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

W. Michael Dismuke [a,1], Jin Liang [b,1], Darryl R. Overby [c], W. Daniel Stamer [a,b,*]

[a] Department of Ophthalmology, Duke University, Durham, NC, USA
[b] Department of Biomedical Engineering, Duke University, Durham, NC, USA
[c] Department of Bioengineering, Imperial College London, London, UK

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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 analog (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 ± 2.4 μM, SNP = 12.6 ± 8.8 μM), with maximum effects at 100 μM. Interestingly, at concentrations of NO donors above 100 μM, the relaxing effect was lost. Relaxation caused by DETA-NO (100 μM) was dose dependently blocked by the soluble guanylate cyclase specific inhibitor ODQ (IC_{50} = 460 ± 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 ± 40 μM). ET-1 caused a dose-dependent contraction of human TM cells (EC_{50} = 1.5 ± 0.5 pM), with maximum effect at 100 pM (56.1%) and this contraction was reversed by DETA-NO (100 μM). 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.

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 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 (AGIS Investigators, 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 (Braughler 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

* Corresponding author. Duke University, DUMC 3802, Durham, NC 27710, USA. Tel.: +1 919 684 3745.
E-mail address: dan.stamer@duke.edu (W.D. Stamer).
[1] These authors contributed equally to the work.

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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, 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, 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 EC_{50} 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

2.1. 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).

2.2. 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, 0.1 mg/ml streptomycin and 0.29 mg/ml glutamine and maintained in humidified air containing 5% CO_2 at 37 °C.

2.3. Contractility assay and treatments

Collagen gels (1.41 mg/mL) were prepared in 24 well plates from rat tail collagen type I (BD Bioscience, Bedford MA) following the manufacturer's instructions (BD Bioscience, Bedford MA). The contraction assay protocol from Luna et al. (2012) with some

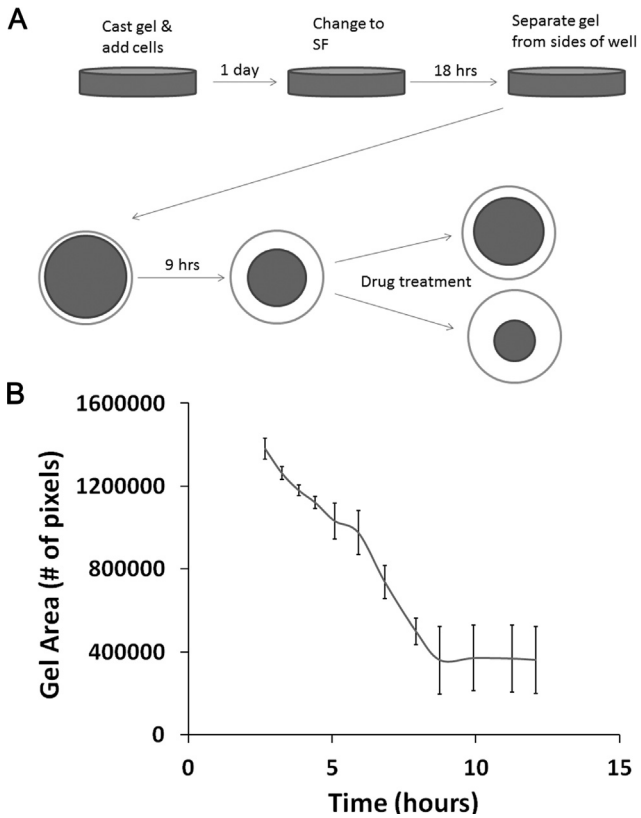

Fig. 1. Human Trabecular Meshwork Gel Contractility Assay. A) Schematic of experimental design. Collagen gels were cast, human TM cells were seeded onto the gel surface and allowed to attach and grow for one day. Cells were then serum starved in serum-free (SF) medium overnight (18 h) before gels were 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 area of the gel incrementally for 9 h (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.