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Concentration-related effects of nitric oxide and endothelin-1 on human trabecular meshwork cell contractility

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Abstract

The contractility status of trabecular meshwork (TM) cells influences aqueous humor outflow resistance and intraocular pressure. Using human TM cells as a model, the goal of the present study was to examine concentration-response relationships of two prototypical molecules, nitric oxide (NO) and endothelin-1 (ET-1), known to differentially influence vascular smooth muscle contractility. Efficacy of ET-1, two NO donors (DETA-NO and SNP) and a cGMP analogue (8-Br-cGMP) were assessed using two complementary methods: functionally in a gel contraction assay and biochemically using a myosin light chain phosphorylation assay. The NO donors DETA-NO and SNP dose dependently relaxed cultured human TM cells (EC_{50} for DETA-NO = $6.0 \pm 2.4 \mu M$, SNP = $12.6 \pm 8.8 \mu M$), with maximum effects at $100 \mu M$. Interestingly, at concentrations of NO donors above $100 \mu M$, the relaxing effect was lost. Relaxation caused by DETA-NO ($100 \mu M$) was dose dependently blocked by the soluble guanylate cyclase specific inhibitor ODQ ($IC_{50} = 460 \pm 190 nM$). In contrast to the NO donors, treatment of cells with the cGMP analog, 8-Br-cGMP produced the largest relaxation (109.4%) that persisted at high concentrations ($EC_{50} = 110 \pm 40 \mu M$). ET-1 caused a dose-dependent contraction of human TM cells ($EC_{50} = 1.5 \pm 0.5 pM$), with maximum effect at $100 pM$ (56.1%) and this contraction was reversed by DETA-NO ($100 \mu M$). Consistent with functional data, phosphorylation status of myosin light chain was dose dependently reduced with DETA-NO, and increased with ET-1. Together, data show that TM cells rapidly change their contractility status over a wide dynamic range, well suited for the regulation of outflow resistance and intraocular pressure.

Keywords

Glaucoma; Aqueous Humor; Schlemm's canal; Conventional Outflow

1. Introduction

A leading cause of blindness worldwide is glaucoma, a heterogeneous group of eye diseases characterized by a permanent loss of vision due to death of retinal ganglion cells. The most

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common form of glaucoma is primary open angle glaucoma (POAG)(Quigley, 1996), in which age and elevated intraocular pressure (IOP) are the two major risk factors. Elevated IOP in POAG is caused by incompletely understood dysfunction in the primary (conventional) drainage route for aqueous humor from the eye(Grant, 1951). Lowering IOP has been shown to prevent progression of vision loss (2000) but current pharmacological therapies do not target the diseased conventional outflow pathway. Efforts are underway to identify druggable targets in the conventional outflow pathway to decrease outflow resistance in the juxtacanalicular region, where IOP is controlled and trabecular meshwork (TM) and Schlemm's canal (SC) cells interact. The paracrine signaling relationship between the TM and SC may be analogous to that of vascular smooth muscle and endothelium, which work together to control vascular tone.

Two mediators that have opposing effects on vascular tone/endothelial permeability and possibly conventional outflow facility are nitric oxide (NO) and endothelin-1 (ET-1) (Pang and Yorio, 1997; Underwood et al., 1999; Wiederholt et al., 2000). ET-1 is a peptide released by the vascular endothelium (O'Brien et al., 1987) that is both a potent vasoconstrictor (Yanagisawa et al., 1988) and inhibitor of endothelial permeability (Filep et al., 1991). ET-1 signals through the G-protein-coupled ETA and ETB receptors, affecting intracellular calcium signaling, vascular tone(Sumner et al., 1992) and permeability(Filep et al., 1993). In contrast, NO's effects on the vasculature are not through a traditional receptor, but via activation of the enzyme soluble guanylate cyclase (Braugher et al., 1979). NO is a gas that freely and rapidly diffuses across cell membranes. Once NO binds to the heme moiety of sGC, the enzyme catalyzes the conversion of GTP to the second messenger cGMP. Increases in intracellular cGMP mediates many of the effects on the vasculature, including vasorelaxation (Gruetter et al., 1981; Kukovetz et al., 1979; Napoli et al., 1980) and altered vascular permeability (Draijer et al., 1995; Meyer and Huxley, 1992).

TM tissue and cells possess contractile properties that are responsive to NO and ET-1, similar to vascular smooth muscle cells. Isolated bovine TM strips in organ bath experiments have been used to study tissue contractility. In these studies, pilocarpine or carbachol elicited potent contractions of the TM strips, and this contraction was reversed with the application of NO donors or the cGMP analog 8-Br-cGMP(Wiederholt et al., 1996; Wiederholt et al., 1994). These results are consistent with the effects of these drugs on isolated aortic rings or arteries(Luscher, 1990). Both vascular smooth muscle and TM cell contraction has been attributed to activation of intracellular kinases like protein kinase C and Rho-associated protein kinase, which regulate myosin light chain phosphorylation through mechanisms partially independent of intracellular calcium concentration(Renieri et al., 2008). In both cell types the large conductance calcium activated potassium (BK_{Ca}) channel plays a major role in regulating the contractile state of the cells(Holland et al., 1996; Stumpff et al., 1997). In fact, direct activation of the BK_{Ca} channel results in cell relaxation and increased outflow in the eye(Dismuke and Ellis, 2009).

Similarly, NO increases outflow facility and lowers IOP in the eye. Administration of NO donors or overexpression of NO producing enzyme endothelial nitric oxide synthase (eNOS) results in increased outflow facility and/or decreased IOP in a number of animals including humans (Dismuke et al., 2008; Heyne et al., 2013; Larsson et al., 1995; Nathanson, 1992; Stamer et al., 2011). Nitric oxide synthase has also been detected in the tissues of the outflow pathway (Nathanson and McKee, 1995) and inhibition of this enzyme results in decreased outflow (Schneemann et al., 2002). Compared to NO, much less is understood about ET-1's effect on outflow facility and IOP. ET-1 mRNA transcripts and protein have been found in numerous tissues of the eye(Fernandez-Durango et al., 2003; Wollensak et al., 1998), aqueous humor contains ET-1 (Kallberg et al., 2002; Kallberg et al., 2007; Lepple-Wienhues et al., 1992), and the ciliary muscle, Schlemm's canal endothelium and trabecular

meshwork express endothelin receptors (Fernandez-Durango et al., 2003). Thus far, a consensus on the effect of ET-1 on conventional outflow and IOP has not been reached, which may be due to species differences (Millar et al., 1998; Taniguchi et al., 1994), a biphasic effect (Okada et al., 1995), effects on the TM versus the ciliary muscle (Cellini et al., 2006; Renieri et al., 2008) and/or the presence of multiple endothelin receptors (Taniguchi et al., 1996; Wang et al., 2011). More work needs to be done to resolve these apparent inconsistencies.

In a systematic manner, the concentration-response relationships between NO and ET-1 on human TM cell contraction are established in the present study. Using a functional collagen gel contraction assay and a biochemical myosin light chain phosphorylation assay, we constructed concentration-response curves and estimated EC₅₀ values for two different classes of NO donors, 8-Br-cGMP, ET-1 and the sGC inhibitor, ODQ. By determining the concentration-response relationship between these compounds and the TM cell contraction state, we provide a pharmacological framework for future experiments examining outflow physiology.

2. Methods

Materials and Reagents

The drugs used in the present study include: diethylenetriamine/nitric oxide adduct (DETA-NO), sodium nitroprusside dehydrate (SNP), endothelin-1 (ET-1), 8-bromoguanosine 3'5'-cyclic monophosphate sodium Salt (8-Br-cGMP) and 1-H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) are from Sigma-Aldrich. The antibodies (p-MLC and MLC) are from Cell Signaling (Danvers, MA).

Human Trabecular Meshwork Cell culture

Human TM cells were isolated from donor eyes using a blunt dissection technique followed by an extracellular matrix digestion protocol (Stamer et al., 1995). Four cell strains (TM 86 from 3 months donor, TM96 from 28 years donor, TM98 from 54 years donor and TM93 from 35 years donor) were characterized as described previously. Cells were cultured in low glucose DMEM (Dulbecco's modified Eagles's medium), containing 10% FBS (fetal bovine serum), 100 U/ml penicillin, 0.1mg/ml streptomycin and 0.29mg/ml glutamine and maintained in humidified air containing 5% CO₂ at 37°C.

Contractility assay and treatments

Collagen gels (1.41mg/mL) were prepared in 24 well plates from rat tail collagen type I (1.5mg/ml) (BD Bioscience, Bedford MA) following the manufacturer's instructions (BD Bioscience, Bedford MA). The contraction assay protocol from Luna, C. *et al* (Luna et al., 2012) with some modifications was followed (diagramed in Figure 1A). Briefly, trypsinized human TM cells in DMEM containing 10% FBS were seeded (1.5×10^5 /cm²) onto a collagen gel. The next day the media was changed to warmed serum-free. After 24 hours of incubation, the edge of the gel was gently detached from around the sides of the culture plate well using a pipette tip. The area of the gel was imaged (Syngene) every hour for 15 hours to determine the time until cessation of "natural contraction" of gel by TM cells (Figure 1B). Data show that after 9 hours the gel area stabilizes. This "natural contraction" is a phenomenon that has been widely observed in several cell types and has been shown to involve mechanotransduction requiring integrins (Seltzer et al., 1994). Drugs that were serially diluted with pre-warmed serum free media to final volume (3mL for each well) were then added to cells, and images were captured at 60 min intervals for 5 hours. Gel area was calculated using Image J software and represented as a percent change in gel area from the pretreatment to the post treatment time points.

Normalization of contraction data and calculation of EC₅₀ and IC₅₀ values

To compare efficacy, calculate EC₅₀/IC₅₀ values for the drugs tested and because gel area after 9 hours of precontraction varied with experiment, data are presented as percentage increase in area (relaxation) or decrease in area (contraction) from steady state "precontracted" area. Specifically, the data was normalized in the following ways: For inhibitor studies with ODQ, resulting changes in gel area were normalized to the maximum relaxation observed with 100μM DETA-NO (63.6±10.3% increase in gel area, mean ± SD). For contraction studies with ET-1, changes in gel area resulting from ET-1 treatment were normalized to the maximum gel contraction observed (56.1±7.7% decrease in gel area, 100pM ET-1, mean±SD). EC₅₀ and IC₅₀ values were calculated based on the dose-response curves normalized to the highest effect and the control effect, as 100% and 0% respectively, and then normalized curves were analyzed by Prismgraph software.

Myosin light chain phosphorylation assay

Human TM cells were seeded at confluence into 6 wells plates. After 24 hrs, media was changed to serum-free for 24 hrs and then cells were treated with drugs for 5 minutes, placed on ice, rinsed with ice-cold PBS and harvested by scraping into hot Laemmli sample buffer. Proteins were loaded onto 12% gel slabs, were fractionated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Antibodies (1:1000 for both anti-MLC and P-MLC IgGs) were used to detect MLC and phospho-MLC. The blots were washed three times (10 minute in TBST), followed by incubation with goat anti-rabbit-HRP secondary antibodies (1:5000) for 2 hours, then washed three times (10 minute in TBST). Blots were developed using Chemiluminescent HRP system (Denville Scientific Inc, South Plainfield, NJ) and subsequent exposure to autoradiography film (Genesee Scientific). Densitometry of protein bands were measured by Image J software from the scanned films, and data used to calculate the ratio P-MLC to MLC.

Statistical analysis

In order to ensure that observer bias did not affect the outlining of the collagen gels for area measurements, measurement of the gel area was conducted by a masked second observer. Using a Chi-squared test, it was determined that a random sample of 300 of the 1091 total images, could be used for gel area measurements, giving a 95% confidence interval and a 5% margin of error. The paired Student's t-test was used to compare the area measurements made by the masked volunteer and the researcher (JL). This test yielded a p-value of 0.0006, indicating that the area measurement results were not highly influenced by the researcher's potential bias. For comparison of mean changes in gel area, a paired Student's T-test was used. For analysis of myosin light chain phosphorylation status, p-MLC to total MLC ratios were calculated for each individual experiment. Mean p-MLC to total MLC ratios from 3 experiments were compared by Student's T-test to determine the effect of drug treatment.

3. Results

3.1 Collagen gel contraction assays

To functionally test concentration-related effects of the potent vasodilator, nitric oxide (NO) on human TM cell contractility, collagen gels precontracted without drugs by human TM cells for 9 hours (figure 1b) were treated with increasing doses of two NO donors (DETA-NO and SNP), and changes in area of collagen gels were monitored over time. To examine the downstream second messenger product of NO-stimulated soluble guanylate cyclase (sGC) activation, cGMP, we used the membrane permeable, non-hydrolyzable, 8-Br-cGMP to mimic sGC activation. In preliminary experiments using 100μM DETA-NO we observed that maximal relaxation (increase in gel area compared to baseline) occurred at 5 hours (data

not shown), which was used as reference time point for all contractility experiments. Figure 2 shows the dose-response relationships for these three compounds (8-Br-cGMP, DETA-NO and SNP). Data are presented as a percent change in the initial area of the hTM seeded collagen gels (% relaxation). Maximum relaxation for each compound were as follows; $109.4 \pm 7.1\%$ for 10mM 8-Br-cGMP, $100.1 \pm 13.1\%$ for 100 μ M SNP and $62.9 \pm 5.3\%$ for 100 μ M DETA-NO. Using these curves, we calculated EC₅₀ values (mean $\pm$ SD) for 8-Br-cGMP ($110 \pm 40 \mu$ M), SNP ($12.6 \pm 8.8 \mu$ M) and DETA-NO ($6.0 \pm 2.4 \mu$ M).

Effects of the NO donating compounds appear to be biphasic in the gel contraction assay. At higher concentrations (200 and 500 μ M), DETA-NO (a slow liberator of NO) appeared to be significantly less effective at relaxing TM cells on precontracted gels ($42.6 \pm 4.8\%$ for 200 μ M, $9.2 \pm 4.1\%$ for 500 μ M, % increase in gel area, mean $\pm$ SD) compared to maximum effects at 100 μ M ($62.9 \pm 5.3\%$ increase in gel area, mean $\pm$ SD). We observed a similar concentration-response profile at higher concentrations with SNP, a compound that rapidly releases NO ($28.1 \pm 2.2\%$ for 1mM versus $100.1 \pm 13.1\%$ for 100 μ M, % increase in gel area, mean $\pm$ SD) (Figure 2).

To specifically examine the involvement of sGC in NO-mediated relaxation in the gel contraction assay, we tested the effects of increasing concentrations (0.25 μ M to 10 μ M) of the sGC inhibitor, ODQ (Schrammel et al., 1996) in the presence of a fixed concentration (100 μ M) of DETA-NO. Figure 3 shows that ODQ dose-dependently inhibited the DETA-NO induced relaxation of HTM cells with a calculated IC₅₀ value of 460 ± 190 nM. Effects of DETA-NO on human TM cell relaxation were completely inhibited at 10 μ M ODQ. When tested by itself, ODQ no significant effect on hTM cell contraction (data not shown).

To examine the dynamic range in human TM cell contractility, we tested the effects of a potent vasoconstrictor, endothelin-1 (ET-1) in the collagen gel contraction assay. Increasing concentrations (100 fM-500 pM) of recombinant human ET-1 were added to human TM cell monolayers on collagen gels and changes in gel area were quantified after 5 hours of treatment. Figure 4 shows that ET-1 dose-dependently and potently enhanced human TM cell contraction ($56.1 \pm 7.7\%$ maximum decrease in gel area, 100pM ET-1, mean $\pm$ SD), with a calculated EC₅₀ value in the low picomolar range (1.5 ± 0.5 pM).

Next we used the collagen gel contraction assay to test whether the ET-1 induced contraction of human TM cells could be reversed by NO. In this set of experiments we used the maximum effective concentrations of ET-1 and DETA-NO, as determined in figures 2 and 4, 100pM and 100 μ M, respectively. ET-1 alone produced a potent contraction that plateaued after 6 hours. However, Addition of DETA-NO at the 6 hour time point reversed the ET-1 induced contraction (Figure 5).

3.2 Myosin light chain phosphorylation assays

In the next set of experiments, we chose to examine the effect of NO and ET-1 on the contractile machinery of human TM cell monolayers. Since cellular contraction is mediated through actin/myosin interactions requiring phosphorylation of myosin light chain (MLC), we examine drug effects on MLC phosphorylation status by Western blot analysis, comparing phosphorylated MLC as a percentage of total MLC protein. Results in figure 6A show that DETA-NO treatment of human TM cells resulted in a concentration-dependent (1 μ M to 100 μ M) reduction in MLC phosphorylation (Figure 6A). Conversely, treatment of human TM cell monolayers with increasing concentrations of ET-1 (0.1pM to 100pM) resulted in concentration-dependent increases in MLC phosphorylation levels (Figure 6B).

4. Discussion

By examining classic vasomodulators in two different assays (functional contractility assay and biochemical phosphorylation assay), we establish the dynamic contractility range for human TM monolayers. For the first time the concentration-response relationships and EC₅₀ values of two NO donors, a cGMP analog and ET-1 are established for human TM cell monolayers. Additionally, we have characterized the inhibitory dose-response relationship and estimate the IC₅₀ value for the sGC inhibitor ODQ. Taken together, data show that human TM cells behave much like vascular smooth muscle in terms of sensitivity to NO and ET-1 (Figure 7) and involvement of sGC in mediating NO responses.

All results were obtained using several established and characterized human TM cell strains. For each drug tested, we used either 2 (8-Br-cGMP), 3 (SNP, ET-1) or 4 (DETA-NO) different human TM cell strains isolated from different human donor eyes. In the present study TM cells isolated from eyes of human donors aged 3 months (HTM86), 28 years (HTM96), 54 years (TM98) and 35 years (TM93) of age at time of death were used. Results obtained from a single drug between cell strains were remarkably similar. For example, the maximum standard deviation for DETA-NO studies using 4 different cells strains was just $\pm 9.9\%$ and $\pm 7.7\%$ for ET-1 using 3 different strains. Thus, the observed drug effects on contractility were not specific to (or dominated by) a certain cell strain or unique to a particular assay.

We established that both DETA-NO and SNP relax HTM cells. The EC₅₀ values we derived from the dose response curves are within the range of reported EC₅₀ values in the literature where gel contraction or vascular relaxation assays were used. However, the reported EC₅₀ values in the literature vary over a wide range (DETA-NO; 30 μ M-379 μ M, SNP; 4.6 μ M-5.1mM), most likely due to species/cell type differences and/or experimental setup (Kimura et al., 1998; Perez Vallina et al., 1998). NO impacts cells in a number of ways, involving the sGC activation/cGMP pathway, thought to primarily mediate cellular relaxation (Wanstall et al., 2005). Not surprisingly, by bypassing endogenous cGMP production and degradation pathways with the non-hydrolysable cGMP analog, 8-Br-cGMP we observed a maximum relaxation (109.4 $\pm$ 7.1%). Similar to the NO donors, the reported EC₅₀ values for 8-Br-cGMP induced vascular relaxation in the literature vary considerably (7.5 μ M-327 μ M) (Perez Vallina et al., 1998; Qi et al., 2007), but our derived EC₅₀ value (110 $\pm$ 40 μ M) is within this range. Interestingly, SNP nearly reached the same level of relaxation as the 8-Br-cGMP, which was significantly higher than the DETA-NO induced maximum. This difference is most likely due to the chemical character of the compounds that determines the rates at which NO is released; T_{1/2} = ~14 min versus ~20 hours for SNP and DETA-NO respectively (Mooradian et al., 1995; Vesey et al., 1990).

To definitively show that sGC activation/cGMP production is required for the NO-induced effects on human TM cells, NO-induced relaxation was completely blocked with the sGC-specific inhibitor, ODQ (Schrammel et al., 1996). These results are consistent with a previous report showing ODQ blockade of NO-induced increase in conventional outflow facility (Ellis et al., 2009). Thus, sGC activation and concomitant increases in cGMP in the TM, account in part for NO-induced increases in outflow facility (Dismuke et al., 2008; Heyne et al., 2013; Schneemann et al., 2002; Stamer et al., 2011). The contribution that NO/sGC/cGMP has on permeability changes of inner wall of SC and outflow facility still need to be determined.

We observed a biphasic concentration response for two different NO donors with different release kinetics in our gel contraction assay. The NO-induced relaxation increased with increasing amounts of NO donor, reaching maximum effects at 100 μ M. At concentrations of

NO donor above 100 μ M we noticed a reduced effect on relaxation. These *in vitro* data are consistent with previous reports *in vivo*, showing a biphasic effects on IOP lowering in rabbits with NO donors (Nathanson, 1992). Moreover, a biphasic effect of NO on different cellular processes has been observed previously in other systems (Calabrese, 2001; Ridnour et al., 2006) and has been attributed to post-translational nitrosylation of proteins in conditions of high NO concentrations (Paolocci et al., 2000). While we did not test for nitrosylation of proteins following DETA-NO and SNP treatments, we found that 8-Br-cGMP did not have a biphasic response. These data suggest sGC-independent effects of NO, such as nitrosylation, account for decreased efficacy at higher doses. Thus, physiological NO concentrations in the TM is an important caveat to consider for development of future NO-based ocular hypotensive therapies, such as NCX 116 (Nicox compound) in phase III clinical trials.

ET-1 has been detected in aqueous humor from dogs, rats and humans (Kallberg et al., 2007; Lepple-Wienhues et al., 1992; Prasanna et al., 2005) and endothelin receptors have been identified in the conventional outflow pathway (Choritz et al., 2005; Rosenthal et al., 2007; Tao et al., 1998), but the reported effects of ET-1 on outflow facility are conflicting. With multiple ET-1 receptors which produce opposing responses in cells, further work into the distribution of specific ET-1 receptor subtypes in the outflow pathway will be necessary to fully understand the role ET-1 plays in outflow resistance. Specifically, distribution of the ETA and ETB receptor subtypes which mediate cellular contraction (Maguire and Davenport, 1995) and relaxation (Mazzuca and Khalil, 2012) respectively, may play an important role in the proposed antagonism between the ciliary muscle and the TM. Interestingly, our calculated EC₅₀ value (1.5 $\pm$ 0.5pM) is lower than values reported in the literature (3.2–18.3nM) (Eglen et al., 1989; McKay et al., 1991; Ohlstein et al., 1989) but near the reported concentration of ET-1 in human aqueous humor and selectivity for the ETB receptor (6.26 pM) (Lepple-Wienhues et al., 1992). While our results show a dose dependent contraction of HTM cells with ET-1, future studies using ETA and ETB specific antagonist will need to determine what ET-1 receptor subtypes are mediating this effect in the HTM cells *in vitro* and whether this receptor subtype is present *in vivo*.

The site(s) and regulation of synthesis for ET-1 and NO that impact the TM are largely unknown. ET-1 is a peptide secreted from non-pigmented ciliary epithelial cells (Lepple-Wienhues et al., 1992; Prasanna et al., 1998), which participate in the production of aqueous humor (Civan and Macknight, 2004). Interestingly, ET-1 levels in POAG have been reported to be elevated (Noske et al., 1997). Uncertain is whether inner TM cells also secrete ET-1, adding to aqueous humor levels as it flows through the conventional outflow pathway, impacting the physiology of TM and Schlemm's canal cells in the resistance-generating JCT region. In contrast to ET-1, NO is a highly diffusible gas with a short half-life. Because its effective range of activity is limited, NO must be produced by cells adjacent to or in the JCT region, where resistance is generated. While the TM is sensitive to NO (Ignarro, 1990) some evidence suggest that TM cells produce little NO (Ellis et al., 2009). Like vascular endothelium (Cheng et al., 2005; Ziegler et al., 1998), recent data suggest that Schlemm's canal endothelial cells produce NO, and overexpression of endothelial NO synthase increases outflow facility. Interestingly, akin to vascular endothelia it appears that NO production is shear-dependent, likely increasing at elevated IOPs when Schlemm's canal begins to collapse and shear stress dramatically increases (Stamer et al., 2011). While Schlemm's canal lies downstream of the TM, the two cell types are very close to each other, physically interacting. NO production by Schlemm's canal theoretically can rapidly diffuse upstream against aqueous flow, relax the TM; providing an elegant negative feedback loop for IOP regulation.

5. Conclusions

Contractility of the TM is thought to be one mechanism by which the TM regulates conventional outflow resistance. The findings presented here for the first time, establish a pharmacological guide, not only for interpreting disparate results seen in past studies, but also for rational experimental design of future studies examining NO or ET-1 and their effects on outflow facility. Our results demonstrate a wide dynamic range of contraction from ET-1 stimulation in the TM that is comparable to other smooth muscle cells, in terms of magnitude and sensitivity. Conversely, the magnitude of relaxation from NO is greater in the TM than reported for other vascular smooth muscle cells (Figure 7). These findings show that the potent vasomodulators, ET-1 and NO, play an equally important role in regulating TM contractility. These comparisons highlight the importance of therapeutically modulating contractility of TM and suggest that dysfunction in contractility may underlie the development of ocular hypertension in glaucoma.

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- Nitric oxide and endothelin-1 effects on human TM cell contractility are quantified.
- Dose response curves and EC50 values are generated using a gel contraction assay.
- Endothelin-1-mediated contraction of human TM cells is reversed by nitric oxide.
- TM contractility is compared to smooth muscle cells for magnitude/sensitivity.

Figure 1. Human Trabecular Meshwork Gel Contractility Assay

A) Schematic of experimental design. Collagen gels were cast, human TM cells were seeded onto the gel surface and allowed to attach and grow for one day. Cells were then serum starved in serum-free (SF) medium overnight (18 hrs) before gels were separated from the sides of the well, allowed to stabilize and treated with drug. B) Following detachment of the collagen gel from the side of the well, human TM cells contract the gel *in the absence of treatment*, reducing the surface area of the gel incrementally for 9 hours (shown schematically in panel A and experimentally in panel B). Data shown were obtained from digitized images of gels (3 different experiments using TM86 and TM 96 cell strains) used to calculate are using Image-J software.

Figure 2. Nitric oxide effects on human TM cell contractility

Shown are concentration-response curves obtained from three different compounds (DETA-NO, SNP and 8-Br-cGMP). In a dose-dependent manner, treatment with the two NO donors DETA-NO and SNP or 8-Br-cGMP increased the area of collagen gels precontracted (without drug) for 9 hours by human TM cells. 8-Br-cGMP was the most efficacious and potent of the three compounds. For 8-Br-cGMP experiments, 2 cell strains (TM86, TM96) were tested in four different experiments. For DETA-NO (0.1–100 μ M), 4 cell strains (TM86, TM98, TM96, TM93) were examined in 9 different experiments and for (200 and 400 μ M) 2 cell strains (TM86, TM96) were examined in three different experiments. For SNP (0.1–100 μ M), 3 cell strains (TM86, TM96, TM93) were evaluated in 4 different experiments and for (1mM) 2 cell strains (TM86, TM93) were tested in three different experiments. Data shown are the mean ($\pm$ SD).

Figure 3. Soluble guanylate cyclase mediation of nitric oxide-induced relaxation of human TM cells

Human TM cells grown on collagen gels were incubated with the sGC inhibitor, ODQ at increasing concentrations (0.25, 0.5, 1, 5 and 10 μM). After a 5 minute incubation with ODQ, DETA-NO (100 μM) was added to media containing ODQ for 5 hours. Changes in gel area resulting from DETA-NO/ODQ treatment were quantified and normalized to untreated controls. Data shown is the mean ($\pm$ SD) of 4 experiments using 2 different cell strains (TM86, TM96).

Figure 4. Endothelin-1 dose-dependently enhances human TM cell contraction

Recombinant human ET-1 was incubated with human TM cells grown on collagen gels for 5 hours. Changes in gel area resulting from ET-1 treatment were quantified and normalized to untreated controls. Data are the mean ($\pm$ SD) values obtained from six experiments using 3 cell strains (TM86, TM96, TM93).

Figure 5. Reversal of endothelin-1 induced contraction of human TM cell by the nitric oxide donor DETA-NO

Recombinant human ET-1 (100pM) was incubated with human TM cells grown on collagen gels for 12 hours. In 3 of 6 experiments, DETA-NO (100μM) was added 6 hours after ET-1. Data are mean (±SD). N=3 for ET-1 treated and N=3 for ET-1 + DETA-NO treated. * p<0.05, ET-1 + DETA-NO versus ET-1 only. # p=0.49, 12hr versus initial gel area.

Figure 6. Dose-dependent effects of nitric oxide and endothelin-1 on myosin light chain phosphorylation status in human TM cells

Human TM cell monolayers were treated with increasing concentrations of DETA-NO (panel A) or ET-1 (panel B). Cell lysates were prepared and analyzed in duplicate by SDS-PAGE followed by western blotting using antibodies that recognize either phospho-MLC (blot 1) or total MLC (blot 2). Band intensity was quantified by densitometry, and expressed as a ratio (mean $\pm$ SD) of p-MLC to total MLC. Blots shown (inset) are representative of results observed for 3 independent experiments using TM 86, TM 96 and TM93 cell strains.

* $p < 0.05$ vs no drug

Figure 7. Dynamic range of human TM versus vascular smooth muscle cell contractility
 A) Shown are representative images of human TM cells grown on collagen gels and treated with DETA-NO or ET-1 compared to untreated controls. After 5 hours of drug treatment, visible changes in collagen gel area are observable and % changes in the individual gels over time are shown below. B) The results obtained in the present study for human TM cellular contractility (from DETA-NO, SNP and ET-1) are compared to published cellular contractility of smooth muscle cells resulting from ET-1 or NO donors (Bourke et al., 2011; Dallot et al., 2003; Defawe et al., 2010; Kotlo et al., 2011; Lee et al., 1998). All contractions or relaxations shown were results using similar gel contraction assays.