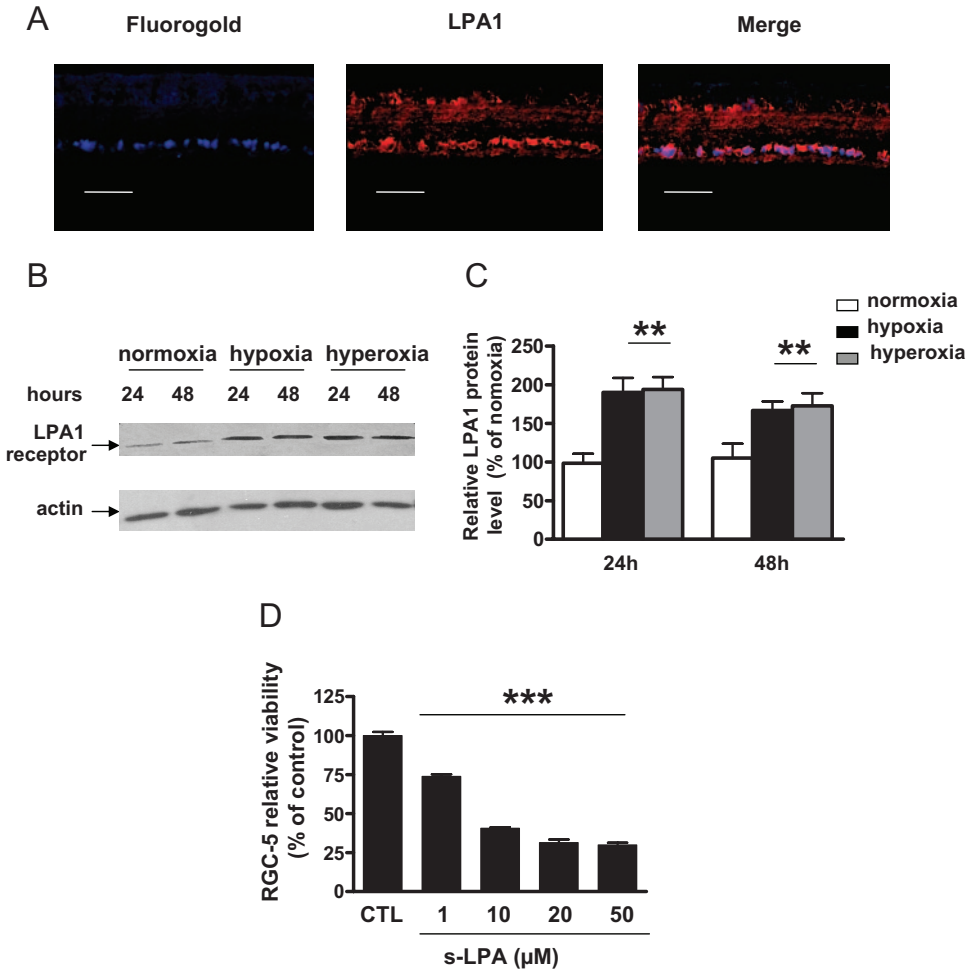

FIGURE 2. Expression of LPA_1 receptor in RGCs and effects of LPA on RGC-5 cell viability. (**A**) Immunohistochemical staining depicting Fluorogold retrograde-labeled RGCs (*left*), LPA_1 receptor (*middle*), and merged images (*right*) from normal retina tissue (scale bar, 50 μm). (**B**) Western blot and (**C**) quantification of LPA_1 receptor normalized to β-actin from RGC-5 cells exposed to hypoxia or hyperoxia for 24 and 48 hours. (**D**) Effects of indicated concentrations of stearoyl-LPA (s-LPA; 24 hours) on RGC-5 cell viability as determined by MTT assay. $^{**}P < 0.01$ vs. normoxia. $^{***}P < 0.001$ vs. control.

normalized to 18S, and the percentage of reduction was calculated according to the formula described.[18]

Neurite Outgrowth and Cell Morphology

Primary RGCs were cultured for 5 days on laminin-coated glass coverslips to permit neurite growth. Neurite length was measured before and after treatment with 20 μM s-LPA or after 24 hours of normoxia or hypoxia. Staurosporine induces differentiation and neurite outgrowth of RGC-5 cells in a dose-dependent manner.[16] RGC-5 cells were treated with 1 μM staurosporine in serum-free medium for 24 hours to induce neurite outgrowth and then were exposed to hypoxia. Photomicrographs were taken under an inverted microscope (Axiovert 200M; Zeiss) at 200× total magnification. Twelve primary RGCs and 30 RGC-5 cells from each condition were analyzed to assess neurite development.[19]

Statistical Analysis

Statistical analyses were performed with ANOVA, and comparison of means was performed with the appropriate post hoc test. Comparisons between two groups were made by Student's unpaired *t*-test. Values are presented as mean ± SEM. Statistical significance was set at $P < 0.05$.

Results

Effect of OIR on RGC Survival

We first intended to elucidate the impact of ROP on RGC survival by using a well-established model of OIR. Rat pups subjected to OIR had significantly reduced numbers of RGCs (26%) compared with their room air-raised counterparts ($P < 0.01$; Fig. 1).

Effects of Oxygen Stress on LPA$_1$ Receptor Expression and Impact of LPA on RGC-5 Cell Survival

Consistent with previous reports, immunolocalization of LPA$_1$ receptor was detected in the normal retina and was colocalized with Fluorogold-labeled RGCs (Fig. 2A). An in-

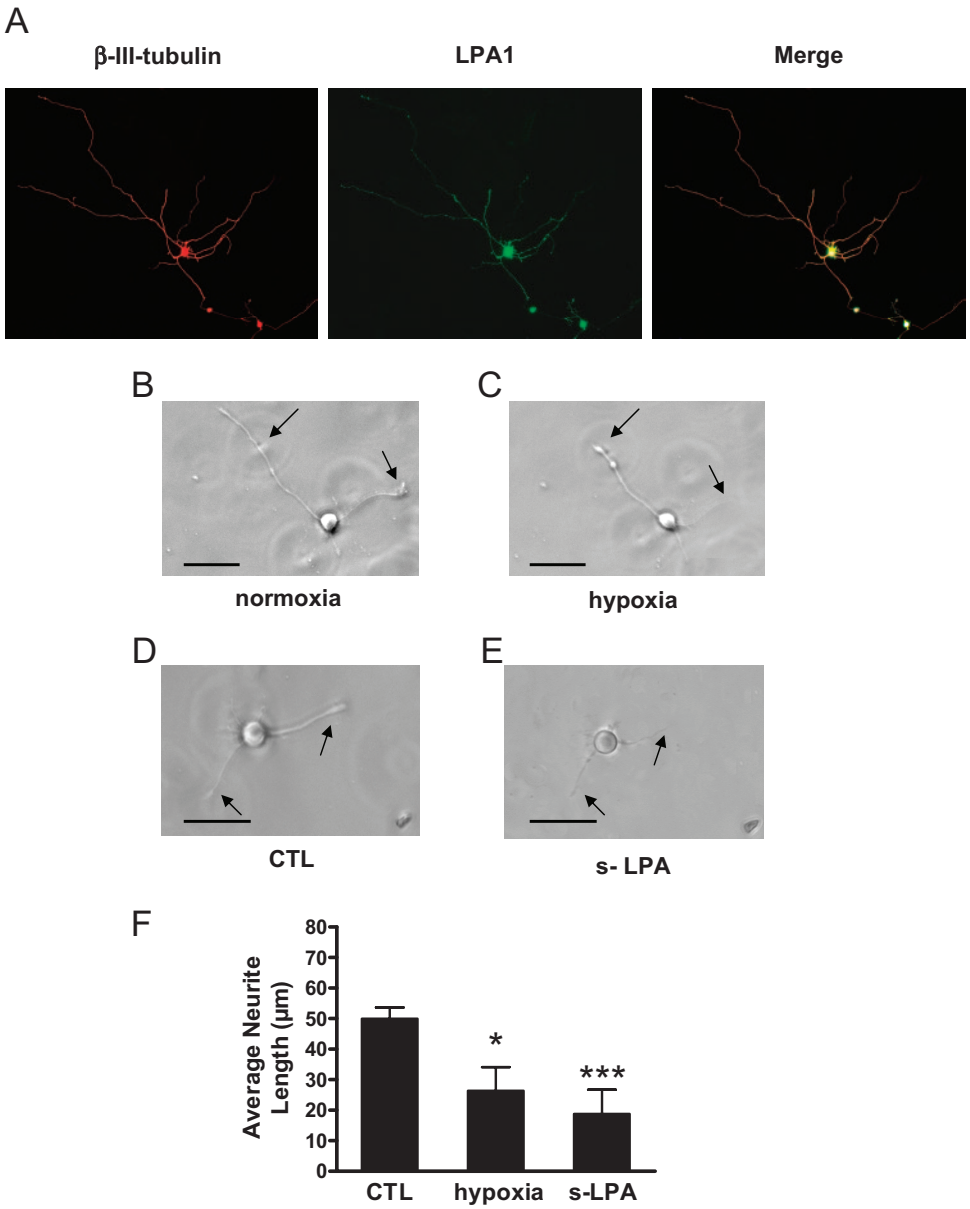

Figure 3. Effect of hypoxia and s-LPA on neurite outgrowth in primary RGCs. (**A**) Immunohistochemical staining demonstrating LPA$_1$ expression in primary RGCs. Representative photographs of cultured RGCs in (**B**) normoxia and (**C**) hypoxia (**D**) before and (**E**) after s-LPA treatment (20 μM, 24 hours). Neurite length is expressed as the average ± SEM of the projections of 12 cells (**F**). $^*P < 0.05$ vs. CTL, $^{***}P < 0.001$ vs. CTL. Scale bar, 20 μm.

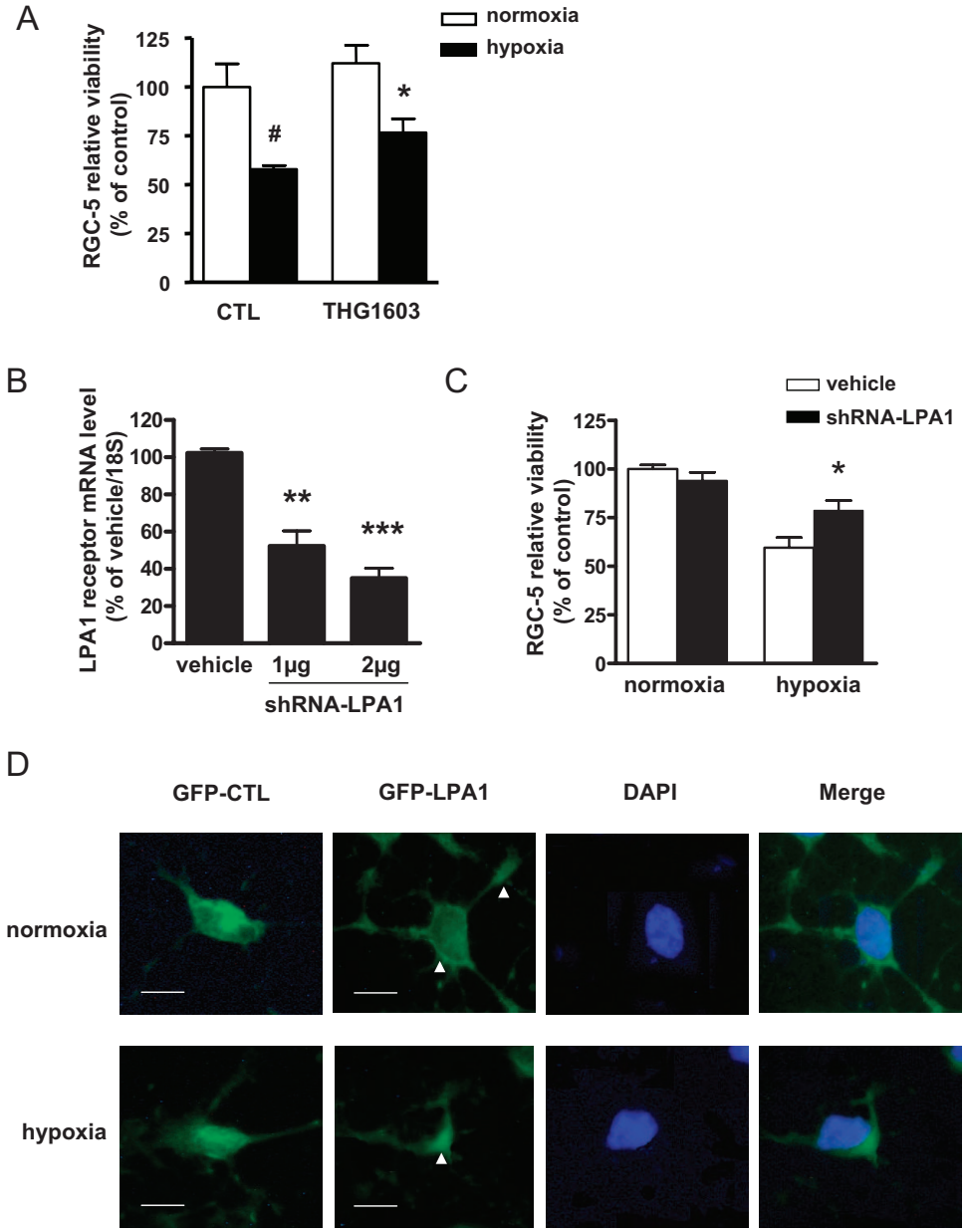

FIGURE 4. Impact of hypoxia on RGC-5 cell viability and implications of LPA_1 receptor signaling. (**A**) RGC-5 cells were exposed to hypoxia (24 hours) in the absence or presence of a specific LPA_1 receptor antagonist THG1603 (100 μM), and cell viability was assessed by MTT assay. $^{\#}P < 0.05$ vs. normoxia; $^{*}P < 0.05$ vs. normoxia + THG1603. (**B**) Quantitative RT-PCR analysis depicting LPA_1 receptor mRNA levels (relative to 18S) in RGC-5 cells transfected with shRNA-LPA_1 vectors (1 μg, 2 μg). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle control. (**C**) RGC-5 cells were transfected with shRNA-LPA_1 vector (2 μg) and subjected to normoxia or hypoxia, and cell viability was determined by MTT assay. $^{*}P < 0.05$ vs. normoxia vehicle. (**D**) RGC-5 cells were transiently transfected with GFP-CTL (*left*) or GFP-LPA_1 retroviral vector and exposed to normoxia (*upper*) or hypoxia (*lower*). Nuclei staining is depicted by DAPI (*middle*), and merged images are represented on the right. *Arrowheads* and *arrows* indicate plasma membrane and intracellular localization of GFP-LPA_1, respectively. Scale bar, 10 μm.

vestigation into the effects of oxygen stress revealed that LPA_1 protein expression was significantly elevated in hypoxia- and hyperoxia-exposed RGC-5 cells at 24 and 48 hours ($P < 0.01$; Figs. 2B, 2C). Additionally, the effects of LPA on RGC survival were evinced by dose-dependent decreases in cell viability on RGC-5 exposure to the LPA_1 receptor agonist s-LPA ($P < 0.001$; Fig. 2D).

Effect of Hypoxia and LPA on Primary RGC Neurite Outgrowth

Primary neonatal RGCs were purified by Thy1.1 antibody and characterized with the use of antibodies against β-III-tubulin and LPA_1; results confirmed that primary RGCs express LPA_1 (Fig. 3A). To further evaluate the effect of hypoxia and LPA on RGC viability, neurite length was determined. Data show that hypoxia and s-LPA significantly induced RGC neurite retraction (Figs. 3B-F; $P < 0.05$, $P < 0.001$ vs. normoxia, respectively).

Roles of Hypoxia and LPA_1 Receptor in Eliciting RGC-5 Degeneration

RGC-5 cells were exposed to hypoxia or hyperoxia for 24 hours, and their viability was assessed by MTT assay. Hypoxia markedly reduced RGC-5 cell viability ($P < 0.05$; Fig. 4A), whereas hyperoxia had no significant effect (data not shown). Intriguingly, and in agreement with the results obtained with s-LPA, pretreatment of RGC-5 cells with a specific LPA_1 receptor antagonist, THG1603 (100 μM), substantially attenuated hypoxia-evoked RGC-5 cell loss ($P < 0.05$; Fig. 4A). To further confirm the contribution of LPA_1 receptor, we knocked down its expression using shRNA-LPA_1 retroviral vector. The efficiency of LPA_1 mRNA downregulation was 40% and 60% in RGC-5 cells treated with 1 μg and 2 μg shRNA-LPA_1 vector, respectively ($P < 0.01$; $P < 0.001$; Fig. 4B). Accordingly, under hypoxia, RGC-5 cells incubated with 2 μg shRNA-LPA_1 exhibited 28% increased cell viability compared with the GFP-CTL–transfected group ($P < 0.05$; Fig. 4C).

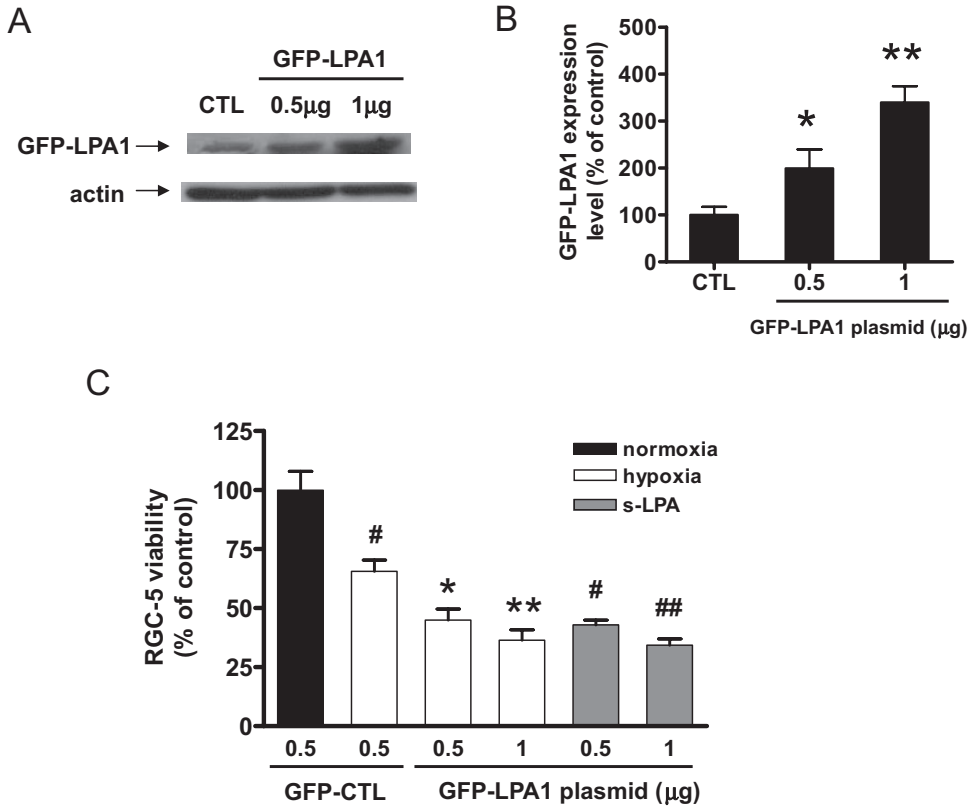

FIGURE 5. Effect of overexpressing LPA$_1$ receptor on RGC-5 viability. (**A**) GFP-LPA$_1$ protein was detected in the transfected RGC-5 cells by Western blot and (**B**) presented as the percentage of the control (transfected with GFP-CTL). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with GFP-CTL. (**C**) Hypoxia and s-LPA significantly reduced cell viability of GFP-LPA$_1$-transfected groups compared with the GFP-CTL group, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with GFP-CTL hypoxia; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with GFP-CTL normoxia.

Our data thus far implied an elusive relationship between hypoxia and LPA$_1$ receptor signaling. To explore this hypothesis, we designed and transiently transfected RGC-5 cells with a GFP-LPA$_1$ receptor expression plasmid. As shown in the confocal images in Figure 4D, GFP-LPA$_1$–transfected cells displayed the classic neuron morphology and membrane localization of LPA$_1$ receptor. Conversely, under hypoxia, RGC-5 cells adopted a rounded phenotype with preferential redistribution of LPA$_1$ receptor to the cytoplasm, which was clearly different from the morphology of GFP-CTL–transfected cells.

Effect of Overexpressing LPA$_1$ Receptor on Hypoxia-Induced RGC Degeneration

To corroborate the hypothesis that LPA$_1$ mediates hypoxia-elicited RGC degeneration, we overexpressed the LPA$_1$ receptor in RGC-5 cells. The success of the overexpression system was demonstrated by a dose-dependent increase in LPA$_1$ protein levels compared with control (Figs. 5A, 5B). As shown in Figure 5C, overexpressing LPA$_1$ markedly increased the susceptibility of RGC-5 to hypoxia and s-LPA treatment (Fig. 5C; $^{*}P < 0.05$ vs. GFP-CTL hypoxia; $\#P < 0.05$ vs. GFP-CTL normoxia).

Implications of Caspase and Oxidative Stress Mechanisms in Hypoxia-Induced RGC-5 Cell Degeneration

It has been postulated that hypoxia/ischemia-induced RGC death operates through caspase-mediated apoptotic[2,15] and oxidative stress[20–22] mechanisms. Here we pretreated RGC-5 cells with a broad caspase inhibitor, Q-VD-OPh, before hypoxia exposure and observed that Q-VD-OPh significantly and dose dependently increased RGC-5 cell viability compared with hypoxia ($P < 0.01$; Fig. 6A). Optimal effects were achieved at 10 μM, which is within the range at which Q-VD-OPh does not exhibit toxicity.[23] Moreover, NAC (10 μM), a potent antioxidant with proven protection against oxidant stress-induced neuronal death,[24] significantly abrogated the adverse effects of hypoxia on RGC-5 cell viability ($P < 0.05$; Fig. 6B).

Effects of Hypoxia and LPA$_1$ Receptor and ROCK Signaling on RGC-5 Neurite Outgrowth

Given that RGCs are neuronal cells, we evaluated whether hypoxia hinders their neurite outgrowth. RGC-5 neurite length was substantially reduced under hypoxia ($P < 0.05$ vs. normoxia; Figs. 7A, 7B); this effect was prevented by THG1603 (100 μM; $P < 0.05$ vs. hypoxia; Figs. 7A, 7B). Because Rho kinase (ROCK) is a downstream effector of LPA$_1$ receptor with demonstrated roles in actin reorganization and cell motility,[25] we pretreated RGC-5 cells with the specific ROCK inhibitor Y27632 (10 μM; 24 hours) and observed a preservation of neurite length compared with hypoxia alone ($P < 0.05$ vs. hypoxia; Figs. 7A, 7B).

Effects of LPA$_1$ Receptor and ROCK Inhibition on RGC Survival during OIR

Finally, we questioned whether antagonizing LPA$_1$ receptor or ROCK signaling was neuroprotective against OIR-induced RGC loss. Rat pups were intravitreally injected with saline, THG1603 (20 mM), or Y27632 (2 mM) and were subjected to normoxia or hyperoxia/hypoxia. Consistent with our in vitro findings, THG1603 and Y27632 significantly prevented OIR-evoked RGC loss ($P < 0.05$; Figs. 8A, 8B).

DISCUSSION

Although previous studies proposed that hypoxia induces RGC death[1,2] and apoptosis in the inner retinal layers,[26,27] direct evidence linking hypoxia to RGC loss was lacking. Herein we report for the first time that RGC density is significantly dimin-

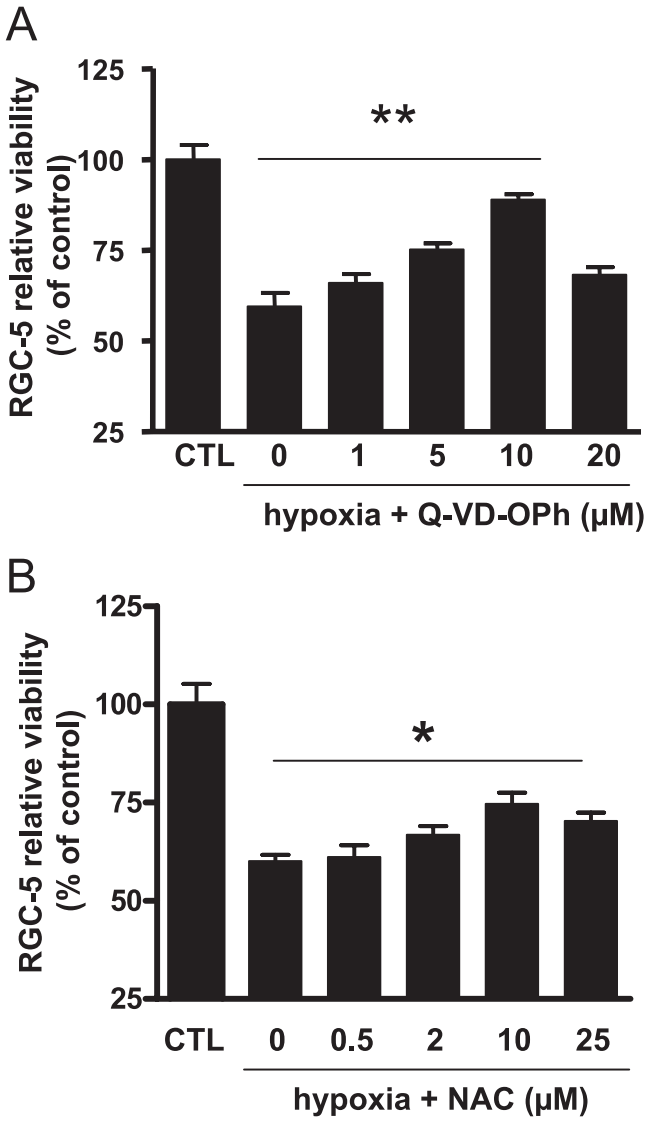

FIGURE 6. Contributions of caspase 3 and oxidative stress mechanisms in hypoxia-induced RGC loss. Graphs illustrating the relative viability of RGC-5 cells after exposure to normoxia or hypoxia in the absence or presence of increasing concentrations of (**A**) broad caspase inhibitor (Q-VD-Oph) or (**B**) NAC. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

ished in rat pups subjected to OIR (Fig. 1). RGCs were labeled with the retrograde fluorescent tracer Fluorogold by injection into the superior colliculus of neonatal rats. This allowed for specific identification of RGCs because fluorescence does not appear in retinal layers other than the RGC layer.[11,28]

Although some may argue that primary RGC cultures are more appropriate for in vitro studies than the immortalized RGC-5 cell line used in our study, it is well appreciated that the viability of purified RGCs is limited.[29] Of relevance, short-term exposure to a broad-spectrum kinase inhibitor, staurosporine, induces RGC-5 cells to adopt characteristic morphologic, postmitotic, electrophysiological, and antigenic features of mature RGCs without inducing apoptosis.[19] Moreover, RGC-5 cells have been routinely used to test the effects of various factors on cell survival and regeneration.[30] Having demonstrated that RGC-5 cells and primary RGCs express the LPA_1 receptor (Figs. 2B, 3A) and that the in vivo deleterious consequences of hypoxia are reproducible in these cells (Figs. 3B–E, 4A–C), we are confident that the RGC-5 cell line is suitable for studying the role of LPA_1 receptor in RGC pathophysiology.

Mediators of oxygen-induced cell death are complex and not fully understood. Our studies focused on LPA_1 receptor for several reasons. First, LPA, a key intermediate in glycerolipid synthesis, is particularly abundant in the brain,[31] and its concentration increases during injury.[32] Second, LPA receptors are present on various cell types of the central nervous system and mediate diverse biological functions.[5] Third, the expression of LPA receptors is upregulated in the ischemic retina.[9] Nonetheless, despite this evidence, defining the precise role for LPA_1 receptor in RGC degeneration has remained elusive. To our knowledge, this is the first demonstration that OIR-induced RGC loss is LPA_1 receptor dependent. Although our data show that LPA_1 receptor expression was augmented by exposure to hypoxia and hyperoxia (Figs. 2B, 2C), RGC-5 cell viability was

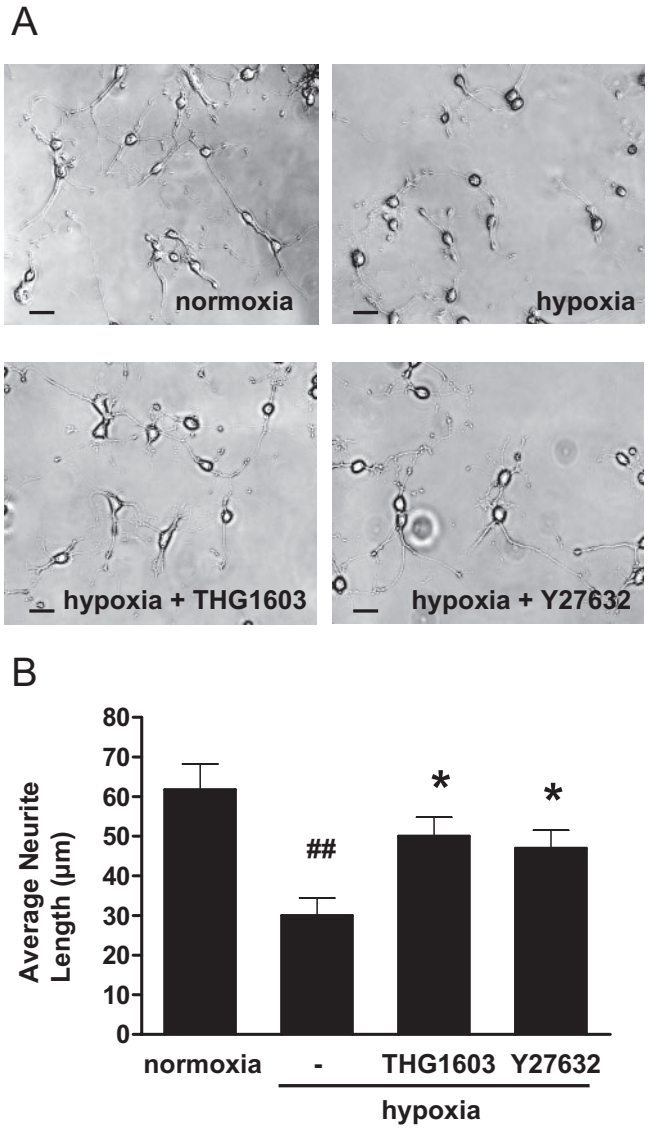

FIGURE 7. Effects of hypoxia, LPA_1 receptor, and ROCK on RGC-5 neurite outgrowth. (**A**) Representative phase-contrast images and (**B**) graph of RGC-5 neurites pretreated with a LPA_1 antagonist (100 μM THG1603) or ROCK inhibitor (10 μM Y27632) and exposed to normoxia or hypoxia. Neurite differentiation was induced by incubating RGC-5 cells with staurosporine (1 μM). (**B**) Neurite length was expressed as the average ± SEM of the projections of 30 cells. $^{\#\#}P < 0.01$ vs. normoxia; $^{*}P < 0.05$ vs. hypoxia. Scale bar, 20 μm.

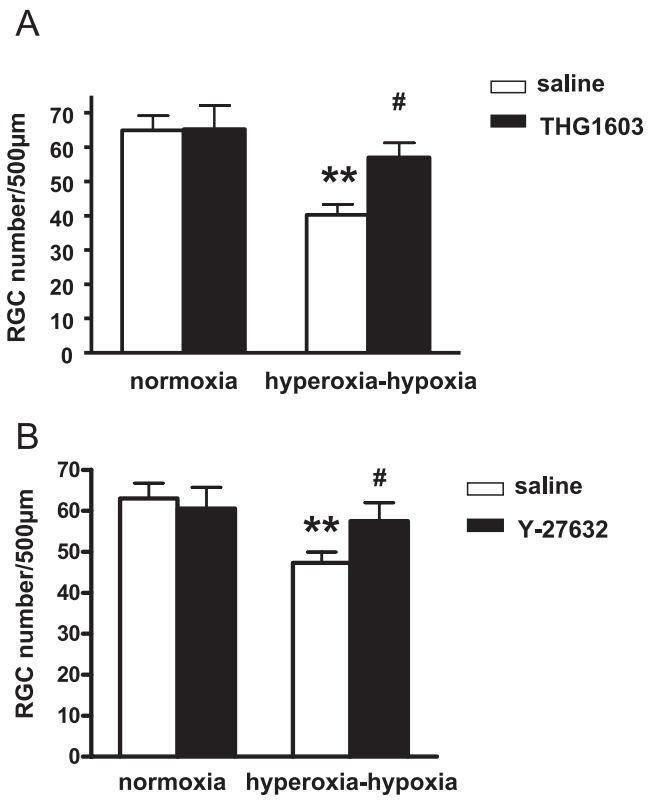

FIGURE 8. Roles of LPA$_1$ receptor and ROCK signaling on RGC survival in vivo. Rat pups were subjected to normoxia or hyperoxia/hypoxia from P0 to P14. Intravitreal injections were performed on P3, P6, P9, and P12 with saline in the contralateral eye ($n = 20$ retinas) and (**A**) LPA$_1$ antagonist (20 mM THG1603; $n = 8$ retinas) or (**B**) ROCK inhibitor (2 mM Y27632; $n = 12$ retinas) in the opposite eye. RGC density was determined by Fluorogold retrograde labeling. $^{**}P < 0.01$ vs. normoxia-saline; $^{\#}P < 0.05$ vs. hyperoxia/hypoxia.

unaffected by the latter (data not shown), suggesting that during OIR, the hypoxic phase is primarily responsible for inducing RGC degeneration.

Ample studies suggest that LPA receptors exhibit mitogenic or antiapoptotic effects in various cell types.[33–36] Conversely, we propose that LPA$_1$ receptor mediates RGC loss (Figs. 2D, 4A, 5C) and impedes neurite outgrowth (Figs. 3B-F, 7), which is in agreement with reports that LPA induces apoptosis of cultured hippocampal neurons, PC12 cells, and endothelial cells.[6,37,38] Therefore, our findings highlight the premise that LPA receptor signaling is diverse and that different receptors have cell type-specific roles.[39,40]

Members of the caspase family are major determinants of inflammation and apoptosis.[41,42] In our study, broad caspase inhibition prevented hypoxia-induced RGC loss (Fig. 6A), which is corroborated by studies reporting the activation of caspase 3 and 8 in ischemia exposed RGCs.[2,15] Increasing lines of evidence also suggest a key role for oxidative stress in the pathogenesis of neurodegenerative diseases. Oxidative stress is known to decrease cellular bioenergetic capacity, which results in increased reactive oxygen species production and consequent cellular damage and apoptosis.[24] Our results indicating the neuroprotective effects of the antioxidant GSH precursor NAC (Fig. 6B) support the view that hypoxia-induced RGC loss involves oxidative stress mechanisms.

The LPA$_1$ receptor C-terminal tail is presumably involved in ligand-induced receptor desensitization and internalization.[43] Our studies unveil novel hypoxia-evoked internalization of LPA$_1$ receptor (Fig. 4D), suggesting that hypoxia activates LPA$_1$ receptor signaling. The biological activities of LPA$_1$ receptor are exerted through multiple signal transduction pathways, including those initiated by the small GTPase Rho,[44] which in turn stimulates several downstream kinases, including ROCK.[45,46] Of interest, numerous studies have revealed that LPA receptors promote neurite retraction and cell rounding by Rho-A–dependent and Rho-A–independent pathways[47] and that the inhibition of ROCK promotes nerve regeneration.[48] Along these lines, we show that the inhibition of ROCK preserves RGC neurite outgrowth (Fig. 7) and survival (Fig. 8) after hypoxia and OIR, respectively.

In summary, we provide compelling evidence that OIR-elicited RGC degeneration is in part mediated by the LPA$_1$ receptor signaling pathway. Given that inhibitors of LPA$_1$ receptor and its downstream effector ROCK were neuroprotective and enhanced RGC survival during OIR, antagonists of this pathway may represent promising therapeutic alternatives for managing retinal diseases associated with RGC degeneration, such as ROP and glaucoma.

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