EFFECTS OF VARIOUS NITRIC OXIDE SYNTHASE INHIBITORS ON THE CHOROIDAL AND GROWTH RESPONSES IN EMMETROPIZATION IN CHICKS

A thesis presented to the graduate faculty of
The New England College of Optometry in partial fulfillment
of the requirements for the degree of Master of Science

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Abstract

Introduction

Chick eyes compensate for image defocus on the retina through changes involving the sclera and the choroid. The rapid choroidal changes in thickness that occur in response to myopic defocus may be important in the process of emmetropization, and because they are consistently associated with the slower changes in the sclera (eye growth), they may form part of this scleral response. Previous work with the non-specific nitric oxide synthase inhibitor L-NAME has supported a role for nitric oxide as a potential mediator in this process. The purpose of this study is to elucidate the source of the nitric oxide involved in the response of the chick eye to myopic defocus through the use of various inhibitors of the three isoforms of nitric oxide, and in doing so, clarify the role of nitric oxide in emmetropization.

Methods

Subjects were White Leghorn chicks (*Gallus gallus domesticus)*. Myopic defocus was induced with prior form deprivation or with +10D lens wear. NOS inhibitors were injected monocularly under anesthesia after 7 days of prior form deprivation (TRIM, Saline), or immediately prior to start of positive lens wear (TRIM, N^{ω}-propyl-L-arginine, L-NIL, L-NIO, Saline, L-NMMA). Trim was also injected monocularly into eyes untreated with lenses or prior form deprivation. Ocular dimensions were measured using high-frequency A-scan ultrasonography at the start of the experiment, and at 7, 24 and 48 hours after. Refractions

were obtained using a Hartinger's refractometer following the 48 hour measurement. Chicks were sacrificed and scleral punches were obtained, digested and assayed to measure scleral glycosaminoglycan synthesis at the end of the experiments.

Results

The nNOS inhibitor N^{ω}-propyl-L-arginine had the same inhibitory effects on the choroidal response, and the same dis-inhibition of the growth response, as did L-NAME. Neither L-NIL nor L-NIO had any effect on this response. N^{ω}-propyl-L-arginine prevented the inhibition of scleral GAG synthesis in response to plus lenses. TRIM, presumably another nNOS inhibitor, did not prevent choroidal thickening or dis-inhibit ocular growth in response to either plus lens-wear or prior form deprivation, in fact it tended towards the opposite: choroids of TRIM injected eyes tended to be even thicker than saline injected eyes. There was no significant effect of TRIM on axial length in either treatment group, however there was an inhibitory effect of TRIM on the anterior chamber growth at 24 hours, similar to that seen with L-NAME. TRIM also produced *higher* amounts of NO in tissue culture after 7 hours, and therefore does not act as a NOS inhibitor in this system. Finally, injections of TRIM in untreated eyes led to rapid increases in choroidal thickness and a non-significant trend towards decreased ocular growth.

Conclusion

The choroidal compensatory response is inhibited by nNOS, possibly from the intrinsic choroidal neurons, or parasympathetic innervation from the ciliary and/or pterygopalatine

ganglia. It is possible that the action of NO in the choroid influences axial growth of the eye. TRIM acted in a manner inconsistent with that of a NOS inhibitor, however the fact that TRIM induced both an increase in NO and choroidal thickening in untreated eyes strengthens the link between choroidal thicknening, ocular growth inhibition and NO.

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Introduction

Background

Similar to many other species, including primates and humans, chick eyes start out ametropic, and through an active, visually guided process, move toward emmetropia. The precise length and shape of the eye are critical for sharp focus of light on the retina. Chick eyes utilize form vision in the emmetropization process, and if deprived of form vision, grow abnormally and become myopic (Wallman, 1978). The process of emmetropization involves compensation for visual defocus through mechanisms affecting vitreous chamber depth, including moving the retina toward the image plane to provide a clear focus (review: Wallman, 1993). Gaining a better understanding of the process of emmetropization may lend insight into the problem of the development of myopia, and potentially provide clues towards its prevention.

Chick eyes compensate for image defocus through changes involving the sclera and the choroid. This occurs through a two-part mechanism, whereby if a positive lens is placed in front of the eye, the choroid thickens (fast, transient response) and the axial growth slows or halts (slow, long term response). The choroidal thickening pushes the retina forward. The axial length is adjusted through changes in the scleral matrix, altering the size of the eye. Together, these two mechanisms bring the retina toward the image plane. Likewise, the eyes compensate for hyperopic defocus through transient choroidal thinning and an increase in axial growth, pulling the retina out to the image plane (Schaeffel et al., 1988; Schaeffel and Howland, 1991; Smith 1991; Irving et al., 1992; Wilsoet and Wallman, 1995; Siegwart and

Norton, 1993). When the defocusing lenses are removed, the opposite compensatory responses occur, and the chick eye readjusts its length to bring the retina back in line with the image plane created by the optics of the eye (Wildsoet & Wallman, 1995).

The choroidal changes that occur in response to defocus are thought to be important in the process of emmetropization. The rapid thickening of the choroid acts as a fast adaptive response, bringing the retina closer to the image plane, similar to the way an accommodative change of the lens brings the image plane closer to the retina. It has been hypothesized that the choroidal response may also act as a signal, transmitting the information that growth should be slowed to obtain correct image focus from the retina to the sclera (Wallman and Adams, 1987; Marzani and Wallman, 1997).

It has been shown that L-NAME, a non-specific nitric oxide synthase inhibitor prevents choroidal thickening in the presence of myopic defocus and also prevents the inhibition of scleral growth, indicating that perhaps preventing choroidal thickening in turn prevents the inhibitory effect of myopic defocus on the sclera (Nickla and Wildsoet, 2004).

The Role of the Choroid

There are two main components of the chicken choroid: the choroicapillaris, located immediately posterior to the RPE and consisting of a network of fenestrated capillaries, and the main part of the choroid, situated behind the choriocapillaris and consisting of larger blood vessels and large endothelial-lined fluid reservoirs (lacunae). These lacunae make up the majority of the volume of the choroid. The multilayered membrane fusca consists of elongated cells connected by adherent junctions, tight junctions, and perhaps also gap

junctions. The structures of the choroid are suspended in a trabecular support system of intracellular matrix, fusiform smooth muscle fibers, fibroblasts and pigmented cells (Meriney & Pilar, 1987) (DeStefano & Mugnaini, 1997).

The classic role for the choroid is the supply of oxygen and nutrients to the outer retina, as well as functioning as a heat sink (Bill, 1985). There is no central retinal artery in the avian eye, therefore regulation of choroidal blood flow is particularly important in the chick eye (Meriney & Pilar, 1987). However, the diverse pattern of neurologic innervations to the choroid has suggested that the choroid has additional functions. The choroid receives autonomic input from the ciliary, superior cervical and pterygopalatine ganglia as well as the oculomotor, trigeminal and facial nerves. Sympathetic innervation leads to vasoconstriction and parasympathetic innervation to vasodilation in the chicken choroid (Bill, 1985).

There are several hypotheses that have been put forward to explain the underlying mechanism for choroidal expansion. One possibility is that the choroid swells due to fluid influx achieved through an increase in the amount of charged proteoglycans in the choroidal extracellular matrix (Myers, Armstrong and Mow, 1984; Wallman, 1995; Junghans, 1999). This is supported by the finding that choroids thickened by positive lens wear synthesize more glycosaminoglycans (GAGs) than normal, and eyes thinned by negative lens wear synthesize fewer GAGs than normal (Nickla et al 1997). Another possibility is that modulation of the degree of fenestration of choroidal capillaries may regulate the entry of large osmotically active molecules into the extracellular space (Korte, Burns & Bellhorn, 1989; Wallman 1995, Nickla 1997). The non-vascular smooth muscle of the chick choroid

may also contribute to the control of choroidal thickness through the degree of contraction (Wallman et al 1993; Wallman 1995; Poukens, 1998).

The choroidal response to visual input is rapid and temporary: responding quickly, within hours, to myopic defocus and returning towards its previous thickness when ocular elongation has slowed sufficiently (Wildsoet & Wallman, 1995). One hypothesis for the function of this temporary choroidal response is that it may act as a signal, driving the scleral response, supported by the co-culturing of choroidal and scleral tissue. When thick choroids are cultured together with scleral tissue from normal eyes, proteoglycan synthesis in these scleras is inhibited relative to tissue co-cultured with normal choroids. The reverse is also true, scleras co-cultured with thin choroids show increased proteoglycan synthesis (Marzani and Wallman, 1997).

Viewing the choroidal response as a signal for the scleral response would help to explain how the signal from the retina, in response to visual manipulations, reaches the sclera. It is suggested that the signal must be carried internally in the eye, as experiments employing optic nerve section or tetrodotoxin to disconnect the retina from the brain show that the eye is able to compensate for myopic defocus independent of the brain (Wildsoet & Wallman, 1995).

The Signal Cascade

Much work has been done to determine the signal cascade from the retina, to the choroid, to the sclera. Candidates for signal molecules connecting retinal defocus to the growth response include dopamine (Stone et al., 1989; Iuvone et al. 1991) and the immediate

early gene ZENK (Fisher et al., 1999). Candidates for signal molecules connecting the choroidal response to the scleral response include retinoic acid (Mertz & Wallman, 2000) and nitric oxide (Nickla & Wildsoet, 2004).

In 1987, nitric oxide was recognized to be the molecule previously known as compound EDRF (endothelial derived relaxing factor) (Palmer, et al, 1987; Ignarro et al, 1987): the vasodilator known to be produced in vascular endothelium and thought essential in enacting acetylcholine-induced regulation of vascular tone (Furchgott and Zawadzki, 1980). It was demonstrated that the vasodilatory effects of nitroglycerine and other nitrates were mediated by a common degradation product, the unstable nitric oxide, through the activation of guanylyl cyclase (Murad et al 1978). This discovery revealed the role of NO as a potent smooth muscle relaxant and a second messenger, and the field exploded with research on the unique molecule.

NO is soluble in tissues, and due to its small size, is freely diffusible across cell membranes. It has a short half life (10 to 60 seconds) (Kiechle & Malinski, 1993) and radical nature, and therefore cannot be stored in vivo, instead regulated at the level of biosynthesis. This regulation, and the rapid diffusion of the molecule, makes it optimally suitable for use in cell to cell transmission, and it is found to be involved in feedback loops throughout the body (Schmetterer and Polak, 2001).

Since the initial discovery, nitric oxide had been shown to be a ubiquitous molecule in the eye, found in all ocular tissues. It acts in myriad ways on a multitude of sites- and our knowledge of the roles of this molecule has grown exponentially in the last 20 years.

However, due to this extensive distribution throughout the eye, these varied functions and interactions are not yet entirely understood.

NO is synthesized from L-arginine and oxygen and the reduction of inorganic nitrate. The essential enzyme in the synthesis of NO is nitric oxide synthase (NOS), of which there are three distinct isoforms. These isoforms were previously categorized as inducible or constitutive, but have since been demonstrated to be regulated dynamically. Two of these isoforms, neuronal NOS and endothelial NOS, are activated via the calcium/calmodulin complex, and the third, "inducible" isoform of NOS is independent of calcium and is believed to be important in immunological and inflammatory functions. The isoforms are products of three different genes and share common cofactors essential for the production of NO, including NADPH.

This common element has allowed for NADPH-diaphorase to be used as a histochemical marker for the presence of NOS. This has been especially useful in demonstrating the presence of NOS in the retina (Koistinaho, 1996): in the avian retina, there is diaphorase staining in photoreceptor ellipsoids, Mueller cells, amacrine cells, ganglion cells, efferent fibers, and axonal processes in both plexiform layers (Fischer, 1999). Furthermore, diaphorase staining demonstrates the presence of NOS in the avian RPE, choroid and sclera. However, NADPH-diaphorase does not distinguish between the isoforms of NOS, and in fact my also label some cells not containing NOS.

Inmmunohistochemical labeling has been used to define the locations of these isoforms. nNOS has been found in amacrine cells, ganglion cells, efferent fibers and axonal processes in both plexiform layers of the retina (Fisher, 1999), as well as in the

pterygopalatine (Cuthbertson 1997) and ciliary (Sun, 1994) neurons, and in the terminals of these neurons in the choroid. iNOS has been located in the outer segments of the photoreceptors, the RPE and choroid (Fujii, 1998), and in Mueller cells (Goureau, 1994). iNOS activity is also found in machrophages and neutrophils in the retina (Goldstein, 1996). eNOS is found in the vascular endothelium of the choroid, but has not been found in the retina.

In the bird retina, NO is involved in visual transduction, synaptic transmission and horizontal cell coupling (Koistinaho, 1996). Nitric oxide is thought to regulate blood flow in the choroid, mediated by the endothelial isoform in the vasculature, and by the neuronal isoform through the parasympathetic nervous system (Nilsson 1996). Koss (1998) demonstrated through the use of laser Doppler flowmetry that the endothelial NO system is responsible for maintaining ocular vasodilator tone, which plays an important role in the maintenance of anterior choroidal blood flow in rats. Koss (1998) showed that a neuronal NOS inhibitor (7-nitroindazole) did not cause depression of choroidal blood flow, but did block vasodilatory responses elicited by stimulation of the Edinger-Westphal nucleus of the rats. NO in the trabecular meshwork may be involved in the regulation of intraocular pressure (Behar-Cohen, 1996; Schuman, 1994; Larsson, 1995; Krupin, 1977; Kiel, 2001). The inducible isoform is located in the RPE, and may be involved in the degradation and phagocytosis of shed outer segments disks (Becquet, 1994).

Another potential role for NO is as an important mediating molecule in the choroidal response to defocus (Nickla, 2004), and perhaps also in regulating the diurnal changes in choroidal thickness (Nickla 1998). The suggestion that NO is an important molecule in visual

modulation of eye growth is especially relevant in the model of the regulation of choroidal thickness involving changes in tonus of non-vascular smooth muscle. NO is a potent smooth muscle relaxant, and NO-positive axon terminals have been found closely apposed to smooth muscle fibers in the choroid (Poukens, et al 1998; Schrodl, et al 1998). Clusters of NO positive neurons with functions as yet undetermined have been found in the choroids of birds (Cuthbertson, et al 1997; Schrodl, et al 2001; Bergua, et al 1998) and primates (Flugel, et al 1994). The association of NO positive axon terminals with myofibroblasts in the choroids of birds and primates suggests non-vasoregulatory functions of NO (Cuthbertson, et al 1997, Poukens, et al 1998, Nickla and Wildsoet 2004).

Nickla and Wildsoet (2004) used L-NAME (N^G-nitro-l-arginine methyl ester), a non specific inhibitor of nitric oxide synthase (NOS) to study potential effects of NO in modulating choroidal thickness. L-NAME inhibited choroidal thickening in eyes recovering from form deprivation and in response to myopic defocus, in a rapid, transient, dose dependant manner. Nickla et al. (2005) demonstrated that inhibition of the transient choroidal response to visual manipulations through the use of this non specific NOS inhibitor has predictable, disinhibitory effects on ocular growth rate, whereby inhibition of the choroidal compensation prevents ocular growth rate compensation. Furthermore, Nickla et al. (2005) demonstrated that this effect is localized to the back of the eye; L-NAME has an inhibitory effect (opposite effect) on the growth of the anterior chamber. When L-NAME is injected *after* a period of visual exposure, after the choroid has already responded, there is no effect on ocular growth at the back of the eye, indicating that the effect of NO is via the visual (i.e. choroidal) effect, and not downstream of it. However, there is still an inhibitory

effect on the anterior chamber, demonstrating the independent mechanisms working on the anterior and posterior aspects of the eye (Nickla, et al. 2005).

The first step towards locating the source of the NO involved in the response is to determine which of the NOS isoforms are involved. eNOS and nNOS require calcium to bind to calmodulin for activation, resulting in short-lived release (minutes to hours). These two isoforms are more likely candidates for the transient choroidal response than iNOS, which is involved in immune responses, and in pathology such as retinal ischemia. iNOS activation, controlled at the level of transcription, is much longer lasting than the other forms, lasting for days at a time (For a review see: Dawson, 1994, Stewart 1994, Snyder 1992). The likelihood of eNOS versus nNOS as the source of the NO depends on the mechanism of the choroidal response. If the mechanism is mediated via smooth muscle, then the more probable candidate is nNOS, which has been localized in both the pterygopalatine terminals innervating the choroid and in intrinsic choroidal neurons (Schroedl, 1998; Poukens, 1998). If the mechanism involves vascular permeability or blood flow, then eNOS would be the more plausible candidate, as eNOS has been located in the vascular endothelium of the choroid.

Experimental Issues:

The purpose of this study was to use various relatively more specific inhibitors of the three isoforms of NOS to elucidate the source of the NO involved in the response of the chick eye to myopic defocus. In doing so, we hoped to clarify the role of NO in the signal

cascade in the visual regulation of eye growth. Localizing the site of this effect is a crucial step towards the development of pharmacological interventions in the progression of myopia.

Methods

Subjects:

Subjects were White Leghorn chicks (*Gallus gallus domesticus)*, hatched in an incubator and raised in temperature-controlled brooders. The light cycle was 12L/12D (8:00 am to 8:00 pm). Food and water were supplied ad libitum. In all experiments, the right eye was treated and the left eye served as the untreated control. Care and use of the animals conformed to the ARVO Resolution for the Care and Use of Animals in Research.

Drugs and Injections:

All drugs were dissolved in 0.9% physiological saline, and eyes were injected intravitrally using a Hamilton syringe and a 30G needle under isoflurane inhalation anesthesia. Injections were made by puncturing the sclera about 45 degrees superior to temporal. In the case of multiple injections, care was taken to use the same injection site. In cases of obvious ocular damage due to the injections (e.g. cloudy cornea or lens) the data were not used.

Experiment 1: Effect of TRIM on ocular dimensions in response to myopic defocus

A. Myopic defocus induced by prior form deprivation

One week old birds had a plastic diffuser glued to the feathers around one eye. After 7 days, the diffusers were removed and the experimental eyes received one injection of TRIM (0.3 µmole; n=4) or saline (n=4). Ocular dimensions (axial length, choroidal thickness, and anterior chamber depth) were measured with high-frequency A-scan ultrasonography under isoflurane inhalation anesthesia (ultrasound methodological details in Nickla et al., 1998) at time 0 (immediately prior to the injection), and at 7 hours, 24 hours and 48 hours post-injection. Refractions were measured with a Hartinger's refractometer at the end of the experiment.

B. Myopic defocus induced by positive spectacle lens wear

+10 D lenses were fitted to one eye of 2-week old chicks by attaching them to Velcro rings, the matching ring of which was glued to the feathers around the eye. One intravitreal injection of TRIM (1-(2-trifluoromethylphenyl) imidazole) (dose: 0.3 µmoles (n=7), 0.03 µmoles (n=8) and 0.003 µmoles (n=8)) or saline (n=12) was given immediately following lens-wear. Ocular dimensions and refractive errors were measured using the same protocol as above.

Experiment 2: Effect of TRIM on normal (untreated) eyes

Ocular dimensions

TRIM (30 µl 0.3 µmoles) was injected into normal (untreated) eyes of 2 week old chicks daily for 3 days (n=10). High frequency A-scan ultrasonography was done at time 0 (pre-injection) and at 7, 24 and 48 hours. Refractions were done using a Hartinger's refractometer at the end of the experiment. Multiple injections were used in order to assess a potential growth effect of the drug that might not be seen using only one injection.

Scleral GAG synthesis and choroidal NO content

Chicks received a single intravitreal injection (30 µl) of TRIM (dose: 0.3 µmoles (n=6) or physiological saline (n=4). Ocular dimensions were measured with high-frequency A-scan ultrasonography at time 0, and at 7hrs post-injection. Following the second ultrasound, the chicks were sacrificed, the eyes were dissected out, and a 7mm punch of the choroid and sclera was taken from the posterior pole of each eye. The choroids were cultured in DMEM for 4 hrs. Nitrates were measured using the inNO system (Innovative Instruments, Inc., Tanmpa, Fla.) as an indirect measure of NO levels in the tissue. The scleras were cultured in DMEM with radiolabeled Na_2SO_4 (S^{-35}; approximately 20µCi/ml; ARS, St. Louis, MO) at 37 degrees for 6 hours. Each sclera was placed in an eppendorf tube with proteinase K solution and placed in a 60 degree waterbath overnight to digest the tissue. Following digestion, glycosaminoglycans were precipitated with 0.05% cetylpyridinium chloride. The precipitated sample was collected on Whatman filters using a Millipore 12-port manifold. Radioactivity

measured by liquid scintillation counting was used as an index of proteoglycan synthesis. (Methodological details in Nickla et al., 1999)

Experiment 3: Effects of the non-specific NOS inhibitor L-NMMA on eyes responding to myopic defocus

At 12 days of age, chicks received a single intravitreal injection (30 µl) of N-methyl-L-arginine (L-NMMA) (dose: 0.12 µmoles (n=5), 1.2 µmoles (n=10), 4.8 µmoles (n=9), 20 µmoles (n=6) or physiological saline (n=6). +10 D lenses were immediately fitted to eyes following the injection. Ocular dimensions were measured with high-frequency A-scan ultrasonography at time 0, and at 7 hours, 24 hours, and 48 hours post-injection.

Experiment 4: Effects of other more specific NOS inhibitors on eyes responding to myopic defocus

At 5 days of age, chicks received a single intravitreal injection (30 µl) of either of the following: the nNOS inhibitor N-omega-L-arginine (dose: 0.6 µmoles, n=12) the iNOS inhibitor L-NIL (dose: 0.6 µmoles, n=5; 6.0 µmole, n=6), or the eNOS inhibitor L-NIO (dose: 0.6 µmoles, n=10; 6.0 µmoles, n=5), or physiological saline (n=18). +10 lenses were immediately fitted to eyes following injection. Ocular dimensions were measured with high-frequency A-scan ultrasonography at time 0, and at 7 hrs, and 24 hrs, and 48 hours post-injection. Refractions were measured using a Hartinger's refractometer at the end of the experiment.

Scleral GAG synthesis

Following the final measurement, eyes were dissected out and a 7mm punch was taken from the posterior scleras and incubated in medium for 2-4 hours. Scleral GAG synthesis was assayed as described above.

Statistical Analysis

Unpaired (two-tailed) t-tests were used in all comparisons between groups of eyes. In some cases ANOVAs were used to describe between-group differences.

Results

I. The effects of TRIM on eyes responding to myopic defocus

Choroidal Thickness and Refractive Error

Unlike L-NAME, injections of TRIM did *not* prevent the choroidal thickening in response to myopic defocus induced by either positive lenses (0.3 µmoles at 7hrs: vs saline controls: 114 µm vs 92 µm, p=0.56; Figure 1A) or prior form deprivation (drug vs saline at 7 hrs: 160 µm vs 128 µm, p=0.42; Figure 1B). In fact, at 24 hours, choroids tended to be even thicker than those injected with saline in both experimental groups (lens: 165 µm vs 105 µm, p=0.19; recovery: 239 vs 173 µm, p=0.29; figure 1) although neither difference was significant. Consistent with the suggestion that TRIM actually enhances choroidal thickening in response to myopic defocus (as opposed to having no effect at all) is the finding of a tendency for a dose-response effect when looking at data from the highest and lowest doses, and saline controls (figure 1C).

In accordance with the absence of a drug-induced effect on the choroidal response, the eyes responded to the myopic defocus induced by both lens-wear and prior form deprivation by becoming hyperopic (figure 2). There was no significant difference between drug- and saline-injected eyes in either group (figure 2A: lenses: 5.2 vs 5.8 D; Figure 2B: recovery: 8.5 vs 8.0 D). An ANOVA for drug, saline and fellow untreated eyes was significant in both groups ($p<0.001$ for both).

Ocular Elongation.

Again, in accord with the lack of choroidal inhibition, there was no significant difference between TRIM- and saline injected eyes in the rate of axial elongation. Both saline and TRIM-injected eyes inhibited their growth rate in response to lens-induced myopic defocus (48 hrs: 84 vs 53 µm; Figure 3A) and prior form deprivation-induced defocus (238 vs 146 µm; Figure 3B). (Note that the difference in rates between the two experimental groups reflects the fact that the recovering eyes begin with a much greater amount of myopia from the form deprivation than the lens-wearing eyes do and so are growing at a significantly

higher rate at the beginning of the defocus period). Interestingly however, the effect of TRIM was similar to that of L-NAME in having an initial inhibitory effect on the growth of the anterior chamber (figure 4A). At 24 hours, in the lens-wearing eyes, TRIM tended to result in a cessation of growth (drug vs saline: -3 vs 66 μm; p=0.06); this effect was gone by 48 hours. This transient inhibitory effect was dose-dependent (figure 4B), having an ED50= 10 μmole.

II. The effects of TRIM on normal eyes

Nitric Oxide Content and IOP

The fact that TRIM had anomalous effects on the axial components (choroidal and growth responses) in the above experiments suggested that it is not working as a NOS inhibitor in our system. To test this, we measured NO in choroids of untreated TRIM-injected eyes and saline-injected control eyes. We found that eyes treated with TRIM did indeed produce higher (as opposed to lower) amounts of NO in culture 7 hrs after injection (drug vs control, 20 vs 8.9 nmol; $p<0.05$; figure 5A). Therefore, it appears that TRIM does NOT act as a NOS inhibitor in this system.

Because some NOS inhibitors have an inhibitiory effect on IOP, we measured IOP in TRIM-injected eyes and found that TRIM resulted in a small but significant decrease in IOP at 24 hours post-injection (pre- vs post-injection: 15.3 vs 11.9 mmHg; figure 5B). This effect might play a role in the inhibition of ocular elongation.

Choroidal thickness and Refractive error

Injections of TRIM into untreated eyes resulted in a very rapid increase in choroidal thickness, showing significant thickening within one hour (change in thickness, drug vs fellow controls: 67 vs –32 μm, $p<0.05$; figure 6). The effect was sustained for at least 7 hours (drug vs fellow and saline controls: 84 vs18 and –17 μm, respectively, $p<0.01$ for both, figure 6). At 24 hours the choroid had become thinner than the pre-treatment measurement (drug vs fellow: -34 vs –7 μm). There was no significant effect of TRIM on refractive error in these eyes (data not shown).

Ocular Elongation

At 24 hours, the axial elongation in TRIM-injected eyes was lower than saline-injected controls, however, the effect did not reach significance (74 vs 106 μm; p=0.1; figure 7). At 48 hours however, the inhibition was significant when comparing the drug-injected to their fellow controls (84 vs 229 μm; p<0.0001) (note: saline data not available from this experiment). By contrast to its effect on eyes exposed to myopic defocus, TRIM did *not* have any effect on the growth of the anterior chamber at 24 hours (drug vs. saline: 117 vs 71 μm, p=0.2; data not shown).

Proteoglycan Synthesis

If the final pathway in the underlying mechanism for growth inhibition by TRIM is similar to all other visual means of inducing the decrease, then TRIM should result in an inhibition in scleral proteoglycan synthesis. We measured scleral proteoglycan synthesis in these eyes at 7 hours after the injection, and at 24 hours. We found that at 7 hours, scleral PG synthesis tended to be lower than saline-injected controls (392 vs 688 CPMs, p=0.07, data not shown). This effect was significant at 24 hours after the injection (7752 vs 11128 DPMs, p<0.005, figure 8).

III. The effects of some specific NOS inhibitors on eyes responding to myopic defocus

Choroidal thickness and Refractive error

We tested three other more specific NOS inhibitors of the different isoenzymes; these data are presented together. Eyes injected with N-omega-propyl-L-arginine resulted in significant inhibition in the choroidal response to myopic defocus induced by positive lenses at 7 hours (drug vs saline controls: 13 vs 116 μm, p<0.01; Figure 9). By 24 hours, while the difference between drug and saline controls was still significant, there was a trend toward a choroidal response (57 vs 127 μm, p=0.04). There was no significant difference between eyes injected with N-omega and fellow control eyes (drug vs fellows: 13 vs –12 μm at 7hrs; -

6 vs –27μm at 24 hrs). Neither L-NIL nor L-NIO had this inhibitory effect: choroids of these eyes showed an increase in thickness in response to the myopic defocus similar to that of saline treated eyes at both 7 and 24 hours (L-NIO and L-NIL vs saline controls at 7 hrs: 75, 103 and 116 μm; figure 9). All of these were significantly different from fellow contol eyes at $p<0.01$.

In accord with this inhibition of the choroidal response by N-omega-propyl-L-arginine , the refractive response of these eyes was also attenuated at 48 hours (i.e. they did not become hyperopic in reponse to the +10D lens) (drug vs saline: +1.4D vs +5.2D; $p<0.01$; Figure 10). There were no significant differences between eyes injected with N-omega-propyl-L-arginine and fellow control eyes (1.4D vs 0.8D). Neither of the other specific inhibitors of NOS showed this effect; there were no significant differences between eyes injected with L-NIO, L-NIL, or saline controls at 48 hours (respectively: +4.2 D, +3.6 D, +5.2D).

Ocular Elongation

As expected if N-omega-propyl-L-arginine mimicked the effects of L-NAME (i.e. that the nNOS inhibitor was the relevant one), eyes injected with this drug *disinhibited* axial growth; the rate of eye growth was similar to that of fellow controls (Figure 11: 102 and 115 μm/24 hours, n.s.) and significantly *higher* than saline injected ones (102 vs 48 μm, $p<0.05$). Neither L-NIO nor L-NIL demonstrated this effect; their rate of growth did not differ from that of saline controls (respectively: 52, 37 and 48 μm; Figure 11). At 48 hours, there were no significant differences.

By contrast to the effect of TRIM, there were no significant differences in the growth of the anterior chamber over the times measured (data not shown).

Proteoglycan synthesis

N-omega prevented the inhibition of scleral GAG synthesis in response to plus lenses; these did not differ from untreated fellow controls (6571 dpm vs 6338 dpm; n.s.; figure 12A) but were significantly higher than that of both saline (4816 dpm; $p<0.05$) and L-NIL-injected eyes (3994 dpm; $p=0.05$). Similarly, L-NIO inhibited PG synthesis relative to fellow controls

(there were no saline controls in this experiment; figure 12B; $p<0.05$). Therefore, the disinhibition by N-omega-propyl-L-arginine is presumably effected at the level of scleral proteoglycan synthesis, as is the TRIM growth *inhibition*.

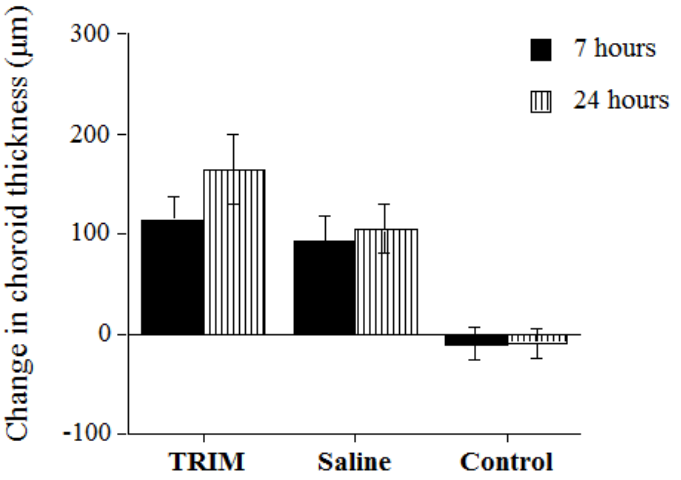
IV. The effects of another non-specific NOS inhibitor (L-NMMA)

Another L-arginine analog that acts as a non-specific NOS inhibitor is L-NMMA. This drug had similar effects on the choroid as did L-NAME: it inhibited the choroidal expansion in response to myopic defocus induced by positive lenses (figure 13A). However, the effect was not as rapid or strong as that of L-NAME: at 7 hours when L-NAME shows complete inhibition (no response), there was no difference between L-NMMA and saline (74 vs 82 μm). The effect reached significance at 24 hours (84 vs 171 μm; $p<0.05$), whereas the effect is reversed by 24 hours for L-NAME, and was gone at 48 hours (186 vs 225 μm). The effect at 24 hours was dose-dependent with an ED50=2.3 umoles. (figure 13B).

There was also a significant inhibition in the growth of the anterior chamber, similar to that of L-NAME, at one of the high doses (4.8 μmoles: 25 vs 85 μm, one-tailed t-test $p<0.05$; figure 14). However, regarding the effect on axial elongation, while the drug-injected eyes tended to grow faster over 48 hours, the difference was not significant (204 vs 142 μm; $p=0.3$; data not shown).

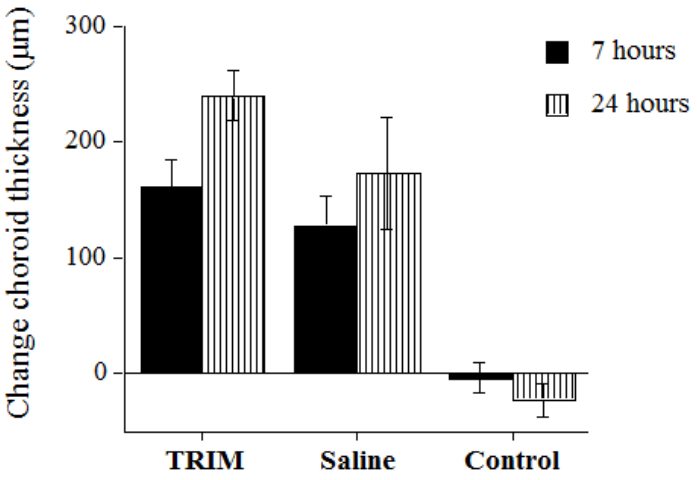
Figure 1

A.



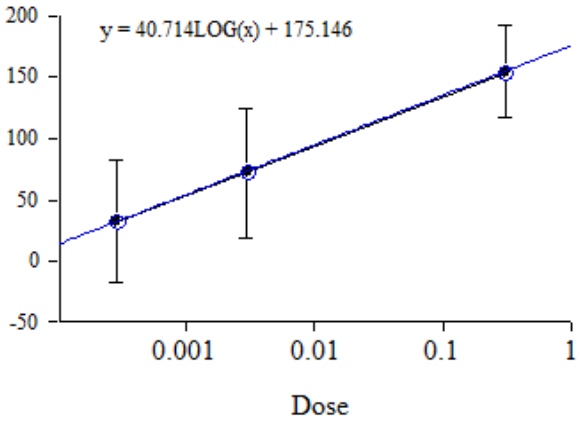
1a. Change in choroidal thickness from baseline in plus lens wearing eyes following injection of TRIM or Saline versus fellow control eyes. TRIM did not prevent choroidal thickening in response to myopic defocus (0.3 umoles at 7 hrs: vs saline controls: 114 um vs 92um, p=0.56). Choroids of TRIM injected eyes tended to be thicker than those injected with saline at 24 hrs.

B



1b. Change in choroidal thickness from baseline in eyes recovering from prior form deprivation following injection of TRIM or Saline versus fellow control eyes. TRIM did not prevent choroidal thickening in response to myopic defocus (drug vs saline controls at 7hrs: 165 um vs 105um, p=0.29). Choroids of TRIM injected eyes tended to be thicker than those injected with saline.

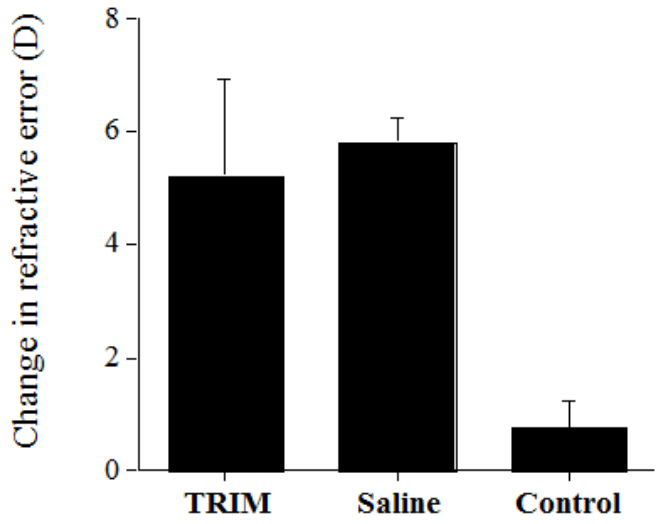
C.



1c. Tendancy towards a dose-response effect, with dose of TRIM shown on the X axis, and change in choroidal thickness over 24 hours on the Y axis.

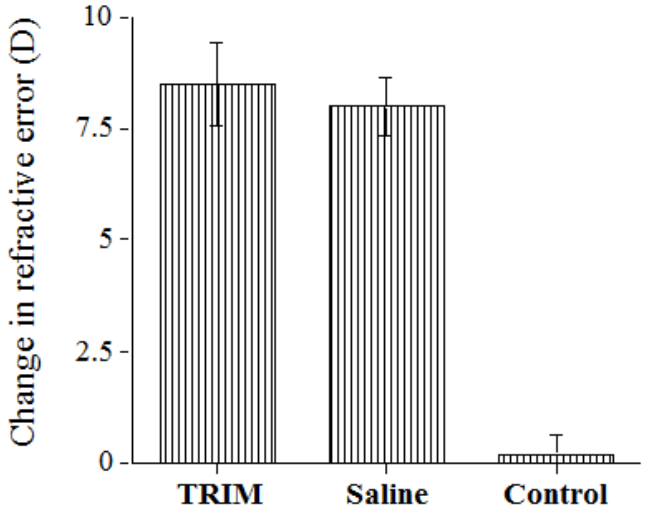
Figure 2

A.



2a. Change in refractive error from baseline after 48 hours of plus lens wear in eyes injected with either TRIM or Saline versus fellow control eyes. There is no significant difference between saline and TRIM injected eyes (+5.2 vs +5.8D), both became significantly hyperopic relative to untreated fellows.

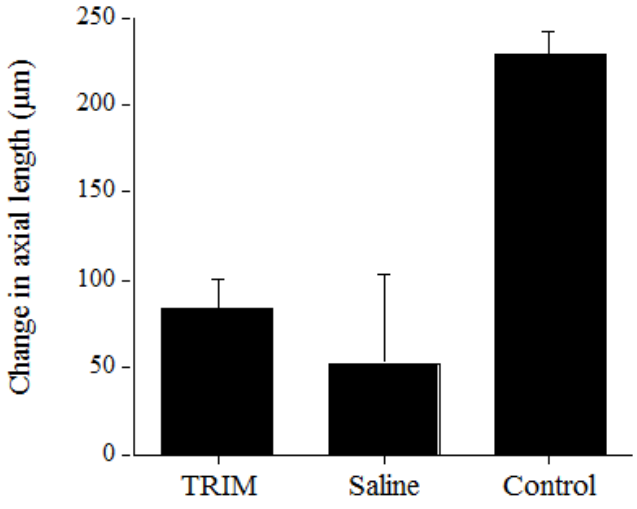
B.



2b. Change in refractive error from baseline 48 hours following removal of diffusers and injection with either TRIM or Saline versus fellow control eyes. There is no significant difference between saline and TRIM injected eyes (+8.5 vs +8.0D), both became significantly hyperopic relative to untreated fellows.

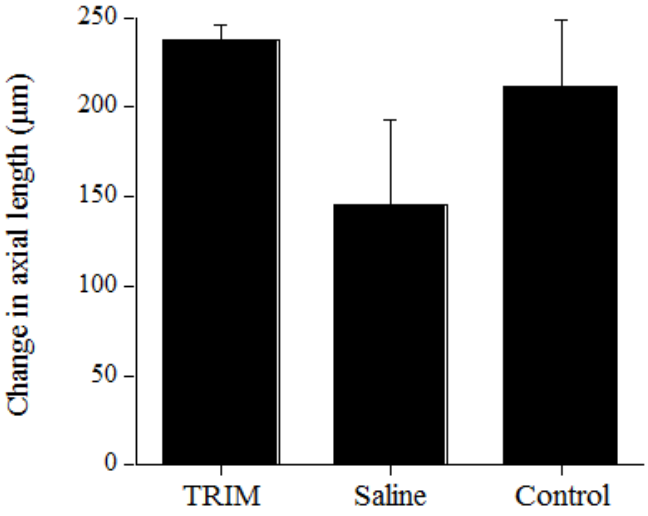
Figure 3

A



3a. Change in axial length from baseline over 48 hours in plus lens wearing eyes following injection of TRIM or Saline vs fellow control eyes. There is no significant difference between TRIM and Saline injected eyes. (84 vs 53um)

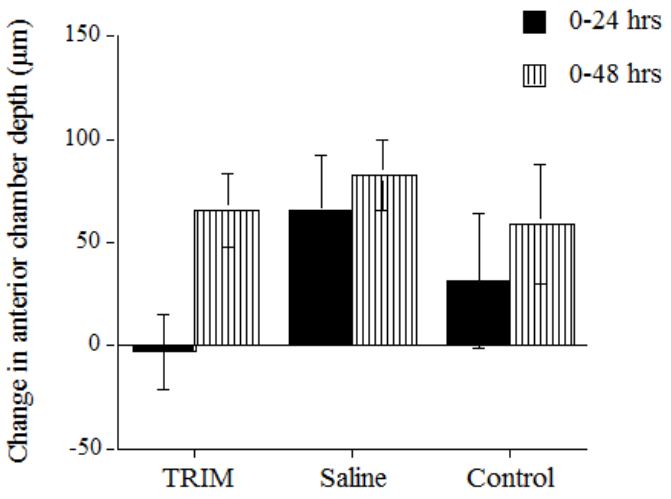
B.



3b. Change in axial length from baseline over 48 hours following removal of diffusers and injection of TRIM or Saline vs fellow control eyes. There is no significant difference between TRIM and Saline injected eyes. (238 vs 146 um)

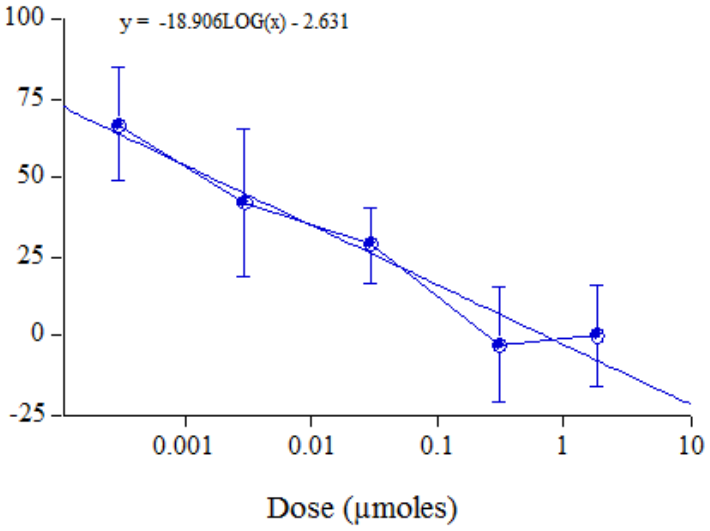
Figure 4

A.



4a. Change in anterior chamber depth from baseline in lens wearing eyes injected with TRIM or Saline vs untreated fellows. Trim resulted in cessation of growth at 24 hours, the effect was gone by 48 hours.

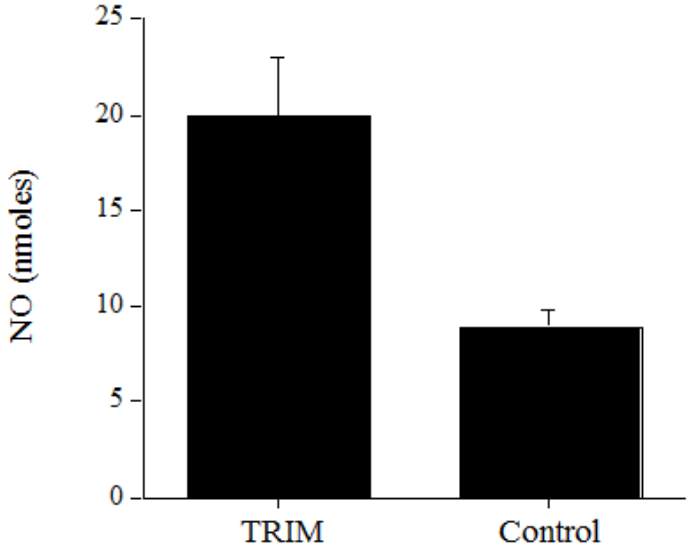
B.



4b. This effect was dose dependant. Dose of TRIM is shown on the X-axis, change in anterior chamber depth in um over 24 hours is shown on the Y-axis.

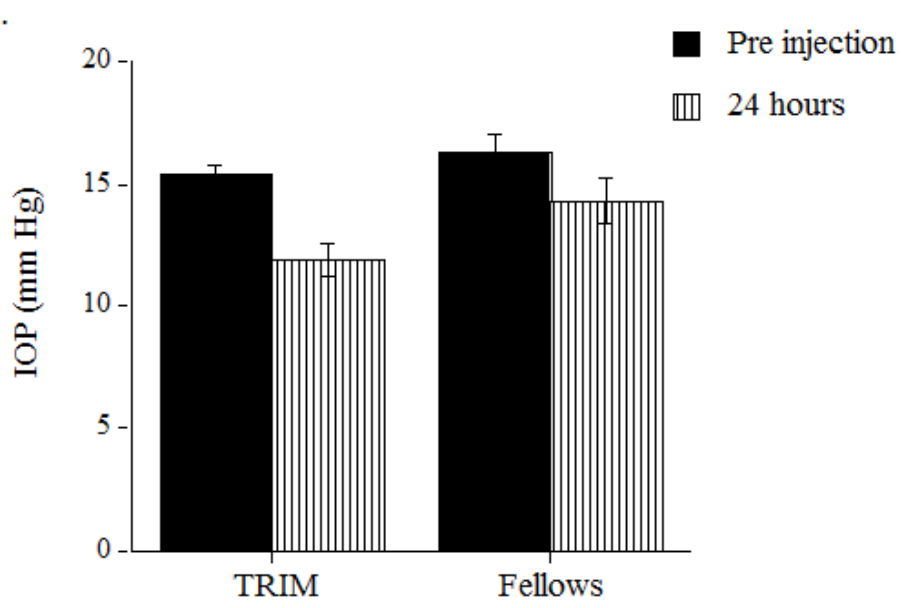
Figure 5

A



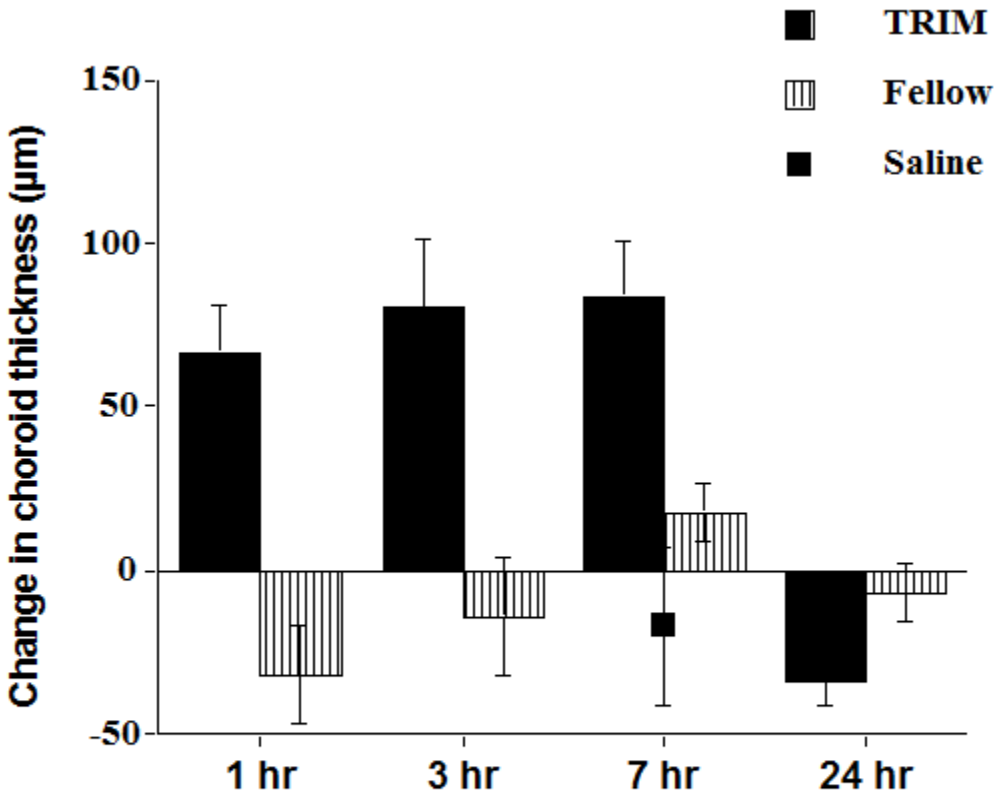
5a. Amount of NO in culture 7 hours after injection in Trim injected vs control eyes. (20 nmol vs 8.9 nmol; $p<0.05$)

B.



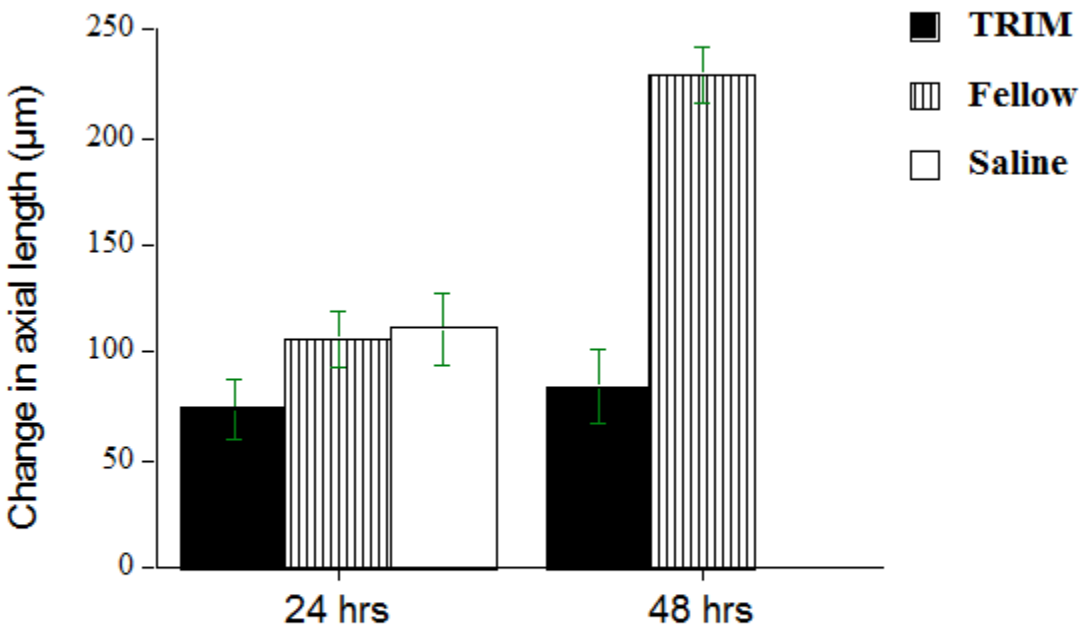
5b. IOP in mm Hg, measured pre injection and 24 hours post injection of TRIM vs fellow controls.

Figure 6



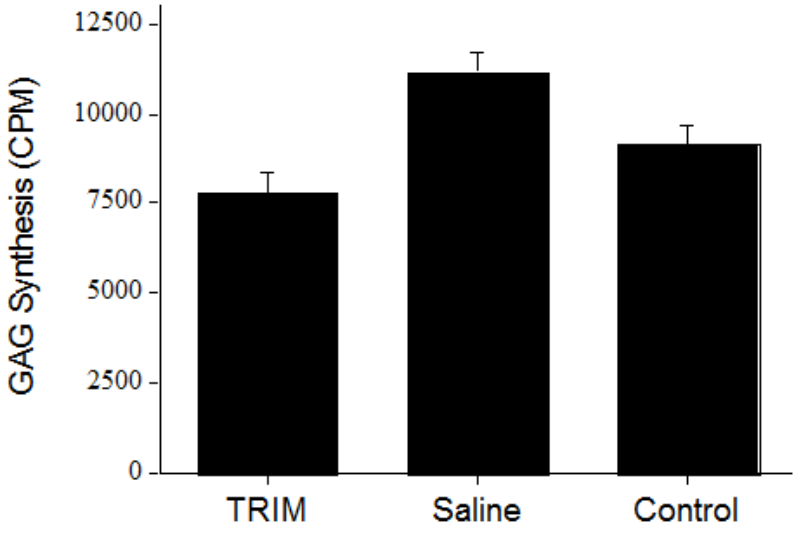
6. Change in choroidal thickness from baseline in untreated eyes injected with TRIM vs fellow control eyes. Saline data dot shown at 7 hours. TRIM injection resulted in rapid increase in choroidal thickness sustained for at least 7 hours. (67 vs -32 um at 1 hour, $p<0.05$).

Figure 7



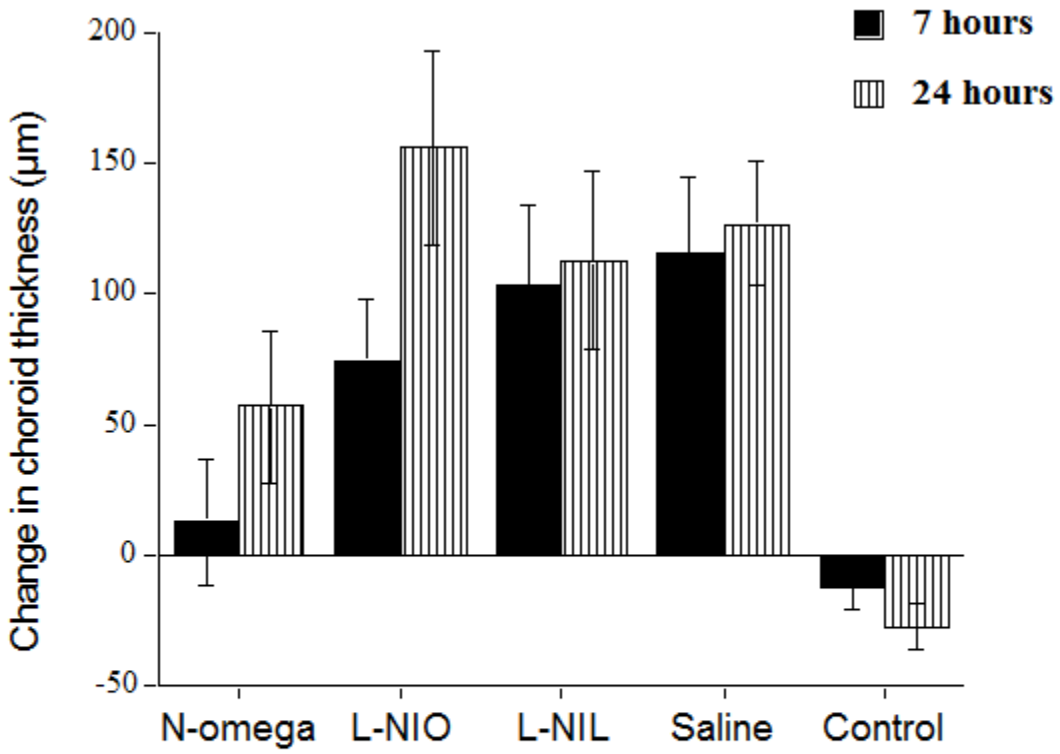
7. Change in axial length from baseline in untreated eyes injected with TRIM or saline vs fellow controls. Axial elongation is lower in TRIM vs saline injected eyes, the effect is not significant at 24 hours (74 vs 106 um; p=0.1), and is significant at 48 hours (84 vs 229 um; p<0.0001).

Figure 8



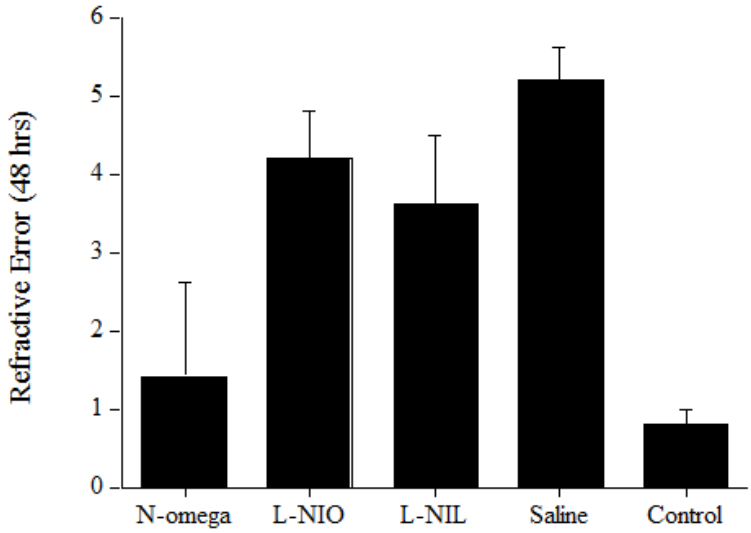
8. Scleral proteoglycan synthesis 24 hours after injection of TRIM or saline in untreated eyes vs control eyes, in CPM. Scleral PG synthesis is lower in TRIM vs saline injected eyes. (392 vs 688 CPM, p=0.07)

Figure 9



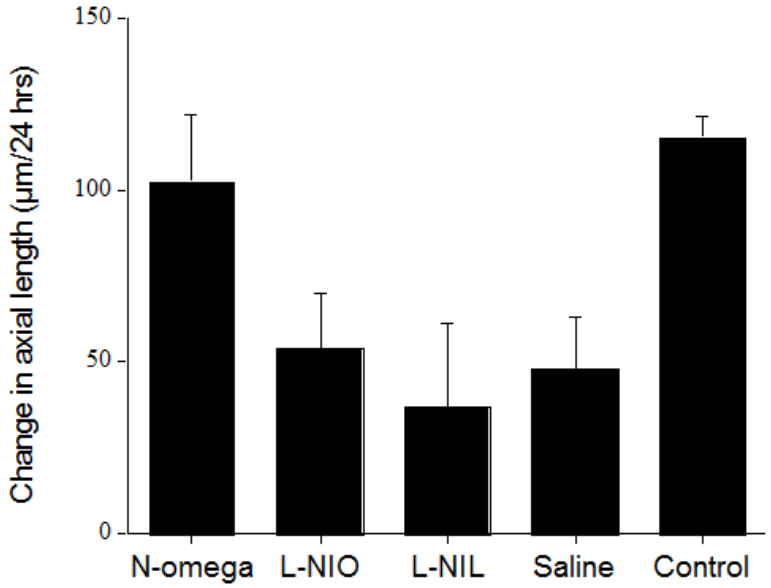
9. Change in choroidal thickness from baseline of plus lens wearing eyes injected with drug or saline vs fellow untreated controls. N-Omega shows significant inhibition of choroidal thickening at 7 hours vs saline controls. (13 vs 116 um, $p<0.01$). There was no significant difference between eyes injected with N-Omega vs fellow controls.

Figure 10



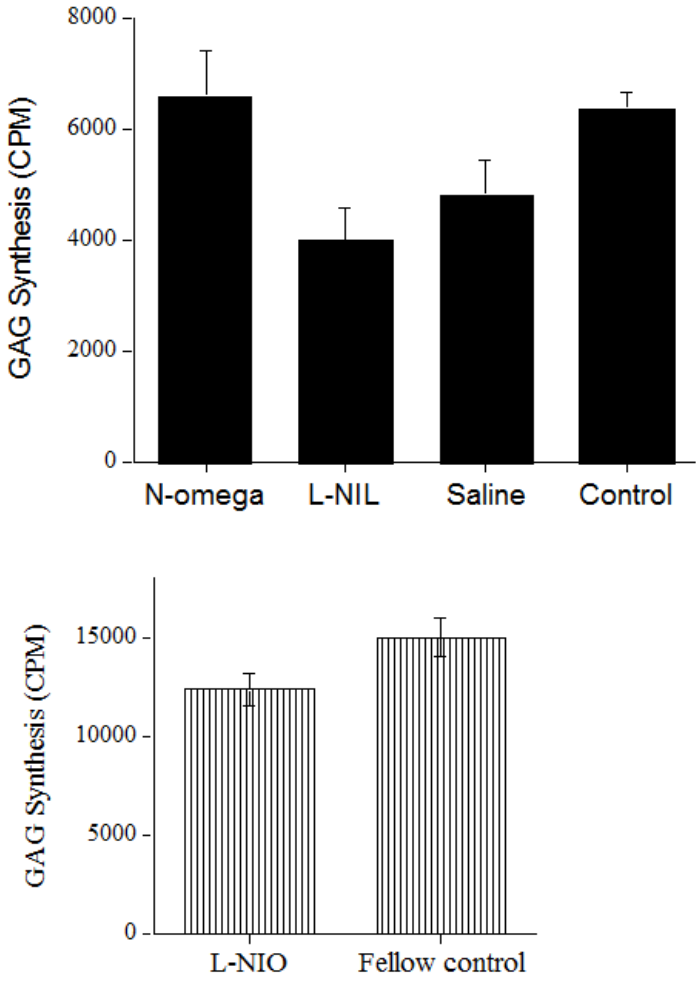
10. Refractive error at 48 hours of plus lens wearing eyes injected with drug or saline vs fellow untreated controls. Eyes injected with N-Omega showed significantly attenuated refractive compensation to plus lens wear relative to saline treated eyes by 48 hours. (+1.4D vs +5.2D; $p<0.01$)

Figure 11



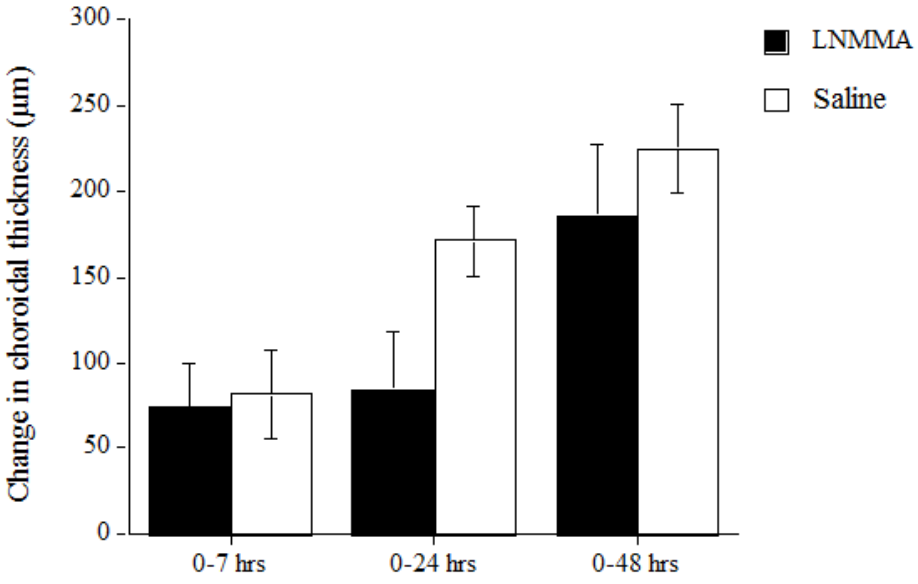
11. Change in axial length from baseline over 24 hours in plus lens wearing eyes injected with drug or saline vs fellow untreated controls. There is no significant difference between eyes injected with N-Omega and fellow untreated controls (102 vs 115 um), and significantly more growth of eyes injected with N-Omega vs saline injected lens-wearing eyes (102 vs 48um, $p<0.05$)

Figure 12



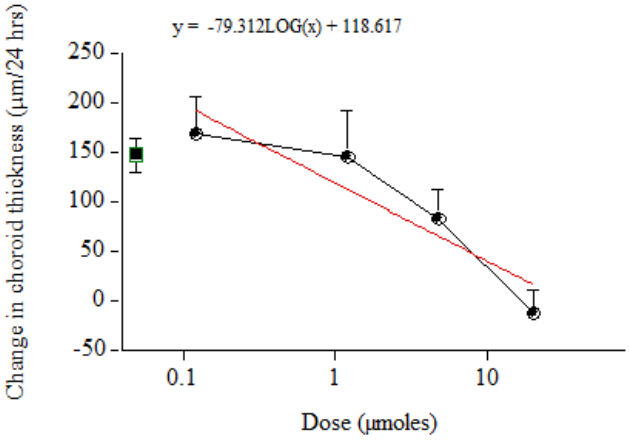
12. Scleral proteoglycan synthesis in plus lens wearing eye injected with drug or saline vs fellow untreated eyes. N-Omega prevented inhibition of scleral GAG synthesis: there was no significant difference between N-Omega injected eyes and fellow controls (6571 vs 6338 cpm) .

Figure 13



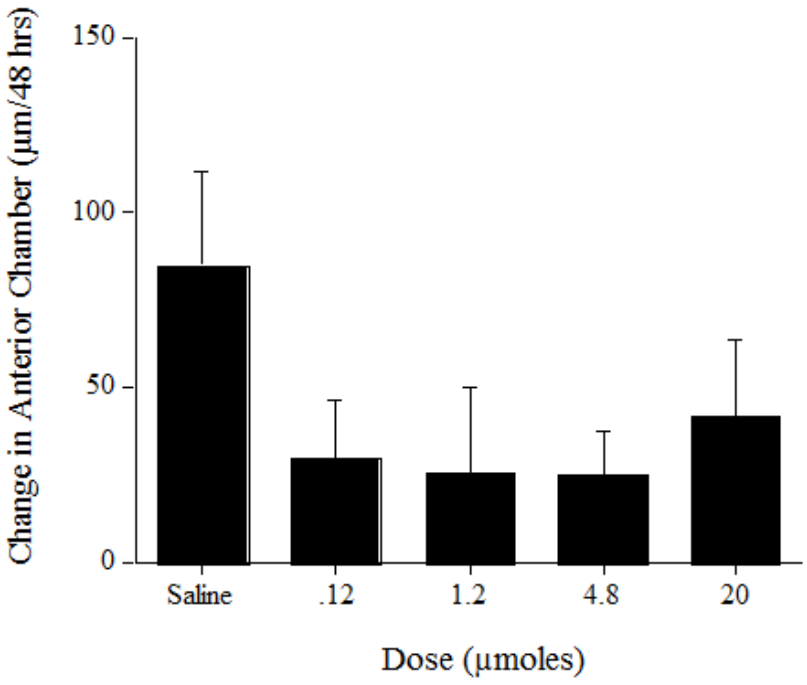
13a. Change in choroidal thickness of plus lens wearing eyes injected with L-NMMA vs saline. LNMMA inhibited choroidal thickening relative to saline treated eyes at 24 hours (84 vs 171 um, $p<0.05$).

13B



13b. The choroidal response to LNMMA is dose dependant at 24 hours.

Figure 14



14. Change in anterior chamber depth over 24 hours in lens wearing eyes injected with saline or L-NMMA. There is significant inhibition in growth of the anterior chamber at one of the high doses vs saline. (4.8 umoles: 25 vs 85um, $p<0.05$).

Discussion

The central finding of this project is that injection of N $^{\omega}$-propyl-L-arginine had results consistent with that of the non-specific NO inhibitor L-NAME. Injection of N $^{\omega}$-propyl-L-arginine prevented chick eyes from compensating to myopic defocus. These eyes did not show the choroidal thickening or halted axial growth normally seen in response to plus lens wear, and therefore a change in refractive error did not occur. N $^{\omega}$-propyl-L-arginine is a specific inhibitor of neuronal nitric oxide synthase, indicating that compensation to myopic defocus does not occur when the production of neuronal nitric oxide is prevented. These results are consistent with the hypothesis that neuronal nitric oxide has a modulatory role in this response.

There are a few different candidates for the specific source of the neuronal nitric oxide involved in the response. A local source, from within the choroid, is more plausible than a retinal source, which would require NO to traverse half the thickness of the retina and to cross both the RPE and Bruchs membrane in order to reach the choroid. Possible candidates for a choroidal source are the intrinsic choroidal neurons, parasympathetic innervations from the ciliary ganglion, or parasympathetic innervations from the pterygopalatine ganglion.

Work has been done to elucidate whether one or both parasympathetic ganglia are involved in regulation of choroidal thickness. While removal of ciliary input via ciliary ganglionectomy resulted in increased choroidal thickness in normal eyes, it had no effect on the choroidal response of eyes responding to prior form deprivation (Schmid, 1992). Likewise, transsection of CN VII, removing pterygopalatine input, did not prevent the

choroidal response the myopic defocus (Schroedl, 2006) This suggests that neither ganglion alone strongly influences the compensatory choroidal response, yet the response is significantly attenuated when *both* ganglia are lesioned (Nickla, 2008). There is likely redundancy in the two pathways, therefore, with the two sharing control of the choroidal response, and potentially the growth response.

It has not yet been determined whether the intrinsic choroidal neurons are also involved in the response. Further study necessitates the development of a pharmacological means of preventing the action of these cells.

The other key finding in this study is the concurrence of axial growth dis-inhibition with the prevention of choroidal thickening following injection of N $^{\omega}$-propyl-L-arginine in the setting of myopic defocus. As with studies involving L-NAME, these responses appear mechanistically linked, whereby the choroidal response always precedes the axial response; and prevention of the choroidal response also consistently results in prevention of the axial growth response. The link between these two responses is not yet understood: NO could act directly on the sclera to disinhibit scleral GAG synthesis, or it may act as part of a signal cascade, whereby it influences another molecule leading to this effect. Summers-Rada et al demonstrated that thickened choroids recovering from form deprivation myopia show significantly increased permeability, and that suprachoroidal fluid recovered from these eyes significantly inhibits scleral GAG synthesis, supporting a role for the delivery of a signal molecule to the sclera (2007). A potential candidate for a second molecule in the signal cascade is retinoic acid. Retinoic acid is a potent inhibitor of scleral GAG synthesis, levels of this molecule are increased in choroids that have thickened in response to myopic defocus,

and decreased in the thinner choroids of eyes rapidly growing towards myopia (Mertz, 1999). Injection of L-NAME has been shown to reduce levels of choroidal retinoic acid supports the suggestion of a causal link between the two molecules (Nickla, 2003).

The results of this study demonstrated that an injection of TRIM had an anomalous effect on NO production in chick eyes, producing *increased* levels of NO in eyes injected with the drug. TRIM did not act as a NO inhibitor in these eyes. It is not surprising, therefore, that injections of TRIM did not produce the inhibition of the compensatory response to myopic defocus demonstrated in eyes injected with L-NAME.

It is interesting to note that TRIM injections did have an effect on eyes not treated with lenses, demonstrating an increase in choroidal thickness in these eyes, and a concomitant decrease in scleral proteoglycan production. Although TRIM did not act as a nitric oxide synthase inhibitor in the posterior chamber, the result that injection of TRIM leads both to increased NO production as well as increased choroidal thickness and slowed ocular growth provides further evidence linking these responses. TRIM injected eyes responded similarly to non-injected eyes receiving myopic defocus in regards to choroidal thickening and inhibition of proteoglycan synthesis, yet these eyes showed an inhibition of anterior chamber growth not seen in non-injected eyes responding to myopic defocus. The disparate effects of TRIM on the anterior chamber and overall axial length of the eye suggest that there may be separate mechanisms influencing the anterior and posterior segment reactions to myopic defocus.

The suggestion that there are separate mechanisms influencing the anterior and posterior segment responses has support in the response to N $^{\omega}$-propyl-L-arginine. While

both L-NAME and L-NMMA inhibit the growth of the anterior chamber in the setting of myopic defocus, N $^{\omega}$-propyl-L-arginine had no effect on anterior chamber growth. On first thought one might suggest that these less specific inhibitors impact the anterior chamber due to their actions on the other sources of NO, however neither L-NIL nor L-NIO, specific inhibitors of iNOS and eNOS respectively, had any significant effect on growth of the anterior chamber. Therefore, L-NAME and L-NMMA must have some other action in the anterior chamber. L-NAME and L-NMMA may act as muscarinic antagonists in this portion of the eye (Buxton, 1993), thereby acting on an entirely separate signaling system. Known muscarinic antagonists, atropine and pirenzapine, inhibit growth at the back of the eye, demonstrating both that it is plausible that a muscarinic antagonist would have an inhibitory effect on the growth of the anterior chamber while simultaneously providing evidence that L-NAME and L-NMMA are *not* acting as on muscarinic receptors in the posterior segment.

Other studies have shown findings consistent with separate mechanisms at the front and back of the eye, through both visual and pharmacological manipulations. For example, constant light results in corneal flattening and elongation of the vitreous chamber (Lauber, 1961; Li, 1995). Similar results are obtained with pharmacological studies: topical melatonin, for instance, leads to a decrease in anterior chamber depth without a resulting decrease in axial length: results instead show a (non-significant) increase in vitreous chamber depth (Summers Rada, 2006).

In conclusion the results of this study support the hypothesis that neuronal nitric oxide synthase is the principal source of the NO involved in the choroidal response to myopic defocus. Furthermore, it is possible that NO in the choroid regulates axial growth of

the eye, potentially through interactions with retinoic acid. The source of this neuronal nitric oxide may be the axon terminals of the parasympathetic ganglia innervating the choroid, or the intrinsic choroidal neurons.

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