

# PARP Inhibitor Protects Against Chronic Hypoxia/Reoxygenation-Induced Retinal Injury by Regulation of MAPKs, HIF1 $\alpha$ , Nrf2, and NF $\kappa$ B

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**PURPOSE.** In the eye, chronic hypoxia/reoxygenation (H/R) contributes to the development of a number of ocular disorders. H/R induces the production of reactive oxygen species (ROS), leading to poly(ADP-ribose) polymerase-1 (PARP1) activation that promotes inflammation, cell death, and disease progression. Here, we analyzed the protective effects of the PARP1 inhibitor olaparib in H/R-induced retina injury and investigated the signaling mechanisms involved.

**METHODS.** A rat retinal H/R model was used to detect histologic and biochemical changes in the retina.

**RESULTS.** H/R induced reductions in the thickness of most retinal layers, which were prevented by olaparib. Furthermore, H/R caused increased levels of Akt and glycogen synthase kinase-3 $\beta$  phosphorylation, which were further increased by olaparib, contributing to retina protection. By contrast, H/R-induced c-Jun N-terminal kinase and p38 mitogen-activated protein kinases (MAPK) phosphorylation and activation were reduced by olaparib, via mitogen-activated protein kinase phosphatase 1 (MKP-1) expression. In addition, H/R-induced hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) levels were decreased by olaparib, which possibly contributed to reduced VEGF expression. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression was slightly increased by H/R and was further activated by olaparib. Nuclear factor- $\kappa$ B (NF $\kappa$ B) was also activated by H/R through phosphorylation (Ser536) and acetylation (Lys310) of the p65 subunit, although this was significantly reduced by olaparib.

**CONCLUSIONS.** Olaparib reduced H/R-induced degenerative changes in retinal morphology. The protective mechanisms of olaparib most probably involved Nrf2 activation and ROS reduction, as well as normalization of HIF1 $\alpha$  and related VEGF expression. In addition, olaparib reduced inflammation by NF $\kappa$ B dephosphorylation/inactivation, possibly via the PARP1 inhibition-MKP-1 activation-p38 MAPK inhibition pathway. PARP inhibitors represent potential therapeutics in H/R-induced retinal disease.

Keywords: retinal ischemia, MAPKs, NF $\kappa$ B, PARP, transcription factors

Retinal ischemia-reperfusion injury occurs in several eye diseases, among them probably glaucoma, diabetic retinopathy, and other ocular vascular disorders<sup>1-3</sup> are the most important. Reactive oxygen species (ROS) production in retinal ischemia-reperfusion injury leads to lipid oxidation, protein disorder, and DNA damage.<sup>4,5</sup> Ischemic injury leads to oxidative stress initiating mitochondrial damage, which can activate cell death pathways.<sup>6-8</sup> ROS and peroxynitrite can initiate DNA breaks followed by activation of poly(ADP-ribose) polymerase-1 (PARP1), a nuclear enzyme involved in DNA repair.<sup>9</sup> PARP1 catalyzes PARylation of nuclear proteins that leads to alteration of signaling pathways and transcription factors.<sup>10</sup> PARP activation suppresses protein kinase B (Akt) activation<sup>11</sup> and

activates c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs),<sup>12</sup> which can activate inflammatory processes,<sup>13</sup> destabilize mitochondria, and induce cell death.<sup>12</sup> A modest activation of PARP can reduce NAD<sup>+</sup> levels, as well as the activity of sirtuins (SIRT's), which play an important role in epigenetic regulation of gene expression.<sup>14</sup> However, overactivation of PARP1 depletes the cellular pools of NAD<sup>+</sup> and ATP, eventually leading to necrotic cell death.<sup>13</sup> Consequently, PARP inhibitors may have a protective role in oxidative stress-related disorders, including ischemic retinal disease.

It has been reported that neuronal cells of the inner retinal layers (retinal ganglion and amacrine cells) are the most

susceptible to hypoxia,<sup>4-7</sup> leading to a reduced thickness of the inner retinal layers.<sup>5,8</sup> PARP1 is one of the most important regulators of cell death processes from a variety of ambient stimuli.<sup>9</sup> In the retina, increased PARP activation leads to optic nerve transection-induced retinal ganglion cell death<sup>15</sup> and is involved in photoreceptor degeneration in a transgenic mouse model<sup>16</sup> and oxidative stress-induced ganglion cell loss.<sup>17</sup> On the other hand, inhibition of PARP protects against chronic hypoperfusion-induced neurodegeneration,<sup>18</sup> diabetic retinopathy,<sup>19</sup> and *N*-methyl-D-aspartate-induced cell death.<sup>20</sup>

Under stress conditions for the retina such as ischemia-reperfusion, inflammation can cause injury progression, although it usually helps in repair. Chronic hypoxia/reoxygenation (H/R) causes excess ROS production and increases intracellular calcium and mitochondrial damage, leading to PARP1 activation and further tissue damage. In addition, H/R activates MAPKs, nuclear factor  $\kappa$ B (NF $\kappa$ B), and hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) that contribute to inflammation and revascularization, thus causing serious problems in the retina.<sup>21,22</sup>

In this study, we used a rat model in which 2 weeks of retinal hypoxia resulting from systemic hypoxia was established, followed by 2 weeks of reoxygenation. This chronic model provided a better simulation of the conditions of disease development than those of most previous studies and represented a reliable method for simulating hypoxia-related disease processes.<sup>23</sup> We analyzed the effect of the PARP inhibitor olaparib on retinal damage in this model and described its protective role on retinal structure, cytokine and chemokine expression, kinase cascades (mammalian target of rapamycin [mTOR]/phosphoinositide 3-kinase [PI3K]/Akt, and MAPKs), and activation of related transcription factors, including NF $\kappa$ B, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and HIF1 $\alpha$ . Our findings indicated that PARP inhibitors could be potential drug candidates in the therapy of ischemic retinal damage.

## MATERIALS AND METHODS

### Animals

Three-month-old male Wistar rats ( $n = 48$ ) weighing 250 to 300 g were used. The experiment was approved by the Animal Research Review Committee of the University of Pécs Medical School, Hungary (Permit number: BA02/2000-24/2011). The animals received care according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication number 85-23, revised 1996) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were maintained under a 12-hour light/dark cycle, with free access to standard rat chow and water.

### H/R and Olaparib Treatment

Rats were divided randomly into four groups ( $n = 12$  in each group). The control group (C) consisted of rats held under normal pressure (101.3 kPa) and oxygen concentration (21%). The control + olaparib group (C + Olap) contained rats held under normal pressure (101.3 kPa) and partial oxygen concentration (21%), receiving olaparib in their drinking water (estimated 4 mg/kg daily) for 28 days. The H/R group was composed of rats exposed to 50-kPa air pressure and a 10% oxygen concentration (equal to that at an altitude of 5000 m above sea level) for 14 days and then returned to normal pressure (101.3 kPa) and oxygen concentration (21%) for an additional 14 days. Finally, the H/R + olaparib group (H/R +

Olap) consisted of rats exposed to an air pressure of 50 kPa and 10% oxygen concentration for 14 days and then returned to normal pressure (101.3 kPa) and oxygen concentration (21%) for an additional 14 days, with olaparib in their drinking water (estimated 4 mg/kg daily) throughout the experiment. At the end of the experiment, rats were anesthetized with 1% sodium pentobarbital (40 mg/kg) intraperitoneally and euthanized by cervical dislocation. During the experiment, the animals were observed twice daily, and all efforts were made to minimize their suffering. No accidental deaths occurred during the experiment.

### Histologic Analysis

After euthanizing the animals, their eyes ( $n = 6$  per group) were dissected into 0.1 M PBS and fixed in 4% paraformaldehyde (PFA) dissolved in 0.1 M PBS (Sigma-Aldrich Corp., Budapest, Hungary). Eyecup tissue was embedded in Durcupan ACM resin (Fluka, Buchs, Switzerland) and then dissected. Two-micrometer sections were stained with toluidine blue (Sigma-Aldrich Corp.) and mounted in distyrene-plasticizer-xylene medium (Sigma-Aldrich Corp.). Images of retinas were captured with a Nikon Eclipse 80i microscope, using the Q-Capture Pro7 program (Nikon, Tokyo, Japan). Tissue blocks were prepared (four per animals), and central retinal areas within 1 mm of the optic nerve were examined (five measurements per tissue block). For the retinal cross sections from the outer limiting membrane to the inner limiting membrane (OLM – ILM), measurements were made of the width of the outer nuclear layer (ONL), the inner nuclear layer (INL), the outer plexiform layer (OPL), and the inner plexiform layer. In addition, the cell number per 100- $\mu$ m section length in the ganglion cell layer (GCL) was determined.

### Cytokine, Chemokine, and Growth Factor Measurements

After removal, rat retinas ( $n = 6$  per group) were pooled and analyzed semiquantitatively using the Proteome Profiler Rat Cytokine Array Kit, Panel A (R&D Systems, Biomedica, Budapest, Hungary), according to the manufacturer's protocol. Samples were homogenized in PBS containing protease inhibitor cocktail (1:1000; Sigma-Aldrich Corp.), and Triton X-100 was added to a final concentration of 1%. Detection was achieved with streptavidin-horseradish peroxidase and a chemiluminescent detection reagent (Amersham Biosciences, Budapest, Hungary). Images were captured on X-ray film, which were scanned and analyzed with Protein Array Analyzer for ImageJ software (National Institutes of Health, Bethesda, MD, USA). Assays were repeated three times.

### Assessment of Oxidative DNA Damage

Retina samples ( $n = 6$  per group) were homogenized in chilled nuclei lysis solution from the Wizard Genomic DNA Purification Kit (Bio-Science Ltd., Budapest, Hungary) and processed according to the manufacturer's protocol. Oxidative damage to the purified genomic DNA samples was assessed using the HT 8-oxo-dG ELISA Kit (Bio-Techne, R&D Systems Ltd., Budapest, Hungary), according to the manufacturer's protocol. Assays were repeated three times.

### Immunoblotting

Retina samples ( $n = 6$  per group) were homogenized in ice-cold homogenization buffer, containing 50 mM Tris, pH 8.0, and phosphatase and protease inhibitor cocktails (Sigma-

Aldrich Corp.). Alternatively, nuclear protein extracts were prepared from retina samples as described previously.<sup>24</sup> Precipitated proteins were dissolved in Laemmli sample buffer, separated on 7%, 10%, or 15% SDS-PAGE gels, and transferred onto nitrocellulose membranes. Membranes were blocked in 3% nonfat milk for 2 hours, and then they were probed overnight at 4°C with antibodies recognizing the following antigens (1:500 dilution; Cell Signaling Technology, Danvers, MA, USA): PARP1, phospho-(p)Akt<sup>S473</sup>, pmTOR<sup>Scr2448</sup>, p-glycogen synthase kinase 3β (pGSK-3β)<sup>Scr9</sup>, pNFκB p65<sup>Scr536</sup>, Nrf2, total Akt, and NFκB p65. Membranes were also probed with antibodies recognizing the following antigens (1:1000 dilution; GeneTex, Irvine, CA, USA): poly(ADP-ribose) (PAR), p-extracellular signal-regulated kinase (pERK)1/2<sup>Thr202/Tyr204</sup>, pp38 MAPK<sup>Thr180</sup>, pJNK1/2<sup>Thr183/Tyr185</sup>, acetyl-(Ac)NFκB p65<sup>Lys310</sup>, p38 MAPK, JNK1/2, ERK1/2, HIF1α, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mitogen-activated protein kinase phosphatase 1 (MKP-1). Appropriate horseradish peroxidase-conjugated secondary antibodies were used: goat anti-rabbit antibody (1:3000 dilution; Bio-Rad, Budapest, Hungary) or rabbit anti-mouse antibody (1:3500 dilution; Sigma-Aldrich Corp.). Protein bands were visualized with the ECL immunoblotting detection system (Amersham Biosciences, Piscataway, NJ, USA). Films were scanned, and pixel densities of the bands were determined using ImageJ software. Each experiment was repeated four times.

### Immunofluorescence

Immunolocalization of nitrotyrosine residues was performed using standard immunofluorescence techniques. Briefly, eye-cups ( $n = 6$  per group) were prepared as described above, fixed in 4% PFA for 2 hours at room temperature, and cryoprotected in 15% and 30% sucrose solutions at 4°C until the tissues sank. Retinal cryosections were cut to a thickness of 12 μm and stored at -20°C until used. Slides were incubated with anti-nitrotyrosine primary antibody (1:150; Millipore, Burlington, MA, USA) overnight at room temperature. The next day, they were incubated for 2 hours with Alexa Fluor 488 secondary fluorescent antibody (1:1000; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Omission of the primary antibody from the incubation steps resulted in no labeling (data not shown). Photographs were taken with an Olympus Fluorview FV-1000 laser confocal scanning microscope (Olympus, Tokyo, Japan). Images were only adjusted for contrast and intensity.

### Statistical Analysis

Data were expressed as mean ± SEM. The Kolmogorov-Smirnov normality test, followed by 2-way ANOVA and Fisher's least significant difference (LSD) post hoc analysis, was used to determine differences (OriginPro, OriginLab, Northampton, MA, USA). Differences were considered significant at  $P \leq 0.05$ .

## RESULTS

### Effects of Olaparib Treatment on Histologic Changes of the Retina

Except for a decrease in INL thickness, olaparib treatment did not cause any morphologic alterations to the retinal layers in control animals (Figs. 1A, 1B). By contrast, chronic H/R animals showed signs of severe retinal degeneration compared with controls, with all layers, except OPL, being significantly thinner. In addition, several empty cell body-shaped spaces were seen in the ONL, indicating cell death (Figs. 1A, 1B).

However, the number of cells in the GCL did not change under H/R and/or olaparib treatment (Figs. 1A, 1B). The reduction of the individual retinal layers contributed to the severe reduction of the retinal thickness between the OLM and the ILM (Figs. 1A, 1B). Olaparib treatment ameliorated the aforementioned effects of chronic H/R and effectively preserved the whole retinal distance: OLM – ILM (Figs. 1A, 1B).

### Effects of Olaparib Treatment on PARylation, PARP1, and Cellular Oxidative Stress Status in the Retina

To confirm that olaparib could pass through the blood-retina barrier, we analyzed PARylation and PARP1 levels in the retina samples. Indeed, we found that the PARP inhibitor olaparib could effectively decrease PARP1 levels both in the control and the H/R groups (Figs. 2A, 2B). Furthermore, olaparib treatment significantly decreased H/R-induced PARylation of the retinal proteins (Figs. 2A, 2B).

To confirm that the status of cellular oxidative stress had changed under the different conditions examined, tyrosine nitrosylation was studied using immunofluorescence. We used a specific primary antibody against the nitrotyrosine residues, followed by a fluorescent secondary antibody, on retina sections. Besides a weak labeling of the photoreceptor layer under all conditions examined, prominent staining could be observed in the INL in H/R conditions (Fig. 2C). Olaparib significantly reduced the nitrotyrosination process when applied in H/R conditions (Fig. 2C). In addition, to assess oxidative DNA damage, we determined 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) levels in the retina samples using a specific ELISA. H/R was found to increase the oxidative DNA damage; however, this was reduced close to control levels by olaparib (Fig. 2D).

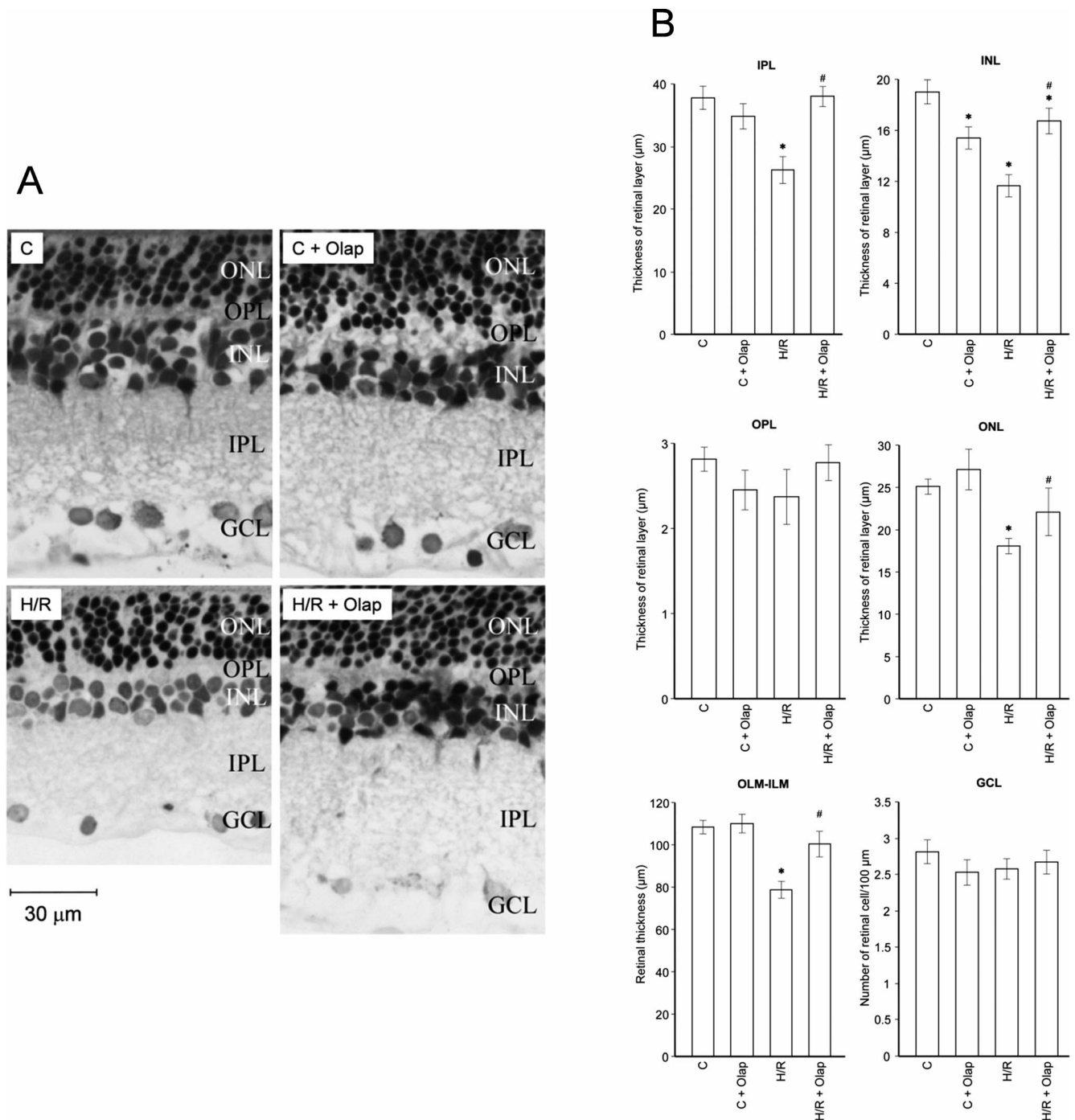
### Effect of Olaparib Treatment on Cytokine, Chemokine, and Growth Factor Expression

We measured 29 different cytokines, chemokines, and growth factors with the Proteome Profiler Rat Cytokine Array Kit, Panel A (R&D Systems, Minneapolis, MN, USA). Among them, five showed alterations after H/R and/or olaparib treatment (Fig. 3A). Olaparib alone did not cause any substantial change in most of the cytokine levels; however, ciliary neurotrophic factor (CNTF), soluble intercellular adhesion molecule-1 (sICAM-1), and reduced thymus chemokine (CXCL7) did show elevated levels (Fig. 3B). On the other hand, no significant increase was observed in levels of CNTF, fractalkine (CX3CL1), sICAM-1, CXCL7, and VEGF in retinas subjected to H/R conditions compared with the control group (Fig. 3B). Olaparib treatment attenuated the activation of CNTF, fractalkine, sICAM-1, and VEGF induced by H/R (Fig. 3B).

### Effects of Olaparib Treatment on mTOR/PI3K/Akt/and MAPK Pathways in H/R-Induced Retinal Injury

Olaparib treatment elevated the phosphorylation (activation) of mTOR<sup>Scr2448</sup> (Figs. 4A, 4B). However, H/R increased mTOR phosphorylation to a much higher extent than olaparib and was diminished by the addition of olaparib (Figs. 4A, 4B). Phosphorylation (activation) of Akt and phosphorylation (inactivation) of its downstream target GSK-3β were significantly elevated following olaparib treatment in control retinas (Figs. 4A, 4B). H/R also enhanced phosphorylation of these proteins compared with the control, although in a much lower extent than olaparib. However, H/R + Olap treatment caused



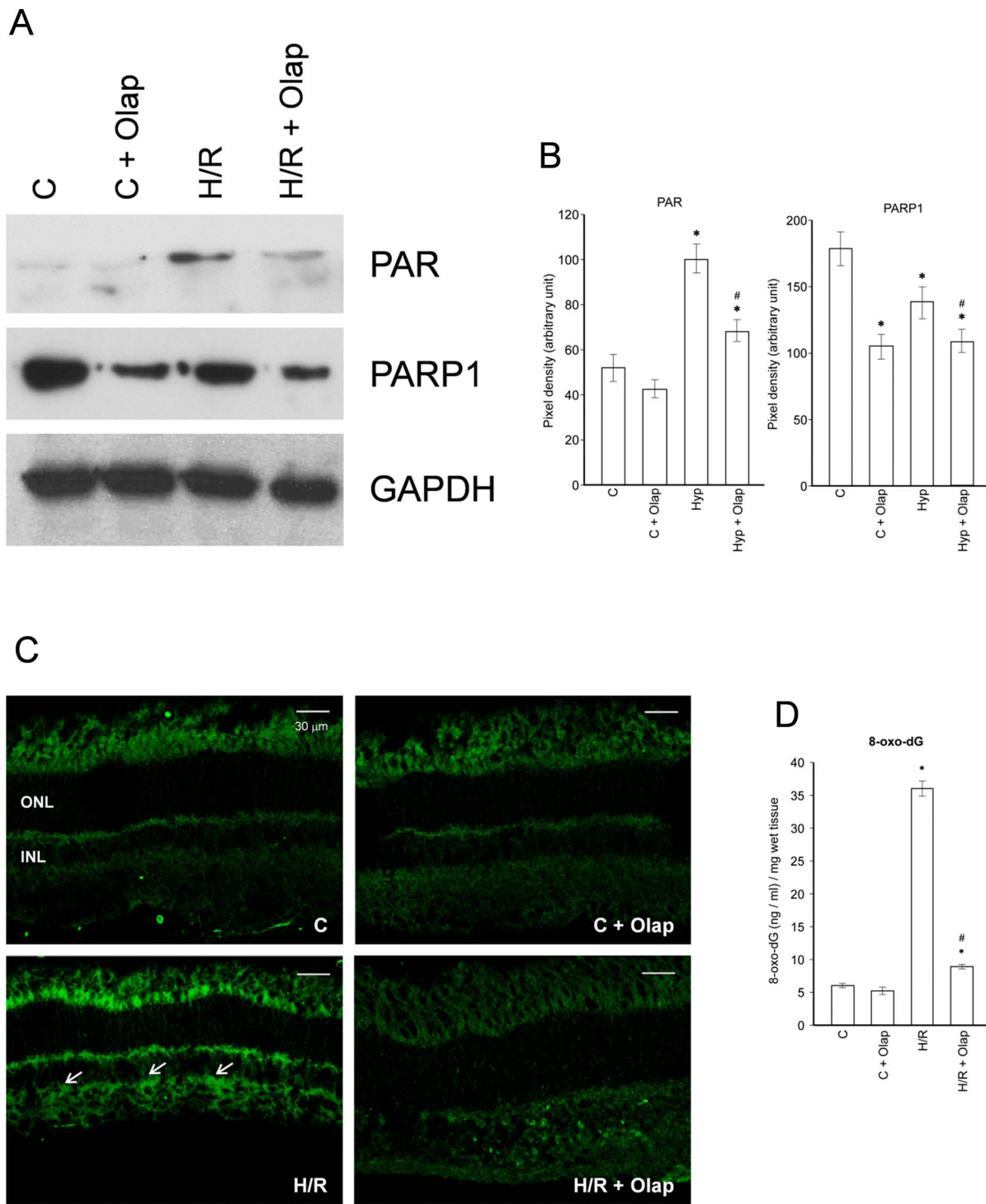


**FIGURE 1.** Effect of olaparib treatment on histologic changes of the retina induced by H/R. (A) Light microscopic images of retinal cross sections stained with toluidine blue. Representative images are presented ( $n = 6$  per group). (B) Quantitative analysis of the thickness of retinal layers: IPL, INL, ONL, and OPL, cross section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM), and the number of cells/100  $\mu\text{m}$  in the GCL. Data are presented as mean  $\pm$  SEM of four independent measurements. \* $P \leq 0.05$  compared with control group; # $P \leq 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis).

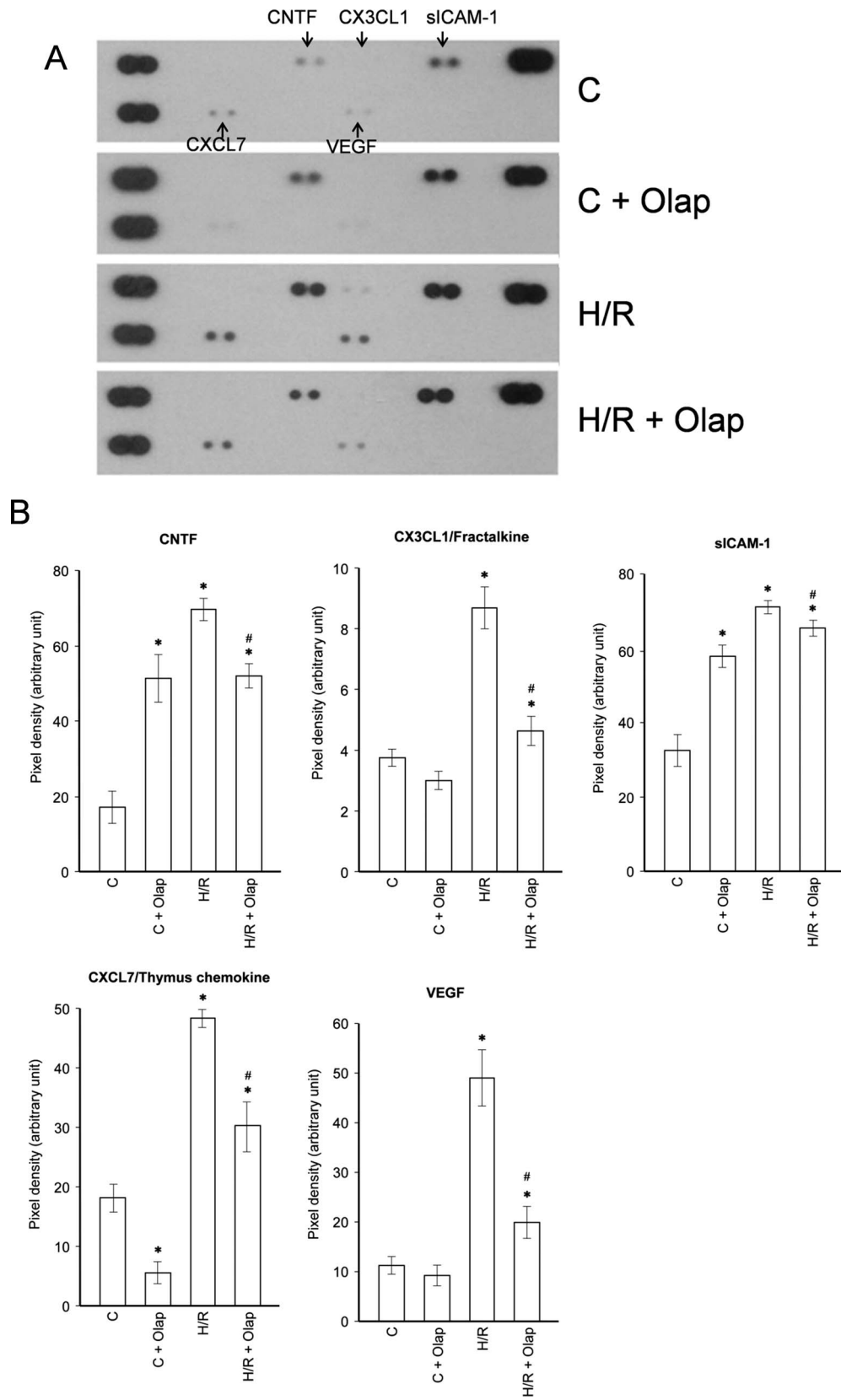
an increase in the phosphorylation of Akt and GSK-3 $\beta$  that exceeded that of C + Olap (Figs. 4A, 4B). These patterns were consistent with the notion that Akt-mediated phosphorylation and inactivation of GSK-3 $\beta$  could be protective in retinal diseases.<sup>25</sup>

We also examined the expression level of MAPKs under our experimental conditions (Fig. 5A). ERK1/2 phosphorylation was increased after H/R and remained elevated after H/R +

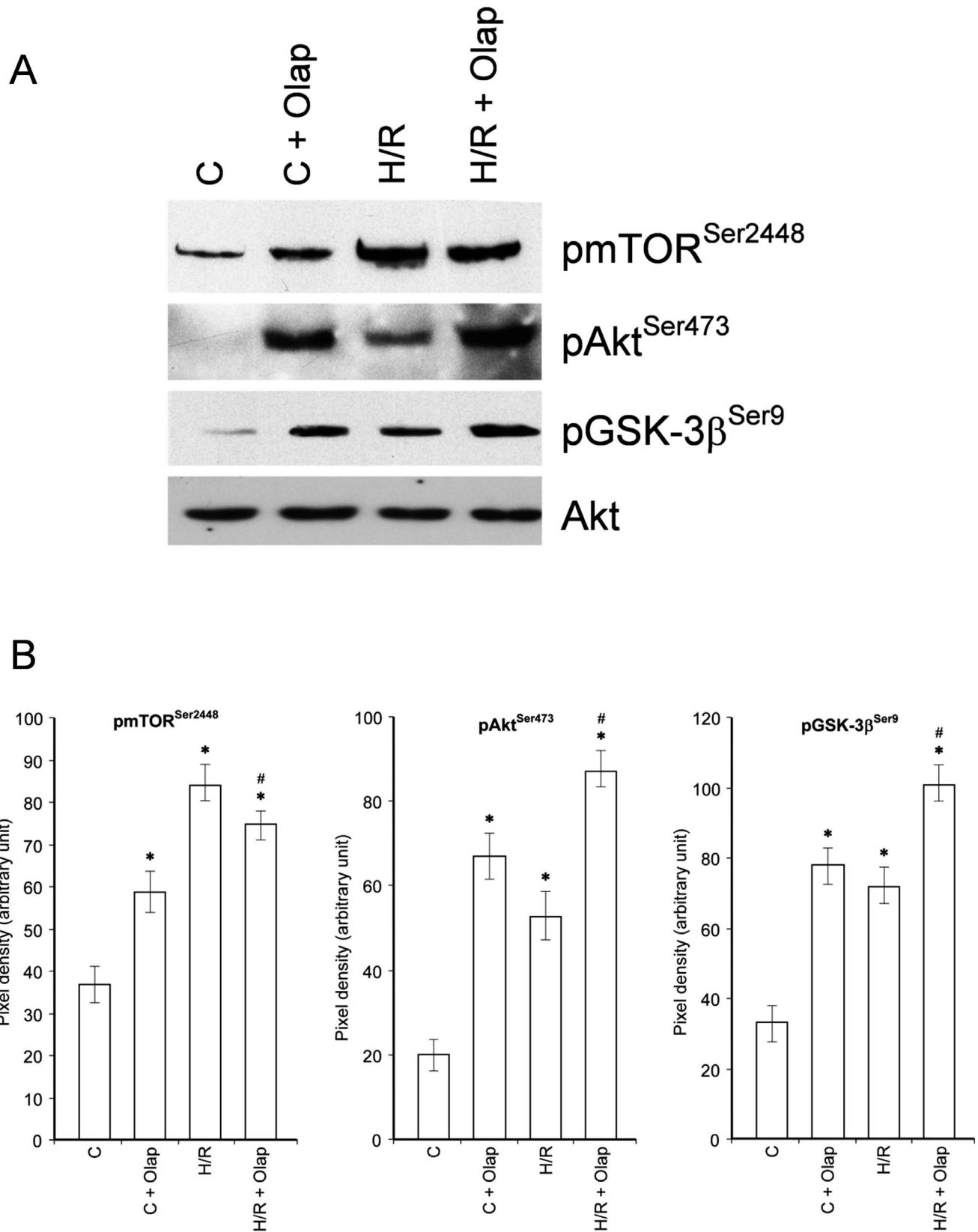
Olap treatment (Figs. 5A, 5B). Olaparib treatment did not affect either the phosphorylation of p38 MAPK or JNK. By contrast, H/R increased pp38 MAPK and pJNK levels that were significantly diminished by olaparib (Figs. 5A, 5B). Because MKP-1 was a negative regulator of p38 MAPK and JNK,<sup>26</sup> we analyzed its expression. H/R had no effect on MKP-1 levels; however, olaparib treatment increase MKP-1 levels in the control, as well as in the H/R groups (Figs. 5A, 5B).



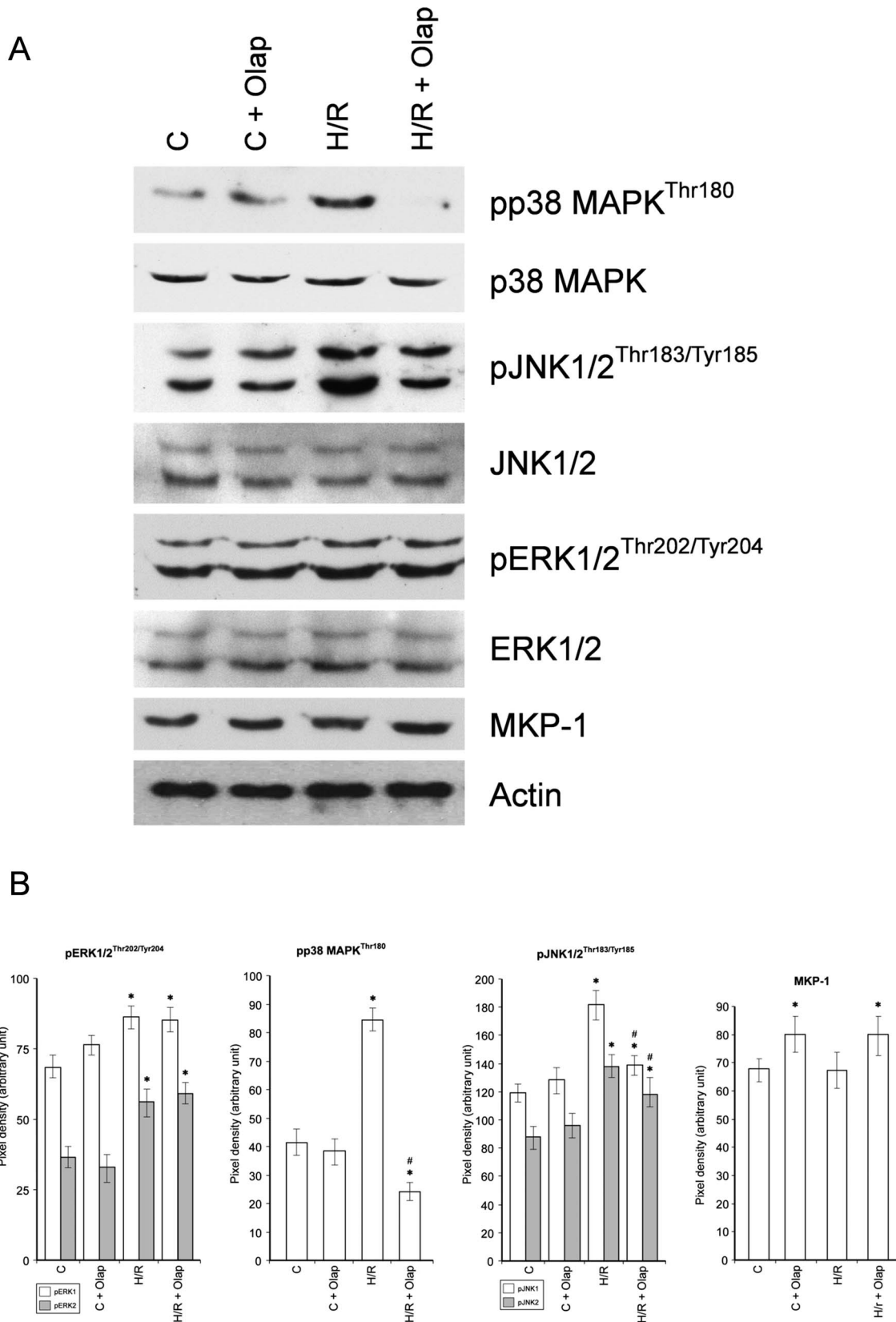
**FIGURE 2.** Effect of olaparib treatment on PARylation, PARP1, nitrotyrosine, and 8-hydroxy-2'-deoxyguanosine levels in H/R-induced retinal injury. **(A)** Expression of PAR and PARP1 determined by immunoblotting using protein-specific primary antibodies. **(B)** Densitometric analysis of immunoblots is presented in bar diagrams. GAPDH is used as an internal control. Data are presented as mean  $\pm$  SEM of four independent measurements. \* $P \leq 0.05$  compared with control group; # $P \leq 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis). **(C)** Cryosections stained with anti-nitrotyrosine antibody. A light background staining in the ONL is characteristic of all four experimental conditions. Elective staining of the INL can be seen for H/R conditions. Several cell bodies of presumed amacrine cells (*arrows*) are particularly strongly labeled. Olaparib reduces H/R-induced nitrosylation. Representative images are presented ( $n = 6$  per group). **(D)** Oxidative DNA damage assessed with an 8-oxo-dG ELISA Kit. The results are presented as a bar diagram, mean  $\pm$  SEM of three parallel measurements of six independent samples in each group. \* $P \leq 0.05$  compared with control group; # $P \leq 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis).



**FIGURE 3.** Effect of olaparib treatment on cytokine, chemokine, and growth factor expression in H/R-induced retinal injury. **(A)** Nitrocellulose-based rat cytokine array assay performed on retina homogenates ( $n = 6$  per group). **(B)** Quantitative analysis of CNTF, CX3CL1/fractalkine, sICAM-1, CXCL7/thymus chemokine, and VEGF expression. Data are presented as mean  $\pm$  SEM of three independent experiments. \* $P \leq 0.05$  compared with control group; # $P \leq 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis).



**FIGURE 4.** Effect of olaparib treatment on the PI3K/Akt/mTOR pathway in H/R-induced retinal injury. **(A)** Expression and phosphorylation of mTOR, Akt, and GSK-3 $\beta$  determined by immunoblotting using phosphorylation-specific primary antibodies. Representative images are presented ( $n = 6$  per group). **(B)** Densitometric analysis of immunoblots is presented in bar diagrams. Bands were normalized to the appropriate Akt and GSK-3 $\beta$  bands. Data are presented as mean  $\pm$  SEM of three independent experiments. \* $P < 0.05$  compared with control group; # $P < 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis).



**FIGURE 5.** Effect of olaparib treatment on the MAPK pathways in H/R-induced retinal injury. **(A)** Expression and phosphorylation of p38 MAPK, JNK, and ERK1/2, as well as expression of MKP-1, determined by immunoblotting using phosphorylation-specific primary antibodies. Representative images are presented ( $n = 6$  per group). **(B)** Densitometric analysis of immunoblots is presented in bar diagrams. Bands are normalized to the appropriate p38 MAPK, JNK1/2, and ERK1/2 bands. GAPDH is used as an internal control. Data are presented as mean  $\pm$  SEM of three independent experiments. \* $P \leq 0.05$  compared with control group; # $P \leq 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis).



## Effect of Olaparib Treatment on HIF1 $\alpha$ , Nrf2, and NF $\kappa$ B Activation, in Addition to Phosphorylation and Acetylation of NF $\kappa$ B p65 in H/R-Induced Retinal Injury

The abovementioned signaling pathways regulated the expression of numerous transcription factors, among which, HIF1 $\alpha$ , Nrf2, and NF $\kappa$ B levels were assessed. HIF1 $\alpha$  levels were significantly increased after olaparib treatment (Figs. 6A, 6B). Nevertheless, the increase was more pronounced in the H/R group and was appreciably attenuated by olaparib (Figs. 6A, 6B). Olaparib-treatment (C + Olap) and H/R treatment alone did not increase the level of Nrf2 levels significantly; however, they rose dramatically and significantly in H/R + Olap retinas (Figs. 6A, 6B). Even so, the treatments did not affect steady-state NF $\kappa$ B levels (Fig. 6A). However, nuclear translocation rather than changes in steady-state levels would indicate activation of Nrf2 and NF $\kappa$ B. Therefore, we assessed nuclear translocation of these transcription factors from nuclear protein extracts of retinas by immunoblotting. C + Olap and H/R treatments, and H/R + Olap treatment to a greater extent showed an increase in nuclear translocation of Nrf2 (Figs. 6A, 6B). By contrast, olaparib reduced, whereas H/R elevated, NF $\kappa$ B nuclear translocation, and the latter effect was abolished by olaparib (Figs. 6A, 6B).

It was well established that NF $\kappa$ B activation was regulated by various kinase signaling pathways, including the abovementioned PI3K-Akt and MAPK pathways.<sup>27</sup> We observed increased phosphorylation, thereby activation of NF $\kappa$ B p65<sup>Ser536</sup> in H/R retinas, which was diminished by olaparib treatment (Figs. 6A, 6B). These data showed that the PARP inhibitor olaparib could regulate NF $\kappa$ B activation by regulating MAPKs. NF $\kappa$ B p65<sup>Lys310</sup> acetylation, and thereby activation, was also determined. Acetylated NF $\kappa$ B p65<sup>Lys310</sup> levels were significantly elevated under H/R conditions, whereas olaparib treatment decreased acetylation in H/R-treated retinas (Figs. 6A, 6B), thereby reducing NF $\kappa$ B activation as previously suggested.<sup>28</sup>

## DISCUSSION

ROS overproduction during ischemia-reperfusion can cause DNA damage that triggers PARP1 activation, which is one of the most important regulators in cell death processes for numerous diseases.<sup>29-31</sup> Elevated PARP activation has been detected in several types of retinal degeneration, such as diabetic retinopathy, retinitis pigmentosa, and ischemia-reperfusion-induced cell death in the retina.<sup>18,19,32-37</sup> Recent data indicate that PARP inhibitors provide therapeutic benefits in cancer treatment,<sup>38,39</sup> and PARP has been proposed as a potential target in nononcologic diseases.<sup>40</sup> Inhibition of PARP protected the retina in the bilateral common carotid artery occlusion (BCCAO) model,<sup>18,41</sup> as well as in the hypoxia-reperfusion model elicited by raising IOP.<sup>42,43</sup> In the BCCAO model, PARP inhibition activated one of the most important cytoprotective pathways, the PI3K-Akt, and suppressed the p38 MAPK and JNK pathways.<sup>18</sup>

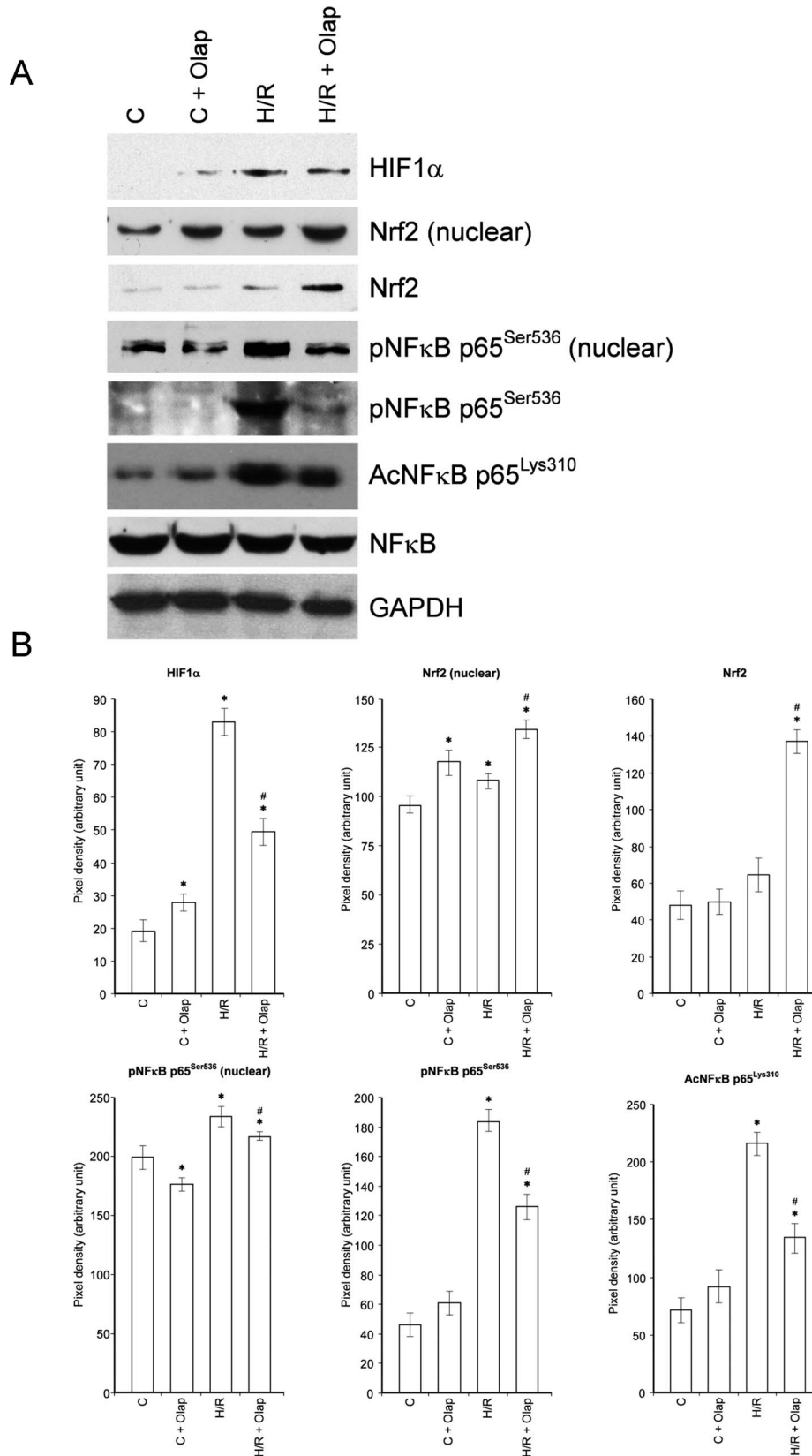
In this study, we analyzed kinase signaling in the retina under chronic H/R and found that a PARP inhibitor induced the activation of Akt, thereby inactivating GSK-3 $\beta$ ,<sup>18</sup> processes that may play a protective role in retinal H/R. These results are consistent with previous findings that inhibition of GSK-3 $\beta$  has protective effects in the retina,<sup>44</sup> and Akt activation is cytoprotective in oxidative stress situations.<sup>45,46</sup> In oxidative stress, PARP inhibition is reported to downregulate JNK and p38 MAPKs<sup>12,26</sup> that can also have a protective role in the retina. JNK initiates mitochondrial fission and a variety of

programmed cell death mechanisms,<sup>47,48</sup> whereas activation of p38 MAPK predominantly contributes to inflammation.<sup>49</sup>

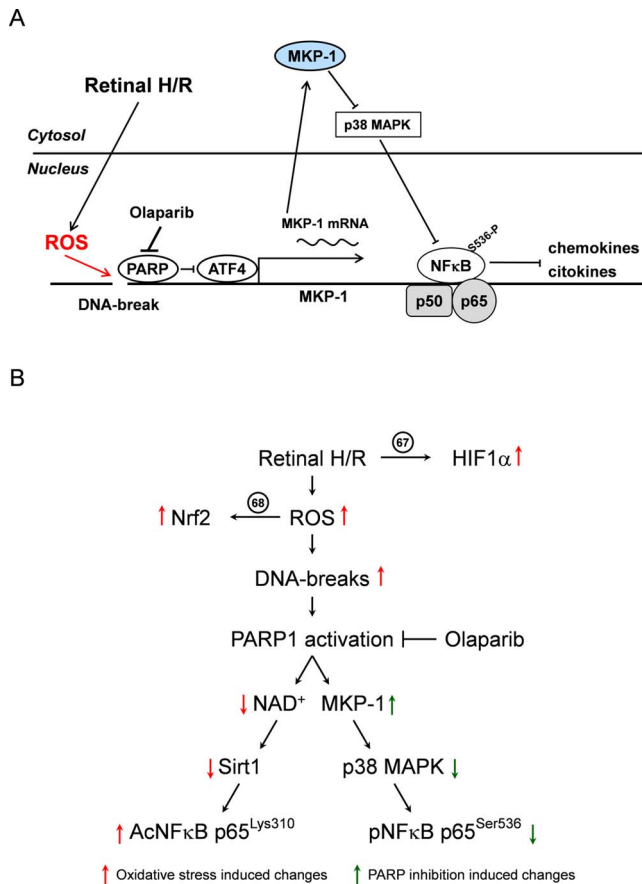
Another potential effect of PARP inhibitors is in anti-inflammation.<sup>50</sup> Besides elevating ROS, H/R increases cytokine/chemokine levels.<sup>51,52</sup> As such, we used a rat cytokine array to detect H/R-induced changes and the effect of olaparib treatment on them. Of the 29 cytokines, chemokines, and growth factors that were examined, 5 showed marked changes in our experiment, namely CNTF, fractalkine, sICAM-1, CXCL7, and VEGF. CNTF, a member of the IL6 family, is one of the most studied neurotrophic agents in retinal diseases.<sup>53</sup> Preclinical studies in more than 12 animal models from four different species provide strong support for the neuroprotective effect of CNTF on photoreceptors and ganglion cells in the retina.<sup>54-57</sup> Intravitreal injections of recombinant CNTF have also been shown to promote capillary regrowth and attenuate preretinal neovascularization in a mouse model of oxygen-induced retinopathy.<sup>58</sup> Normal neurons in the central nervous system, as well as in the retina, constitutively express fractalkine.<sup>59</sup> Disruption of fractalkine signaling influences microglial physiology in disease conditions.<sup>60</sup> VEGF, as an important angiogenic factor, plays a role in several retinopathies, such as retinopathy of prematurity and diabetic retinopathy. However, it could also have a neuroprotective function. In retinal ischemia, the influence of its expression depends on timing, localization, and reperfusion.<sup>61,62</sup> In the current study, olaparib treatment significantly reduced VEGF levels in hypoxic retina, which could be related to PARP inhibitor-induced reduction of HIF1 $\alpha$  and NF $\kappa$ B levels (Fig. 6).<sup>63</sup>

Most of the aforementioned cytokine and chemokine expressions were regulated by NF $\kappa$ B, which was reported to activate a number of cytokine and growth factor genes associated with ischemia-reperfusion injury in the retina.<sup>64,65</sup> NF $\kappa$ B is retained in the cytoplasm in an inactive form by its inhibitor, I $\kappa$ B, which is subjected to phosphorylation-mediated degradation. A number of signaling kinases, including MAPKs, phosphorylate and thereby activate I $\kappa$ B kinase. After removal of I $\kappa$ B, a nuclear localization signal on NF $\kappa$ B becomes unmasked, and the transcription factor translocates to the nucleus, where it is activated by phosphorylation and acetylation to stimulate NF $\kappa$ B-dependent gene expression.<sup>66</sup> H/R activates MAPKs, including p38 MAPK, which is responsible for the phosphorylation of p65<sup>Ser536</sup>. Therefore, the MKP-1 expression inducing effect of PARP1 inhibition<sup>26</sup> explains how olaparib can reduce phosphorylation of p65<sup>Ser536</sup>, thereby attenuating inflammation during H/R. We found that acute H/R activated the acetylation of subunit p65<sup>Lys310</sup> of NF $\kappa$ B, contributing to the activation of this transcription factor (Fig. 6). It is not surprising that H/R increases p65 acetylation because SIRT enzymes (NAD<sup>+</sup>-dependent deacetylases) are responsible for the deacetylation of p65. Upon activation, PARP1 consumes NAD<sup>+</sup>, thereby reducing SIRT activity (Fig. 7). Inhibition of PARP maintains NAD<sup>+</sup> levels and consequently SIRT activity, resulting eventually in significantly reduced p65<sup>Lys310</sup> acetylation (Figs. 6, 7).

As expected, H/R caused considerable cell death, as was revealed by a decrease in the thickness of all retinal layers but OPL and the presence of observable lesions (Fig. 1). It also induced significant protein (Fig. 2C) and DNA damage (Fig. 2D). All these effects were significantly attenuated by olaparib (Figs. 1, 2). In addition to the aforementioned protective effects, olaparib treatment dramatically increased Nrf2 levels (Fig. 6), the main transcription factor activating the expression of antioxidant enzymes. This was the first report indicating that PARP inhibition could contribute to better protection against H/R-related oxidative stress, possibly by elevating Nrf2.



**FIGURE 6.** Effect of olaparib treatment on HIF1 $\alpha$  and Nrf2 expression and on NF $\kappa$ B p65 phosphorylation and acetylation in H/R-induced retinal injury. **(A)** Expression of HIF1 $\alpha$ , Nrf2, and NF $\kappa$ B p65, as well as the phosphorylation and acetylation of NF $\kappa$ B p65 determined by immunoblotting using phosphorylation- and acetylation-specific primary antibodies. Representative images are presented ( $n = 6$  per group). **(B)** Densitometric analysis of immunoblots is presented in bar diagrams. Bands are normalized to the appropriate GAPDH and NF $\kappa$ B internal control. Data are presented as mean  $\pm$  SEM of three independent experiments. \* $P \leq 0.05$  compared with control group; # $P \leq 0.05$  compared with H/R group (ANOVA with Fisher's LSD post hoc analysis).



**FIGURE 7.** Possible molecular mechanism of the protective effects of PARP inhibitor olaparib in chronic H/R-related retinal injury. References for mechanisms not investigated in this study are indicated by circled numbers.<sup>67,68</sup>

In conclusion, we demonstrated the possible protective effects of the PARP inhibitor olaparib in a chronic retina H/R model, characterized by histologic changes (Fig. 1), cytokine expression patterns (Fig. 3), signaling cascades (Figs. 4, 5), and transcription factor activation (Fig. 6). These findings provided further insight into the neuroprotective mechanism of olaparib in a systemic H/R model. They also showed that olaparib treatment ameliorated ischemic retinal injury involving anti-inflammatory actions by diminishing NFκB p65 phosphorylation (Ser 536) and thereby activation most probably via the PARP1-MKP-1-p38 MAPK-NFκB p65 pathway, and by promoting deacetylation (Lys310), thereby inactivation of NFκB p65, possibly via the PARP1-NAD<sup>+</sup>-SIRT1 pathway (Fig. 7). In addition, we found that the PI3K-Akt pathway and JNK downregulation via activation of MKP-1 expression might also have contributed to a protective effect of the PARP inhibitor against oxidative stress-induced retinal cell death. Taken together, these data suggest a therapeutic potential for olaparib or other nontoxic PARP inhibitors in retinal disease.

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