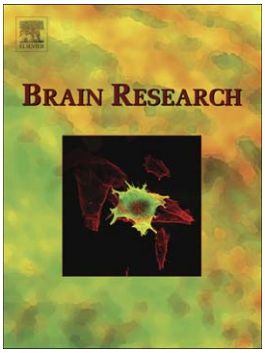

Protective role of endothelial nitric oxide synthase following pressure-induced insult to the optic nerve

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TITLE: PROTECTIVE ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE FOLLOWING PRESSURE-INDUCED INSULT TO THE OPTIC NERVE

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Abstract

Although intracranial pressure (ICP) elevation can induce significant structural and functional changes within the central nervous system (CNS), almost complete neuronal recovery is possible if ICP and associated pathogenic factors are restored in the acute phase of the disease process. Nitric oxide synthase (NOS) isoforms have been implicated in the pathogenesis of many CNS diseases and may play an important role in the development of neuronal tolerance in the early stages of pressure elevation. In this paper we use the pig optic nerve, a typical central white matter tract, to study the time-dependent sequence of NOS isoform change following pressure elevation. The timing of NOS isoform change in relationship to structural and functional changes to axons and glial cells is also discussed. This study demonstrates that endothelial cell nitric oxide synthase (ecNOS), an enzyme that plays a protective role in the CNS, is up-regulated in a time-dependent manner after pressure elevation. ecNOS levels increase after axonal and astrocyte injury, suggesting that it might be a compensatory response that is initiated in an effort to preserve CNS function. Inducible NOS (iNOS) and neuronal NOS (nNOS), which are known to have a deleterious effect on the CNS, were not detected in this study. The increase in ecNOS demonstrated in this study is significantly different to the increase in iNOS and nNOS previously demonstrated following traumatic brain injury. Changes in ecNOS levels may therefore be important in the development of neuronal tolerance in the early stages of CNS diseases such as hydrocephalus.

1. Introduction

Raised intracranial pressure (ICP), which is commonly associated with traumatic brain injury (TBI) and hydrocephalus, can have a deleterious impact on central nervous system (CNS) structure and function (Prins et al. 1996;Sainte-Rose C 1996). Neuronal cytoskeleton disruption and axonal transport alteration, which are hallmarks of CNS injury, have been demonstrated in the early stages of both TBI and hydrocephalus (Chovanes et al. 1988;Aoyama et al. 2006;Kupina et al. 2003). However, the neuronal prognosis and long term neurological morbidity for each of these conditions is known to be vastly different. ICP control is the mainstay of hydrocephalic management and neuronal recovery can occur if ICP is restored to the normal range in the early stages of the disease process (Aoyama et al. 2006). The magnitude of neuronal restoration in hydrocephalus is known to be inversely proportional to the duration of pressure elevation, suggesting that the phenomenon of neuronal tolerance to irreversible injury is lost over time (Boillat et al. 1997). In contrast to hydrocephalus, normalisation of ICP remains a controversial issue in the management of TBI (Adamides et al. 2006), implying that pathogenic factors apart from ICP elevation are important in mediating neuronal damage in this condition. Differences in the biochemical systems that are activated in TBI and hydrocephalus may explain why neurons in TBI are predisposed to ongoing damage while neurons in hydrocephalus are initially conferred some level of protection from irreversible injury.

Nitric oxide synthase (NOS) isoforms are a closely related family of enzymatic proteins that synthesise and regulate nitric oxide levels in the CNS (Dawson and Dawson 1996). By controlling nitric oxide concentrations within the brain, NOS

enzymes have the capacity to modulate critical homeostatic processes (Szabo 1996). Three isoforms of NOS enzyme have been identified: neuronal nitric oxide synthase (nNOS) or NOS-1, immunologic nitric oxide synthase (iNOS) or NOS-2 and endothelial cell nitric oxide synthase (ecNOS) or NOS-3. Because NOS isoforms regulate CNS function, an alteration in the concentration of these enzymes can result in neurodegenerative disease (Szabo 1996).

The activity of NOS enzyme systems are subject to carefully controlled activation and inactivation pathways that are influenced by numerous biochemical and cellular factors (Nathan and Xie 1994). Axonal transport processes, cytoskeleton proteins, glial cells and inflammatory cells are important mediators of local NOS enzyme levels (Chou et al. 1996;Murphy et al. 1993;Gahm et al. 2002). We have previously used the optic nerve, a typical central white matter tract, to report the time-dependent sequence of axonal transport, cytoskeleton protein and astrocyte change after pressure elevation (Balaratnasingam et al. 2007;Balaratnasingam et al. 2008a;Balaratnasingam et al. 2008b). Our experimental model of pressure elevation that involved precise control of pressure in compartments surrounding the optic nerve including the intraocular compartment, cerebrospinal fluid (CSF) compartment and vascular compartment allowed us to induce neural pressure elevation without mechanically disrupting CNS structures. Using micro-electrode technology we have also previously demonstrated that neural pressure in the anterior (pre laminar) region of the optic nerve is equal to intraocular pressure (IOP) and neural pressure in the posterior portion (post laminar region) of the optic nerve is determined by cerebrospinal fluid pressure in the subarachnoid space (Morgan et al. 1995;Morgan et al. 1998). A significant pressure gradient exists between the anterior and posterior

compartments of the optic nerve and this occurs at the lamina cribrosa (Morgan et al. 1995). Absolute pressure alteration and pressure gradient change can influence NOS enzyme levels (Kim et al. 2005). The optic nerve is therefore an ideally suited model to simultaneously study the change in NOS isoform levels between regions of pressure increase and pressure gradient alteration.

NOS isoform changes have been shown to be intimately linked to the time-dependent sequence of neuronal injury in TBI (Gahm et al. 2000;Gahm et al. 2002). With regards to pathogenic factors, it is widely accepted that in addition to ICP elevation, some of the neuro-pathological changes in TBI are a consequence of mechanical brain damage (Cortez et al. 1989). Because multiple pathogenic factors are acting simultaneously in TBI, it has been difficult to elucidate if NOS enzyme changes in TBI are a result of mechanical damage or ICP elevation. Animal models of hydrocephalus have been used to isolate the effects of ICP elevation on NOS isoform expression (Klinge et al. 2003;Klinge et al. 2002). These studies have demonstrated that nNOS plays an important role in mediating neuronal damage after two weeks of ICP elevation (Klinge et al. 2003). However, these studies have not examined NOS isoform changes at earlier time points of pressure elevation. Therefore we have limited information regarding NOS isoform alteration during the critical period when neuronal tolerance to irreversible injury is believed to occur. The patho-physiological basis for the difference in neuronal prognosis between TBI and hydrocephalus remains unclear. A distinctly separate sequence of NOS isoform alteration and hence nitric oxide mediated toxicity/protection may play an important role.

In this paper we report time-dependent NOS isoform changes following pressure elevation using optic nerves where axonal transport, cytoskeleton protein and astrocyte changes have been previously documented. Our experimental design has allowed us to correlate the sequence of neuronal and glial cell changes with individual NOS isoform changes following pressure elevation. By comparing changes between regions of absolute pressure increase and regions of pressure gradient increase we have also been able to determine the influence of important physiological factors on NOS enzyme expression. Taken together with other reports (Gahm et al. 2000;Gahm et al. 2002), the results from this paper demonstrates important differences in the sequence of NOS isoform expression between TBI and hydrocephalus, two conditions that are commonly associated with ICP rise. This work focuses on NOS enzyme changes in the first 12 hours of pressure elevation and is useful in understanding why the phenomenon of neuronal tolerance occurs in hydrocephalus, while TBI is often associated with catastrophic and irreversible neuronal injury.

2. Results

2.1 General

The mean systolic blood pressure for all 12 pigs was 85.2 ± 1.6 mmHg. Average arterial pO_2 was 101.3 ± 2.3 mmHg, pCO_2 was 37.7 ± 0.9 mmHg, and pH was 7.5 ± 0.0 on blood gas analysis. The mean CSFp was 5.3 ± 1.2 mmHg. The average left-eye and right-eye IOP was 43.5 ± 0.4 and 12.9 ± 0.4 mmHg respectively. The average differences between the IOP and CSFp in the left and right eyes were 38.2 ± 1.1 and 7.6 ± 1.0 mmHg respectively. Mean data for the 6 and 12 hour groups are presented in Table 1. There was no statistical difference in blood pressure, CSFp,

left-eye IOP, right-eye IOP, pO_2, pCO_2 and pH between the 6 and 12 hour groups (all $P > 0.350$).

2.2 NOS enzyme staining in the normal porcine eye

NOS enzyme staining in optic nerve, positive control and negative control tissues is presented in Figure 1. Strong staining for nNOS, iNOS and ecNOS was present in the anterior segment ciliary processes of positive control tissue. There was no nNOS or iNOS staining in the optic nerve head but strong immunoreactivity for ecNOS was found. Although specific co-labelling was not performed, the expression pattern of ecNOS was consistent with previous reports that have described the normal distribution of retinal, choroidal and optic nerve vascular endothelium in the pig eye (Rootman 1971;Simoens P 1985).

2.3 NOS enzyme changes following pressure elevation

Four different regions of the optic nerve were analysed for NOS isoform changes (Fig. 2). We did not detect any nNOS or iNOS staining in normal-pressure and high-pressure nerves in any of the experiments. Changes in ecNOS enzyme stain following 6 and 12 hours of pressure elevation is presented in Figure 3. After 6 hours there was no visible difference in the intensity of ecNOS stain between normal pressure and high-pressure nerves. Quantitative analysis revealed no difference in mean pixel intensity per μm^2 in any of the laminar regions between the normal-pressure and high-pressure nerves after 6 hours ($P = 0.879$; Fig. 4A).

After 12 hours of pressure elevation, the intensity of ecNOS staining was visibly greater in the pre laminar and lamina cribrosa regions of high-pressure nerves (Fig. 3). The expression pattern of increased ecNOS staining was consistent with the expression pattern of optic nerve endothelium. Quantitative analysis revealed a significant difference in mean pixel intensity per μm^2 between normal-pressure and high-pressure nerves ($P < 0.001$; Fig. 4B). Post-hoc analysis revealed this difference was significant in the pre laminar ($P = 0.013$) and lamina cribrosa ($P < 0.001$) regions. Although the mean pixel intensity per μm^2 of tissue in the immediate post laminar region of high pressure nerves was greater that normal-pressure nerves this difference did not reach statistical significance ($P = 0.569$). There was no difference between the normal and high pressure nerves in the post laminar region 1mm behind the posterior boundary of the lamina cribrosa ($P = 0.982$).

When we compared 6 hour and 12 hour data we found a statistical difference in mean pixel intensity per μm^2 between the two groups ($P = 0.018$), with post-hoc analysis revealing that the difference was greatest at the lamina cribrosa ($P = 0.032$). The total area of optic nerve that was analysed for NOS enzyme changes was not different between normal and high-pressure eyes ($P = 0.051$).

2.4 Reproducibility of histological measurements

The mean co-efficient of variation (CV) for ecNOS stain intensity measurement was small, at 10.7% (SD 8.6%), with no significant difference in the CV between the different optic nerve regions ($P = 0.099$). Testing the influence of individual pig, optic nerve region and whether high-pressure or normal-pressure revealed no significant difference in CV between the two nerves ($P = 0.953$). Repeated measurements on six pairs of ecNOS intensity data found no relationship between day

of measurement and stain intensity in any of the optic nerve regions ($P = 0.681$). With regard to the area of the laminar regions measured for ecNOS images, the mean CV was 14.4% (SD 9.0%) with no difference in the CV between the different optic nerve regions ($P = 0.713$). Testing the influence of individual pig, optic nerve region and whether high-pressure or normal-pressure revealed no significant difference in CV for area between the two nerves ($P = 0.756$). Repeated measurements on 6 pairs of ecNOS area data found no relationship between day of measurement and regional area in any of the optic nerve regions ($P = 0.991$).

3. Discussion

This study reports the temporal sequence of NOS isoform change in the optic nerve, a typical central white matter tract, following pressure elevation. The major findings are: (1) Endothelial cell nitric oxide synthase is the only NOS isoform in the normal porcine optic nerve. (2) A rise in CNS pressure does not result in a detectable increase in iNOS or nNOS levels in the first 12 hours. (3) A time-dependent increase in ecNOS levels occurs in regions of absolute pressure increase and pressure gradient elevation. (4) The magnitude of ecNOS elevation following pressure rise is proportional to the degree of axonal and astrocyte injury. (5) Pressure-induced neuronal and glial cell injury in the form of cytoskeleton alteration, axonal transport retardation and astrocyte swelling precede changes in ecNOS levels.

NOS isoforms control nitric oxide production and therefore are critically placed to regulate CNS activity (Dawson and Dawson 1996). In neuro-pathological disease, endothelial nitric oxide synthase has a beneficial effect on the CNS and promotes

vessel dilatation and inhibits micro-vascular plugging by platelets and inflammatory cells (Hlatky et al. 2003). Neuronal nitric oxide synthase and inducible nitric oxide synthase antagonise the effects of ecNOS and cause widespread neural damage by activating cytokine systems and promoting the destructive activity of inflammatory cells (Dudzinski et al. 2006). Because the activity of nitric oxide is dependent of the source from which it is produced, the selective inhibition of NOS isoforms is a potential therapeutic target in the treatment of CNS disorders.

Extensive work has focussed on the temporal sequence of NOS isoform expression in the early stages of TBI (Gahm et al. 2000;Gahm et al. 2002). In these reports an elevation of all three NOS isoforms has been detected in the first 12 hours of TBI. An increase in both nNOS and iNOS occurs within 6 hours of TBI and remains elevated post-injury for 12 hours and 36 hours respectively (Gahm et al. 2000). ecNOS elevation however is induced early and only momentarily, reaching peak levels at 6 hours and returning to baseline levels by 12 hours. This suggests that the transient beneficial role of ecNOS in the post-injury preservation of blood flow is quickly offset by the sustained neurotoxic activity of iNOS and nNOS. The early expression of iNOS and nNOS, which often occurs before structural and functional axonal changes are evident, also implies that these enzymes play an important role in mediating some of the irreversible CNS changes following trauma (Chatzipanteli et al. 2002).

Experimental models of hydrocephalus have been used to isolate the role of NOS isoforms on pressure-induced neuronal change (Klinge et al. 2002;Klinge et al. 2003). These studies have clearly demonstrated that the sequence of NOS isoform expression

in TBI and hydrocephalic brains are different. They have also been important in identifying nNOS as a significant mediator of neuronal damage following prolonged periods of pressure elevation in hydrocephalus (Klinge et al. 2002). However, these published reports have provided little information about NOS isoform alteration immediately after pressure elevation. The advantage with our experimental design is that accurate control of pressures surrounding the optic nerve was achieved with precise surgical technique. The availability of veterinary anaesthetic expertise for this work also allowed us to maintain experiments within defined physiological parameters for extended periods of time. This in turn has allowed us to report NOS enzyme changes in the first 12 hours of pressure elevation, the critical period when neuronal tolerance to irreversible injury may develop.

In this work we were able to demonstrate that ecNOS is the only NOS isoform expressed in the normal porcine optic nerve. Our finding is consistent with previous work that demonstrates selective expression of NOS isoforms through out the mammalian central nervous system (Bredt et al. 1991). We did not find a change after 6 hours of pressure elevation but demonstrated an increase in ecNOS levels in the pre laminar and lamina cribrosa regions after 12 hours of pressure elevation. We have previously reported time-dependent axonal and astrocyte injury in the same optic nerves that were used for this study (Balaratnasingam et al. 2008a;Balaratnasingam et al. 2008b). Taken together with those previous reports, this study demonstrates that an elevation of ecNOS occurs only after optic nerve axonal and astrocyte injury. This suggests that ecNOS elevation is a compensatory response that is initiated following cellular damage and may be a biochemical measure that attempts to minimise ongoing

injury. This is an important difference from TBI, where changes in NOS isoforms, particularly iNOS and nNOS, precede neuronal injury.

The greatest increase in ecNOS occurred at the lamina cribrosa. In our previous reports we have demonstrated that the lamina cribrosa is the site of greatest axonal and astrocyte injury (Balaratnasingam et al. 2008a;Balaratnasingam et al. 2008b). Taken together with our previous findings, the results from this paper imply that ecNOS expression increases most in regions where axonal transport inhibition, neuronal cytoskeleton and glial cell injury are greatest. In this study we were unable to isolate the stimulus for ecNOS increase. Previous work has shown that cytoskeleton proteins, axonal transport, astrocytes and pressure changes can all regulate local ecNOS expression by modifying intracellular calcium levels (Chou et al. 1996;Murphy et al. 1993;Gahm et al. 2002). Because astrocyte, axonal and pressure changes had all preceded changes in ecNOS levels we were unable to isolate a single causative factor in this report. It is interesting that we did not detect a significant increase in ecNOS levels in the post laminar region of the optic nerve although we have previously demonstrated that axonal and astrocyte injury did occur in this region following 12 hours of IOP elevation (Balaratnasingam et al. 2008a;Balaratnasingam et al. 2008b). The magnitude of axonal injury in the post laminar region is known to be less than the pre laminar and lamina cribrosa region and may not have been enough to stimulate an increase in ecNOS levels after 12 hours (Balaratnasingam et al. 2008a). It is possible that an increase in ecNOS levels may occur in this region following a longer duration of IOP elevation. Alternatively, the reason why ecNOS levels did not change in post laminar tissue may have been because there was no associated change in pressure in this region. If this is correct it

suggests that an absolute pressure increase and pressure gradient change plays an early and significant role in modulating ecNOS levels in the optic nerve.

This report demonstrates that ecNOS levels in the optic nerve increases in a time-dependent manner immediately after pressure elevation. This report also demonstrates that the neurotoxic effects of nNOS and iNOS are not induced during the period when ecNOS levels are elevated. We speculate that the net effect of ecNOS elevation results in a cellular environment that is favourable towards neuronal survival. It is possible that nNOS and iNOS changes occurred immediately after pressure elevation and returned to normal levels by 6 hours. However this is unlikely as both iNOS and nNOS are known to remain elevated for extended periods of time following induction (Nathan and Xie 1994). Sustained elevation of iNOS and nNOS, following prolonged periods of IOP rise, are known to mediate optic nerve injury. This has been demonstrated in the optic nerve head of human glaucoma eyes where increased levels of iNOS are produced by neuro-toxic glial cells (Yuan and Neufeld 2001;Liu and Neufeld 2000). Experimental work involving rodent models of ocular hypertension have reinforced human post mortem studies and have demonstrated increased levels nNOS and iNOS after 3 months of IOP elevation (Vidal et al. 2006;Shareef et al. 1999). The difference in the sequence of NOS isoform expression reported in TBI studies and our report may provide an important explanation why neuronal tolerance develops in hydrocephalus and not TBI, despite an increase in pressure in both conditions.

4. Materials and Methods

4.1 Experimental design

All experiments were conducted in accordance with the European Communities Council Directive for animal treatment. The study was approved by the University of Western Australia Animal Ethics Committee. All appropriate measures were taken to minimize pain or discomfort. A total of 12 White Landrace pigs were used for this work. A pig model of acute pressure elevation with continuous IOP, CSF pressure and blood pressure monitoring was used for this work. For all experiments the right nerve was used as the normal-pressure nerve, with the IOP maintained between 10 and 15 mmHg, while the left nerve was the high-pressure nerve, with the IOP between 40 and 45 mmHg. These experiments comprised of 2 separate groups based on the duration of IOP elevation. Animals had their IOP elevated for either 6 hours (n=6) or 12 hours (n=6) before euthanasia. We have previously given detailed descriptions of the anaesthetic and surgical techniques used for these acute pressure elevation experiments (Balaratnasingam et al. 2007;Balaratnasingam et al. 2008a). Most of the animals used for these experiments were also used for astrocyte, axonal transport and cytoskeleton studies previously reported (Balaratnasingam et al. 2007;Balaratnasingam et al. 2008a).

4.2 NOS Enzyme Immunohistochemistry

Following euthanasia, eyes together with their optic nerves were enucleated and sectioned into 12 μm longitudinal specimens on a cryotome set at -30ºC. Previously described measures were adopted to orientate sections and to ensure that the levels of the sections used for comparison between normal-pressure and high-pressure nerves

within each animal were reliably matched (Balaratnasingam et al. 2007). Sections taken from the central optic nerve were used for this report.

The distribution of nitric oxide synthase isoforms in the porcine eye has been previously described (Meyer et al. 1999). The immunohistochemical staining protocol for this work was adopted from this previous report. Primary antibodies for NOS enzyme labelling include inducible NOS antibody (1:10, Product No. 610328, Transduction Labs, Lexington, USA), neuronal NOS antibody (1:25, Product No. 610308, Transduction Labs, Lexington, USA) and endothelial NOS antibody (1:100, Product No. 610296, Transduction Labs, Lexington, USA). The specificity of all primary antibodies were characterised by routine testing and stained a single band of molecular weight 130 kDa, 155 kDa and 140 kDa for iNOS, nNOS and ecNOS respectively on western blot analysis.

All primary antibodies used for this work were made into solution with 1% goat serum (G9023; Sigma-Aldrich) and 1% bovine serum albumin. Triton X-100 (0.1%; Sigma-Aldrich) was also used for all primary antibody incubations to improve permeability. All primary antibodies were incubated for 24 hours. Slides were then given 4 washes over 20 minutes in a 4ºC wash solution (0.01M PBS, pH 7.4 and Tween 20) and then incubated overnight with a goat anti-mouse IgG secondary antibody (1:400, Alexa Fluor 488, A11029; Invitrogen-Molecular Probes, Eugene, OR). After secondary antibody incubation all specimens were washed four times over 20 minutes in a 4ºC wash solution. Slides were then mounted in glycerol and immediately viewed with the confocal microscope.

Previous work has demonstrated that conjunctiva, iris, ciliary body and ciliary processes of the normal porcine eye has a high concentration of all three NOS enzymes (Meyer et al. 1999). For this reason we used the anterior segment of the eye as our positive control. Negative controls for this work consisted of replacing the primary antibody solution from optic nerve and anterior segment sections with 0.01M PBS with the remainder of the staining protocol being the same. Two optic nerve negative control sections were used for each experiment.

4.3 Confocal Microscopy

Digital images of labelled tissue were captured using a BioRad MRC 1000/1024 UV confocal laser scanning microscope controlled by COMOS image acquisition software. Visualisation of sections was achieved by laser excitation at a 488 nm line from an argon laser with emissions detected through a 522/35 nm band pass filter. A Nikon 20x (NA 0.4) dry lens was used to view all slides. Confocal microscope images of the control and high pressure nerve labelled with the same primary antibody were acquired immediately after each other using identical instrument settings being sure to avoid saturation of the brightest pixels. To allow for background correction during image analysis two negative control slides were also montaged with the confocal microscope.

Using a motorised stage and a macro written in MPL under COMOS a series of Z stacks were captured for each slide beginning in the pre laminar region and extending through to the post laminar tissue. Images were collected in gray scale (on a scale of

0 -255). Each z stack consisted of a depth of optical sections collected at 2 µm increments along the z plane. Each z stack consisted of 7 sequential images collected using Kalman averaging. Z stacks were then montaged using an Image J plug-in (ver. 1.36; http:/rsb.info.nih.gov/ij/ developed by Wayne Rasband, National Institute of Health, Bethesda, MD) to give a montage of each slide. Each montage commenced in the pre laminar region and extended 2000 µm into post laminar tissue. Creating a montage allowed us to study spatial changes in the pre laminar, lamina cribrosa and post laminar regions of the optic nerve in detail.

After slides were montaged using the confocal microscope, a light microscope image of the same section was captured using a high resolution digital camera (DXM 1200; Nikon) attached to a microscope (Eclipse E80; Nikon). The light microscope image was used as the reference image during image analysis to accurately demarcate each of the optic nerve regions in the accompanying confocal microscope image.

4.4 Image analysis

Quantitation of all images was performed using computer software (Image Pro Plus, ver. 5.1; Media Cybernetics). All images for the manuscript were prepared using Adobe Photoshop (version 8.0, Adobe Systems Inc.) and Adobe Illulstrator CS2 (version 12.0, Adobe Systems Inc.). Confocal images for the manuscript were false coloured using Look Up Tables available on Image J (version 1.36, National Institute of Health, USA, http:/rsb.info.nih.gov/ij).

4.4.1 Image randomisation and segmentation

Prior to analysis all images were randomised such that treatment and time groups were unknown by the observer. Confocal images were analysed in gray scale (0 – 255 pixel intensity) with the average projection of each montaged Z stack being used for analysis. Each image was divided into 4 regions – pre laminar, lamina cribrosa, proximal 400 μm of immediate post laminar tissue and a second region of post laminar tissue that commenced 1 mm behind the lamina cribrosa and extended 400 μm posteriorly. A light microscope image of the section that was viewed with the confocal microscope was used to delineate each of the optic nerve regions prior to image analysis. Figure 2 demonstrates the method used to divide each confocal image for quantitative analysis.

4.4.2 Quantitative analysis of NOS enzyme change

To detect NOS enzyme differences between normal-pressure and high-pressure nerves we used previously reported techniques for quantifying pixel intensity with back ground correction from NOS microscope images (Burns et al. 2007). The mean value of pixels per μm^2 of tissue in each of the laminar regions in each confocal image was determined and used for statistical analysis. The mean value of pixels per μm^2 in each of the laminar regions was determined using the following steps:

i. Each confocal image was divided into 4 optic nerve regions (pre laminar, lamina cribrosa, immediate post laminar and 1 mm post laminar) and each region was analysed separately.
ii. The total area of each laminar region (in μm^2) was determined.

iii. The area occupied (in μm^2) by each pixel value (from 0 – 255) within each laminar region was determined.

iv. Each pixel value (0 – 255) was multiplied by the area it occupied, and the sum of these 256 measurements calculated. This determined the total pixel intensity in each of the laminar regions and was used as a measure of the total amount of fluorescence.

v. The mean fluorescence in each laminar region was then calculated by dividing the total pixel intensity within the laminar region (step iv) by the total area of that laminar region (step ii). This result was defined as the *mean pixel intensity per μm^2 of tissue*.

vi. Steps (i) to (v) were performed on confocal images from normal-pressure, high-pressure and negative control optic nerve slides from the same animal.

vii. To correct for background intensity, the mean pixel intensity per μm^2 of tissue in the negative control slide was subtracted from the mean pixel intensity per μm^2 of tissue in the treated slide (normal-pressure or high-pressure) for each laminar region.

viii. The final result was used for statistical analysis.

4.5 Statistical Analysis

All statistical testing was performed using commercial software (SigmaStat, ver. 3.1; SPSS, Chicago, IL). Kolmogorov-Smirnov testing was performed on all data prior to analysis to determine if data was normally distributed. Normally distributed data was analysed using analysis of variance (ANOVA) with post hoc factor comparison performed using a paired Student's *t*-test with Bonferroni correction. Non-normally

distributed data was analysed using ANOVA on ranks with the Tukey test employed for post hoc paired analysis. ANOVA was used to determine if mean pixel intensity (of NOS enzyme) per μm^2 of tissue was significantly different between normal-pressure and high-pressure nerves and also between 6 hour and 12 hour groups. ANOVA was also used to determine if the area of optic nerve tissue analysed in normal-pressure eyes was significantly different from high-pressure eyes. The latter analysis was used to determine if we had analysed more/less optic nerve tissue in one group with respect to the other. Results are expressed as mean ± standard error.

4.6 Reproducibility of NOS measurements

To determine observer reproducibility, the different laminar regions of 12 ecNOS images (6 pairs) were quantified as described above on 3 separate occasions, each at least 1 week apart, by the same masked observer who performed all the data analysis. Three way ANOVA was used to test whether the *mean pixel intensity per μm^2 of tissue* and *total area* within each laminar region for each measurement day was statistically significant. ANOVA was also used to determine if the co-efficient of variation between normal-pressure and high-pressure eyes was different.

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Figure Legends

Table 1 – The mean (± SE) for each physiological variable for 6 hour and 12 hour experiments are provided. All measurements apart from pH are in mmHg. There was no significant difference between the 2 groups for any of the physiological variables (all $P > 0.350$).

Figure 1 – Confocal images of endothelial nitric oxide synthase (ecNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) labelled positive control (anterior segment ciliary processes) and normal pig optic nerve tissue. There was strong immunostaining of all NOS enzymes in ciliary processes (A, B and C). No staining was seen in anterior segment negative control tissue when primary antibodies were replaced with PBS solution (D, E and F). The vascular endothelium in the optic nerve vessels showed strong immunoreactivity to ecNOS (G). There was no detectable nNOS (H) or iNOS (I) staining in optic nerve tissue. Scale bar = 100 μm.

Figure 2 – NOS stain was quantitated in four different regions of the optic nerve. A light microscope image (A) of the same section that was viewed with the confocal microscope (B) was used to accurately divide each confocal image into pre laminar region, lamina cribrosa region, immediate post laminar region (which commenced immediately after the posterior boundary of the lamina cribrosa and extended 400μm posteriorly) and 1 mm post laminar region (which commenced 1 mm behind the posterior boundary of the lamina cribrosa and extended 400μm posteriorly). Dotted lines demarcate each of the four regions that were analysed. Confocal microscope image in this figure is stained with ecNOS. All confocal images were false coloured following analysis using a colour look up table.

Figure 3 – Temporal sequence of optic nerve ecNOS change following pressure elevation. Left and right panel of images represent normal-pressure and high-pressure nerves respectively from 6 hour (A, B, C and D) and 12 hour (E, F, G and H) experiments. Corresponding light microscope images of optic nerve are provided below each confocal image. There was no difference in ecNOS stain between normal-pressure and high-pressure nerves in the pre laminar (PL), lamina cribrosa (LC) and post laminar (PoL) regions after 6 hours of pressure elevation. After 12 hours, there was visibly more stain in the PL and LC regions of the high-pressure nerve. Confocal images were false coloured with a colour look up table with an intensity scale bar provided on the right of the image. Scale bar = 300μm

Figure 4 – Quantitative analysis of ecNOS intensity for each optic nerve region in 6 hour and 12 hour groups. There was no difference between normal-pressure and high-pressure nerves in any of the optic nerve regions after 6 hours of pressure elevation (A). Intensity was greater in the pre laminar (PL) and lamina cribrosa (LC) regions of the high-pressure nerve after 12 hours of pressure elevation but no difference was found in the immediate post laminar (IPoL) and 1mm post laminar (1mmPoL) segments (B). ** Significant difference with $P < 0.05$ as determined by ANOVA.

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	BP	(L) IOP	(R) IOP	CSFp	pO2	pCO2	pH
6 hour	83.1 ± 5.9	43.7 ± 1.1	13.2 ± 0.9	5.5 ± 1.8	100.2 ± 3.0	36.8 ± 0.8	7.5 ± 0.0
12 hour	86.6 ± 2.1	43.3 ± 0.7	12.6 ± 0.7	4.8 ± 2.8	101.7 ± 3.6	38.6 ± 1.6	7.5 ± 0.0

Table 1