Characterization of Soluble Guanylate Cyclase in NO-Induced Increases in Aqueous Humor Outflow Facility and in the Trabecular Meshwork

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PURPOSE. Nitric oxide (NO) increases the rate at which aqueous humor exits the eye; however, the involvement of soluble guanylate cyclase (sGC) is unknown. This study investigated the role of sGC in mediating the NO-induced increases in outflow facility.

METHODS. Outflow facility was measured in porcine eyes using the anterior segment organ culture perfusion system. sGC activity was assessed by cGMP production in low-passage porcine and human trabecular meshwork (TM) cells and transformed human TM cells, as measured by enzyme immunoassay. sGCα and sGCβ isoform expression were determined using Western blot analysis.

RESULTS. Activation of sGC is necessary for the NO-induced increases in outflow facility (0.3215 μL/min per mm Hg [baseline outflow facility] ± 0.0837 [SEM]). NO resulted in increased sGC activity that was abolished by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-1 (ODQ). Western blot analysis of total protein demonstrated an equivalent ratio of sGCα and sGCβ subunit expression. In transformed cell fractions, however, the level of cytoplasmic sGCα subunit expression was decreased compared with low-passage human TM cells.

CONCLUSIONS. Activation of sGC is involved in the NO-induced increases in outflow facility. The expression of α and β sGC subunits in an equivalent ratio would suggest a functional sGC heterodimer because DETA-NO increased cGMP levels in low-passage human and porcine TM cells. However, the inability of DETA-NO to cause increases in cGMP levels in transformed TM cells suggests that though the sGC heterodimer is necessary, it is not sufficient and may require other factors not present in transformed cells. (Invest Ophthalmol Vis Sci. 2009;50:1808–1813) DOI:10.1167/iovs.08-2750

The trabecular meshwork (TM)1-2 and Schlemm’s canal3-5 constitute the major route for the outflow of aqueous humor from the eye. The TM, in concert with the Schlemm’s canal, regulates resistance to aqueous humor outflow and thus regulates intraocular pressure (IOP). Improper regulation of IOP may result in high IOP, which is a risk factor for glaucoma, a blinding disease that affects millions of people worldwide. Two distinct cell populations6 are consistent with the identified regions of the TM, the cribiform or juxtacanalicular region and the uveal/corneoscleral region.7,8 TM cells play a functional role in regulating aqueous humor outflow, and cellular mechanisms attributable to regulating outflow include changes in TM cell volume9 and contractility,10 both of which are regulated by the nitric oxide (NO) system.

The outflow of aqueous humor is regulated by complex hormonal mechanisms to maintain proper IOP. Functionally important nitrergic and adrenergic innervations of the TM are known,11-13 and decreases in IOP by the activation of the NO and sGC system14-16 and the adenylate cyclase system17 have been documented. In spite of the role of sGC as the principal target for NO, little attention has been paid to the role sGC plays in mediating NO-induced decreases in IOP.

sGC comprises an α subunit and a smaller heme-containing β subunit.18,19 The α and the β subunits constitute the active enzyme, and heterodimers are activated by NO binding to the heme moiety, whereas homodimers exhibit little or no synthetic activity, even in the presence of NO.20 Binding of NO to sGC results in the formation of 3′,5′-cyclic guanosine monophosphate (cGMP) from guanosine 5′-triphosphate. Increased cGMP activates protein kinase G,21 which phosphorylates target proteins.

In these studies we examine the role of sGC in the NO-induced increases in outflow facility and the expression and enzymatic activity of sGC in TM cells.

METHODS

Cell Culture

Cells were grown from human donor eyes with no history of ocular disease or surgery and were obtained from Lions Eye Institute (Tampa, FL) or from human TM explants obtained from corneal scleral rims stored in medium (Optisol or Dexol; Chiron Ophthalmics, Irvine, CA) at 4°C. Transformed TM cells, GTM 3,22 and HTM 5 were generous gifts of Abbot F. Clark (Alcon Laboratories, Fort Worth, TX).

Primary human TM cell lines (numbers represent donor ages) HTM36, HTM80, and HTM86 were developed. For our experimental protocols, cells from early passages (passages 3 to 5) were used. Porcine eyes were obtained from the local abattoir within 1 hour of death and were maintained on ice. The eyes were bisected, and the cornea, iris, lens, and ciliary body were removed. TM cells were isolated after collagenase digestion of TM explants.23 Collagenase-treated and transformed cells were grown in low-glucose (1 g/L) and high-glucose (4.5 g/L) DMEM (Mediatech, Herndon VA), respectively, in the presence of 10% fetal bovine serum (Mediatech), 100 U/mL penicillin and 100 μg/mL streptomycin (Mediatech). We validated human TM cells by their morphology and the presence of dexamethasone-induced myocilin expression.24 Porcine TM cells were identified by their ability to take up acetylated low-density lipoprotein and to secrete tissue plasminogen activator.

Outflow Facility Measurements

Anterior segment organ culture perfusion was used to measure outflow.25 Porcine eyes were obtained and bisected as described. Anterior segments were cultured at 37°C in 100% humidity at 5% CO2 atmosphere.
sphere and were perfused at a constant pressure of 14 mm Hg.26-27 Outflow rates were determined gravimetrically as changes in weight of the media as the eyes were perfused over time. Data were captured at 1-minute intervals by serial data collection software (WinWedge; TALtech, Philadelphia, PA) attached to the balance and recorded on a spreadsheet (Excel; Microsoft, Redmond, CA). Outflow facility was expressed as microliter per minute per millimeter of mercury perfusion pressure. Eye anterior segments were allowed to adapt to their new environment, during which time outflow facility increased for the first 3 to 8 hours, a phenomenon referred to as washout,27,28 after which baseline outflow facility remained stable.29 Because of the stability of the outflow facility baseline after the initial washout period, it was not necessary to correct for nondrug-related changes in outflow facility in our experimental protocol. After a stable baseline was achieved the unpaired eyes were perfused with DMEM with the NO donor, diethylentriamine nitric oxide (DETA-NO; 100 μM)30,31 in the presence or absence of the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-1 (ODQ) (1 μM).31

Measurement of sGC Activity

Soluble GC activity was assessed by measuring cGMP levels in TM cells and tissue in response to DETA-NO treatment. For cGMP measurements, cells were grown in 12-well culture dishes (Nalge Nunc International, Rochester, NY) in a tissue culture incubator at 37°C in 5% CO₂, as described. Two days before experiments, the cells were exposed to serum-free media. cGMP was assayed by enzyme immunoassay (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s protocol.

Soluble GC α and β Subunit Expression

Human and porcine low-passage TM cells, GTM3, and HTM5 cells were grown to confluency in a tissue culture incubator at 37°C in 5% CO₂, as described. The cells were then lysed, and protein concentrations were determined by the method of Lowry. Preliminary experiments determined that at 30 μg protein, the β1 sGC antibody binding was linear, and this concentration of protein was used subsequently for the detection of the sGCβ and sGCα antibodies. Proteins were separated by gel electrophoresis and electrophoretically transferred to nitrocellulose membrane. For detection of the α and β sGC subunits, blots were exposed to the α or β sGC polyclonal antibody (Cayman Chemicals, Ann Arbor, MI) in the presence or absence of their respective blocking peptides (Cayman Chemicals). Blots were then stained with horseradish peroxidase-conjugated secondary antibody and developed with luminol reagent.

To determine the subcellular localization of sGC in TM, cellular components were separated by centrifugal force at 4500g, 100,000g, and 500,000g. Supernatants and pellet fractions were collected. Blots were exposed to α or β sGC polyclonal antibodies in the presence or absence of their respective blocking peptides, which served as controls.

Materials and Reagents

Routine reagents and 3-isobutyl-1 methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). ODQ and DETA-NO were obtained from σ-RBI (Natick, MA), and cGMP assay kits for enzyme immunoassay were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Statistical Analysis

Statistical comparisons were performed by ANOVA, followed by the Holm-Sidak method for comparisons of significant difference among different means.

RESULTS

The porcine anterior eye segment organ perfusion model can be correlated with the human perfusion system26 and is routinely used to test the effects of NO on aqueous humor outflow. Cellular mechanisms by which these agents allow for changes in outflow facility are often studied in cultured TM cells; therefore, it was necessary to correlate organ perfusion studies with parallel studies in primary or low-passage porcine and human TM cells. Additionally, because transformed TM cells are also used to characterize the involvement of the sGC/cGMP system in TM cell functions22,32 in pathologic and physiological states, we characterized the sGC system in these cells.

Soluble GC Mediation of the NO-Induced Increase in Outflow Facility

To test the involvement of the sGC system in porcine outflow pathway, porcine anterior eye segments were perfused with DMEM, and a stable basal outflow facility of 0.3215 μl/min/mm Hg ≥ 0.0837 was maintained before drug treatments. The eyes were then perfused with ODQ (1 μM) for 50 minutes, after which DETA-NO (100 μM), known to cause increases in outflow facility, was added to the perfusate. Figure 1 demonstrates that in eyes pretreated with ODQ, DETA-NO was unable to increase outflow facility.

To test the viability of the eye segment and to determine that the effects of ODQ on outflow facility were not artifactual, eyes were perfused with DETA-NO (100 μM). Addition of DETA-NO (100 μM) resulted in a 220% increase in outflow facility over baseline values (Fig. 1, representative of three experiments). As in a previous study,5 outflow facility increased within 20 to 40 minutes of the application of drugs, and the increases were sustained for approximately 2 hours, after which outflow facility returned to baseline values.

Soluble GC Expression in TM

Because sGC mediates the NO-induced increases in aqueous humor outflow, we wanted to assess the expression of the sGCα isoform in low-passage porcine cells and then conduct parallel experiments in low-passage human TM cells. We also characterized sGCs in transformed TM cells. Nitrocellulose blots containing separated proteins were exposed to the sGCα antibody in the presence or absence of the sGCα blocking
peptide, used as a negative control; the brain cytoplasmic preparation served as a positive control. Figure 2A demonstrates that the sGCα isoform is expressed in porcine and human low-passage TM cells and transformed GTM3 and HTM5 cells. Interestingly, the α subunit was expressed as doublets in both transformed human cell lines. As with the α subunit, blots were exposed to sGCβ1 polyclonal antibody in the absence of soluble guanylate cyclase β1 blocking peptide (BP). Immunoblot demonstrates that the sGCβ1 subunit was expressed in human and porcine low-passage TM cells and in GTM3 and HTM5 cells (Fig. 2B). As was the α subunit, the β subunit of sGC was expressed as doublets in transformed cells.

### Soluble GC Activity in TM Cells

Activation of sGC by NO results in increases in cGMP; therefore, cGMP was measured in low-passage human and porcine TM cells and transformed human TM cells. TM cells were exposed to DETA-NO (100 μM) in the presence or absence of ODQ (1 μM), and cGMP levels were measured. Because cGMP can be degraded by phosphodiesterases, IBMX, the nonspecific phosphodiesterase inhibitor, was added to the cells after drug incubation. Figure 3A demonstrates sixfold and ninefold increases in cGMP levels in low-passage human and porcine TM cells, respectively, with their controls. ODQ abolished the ability of DETA-NO to increase cGMP (Fig. 3A). ODQ alone had no effect on cGMP levels. Unlike low-passage TM cells, the exposure of transformed TM cells to DETA-NO did not result in statistically significant increases in cGMP levels (Fig. 3B).

### Subcellular Localization of sGC Expression in TM

Western blot analysis and densitometric analysis demonstrated equivalent abundance of α and β sGC subunits in all cell types tested. Therefore, we were surprised that cGMP levels were unchanged in response to NO treatment in transformed human TM cells, even in the presence of IBMX. We sought to assess subcellular levels of expression of the α sGC isoform in low-passage human TM cells compared with transformed TM cells. Nitrocellulose blots containing separated proteins were exposed to sGC α antibody in the presence or absence of the α sGC blocking peptide, used as a negative control.

Western blot analysis (Figs. 4B, 4D) demonstrated that in GTM3 (Figs. 4A, 4B) and HTM5 (Figs. 4C, 4D) cells, the α sGC isoform was detected in the pellet and supernatant of the 4500g and the 100,000g centrifugations. However, the expression level of the α subunit was decreased in the supernatant of the 300,000g centrifugation in TM cells compared with transformed TM cells. Different subunits of sGC were expressed in these cells, even in the presence of IBMX. We sought to assess subcellular levels of expression of the α sGC isoform in low-passage human TM cells compared with transformed TM cells. Nitrocellulose blots containing separated proteins were exposed to sGC α antibody in the presence or absence of the α sGC blocking peptide, used as a negative control.

As with the α isoform in the transformed cells, Western blot analysis (Figs. 5B, 5D) and densitometric analysis (Figs. 5A, 5C) demonstrated that the β1 sGC isoform was detected in the pellet and supernatant of the 4500g and the 100,000g centrifugations. Unlike the α sGC antibody, the β1 sGC antibody was present in the supernatant and the pellet of the 300,000g centrifugation in both transformed TM cells.

### DISCUSSION

These studies demonstrated the involvement of sGC in mediating the NO-induced regulation of TM cell function. Specifically, sGC mediated the DETA-NO-induced increases in outflow facility. NO increased sGC activity in human and porcine low-passage TM cells as determined by changes in cGMP levels but not in transformed human cells. Western blot analysis demonstrated that both the α and β sGC isoforms are expressed in all cell types. However, in the transformed cells,
though the \( \beta 1 \) isofrom is expressed in the nuclear, cytoplasmic, and membrane components of the cells, \( \alpha \) isofrom expression levels are lower in the cytoplasmic component of the cell.

**Anterior Segment Perfusion Studies**

Our studies demonstrated that the activation of sGC is obligatory for the NO-induced increases in outflow facility. As previously reported, DETA-NO increased outflow facility in porcine eye anterior segments.\(^9\) Other studies in monkeys\(^{33}\) and rabbits\(^{16}\) demonstrated the involvement of NO in increasing outflow facility. Although many of the actions of NO involve the activation of sGC, studies have demonstrated that some effects of NO are sGC independent.\(^{34}\) Therefore, it was important to determine its involvement in the NO-induced increases in outflow facility. In anterior eye segments that were preincubated with ODQ, DETA-NO was unable to cause increases in outflow facility, demonstrating that the activation of sGC mediated the DETA-NO response.

In addition to the ability of the NO/sGC system to regulate IOP by increasing outflow facility, the NO/sGC system has been shown to regulate IOP by decreasing NaK-ATPase activity.
GTM3 and HTM5

as arbitrary units and represent the mean ± SEM of three experiments.

in ciliary processes, thereby decreasing aqueous humor secretion.30 Although other studies have demonstrated that NO decreases IOP, one report by Krupin et al.35 demonstrated an increase in IOP in rabbits in response to the NO donor sodium nitroprusside. Although we do not understand the reason for this discrepancy, it is may be explained by concentration-dependent effects of NO on IOP. Higher concentrations of NO donors result in increases in IOP, whereas lower concentrations result in decreases in IOP.14,36 Additionally, repeated use of the organic nitrate, nitroglycerin, resulted in tolerance, whereas chronic use of the nucleophile hydralazine did not result in tolerance.14

sGC Isoform Expression and Activity in TM Cells

Outflow facility studies in eye anterior segments are often correlated with cellular studies in TM cells in culture. Therefore, the ability of ODQ to abolish the NO-induced increases in outflow facility suggests the involvement of sGC in TM cell function. In these studies, we tested sGC activity in several cell lines including porcine, low-passage, and transformed human cells.

In these studies we used the NO donor, DETA-NO, which activates sGC presumably by spontaneous release of NO. The ability of DETA-NO to increase cGMP levels in low-passage human and porcine TM suggests that sGC is expressed in these tissues and that activation of the NO system and the subsequent binding of NO to sGC results in cGMP production. Furthermore, the ability of ODQ to abolish the DETA-NO-induced increases in cGMP in TM cells suggests that a direct consequence of NO stimulation is the activation of sGC. ODQ alone had no effect on basal cGMP levels, suggesting that ODQ does not act on sGC but, rather, has the ability to block NO action on sGC and inhibit its activity. Additionally, in these studies, the time course for the NO-induced increases in outflow facility correlated with the time course for the NO-induced increases in cGMP levels. Taken together, these data suggest that a direct consequence of NO stimulation in the outflow pathway is the activation of sGC, increased cGMP levels, and subsequent increases in outflow facility.

We did not observe changes in cGMP levels in response to DETA-NO treatment in transformed cells. We do not know the reason(s) for alterations in the NO/cGMP system in transformed cells. Because cGMP concentration in the cell is dependent on the presence or activation of cGMP-hydrolyzing phosphodiesterases.32 we added IBMX immediately after the DETA-NO incubation to prevent the hydrolysis of cGMP by phosphodiesterases. However, in our hands, there were no observable increases in the levels of cGMP in DETA-NO treated transformed cells in the presence or absence of IBMX compared with control. In other studies using the transformed cell line, GTM3, the NO donor, sodium nitroprusside increased cGMP levels in the presence of IBMX22 or the phosphodiesterase 5 inhibitor E4021,32 suggesting that factors other than phosphodiesterase hydrolysis of cGMP may be responsible for the lack of cGMP increases in transformed cells in response to DETA-NO.

Lack of enzyme activity may be attributable to changes in gene expression, enzyme inactivation, protein degradation, or a combination of these. To correlate protein levels of the α and β subunits with protein activity, Western blot and densitometric analyses were performed. We examined protein expression in crude cell lysates and demonstrated relative equivalent sGC content in all cell types examined. However, unlike crude cell lysates, levels of expression of the α sGC subunit in the cytoplasm of the transformed cells were low compared with the α subunit expression in low-passage human TM cells. The functional enzyme consists of the holoenzyme; the absence of sufficient α subunits to form functional enzymes with the β subunits suggests that the cytoplasmic localization of the subunits is important for enzyme activation in these cells. In other studies, Nathanson et al.37 observed increased sGC activity in membrane and soluble cerebellar fractions; this may not be the case in transformed TM cells because quantitative detection of crude homogenates showed that the α and β sGC subunits are expressed in equivalent ratios. Additionally, it should be noted that the pathways involved in the formation of NO are different for different classes of NO donors.38–40 Whether these differences are physiologically and pharmacologically relevant to our observations is unclear. However, it should be noted that GTM3 and HTM5 cells are not ideal to use as correlates for the study of DETA-NO-induced regulation of aqueous humor outflow facility.

We conclude that sGC mediates the actions of NO in increasing outflow facility and that the functional sGC enzyme produces cGMP, a third messenger mediating the NO-induced response in low-passage human and porcine TM cells.
References


