

Alterations in Tight- and Adherens-Junction Proteins Related to Glaucoma Mimicked in the Organotypically Cultivated Mouse Retina Under Elevated Pressure

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PURPOSE. To scrutinize alterations in cellular interactions and cell signaling in the glaucomatous retina, mouse retinal explants were exposed to elevated pressure.

METHODS. Retinal explants were prepared from C57bl6 mice and cultivated in a pressure chamber under normotensive (atmospheric pressure + 0 mm Hg), moderately elevated (30 mm Hg), and highly elevated (60 mm Hg) pressure conditions. The expression levels of proteins involved in the formation of tight junctions (zonula occludens 1 [ZO-1], occludin, and claudin-5) and adherens junctions (VE-cadherin and β -catenin) and in cell-signaling cascades (Cdc42 and activated Cdc42 kinase 1 [ACK1]), as well as the expression levels of the growth-factor receptors platelet-derived growth factor receptor beta and vascular endothelial growth factor receptors 1 and 2 (VEGFR-1, VEGFR-2) and of diverse intracellular proteins (β -III-tubulin, glial fibrillary acidic protein transcript variant 1, α -smooth muscle actin, vimentin, and von Willebrand factor VIII), were analyzed using immunohistochemistry, western blotting, and quantitative real-time polymerase chain reactions.

RESULTS. The retinal explants were well preserved when cultured in the pressure chambers used in this study. The responses to pressure elevation varied among diverse retinal cells. Under elevated pressure, the expression of ZO-1 increased in the large vessels, neuronal cells began to express VEGFR-1, and the Cdc42 expression in the optic nerve head was downregulated. Overall we found significant transcriptional downregulation of VE-cadherin, β -catenin, VEGFR-1, VEGFR-2, vimentin, Cdc42, and ACK1. Western blotting and immunohistochemistry indicated a loss of VE-cadherin with pressure elevation, whereas the protein levels of ZO-1, occludin, VEGFR-1, and ACK1 increased.

CONCLUSIONS. The pressure chamber used for cultivating mouse retinal explants can serve as an in vitro model system for investigating molecular alterations in glaucoma. In this system, responses of the entire retinal cells toward elevated pressure with conspicuous changes in the vasculature and the optic nerve head can be seen. In particular, our investigations indicate that changes in the blood–retina barrier and in cellular signaling are induced by pressure elevation.

Keywords: retina explants, mouse, elevated pressure, cell contacts, cell signaling

Glaucoma, one of the leading causes of blindness worldwide, is characterized by progressive degeneration of optic nerve axons and the retrograde death of retinal ganglion cells. The primary site of the intraocular pressure (IOP)-induced insult in glaucoma is at the optic nerve head.¹ Although elevated IOP is a major risk factor for glaucoma, the disease progresses despite normalization of the IOP.² Thus, the pathophysiology of glaucoma and therewith its molecular and cellular interactions are merely understood. More research is needed to decipher these changes and find IOP-independent treatments.

Animal models of IOP elevation and in vitro models of excitotoxicity have revealed that glaucomatous neuropathy

affects neurons, glial cells, capillary endothelial cells, microglial cells, and also the surrounding biomechanical environment consisting of extracellular matrix.² It remains unclear which spatiotemporal cascade is initiated by the onset of the disease and which cells are affected first to subsequently influence neighboring cells. Increased attention, therefore, is being paid to the role of cell–cell interactions within organically cultured retinal tissue.

We hypothesize that elevated pressure induces alterations in cellular interactions and cell signaling. Within the retina in vivo, cells are embedded hierarchically in highly organized units in which intimate contact and noncontact interactions occur. Molecules participating in cell–cell interactions,

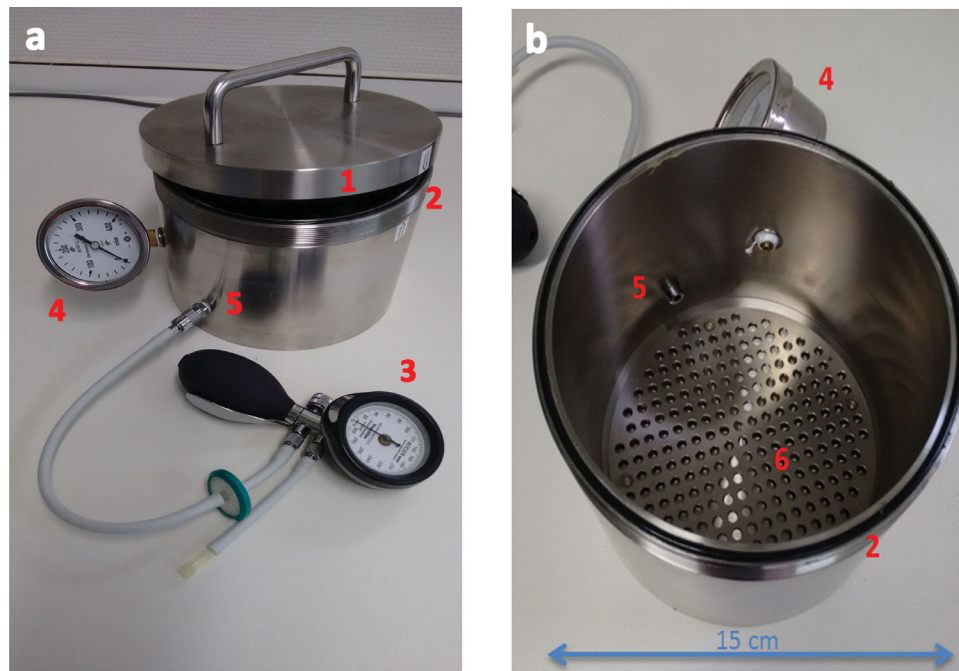


FIGURE 1. (a) Prototype of the in vitro cultivation elevated-pressure chamber fabricated to simulate an elevated IOP. The chamber was constructed from stainless steel and is able to maintain pressures up to 200 mm Hg for several days. The lid (1) is screwable and includes a sealing ring (2) to prevent air leakage. An external, removable manometer (3) is used to adjust the pressure via a unidirectional valve (5). A second manometer (4) continually monitors the pressure within the chamber. (b) The interior bottom of the chamber is comprised of a perforated plate (6) to allow aeration of the cultures on petriPERM dishes or multiwell plates. To equilibrate the air inside the chamber, it is preincubated with the lid open within a commercial cell culture incubator. Cells or retinal explants were incubated under either normal (30 mm Hg) or hypertensive (60 mm Hg) conditions in identical pressure chambers in parallel.

adhesion, and cell integrity involving zonula occludens 1 (ZO-1), occludin, VE-cadherin, β -catenin, claudin, vascular endothelial growth factor receptors (VEGFRs), and intracellular vimentin, as well as signaling cascade members, have been used to identify cells that interact during either physiological or pathological conditions.

A culture chamber model has recently been developed that makes it possible to create conditions mimicking those within the eye under an abnormally elevated IOP. In the present study, we induced an elevated intrachamber pressure (ICP) in vitro and cultured mouse retinal explants under normal atmospheric pressure (10 mm Hg, controls) or ICPs of 30 or 60 mm Hg imitating normal and pathological pressure conditions, respectively, in vivo, copying chronic glaucoma and the highly increased pressure threatening optic nerve perfusion. Here we describe the differential responses of the retinal tissue in the context of adhesion molecules, cell receptors, cell contacts, and intercellular signaling.

MATERIALS AND METHODS

Cultivation of Retinal Explants

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Young adult C57bl6 mice (8–12 weeks old) were sacrificed and their eyes were excised and sterilized by incubating them for 3 minutes in povidone-iodine (Betaisodona). The eye removal after euthanasia was approved by the local institution LANUV (Landesuntersuchungsamt) in accordance with the protection of animals act. The anterior segment and the sclera were removed,

and then the retina including the optic nerve head was incised and flat-mounted with the photoreceptor side facing downward on sterile nitrocellulose filters (Sartorius AG, Göttingen, Germany). The vitreous body was removed with sterile Whatman filters. The retinal explants were transferred to petriPERM dishes (Sigma-Aldrich, St. Louis, MO, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM/F12) supplemented with 10% fetal calf serum (FCS), 15-mM HEPES, and 1% penicillin/streptomycin for 24 to 72 hours at 37°C and 5% CO₂ in a humidified atmosphere.

Elevated-Pressure Incubation Chamber

A custom-made pressure chamber was designed for culturing cells or retinal explants under normo- or hypertensive conditions (Fig. 1).³ The chamber was constructed from stainless steel and is able to maintain pressures up to 200 mm Hg for several days. The lid is screwed on and has a sealing ring to prevent air leakage. An external removable manometer is used to adjust the pressure through a unidirectional valve. Pressure can be readjusted at distinct time points to prevent hypoxic conditions. A second manometer was used to continually monitor the pressure within the chamber. Inside the chamber, a perforated plate at the bottom allows aeration of retinal cultures on petriPERM dishes with gas-permeable bases. To equilibrate the air inside to 5% CO₂ and 37°C, the chamber was preincubated with the lid open in a commercial cell-culture incubator (Heraeus, Hanau, Germany). Identical pressure chambers were used so that experiments involving different pressure conditions (atmospheric + 0 mm Hg, 30 mm Hg, or 60 mm Hg) could be performed in parallel.

Western Blotting

Mouse retinal explants were transferred into 1.5-ml Eppendorf tubes, washed twice with PBS, and resuspended in a nuclear cell extraction buffer containing 50-mM Tris-HCl, pH 7.4; 150-mM NaCl; 2-mM EDTA; 0.5% sodium deoxycholate; 1% Triton X-100 (Sigma-Aldrich); 10% glycerin; 1-mM 1,4-dithiothreitol (Sigma-Aldrich); and 0.25% SDS, supplemented with 1-mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche, Basel, Switzerland), and phosphatase inhibitors (Sigma-Aldrich) directly before use. The samples were then incubated on ice for 30 minutes, sonicated using an ultrasonic bar, and centrifuged for 30 minutes at 15,800g at 4°C. Supernatants were collected as whole-cell extracts.

The protein concentrations were quantified using the Bradford assay with BSA as the standard. Equal amounts of protein extracts were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. These were blocked with 3% fat-free dry milk in Tris-buffered saline, pH 7.6, for 1 hour and then incubated with the first antibody overnight at 4°C. Subsequent incubation with the horseradish-peroxidase-conjugated secondary anti-mouse (A3682), anti-rabbit (A9169), or anti-goat antibody (A4187; Sigma-Aldrich) at a dilution of 1:40,000 or 1:5000 (7076 and 7074; Cell Signaling Technology Europe BV, Leiden, Netherlands) was performed for 1 hour at room temperature.

The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; G9545, 1:100,000; Sigma-Aldrich), anti- β -tubulin (ab6046, 1:8000; Abcam, Cambridge, UK), and anti-TATA-binding protein (TBP; #8515, 1:1000; Cell Signaling Technology) control antibodies were used for normalization of cytoplasmic and nuclear proteins. Blots were developed using enhanced chemiluminescent detection reagent (Amersham Biosciences, Little Chalfont, UK) and analyzed with the ChemiDoc system (BioRad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Mouse retinal explants were washed with PBS for 10 minutes and then fixed in 4% paraformaldehyde for 1 hour, washed twice in PBS, and placed in 30% sucrose in PBS (pH 7.4) at 4°C for 24 hours. The tissue was then washed with PBS and rapidly frozen in *N*-methyl-butane/liquid nitrogen and transferred into PBS for immunohistochemistry (IHC) staining. After blocking the specimens with 10% FCS containing 0.4% Triton X-100 for 1 hour, incubation with the first antibody was carried out in 10% FCS containing 0.4% Triton X-100 for 2 to 3 days at 4°C. The specimens were then washed for 15 minutes twice with PBS and incubated with the secondary antibody (Alexa Fluor 488/594 donkey anti-rabbit/-goat, 1:500; Thermo Fisher Scientific, Waltham, MA) diluted in 10% FCS containing 0.4% Triton X-100 for 2 to 3 days at 4°C. After three washes in PBS for 15 minutes each, the specimens were embedded in Mowiol (Sigma-Aldrich) and cover-slipped for fluorescence microscopy (Axio Imager M2, ApoTome.2, ZEN 2012 software; Carl Zeiss AG, Jena, Germany).

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated with the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's protocol. The amount of isolated RNA was measured

with an ultraviolet/visible spectrophotometer (NanoDrop ND-1 000; Peqlab, Erlangen, Germany). cDNA was synthesized from 0.5 to 1.0 μ g of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

The subsequent qRT-PCR analysis used the primer pairs listed in the Table. For qRT-PCR, the SYBR Green PCR Kit (Applied Biosystems) was used according to the manufacturer's protocol. The relative expression was calculated as $2^{-\Delta\Delta Ct}(\text{specific gene})/2^{-\Delta\Delta Ct}(\text{mean})$, using the *GAPDH* gene as the endogenous housekeeping control gene. The relative expression was calculated and is presented as the change in the expression level relative to the control group.

Statistical Analysis

Mean \pm SD values of the obtained data were analyzed with the Kolmogorov-Smirnov test to confirm that they conformed to a Gaussian distribution. The independent-samples *t*-test and the Kruskal-Wallis *H* test were used for Gaussian and non-Gaussian distributions, respectively. Local *P* values were corrected for multiple comparisons using the Holm-Bonferroni method. Figures were prepared using standard image-processing software (Photoshop; Adobe Systems, San Jose, CA, USA), and the overall brightness and contrast were adjusted. Data are presented as mean \pm SEM values, and the cutoff for statistical significance relative to an unaffected control was set at *P* < 0.05.

RESULTS

Within the culture chamber fabricated for this study, in microscopic examinations all of the explants showed normal histological morphologies after 3 days in vitro (data not shown). The three chambers used in parallel allowed for an identical environment for the cultivation of the tissues to be compared, with well-defined pressures of 10, 30, and 60 mm Hg as monitored on the manometers (Fig. 1).

Constant Expression of Cell-Specific Markers

We first examined different cell-specific markers by IHC of retinal explants cultured under the aforementioned pressure conditions to check for the preservation and integrity of the retina in the culture chambers. The typical vascular cell markers used—pericyte-specific α -smooth muscle actin (α SMA) (Figs. 2a–2f) and endothelial-cell-specific von Willebrand factor VIII (vW) (Figs. 2e–2l)—exhibited unchanged fluorescence intensities, as was the case for the neuron-specific marker β -III-tubulin (Fig. 2). This indicates that the characteristics of the cells remained unchanged within the explants independently of the ICP and that the model used preserves the retinal cells and is suitable for studying cellular responses to elevated pressure.

Changes in Adherens Junctions

We next focused on the expression of the two adhesion junction (AJ) proteins vascular endothelium (VE)-cadherin and β -catenin. When explants cultured under different pressure conditions were whole mounted and immunohistochemically labeled for VE-cadherin, which is the main determinant of endothelial cell contact integrity,¹ a lower fluorescence of vessel walls was seen with increased ICP (Figs. 3a–3c),

TABLE. Primer Used for qRT-PCR

Mouse Gene	Accession Number	Forward	Reverse
<i>ACK1</i>	NM_016788.3	TTCGACTGGAACCTAGAGCAA	CAGTCAACCATCAGCGTTTG
<i>β-catenin</i>	NM_007614.3	GCCGCGCCGCTTATAATC	CTTCACAGGACACGAGCTGA
<i>Cdc42</i>	NM_009861.3	GAGTGCTCTGCCCTCACAC	GGCTCTTCTCGGTCTCTGG
<i>claudin 5</i>	NM_013805.4	TTAAGGCACGGGTAGCACTC	GTTGGCGAACCAGCAGAG
<i>endothelin-1</i>	NM_010104.3	GCGGCGGAGAAGTGTCCGAG	AGCGCGGTCTCAGCAAGTG
<i>ETRA</i>	NM_010332.2	CACCTCAAACAGCGTCGAGA	TGCGGCTTAAGTGAAGAGGG
<i>GAPDH</i>	NM_008084.3	CATGGCAAATACGGCA	TGCCTCTGGTGGTGA
<i>GFAPV1</i>	NM_001131020.1	CAGATCCGAGGGGCAAAAAG	TGGCAGGGCTCCATTTTCAA
<i>GFAPV2</i>	NM_010277.3	GCGGGATGGTGAGGTCATTA	TAAGCTTTGGGCCCTCACAC
<i>occludin</i>	NM_008756.2	GTCCGTGAGGCCTTTTGA	GGTGCATAATGATTGGGTTTG
<i>Pdgfrb</i>	NM_001146268.1	CCCCTGATTCTCTCAGGGTTT	TGGTGTCCGGGCTACAGA
<i>VE-cadherin</i>	X83930.2	ATTGGCCTGTGTTTTTCGCAC	CACAGTGGGGTCATCTGCAT
<i>VEGFR-1</i>	NM_010228.3	TACCTCACCGTGCAAGGAAC	AAGGAGCCAAAAGAGGGTCG
<i>VEGFR-2</i>	X70842.1	TGTGAAGCCATCAACAAAGC	CAGGTGTCACAGTAATTCAGG
<i>vimentin</i>	NM_011701.4	TTTGCTGACCTCTCTGAGGC	CTCCAGGACTCGTTAGTGC
<i>ZO-1</i>	NM_009386.2	GAGCCTGCTAAGCCAGTCC	CAGCATCAGTTTCGGGTTTT

indicating the potential downregulation of this antigen. Indeed, western blot analysis revealed a significant downregulation of VE-cadherin to $53.19 \pm 7.4\%$ of the usual expression level when ICP was increased to 60 mm Hg ($P < 0.05$) (Figs. 3m, 3n). Moreover, further analysis using qRT-PCR revealed downregulation of VE-cadherin at the mRNA level ($63.18 \pm 0.03\%$; $P < 0.05$) (Fig. 3o). On the other hand, β -catenin, which plays an important role in the regulation of VE cell-cell adhesion and barrier function by linking VE-cadherin to the cytoskeleton,⁴ remained almost unchanged under the different pressure conditions (Figs. 3d–3f) as detected by IHC and western blot analysis (p30: $103.4 \pm 3.51\%$, $P = 0.7$; p60: $116.88 \pm 10.48\%$, $P = 0.7$) (Fig. 3h), whereas its mRNA was significantly downregulated (p30: $52.9 \pm 0.06\%$, $P < 0.05$; p60: $54.01 \pm 0.06\%$, $P < 0.05$) (Fig. 3i).

Site-Dependent Changes in Tight Junctions

We were also interested in changes in tight junction (TJ) proteins, which help to control the paracellular passage of ions and solutes between cells and are important for the rigidity of the endothelial barrier.⁴ Immunostaining of the TJ transmembrane protein occludin produced a higher fluorescence at 60 mm Hg than in the control condition (data not shown). In western blots, both occludin and phosphorylated occludin were slightly upregulated ($128.84 \pm 9.62\%$, $P = 0.27$ and $138.01 \pm 9.44\%$, $P = 0.16$, respectively) (Fig. 3h), whereas the mRNA showed no ICP-related changes (Fig. 3i). The cytoplasmic TJ protein ZO-1, which links occludin to the cytoskeleton,²⁵ was detectable by IHC on the side of the retinal pigment epithelium (RPE) facing the retinal explants in the controls (Figs. 4a–4c) and decreased when the ICP was increased to 60 mm Hg (Figs. 4d–4f). When this staining was studied on the retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL) of the explants, a marked increase in the ZO-1 fluorescence was seen in blood vessels and capillaries (Figs. 4g–4l), indicating different responses of the outer and inner aspects of the retina. These data indicate that the response to an increased ICP differs between the RPE and the VE of the inner retina. Unfortunately, these changes could only be quantified for whole retina protein extracts by western blotting. As shown in Figures 4m and 4n, the protein expression of ZO-1 increased significantly in the presence

of elevated pressure (186.96 ± 8.63 ; $P < 0.05$), whereas the mRNA expression of ZO-1 was almost unchanged (data not shown).

Influences on Signaling Cascades

These data indicate pressure-induced changes in the TJ and AJ proteins that mediate vascular stability and can regulate endothelial responses by directly activating signaling molecules such as phosphatidylinositol-3-kinase, the formation of complexes with growth-factor receptors, the organization of the actin cytoskeleton, and the regulation of gene transcription by modulating transcriptional cofactors such as β -catenin.²⁶ Phosphorylation of proteins along the intracellular cascade of TJ and AJ assembly and responses probably involves both tyrosine-dependent and -independent kinases.

We next examined the intracellular kinase Cdc42, a member of the Rho-GTPase family, which regulates AJ permeability by controlling the interaction of α - and β -catenin and thereby the interaction of VE-cadherin with the actin cytoskeleton²⁷ and is an important regulator of cytoskeletal dynamics, cell migration, and polarity, as well as cell-cycle progression and survival.^{28,29} IHC staining of Cdc42 was strongly co-localized with β -III-tubulin both within the optic nerve head and within the RNFL (Figs. 5g–5l). The staining showed striking differences in the optic nerve head at ICPs of 0 and 60 mm Hg that indicated its sensitivity to elevated pressure. The overall staining of Cdc42 in the retina did not change significantly with pressure elevation. Western blot and qRT-PCR analyses of Cdc42 showed inconsistent changes in its protein and mRNA expression in the whole retina under different pressure conditions (data not shown). Furthermore, the nonreceptor tyrosine kinase ACK1, which was initially identified as a downstream effector of Cdc42,¹⁹ was also examined because it reportedly plays a role in central nervous system development and synaptic function and plasticity.²² Co-staining of ACK1 and β -III-tubulin revealed considerably increased staining of the former at 60 mm Hg (Figs. 5a–5f), indicating ACK1 upregulation in explants exposed to an elevated ICP. Western blots (Figs. 5m, 5n) also revealed upregulation of ACK1 at the protein level ($239.52 \pm 34.9\%$; $P = 0.3$), whereas the mRNA expression remains almost constant (data not shown).

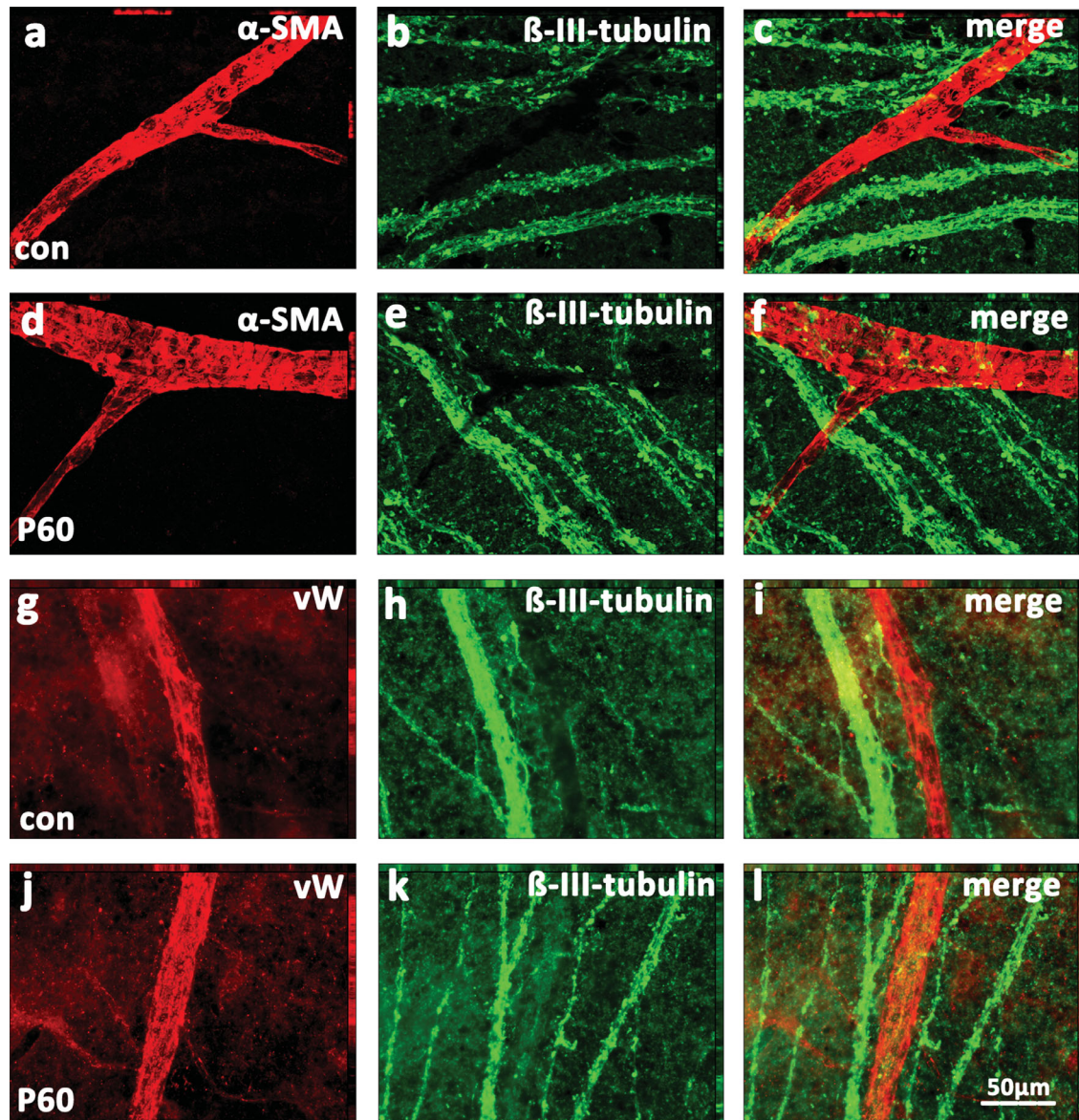


FIGURE 2. Retinal explants stained for the pericyte marker α -smooth muscle actin and the neuronal marker β -III-tubulin. Images were made with the Zeiss Axio Imager M2 using an ApoTome.2 and stacks of two Z-layers. (a–f) Representative images showed unchanged fluorescence under normotensive and elevated pressure cultivation conditions. (g–l) The endothelial cell marker von Willebrand factor VIII (vW) also showed unchanged patterns of staining under these culture conditions. Scale bar: 50 μ m. con, control; p60, 60 mm Hg.

Diverse Changes in Growth Factor Receptors and Other Proteins Associated with TJs

VEGFRs are associated with TJ proteins and play a role in blood–brain barrier permeability.³⁰ We therefore examined VEGFR-1 in explants, which revealed different responses at both the protein and mRNA levels. VEGFR-1 staining of retinal whole-mounts showed a strikingly increased incidence of this receptor in neuronal cells at an elevated ICP (Figs. 6a–6f). This finding was confirmed by western blot analysis, which revealed a seven- to eightfold upregulation of VEGFR-1 by pressure elevation ($P < 0.05$ for 30 mm Hg and $P = 0.06$ for 60 mm Hg) (Figs. 6g, 6h). Analysis of the corresponding mRNA surprisingly revealed significant downregulation to 60% at an ICP of 60 mm Hg ($P < 0.05$) (Fig. 6i). In contrast to VEGFR-1, no pressure-dependent

regulation was observed for VEGFR-2 at the protein level, but there was a downregulation to 64% at the mRNA level at an ICP of 60 mm Hg ($P = 0.08$) (data not shown).

In addition to the above-mentioned proteins examined by IHC, western blotting, and qRT-PCR, some other molecules were examined at either level only. Figure 7 shows the qRT-PCR results for other regulated mRNAs. Significant pressure-dependent upregulation was found for platelet-derived growth factor receptor beta (PDGFR β) ($135.09 \pm 0.1\%$; $P < 0.05$) and glial fibrillary acidic protein transcript variant 1 (GFAPV1) ($128.64 \pm 0.08\%$; $P < 0.05$). Also, significant downregulation was observed for vimentin ($55.28 \pm 0.09\%$; $P < 0.05$) and GFAPV2 ($58.29 \pm 0.13\%$; $P < 0.05$), as well as slight downregulation for endothelin receptor type A ($88.66 \pm 0.02\%$; $P < 0.05$). The endothelium-specific TJ protein claudin-5 showed no pressure-dependent

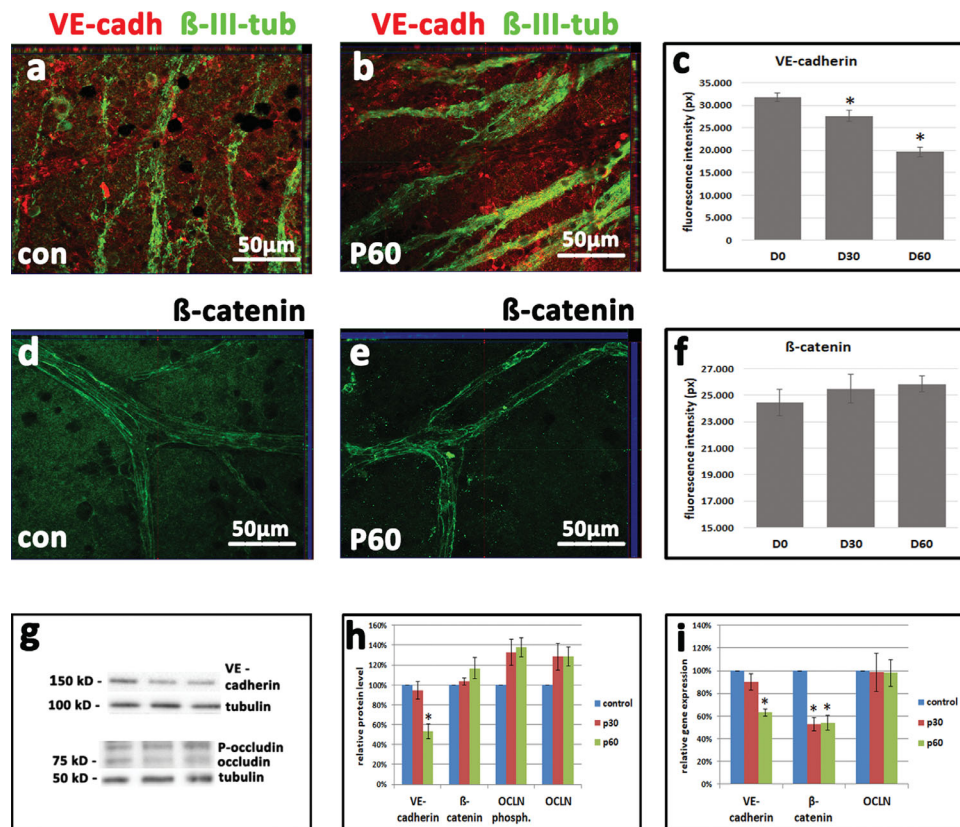


FIGURE 3. (a–c) Immunohistochemistry of retinal explants with VE-cadherin (red) and β -III-tubulin (green) under control and elevated-pressure culture conditions and quantitative analysis of VE-cadherin (c). VE-cadherin is significantly downregulated at pressures of 30 mm Hg and 60 mm Hg compared to control. IHC of retinal explants with β -catenin shows no significant changes with pressure elevation (d–f). These IHC data were confirmed using western blotting with β -tubulin as the loading control (g, h) and qRT-PCR (i). Two Z-layers. Scale bar: 50 μ m. con, control; p30, 30 mm Hg; p60, 60 mmHg.

regulation in these investigations. These data indicate that an elevated ICP influences the mRNA expression of diverse proteins involved in either vascular regulation or cytoskeleton arrangement.

DISCUSSION

The main finding of this study is that culturing retinal tissue under elevated pressure inside a cultivation chamber results in detectable changes in both AJ and TJ components and intracellular signaling. The application of IHC, western blotting, and qRT-PCR revealed changes in the AJ proteins VE-cadherin and β -catenin, the TJ proteins occludin and ZO-1, and kinases associated with the arrangement of the cytoskeleton related to actin and microtubules such as ACK1 and Cdc42. Moreover, our data indicate a conspicuous upregulation of VEGFR-1 by elevated pressure with a strikingly higher occurrence of this receptor in neuronal cells. Finally, the mRNAs of the intermediate filaments GFAP and vimentin and the growth-factor receptor PDGFR β were regulated under an elevated incubation pressure.

The culture chamber prototype model used in this study allows the generation of culture pressures adjusted according to the scope of the study and also the investigation of associated molecular changes. The chamber was able to maintain a constant ICP over the study period. It is very likely that even longer periods of incubation ranging for several days to weeks are possible. This offers advantages

over *in vivo* models, such as (1) applicability to cell cultures and organotypic explants, and (2) providing the opportunity to monitor the ICP continuously with the aid of a manometer. The retina is a complex tissue consisting of neuronal, glial, vascular, microglial, and pigment epithelial elements, which together with the extracellular matrix form the functional retina unit. TJs and AJs are cell–cell adhesion complexes in epithelial and endothelial cells. We found that several members of these proteins are regulated during the period of retinal explant cultivation under an elevated ICP.

VE-cadherin is a classic Ca²⁺-dependent type-II cadherin that plays important roles in vessel functions, and it was downregulated at both the protein and mRNA levels. VE-cadherin and its association with catenins are essential for full control of endothelial permeability and junction stabilization,³¹ which points to a weakening of AJs and thereby a loss of vascular integrity. This interpretation coincides with the previous observation that administering anti-VE-cadherin antibodies led to dramatic increases in permeability, vascular fragility, and hemorrhages.³² It has previously been reported that VE-cadherin participating in stable junctions excludes β -catenin from the nucleus, whereas when junctions are weak β -catenin is stabilized and translocates into the nucleus via the Wnt signaling pathway, where it is transcriptionally active. In the nucleus, β -catenin heterodimerizes with one of the T-cell factor/lymphoid enhancer factor family of transcription factors to affect the transcription of various target genes, particularly regulators

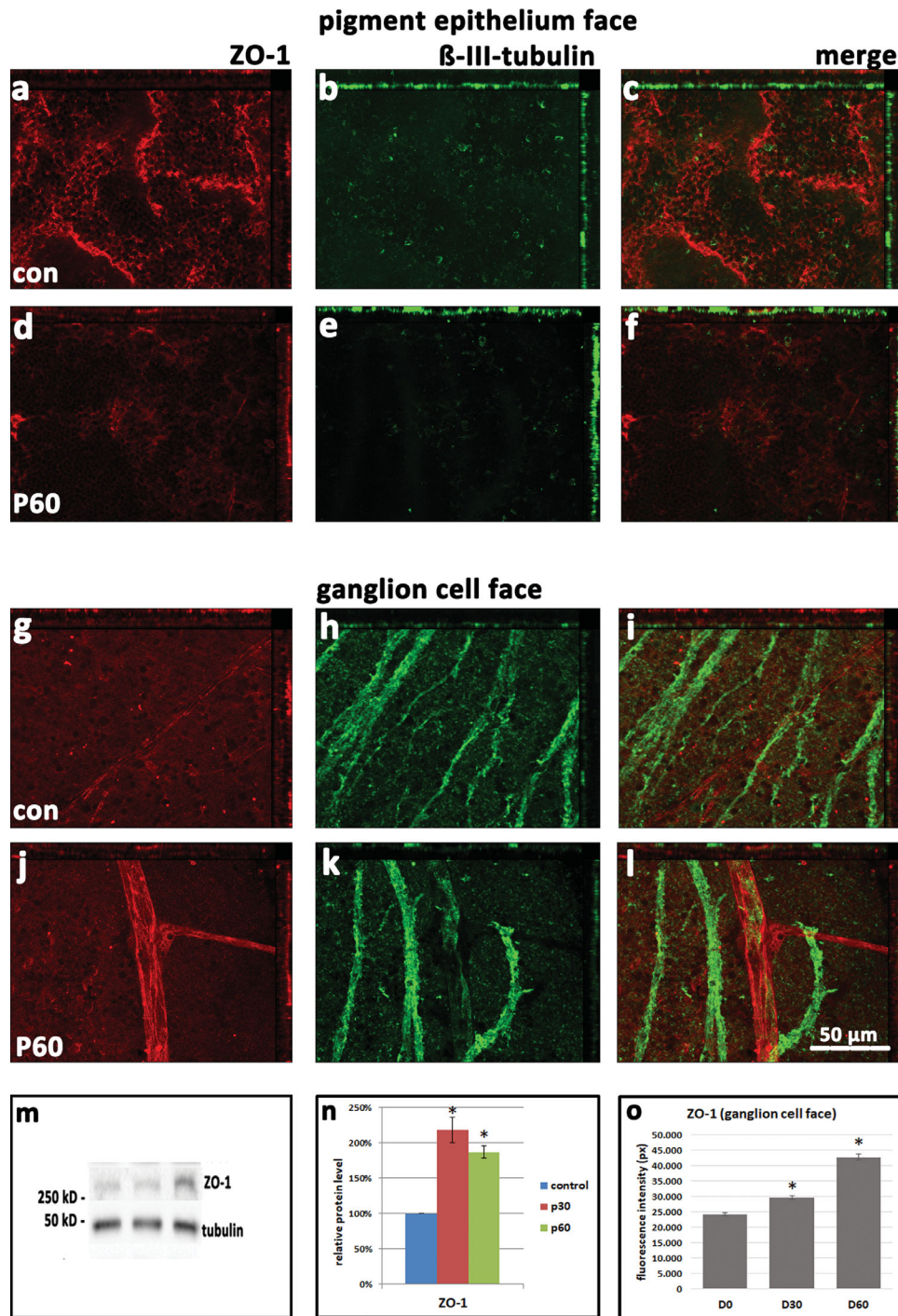


FIGURE 4. Immunohistochemistry for ZO-1 and β -III-tubulin. (a–f) The photoreceptor face of the explant shows no expression of β -III-tubulin, as expected, but decreased staining of ZO-1 between the control (a–c) and elevated-pressure (d–i) cultured retinas (g–l). The same arrangements viewed from the ganglion cell face show increased expression of ZO-1 in retinal vessels under an elevated pressure of 60 mm Hg (j–l). Fluorescence intensity of ZO-1 at the ganglion cell face was quantitatively analyzed (o). These findings were confirmed in western blots shown in (m) and (n). Two Z-layers. Scale bar: 50 μ m. con, control; p30, 30 mm Hg; p60, 60 mm Hg.

of cell growth and proliferation, such as myc and cyclin D1, and regulators of cell–cell communication, such as connexin-43 and metalloproteinases.^{33,34} The stable protein levels β -catenin in spite of downregulation of its mRNA may therefore indicate stabilization of this protein and subsequent changes in transcriptional regulation in the glaucomatous retina. This hypothesis is supported by the observed upreg-

ulation of PDGFR β by elevated pressure in conjunction with the report that PDGF induces an increase in β -catenin transcriptional activity via the formation of an ACK1/PDGFR β complex and AKT activation.³⁵

It is also noteworthy that the regulation of ZO-1 protein differs between the outer retina (particularly within the RPE) and the inner retina. ZO-1 is downregulated in the RPE

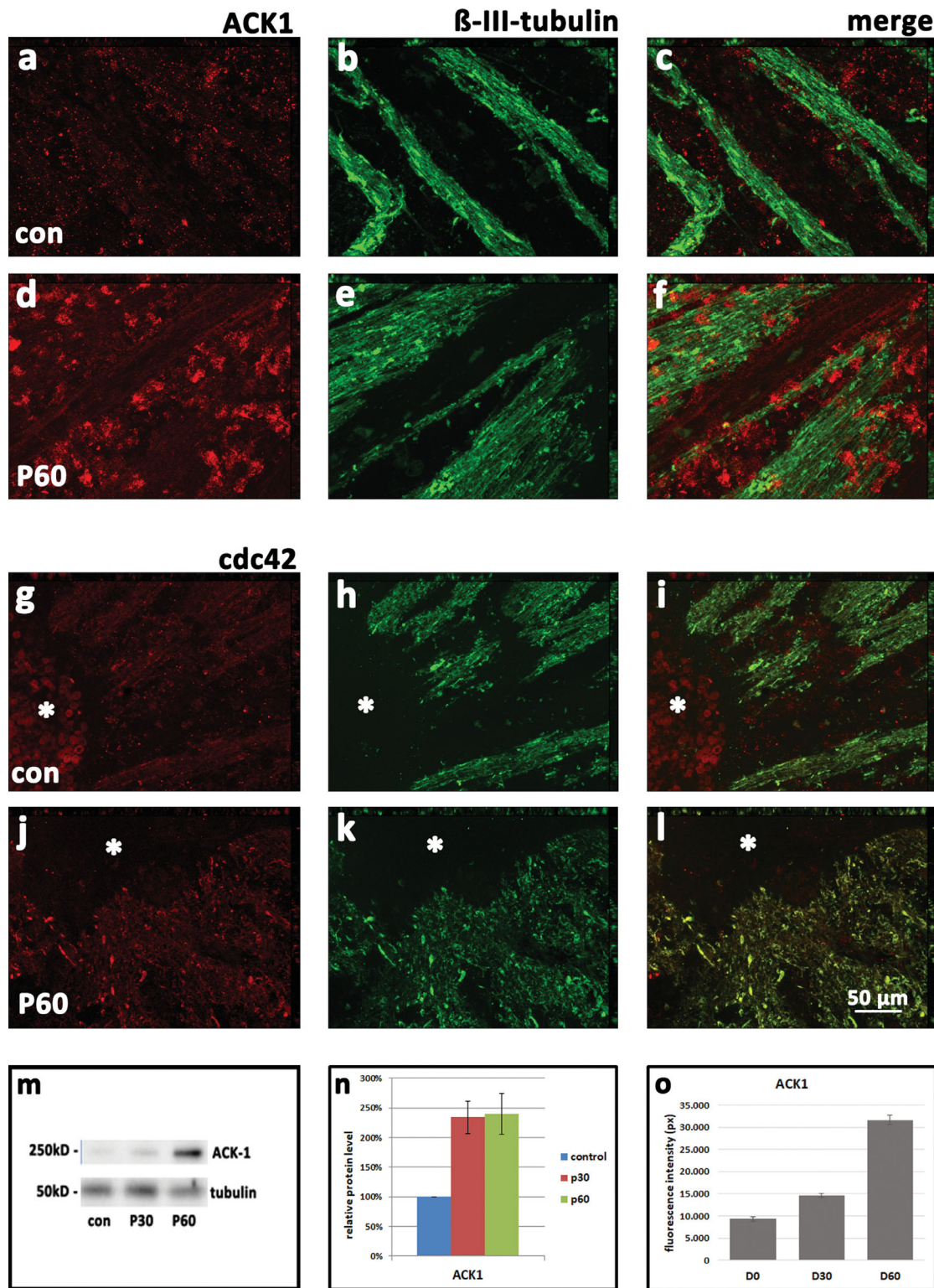


FIGURE 5. (a–f) Immunohistochemistry for ACK1 shows a conspicuously enhanced staining under pressure elevation up to 60 mm Hg. (g–l) IHC for Cdc42 shows decreased Cdc42 staining within the optic nerve head (*) under increased pressure conditions. (m, n) The ACK1 increase was confirmed by western blotting. (o) Quantitative analysis of fluorescence intensity of ACK1. Two Z-layers. Scale bar: 50 μm. con, control; p30, 30 mm Hg; p60, 60 mm Hg.

at an elevated ICP but upregulated in the GCL and RNFL. ZO-1 is localized to TJs and can associate with cadherin-based AJs and with gap junctions. ZO-1 further links

these junctions to the actin cytoskeleton and functions in signal transduction pathways via interactions with diverse signaling molecules and transcriptional regulators.^{9,36} These

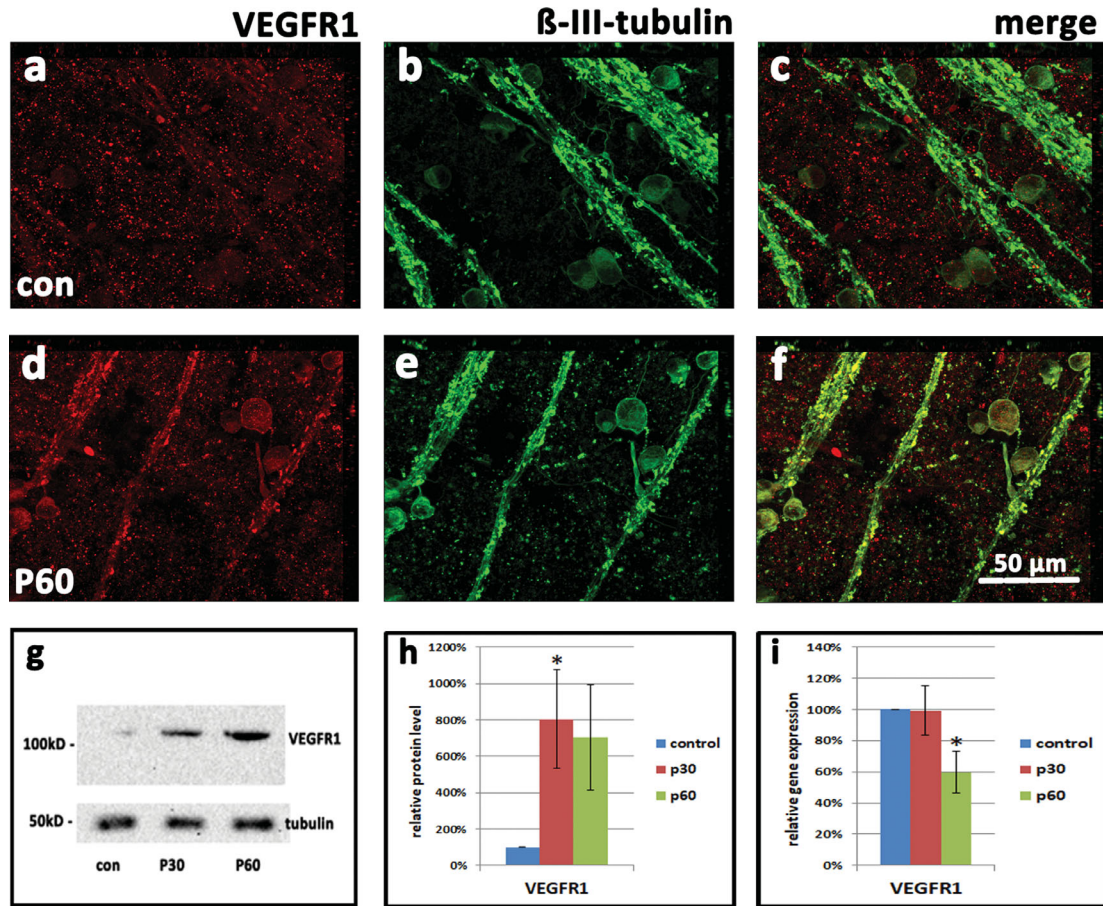


FIGURE 6. (a–f) Immunohistochemistry for VEGFR-1 showed clear upregulation of the VEGFR-1 protein in neuronal cells. (g, h) Western blots confirmed this change. (i) The VEGFR-1 mRNA was unchanged at 30 mm Hg and significantly reduced at 60 mm Hg. Two Z-layers. Scale bar: 50 μ m. con, control; p30, 30 mm Hg; p60, 60 mm Hg.

differences in pressure-dependent regulation may derive from specialized functions of ZO-1 in epithelial cells of the RPE, which forms the outer blood–retinal barrier (BRB), and in endothelial cells of vascular tubes in the inner retina that form the inner BRB. ZO-1 may therefore subserve different functions associated with either TJ function or signal transduction within epithelial and endothelial cells. However, the function of ZO-1 in the retina is largely unknown, and so further studies are needed to understand its observed pressure-dependent regulation.

The other TJ protein examined in our study, occludin, was found to be slightly upregulated at the protein level, with the phosphorylated form showing a larger increase than the non-phosphorylated form. Occludin is predominantly expressed at TJs of epithelial and endothelial cells, and its expression is correlated with the barrier integrity in different tissues.³⁷ Its localization at TJs depends on binding to ZO-1.³⁸ Occludin can be phosphorylated at serine, threonine, or tyrosine residues, with its cellular localization and function thereby being modulated. Depending on the kind of phosphorylation, the endothelial or epithelial permeability may increase or decrease.^{15,37,39,40} Our results, together with the observed upregulation of ZO-1 in retinal vessels, may indicate an increase in TJ integrity in endothelial cells. More detailed information about the pressure-dependent regulation of occludin and ZO-1 in different retinal tissues

could probably be obtained by examining diverse cells cultivated under elevated pressure.⁴¹

Crosstalk is present among various components of the TJ proteins and the intracellular signaling pathways, including those that undergo tyrosine or non-tyrosine phosphorylation. Among them we found that the nonreceptor tyrosine kinase ACK1 is upregulated under an elevated ICP, whereas the cyclin-dependent kinase Cdc42 shows no consistent pressure-dependent regulation throughout the retina, except for its downregulation in the optic nerve head. Cdc42 is known to generate independent signals that regulate the rearrangement of the actin cytoskeleton and gene transcription. It is therefore not surprising that ICP elevation downregulates Cdc42 in the most active parts of the retina, which are the ganglion cell axons within the RNFL and the optic nerve head. The autoinhibited ACK1 can be activated by cell adhesion, interaction with Cdc42, and multiple growth-factor receptors, including epidermal growth factor receptor and PDGFR.⁴² A particularly interesting finding is that ACK1 is activated for nuclear translocation by Cdc42, which then remains in the cytoplasm, and it has been shown that overexpression of ACK1 blocks Cdc42 signaling and leads to impaired cell growth and wound healing.⁴³ This led us to hypothesize that the increased expression of ACK1 under elevated pressure impairs the regeneration of glaucomatous insults in the retina. The observed

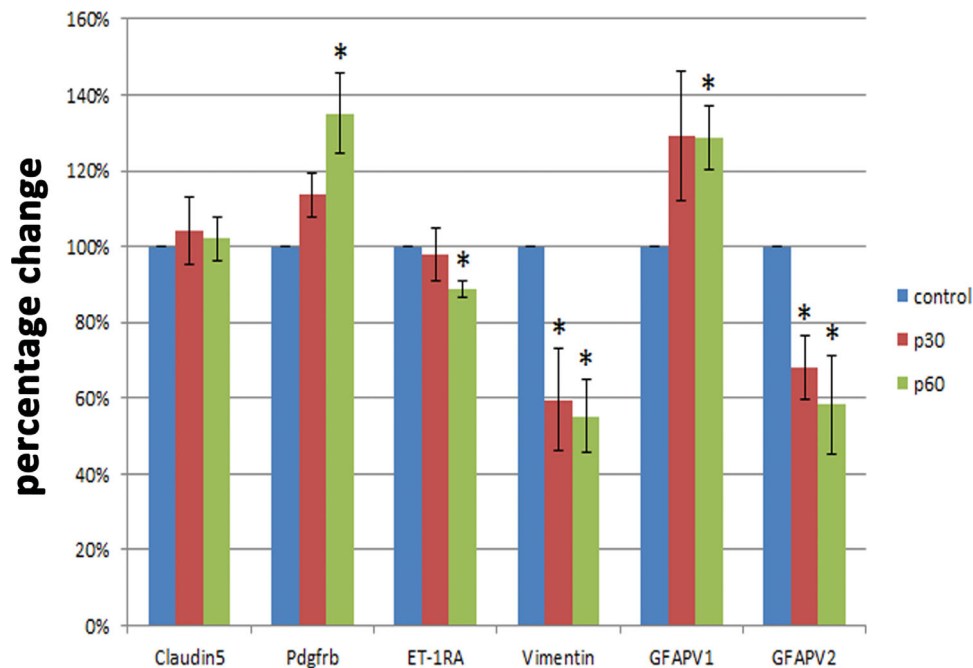


FIGURE 7. Results from qRT-PCRs for mRNAs up- or downregulated by elevated pressure for claudin-5, PDGFR β (Pdgfrb), ET-1RA, vimentin, GFAPV1, and GFAPV2. p30, 30 mm Hg; p60, 60 mm Hg.

pressure-dependent downregulation of vimentin may support this view. Because GFAP is known to play a role in repair after central nervous system injury by the formation of a glial scar but is also involved in cell communication and functioning of the blood–brain barrier, it would be worthwhile to elucidate the effect of the differently pressure-regulated GFAPV1 and GFAPV2 in the glaucomatous retina.

It has been reported that VEGFRs are not exclusively expressed in the vasculature, as they are also present in Schwann cells, Müller cells, and peripheral neurons in the mouse neural retina.⁴³ We detected an increased neuronal expression of VEGFR-1 after applying pressure elevation to cultivated mouse retinal explants. It is possible that VEGFR-1 redirects VEGF in order to ensure an appropriate vascular supply to pressure-afflicted neurons, as has been described for VEGFR-2 in retinal development and pathology.⁴⁴

CONCLUSIONS

Our mouse glaucoma model revealed pressure-induced changes related to a weakening of AJs and the BRB, altered signal transduction, and impairment of tissue integrity and retinal regeneration *in vitro*; however, these findings require further investigation. Our results reflect only *in vitro* findings over a relatively short period of pressure elevation. Next steps would be to use *in vivo* models to investigate those changes and to look at the reversibility of those changes, as well as at a longer exposure time for elevated pressure or very high pressure elevations. We assume that the changes would be irreversible and more pronounced if exposed to a longer period of elevated IOP.

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