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ORIGINAL ARTICLE

Ischemia–reperfusion injury induces occludin phosphorylation/ubiquitination and retinal vascular permeability in a VEGFR-2-dependent manner

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Retinal ischemia–reperfusion (IR) induces neurodegenaration as well as blood–retinal barrier (BRB) breakdown causing vascular permeability. Whereas the neuronal death has been extensively studied, the molecular mechanisms related to BRB breakdown in IR injury remain poorly understood. In this study, we investigated the early changes in tight junctional (TJ) proteins in response to IR injury. Ischemia–reperfusion injury was induced in male rat retinas by increasing the intraocular pressure for 45 minutes followed by natural reperfusion. The results demonstrate that IR injury induced occludin Ser490 phosphorylation and ubiquitination within 15 minutes of reperfusion with subsequent vascular permeability. Immunohistochemical analysis revealed a rapid increase in occludin Ser490 phosphorylation and loss of Zonula occludens-1 (ZO-1) protein, particularly in arterioles. Ischemia–reperfusion injury also rapidly induced the activation and phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) at tyrosine 1175. Blocking vascular endothelial growth factor (VEGF) function by intravitreal injection of bevacizumab prevented VEGFR-2 activation, occludin phosphorylation, and vascular permeability. These studies suggest a novel mechanism of occludin Ser490 phosphorylation and ubiquitination downstream of VEGFR2 activation associated with early IR-induced vascular permeability.

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Keywords: blood–retinal barrier; ischemia–reperfusion injury; occludin phosphorylation; tight junction; vascular endothelial growth factor; vascular permeability

INTRODUCTION

Retinal ischemia–reperfusion models several components of various eye disease pathologies such as retinal vein occlusion, diabetic retinopathy, and glaucoma.[1–6] The IR model has been widely used for studying retinal neuronal cell damage after ischemic insult[6] and consists of transient ischemia followed by natural reperfusion leading to an inflammatory and neurodegenerative response in the intact retina.[7] Histologic analysis demonstrated that the IR injury causes selective neuronal loss indicated by reduced thickness of retinal layers including the ganglion cell layer, inner nuclear layer, and inner plexiform layer.[8,9] A recent study demonstrated that IR also induced vascular abnormalities such as capillary dropout after 8 to 14 days of reperfusion and concluded these vascular changes occurred after neuronal loss.[6] Recently IR was shown to increase retinal vascular permeability in a vascular endothelial growth factor (VEGF)-dependent manner, suggesting that this model could be used to investigate the mechanisms and drugs targeting VEGF-induced permeability.[3] Vascular endothelia growth factor, a hypoxia-responsive angiogenic and vasopermeability factor, contributes to vascular leakage in multiple retinal pathologies.[10] Although many studies have demonstrated perturbations of retina glial and neuronal elements in IR injury, no studies have investigated the mechanism of vascular breakdown during the early (24-hour post ischemia) injury phase of IR.

The blood–retinal barrier (BRB) serves as a selective barrier that regulates the local environment of the neural retina.[11] The BRB possesses a well-developed tight junction (TJ) complex that helps to control the flux of fluids and solutes across the endothelial cell barrier creating a defined microenvironment and establishing the retina as an immunologically privileged site.[11–14] Tight junctions include at least 40 proteins including both proteins that contribute to the organization and structure of the TJ complex and regulatory proteins. Claudin family proteins in the BRB and blood–brain barrier (BBB) include claudins 1, 3, 5, and 12 with genetic evidence for claudin 5 as necessary for the BBB.[15,16] Zonula occludens proteins are required for formation of the junctional complex and are an excellent means to monitor junctional organization.[17]

Alterations of TJ complex proteins during retinal diseases may lead to BRB breakdown and subsequent vascular permeability and macular edema.[18] Several studies have demonstrated that changes in occludin content and distribution are associated with endothelial cell barrier properties.[4,19–22] Although cells do not require occludin for TJ formation,[23] occludin phosphorylation on specific sites regulates barrier properties.[19,24] Treatment of endothelial cells with VEGF increases occludin phosphorylation and alters its distribution from cell border to intracellular puncta.[25,26] Mutational analysis demonstrates phosphorylation at Ser490 is required for VEGF-induced occludin ubiquitination, endocytosis

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and endothelial permeability.[24,25,27] Further, intravitreal injection of VEGF induces occludin Ser490 phosphorylation, ubiquitination, and redistribution from the BRB *in vivo*, which is associated with increased permeability.[14]

In the present study, we test the hypothesis that IR induces rapid alterations to the tight junction complex, specifically occludin posttranslational modifications and Zonula occludens-1 (ZO-1) protein reorganization in a VEGF-dependent manner that may account for the rapid VEGF-induced alterations in junctional permeability. We demonstrate that IR induces rapid occludin Ser490 phosphorylation and ubiquitination in response to IR injury, coinciding with vascular endothelial growth factor receptor-2 (VEGFR-2) activation. Further, ZO-1 organization in the arteriolar vessels was lost at the junctional complex without apparent loss of total cellular ZO-1 protein content. Ischemia–reperfusion-induced occludin Ser490 phosphorylation was relatively transient peaking at 15 minutes and returning to normal by 4 hours, while alteration of ZO-1 organization persisted over this time period. In addition, we report that the intravitreal injection of bevacizumab, an anti-VEGF monoclonal antibody, blocked early IR-induced vascular permeability. This study thus provides a molecular mechanism of BRB breakdown in early IR injury, and demonstrates a role for occludin in regulation of vascular permeability *in vivo*.

MATERIALS AND METHODS

Animal Model

Male Long-Evans rats (Charles River Laboratories, Wilmington, MA, USA) weighing 175 to 225 g were maintained in specific pathogenic-free conditions, monitored by quarterly sentinel testing and treated in accordance with the guidelines of the University of Michigan Committee on Use and Care of Animals (UCUCA) and consistent with ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.[13] All animal procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Ischemia–Reperfusion

Animals were anesthetized with intramuscular injection of ketamine and xylazine (66.7 mg/kg and 6.7 mg/kg body weight, respectively). Ischemia was applied to the eye by increasing the intraocular pressure to cut off the blood supply from the retinal artery as described earlier.[3] Constant intraocular pressure was maintained by continuous flow (40 μl/minute) of sterile saline through a 32-gauge needle inserted into the anterior chamber of the eyes through the corneas and connected to a syringe pump. Blanching of the retina was monitored to confirm the loss of blood flow. For sham-treated group, contralateral eyes were treated by briefly inserting a 32-gauge needle into the anterior chamber of the eye through the cornea. Intraocular pressures were monitored using a microtonometer specially designed for use on rodent eyes (TonoLab, Icare, Helsinki, Finland). Pressures ranging from 9 ± 1 to 11 ± 1 mm Hg were observed in sham-treated eyes while pressures of 90 ± 3 to 94 ± 4 mm Hg were observed in eyes undergoing ischemia. For pretreatment with bevacizumab (Avastin, Genentech, South San Francisco, CA, USA), anesthetized rats received intravitreal injections of 50 μg per eye in 2 μL in both eyes or of 2 μL of saline as vehicle control 48 hours before IR injury using a 33-gauge needle.

Evans Blue Assay

Retinal vascular permeability was measured by the accumulation of albumin-binding dye, Evans blue, as described previously.[3] The Evans Blue content in blood plasma after 2 hours of circulation was used for normalization, and values expressed as microliters of plasma per gram of retina (dry weight) per hour of circulation.

Primary Bovine Retinal Endothelial Cell Culture

Bovine retinal endothelial cells were isolated and cultured as described previously.[20,28] Bovine retinal endothelial cells were grown to confluence and then cell culture media was changed to MCDB-131 medium with 0% fetal bovine serum and incubated overnight before experiment. The cells were treated with 50 ng/mL of human recombinant VEGF (R&D systems, Minneapolis, MN, USA) for 15 minutes, then cells were washed with sterile phosphate-buffered saline twice and harvested in lysis buffer containing protease and phosphatase inhibitors as previously described.[14,29]

Western Blotting

Immediately after euthanization, retinas were excised, carefully cleaned of all vitreous and retinal pigmented epithelium and then placed in immunoprecipitation buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 2 mmol/L *N*-ethylmaleimide, 1 mmol/L NaVO4, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1 mmol/L benzamidine, and complete protease inhibitor cocktail) as previously described.[14] Retinas were gently sonicated followed by 20-minute incubation at 4°C and a 10-minute centrifugation at 16,000 *g* and 4°C to sediment insoluble material. Protein concentrations in cleared lysates were determined using the Micro BCA Protein colorimetric assay (Pierce, Rockland, IL, USA). For western blotting, 4% to – 12% NUPAGE Bis-Tris gel (Invitrogen, Grand Island, NY, USA) was used as described previously.[14] For transferring high molecular weight proteins such as VEGFR-2, 0.05% SDS was included in transfer buffer containing 10% methanol. The primary antibodies used at a dilution of 1:1,000 included the following: rabbit polyclonal anti-occludin, anti-ZO-1, and anti-claudin-5 (all from Invitrogen), rabbit anti-VEGF, mouse monoclonal anti-phosphotyrosine (pY20), and rabbit polyclonal anti-VEGFR-2/KDR (all from EMD Millipore, Bedford, MA, USA), rabbit anti-VE-Cadherin (Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit anti-VEGFR-2 phosphoTyr1175 (Cell Signaling Technology, Danvers, MA,USA). For internal standard or loading control, mouse monoclonal anti-β-actin antibody (1:10,000, Cell Signaling Technology, Danvers, MA, USA) were used. Bound primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G secondary antibodies and ECL Advance chemiluminescent substrate (GE Healthcare, Piscataway, NJ, USA). Densitometry analysis was conducted on digital images of the blots using the AlphaView 3.1 software (ProteinSimple, Santa Clara, CA, USA).

Immunoprecipitation and Detection of Ubiquitination

Bovine retinal endothelial cells or retinal lysates were prepared in immunoprecipitation buffer, centrifuged for 10 minutes at 16,000 *g* and 4°C, the supernatants precleared with Protein G beads (GE Healthcare), and an aliquot of each cleared lysate was saved to be used as an input control. To immunoprecipitate total or Ser-490-phosphorylated occludin, respective antibodies were added to lysates and incubated overnight at 4C, followed by addition of Protein G beads and incubation for an additional 1 hour. The beads were centrifuged and washed four times with lysis buffer and then boiled in Laemmli buffer. Eluted proteins were subjected to western blotting with mouse monoclonal anti-ubiquitin antibody (Enzo Life Sciences, Plymouth Meeting, PA, USA) followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin G and chemiluminescence with ECL advance. The same method was employed to immunoprecipitate the phosphorylated forms of VEGFR-2 in BREC or retinal lysates using a mouse monoclonal anti-phosphotyrosine antibody (pY20, Millipore), followed by immunoblotting with rabbit anti-VEGFR-2 antibodies (EMD Millipore).

Immunocytochemistry

Immunolocalization of occludin and ZO-1 in retinal vessels was performed as previously described.[21] Retinas were incubated with monoclonal anti-occludin antibody, rabbit anti-ZO-1 antibody (each at 1:50 dilution) or with rabbit polyclonal antibodies specific to occludin phosphorylated at the Ser490 site[24] (pSer490-occludin; 1: 50 dilution) for 3 days at 4°C. Primary antibodies bound to the retinal vessels were detected with Alexa Fluor 488- or 594-conjugated anti-mouse or anti-rabbit immunoglobulin G secondary antibodies (Invitrogen) incubated overnight at 4°C. After several washes, retinas were mounted on AES-coated glass slides with Prolong Gold anti-fade mounting media (Invitrogen), and analyzed on a Leica TCS SP5 AOBS confocal microscope. A confocal *Z*-stack of 10 images were collected over a depth of 5 μmol/L and projected as one image. Image intensity of the anti-pSer490-occludin antibodies staining in the retinas was quantified using Imaris software (Bitplane, South Windsor, CT, USA). For each condition, the scoring intensities for four images representative of four independent experiments were summed. Occludin and ZO-1 border staining were quantified by a semi-quantitative ranking score system based on a scale of 1 to 5:1 for near complete loss of border staining (0% to 25%), 2 for 25% to 50% continuous border staining, 3 for 50% to 75%

continuous border staining, 4 for 75% to 100% continuous border staining, and 5 for completely continuous border staining. Scoring was completed in a masked fashion by three impartial observers provided scoring standard images for comparison. For each condition, the results of eight images representative of three independent experiments were summed and the frequency of each ranking score was calculated and plotted.

Statistical Analysis

Results are expressed as mean ± s.e.m. Two-tailed student's *T*-test was performed to assess the statistical difference between two conditions. One-way analysis of variance was employed to calculate the statistical difference between three or more groups using Prism 4.0 (GraphPad Software, San Diego, CA, USA) where values of $P < 0.05$ were considered as significant.

RESULTS

Ischemia–Reperfusion Induced Blood–Retinal Barrier Breakdown

The effect of IR injury on BRB breakdown was examined by inducing a mild ischemic insult in the rat retina as previously described.[3] Rat retinas were subjected to 45-minute ischemia followed by natural reperfusion of blood for a period of 15 minutes or 4 hours with determination of the accumulation of albumin-bound Evans Blue dye over the following 2-hour period. Relative to retinas from sham-treated eyes, vascular permeability was significantly increased by 2.7-fold in IR retinas evaluated 15 minutes to 2 hours and 15 minutes after ischemia, and by 2.4-fold in IR retinas evaluated 4 to 6 hours after ischemia (Figure 1A). The increase in the vascular permeability starting at 15 minutes of reperfusion suggests a rapid effect of IR injury on endothelial TJ function. Fluorescein angiography was also performed using intravenous injected FITC-BSA to assess the locations of vascular permeability. The IR retinas showed increased FITC-BSA leakage near the optic nerve head region, along the large vessels and in capillary bed compared with sham-treated retinas (Figures 1B–1E).

Ischemia–Reperfusion Altered Tight Junction Protein Distribution at the Blood–Retinal Barrier

To determine the effect of IR injury on retinal vascular tight junction organization, retinas were removed after 15 minutes, 1 hour, or 4 hours of reperfusion, flat mounted, immunolabeled for occludin and ZO-1 proteins, and imaged by confocal microscopy. In sham retinas, intense immunostaining of occludin and ZO-1 was observed at the junctions of the cell membranes in the vascular endothelium (Figures 2A and 2B). In eyes subjected to IR injury, a reduction of ZO-1 immunostaining at the border of endothelial cells in arterioles and meta-arterioles was consistently evident in retinas harvested after 15 minutes, 1 hour, and 4 hours of reperfusion, whereas ZO-1 staining in connecting capillaries of these retinas appeared largely unaffected. No differences in claudin-5 immunoreactivity at the cell border were observed after 4 hour of reperfusion. The extent of ZO-1 organization at cell borders were graded by masked observers with a score of 1 representing a complete loss of TJ border staining and a score of 5 representing intact border staining for the overall image. The results demonstrated a significant change in ZO-1 organization. Further, intermittent loss of occludin distribution was noticed in the IR retinas at 15 minutes but did not reach statistical significance (Figure 2C). The results suggest that the IR injury alters the distribution of specific tight junction proteins at the BRB, and the resultant altered organization of TJ proteins leads to vascular permeability.

Ischemia–Reperfusion Induced Phosphorylation of Occludin at S490

We tested the hypothesis that IR induces phosphorylation of occludin Ser490, thus resulting in the observed IR-induced BRB breakdown. The effects of IR injury on the retinal content of tight junction proteins occludin, ZO-1, and claudin 5 and 10 as well as

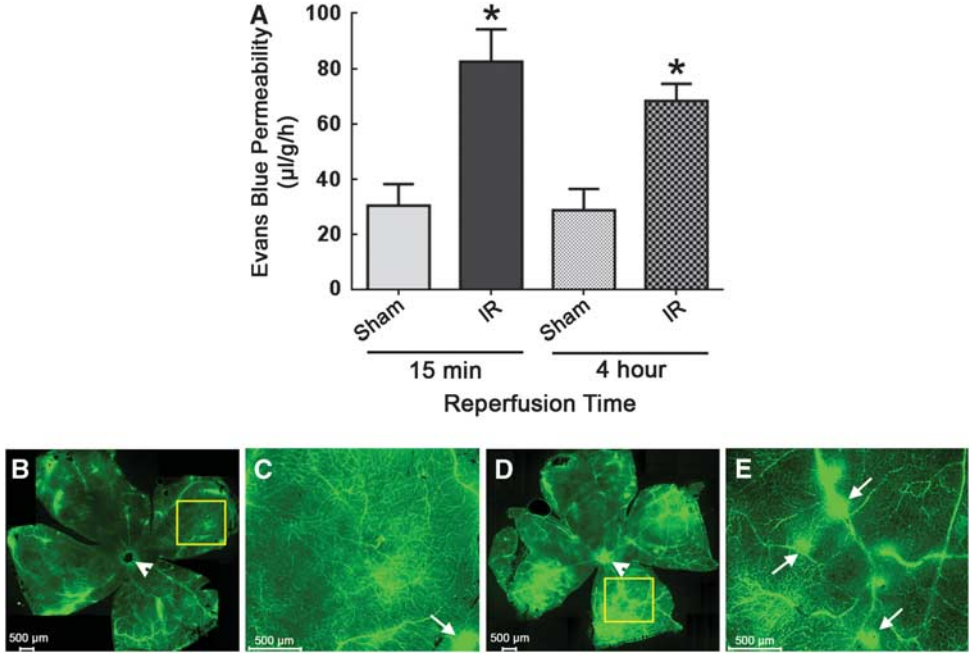

Figure 1. Retinal ischemia–reperfusion (IR) injury caused rapid vascular permeability. One eye of each animal was subjected to retinal ischemia for 45 minutes, followed by natural reperfusion. The contralateral eyes were subjected to needle puncture and served as sham controls. (**A**) Evans Blue dye was injected and allowed to circulate 2 hours before flushing and determination of dye accumulation in retinal tissue. (**B**–**E**) Vascular leakage after 4 hours. Ischemia–reperfusion was visualized by intravenous FITC-BSA administration with 20-minute circulation before retina removal and imaging. (**B**) Sham control. (**C**) Magnified portion of inset in panel **B**. (**D**) IR-induced retina. (**E**) Magnified portion of inset in panel **D**. Arrows indicate vascular leaks; arrowhead indicates optic nerve head. Data are expressed as mean ± s.e.m. ($n = 8$ animals). * indicates statistically different from sham control ($P < 0.05$).

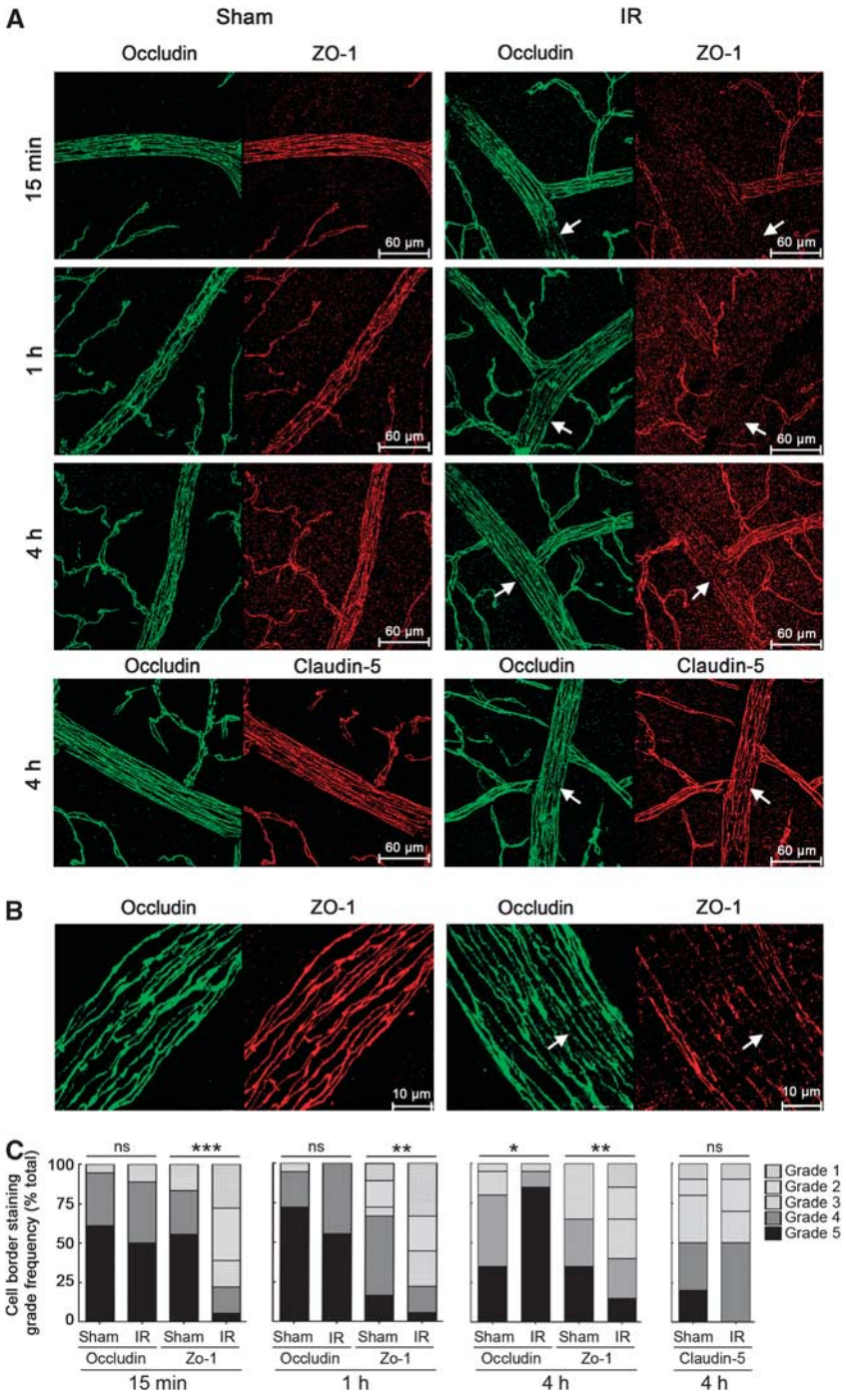

Figure 2. Retinal ischemia–reperfusion (IR) altered the distribution of tight junction proteins at the blood–retinal barrier (BRB). Rat retinal whole mounts obtained from control and IR rats were analyzed for immunoreactivity of occludin, Zonula occludens-1 (ZO-1) and claudin-5. (**A**) Immunohistochemistry for occludin and ZO-1 or claudin 5 in retinal whole mounts over a time course of reperfusion. (**B**) High magnification image of retinal whole mount. (**C**) Histogram represents the scoring of occludin and ZO-1 loss. Staining of occludin and ZO-1 at the tight junction border was quantified as described in Materials and Methods. For each condition, results of eight images representative of three independent experiments were summed and the frequency of each ranking score was calculated and plotted on a bar chart. Arrows indicate regions exhibiting loss of tight junction proteins. Data are expressed as mean ± s.e.m. ($n = 3$ animals). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ indicates statistically different from sham ($P < .05$). NS, nonsignificant.

adherens junction protein VE-cadherin were examined at various times after reperfusion up to 7 d after ischemia. Ischemia–reperfusion injury did not cause an observable change in the content of ZO-1, VE-cadherin (Figures 3B and 3D) or claudins 5, and 10 (Supplementary Figure S1). No changes in the total occludin content were observed after 15 minutes and 60 minutes of reperfusion (Figure 3A). However, there was a significant 12% reduction in the occludin content 4 hours after IR ($n = 4$; $P < 0.01$; data not shown). In contrast, IR induced a rapid transient increase in Ser490-phosphorylated occludin (pS490) content relative to

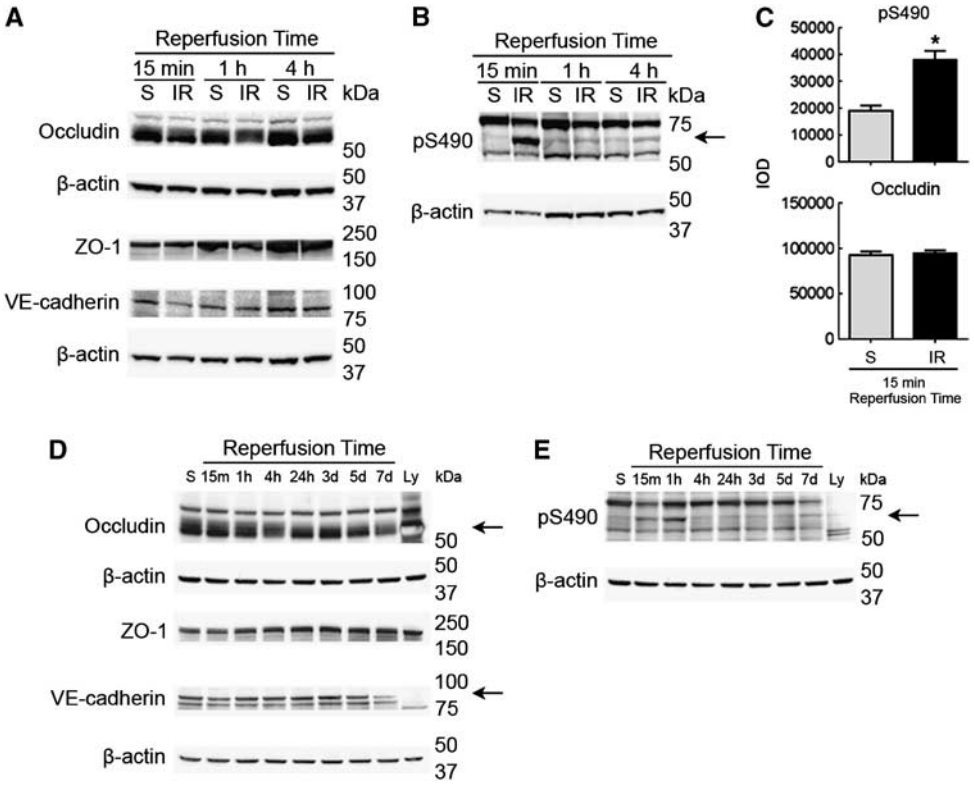

Figure 3. Retinal ischemia–reperfusion (IR) caused rapid phosphorylation of occludin at Ser490. (**A**) Western analysis of retinal occludin, Zonula occludens-1 (ZO-1), VE-cadherin, and β-actin at early times retinal IR injury. (**B**) Western analysis of pS490-occludin and β-actin in early IR. (**C**) Histogram represents the quantification of pS490-occludin and basal occludin in panels **A** and **B**. Data are expressed as mean ± s.e.m. ($n = 8$ animals). * indicates statistically different from sham ($P < 0.05$). (**D**) Western analysis of retinal occludin, ZO-1, VE-cadherin, and β-actin at extended times after IR injury. (**E**) Western analysis of pS490-occludin and β-actin in later IR. Arrows indicate the positions of occludin and pS490-occludin. S, sham.

sham eyes that was increased by fourfold at 15 minutes after reperfusion and declined thereafter (Figures 3B and 3C). The apparent molecular weight of the pS490 band (~60 to 65 kDa) was similar to that previously identified in endothelial cells.[19] An additional band was observed at 75 kDa but did not vary in any condition and was not observed in endothelial cell lysates. Rapid phosphorylation of occludin at Ser490 after IR injury was further confirmed by immunoprecipitation of occludin followed by blotting for pS490 (Supplementary Figure S2).

To confirm the changes of pS490-occludin content in response to IR and identify the location of the pS490-occludin in the retinal vasculature, retinal whole mounts were immunolabeled with the pS490-specific antibody. Immunoreactivity of pS490 was observed only in IR retinas and localized to the arterioles, meta-arterioles, and small capillaries (Figure 4). These studies suggest that IR causes rapid phosphorylation of occludin at Ser490 that coincides with altered organization of TJ proteins and increased vascular permeability.

Ischemia–Reperfusion Induced Occludin Ubiquitination

Previous studies demonstrated that phosphorylation of occludin at Ser490 is required for subsequent ubiquitination of occludin and permeability induced by VEGF treatment.[19] Because occludin Ser490 phosphorylation was observed after IR, the effect of IR on ubiquitination of occludin was tested. Retinas were harvested after 15 minutes of reperfusion and immunoprecipitated occludin protein was probed by immunoblotting with an antibody to ubiquitin. Whereas occludin from sham retinas showed little evidence of ubiquitination, occludin from IR-injured retinas exhibited a series of ubiquitin-containing bands ranging from 65 kDa to 150 kDa, suggesting its polyubiquitination (Figure 5). These data suggest that IR induces Ser490 phosphorylation and subsequent ubiquitination of occludin in retinal vascular endothelial cells.

Ischemia–Reperfusion Increased Phosphorylation of a Vascular Endothelial Growth Factor Receptor-2 Activation Site

Because occludin Ser490 phosphorylation and ubiquitination, as observed after retinal IR injury, are also observed after VEGF treatment both *in vitro* and *in vivo*,[14,19] we examined the possibility that IR leads to activation of the VEGF receptor VEGFR-2. Lysates from sham and IR retinas were immunoblotted with antibodies specific to VEGFR-2 phosphorylated at tyrosine 1175, (pTyr1175), a modification that is indicative of VEGFR-2 activation after VEGF binding.[30] The retinal content of pTyr1175 was increased 15 minutes after ischemia and declined after 1 and 4 hours of reperfusion (Figure 6A), whereas the levels of total VEGFR-2 protein were unchanged. Appearance of an immunoreactive band at the location corresponding to VEGFR-2 was also observed in IR samples after blotting with a non-specific antibody to phosphotyrosine (pY20) (Figure 6A). To confirm these results, tyrosine phosphorylated proteins in lysates from IR retinas and sham-treated retinas harvested after 15 minutes of reperfusion were immunoprecipitated with pY20 antibody and then blotted with antibodies specific to pTyr1175 or total VEGFR-2. The results indicated that a twofold increase in phosphorylation of VEGFR-2 at

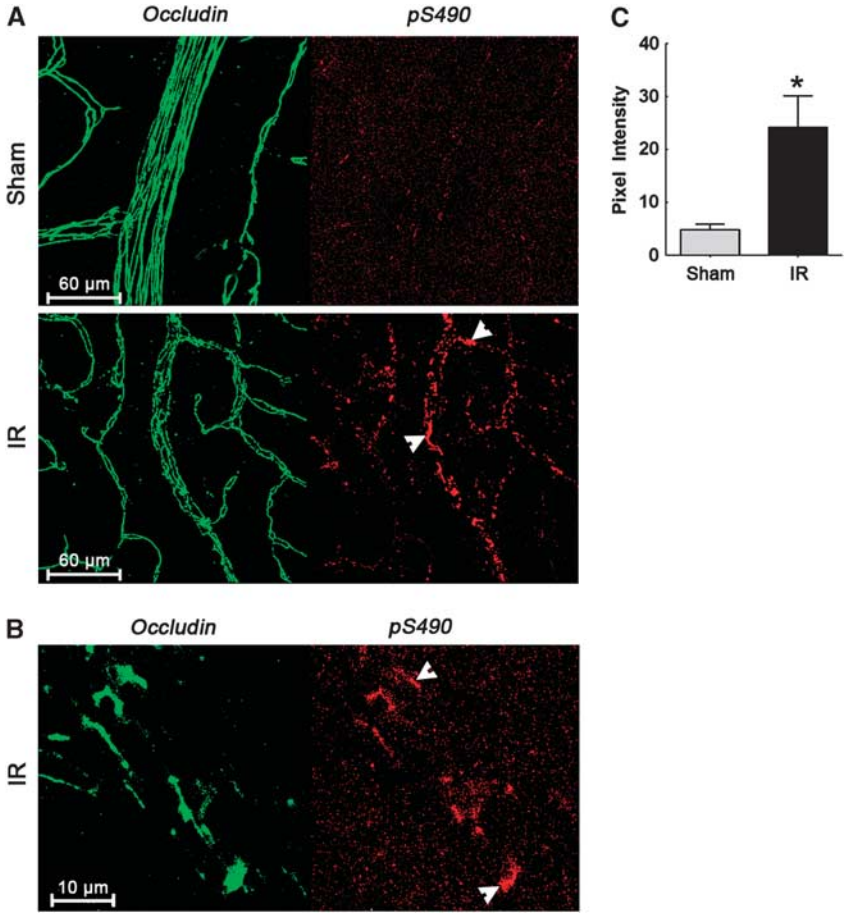

Figure 4. Retinal ischemia–reperfusion (IR) induces rapid phosphorylation of occludin at Ser490. Immunohistochemistry of occludin and pS490-occludin in early IR retinal whole mount. (**A**) Whole mounts of IR and sham retinas after 15 minutes of reperfusion were analyzed for immunoreactivity to pS490-occludin and occludin. (**B**) High magnification image from a separate retina. (**C**) Histogram represents the scoring of pS490-occludin immunoreactivity. Arrowheads indicate the immunostaining of pS490-occludin. Data are expressed as mean ± s.e.m. ($n = 3$ animals). * indicates statistically different from sham ($P < 0.05$).

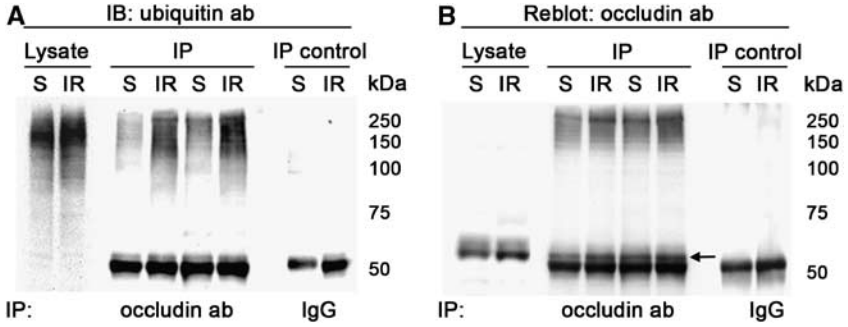

Figure 5. Retinal IR ischemia–reperfusion induced a rapid increase in occludin ubiquitination. (**A**) Lysates from IR and sham retinas after 15 minutes of reperfusion were immunoprecipitated with antibody to occludin and immunoblotted with antibodies to ubiquitin. (**B**) Membranes were stripped and reblotted with antibody to occludin. $n = 3$. IB, immunoblot; IgG, immunoglobulin G; IP, immunoprecipitation; S, sham.

the Tyr1175 site was caused by IR (Figures 6B and 6C). However, it should be noted that several experiments measuring VEGF protein levels immediately after IR injury found no significant increases in retinal VEGF protein content after IR injury (for example see Supplementary Figure S4). Therefore, we cannot conclude that the rapid VEGFR-2 activation observed after IR is caused by increased expression of VEGF protein.

Blockade of Vascular Endothelial Growth Factor Receptor-2 Activation by Bevacizumab Reduces the Occludin Phosphorylation and Retinal Vascular Permeability

We previously demonstrated that intravitreal injection with a neutralizing VEGF antibody, bevacizumab, before IR injury inhibited the vascular permeability response measure after 48 hours of reperfusion.[3] To determine whether the early vascular

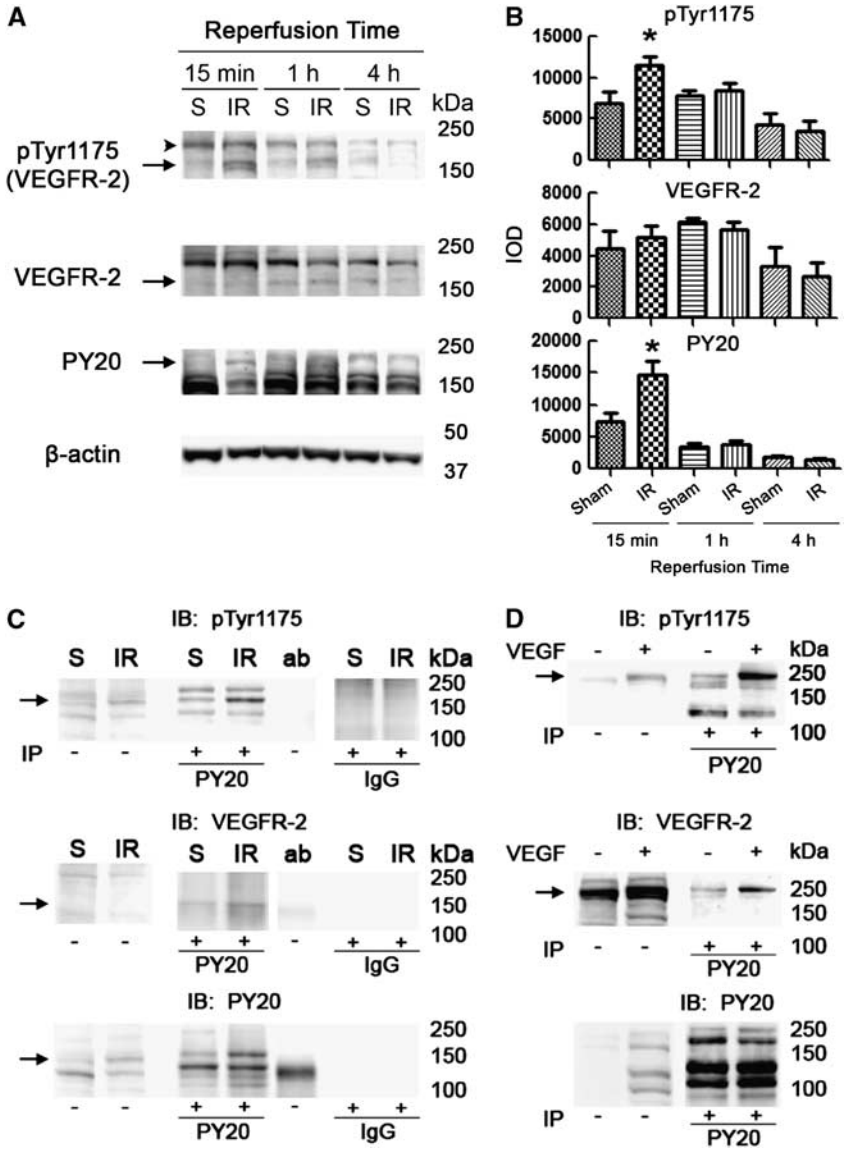

Figure 6. Retinal ischemia–reperfusion (IR) induces a rapid increase in phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2). (**A**) Western analysis of phospho-VEGFR-2 (pTyr1175) in IR and sham retinas at indicated times after ischemia. (**B**) Histogram representing the quantification of western blotting results from three separate experiments. (**C**) Immunoprecipitation of phosphorylated tyrosine proteins in IR and sham retinas after 15 minutes of reperfusion. (**D**) Immunoprecipitation of phosphorylated tyrosine proteins in lysates from bovine retinal endothelial cell (BREC) treated with and without vascular endothelial growth factor (VEGF) as a positive control for VEGFR-2 expression in endothelial cells. In (**C**, **D**) lysates were immunoprecipitated with PY20 antibody to phosphotyrosine and then immunoblotted with antibody to VEGFR-2 pTyr1175. Blots were stripped and reblotted with antibody to total VEGFR-2. Arrow indicates the changes in VEGFR-2 protein content in response to IR. Arrowhead indicates a high molecular weight VEGFR-2 protein band that was not responsive to IR. Data are expressed as mean ± s.e.m. ($n = 10$ animals). * indicates statistically different from sham ($P < 0.05$). IB, immunoblot; IP, immunoprecipitation; S, sham.

permeability response to IR injury is VEGF mediated, eyes were pretreated with bevacizumab 48 hours before IR. Bevacizumab pretreatment prevented the IR induced early vascular permeability measured by Evans Blue dye injection beginning after only 15 minutes of reperfusion (Figure 7A). In addition, bevacizumab pretreatment inhibited IR-induced pS490 phosphorylation of occludin and VEGFR-2 phosphorylation at Tyr1175 (Figures 7B and 7C and 7D and 7E). These results strongly suggest that the BRB breakdown during the early injury phase of IR is mediated by VEGF signaling through VEGFR-2. To further test the possibility of VEGF-mediated VEGFR-2 activation, the phosphorylation of signaling proteins downstream of this receptor, MAPK-ERK1/2 and AKT, was examined. Indeed, IR caused rapid phosphorylation of MAPK-ERK1/2 and AKT (Supplementary Figure S3), consistent with rapid activation and downstream signaling of VEGFR-2 after IR injury. However, bevacizumab pretreatment did not alter the IR-induced MAPK-ERK1/2 phosphorylation response (Figure 7D), suggesting that this response to IR could be mainly because of MAPK-ERK1/2 activation in neuronal cells VEGF-independent mechanisms.

DISCUSSION

The data herein reveals for the first time occludin Ser490 phosphorylation and ubiquitination-induced permeability in an

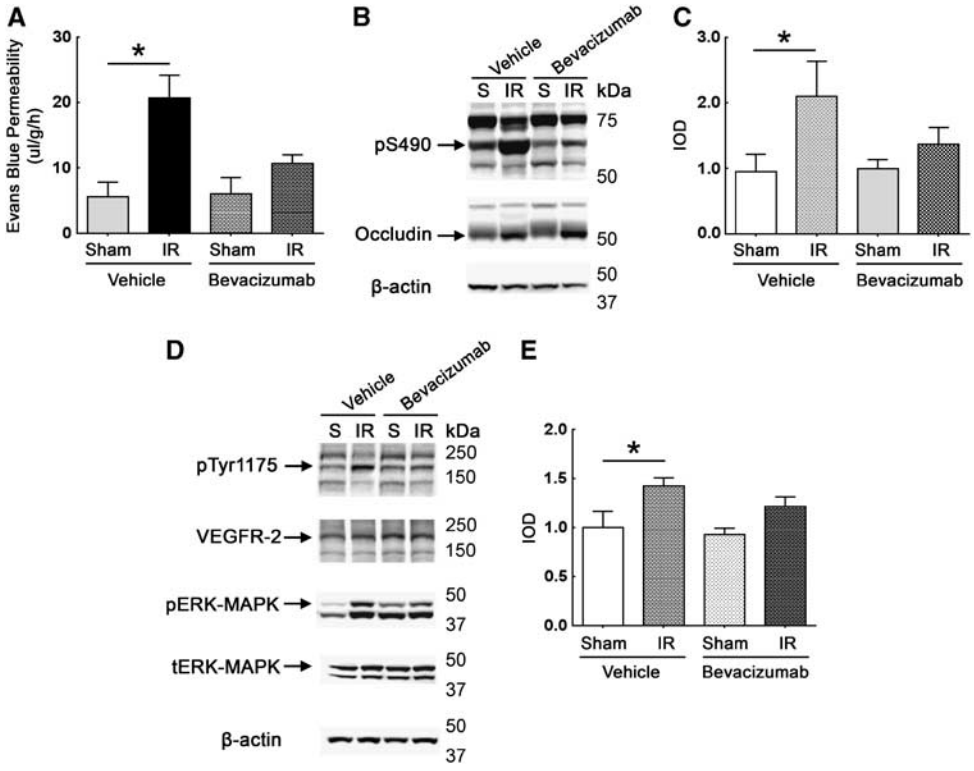

Figure 7. Intravitreal bevacizumab attenuates retinal IR injury-mediated vascular permeability by blocking vascular endothelial growth factor receptor-2 (VEGFR-2) activation and occludin phosphorylation. (**A**) Evans blue permeability assay. Rats received pretreatment with intravitreal bevacizumab (50 μg per eye) in both eyes or with saline vehicle in both eyes. Forty-eight hours after pretreatment, one eye of each animal was subjected to IR injury and the contralateral eye subjected to sham treatment. Retinal vascular permeability was assayed by Evans Blue leakage from 15 minutes to 2.25 hours after ischemia. (**B**) Western analysis of pS490 contents in IR and sham retinas with or without intravitreal bevacizumab pretreatment. (**C**) Histogram of the quantification of results obtained in three experiments as shown in panel **B**. (**D**) Representative western blot of VEGFR-2 pTyr1175 content. (**E**) Histogram of the quantification of results obtained in three experiments as shown in panel **D**. Data are expressed as mean ± s.e.m. ($n = 8$ animals). * indicates statistically different from sham ($P < 0.05$). S, sham.

animal model of ischemic disease. Ocular pressure-induced retinal IR injury represents a useful model for the study of vascular and neuronal damage.[3] Our previous study[3] demonstrated a VEGF-dependent mechanism of IR-induced permeability; however, the molecular mechanisms driving this permeability were not investigated. Here, we demonstrate that IR injury induces VEGFR-2 activation, occludin phosphorylation at serine 490, and occludin ubiquitination within minutes of reperfusion, resulting in an early vascular permeability response. In addition, inhibition of VEGF function blocks both VEGFR-2 activation and the early permeability response. As previous studies have demonstrated that occludin phosphorylation and ubiquitination are required for VEGF-induced permeability in cell culture,[19] we conclude that VEGF-induced occludin phosphorylation contributes to changes in vascular permeability during the early injury phase of IR. Our data thus reveal an important mechanism of IR injury-mediated occludin phosphorylation and ubiquitination that regulates TJ distribution and BRB permeability in a VEGFR-2-dependent manner within minutes of reperfusion.

Integrity of the BRB and BBB is achieved through well-developed tight junctions and alterations of BRB and BBB TJ protein distribution during pathologic conditions may cause changes in barrier permeability.[4,31] Decreased protein contents of occludin (~30%) and claudin-5 (~25%) were observed in the cerebral cortex of sheep fetus after 4 hours of ischemia and were suggested to cause BBB permeability.[32,33] In the present study, no change in the content of claudins, VE-cadherin, or ZO-1 was observed in early or late reperfusion. However, a dramatic change in the immunolocalization of ZO-1 organization at the cell border in arterioles and meta-arterioles occurred within minutes after IR injury. Further, we observed increased BRB permeability in association with a rapid increase in occludin phosphorylation and ubiquitination and a subsequent minor (15%) reduction in the occludin content that recovered after 24 hours of reperfusion. The magnitude of this reduction may be diminished because changes in occludin phosphorylation occurred only in arterioles and meta-arterioles and not in other types of vessels within the retina. Previous studies suggested that Ser490 phosphorylation reduces the association of occludin with ZO-1 and promotes Itch-dependent ubiquitination.[19] Similar events were observed recently *in vivo* during VEGF-induced BRB breakdown.[14] Collectively, these data suggest that the posttranslational modifications to occludin contribute to alterations in tight junction organization and vascular permeability. Others have observed different occludin phosphorylation events associated with BBB permeability. A marked increase in tyrosine phosphorylation of occludin in association with decreased levels of occludin and ZO-1 have been reported to coincide with increased BBB permeability in rat brain capillaries after microsphere-induced cerebral embolism.[33]

It was particularly noteworthy that ZO-1 immunostaining was reduced particularly at the cell border of arterioles and meta-arterioles. Others have observed altered localization of tight junction protein after ischemia. Vascular changes that occur mainly in arterioles also resulted in increased cerebrovascular permeability induced by platelet-derived growth factor-CC or tissue plasminogen activator.[34] Jiao *et al*[35] also observed changes

in ZO-1 immunoreactivity that was specifically located at arterioles and meta-arterioles. In that study, occludin, claudin 5, and ZO-1 were altered with no recovery of any of the TJ components in the cerebral microvessels of rats even after 120 hours of reperfusion after middle cerebral artery occlusion.[35] In fetal sheep brain, ZO-1 and ZO-2 were reduced at the cell border and remained reduced for 72 hours after ischemia.[32] In contrast, the present study found that ZO-1 content at the cell border remained reduced whereas occludin immunostaining soon normalized (Figures 2A and 2B). Occludin and ZO-1 directly interact through the occludin acidic head of the coiled–coil domain and the guanylate kinase homology domain of ZO-1.[36] The observation that occludin was restored at the cell border without ZO-1 was therefore surprising. Further, vascular permeability remained elevated after the observed recovery of occludin at the cell borders. Clearly, additional mechanistic studies are required to determine how occludin returns to the cell border while ZO-1 fails to recover and how the lack of ZO-1 contributes to permeability in the later stages of IR.

Intravitreal injection of bevacizumab significantly attenuated IR-mediated vascular permeability both early (this study) and 48 hours after ischemia.[3] Elevated levels of VEGF with increased VEGFR-2 activation is well known in animal models of ischemic retinopathy, such as oxygen-induced retinopathy[37] and cerebral ischemic injury,[38,39] yet the role of VEGF and VEGFR-2 in the physiologic response to ischemic insult is not well characterized. Blocking VEGF signaling with a VEGFR-2 inhibitor has been shown to prevent cerebral vascular permeability in IR models.[40] Although we could not detect an increase in retinal VEGF protein levels after IR, we clearly demonstrated increased phosphorylation of VEGFR-2 at tyrosine 1175 at 15 minutes after IR, which decreased after 60 minutes and 4 hours of reperfusion. Further, the intravitreal injection of bevacizumab attenuated the rapid IR-induced VEGFR-2 activation and occludin Ser490 phosphorylation suggesting that the early IR injury-induced vascular permeability is VEGF-dependent and leads to occludin phosphorylation, ubiquitination, and vascular permeability. The release of presynthesized VEGF or other mechanisms that control VEGF/receptor interactions independent of increased VEGF synthesis may result in VEGFR activation after IR. Further, the possibility of an off-target effect of the anti-VEGF-A antibody on a similar ligand that can activate the VEGFR cannot be dismissed. Regardless, this study strongly suggests VEGFR activation and occludin posttranslational modifications associated with IR-induced permeability. In addition to VEGFR signaling, other mechanisms likely contribute to vascular permeability after IR injury including a well-documented inflammatory response.[18]

In conclusion, the present study demonstrated that early IR-induced occludin phosphorylation followed by ubiquitination contributes to vascular permeability in a VEGFR-2-dependent manner and suggest that occludin phosphorylation and ubiquitination promotes intercellular trafficking of TJ proteins such as ZO-1 leading to breakdown of the BRB. These findings strongly support a vital role for occludin posttranslational modifications in the regulation of vascular permeability *in vivo* after IR injury.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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