Connexin43 mimetic peptide reduces vascular leak and retinal ganglion cell death following retinal ischaemia

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Connexin43 gap junction protein is expressed in astrocytes and the vascular endothelium in the central nervous system. It is upregulated following central nervous system injury and is recognized as playing an important role in modulating the extent of damage. Studies that have transiently blocked connexin43 in spinal cord injury and central nervous system epileptic models have reported neuronal rescue. The purpose of this study was to investigate neuronal rescue following retinal ischaemia-reperfusion by transiently blocking connexin43 activity using a connexin43 mimetic peptide. A further aim was to evaluate the effect of transiently blocking connexin43 on vascular permeability as this is known to increase following central nervous system ischaemia. Adult male Wistar rats were exposed to 60 min of retinal ischaemia. Treatment groups consisted of no treatment, connexin43 mimetic peptide and scrambled peptide. Retinas were then evaluated at 1–2, 4, 8 and 24 h, and 7 and 21 days post-ischaemia. Evans blue dye leak from retinal blood vessels was used to assess vascular leakage. Blood vessel integrity was examined using isolectin-B4 labelling. Connexin43 levels and astrocyte activation (glial fibrillary acidic protein) were assessed using immunohistochemistry and western blot analysis. Retinal whole mounts and retinal ganglion cell counts were used to quantify neurodegeneration. An in vitro cell culture model of endothelial cell ischaemia was used to assess the effect of connexin43 mimetic peptide on endothelial cell survival and connexin43 hemichannel opening using propidium iodide dye uptake. We found that retinal ischaemia-reperfusion induced significant vascular leakage and disruption at 1–2, 4 and 24 h following injury with a peak at 4 h. Connexin43 immunoreactivity was significantly increased at 1–2, 4, 8 and 24 h post ischaemia-reperfusion injury co-localizing with activated astrocytes, Muller cells and vascular endothelial cells. Connexin43 mimetic peptide significantly reduced dye leak at 4 and 24 h. In vitro studies on endothelial cells demonstrate that endothelial cell death following hypoxia can be mediated directly by opening of connexin43 hemichannels in endothelial cells. Blocking connexin43 mediated vascular leakage using a connexin43 mimetic peptide led to increased retinal ganglion cell survival at 7 and 21 days to levels of uninjured retinas. Treatment with scrambled peptide did not result in retinal ganglion cell rescue. Pharmacological targeting of connexin43 gap junction protein by transiently blocking gap junction hemichannels following injury provides new opportunities for treatment of central nervous system ischaemia.
Introduction

Connexin43, a ubiquitous CNS gap junction protein expressed in astrocytes and the vascular endothelium, is recognized as playing an important role in modulating the response to CNS injury. Six connexin units form a connexon, or hemichannel, that binds to an analogous structure on a neighbouring cell to form a gap junction. Gap junctions couple astrocytes to form a glial syncytium (Naus et al., 1991; Rouach et al., 2002). Connexin43 gap junctions also contribute to the coupling and coordinated response produced by the endothelial cell network (DePaola et al., 1999; Yeh et al., 2000). Under normal physiological conditions, the glial network is said to be responsible for the spatial buffering of ions and signalling molecules to maintain homeostasis (Nagy and Rash, 2000; Zabs et al., 2003).

Investigations have demonstrated that connexin43 gap junctions are involved in the earliest cellular responses to injury, although the precise role remains controversial. Some studies have reported that connexin43 gap junctional communication is associated with the spread of cell death signals (Garcia-Dorado et al., 2000; Zahs et al., 2003). Under normal physiological conditions, the glial network is responsible for the spatial buffering of ions and signalling molecules to maintain homeostasis (Nagy and Rash, 2000; Zabs et al., 2003).

An increase in connexin43 immunoreactivity levels has been described following stroke (Nakase et al., 2004), brain injury (Hossain et al., 1994; Haupt et al., 2007), traumatic brain injury (Ohsumi et al., 2006) and spinal cord injury (Lee et al., 2005).

Recently, gap junction modulation has been identified as a potential neuroprotective target (Frantseva et al., 2002a, b; Cronin et al., 2008; Danesh-Meyer et al., 2008; O’Carroll et al., 2008; De Bock et al., 2011) with gap junction inhibitors protecting retinal neurosensory cells from ischaemia in a cell culture model (Das et al., 2008). More specific blockers of connexin43 have also been investigated. Transient blocking of connexin43 following injury has been demonstrated to show some neuroprotective effects. In an in vitro interphase organotypic culture model of optic nerve ischaemia, application of connexin43 antisense oligodeoxynucleotide treatment downregulated neuroinflammation (Danesh-Meyer et al., 2008). Application of the connexin43 antisense oligodeoxynucleotide has also been shown to reduce spinal cord swelling and disruption with better behavioural scores in two models of spinal cord injury (Cronin et al., 2008). A different approach has been to use mimetic peptides, small amino acid sequences designed to bind to extracellular regions of the connexin43 protein thereby inhibiting hemichannel opening and gap junction communication. Connexin43 mimetic peptides have proven successful in preventing hemichannel opening-dependent dye uptake and reducing the spread of damage following CNS injury (O’Carroll et al., 2008) and cardiac ischaemia (Hawat et al., 2010), and in preventing vascular leak both in vitro and in vivo after application of the inflammatory peptide bradykinin (De Bock et al., 2011). In an ex vivo model of hippocampal slice cultures induced to demonstrate epileptiform activity, connexin43 mimetic peptide resulted in decreased epileptiform activity (Yoon et al., 2010).

The current investigation set out to assess whether blocking connexin43 channels with a systemically delivered connexin43 mimetic peptide produces neuroprotective effects for retinal ganglion cells in a model of retinal ischaemia-reperfusion. Similar to the brain and spinal cord, connexin43 is expressed on astrocytes in the retinal ganglion cell layer and optic nerve, Muller cells, and endothelial cells in the retinal and choroidal vasculature (Kerr et al., 2010). The retinal ganglion cell death that occurs with retinal ischaemia is implicated in the pathology of conditions such as central retinal artery/vein occlusion, diabetic retinopathy and glaucoma. Retinal ischaemia-reperfusion has been shown to produce an increased vascular permeability of the endothelial cell network at the blood–retinal barrier evident from 4 h (Wilson et al., 1995; Zheng et al., 2007; Abcouwer et al., 2010), with subsequent retinal ganglion cell degeneration (Osborne et al., 2004; Zheng et al., 2007).

We hypothesized that the upregulation of connexin43 that occurs following retinal ischaemia-reperfusion in retinal astrocytes, Muller cells and the vascular endothelium is involved in the induction of neuronal cell death and that application of a connexin43 mimetic peptide blocker would lead to neuronal rescue. Following retinal ischaemia-reperfusion animals were randomized into three groups: no treatment, connexin43 mimetic peptide or scrambled peptide. In addition, in vitro assays were performed on primary rat brain microvascular endothelial cells and human dermal microvascular endothelial cells (HMEC-1) to investigate the effect of connexin43 mimetic peptide on endothelial cell survival after ischaemia. We discovered that retinal ischaemia causes connexin43 mediated vascular leakage that leads to the loss of retinal ganglion cells by Day 21. Blocking connexin43 hemichannels using a connexin43 mimetic peptide rescued retinal ganglion cells with no significant difference to uninjured retinas.

**Keywords:** connexin43; central nervous system ischaemia; retinal ischaemia; retinal ganglion cells; neuroprotection

**Abbreviations:** GFAP = glial fibrillary acidic protein
Materials and methods

Animal model

All procedures were conducted in compliance with the ARVO Statement of Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committee of the University of Auckland. One hundred and fifty adult male Wistar rats weighing 250–300 g were obtained from the Vernon Janson Unit of the University of Auckland and housed with a 12-h light/dark cycle and received food and water ad libitum. Table 1 shows the number of animals used in each experiment.

Connexin43 mimic peptide design

The rat connexin43 mimic peptide design was derived from previous studies (O’Carroll et al., 2008). Connexin43 mimic peptide (sequence VDCFLSRPTEKT) has been shown to block connexin43 gap junction channels following CNS injury (O’Carroll et al., 2008). A scrambled sequence of connexin43 mimic peptide (sequence RFKPSLCTTDEV) was used as a scrambled peptide control. Peptides were synthesized by solid phase using Fmoc chemistries on a Protein Technologies, Symphony® instrument. Peptides were purified by high performance liquid chromatography and the structures confirmed by analytical high performance liquid chromatography and mass spectral analysis.

Table 1 Number of eyes analysed in each experimental group

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Total n = 150.

Retinal ischaemia-reperfusion model and treatment

Animals were anaesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and medetomidine hydrochloride (0.4 mg/kg) and the cornea anaesthetized with oxybuprocaine hydrochloride (0.4%). The technique of retinal ischaemia-reperfusion has previously been described (Sun et al., 2007). Briefly, the left anterior chamber was cannulated with a 30-gauge infusion needle connected by silicone tubing to a reservoir of sterile 0.9% saline. Cannulation was performed using a stereotaxic manipulator arm to avoid injury to the corneal endothelium, iris or lens. The intraocular pressure of the cannulated eye was raised to 120 mmHg for 60 min by elevating the saline reservoir. Retinal ischaemia-reperfusion was confirmed by pallor of the posterior segment. After 60 min, the cannula was removed and reperfusion of the retinal vessels was confirmed by ophthalmoscopy. Three experimental groups were applied at the end of 60 min of ischaemia. These included no treatment, connexin43 mimic peptide, or scrambled peptide. Systemic delivery of 1 ml of a 2-mM connexin43 mimic peptide or scrambled peptide solution diluted in 0.9% saline was achieved through intraperitoneal delivery at the start of reperfusion. A final blood peptide concentration of 100 μM was intended, assuming a blood volume of 20 ml and total systemic uptake of peptide. At various times after reperfusion (1–2, 4, 8 and 24 h, and 7 and 21 days), animals were euthanized with CO2.

Evans blue dye leak

Vascular leak following retinal ischaemia-reperfusion was assessed using Evans blue dye (E2129, Sigma Aldrich), an azo dye that binds to serum albumin and is used to quantify vascular endothelium permeability. It was prepared in 0.9% saline at a concentration of 100 mg/ml and filtered through a 0.22-μm filter prior to administration. The dye solution was injected intraperitoneally at 1 ml/100 g of animal body weight, 10 min before the animal was due to be culled in order to allow absorption of the dye and circulation throughout the body. The dye concentration, volume injected and latent period were based on results from preliminary investigations. Animals were assessed in the control uninjured group, and 4 and 24 h following reperfusion in both treatment groups (connexin43 mimic peptide and no treatment). Animals were culled and eyes enucleated at the respective times. The cornea, lens and vitreous humour were removed, and the remaining retina and sclera fixed in 4% paraformaldehyde in phosphate-buffered saline (BR0014G, Oxoid) for 30 min at room temperature. The fixed retina was carefully removed from the sclera and mounted onto a SuperFrost® Plus slide (Menzel-Glaser) using Citifluor® mounting medium, before imaging.

In vitro assays

In order to establish the mechanism by which vascular leak was occurring and the link with connexin43 expression, an in vitro endothelial cell assay was used with a modified protocol from Zhou et al. (2010). Rat brain microvascular endothelial cells (R840K-05a, Cell Applications) were plated into 24-well plates (1 × 10^5 cells/well) in rat brain microvascular endothelial cell growth media (R819K-500, Cell Applications) and allowed to settle for 16 h. Medium was then removed and replaced with Dulbecco’s Modified Eagle’s Medium/F12 containing 0.5% foetal bovine serum and 1% glutamine. Hypoxia was induced by placing cells in a pre-warmed Billups–Rothenburg Modular Incubator Chamber and flushing with 95% N2, 5% CO2 for 5 min
(201/min). The chamber was placed in a 37°C incubator for 3 h. The chamber was regassed with 95% N₂, 5% CO₂ after the first hour to ensure removal of all the gas that may have been trapped in the plasticware. Following hypoxia, medium was removed and replaced with rat brain microvascular endothelial cell growth media containing either H₂O (no treatment), 100 µM carbeneoxolone (non-specific gap junction channel blocker), 200 µM lanthanum chloride (LaCl₃, non-specific hemichannel blocker), 50 µM connexin43 mimetic peptide or 50 µM scrambled peptide. Cells were incubated at 37°C, 5% CO₂ for 6 h. Cells were then trypsinized, resuspended in medium and the cell suspension mixed with 0.4% Trypan Blue in order to count the number of viable cells. Cells that had not been exposed to low serum and hypoxia were counted as controls. Viable cell counts were expressed as a percentage of control. Six experiments were performed with three wells per treatment in each experiment. Data from three wells were averaged to obtain the average cell count in each treatment, which was then analysed. Human microvascular endothelial cells (plated at 1.3 × 10⁵ cells/well) and subjected to hypoxia-reperfusion as described above. Following 1 h of reperfusion, propidium iodide was added to a final concentration of 20 µg/ml. After 30 min, the cells were washed well to remove all propidium iodide from the media and fixed with 10% paraformaldehyde for 5 min. Cells were then imaged on a Leica DM IRB microscope and Leica DFC 425C camera. Six wells were analysed for each experimental group. Four images were taken from every well at ×10 magnification and the number of propidium iodide-positive cells counted. As fixation of the cells alone led to a low level of propidium iodide uptake, normal cells were treated with propidium iodide for the same period and fixed. These were imaged and used to set a threshold so that only cells with a propidium iodide uptake above this level were counted as propidium iodide-positive. A bright field image was taken of each field to determine the total number of cells. The level of propidium iodide uptake was calculated as the number of cells containing propidium iodide as a percentage of the total (% cells with propidium iodide).

**Immunohistochemistry**

Immunohistochemical techniques were utilized to investigate the effects of ischaemia-reperfusion on connexin43 and GFAP expression and for retinal ganglion cell analysis. Retinas that had one or more quadrants damaged during processing were discarded. For analysis of connexin43 and GFAP, animals were used for each of the following time points after ischaemia-reperfusion and no treatment: 1–2, 4, 8 and 24 h. Retinal whole mounts were used to map retinal ganglion cell counts at 7 and 21 days after ischaemia-reperfusion and no treatment, connexin43 mimetic peptide, or scrambled peptide. Vascular integrity was examined at 4 h after ischaemia-reperfusion and no treatment. Co-localization of connexin43 and vascular endothelial cells was examined in uninjured and ischaemic retinas 8 h after ischaemia-reperfusion. After euthanasia, eyes were enucleated and the cornea, lens and vitreous humour removed. The dorsal aspect of the retina was notched to retain orientation. The retina and sclera were fixed in 4% paraformaldehyde in phosphate-buffered saline for 60 min at room temperature. The retina was then carefully removed from the sclera and permeabilized via incubation in 0.5% Triton® X-100 in phosphate-buffered saline solution for 15 min at −80°C. Following thorough washing with phosphate-buffered saline, free floating retinas were incubated overnight at 4°C in a primary antibody solution, using rabbit anti-connexin43 (C6219, Sigma Aldrich, 1:2000) and mouse anti-GFAP conjugated to Cy3 (GFAP—C9205, Sigma Aldrich, 1:1000) primary antibodies in 10% normal goat serum and 2% Triton® X-100 in phosphate-buffered saline solution to label connexin43, astrocytes and Muller cells. Primary antibody solution containing rabbit anti-connexin43 and isoelectin-B4 conjugated to Alexa594 (I-21413, Molecular Probes, 1:100) was used to double label connexin43 and vascular endothelial cells. Labelling of retinal ganglion cells was achieved with goat anti-Bn3a primary antibody (SC-31984, Santa-Cruz Biotechnology, 1:100) in 2% horse serum and 2% Triton® X-100 in phosphate-buffered saline solution. Vascular integrity was examined by labelling vascular endothelial cells using isoelectin-B4 conjugated to Alexa594. After further washing of the retinas with phosphate-buffered saline, a secondary antibody solution was applied for 2 h at room temperature. A goat anti-rabbit Alexa488 secondary antibody (A11034, Invitrogen, 1:1000) labelled the connexin43 primary antibody while a donkey anti-goat Cy3 secondary antibody (705-165-147, Jackson Immuno Research, 1:500) labelled the Bn3a primary antibody. Retinas were incubated in the nuclear marker DAPI (4',6-diamidino-2-phenylindole) (D9542, Sigma Aldrich) before being mounted onto SuperFrost® Plus slides using Citifluor™ mounting medium, and imaged.

**Imaging and quantification**

Specimens were examined with an Olympus FV1000 confocal laser scanning microscope. For dye leak experiments, the entire retina was imaged using a ×10 air objective. Absorbance and emission spectra for Evans blue dye have been previously characterized (Saria and Lundberg, 1983). Here, 559 nm excitation was used and emission was viewed with a 575- to 675-nm band filter. Dye leak was consistently spherical or ovoid in shape and the area of dye leak was thus measured using the formula:

\[
\text{Area of dye} = r_1 \times r_2 \times \pi,
\]

where \(r_1\) and \(r_2\) are dye leak radii in the \(x\) and \(y\) axes. Total accumulated dye leak from vessels was measured as the sum of individual dye leak areas.

For connexin43, astrocytosis and retinal ganglion cell density immunohistochemical analysis, two fields in each quadrant of each retina were imaged giving a total of eight images per retina. This method insured similar locations were assessed between different eyes and avoided any possible area bias present in the retina. Connexin43 and GFAP label was imaged using a ×60 oil immersion objective lens and retinal ganglion cell labelling was imaged at ×10 magnification. Voltage and offset settings were adjusted to best discriminate individual antibody labelling and to avoid oversaturation of the image. Quantification was performed using automated spot counts in NIH ImageJ software. For connexin43 quantification, each image was converted to a binary image using a threshold of 30. To separate clusters the watershed algorithm was applied. A particle count was then performed to determine the connexin43 spot count per image. For retinal ganglion cell quantification each image was first filtered with a sharpen filter to delineate cell edges before being converted to a binary image using a threshold of 33. Spots of 1 or 2 pixel² that
resulted from noise and artefacts that were clearly not retinal ganglion cell of origin were excluded during particle counts. Retinal ganglion cell density was calculated as the number of retinal ganglion cells per mm².

**Western blotting**

Western blotting was used to determine changes in GFAP expression 8 h after ischaemia-reperfusion injury. Treatment groups included no treatment, systemic connexin43 mimetic peptide perfusion, or scrambled peptide perfusion. Retinas from uninjured animals were used as controls. Two animals were included in each treatment group. The 8-h time point was chosen to coincide with maximum connexin43 upregulation. After euthanasia, eyes were enucleated and the cornea, lens and vitreous humour removed. Retinas were immediately frozen in liquid nitrogen and stored at −20°C until homogenization. Each retina was cut into pieces using iris scissors and placed into 200 µl ice-cold homogenization buffer (150 mM sucrose, 50 mM HEPES pH 7.9, 60 mM KCl, 5 mM EDTA pH 8.0, 1 mM EGTA pH 8.0) containing one complete mini protease inhibitor cocktail tablet (04 693 124 001, Roche) per 10 ml. Tissue was then homogenized using a hand-held homogenizer (5 x 75 mm probe, Pro Scientific Pro 200) for 90 s while being kept on ice. Samples were incubated on ice for 1 h with 1% Triton X-100, centrifuged at 10 000 rpm for 10 min and the supernatant collected for protein concentration assay.

Sample protein concentrations were determined using the Biorad RC DC™ Protein Assay Kit II (500-0122) following kit instructions. Samples were diluted with homogenization buffer to a standard concentration of 1 µg/µl for western blotting. A 10-µl sample in 10 µl of sample loading dye, and 10 µl of BenchMark™ pre-stained protein ladder (10748-010, Invitrogen) were run on a 10% separating bis-acrylamide gel with 4% stacking gel for 50 min at 170 mA. The membrane was blocked with 5% non-fat milk powder in TBS-T (containing 20 mM Tris, 137 mM NaCl, glycine, and 20% v/v methanol). The membrane was blocked with 5% non-fat milk powder in TBS-T (containing 20 mM Tris, 137 mM NaCl, 0.1% Tween-20) for 1 h and thoroughly washed (six times for 5 min each in TBS-T). All membranes were labelled with a cocktail of rabbit anti-GFAP antibody (Z0334, Dako, 1:1000) and anti-GAPDH antibody (G9545, Sigma Aldrich, 1:4000) in antibody solution (TBS-T with 2 mM EDTA pH 8.0 and 1% bovine serum albumin) overnight, thoroughly washed, followed by anti-rabbit Ig horse radish peroxidase linked whole antibody (NA934V, Amersham Biosciences, 1:40 000) in antibody solution, then thoroughly washed again. The signal was detected using an Amersham ECL Plus™ Western Blotting Detection System (RPN2132, GE Healthcare) and a Fujifilm LAS 3000 Imager with the chemiluminescence function. A visible light photo of the protein ladder was also taken without moving the membrane.

Equal loading was controlled for by the amount of a house keeping protein GAPDH. The images taken were then analysed with ImageJ. The integrated intensity of connexin43 bands and GAPDH bands was measured, and a relative integrated intensity calculated by dividing the integrated intensity of connexin43 by GAPDH. The average relative integrated intensity of two samples ± SEM in each treatment group from one western blot run was plotted and presented along with four sample lanes. Western blotting of the same samples was repeated four times to confirm consistency of results.

**Statistical analysis**

Data are given as arithmetic means ± standard error. Statistical comparisons between groups were performed using the Mann-Whitney U test (also known as the Wilcoxon test) since data were not normally distributed and normality cannot be restored by transformation. The difference was considered significant when $P < 0.05$. All statistical analysis was performed in IBM SPSS Statistics 19.

**Results**

**Treatment with connexin43 gap junction channel blocker limits vascular leakage**

Following retinal ischaemia-reperfusion, extravascular dye leakage was observed within an hour of reperfusion, peaked at 4-h post-ischaemia but continued at 24 h, the longest reperfusion period analysed (Fig. 1). The dye appeared in ovoid patches where it had leaked from compromised vascular endothelium. Retinas from animals that had no Evans blue dye injected were examined to ensure that there was no background fluorescence. Uninjured animals that were injected with Evans blue dye did not exhibit dye leak from the retinal vasculature beyond a few patches resulting from handling, but the vessels were clearly demarcated by the dye within them. Isolectin-B4 labelling of retinal vascular endothelial cells showed tubular and continuous vessels forming an anastomosing vascular network (Fig. 1E). By 4 h following ischaemia-reperfusion, isolectin-B4 labelling revealed clumped vascular membrane material, single endothelial cell layers (as opposed to double track or tube-like structures) and label breaks, indicating endothelial cell loss and vessel rupture (Fig. 1F).

Quantification of dye leak showed that the total accumulated area of leaked dye per retina following ischaemia-reperfusion was significantly greater than in uninjured tissue (10 567 ± 2615 µm²) at 1–2 h (87 975 ± 30 675 µm²) and 24 h (601 671 ± 234 163 µm²) and 24 h reperfusion periods (410 143 ± 81 323 µm²) that received no treatment (Fig. 2). Systemically delivered connexin43 mimetic peptide significantly reduced the total accumulated area of leaked dye at 4 h (86 280 ± 20 099 µm²) and 24 h (26 671 ± 5655 µm²) post-ischaemia compared with no treatment at the respective time points. While the total accumulated area of dye leak at 4 h after connexin43 mimetic peptide treatment was still elevated compared to uninjured control animals, it reached only 14% of that in the untreated, ischaemic eyes.

**Endothelial cell death and dye uptake secondary to hypoxia is minimized with connexin43 mimetic peptide in vitro**

In order to investigate mechanisms contributing to vascular leak, rat brain microvascular endothelial cells in vitro were exposed to 3 h hypoxia followed by 6 h reperfusion in culture medium containing connexin43 mimetic peptide, scrambled peptide, carbenebolone, LaCl₃, or no treatment. Following hypoxia and
no treatment or scrambled peptide, ~25% of the endothelial cells died compared to control (100 ± 2.9%; no treatment 77.0 ± 4.2% survival; scrambled peptide 76.7 ± 4.3% survival; P < 0.05) (Fig. 3A) as assessed by viable cell counts. Addition of either the non-specific gap junction channel blocker carbenoxolone (95.0 ± 1.4%), the hemichannel blocker LaCl₃ (95.8 ± 2.0%) or 50 μM connexin43 mimic peptide (95.6 ± 2.6%) prevented cell death (P < 0.05) when compared

Figure 1  Confocal microscope optical slices of flat mounted retinas displaying Evans blue dye fluorescence (A–D) and isolectin-B4 labelled blood vessel endothelial cells (E and F). No endogenous fluorescence was seen in the retina from an animal with no Evans blue dye administered (A). Normal blood vessels from an uninjured animal infused with Evans blue dye are seen in (B), where the vessels are demarcated by the dye but there is no leakage to the extracellular space. Dye leak from blood vessels (arrows), indicating vascular disruption, was evident at both 4 (C) and 24 h (D) following retinal ischaemia-reperfusion. Blood vessel endothelial cell labelling with isolectin-B4 from an uninjured animal is seen in E. The vessels appear tubular and continuous. At 4 h following ischaemia-reperfusion (F) vessels appear fragmented with breaks (arrow), many single membrane structures (arrowhead) and endothelial membrane aggregations (asterisk). Scale bar = 500 μm.
to no treatment. In addition, human microvascular endothelial cells were also analysed in a similar manner. Following hypoxia and no treatment or scrambled peptide, ~30% of the endothelial cells died compared to control (100 ± 1.5%; no treatment

70.9 ± 4.3% survival; scrambled peptide 71.8 ± 6.0% survival; P < 0.05) as assessed by viable cell counts. Addition of either carbenoxolone (97.0 ± 3.6%), LaCl₃ (93.1 ± 4.1%) or 50 μM connexin43 mimetic peptide (93.9 ± 4.8%) prevented cell death (P < 0.05) when compared with no treatment.

Hypoxia resulted in connexin43 hemichannel-mediated propidium iodide dye uptake in 19.9 ± 5.0% cells with no treatment or 15.1 ± 2.5% with scrambled peptide (Fig. 3B). Treatment with either carbenoxolone (1.1 ± 0.6%), LaCl₃ (1.6 ± 0.6%) or 50 μM connexin43 mimetic peptide (2.0 ± 0.6%) prevented dye uptake (P < 0.05) when compared to no treatment.

Connexin43 and glial fibrillary acidic protein response

Quantitative and qualitative changes were observed in connexin43 and GFAP protein expression following retinal ischaemia-reperfusion. Four hours after ischaemia-reperfusion, connexin43 was significantly upregulated in the retina, and co-localized with GFAP positive astrocytes (GFAP also labels Muller cells) and isolectin-B4-positive vascular endothelial cells (Fig. 4). Mean connexin43 spot counts were significantly increased (P < 0.05) in the retina at 1–2 h (1854 ± 140), 4 h (3484 ± 224), 8 h (3536 ± 309) and 24 h (3353 ± 320) after ischaemia-reperfusion compared to uninjured retinas (1443 ± 122) (Fig. 5). Since astrocytes wrap around vessels with foot processes onto endothelial cells, blood vessels were clearly outlined with GFAP labelling (Fig. 6).

Ischaemia-reperfusion caused patches of localized GFAP upregulation and astrocyte disorganization adjacent to blood vessels within 1 h. In those areas where the vascular endothelium had been disrupted, astrocytic foot processes had lost their normal organized
Figure 4 Confocal microscope images of flat mounted retinas labelled for connexin43 (green), GFAP (red, A–C) and isolectin-B4 (red, D and E). An uninjured retina with connexin43 labelling co-localized with astrocyte processes is seen in A. Four hours after ischaemia-reperfusion, connexin43 was significantly upregulated in the retina, and co-localized to activated astrocytes that appeared hypertrophied and expressed increased levels of GFAP (B). A higher magnification image with connexin43 and GFAP co-localization (yellow spots) is shown in (C). Connexin43 labelling (green) was also co-localized with blood vessel endothelial cells (red) in uninjured retina (D) and increased in ischaemic retina (E) 8 h after ischaemia-reperfusion. Scale bar = 40 μm.

Figure 5 Average connexin43 spot counts in uninjured control retinas and following ischaemia-reperfusion. Connexin43 spot counts were significantly increased in retinas following ischaemia-reperfusion injury at 1–2, 4, 8 and 24 h compared to uninjured control animals. Stars denote statistical significance when compared to the control group; $P < 0.05$. 
appearance and the processes appeared within the damaged vessel lumen (seen by sequentially stepping through the confocal z-stack shown in Fig. 6D). Astrocyte disorganization in localized regions adjacent to blood vessels in the retinal tissue was still evident 24 h after reperfusion. These localized areas of astrocytosis appeared similar in distribution to the areas of vessel leak observed after Evans blue dye perfusion and correlated with significant upregulation in connexin43 expression. Western blot analysis 8 h...
Connexin43 mimic peptide treatment results in retinal ganglion cell rescue

Following ischaemia-reperfusion, retinal ganglion cell density (number of cells per mm²) was significantly reduced at both 7 and 21 days in animals that received no treatment (1985 ± 130 and 1971 ± 139, respectively) or treatment with a scrambled peptide (1820 ± 97 and 2094 ± 90, respectively) compared to uninjured controls (3035 ± 258) (Figs 8 and 9). The normal distribution of retinal ganglion cells in flat whole mounts of uninjured retinas is seen in Fig. 8A. Figure 8B shows the retinal ganglion cell distribution in a retina 7 days after ischaemia-reperfusion. Figure 8C and D show the typical appearance of a retina 7 days of animals treated with connexin43 mimic peptide and scrambled peptide, respectively. The retinal ganglion cell densities are plotted against time in Fig. 9. Animals treated with a single injection of connexin43 mimic peptide immediately following ischaemia-reperfusion showed no significant reduction in retinal ganglion cell density at 7 days (2775 ± 158) and 21 days (2611 ± 158) compared to uninjured retinas (Fig. 9).

Discussion

This study provides new information on the pathology of and potential treatment strategies for retinal ischaemia, which may be extrapolated more broadly to CNS ischaemia, chronic inflammatory diseases and trauma. We observed vascular leak peaking at 4-h post ischaemia-reperfusion, which correlated with discrete, localized regions of astrocytosis (and potentially Müller cell activation) and associated connexin43 upregulation. Importantly, this vessel leak was reduced with systemic delivery of connexin43 mimic peptide. Astrocytosis (as determined by GFAP levels) was similarly attenuated. Furthermore, retinal ganglion cell loss seen at 7- and 21-day post ischaemia-reperfusion was significantly reduced by a single intraperitoneal injection of connexin43 mimic peptide given immediately after the ischaemic event. We would expect the severity and duration of the insult to affect the number of retinal ganglion cells lost and in our model this was ~35% following 1 h ischaemia. With systemic mimic delivery, this loss was reduced by two-thirds (down to 9–14%). We suggest that the upregulation of connexin43 immunoreactivity that occurs following ischaemic injury is responsible for the increased vascular leakage and subsequently the inflammatory cascade leading to retinal ganglion cell death. Blocking connexin43 hemichannels has a significant neuroprotective effect.

It is well recognized that the retina, like the brain and spinal cord, responds to ischaemia-reperfusion with both neurodegeneration and increased vascular permeability (Wilson et al., 1995; Zheng et al., 2007; Abcouwer et al., 2010). In a study similar to ours, in which rat retinas were subjected to 45 min of retinal ischaemia followed by reperfusion, Evans blue dye leak from the retinal vasculature was evident at 4 h but continued to 48 h (Abcouwer et al., 2010). An MRI study found that vascular leakage after 60 min of retinal ischaemia lasted for up to 57 days (Wilson et al., 1995). Retinal capillaries were found to start to degenerate, shown with TUNEL (terminal dUTP nick end labelling) cell death labelling, from Day 2, and this became much more pronounced at Day 7 (Zheng et al., 2007). Vascular leakage was also a common feature in studies following hypoxia induced injury to the retina (Kaur et al., 2007, 2008). In the present study, vascular integrity of retinal vessels was shown to be compromised as early as 1 h following ischaemia-reperfusion and continued to the 24-h time point. Administration of connexin43 mimic peptide markedly reduced the total accumulated amount of leaked at the 4- and 24-h time points.

In the retina and the CNS, connexin43 coupled astrocyte end-foot processes have an intimate relationship with the vasculature surrounding each blood vessel to form a comprehensive gliovascular interface (Kim et al., 2006; Kaur et al., 2008; Kerr et al., 2010). Connexin43 expression on astrocytes surrounding the vasculature may play a role in controlling blood vessel permeability (Simard et al., 2003). One possible mechanism is through...
Figure 8  Confocal microscope single optical slice images of flat mounted retinas with Brn3a labelled retinal ganglion cells. In each case, images shown were taken from the mid-periphery of the retina. An uninjured retina is seen with densely packed retinal ganglion cells (A). An ischaemic retina 7 days after ischaemia-reperfusion had a reduced retinal ganglion cell density (B). Systemic delivery of connexin43 mimetic peptide (C) has maintained retinal ganglion cell density at 7 days at almost the same level as uninjured retinas. Scrambled peptide treatment was not neuroprotective and a reduced retinal ganglion cell density was apparent (D), with a retinal ganglion cell pattern being similar to that in the ischaemic untreated control (B). Scale bar = 1000 µm.

Figure 9  Ischaemia-reperfusion injury lead to a significant loss of retinal ganglion cells (RGC) in the retina at both 7 and 21 days compared to uninjured control. Connexin43 (Cx43) mimetic peptide had a significant rescue effect compared to no treatment at both times whereas scrambled peptide did not. Stars denote statistical significance when compared to the control group or compared between groups in brackets; $P < 0.05$. 
astrocytic propagation of calcium waves. Calcium signalling in astrocytes at the blood-retinal barrier is involved in local blood flow regulation and metabolic trafficking (Cornell-Bell et al., 1990; Simard et al., 2003). Calcium waves propagate in the astrocyte-endothelial network via two recognized pathways: inositol trisphosphate spread through connexin43-containing gap junctions between astrocytes and endothelial cells or extracellular ATP spread that may also be facilitated by hemichannel release of ATP (Braet et al., 2001).

Connexin43 expression on endothelial cells (Farahani et al., 2005; Kerr et al., 2010) may, however, independently play an important role in the permeability of blood vessels. Elevation of connexin43 in the walls of small blood vessels has been associated with vascular leakage and extravasation of blood-borne neutrophils within 6 h of a traumatic spinal cord injury (Cronin et al., 2008). Suppression of the connexin43 upregulation using an antisense oligodeoxynucleotide significantly reduced leak of labelled bovine serum albumin and invasion of neutrophils from the blood to the injury site (Cronin et al., 2008). Using an in vitro cell ischaemia model, we have demonstrated here that cell death can be mediated directly by opening connexin43 hemichannels in endothelial cells, occurring in the absence of astrocytes. The effect is not species specific. Endothelial cell death was prevented in endothelial cells of both human and rat origin using both non-specific gap junction and hemichannel blockers and specifically the connexin43 mimetic peptide at a concentration that blocks hemichannels but does not uncouple gap junctions (O’Carroll et al., 2008). Ischaemia induced propidium iodide dye uptake was prevented by connexin43 mimetic peptide, indicating the efficacy of the peptide in blocking connexin43 hemichannels. It is probable that in vivo endothelial cell death and vessel leak may also occur independently of astrocytosis. Conversely, vascular leak appears to trigger astrocytosis. We identified localized patches of connexin43 upregulation associated with abnormal GFAP expression during the same 1–24 h reperfusion periods following ischaemia, and suggest a causal link between vascular dysfunction and the glial inflammatory response.

A number of factors are said to contribute to blood–brain barrier permeability after hypoxia, in particular tight junction disruption (reviewed in Ballabh et al., 2004; Kaur and Ling, 2008; Yang and Rosenberg, 2011). However, connexin43 hemichannel effects on cytosolic Ca$^{2+}$ concentration and cell volume regulation after ischaemia in a number of cell types signal their vital role in cell injury (reviewed in Rodriguez-Sinovas et al., 2007). Expression of connexin43 in cells results in cell swelling and lysis when the extracellular Ca$^{2+}$ concentration is lowered, a condition promoting hemichannel opening (Rodriguez-Sinovas et al., 2007) and conversely, downregulating connexin43 expression after injury reduces the number of connexin43 hemichannels in the membrane leading to a significant reduction in cell swelling (Cronin et al., 2008; O’Carroll et al., 2008; Zhang et al., 2010). In the vascular endothelium, hypoxia leads directly to endothelial cell loss (Petito, 1979; Danesh-Meyer et al., 2008). We have shown here propidium iodide uptake in endothelial cells and subsequent cell death. Recently, De Bock et al. (2011) demonstrated that connexin hemichannel opening leads to blood–brain barrier permeability after a bradykinin-induced inflammatory response.

Bradykinin-induced endothelial cell calcium oscillations were blocked both in vitro by connexin-specific mimetic peptides, and in vivo after systemic peptide delivery. Although they postulate that spatially restricted calcium changes adjacent to tight junctions might be occurring, immunohistochemical localization of occludin and ZO-1 proteins did not reveal tight junction reorganization as the cause of vascular permeability. Regardless, once the blood–brain barrier is breached, astrocytes will become activated (Dietrich et al., 1994; Hirano et al., 1994; Kaur and Ling, 2008) and T cell and monocyte migration augments the inflammatory reaction leading to further tissue damage (Pachter et al., 2003).

Death of retinal ganglion cells following ischaemia-reperfusion is thought to be a result of a complex cascade of events involving glutamate excitotoxicity and inflammatory mediator release (Osborne et al., 2004). Retinal ganglion cells express high levels of kainate and N-methyl-D-aspartate glutamate receptors (Brandstatter et al., 1994; Fletcher et al., 2000; Lin et al., 2002), making them especially susceptible to glutamate excitotoxicity (Brandstatter et al., 1994). The disrupted uptake of glutamate into astrocytes following retinal ischaemia-reperfusion may thus play a part in mediating retinal ganglion cell death (Osborne et al., 2004). Nitric oxide is another important neuromediator in the CNS, with increased production by three forms of nitric oxide synthase, endothelial nitric oxide synthase, (Ju et al., 2001; Cheon et al., 2003), neuronal nitric oxide synthase (Gwon et al., 2001; Cheon et al., 2002) and inducible nitric oxide synthase (Neufeld et al., 2002) following retinal ischaemia-reperfusion injury. Although some studies reported a retinal ganglion cell rescue effect due to expression of nitric oxide synthase, the majority of studies found that expression of nitric oxide synthase exacerbates retinal ganglion cell loss, and that inhibition of nitric oxide synthase was protective for retinal ganglion cells following retinal ischaemia-reperfusion (Geyer et al., 1995; Lam and Tso, 1996; Adachi et al., 1998; Ju et al., 2000; Neufeld et al., 2002). Inducible nitric oxide synthase in particular is produced by resident glial cells disrupted by ischaemia-reperfusion injury and neutrophils, which infiltrate the retina following injury and vascular leakage (Hangai et al., 1996; Neufeld et al., 2002). The mechanism of rescue of retinal ganglion cell cells with connexin43 mimetic peptide may therefore be attributed in some part to inhibition of nitric oxide production by reducing retinal glial cell activation and dysfunction. The primary event, however, appears to be hemichannel mediated breaches in the blood–brain barrier; blocking of endothelial hemichannels prevents vascular leak, and the subsequent inflammatory cascade that leads to retinal ganglion cell loss. We have illustrated this pathway schematically in Fig. 10.

In summary, the present findings support the idea that connexin43 upregulation mediates vascular leakage, which is associated with astrocytosis and subsequent retinal ganglion cell loss following retinal ischaemia. Death of retinal ganglion cells after ischaemia-reperfusion follows a complex cascade of events involving glutamate excitotoxicity and inflammatory mediator release including nitric oxide. Our results, however, indicate that an initiating event is vascular endothelial hemichannel related since blocking these channels curtails subsequent events. Modulation of connexin43 after CNS ischaemic-reperfusion injury alleviates vascular leakage and provides significant neuron sparing.
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References


Figure 10 A schematic drawing depicting the initial events following retinal ischaemia that lead ultimately to retinal ganglion cell loss. (A) Astrocyte processes interface with the vascular endothelium in order to maintain the neuronal environment and regulate blood flow (reviewed by Kimelberg and Nedergaard, 2010). (B) An enlarged view of endothelial cells that are gap junction coupled, with undocked, but closed, hemichannels in the plasma membrane. After ischaemia–reperfusion endothelial connexin expression is upregulated (Kerr et al., 2010 and Fig. 4F) and after insult hemichannels open (Orellana et al., 2009; De Bock et al., 2011). This leads directly to endothelial cell oedema (C) and rupture (D) (Petito, 1979; Rodriguez-Sinovas et al., 2007; Danesh-Meyer et al., 2008; Fig. 1E and F). Astrocytes then become activated (E) (Dietrich et al., 1994a; Hirano et al., 1994; Kaur et al., 2006; Fig. 6) and T cell and monocyte migration augments the inflammatory reaction and leads to further tissue damage (Pachter et al., 2003). The subsequent death of retinal ganglion cells following ischaemia–reperfusion is a complex cascade of events involving glutamate excitotoxicity and inflammatory mediator release including nitric oxide (Osborne et al., 2004). Astrocyte gap junction channels also play a role with ATP and glutamate release; inhibition of astrocytic gap junction permeability by octanol for example restricts the flow of neurotoxins that otherwise exacerbate neuronal damage (Rami et al., 2001). The initiating event appears to be vascular endothelial hemichannel-related since block of these hemichannels with connexin43 mimetic peptides reduces astrocytosis, and subsequent downstream retinal ganglion cell loss.


