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C-type natriuretic peptide protects the retinal pigment epithelium against advanced glycation end-product-induced barrier dysfunction

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Running title: CNP reverses the AGE-effect in the RPE

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Abbreviations: AGE, advanced glycation end-product; Glyc-alb, glycated-albumin; TEER, transepithelial electrical resistance; RPE, retinal pigment epithelium; hRPE, human fetal retinal pigment epithelium; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR2, natriuretic peptide receptor 2

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ABSTRACT

In diabetic retinopathy, vision loss is usually secondary to macular edema, which is thought to depend on the functional integrity of the blood-retina barrier. The levels of advanced glycation end-products in the vitreous correlate with the progression of diabetic retinopathy. Natriuretic peptides (NP) are expressed in the eye and their receptors are present in the RPE. Here, we investigated the effect of glycated-albumin (Glyc-alb), an AGE model, on RPE barrier function and the ability of NP to suppress this response. Transepithelial electrical resistance (TEER) measurements were utilized to assess the barrier function of ARPE-19 and human fetal RPE monolayers. The monolayers were treated with 0.1 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ Glyc-alb in the absence or presence of 1 pM to 100 nM of apical ANP, BNP or CNP. Glyc-alb induced a significant reduction in TEER within two hours. This response was concentration-dependent ($\text{EC}_{50}=2.3$ $\mu\text{g/mL}$) with a maximal reduction of $40\pm 2\%$ for ARPE-19 and $27\pm 7\%$ for hFRPE at 100 $\mu\text{g/mL}$ six hours post treatment. One hour pretreatment with ANP, BNP or CNP blocked the reduction in TEER induced by Glyc-alb (100 $\mu\text{g/mL}$). The suppression of the Glyc-alb response by NP was dependent upon the generation of cGMP and exhibited a rank order of agonist potency consistent with the activation of NPR2 subtype ($\text{CNP} \gg \text{BNP} \geq \text{ANP}$). Our data demonstrate that Glyc-alb is effective in reducing RPE barrier function and this response is suppressed by NP. Moreover, these studies support the idea that NPR2 agonists can be potential candidates for treating retinal edema in diabetic patients.

INTRODUCTION

Advanced glycation end-products (AGE) are formed by non-enzymatic glycation reactions between reducing sugars and the free amino groups on proteins, lipids, and DNA (Li et al., 1996). Although the accumulation of AGE is a consequence of aging, the rate of AGE formation is accelerated in diabetic patients. Diabetic retinopathy is a major complication of diabetes mellitus and a leading cause of visual impairment and blindness in the United States (Aiello, 2003). The progression of diabetic retinopathy is associated with the accumulation of AGE in the retina and vitreous (Yokoi et al., 2005; Yamagishi et al., 2006; Kakehashi et al., 2008).

The responses to AGE are mediated by pattern recognition receptors (Yamada et al., 2006). Previous studies have shown that the retina expresses several of these receptors including the AGE receptor (RAGE) (Yamada et al., 2006); the AGE receptor complex (AGE R1-R3) (Wautier and Guillausseau, 2001); class A scavenger receptor; class B scavenger receptors; and class D scavenger receptors (Horiuchi et al., 2003; Tamura et al., 2003). However, simply blocking the RAGE receptor was not effective in reducing the complications associated with diabetic retinopathy (Peyroux and Sternberg, 2006).

In diabetic retinopathy, visual loss is often secondary to disruption of the blood-retina barrier (BRB) and the development of macular edema (Aiello, 2003). The retinal pigment epithelium (RPE) and blood vessels of the inner retina form the outer and inner blood-retina barrier, respectively. These barriers control the movement of fluid and solutes into the extracellular spaces of the neural retina (Maepea, 1992). Although RPE function is essential to maintaining a dehydrated neural retina environment, the regulation of the RPE barrier in macular edema has

received relatively limited attention.

Natriuretic peptides (atrial, brain, and C-type) primarily control diuresis, natriuresis, and vasodilatory functions of the cardiovascular system (Potter et al., 2006). ANP and BNP activate natriuretic peptide receptor 1 (NPR1), while CNP activates natriuretic peptide receptor 2 (NPR2) (Potter et al., 2006). NPR1 and NPR2 are transmembrane guanylyl cyclase receptors and catalyze the synthesis of cyclic guanosine monophosphate (cGMP) which in turn activates protein kinase G (PKG) and subsequent target genes (Levin et al., 1998). A third natriuretic peptide receptor, NPR3, clears natriuretic peptides through receptor-mediated internalization and degradation (Potter et al., 2006).

Natriuretic peptides and their receptors are expressed in the neural retina and the RPE (Rollin et al., 2004). Although the roles of these peptides and their receptors in the retina are not clear, induction of diabetes in rats causes a down regulation of ANP and NPR3 expression in the retina (Rollin et al., 2005). Moreover, it has been shown recently that ANP can suppress endothelial (Xing et al., 2011) and RPE (Lara-Castillo et al., 2009) leakage. Together these indicate that the natriuretic peptide system in the retina may influence diabetic retinopathy and blood-retina barrier dysfunction. In this manuscript, using ARPE19 cell line to perform initial experiments and confirming the results in the more RPE-like primary hRPE cell model (Ablonczy et al., 2011), we provide new evidence that the administration of glycated-albumin (Glyc-alb), a RAGE agonist, decreased RPE transepithelial resistance and that this response was prevented by pretreatment with natriuretic peptides through a cGMP-dependent pathway.

MATERIALS and METHODS

Tissue culture

ARPE19 cells were obtained from the American Type Culture Collection (Manassas, VA) and hRPE cells were isolated from human fetal eyes acquired from Advanced Bioscience Resources (Alameda, CA). Confluent monolayers of both cell types were established and maintained on permeable membrane inserts (Costar Clear Transwell, 0.4 μm pore, 24 mm; Thermo Fisher Scientific, Fair Lawn, NJ) as described previously (Ablonczy et al., 2011). Transepithelial electrical resistance (TEER), which is inversely proportional to the paracellular permeability of cultured RPE cells and is a reliable assay for the assessment of RPE barrier function (Dunn et al., 1996; Ablonczy and Crosson, 2007; Ablonczy et al., 2009; Ablonczy et al., 2011), was measured by means of a volt/ohm meter equipped with an STX2 electrode or 24 mm endohm chamber (World Precision Instruments, Sarasota, FL). Resistance values for each condition were determined from a minimum of four individual cultures, and corrected for the inherent Transwell resistance within 3 minutes after removing the plates from the incubator. All values represent the mean \pm SE. Data were analyzed using the Student *t* test, and were considered statistically significant at $P < 0.05$. Concentration curves were analyzed using Prism 4.02 software (GraphPad Software; San Diego, CA). Only confluent monolayer cultures with stable TEER values were used in these experiments (40-50 $\Omega\cdot\text{cm}^2$ for ARPE 19 cells and $> 800 \Omega\cdot\text{cm}^2$ for hRPE cells).

Cell treatments

Cells were treated with various concentrations of 0.1 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ of human albumin (Alb) or human glycated-albumin (Glyc-alb; Sigma-Aldrich, St. Louis, MO), the latter of which is a widely used RAGE-agonist (Fritz, 2011), apically and basolaterally. Change in TEER was then followed for six hours post administration. Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) were obtained from Sigma-Aldrich. Natriuretic peptides were administered apically or basolaterally, one hour prior to the administration of 100 $\mu\text{g/mL}$ of Glyc-alb at concentrations that ranged from 1 pM to 100 nM. In peptide studies, TEER was measured prior to natriuretic peptide treatment, one hour after treatment, and then followed for six hours post Glyc-alb administration. Selected cultures were pretreated with isatin (100 $\mu\text{mol/L}$, Sigma-Aldrich) at a concentration high enough to antagonize all three NP receptors (Telegdy et al., 2000; Potter et al., 2004), or KT5823 (5 $\mu\text{mol/L}$, Cayman Chemical, Ann Arbor, MI), one hour before the addition of NP and Glyc-alb. 8-Bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP; Sigma-Aldrich), a cell permeable cGMP analogue, was administered in a similar manner to natriuretic peptides.

cGMP Enzyme-linked immunoassay

24-hours-starved hFRPE monolayers from two different donors were treated with 500 μM of 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 15 minutes prior to the addition of ANP or CNP (100 nM). 15 minutes later, cGMP was extracted and quantified using a cGMP ELISA kit (Cayman Chemical) per provider instruction. cGMP concentrations were then normalized to the cellular protein concentration as determined by protein assay (Bio-Rad, Hercules, CA). Selected cultures were pretreated with isatin (100 $\mu\text{mol/L}$) 15 minutes before the administration of IBMX.

Immunofluorescence

Monolayers of hRPE cells were stained as described previously (Ablonczy et al., 2011). The primary antibodies were mouse anti-ZO-1 (diluted 1:100; Chemicon, Temecula, CA), rabbit anti-NPR2 (1:50, Sigma-Aldrich). The secondary antibodies were Alexa 594-conjugated goat anti-mouse (diluted 1:100, Invitrogen, Grand Island, NY) and Alexa 488-conjugated goat anti-rabbit (1:100; Invitrogen). The resulting samples were analyzed in a Leica TCS RM confocal microscope (Leica Microsystems, Wetzlar, Germany) at 488 and 594 nm excitation using Leica Confocal Software. Stacks of 200 confocal images were collected at successive focal planes (0.11 μm apart) throughout the entire cell monolayer (22 μm).

Immunoblots

Monolayers of ARPE19 and hRPE cultures were washed with ice-cold PBS and lysed (100 μL ; pH 7.5; 2.42 g/L Tris Base, 1 mM EGTA, 2.5 mM EDTA, 5 mM dithiothreitol, 0.3 M sucrose, 1 mM Na_3VO_4 , and 20 mM NaF – all from Sigma; one complete mini protease 8 inhibitor tablet; Roche Applied Science, Indianapolis, IN), scraped, and collected in a centrifuge tube. The samples were sonicated twice for 10 seconds each, centrifuged for 5 min at 10,000g; and the supernatant collected and centrifuged at 50,000g for 90 min. Equal quantities of the samples (determined by protein assay; Bio-Rad) were separated on 4-12% Bis-Tris Gel, transferred to a blotting membrane, blocked with 5% nonfat dry milk, and incubated with anti-NPR2 (Sigma-Aldrich) overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibody for one hour and the lanes were visualized with a VersaDoc 5000 imager (Bio-Rad) after treatment with chemiluminescent reagent (Fisher Scientific).

RESULTS

Glycated-albumin decreases TEER in both ARPE19 and hfRPE cells

To investigate if AGE products alter RPE barrier function, the RAGE agonist, glycated-albumin (Glyc-alb), was utilized. The mean basal TEER for confluent ARPE-19 monolayers was $40 \pm 4 \Omega \cdot \text{cm}^2$. Apical administration of Glyc-alb (100 $\mu\text{g/mL}$) caused a rapid decline in TEER with a maximal response of $40 \pm 2\%$ drop after six hours (**Fig. 1a**). This decline in TEER was concentration dependent with an EC_{50} of 2.3 $\mu\text{g/mL}$ (**Fig. 1b**). Basolateral administration of human Glyc-alb (100 $\mu\text{g/mL}$) did not significantly alter TEER (**Fig. 1b**). The administration of 100 $\mu\text{g/mL}$ of albumin to the apical or basolateral solution did not significantly alter the TEER compared to untreated controls (**Fig. 1a**).

In primary cultures of hfRPE the mean basal TEER for confluent monolayers was $916 \pm 40 \Omega \cdot \text{cm}^2$. Apical administration of 100 $\mu\text{g/mL}$ of human Glyc-alb produced a $27 \pm 7\%$ drop in TEER six hours after treatment. Again, basolateral administration of Glyc-alb or the administration of albumin to apical or basolateral surfaces did not significantly alter TEER (**Fig 2**).

Natriuretic peptides suppress the AGE-induced decrease in TEER

In ARPE-19 monolayers the administration of individual natriuretic peptides alone did not significantly alter the TEER (**Fig. 3a**). However, one hour pretreatment with 1 nM of ANP, BNP or CNP inhibited the reduction in TEER caused by apical Glyc-alb by 52, 60 and 100%,

respectively (**Fig. 3a**). For each NP, the inhibitory response was concentration-dependent. The IC_{50} for ANP, BNP and CNP responses were 2.5 nM, 0.9 nM, and 9.5 pM, respectively (**Fig. 3b**).

In hRPE cells, pretreatment with CNP (100 nM) completely blocked the decrease in TEER induced by Glyc-alb. Pretreatment with ANP or BNP at concentrations of 100 nM produced a partial inhibition of 52% ($11 \pm 6\%$ reduction in TEER) and 33% ($21 \pm 2\%$ reduction in TEER), respectively (**Fig. 4**).

To confirm this evidence that the suppressive action of natriuretic peptide receptors was mediated by NP receptors, we evaluated the effect of isatin, a nonselective natriuretic peptide receptor antagonist (Telegdy et al., 2000; Potter et al., 2004), on CNP-induced changes in TEER in ARPE19 cells. In the presence of isatin (100 μ M), pretreatment with CNP (10 nM) did not significantly alter the reduction in TEER induced by Glyc-alb. The administration of isatin alone did not significantly change the TEER in ARPE19 monolayers (**Fig. 5a**). To determine if the response to natriuretic peptides was polarized, we compared apical and basolateral pretreatments of 1 nM CNP. Basolateral administration of 1 nM CNP did not significantly alter the reduction in TEER induced by Glyc-alb. However, apical pretreatment with 1 nM CNP completely blocked the response to Glyc-alb in ARPE19 monolayers (**Fig. 5b**).

Natriuretic peptide effect is mediated by cGMP

The involvement of cGMP in NP-induced suppression of Glyc-alb was investigated by treating ARPE19 monolayers with 100 μ M 8-Br-cGMP. Pretreatment with 8-Br-cGMP suppressed the Glyc-alb-induced reduction in TEER (**Fig. 6a**). Additional studies demonstrated that

pretreatment with KT5823 (5 μ M), a protein kinase G (PKG) inhibitor, also reversed the protective effect of CNP on the Glyc-alb-induced barrier breakdown.

Treating hRPE monolayers with 100 nM CNP for 15 minutes showed a 4-fold increase in cGMP levels compared to the statistically non-significant increase when treated with ANP. The production of cGMP increased depending on the concentration of CNP (1 nM-100 nM) resulting in an EC₅₀ of 8.69 nM (data not shown). This effect of CNP on cGMP production was reversed by pretreatment with isatin (100 μ M) (**Fig. 6b**).

Immunoanalysis shows the apical localization of NPR2

The rank order of potency for the suppression of AGE-induced reduction in TEER by natriuretic peptides and the synthesis of cGMP provides evidence that this response is mediated by the NPR2 subtype. To confirm the presence of these receptors in ARPE19 and hRPE, Western blot and immunofluorescence were conducted. **Figure 7** (panels **A-H**) shows the immunofluorescence staining for the NPR2 in hRPE; the tight junction marker ZO1, delineating apical and basolateral sides of the cell; and Draq5 (blue) labels the nuclei located close to the basement membrane. Confocal analysis demonstrated that NPR2 is mainly localized on the apical surface of the cells above the basolateral location of Draq5 (**Fig. 7I**). Western blot analysis for the membranous and the cytosolic fractions against the NPR2 showed a single band at \approx 130 kDa only in the membranous fraction, and not in the cytosolic fraction (**Fig. 7J**).

DISCUSSION

Advanced glycation end-products (AGE) have been associated with numerous complications of diabetes (Ahmed, 2005). The levels of AGEs in the blood and vitreous humor of diabetic patients have been correlated with the clinical progression of diabetic retinopathy (Yokoi et al., 2005). Although the RPE expresses several pattern-recognizing receptors activated by AGEs, a direct causal relationship between AGEs and RPE dysfunction has not been addressed before. Using human glycated-albumin, we determined the effect of AGEs on the barrier function of the RPE. The RPE constitutes the outer-blood-retina barrier and is responsible for fluid transport from the neural retina to the choroid. This transport counters the Starling forces across the RPE that drive fluid toward the retina (Maepea, 1992). As a result, increasing RPE permeability can contribute to the development of macular edema, a key component of diabetic retinopathy.

Our experiments demonstrated that the administration of human Glyc-alb reduced TEER in both ARPE-19 and hfRPE monolayers only when administered to the apical surface and the response was concentration-dependent (EC_{50} of 2.3 $\mu\text{g/mL}$ and $\log EC_{50} = -5.63 \pm 0.4$). These data support the idea that this effect is receptor-mediated, and that these receptors for AGE products are localized on the apical side of the RPE monolayers. The EC_{50} for glycated-albumin is consistent with the results for a dose-dependent increase in permeability ($\log EC_{50} = -5.88 \pm 0.3$) seen in retinal microvasculature (Warboys et al., 2009), and is in the same range of Glyc-alb increase seen in the vitreous of STZ-induced diabetic rats (1.92 $\mu\text{g/mL}$) (Cohen et al., 2008).

While our current studies were not designed to characterize the pattern recognition receptor(s) involved in the response, the polarized nature of the response would argue that the receptor for AGE (RAGE) was on the apical side of the cells and is affected by any AGE increase in the vitreous (Yokoi et al., 2005; Yamagishi et al., 2006; Kakehashi et al., 2008). Increase in intravitreal AGEs (which can originate from leakage of blood through the inner retina vessels, or can be generated in the retina *in situ*) would then in turn disrupt RPE barrier function causing further increase in AGE accumulation in the ocular environment. AGE can also accumulate at the basolateral surface of the RPE most likely diffusing from the choroid (Yamada et al., 2006). Moreover *in vivo* RAGE was found to colocalize with AGE at the basal deposits, however its physiological implications have not been shown. Our polarized acute AGE response may indicate just a difference between short- and long-term AGE exposure or that the expression of RAGE might be under the control of its agonist.

Natriuretic peptides are important regulators of cardiovascular function influencing fluid balance, vasodilatation and vascular permeability (Potter et al., 2006). In the eye, studies have shown that NP receptors are present in the mammalian neural retina (Rollin et al., 2004) and RPE (Fujiseki et al., 1999); and the activation of these receptors can influence VEGF-induced permeability changes in the RPE (Ablonczy and Crosson, 2007; Lara-Castillo et al., 2009). However, the receptor subtype responsible for this response has not been investigated. In the current study we investigated if natriuretic peptides can suppress the permeability changes induced by AGEs. As shown in **Figures 3 and 4**, pretreating RPE monolayers with ANP, BNP, or CNP suppressed the decrease in TEER induced by Glyc-alb in a concentration-dependent fashion. While 100 nM of all three NP peptides was able to reverse the effect of Glyc-alb on the

TEER in ARPE19 cells, only 100 nM CNP was able to do so in hRPE cultures indicating that the two cell lines respond a little differently to NPs. Although the responses in hRPE cells are expected to better represent properties *in vivo*, both models showed the same rank order of potency with $\text{CNP} \gg \text{BNP} \geq \text{ANP}$. In addition, the non-selective NPR antagonist, isatin, blocked this response. Together these data provide pharmacological evidence that response is primarily mediated through the NPR2 sub-type.

We used isatin primarily to show that the action of NPs was a receptor-mediated process. Although several studies have used isatin as a selective NPR-A antagonist (Katoli et al., 2010), it has also been shown that at high enough concentration it can antagonize all three NP receptors (Telegdy et al., 2000; Potter et al., 2004; Katoli et al., 2010). Isatin is an indole molecule, nevertheless it has little effect on the many neurotransmitter and hormonal receptors in the rat hippocampus, acting primarily as an inhibitor of atrial natriuretic peptide binding (Telegdy et al., 2000). Although changes in serotonin and melatonin levels or the activation of their receptors have not been investigated, isatin by itself had no effect on RPE survival or barrier function (**Fig. 5**), implying that any effect other than blocking NP receptors, was not relevant.

As only apical treatment with natriuretic peptides suppressed the AGE-induced reduction in RPE resistance, we conclude that the receptors (NRP2) are localized on the apical surface of the RPE. Consistent with this conclusion and results from other laboratories (Wistow et al., 2002), Western blot analysis confirmed that NPR2 receptors are expressed in RPE monolayers. Confocal analysis of immune-localization studies confirmed the apical orientation (**Fig. 7I**).

The primary second messenger linked to the NPR2 receptor is cGMP (Levin et al., 1998; Potter et al., 2006). Our data provided evidence that natriuretic peptides are effective in stimulating cGMP synthesis in the RPE and that the cell permeable cGMP analogue can suppress AGE-induced RPE barrier dysfunction. Consistent with the data that NPR2 receptor subtype is the primary receptor involved in the NP effect, 100 nM CNP for 15 minutes showed a 4-fold increase in cGMP levels compared to the statistically non-significant increase when treated with 100 nM ANP. Although ANP at 100 nM showed half protection (52%) against the Glyc-alb-induced decrease in TEER; the reason why we don't see a significant increase in cGMP might be as simple as a timing difference between the two experiments. cGMP Levels were evaluated 15 minutes after adding 100 nM of ANP, while we evaluated the pharmacological effect on TEER after 2 hours. Moreover, cGMP might be compartmentalized, resulting in an increase in cGMP with ANP treatment that is too low to be detected by our assay.

Previous studies have shown that ANP and cGMP alone can stimulate the pumping activity of the RPE and increase the rate of reabsorption of subretinal fluid (Marmor and Negi, 1986; Baetz et al., 2012). These data provide evidence that the activation of the cGMP-PKG pathway is important for maintaining the functional integrity of the RPE and removal of fluid from the subretinal environment.

Preclinical studies targeting agents that suppress AGE formation or RAGE-signaling showed encouraging results; however, subsequent clinical trials were disappointing (Peyroux and Sternberg, 2006). In the current study, we demonstrated that administration of RAGE agonist, Glyc-alb, produced significant reductions in RPE monolayer resistance and that pretreatment

with natriuretic peptides suppressed this response. Pharmacological and structural studies provided evidence that the response to natriuretic peptides was mediated by NPR2 receptors in the apical membranes of the RPE, and is dependent on cGMP. Thus, NPR2 agonists or agents that increase cGMP may provide alternative treatment options for diabetic macular edema.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Dahrouj, Crosson, and Ablonczy

Conducted experiments: Dahrouj and Liu

Performed data analysis: Dahrouj, Crosson, and Ablonczy

Wrote or contributed to the writing of the manuscript: Dahrouj, Al Sarraf, Crosson, and
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FOOTNOTES

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- b) **Unnumbered Footnote:** Portions of this work were presented as an abstract at the 2011 annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, FL as Dahrouj M, Ablonczy Z, and **Crosson CE**. Natriuretic peptides protect the RPE from AGE-induced barrier breakdown. *Invest Ophthalmol Vis Sci* 2011; 52:E-Abstract PN 5659.
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- d) **Numbered Footnotes:** N/A.

FIGURE LEGENDS

Figure 1. *Glycated-albumin (Glyc-alb) reduces the TEER of ARPE-19 cells.* **A.** Apical administration (Δ) of 100 $\mu\text{g/mL}$ of Glyc-alb to ARPE-19 cells caused a decrease in TEER with a maximal drop of $40 \pm 2\%$ after six hours. Basolateral administration of Glyc-alb (\circ) (100 $\mu\text{g/mL}$) or apical albumin (\square) (100 $\mu\text{g/mL}$) had no significant effect on TEER after six hours. **B.** Concentration-dependent reduction in TEER six hours after apical administration of Glyc-alb in ARPE19 cells. The percent decrease in resistance was concentration-dependent with an EC_{50} of 2.3 $\mu\text{g/mL}$ ($\log \text{EC}_{50} = -5.63 \pm 0.4$). Values are means \pm SE of individual measurements normalized to the average TEER values at $t = 0$, * $P < 0.05$ ($n = 4-12$).

Figure 2. *Glycated-albumin (Glyc-alb) reduces the TEER of hRPE cells.* Apical administration of 100 $\mu\text{g/mL}$ of Glyc-alb (Δ) decreased TEER with a maximal drop of $27 \pm 7\%$ after six hours. Basolateral administration of Glyc-alb (\circ) (100 $\mu\text{g/mL}$) and apical albumin (\square) (100 $\mu\text{g/mL}$) had no significant effect on TEER after six hours of treatment. Values are mean \pm SE of individual measurements normalized to the average TEER values at $t = 0$, * $P < 0.05$ ($n = 4-6$).

Figure 3. *Apical natriuretic peptides (NP) antagonize the AGE-induced TEER reduction in ARPE-19 cells.* **A.** The effect of 100 $\mu\text{g/mL}$ of apical Glyc-alb on ARPE-19 cells pretreated (1 hour) with 1 nM of ANP, BNP or CNP apically applied. At this concentration, pretreatment with CNP (\square) prevented the drop in TEER, while ANP (\circ) and BNP (\diamond) showed $21 \pm 2\%$ and $16 \pm 2\%$ drop in TEER, respectively, as compared to $40 \pm 2\%$ with no pretreatment (Δ). **B.** Dose-response curves for ANP-, BNP- and CNP-induced inhibition of the reduction in TEER

following the six-hour apical administration of Glyc-alb (100 μ g/mL). The rank-order of agonist potency is CNP (IC_{50} = 9.5 pM) \gg BNP (IC_{50} = 0.9 nM) \geq ANP (IC_{50} = 2.5 nM). Values are mean \pm SE of individual measurements normalized to the average TEER values at t = -1 hour,* P < 0.05 (n = 4-6).

Figure 4. *Apical natriuretic peptides (NP) antagonize the AGE-induced TEER reduction in hfRPE cells.* The effect of 100 μ g/mL of apical Glyc-alb on hfRPE cells pretreated (1 hour) with 100 nM of ANP, BNP or CNP apically applied. CNP provided a complete protection while ANP and BNP showed approximately a 10% drop in TEER after six hours of apical administration of 100 μ g/mL of Glyc-alb. Values are mean \pm SE of individual measurements normalized to the average TEER values at t = 0,* P < 0.05 (n = 4-6).

Figure 5. *Polarized effect of natriuretic peptides on AGE-induced barrier breakdown. A.* Effect of isatin (100 μ M), a non-specific natriuretic peptide receptor antagonist, on CNP-induced inhibition of the Glyc-alb response in ARPE-19 cells. Pretreatment with isatin reversed the inhibitory effect of CNP (10 nM) on the Glyc-alb (100 μ g/mL)-induced decrease in TEER in ARPE19 cells. **B.** Pretreatment with basolateral CNP (1 nM) did not show any inhibition of the AGE-induced effect with $34 \pm 3\%$ decrease in TEER after six hours of Glyc-alb (100 μ g/mL) administration. On the other hand, apical pretreatment with CNP (1 nM) reversed the AGE-induced effect with no significant decrease in TEER six hours following Glyc-alb (100 μ g/mL) administration. Values are mean \pm SE of individual measurements normalized to the average TEER values at t = -1 hour,* P < 0.05. (n = 4-6). Similar results were seen for ANP and BNP.

Figure 6. *The effect of CNP is mediated by cGMP and PKG.* **A.** Pretreatment with 8-Br-cGMP (100 μ M), a cell permeable cGMP analogue, reversed the AGE-induced effect with no significant decrease in TEER six hours following 100 μ g/mL Glyc-alb administration in ARPE19 cells. KT5823 (5 μ mol/L), a PKG inhibitor, reversed the inhibitory effect of CNP (1 nM) on AGE-induced reduction in TEER. **B.** cGMP ELISA showed a 4-fold increase in cGMP levels in hRPE monolayers when treated with 100 nM of CNP. ANP at 100 nM did not cause any significant increase in cGMP production. Isatin, 100 μ M, reversed the effect of CNP on cGMP production. Values are [cGMP] (pmol/mg of protein) plotted as mean \pm SE of individual measurements, * $P < 0.05$ from IBMX only, ** $P < 0.05$ from CNP treatment (n = 6, from two different donors). Values are mean \pm SE of individual measurements normalized to the average TEER values at t = -1 hour, * $P < 0.05$ (n = 4-12).

Figure 7. *Expression and localization of NPR2.* hRPE Monolayers were incubated with Alexa 488-conjugated goat anti-rabbit (**A**) or anti-NPR2 and Alexa 488-conjugated goat anti-rabbit (**E**), anti-ZO1(**B, F**; red, tight junction marker), and Draq5 (**C, G**; blue nuclear dye). **D** shows **A, B** and **C** merged, while **H** shows **E, F** and **G** merged (Bar = 20 μ m). **I.** Cross-sections through the Z-plane confirm the apical localization of the receptor above Draq5 and ZO1. **J.** Immunoblot of NPR2 shows a band in the membranous fraction (M) and not in the cytosolic fraction (C) of both hRPE and ARPE19 cells.

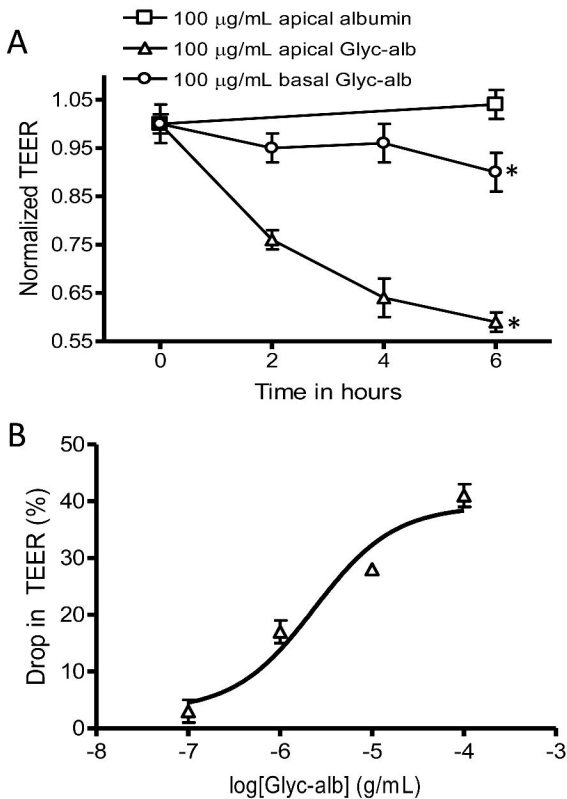


Figure 1

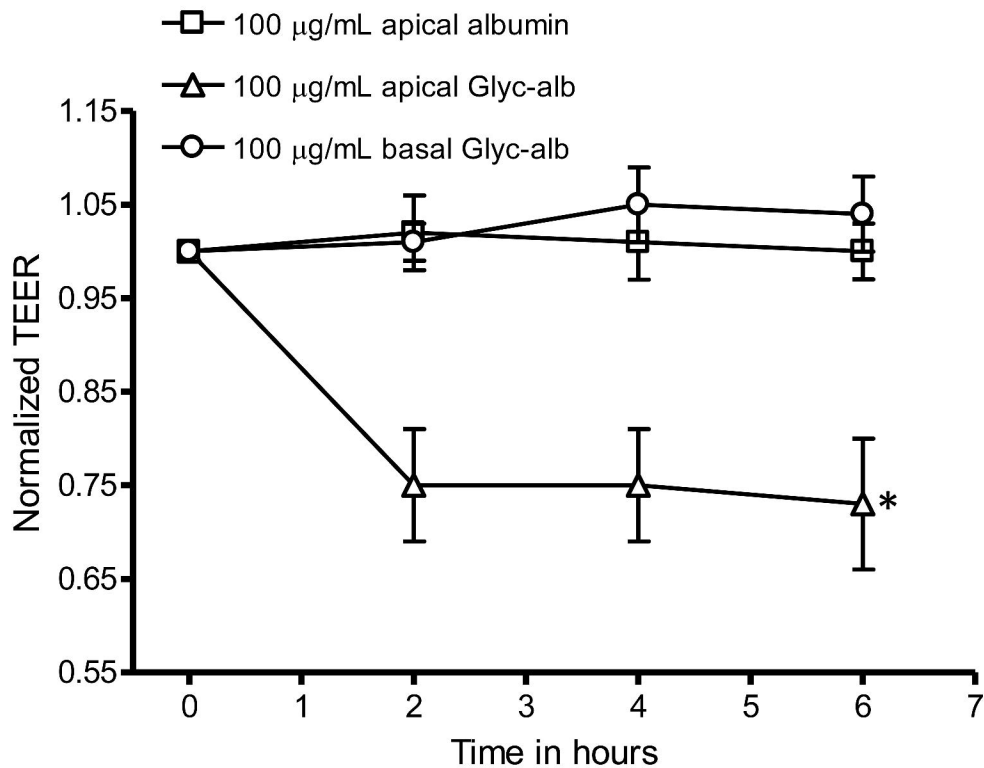


Figure 2

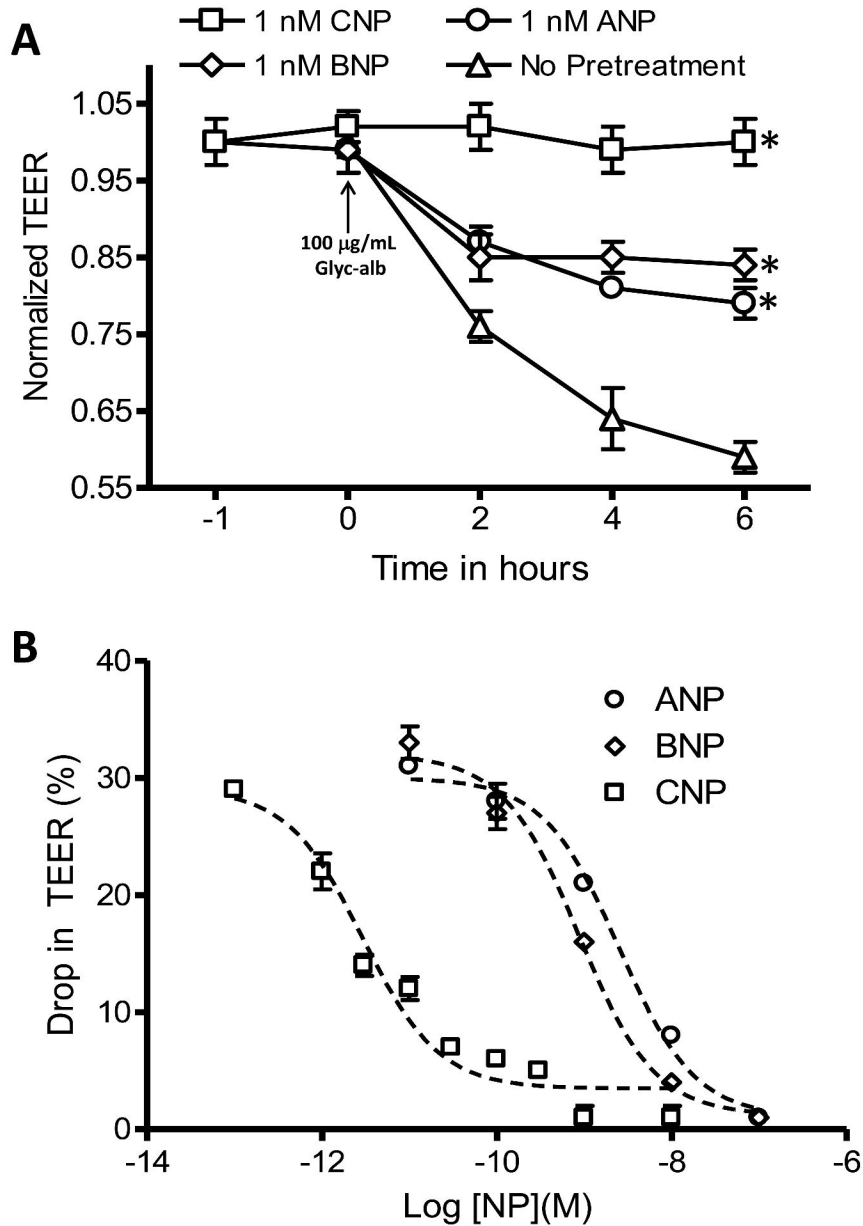


Figure 3

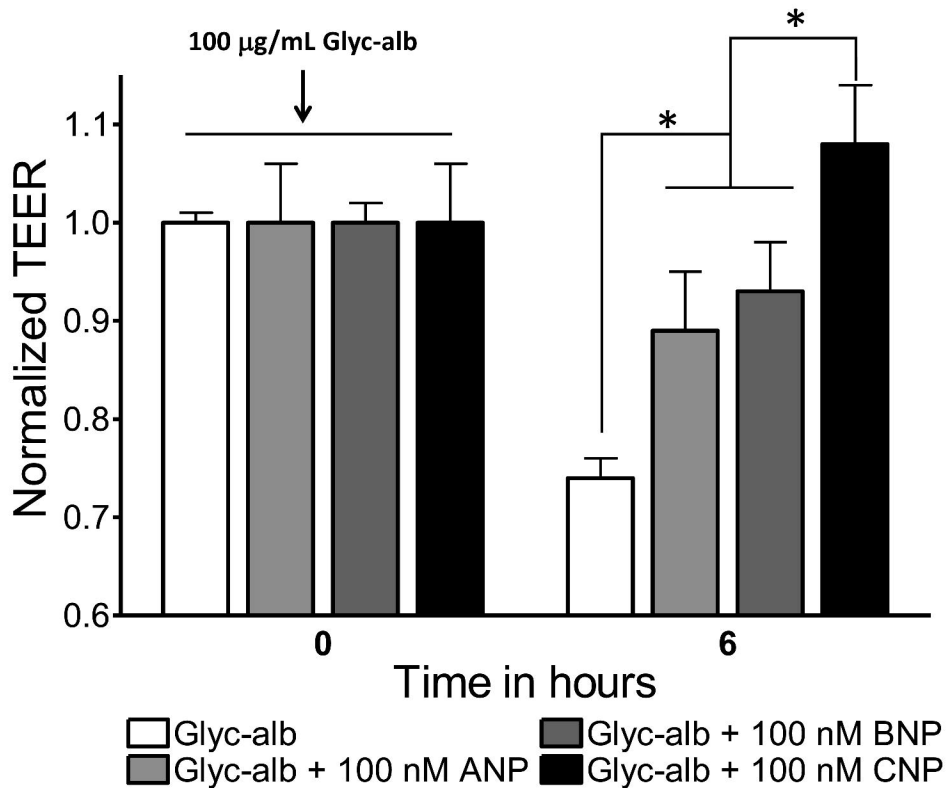


Figure 4

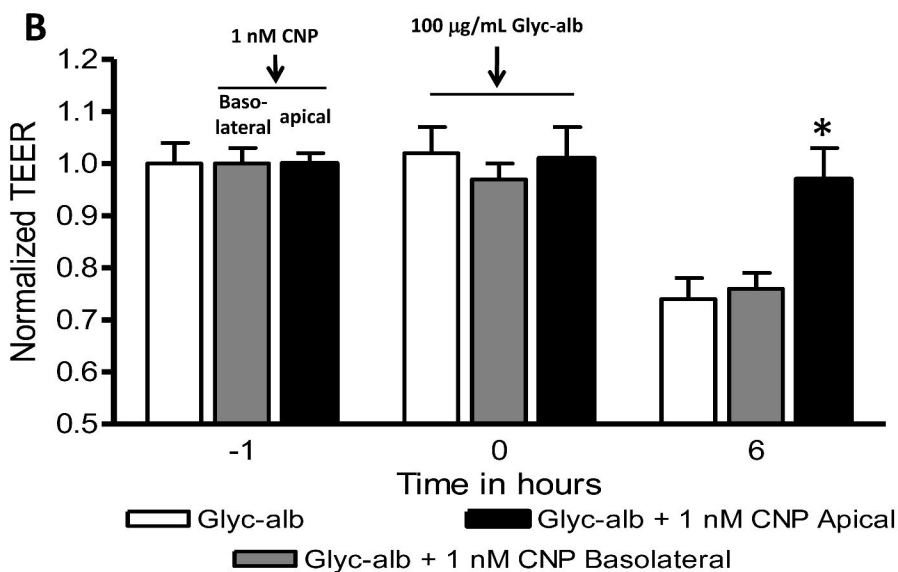
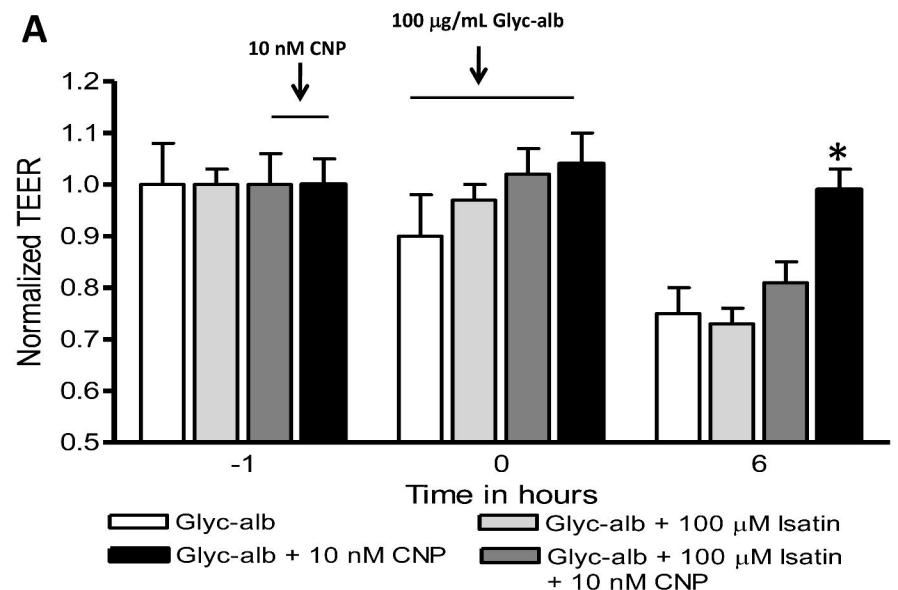


Figure 5

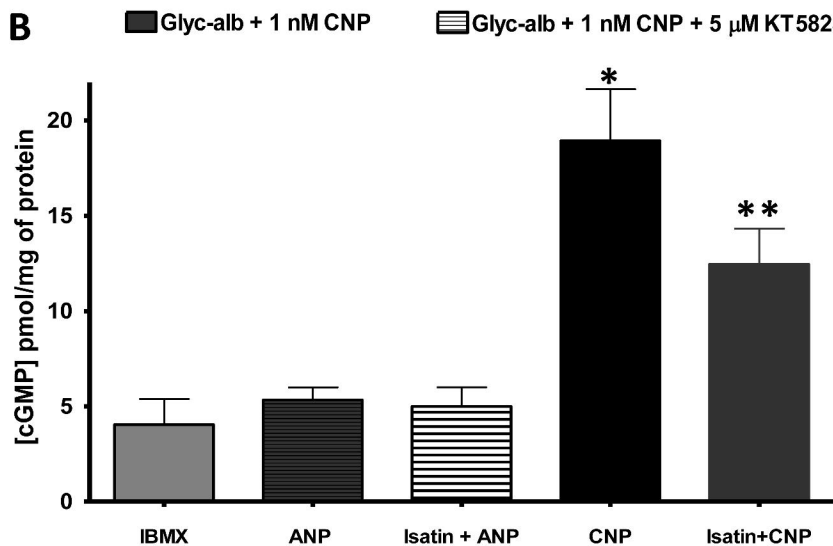
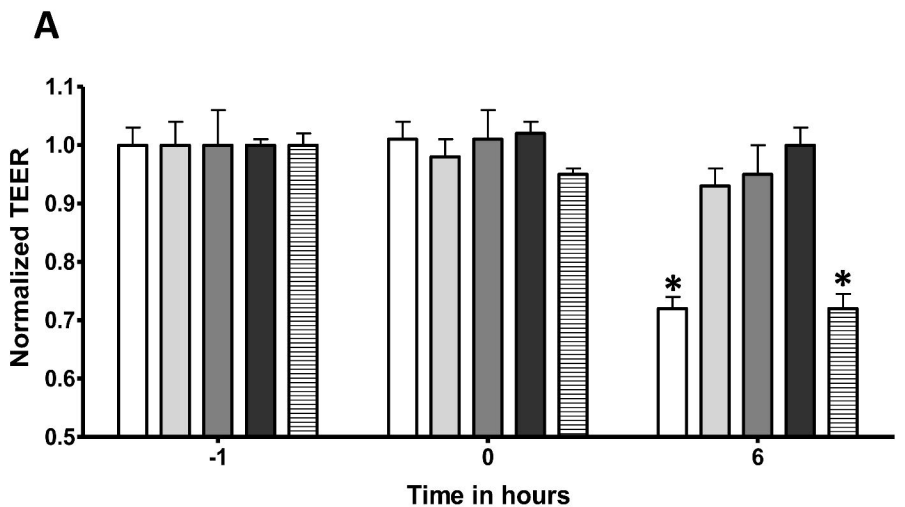


Figure 6

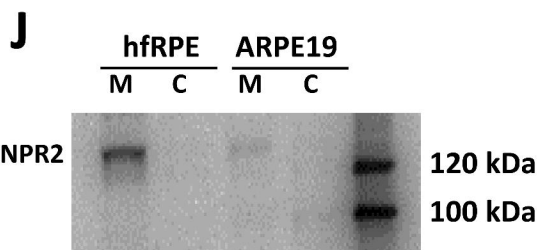
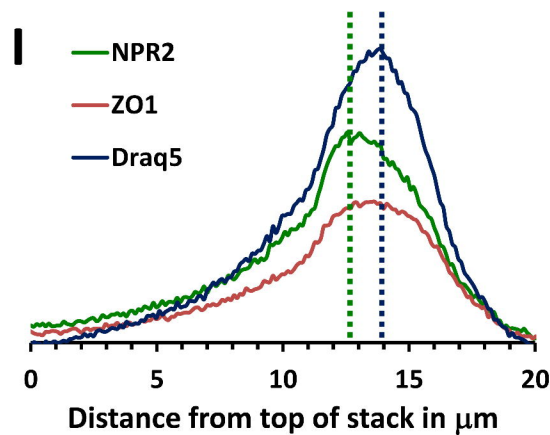
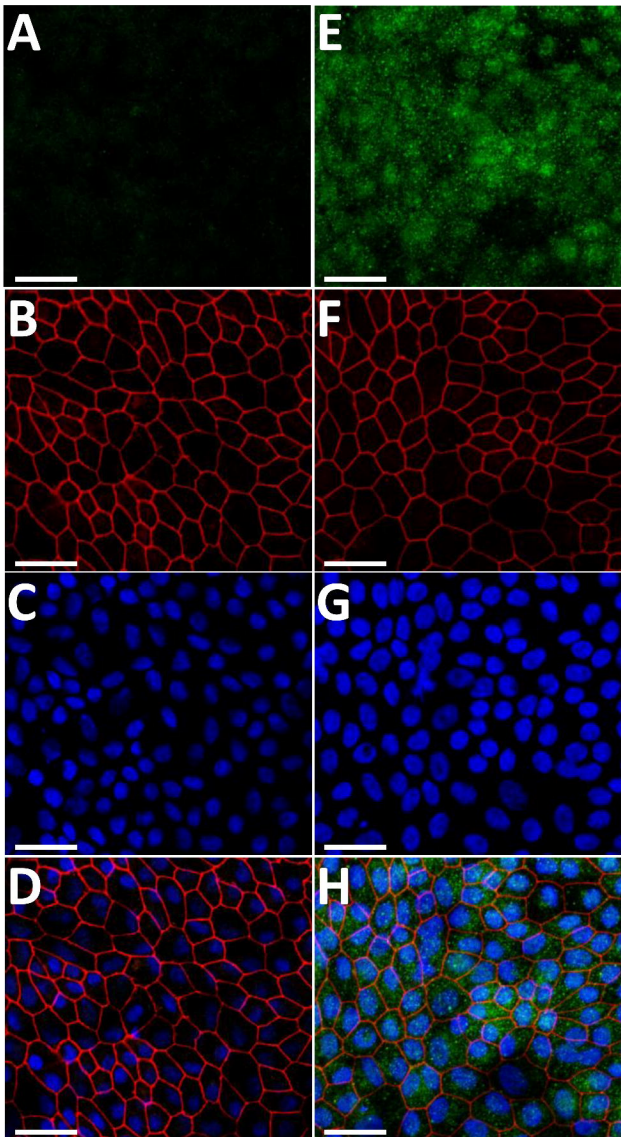


Figure 7