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VEGF-A is necessary and sufficient for retinal neuroprotection in models of experimental glaucoma

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Abstract

Vascular endothelial growth factor A (VEGF-A) is a validated therapeutic target in a number of angiogenic- and vascular permeability-related pathologies, including certain cancers and potentially blinding diseases such as age-related macular degeneration and diabetic retinopathy. We and others have shown that VEGF-A also plays an important role in neuronal development and neuroprotection, including in the neural retina. Antagonism of VEGF-A function might therefore present a risk to neuronal survival as a significant side effect. Here we demonstrate that VEGF-A acts directly on retinal ganglion cells (RGCs) to promote survival. VEGFR-2 signalling via the PI3K/Akt pathway was required for the survival response in isolated RGCs. These results were confirmed in animal models of staurosporine-induced RGC death and experimental hypertensive glaucoma. Importantly, we observed that VEGF-A blockade significantly exacerbated neuronal cell death in the hypertensive glaucoma model. Our findings highlight the need to better define the risks associated with use of VEGF-A antagonists in the ocular setting.

Introduction

VEGF-A was initially identified as a vascular permeability factor and endothelial cell mitogen. Since then, it has been shown to have numerous roles outside the vasculature, perhaps most significantly in the nervous system. Neurons express VEGFR-1 and VEGFR-2, and are able to respond to VEGF-A [1]. Furthermore, neuropilins, which are important receptors for neuronal development and function, are also co-receptors for the heparin-binding VEGF164 and VEGF188 isoforms [2]. Studies have revealed neuro-developmental, neurotrophic and neuroprotective roles for VEGF-A in a variety of nervous tissues. *In vitro,* VEGF-A can protect neurons against hypoxia, glutamate excitotoxicity, and deprivation of serum, oxygen or glucose [3-5], as well as mediate neuronal migration, axonal outgrowth and Schwann cell proliferation [6, 7]. *In vivo*, VEGF-A can rescue retinal neurons following optic nerve axotomy [8], protect neural tissues through hypoxic-preconditioning in ischaemia-reperfusion injury [9], improve function in rodent models of amyotrophic lateral sclerosis [10] and cerebral ischaemia [11], and mediate neuroprotection during development via the co-receptor neuropilins [12]. VEGF-A appears to exert these effects directly on neuronal cells, independently of its vascular actions, and may even be important for maintenance of neuronal circuitry [13].

A better understanding of VEGF-A's roles in the nervous system is critical, given that antagonism of VEGF-A function is used as a therapeutic strategy for numerous pathologies, including various types of cancer, choroidal neovascularization associated with age-related macular degeneration, and macular oedema associated with diabetes mellitus and retinal vein occlusion [14, 15]. This therapeutic strategy is also being explored for additional conditions in which vascular growth and permeability are important, such as neovascular glaucoma and fibrotic complications of glaucoma filtration surgery [16]. Given the functional and protective

roles of VEGF-A in the nervous system, these treatments might have unexpected side effects on neural function, particularly in the eye.

With this in mind, we sought to explore the mechanism by which VEGF-A exerts its neuroprotective effects. We first determined if VEGF-A can act directly on isolated RGCs. Having established that VEGF-A directly prevents RGC apoptosis via VEGFR-2 and phosphoinositide-3-kinase (PI3K)/Akt signalling, we used two different animal models to study RGC death *in vivo*. Our findings suggest a neuroprotective role for VEGF-A in models of acute toxicity and hypertensive glaucoma, and highlight the need for rigorous assessment of the long-term impact of VEGF-A inhibition on retinal neurons.

Methods

Animals

All animals were obtained from Harlan Laboratories and used according to Home Office guidelines.

RGC isolation and culture

We used an immunomagnetic cell separation protocol based on Sappington *et al.*. (2006) [17], with modifications. Retinas from postnatal day 1 Sprague-Dawley rats were dissociated as previously described. To ensure purity of RGCs, we removed macrophages first. The pellet was resuspended in Dulbecco's Modified Essential Media (DMEM; Invitrogen) with rabbit anti-rat-macrophage antiserum (1:100, Accurate Chemical, Westbury, NJ). The solution was then incubated with goat anti-rabbit secondary conjugated to magnetic microbeads, and separated using an autoMACS (Miltenyi Biotec, Germany). The negative fraction was incubated with mouse anti-rat Thy1.1 antibody (1:125, BD Pharmingen, San Diego, CA,

followed by secondary rat anti-mouse IgG_1 antibody conjugated to magnetic beads (Miltenyi Biotec). AutoMACS separation was carried out, leaving Thy1.1 positive RGCs, which are reported to comprise 93% of Thy1.1-positive cells in the retina[18].

Prior to seeding, culture vessels were coated with poly-d-lysine (0.01 mg/ml; Sigma-Aldrich, Dorset, UK), and laminin (0.01 mg/ml; Roche Applied Science, UK). Cells were seeded in 4-well plates (Nunc, Roskilde, Denmark) on 13 mm glass coverslips at $2.5x10^4$ cells/well, and $5x10^5$ cells/well on 12-well plates for real-time PCR. Cells were grown in serum-free Neurobasal-A medium as previously described [17], and maintained at 37°C in 5% CO_2.

Because RGCs require growth factors to survive, it was necessary to dissect protective properties of VEGF-A from those offered by growth factors already present. Cells received full medium at day *in vitro* (DIV) 0 and DIV 1, then no further medium until treatment on DIV 5. This ensured sufficient cells survived for assays without masking the beneficial effects of VEGF-A by other neuroprotectants. Mouse VEGF164, VEGF120 (R&D Systems, Abingdon, UK), VEGF-E (Isolate D1701 with His tag, CRV007, Cell Sciences, Canton, MA), PlGF-1 and PlGF-2 (Peprotech, London, UK), at 2.5 nM final concentration, were added in Neurobasal-A on DIV 5, 24 hours before toxicity treatment. These were added in media minus supplements or growth factors to media covering the monolayer, as removal of all survival factors was too damaging. For H_2O_2 treatment, cell culture medium was removed, and 500 µl 10 µM H_2O_2 +/- VEGF receptor (VEGFR) ligands in Neurobasal-A was added for 5 hours. Due to staurosporine (SSP) potency, it was necessary to add this onto media already present. SSP (1 µM) +/- VEGFR ligands were added for 24 hours in Neurobasal-A. The PI3K inhibitors LY-294,002 (0.1-10 µM) and wortmannin (0.3-30 nM) were added 10 minutes prior to VEGFR agonist pre-treatment in Neurobasal-A. Pan-caspase inhibitors Z-VAD-Fmk and Q-VD-Oph, used individually or in combination, were added simultaneously with H_2O_2

or SSP at 100 µM. Equivalent concentrations of dimethyl sulphoxide (DMSO) were included as controls for SSP, PI3K and caspase inhibitor experiments.

Cell survival assay

Cell survival was determined using calcein AM dye to quantify viable cells remaining following treatments, based on previously published methods[19]. Calcein AM is a cell permeable, fluorogenic esterase substrate, which is hydrolysed by intracellular esterases in living cells and converted into the fluorescent product calcein. We photographed 3 random non-overlapping fields of each well, on duplicate coverslips at 10x magnification using a BX51 epifluorescence microscope with a Retiga SRV camera (QImaging, BC, Canada)**.** At least 200 cells were counted per N, using an automated cell counting programme (Image Pro Plus 6.2, Media Cybernetics, San Diego, CA). Survival rate was expressed as a percentage of the total number of cells in control wells at each time point.

Real-time PCR

For *in vitro* real-time PCR, cells received full media, plus or minus 2.5 nM VEGF164 or PlGF-1, at DIV 1, -2 and -5. At DIV 7, total RNA was isolated using the RNEasy kit (Qiagen, Sussex, UK). For *in vivo* studies, eyes were stored in RNAlater (Invitrogen) until RNA was extracted. Real-time PCR was conducted using the Taq-Man Gene Expression Assay (Applied Biosystems, Warrington, UK). To detect expression of the target gene, the following assays were used: VEGF (Rn00582935_m1), VEGFR-2 (Rn00564986_m1), VEGFR-1 (RN00570815_m1), and β-actin (RN00667869_m1). Expression levels of target genes were determined by the relative quantification method using β-actin as an endogenous control.

TUNEL staining

The terminal deoxynucleotidyl transferase-mediated dUTP nick-labelling (TUNEL) assay quantified apoptotic cells *in vitro* and in whole mount retinas, according to manufacturer's instructions (Promega, Southampton UK). For RGCs *in vitro*, cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes, permeabilised with 0.2% Triton X-100 in PBS (T-PBS), before the TUNEL reaction. Coverslips were mounted on glass slides, and images of 6 non-overlapping fields taken from duplicate coverslips at 10x magnification using an Olympus BX51 microscope (Essex, UK) with Retiga SRV camera (QImaging, BC, Canada). 4',6-diamidino-2-phenylindole (DAPI)- and TUNEL-positive cells were counted; minimum of 500 cells/coverslip. The number of TUNEL-positive cells was subtracted from DAPI-positive cells to give TUNEL-negative (non-apoptosing) cells, and averaged per field. Each N represents independent cell separations.

For whole mounts, animals were CO_2 asphyxiated, then eyes fixed in 4% PFA. Retinas were permeabilised in 3% T-PBS for 2 hours. The TUNEL protocol was carried out, then retinas washed in 0.3% T-PBS with 5 µM DAPI then flat-mounted in Vectashield (Vector Laboratories, Peterborough, UK). To quantify TUNEL-positive neurons, we used a Zeiss 700 confocal microscope (Zeiss, Oberkochen, Germany), taking 10 µm z-stacks through the ganglion cell layer (GCL) at 20x magnification. Morphological criteria discriminated non-neuronal (endothelial and glial) from neuronal cells. We took 3 images on each of the 4 petals; close to the optic nerve, the middle, and periphery of the retina, giving 12 images per whole mount and sampling circa 7000 cells. Areas were selected using only DAPI, and investigators masked to treatment groups.

Acute toxicity model

Male 10-week-old C57/Bl6 mice were anaesthetised with 100 mg/kg ketamine and 0.5 mg/kg xylazine, and pupils dilated with 2.5% phenylephrine hydrochloride and 1.0% tropicamide (Bausch and Lomb, Surrey, UK). For pre-treatment, bilateral intravitreal injections of 4 pmol VEGF120 or PBS vehicle were administered in 1 µl volume prior to injecting SSP or vehicle. Mice recovered for 24 hours after initial injection, then 1 nmol SSP or 10% DMSO vehicle +/- wortmannin in 1 µl was administered intravitreally. Animals were sacrificed 24 hours later by CO_2 asphyxiation. Eyes were enucleated and fixed in 4% PFA for 4 hours for staining. Investigators were masked to treatment groups until analysis was complete.

Ocular Hypertension Model

Experimental glaucoma was induced by elevating intraocular pressure (IOP) via injection of paramagnetic microspheres into the anterior chamber, based on Samsel *et al.*. (2011)[20]. Briefly, 250-300g female ex-breeder Brown Norway rats were housed for one week in a constant low-light environment (40-60 lux) to minimise diurnal fluctuations in IOP. Rats were anaesthetised with 37.5mg/kg ketamine and 0.25mg/kg medetomidine hydrochloride, and a toroidal magnet (Supermagnete, Germany) placed around the eye, before 25 µl of a solution containing 30 mg/ml 8 µm magnetic microspheres (Bangs Laboratories, Fisher, IN) in Hanks balanced salt solution was injected into the anterior chamber. The magnet drew the beads into the iridocorneal angle, to impede aqueous drainage from the trabecular meshwork. Right eyes acted as unoperated controls. IOP measurements were taken in awake animals before bead injection, then every 2-3 days using a TonoLab rebound tonometer (Tiolat, Oy, Finland). IOP was taken as the mean of 5 readings. To investigate VEGF-A neuroprotection, 20 pmol of VEGF120, VEGFR-2 Fc chimera (R&D Systems, Abingdon, UK), IgG or vehicle controls were injected intravitreally on days 3 and 10 post induction. Animals were sacrificed

on day 17 post induction, eyes enucleated and fixed in 4% PFA overnight for TUNEL. All experiments were carried out masked.

Immunostaining

For RGC cultures, cells were fixed in 4% PFA, and blocked with 5% normal goat serum in 0.1% T-PBS for 2 hours. Primary antibodies were rabbit anti-Thy1 (1:200, Santa Cruz, Santa Cruz, CA), rabbit anti-βIII-tubulin (1:500, Abcam, Cambridge, UK), rabbit anti-VEGFR-2 (1:200, Abcam), goat anti-VEGFR1 (1:100, Santa Cruz), rabbit anti-phospho-Akt (1:200, Cell Signaling, Beverly, MA), and rabbit anti-active caspase-3 (1:250, R&D Systems). Specificity for VEGFR-2 was confirmed using blocking peptide (Abcam). Secondary antibodies were goat anti-rabbit or donkey anti-goat conjugated to Alexa Fluor 488 or 594, used at a 1:500 dilution for VEGFR staining and 1:200 dilution for all other experiments. Coverslips were mounted on glass slides in ProLong Gold with DAPI (Invitrogen).

For retinal whole-mounts, animals were sacrificed and retinae prepared as for TUNEL staining. The tissue was blocked for 2 hours in 5% donkey serum, 0.3% T-PBS before primary antibodies were applied overnight. Secondary antibodies were added for 2 hours in 0.3% T-PBS. After secondary incubation, the tissue was rinsed in 0.3% T-PBS plus 5 µM DAPI then flat-mounted in Vectashield (Vector Laboratories, Peterborough, UK). Primary antibodies used were rabbit anti-phospho-Akt (1:500, Cell Signaling), and goat anti-Brn3a (1:200, Santa Cruz); secondary antibodies were donkey anti-rabbit conjugated to Alexa Fluor 633 and donkey anti-goat conjugated to Alexa Fluor 594 (1:1000, Invitrogen). Fluorescein-conjugated *Griffonia simplicifolla* isolectin B4 (1:400 Vector Laboratories, Peterborough, UK) stained blood vessels. Controls were no primary antibody and relevant IgG isotypes. Images of cells and retinas were taken on a Zeiss 700 confocal microscope.

Optic nerve sections

Optic nerves were fixed overnight in Karnovsky's solution at 4^0C. Specimens were osmicated for 2 hrs in 1% (w/v) osmium tetroxide, then dehydrated in 100% ethanol. Optic nerves were then incubated in propylene oxide (PO) for 30 minutes and placed in a 50:50 mixture of PO:araldite overnight. This solution was changed to 100% araldite and cured overnight at 60^0C. Semithin sections (0.75µm) were cut and stained with 1% toluidine blue/borax (TB) in 50% ethanol prior to examination by light microscopy. For quantification, 3 non-overlapping images were taken at 63 x magnification, in the centre, midway and periphery of the optic nerve, using Olympus BX51 microscope (Essex, UK) with Retiga 2000R camera (QImaging, BC, Canada). Non-viable/dying axons with TB accumulated were counted in two sections per optic nerve, averaged per section, then expressed as dying axons per mm^2. At least 3 optic nerves were quantified per treatment group.

Statistical analysis

Statistical analyses were done using Graphpad Prism (Graphpad Software, La Jolla, CA). In all instances one-way ANOVA with Newman-Keul's post-hoc test was used, except for RGC cultures, when ANOVA with repeated measures was used. To analyse real-time PCR results, Ct values were normalised to β-actin and statistics done on ΔCt values. Results are mean ± SEM unless stated, with each N representing an individual cell culture separation or retina; N was at least 3 for each statistical analysis. A P value of less than 0.05 was considered significant.

Results

Characterization of primary RGC cultures

We have previously demonstrated VEGF-A can protect retinal neurons from death induced by ischemia-reperfusion injury [9]. Since this *in vivo* model involves potential indirect effects of blood flow and paracrine-mediated protection from endothelial or other retinal cell types, we used primary RGC cultures to probe the mechanisms of VEGF-A-mediated neuroprotection. RGCs were used as relatively homogenous cultures can be obtained in comparison to other retinal neurons, and they are relevant to many retinal pathologies [21]. We confirmed purity of our primary RGC cultures by immunostaining for the RGC marker Thy-1 and neuron-specific βIII-tubulin. At day *in vitro* (DIV) 5, RGC cultures were > 95% positive for both markers (Supplemental Figure 1). The cells formed dense networks of neurites and were capable of surviving for weeks in culture.

Expression and function of VEGF receptors in cultured RGCs

In vivo, RGCs express both VEGF-A and its receptors [1]. Using RT-qPCR, we demonstrated that VEGFR expression was maintained in our cultured RGCs. VEGFR-2 was the most abundant receptor, with relative levels 17-fold higher than those of VEGFR-1 ($P < 0.001$; Supplemental Figure S2A). When compared with primary rat brain microvascular endothelial cells in culture (kindly donated by Dr. Patric Turowski); a cell type known to express functional VEGF receptors, the relative levels of VEGFR-2 were 5-fold higher and VEGFR-1 4-fold lower in RGCs, and ratios of VEGFR-2 to VEGFR-1 were approximately 17:1 in RGCs and 1:1 in endothelial cells (Supplemental Figure S2A). VEGFR-2 staining in RGCs was predominantly perinuclear (Figure 1B).

To determine if the receptors for VEGF-A are functional in cultured RGCs, we treated cells with VEGF-A isoforms VEGF120 and VEGF164; VEGFR-1-specific PlGF-1 and PlGF-2;

and VEGF-E, which is specific for VEGFR-2. VEGF164 induced a 2-fold increase in VEGFR-2 expression ($P < 0.001$; Figure 1A), which was accompanied by increased VEGFR-2 immunostaining, particularly along the neurites and perinuclear region (Figure 2B). Increased immunostaining was also observed with VEGF120 and VEGF-E treatments (Figure 1B). VEGFR-1 expression rose approximately 1.6-fold ($P < 0.01$) after VEGF164 treatment **(**Figure 1A**)**. PlGF-1 did not significantly modify mRNA expression of either VEGF receptor (Figure 1A), nor was the qualitative immunostaining pattern altered for PlGF-1 or PlGF-2 (Figure 1B). Together these data demonstrate that cultured RGCs express VEGFR-2, and that receptors are functional.

RGC responses to cell death agents

To assess the neuroprotective properties of VEGF-A, it was necessary to identify agents that effectively induce RGC death in culture. We evaluated nine conditions described in the literature [3, 4, 22-28] for inducing RGC death (Table S1). RGCs in culture were surprisingly resilient; agents that induce receptor-mediated apoptosis, including tumor necrosis factor α, Fas ligand, and interleukin-1β, failed to cause RGC death following 24 hours to 5 days of treatment. Excitotoxic ligands N-methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) and glutamate also failed to cause significant cell death, even with 5 days of 500 μM ligand. However, RGCs in culture have previously been shown to be invulnerable to NMDA-mediated cell death [29]. Even hypoxia for up to 24 hours did not induce significant cell death (1% O_2) (Table S1).

Conditions that did cause significant RGC death were growth factor withdrawal and exposure to paraquat, SSP or H_2O_2 (Table S1). From these, H_2O_2, used to model oxidative stress *in vitro*, and SSP, a non-specific protein kinase inhibitor that broadly activates cellular death

pathways [30], were chosen as they were optimal for our assays. Both induced consistent, dose-dependent RGC death ($P < 0.05$).

VEGF-A protects RGCs via VEGFR-2, independent of neuropilins

To examine VEGF-mediated effects on RGC survival, cultures were pre-treated with VEGF family ligands with different binding properties for VEGFR-1, VEGFR-2, and neuropilins. RGCs were pre-treated at DIV 5 for 24 hours with media supplemented with the agonists (final concentration 2.5 nM), followed by addition of 10 μM H_2O_2 for 5 hours, or SSP for 24 hours. Of the different VEGFR ligands tested, VEGF164 ($P < 0.01$), VEGF120 ($P < 0.05$) and VEGF-E ($P < 0.01$) all increased RGC survival by approximately 50% (Figure 2A). In contrast PlGF-1 and PlGF-2 did not protect against H_2O_2-mediated cell death. A similar pattern emerged for SSP treatment. SSP at 1 μM induced approximately 25% death of RGCs, which was completely reversed by 24 hour pre-treatment with VEGF164, VEGF120 or VEGF-E (all $P < 0.001$). Again, both PlGF-1 and -2 failed to offer detectable protection.

These data indicate that VEGFR-2 is required for protection of RGCs, consistent with our findings *in vivo* [9]. It appeared that the heparin-binding domain of VEGF-A was not essential, since the VEGF120 isoform and VEGF-E that lack this domain, rescued cells with similar potency to heparin-binding VEGF164. These data further suggest that neuropilin receptors are not required for protection, since VEGF120 and VEGF-E, which exhibit little or no binding to neuropilin-1 and -2 [2, 31, 32] were protective, whereas PlGF-2, which binds to both neuropilins, did not enhance survival. We decided to use VEGF120 in subsequent experiments, as this isoform produced consistent protection (Figure 2A) and induces fewer adverse effects upon intravitreal injection *in vivo* [9].

VEGF-A is able to protect against apoptotic, caspase-dependent cell death

We sought to characterise the mechanism of VEGF-A-mediated neuroprotection in further detail. TUNEL staining was used to define whether cell death was associated with DNA fragmentation, commonly associated with apoptosis. Inhibitors and immunostaining were also used to determine if modulation of caspase signalling was associated with VEGF-A-mediated neuroprotection. TUNEL staining revealed that H_2O_2 treatment significantly reduced viable (TUNEL-negative) cell number by 42% relative to control ($P < 0.001$), and that VEGF120 dose-dependently augmented survival compared to control – by 31% at 2.5 nM ($P < 0.05$) and 50% at 5.0 nM ($P < 0.05$) (Figure 2B). These effects were also observed with SSP treatment, for which VEGF120 exposure significantly reduced cell death ($P < 0.05$ compared to SSP alone).

Caspase activation is an early step in the initiation of apoptosis. To determine the role of caspases in H_2O_2- and SSP-mediated RGC death, two different caspase inhibitors, Z-VAD-Fmk and Q-VD-Oph, were used. These inhibitors have differential affinities for individual caspases; they therefore must be used in combination to fully differentiate between caspase-dependent and caspase-independent death [33]. Both Z-VAD-Fmk and Q-VD-Oph significantly increased the percentage of viable RGCs, from 39% to 72% and 68%, respectively (both $P < 0.01$), and to 63% ($P < 0.05$) when combined (Figure 2C). Findings were similar for SSP-induced cell death (Figure 2C).

Since caspase activity and DNA fragmentation are involved in H_2O_2- and SSP-induced RGC death, we sought to determine if VEGF-A's neuroprotective effects involve modulation of caspase activation. Immunostaining confirmed an increase in activated caspase 3 levels in the presence of H_2O_2, and pre-treatment of cells with VEGF120 markedly reduced the amount of activated caspase 3 (Figure 2D). Taken together, these data suggest that H_2O_2 and SSP initiate apoptotic, caspase-dependent death in RGCs, and that VEGF-A signalling via VEGFR-2 inhibits caspase-3 activation to promote RGC survival.

PI3K/Akt signalling pathways mediate VEGF-A neuroprotection in vitro

The PI3K/Akt signalling pathway is involved in numerous cellular functions, and has been identified as central for survival of many cell types, including neurons [5]. To determine if VEGF-A mediates neuroprotection via PI3K/Akt in RGCs, we first explored the activation status of Akt in cultured cells. In the presence of H_2O_2, RGC phospho-Akt levels were reduced, an effect prevented by VEGF120 pre-treatment (Figure 3C). Furthermore, pre-treatment of RGCs with the PI3K inhibitor LY294,002 blocked VEGF120-induced Akt phosphorylation. These data indicate that Akt signalling is activated during VEGF120-mediated protection of RGCs.

To confirm that VEGF-A acts via the PI3K/Akt signalling axis, cells were exposed to PI3K inhibitors during VEGF120 pre-treatment and the effects on neuroprotection monitored. LY294,002 and wortmannin alone did not induce RGC death (Supplemental Figure S3A + B). When added to RGCs immediately preceding VEGF120, LY294,002 dose-dependently abolished VEGF120's survival-enhancing properties against H_2O_2 ($P < 0.05$; Figure 3A). Similar results were obtained with wortmannin ($P < 0.05$). When the corresponding experiments were conducted using SSP, attenuation of VEGF120 protection was observed at the highest inhibitor doses tested ($P < 0.05$) (Figure 3B).

VEGF-A protects RGCs in an in vivo acute toxicity model via PI3-kinase dependent pathways

Using primary cell culture we have illustrated the direct neuroprotective function of VEGF-A using RGCs and implicated signalling pathways involved. To examine the applicability of these findings *in vivo*, acute toxin-induced retinal cell death was initiated by intravitreal injection of SSP. Mice were pre-treated with an intravitreal injection of 4 pmol VEGF-A or vehicle for 24 hours before receiving 1 nmol SSP or vehicle for a further 24 hours. Injection of SSP significantly increased the number of TUNEL-positive cells in the GCL (68.9 ± 16.8

cells per retina), compared to saline-injected (12.3 ± 3.3 cells per retina; $P < 0.01$) or DMSO vehicle controls (6.0 ± 1.2 cells per retina; $P < 0.001$) (Figure 4A, C). VEGF120 pre-treatment significantly protected against SSP-induced toxicity, reducing apoptotic nuclei by 57% compared to vehicle control (29.8 ± 6.4 cells per retina; $P < 0.01$). To explore if VEGF-A-mediated neuroprotection is mediated by PI3K signalling *in vivo*, the PI3K inhibitor wortmannin was injected simultaneously with SSP, following VEGF120 pre-treatment. Wortmannin alone did not increase RGC apoptosis compared to controls (Supplemental Figure S3C), but it fully reversed the protective effects of VEGF120 against SSP toxicity (21.79 ± 4.2 vs. 53.29 ± 9.1 cells per retina; $P < 0.05$) (Figure 4B), suggesting a fundamental role for PI3K in VEGF-A-mediated neuroprotection.

VEGF-A protects against RGC death in an ocular hypertension model

The protective effect of VEGF-A was also explored in an *in vivo* model of experimental glaucoma, in which RGC death was induced by mechanically increasing IOP. In patients with ocular hypertensive glaucoma, elevated IOP caused by obstruction of aqueous outflow is a key risk factor in the disease pathophysiology [34]. Animal models have been developed to mimic blockage of the trabecular meshwork, including using magnetic microspheres drawn into the iridocorneal angle to reduce outflow [20]. This model was used here to validate the neuroprotective properties of VEGF-A in rats.

Injection of magnetic beads into the anterior chamber triggered a significant and prolonged rise in IOP. Mean IOP averaged over the full length of the experiment for control, non-bead-injected eyes was 19.8 ± 0.6 mmHg, compared to 43.3 ± 3.3 mmHg for bead-injected eyes ($P < 0.001$, Figure 5A). Peak IOP was 22.8 ± 0.6 mmHg for control eyes vs. 55.2 ± 3.5 mmHg for bead-injected ($P < 0.001$). Intravitreal injection of VEGF120 did not affect IOP in bead-

injected eyes (Figure 5A); mean and peak IOP were all similar for VEGF120- and vehicle-injected eyes.

To investigate if expression of endogenous VEGF-A and its receptors were altered following IOP elevation, retinas were analysed by RT-QPCR. No change in mRNA levels for VEGF-A (Figure 5B), VEGFR-2 or VEGFR-1 (Supplemental Figure S4) was detected between control and retinae from hypertensive eyes.

Cell death in the GCL was assessed using TUNEL staining, which has been shown to increase in patients with glaucoma [35, 36], and correlates with RGC loss and optic nerve degeneration in animal glaucoma models [37-39]. In eyes where magnetic beads were injected, but pressure did not rise (due to incomplete blockage of iridocorneal angle), numbers of TUNEL positive cells were not significantly different to non-bead-injected control (Figure 5C). These eyes were excluded from our studies. In eyes where IOP increased following microspheres injection, there was a significant elevation in TUNEL-positive apoptotic nuclei in the GCL, which is mostly composed of RGCs as reported previously [20, 40]. The number of apoptotic nuclei increased by approximately 16-fold, from 1.9 ± 0.5 to 31.0 ± 10.0 cells per retina ($P < 0.01$; Figure 5C**)** confirming that high IOP leads to apoptosis of RGCs. Treatment with intravitreal injection of 20 pmol VEGF120 on days 3 and 10 following glaucoma induction reduced apoptotic cell counts by 77% ($P < 0.01$), from 31.0 ± 10.0 to 7.0 ± 1.6 cells per retina (Figure 5D, F), indicating that VEGF120 protects retinal neurons against apoptotic cell death in experimental glaucoma. Furthermore, we observed extensive damage to the optic nerve in histologically stained transverse sections from hypertensive eyes. There were approximately 20-fold more degenerating axons in animals with high IOP, from 0.6 ± 0.3 to 12.4 ± 2.8 axons per mm^2 optic nerve ($P < 0.01$). This damage was reduced by 63% ($P < 0.05$) to 4.6 ± 0.94 axons per mm^2 optic nerve in eyes treated with intravitreal VEGF120 (Figure 5E, G).

To determine if VEGF-A affects PI3K/Akt signalling during neuroprotection *in vivo,* we stained whole mount retinae from the ocular hypertension model for phospho-Akt. Immunostaining revealed barely detectable levels of phospho-Akt in control and PBS-treated glaucomatous retinae (Figure 6H). In response to VEGF120 injection, phospho-Akt immunoreactivity in glaucomatous retinae increased considerably, particularly in the cell cytoplasm, suggesting that the PI3K/Akt pathway is involved in mediating VEGF120 protection in this model.

VEGF neutralisation exacerbates cell death in ocular hypertension model

Finally, to probe the role of endogenous VEGF-A on RGC survival in our experimental hypertensive glaucoma model, soluble human VEGFR-2/Fc chimera (sVEGFR-2) was injected intravitreally to neutralise endogenous VEGF-A. Injection of sVEGFR-2 and human IgG control did not influence IOP, when compared to control PBS bead-injected hypertensive eyes (Figure 6A). However, a comparison of cell death in IgG- and sVEGFR-2-treated hypertensive eyes revealed that TUNEL-positive apoptotic cells markedly increased as a result of VEGF neutralisation. Apoptosis in the GCL of the retina was significantly elevated by approximately 3.5-fold above IgG treatment, from 22.0 ± 7.4 to 79.2 ± 26.5 cells per retina ($P < 0.01$) (Figure 6B, C), indicating that neutralisation of endogenous VEGF-A further exacerbates neuronal death in this model.

Discussion

VEGF-A has long been identified as a critical survival factor for endothelium [41], but this role has been significantly expanded over recent years to involve other cell types, including those in both the peripheral and central nervous system. In the retina, VEGF-A has been shown to reduce retinal neuron loss [9, 42], findings that have led to discussion about the long-term

neuronal risk of VEGF-A antagonists [43], which are being widely used to treat various ocular conditions [14]. In this study, we have used several approaches to determine the mechanistic basis of VEGF-A's neuroprotective effects, both *in vitro* and *in vivo.* Our findings highlight the need to further define risks that may be associated with inhibition of VEGF-A in ocular conditions.

In our isolated RGC model, the neuroprotective effects of VEGF-A were mediated by VEGFR-2. This finding is consistent with other published data, including *in vitro* models using hypoxia or serum withdrawal [4, 44], oxidative stress [45] and glutamate toxicity [22], plus *in vivo* models, such as optic nerve transection [8]. A number of studies have suggested that neuropilin-1 may be involved in VEGF-A mediated neuroprotection [46], particularly during embryonic development [12], but it has not yet been established in the adult. Given that VEGF164, VEGF120 and VEGF-E had comparable neuroprotective potency in our study, despite the fact that VEGF-E does not bind and VEGF120 interacts very weakly if at all with neuropilin-1, and that the neuropilin ligand PlGF-2 was not neuroprotective, neuropilin-1 may not be necessary for VEGF-A-mediated neuroprotection.

In terms of mechanisms downstream of VEGFR-2, several pathways have been shown to initiate survival in neuronal tissues. Outside the ocular setting, MEK/MAPK/ERK [47], protein kinase A [4], and PI3K/Akt [5], alone or acting together, have been shown to mediate neuroprotection. In the retina, PI3K/Akt alone [9] and dual activation of PI3K/Akt and ERK-1/2 [8] were shown to enhance survival. We showed that H_2O_2 and SSP triggered RGC death *in vitro* that was caspase-dependent and accompanied by DNA fragmentation, and therefore likely due to apoptosis. VEGF-A-mediated cell rescue was prevented by the PI3K inhibitors LY-294,002 and wortmannin, which was validated using wortmannin in the SSP-induced neuronal cell death model *in vivo*. These PI3K inhibitors have been reported to have off-

target effects[48] however, so future experiments could confirm specificity using cell-specific VEGFR-2 phosphorylation site mutant mice, to prevent PI3K signal transduction following VEGFR-2 activation, and also RGC-selective inducible VEGFR2 inactivation model to confirm the role of VEGFR2 in this pathway. Nevertheless, phospho-Akt staining in the experimental glaucoma model corroborated the results from RGC cultures and further strengthens the case for involvement of PI3K/Akt signalling.

Importantly, our studies using the ocular hypertension model of glaucoma demonstrate that VEGF-A signalling is a critical part of the endogenous response to neural damage. Administration of a VEGFR-2 soluble receptor significantly increased the number of TUNEL-positive cells in the GCL during ocular hypertension. We have previously shown that VEGF-A acts as an endogenous neuroprotective factor as part of the adaptive response to acute (1hr) ischemia [9]. In our experimental glaucoma model, there was no change in VEGF-A or VEGF receptor levels, yet the data demonstrate that VEGF-A is required for neuronal survival during a relatively prolonged insult (> 2 week) to retinal neurons. Taken together, these data suggest that VEGF-A may play a constitutive role in RGC neuroprotection. These data are also consistent with those of previous studies, in which VEGF-A depletion via intravitreal or systemic injection of a neutralizing antibody, or adenoviral transfection of soluble VEGFR-1, did not affect normal adult vasculature but did lead to enhanced apoptosis of neurons of the inner and outer retina [9, 42].

Do the data from animal models of acute and chronic retinal disease suggest there is a risk to the human retina exposed to VEGF-A antagonists? At a minimum, our findings suggest that risks to glaucoma patients may need to be more systematically and rigorously assessed. It was recently reported based on full-field electroretinogram results that VEGF neutralization with bevacizumab regressed neovascularisation, but also reduced photoreceptor function in patients with neovascular glaucoma [49]. Furthermore, a study observing 49 patients with AMD

found that in eyes treated with ranibizumab, nerve fibre layer thickness was significantly reduced after one year of treatment, whereas untreated control eyes displayed no change [50]. However, determining the risk profile in glaucoma as well as in the broader retinal disease population is challenging. First, clinical evidence suggests that 25-35% of RGCs must be lost before there is a significant impact on visual acuity [51], so subclinical retinal neuron death could occur in patients being treated with VEGF-A antagonists. Even if loss of visual acuity is noted in patients, this could be attributed to the natural course of diseases such as neovascular age-related macular degeneration, diabetes mellitus and glaucoma [21, 52, 53]. Given the enormous scale of these diseases and potential increasing use of VEGF antagonists in all of them, even a small effect would be very significant.

Lastly, though focused on inherited disease, experimental analyses suggest that the rate of neurodegeneration in rodents could be as much as two orders of magnitude greater than in humans, and is related to maximum lifespan potential [54]. Therefore, short-term rodent experiments may exaggerate the acute risks, and long-term monitoring of patients may be required. Of note is the SEVEN-UP study, a small-scale (63 patients) follow-up study of patients with exudative age-related macular degeneration. Despite initial success in ranibizumab-treated patients in the first 24 months, after 7-8 years of follow-up and intermittent treatment, 37% of eyes had acuities of 20/200 or worse, with many patients exhibiting geographic atrophy [55]. These data are currently the longest available follow-up of patients treated with VEGF-A antagonists, and need to be expanded upon before strong conclusions can be made.

Given the remarkable impact of anti-VEGF strategies on near-term patient outcomes, one strategy for managing a potential trade-off between the positive vascular outcomes and

longer-term neuronal risk may be to develop combination treatments for neovascular conditions that include neuroprotectants. Further elucidation of the details downstream of VEGF-A receptor activation could be critical in the development of a more holistic strategy for preserving the proper function of retinal neurons.

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Figure Legends

Figure 1. Primary RGC cultures express VEGF-A and its receptors. A) Real-time QPCR analysis of VEGFR-2, VEGFR-1 and VEGF-A. Relative expression levels of VEGFR-1 and -2 were significantly elevated following VEGF164 treatment in comparison to control, whereas PlGF had no effect. In contrast, VEGF-A RNA levels were slightly attenuated with VEGF164 supplementation, and enhanced in response to PlGF-1. **P < 0.01, ***P < 0.001, N = 6. Data = mean +/- SD. B) Immunolabelling of VEGFR-2 (green) and βIII-tubulin (red) in RGCs cultured in control medium or with 2.5 nM PlGF-1, PlGF-2, VEGF120, VEGF164 or VEGF- E for 5 days at 63x magnification. VEGFR-2 immunoreactivity increased following VEGF120, VEGF164 or VEGF-E supplementation, with punctate staining observed both perinuclearly and on neurites. In contrast, VEGFR-2 expression did not increase and remained perinuclear in control and PlGF-1 and -2-treated RGCs. Data Scale bar = 10 μm.

Figure 2. VEGF-A protects against apoptotic, caspase-dependent death via VEGFR-2, independent of neuropilins. A) RGCs at DIV 5 were pre-treated for 24 hours with media +/- VEGF120, VEGF164, VEGF-E, PlGF-1 or PlGF-2 (2.5 nM). H_2O_2 (10 μM, left panel) was added to the cells for 5 hours, and SSP (1 μM, right panel) for 24 hours. VEGF164,

VEGF120 and VEGF-E all increased survival of the cultures, whereas neither PlGF-1 nor PlGF-2 prevented RGC death. *P < 0.05, **P < 0.01, ***P < 0.001, N = 5-6. B) Cells were treated with H_2O_2 (left panel) or SSP (right panel) and TUNEL-stained. Percentage of TUNEL-negative, viable RGCs increased following pre-treatment with 2.5 nM and 5.0 nM VEGF120. *P < 0.05, **P < 0.01, N = 8. Data = mean +/- SEM. C) RGCs were incubated with pan-caspase inhibitors Z-VAD-Fmk and Q-VD-Oph prior to H_2O_2 or SSP exposure. Caspase inhibitors largely abolished the toxic response, both independently and combined. *P < 0.05, **P < 0.01, N = 4-8. D) Immunocytochemistry for active caspase-3 (green) showed increased staining in cells treated with H_2O_2 (middle panel, note condensed caspase-3 staining around apoptotic nuclei), which was reduced following VEGF120 pre-treatment (right panel). Cells were counterstained with βIII-tubulin (red) and DAPI (blue). Magnification = 20x. Scale bar = 10 μm.

Figure 3. Neuroprotection by VEGF120 is PI3K/Akt-mediated. A and B) Increasing concentrations of PI3K inhibitors LY294,002 (left panel) and wortmannin (right panel) were added to RGCs immediately prior to VEGF120 pre-treatment, before H_2O_2 (A) or SSP (B) was added to kill the cells. Both LY294,002 and wortmannin dose-dependently abolished the protective effect of VEGF120. *P < 0.05, N = 5-6. Data = mean +/- SEM. C) Immunocytochemistry revealed cytoplasmic expression of phospho-Akt (green) in control cultures, which was reduced following H_2O_2 exposure. In cells rescued with VEGF120, pAkt reactivity was similar to control, and was reduced with LY294,002 treatment. Cells were counterstained with βIII-tubulin (red) and DAPI (blue). Magnification = 63x. Scale bars = 10 μm.

Figure 4. VEGF120 protects against SSP-induced retinal cell death *in vivo* via the PI3K/Akt pathway. A) VEGF120 protects against SSP-induced cell death in the mouse retina. DMSO vehicle did not increase the number of TUNEL positive cells above PBS vehicle control (N = 6-8), whereas SSP elevated apoptotic nucleus counts by approximately 5.5-fold ($P < 0.01$, N = 12). Pretreatment with VEGF120 protected against SSP toxicity, reducing TUNEL-positive cells by 57%. **$P < 0.01$, N = 10. B) Treatment with 1 nmol of the PI3-kinase inhibitor wortmannin reversed VEGF120-mediated neuroprotection ($P < 0.05$; N = 14). Note: PBS/SSP data in this figure was taken from the experiment shown in Figure 2A. Data = mean +/- SEM. C) Representative images of PBS/PBS- (left), PBS/SSP- (middle) and VEGF120/SSP- (right) injected retinas, stained for DAPI (blue) and TUNEL (green; shown with arrows). Magnification = 20x. Scale bar = 50 μm.

Figure 5. VEGF120 protects RGCs against apoptosis in experimental hypertensive glaucoma, with a corresponding increase in phospho-Akt. A) Bead injection triggers a significant increase in IOP, in both PBS- and VEGF120-bead-injected (B) compared to control, non-bead-injected (NB) rat eyes ($P < 0.001$, N = 10). B) VEGF mRNA remained at control levels in bead-injected eyes (N = 4). C) A significant rise in TUNEL-positive nuclei in the GCL was observed for bead-injected eyes with an increase in IOP, but not for bead-injected eyes where pressure did not rise (N = 5-7). *, $P < 0.05$. D) In PBS bead injected eyes (B) apoptotic cell number rose 16-fold ($P < 0.01$; N = 8) above control (NB), but was markedly reduced by 77% in eyes treated with 20 pmol VEGF120 (N = 8). Data = mean +/- SEM. **, $P < 0.01$. E) VEGF120 also protected against optic nerve damage. Extensive damage was found to the optic nerve in histologically stained transverse sections from hypertensive eyes, as determined by toluidine blue (TB) staining of semithin sectioned nerve segments. Approximately 20-fold more degenerating axons were found in animals with high

IOP, from 0.6 ± 0.3 to 12.4 ± 2.8 axons per mm^2 optic nerve ($P < 0.01$). This was reduced by 63% ($P < 0.05$) to 4.6 ± 0.94 axons per mm^2 optic nerve in eyes treated with intravitreal VEGF120 (Figure 5E). Data = mean +/- SEM. *, $P < 0.05$, **, $P < 0.01$. N = 3-5. F) Representative images of control (left), PBS bead-injected (middle) and VEGF120 bead-injected (right) retinae stained for DAPI (blue) and TUNEL (green), showing that VEGF120 treatment reduces TUNEL staining (arrows) to near-control levels. Magnification = 20x. Scale bar = 50 μm. G) Representative images of optic nerve staining, showing an increase in TB accumulation from control (left), in animals with high IOP (middle), which was reduced with VEGF120 administration (right). Arrows denote degenerating axons. Magnification = 63x Scale bars = 50μm. H) Representative images of whole-mount staining for phospho-Akt (pAkt - red). In control (left) eyes pAkt was barely detectable within the GCL, indicating Akt is not constitutively phosphorylated in RGCs. This staining pattern was also observed in PBS bead injected (middle) groups. However in VEGF120 (right) treated eyes, strong pAkt immunoreactivity was observed around Brn-3a (green) positive RGCs, as well as vessels stained with isolectin B4 (white). These images confirm VEGF120 stimulates pAkt signalling, in correlation with its neuroprotective activity. Magnification = 63x Scale bars = 10μm

Figure 6. Anti-VEGF treatment exacerbates neuronal injury in experimental hypertensive glaucoma. A) Bead injection triggers a significant increase in IOP over time, in both IgG- and sVEGFR-2-bead-injected (B) eyes compared to control, non-bead-injected (NB) rat eyes ($P < 0.001$, N = 8). B) sVEGFR-2 initiated a large rise in TUNEL positive cells in the GCL, above contralateral non-bead injected (NB) (N = 6-9) and IgG vehicle (N = 6-7) groups. Endogenous VEGF-A is therefore neuroprotective under conditions where ocular hypertension provokes neuronal damage. **, $P < 0.01$. Data = mean +/- SEM. C)

Representative images of control (left), IgG bead-injected (middle) and sVEGFR-2 bead-injected (right) retinae stained for DAPI (blue) and TUNEL (green; shown with arrows). Magnification = 20x. Scale bar = 50 μm.

Figure 1

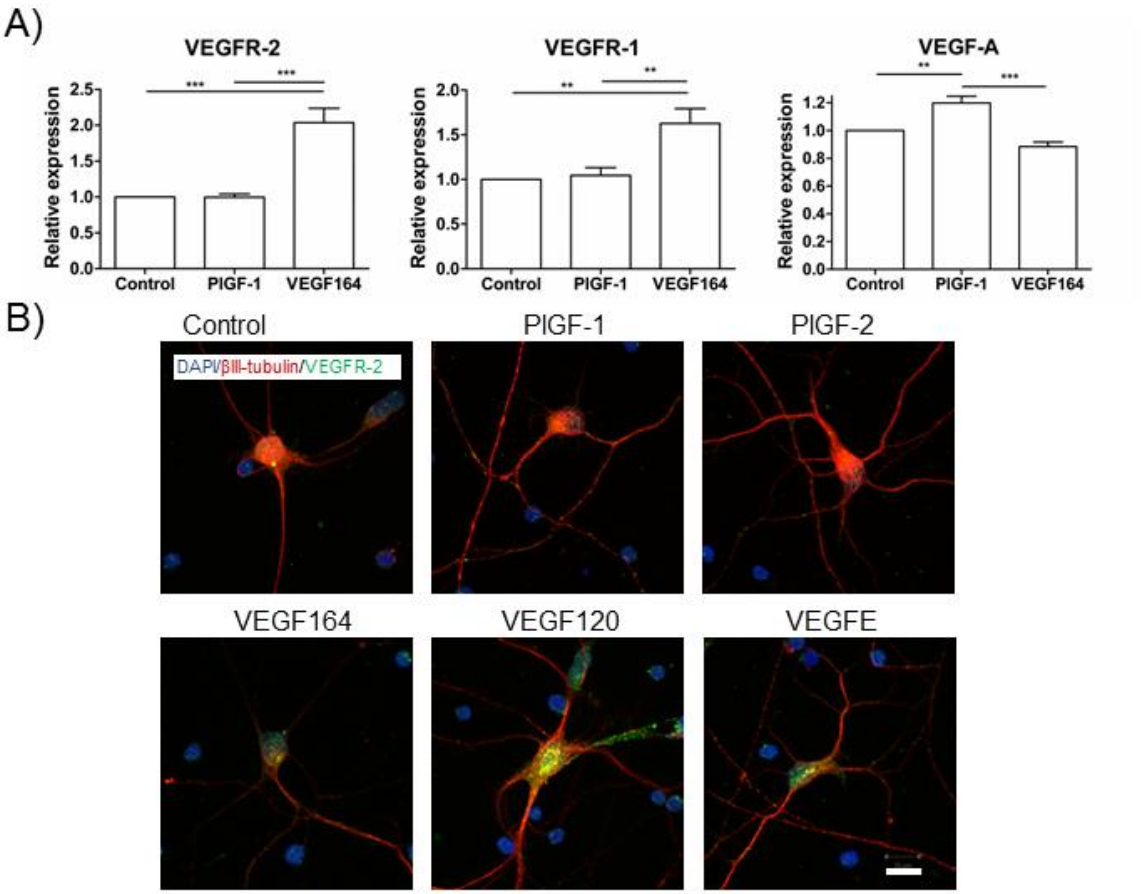

A)
VEGFR-2
VEGFR-1
VEGF-A
Relative expression
Control
PlGF-1
VEGF164
B)
Control
PlGF-1
PlGF-2
DAPI/βIII-tubulin/VEGFR-2
VEGF164
VEGF120
VEGFE

Figure 2

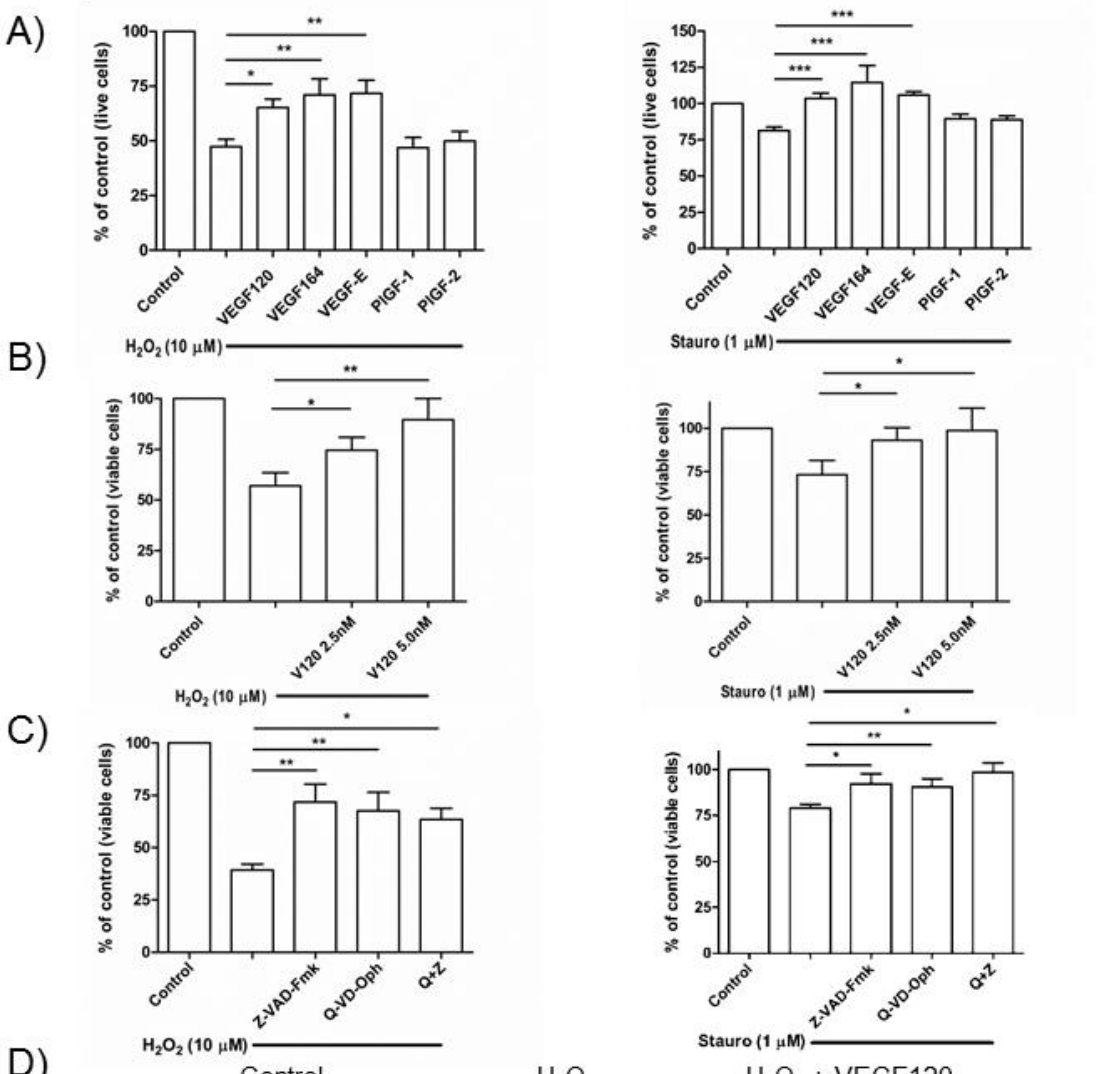

A)
% of control (live cells)
Control
VEGF120
VEGF164
VEGF-E
PlGF-1
PlGF-2
Stauro (1 μM)
B)
H2O2 (10 μM)
% of control (viable cells)
Control
V120 2.5nM
V120 5.0nM
C)
Z-VAD-Fmk
Q-VD-Oph
Q+Z
D)
Control
H2O2
H2O2 + VEGF120
DAPI/βIII-tubulin/
active caspase-3

Figure 3

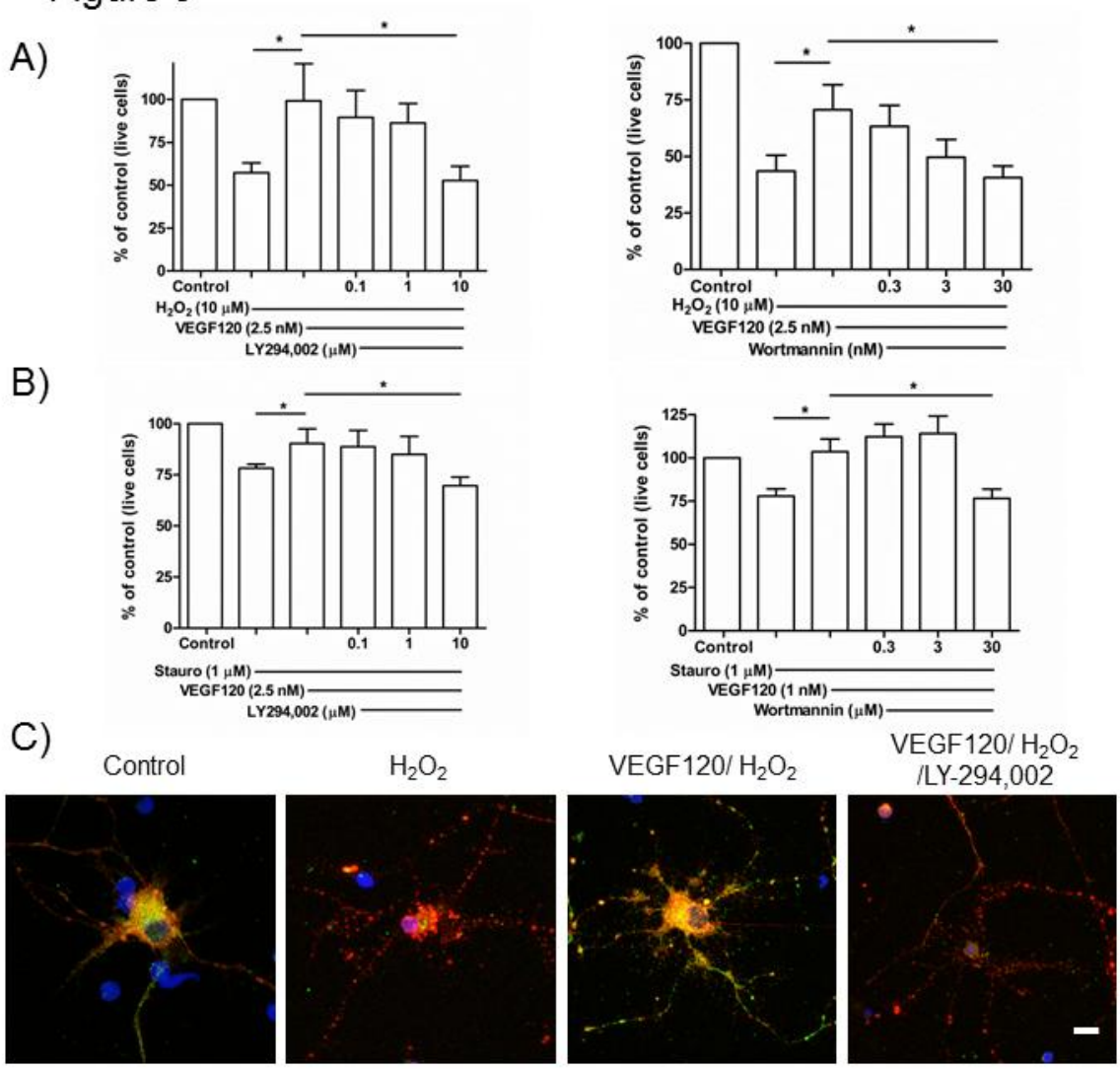

A)
% of control (live cells)
Control
0.1
1
10
H_2O_2 (10 μM)
VEGF120 (2.5 nM)
LY294,002 (μM)
0.3
3
30
Wortmannin (nM)
B)
Stauro (1 μM)
Wortmannin (μM)
C)
Control
H_2O_2
VEGF120/ H_2O_2
VEGF120/ H_2O_2 /LY-294,002

Figure 4

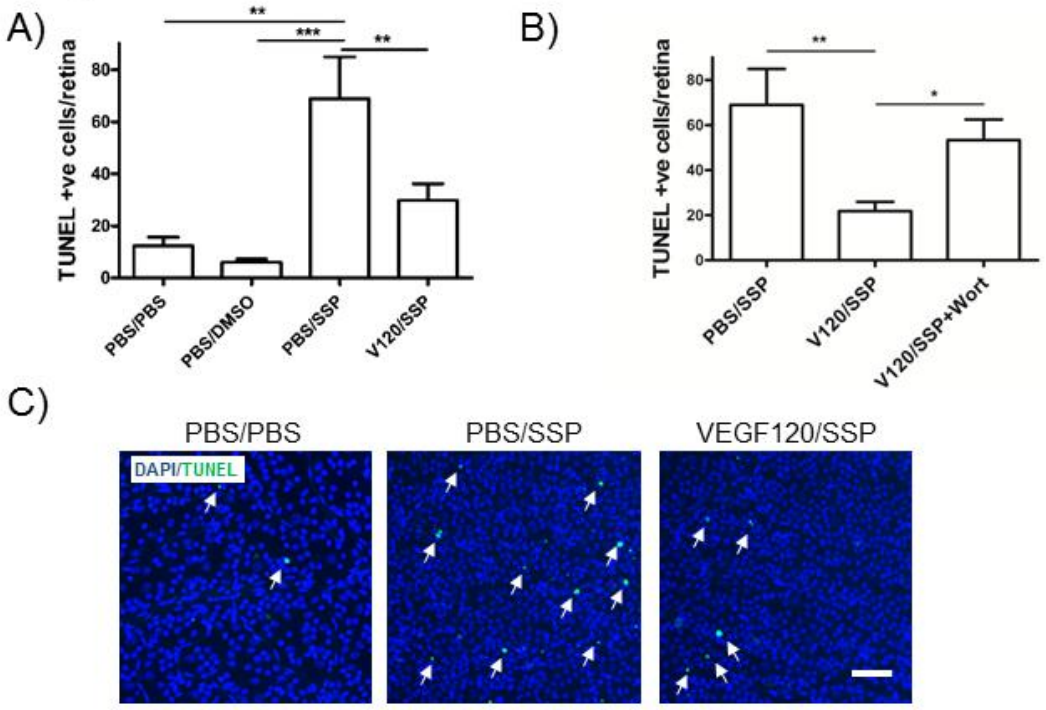

A)
B)
C)
TUNEL +ve cells/retina
PBS/PBS
PBS/DMSO
PBS/SSP
V120/SSP
V120/SSP+Wort
PBS/SSP
VEGF120/SSP
DAPI/TUNEL

Figure 5

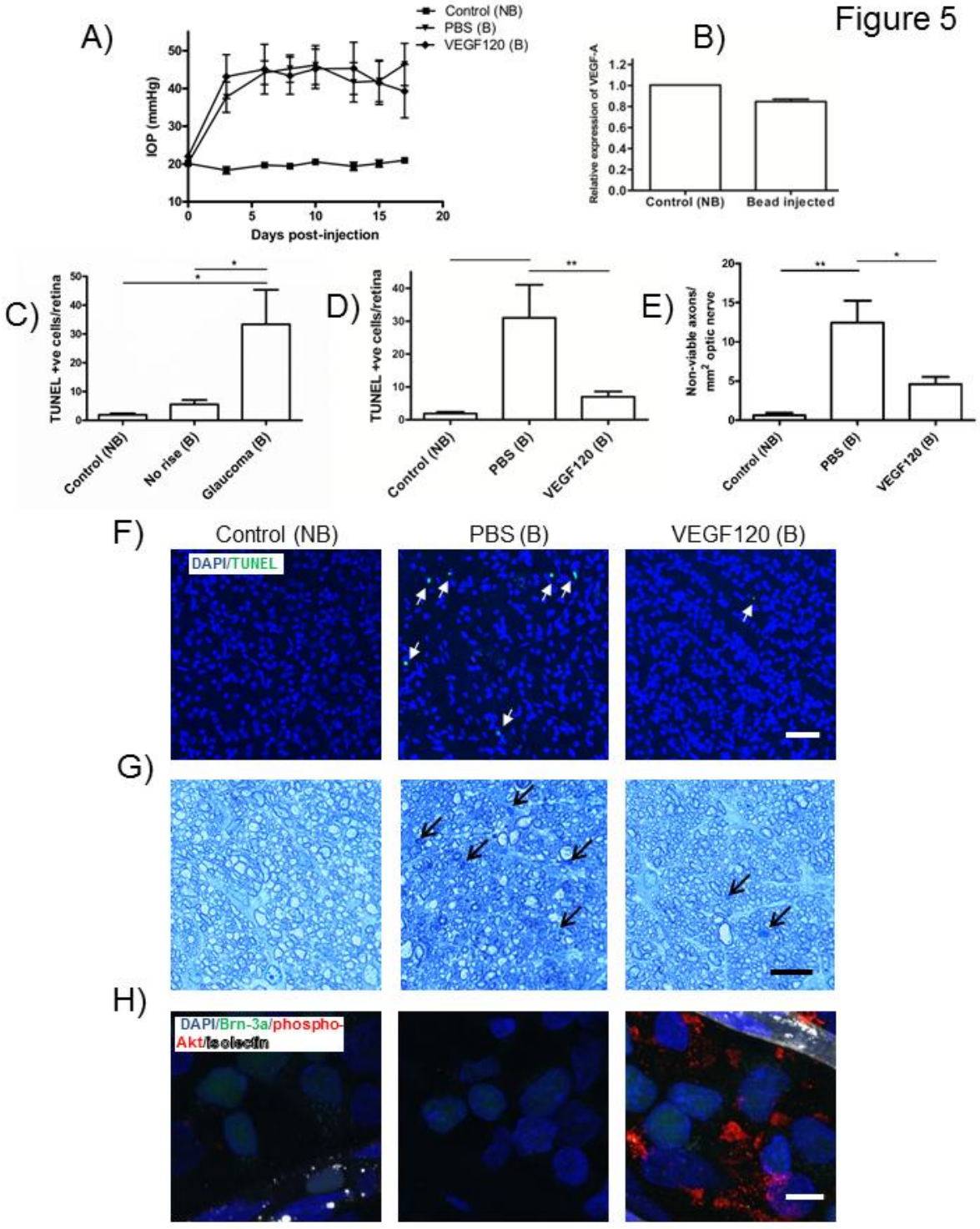
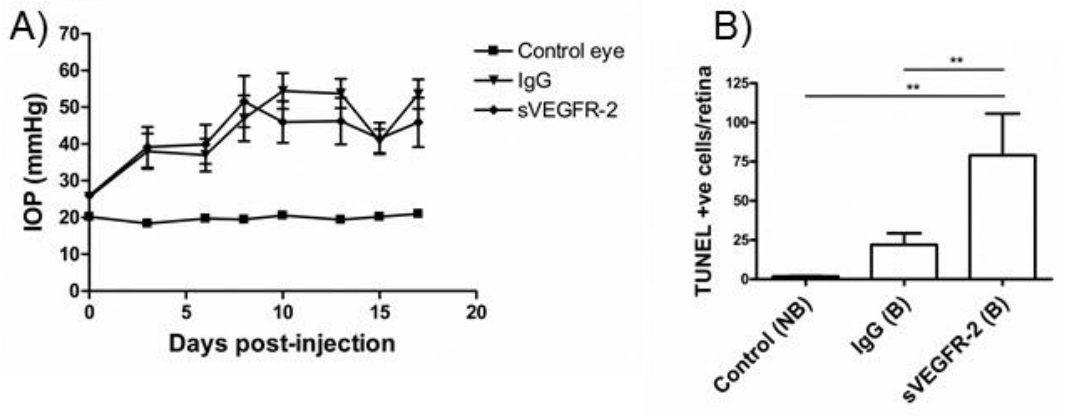
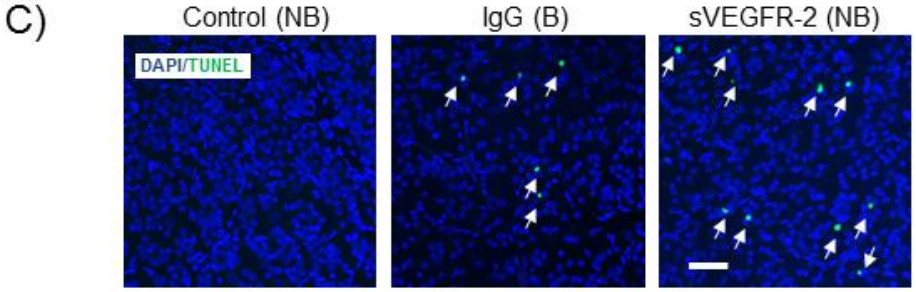

A)
Control (NB)
PBS (B)
VEGF120 (B)
IOP (mmHg)
Days post-injection
B)
Relative expression of VEGF-A
Control (NB)
Bead injected
C)
TUNEL +ve cells/retina
Control (NB)
No rise (B)
Glaucoma (B)
D)
TUNEL +ve cells/retina
Control (NB)
PBS (B)
VEGF120 (B)
E)
Non-viable axons/mm² optic nerve
Control (NB)
PBS (B)
VEGF120 (B)
F)
Control (NB)
PBS (B)
VEGF120 (B)
DAPI/TUNEL
G)
H)
DAPI/Brn-3a/phospho-Akt/Isolectin

Figure 6


A)
IOP (mmHg)
Days post-injection
Control eye
IgG
sVEGFR-2
B)
TUNEL +ve cells/retina
Control (NB)
IgG (B)
sVEGFR-2 (B)
**
**


C)
Control (NB)
IgG (B)
sVEGFR-2 (NB)
DAPI/TUNEL