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Hesperidin stimulates CFTR-mediated chloride secretion and ciliary beat frequency in sinonasal epithelium

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Abstract

Objectives—Pharmacologic agents designed to promote mucociliary clearance (MCC) in chronic rhinosinusitis (CRS) represent a novel therapeutic strategy. The objectives of the present study were to investigate whether the natural bioflavonoid hesperidin 1) increases transepithelial chloride (Cl[−]) secretion *in vitro* and *in vivo*, 2) enhances ciliary beat frequency (CBF), and 3) exerts its mechanistic effects through cAMP/PKA dependent pathways.

Study Design—*In vitro* and *in vivo* study.

Setting—Laboratory.

Subjects and Methods—Transepithelial Cl[−] transport (Ussing chamber) and CBF were investigated in primary murine nasal septal (MNSE) and human sinonasal epithelial (HSNE) cultures. *In vivo* activity was measured using the murine nasal potential difference (NPD) assay. CFTR R-domain phosphorylation and cAMP levels were investigated to rule out a cAMP/PKA dependent mechanism of activation.

Results—Hesperidin significantly increased CFTR-mediated Cl[−] transport (change in short-circuit current, ΔI_{SC}) in both MNSE [13.51 $\pm$ 0.77 vs. 4.4 $\pm$ 0.66 (control); $p < 0.05$] and HSNE [12.28 $\pm$ 1.08 vs. 0.69 $\pm$ 0.32 (control); $p < 0.05$]. Cl[−] transport across *in vivo* murine nasal epithelium was also significantly enhanced with hesperidin [−2.3 $\pm$ 1.0 vs. −0.8 $\pm$ 0.8mV (control), $p < 0.05$]. There was no increase in cellular cAMP or phosphorylation of the CFTR R-domain. Hesperidin significantly increased CBF (ratio of pre to post-treatment) with both basal ((1.31 $\pm$ 0.07 vs. 0.93 $\pm$ 0.06 (control); $p < 0.05$), apical (1.72 $\pm$ 0.09 vs. 1.40 $\pm$ 0.07, control; $p < 0.05$) and basal + apical delivery (2.26 $\pm$ 0.18 vs. 1.60 $\pm$ 0.21, respectively; $p < 0.05$).

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Corresponding Author: Bradford A. Woodworth, MD University of Alabama at Birmingham Otolaryngology – Head and Neck Surgery BDB 563; 1530 3rd Ave S Birmingham, AL 35294 205-934-9765 (Phone) 205-934-3993 (Fax) bwoodwo@hotmail.com . Dr. Sorscher and Dr. Woodworth are inventors on a patent submitted regarding the possible activity of chloride secretagogues for therapy of sinus disease (Provisional Patent Application Under 35 U.S.C. §111(b) and 37 C.F.R. § 1.53(c) in the United States Patent and Trademark Office).

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Conclusion—Our *in vitro* and *in vivo* investigations provide strong support for future testing of this robust Cl^- secretagogue and CBF activator in human clinical trials for CRS.

Keywords

Transepithelial Ion Transport; Hesperidin; CFTR; Chronic Sinusitis; Chloride Secretion; Murine Nasal Culture; Human Sinus Epithelium; Mucociliary Clearance; Ciliary Beat; cAMP; PKA; R-D phosphorylation

Introduction

Ineffective mucociliary clearance (MCC) is a common pathophysiologic process present in airway inflammation and infection. Airway surface liquid (ASL) is a principle component of the mucociliary apparatus and is strongly influenced by the vectorial transport of ions, such as chloride (Cl^-).¹ Dysfunctional Cl^- transport results in dehydration of the ASL and mucus stasis as demonstrated in the severe lower and upper respiratory disease, cystic fibrosis. Patients with inspissated mucus are at high risk for bacterial infection and widespread chronic rhinosinusitis (CRS) refractory to medical management. Conventional interventions for CRS have been limited by bacterial resistance incurred with antibiotic overuse and the deleterious side effects of steroids. Safe, but effective, compounds that enhance Cl^- transport, specifically those that activate the cystic fibrosis transmembrane conductance regulator (CFTR), could provide significant therapeutic advantages in this regard.

Recent drug discovery efforts have identified several exciting small molecules that activate or potentiate CFTR^{2,3} for treatment of CF and other respiratory diseases. CFTR has two transmembrane domains, two nucleotide binding domains (NBDs), and a regulatory domain (R-D). Activation of CFTR Cl^- transport requires phosphorylation of the R-D by cAMP/PKA dependent pathways, as well as heterodimerization of the two NBDs⁴ (Fig 1). Drugs that either activate (phosphorylate the R-D) or potentiate (increase channel open time) are desirable, and continue to be an active area of laboratory and clinical investigation in airway disease. This strategy has shown significant clinical promise in CF and has been highly visible in both the scientific⁵ and lay communities⁶ over the past year. However, the same strategy clearly activates Cl^- secretion in the nasal as well as lower airways, and therefore may benefit subjects with CRS and impaired sinonasal MCC.⁷ Using CFTR activators as a therapeutic intervention in CRS has not been investigated previously, and thus represents a new and leading edge approach to treatment.

Flavonoids are among the most potent activators of CFTR gating,^{8,9} and are excellent candidates for restoring and/or increasing CFTR activity in sinus and nasal epithelium. Flavonoids are a group of ubiquitous plant molecules (based on the backbone of 2-phenylchromen-4-one, Figure 1) that exhibit a number of biological effects critical to human health, and have been shown previously to confer antibacterial, antiviral, anti-inflammatory and antiallergic effects.⁸ The flavonoid, hesperidin, has been investigated as a therapeutic intervention for a wide spectrum of human diseases, including high cholesterol (via impairment in high density lipoprotein, low density lipoprotein, triglycerides, and total lipids),¹⁰ inflammation (hesperidin blocks synthesis of pro-inflammatory arachidonic acid derivatives, including prostaglandins E_2 and F_2 and thromboxane A_2),¹¹ and cancer (the compound inhibits 4-nitroquinoline 1-oxide, a known carcinogen).¹² However, hesperidin and other glycosylated flavonoids were previously reported to have poor Cl^- secretory activity in a colonic cell line.¹³

In this manuscript, we present evidence that hesperidin is a robust Cl^- secretagogue (via CFTR) and CBF activator in sinonasal epithelium, and therefore represents an excellent

candidate for investigating the mechanisms underlying CRS and as part of therapeutic development.

Methods

University of Alabama at Birmingham Institutional Animal Care and Use Committee and Institutional Review Board approval were obtained prior to initiation of the study.

Cell Culture

Cultures were developed from primary murine nasal septal epithelium (MNSE) and human sinonasal epithelium (HSNE) as previously described.¹⁴⁻¹⁸ Cultures with well-differentiated, beating cilia and transepithelial resistances (R_t) > 500 Ω cm² were mounted in Ussing chambers for short-circuit current (I_{SC}) measurements or monitored for ciliary beat frequency as described below.

Insert Summary

A total of 97 MNSE and 12 HSNE cell cultures were used in the completion of these studies. Cultures with transepithelial resistances (R_t) > 500 Ω cm² were used to obtain Ussing chamber measurements and monitor ciliary beating with an inverted microscope. A minimum of 5 wells were used per condition.

Electrophysiology

Solutions and Chemicals—The bathing solution contained (in mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 10 glucose. The pH of this solution is 7.3-7.4 when gassed with a mixture of 95% O₂-5% CO₂ at 37°C. All chemicals were obtained from Sigma (St. Louis, MO). Each chemical was prepared as a 1000X stock and used at 1X in the Ussing chamber. All experiments were performed with low Cl⁻ (6mM) in the mucosal bath. Pharmacologic preparations were as follows: Hesperidin (250 μ M, 500 μ M, 750 μ M, 1.0 mM, 1.5 mM, 2 mM), amiloride (100 μ M), Forskolin (2 μ M), INH-172 (10 μ M), and H-89 (50 μ M) Hesperidin was dissolved in dimethyl sulfoxide (DMSO).

Short Circuit (I_{SC}) Measurements—Transwell inserts (Costar) were mounted in Ussing chambers in order to investigate pharmacologic manipulation of vectorial ion transport. Monolayers were continuously monitored under short circuit conditions following fluid resistance compensation using automatic voltage clamps (VCC 600; Physiologic Instruments, San Diego, CA). Batch solutions for the transwell filters were warmed to 37°C, and each solution continuously gas lifted with a 95%O₂-5%CO₂ mixture. DMSO control solutions were tested for comparison. The I_{SC} was assessed at one current measurement per second. By convention, a positive deflection in I_{SC} was defined as the net movement of anions in the serosal to mucosal direction.

Nasal Potential Difference

A 3-Step protocol was used, as described previously.¹⁹ First, nasal cavities of anesthetized mice (C57/BL6) were perfused sequentially with 1) Ringer's solution containing 140mM NaCl, 5mM KCl, 1mM MgCl₂, 2mM CaCl₂, 10mM HEPES, and amiloride 50 μ M (pH 7.3); 2) amiloride + a low-Cl⁻-containing solution (NMDG, 6 mM Cl⁻, pH 7.3); and 3) amiloride + low-Cl⁻-containing solution + hesperidin, forskolin 20 mM, or DMSO control. Because of the continuous presence of amiloride (50 μ M) and the complete replacement of Na⁺ with a membrane-impermeant cation (140 mM NMDG in the perfusion solution), hyperpolarization in this setting is taken to reflect Cl⁻ secretion rather than sodium

absorption. Each condition was studied for 5 to 10 minutes until a stable signal was achieved. All traces were interpreted in a blinded fashion.

Detection of Regulatory Domain (R-D) Phosphorylation and cAMP levels

Because activation of CFTR anion transport requires phosphorylation of the R-D, an ELISA-based detection kit (Cayman Chemicals, Ann Arbor, MI) was used to measure stimulation of cellular cAMP by hesperidin in MNSE cultures, as previously described.⁸ To confirm absence of a cAMP/PKA dependent mechanism, polyclonal NIH-3T3 cells expressing HA-tagged R-domain were treated with hesperidin for 20 minutes, and compared to forskolin (100 nM) $\times$ 5 minutes as a positive control and DMSO as negative control. Following lysis, equal amounts (50 μ g) of total cell lysate were electrophoresed through a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and immunoblotted with antibody to the HA tag (Covance, Cumberland, VA). Phosphorylation of the R-domain was visualized as a 2-4 kD shift in migration, as previously described.⁸

Ciliary Beat Frequency

Images were visualized using a 20X objective on an inverted scope (Fisher Scientific, Pittsburgh, Pa.). Data was captured using a Model A602f-2 Basler area scan high-speed monochromatic digital video camera (Basler AG, Ahrensburg, Germany) at a sampling rate of 100 frames per second and a resolution of 640 $\times$ 480 pixels. The Sisson-Ammons Video Analysis (SAVA)²⁰ system version 2.1.6 was used to analyze each image. For each experiment, a large area of beating cilia was identified. The digital image signal was then routed from the camera directly into an acquisition board (National Instruments) within a Dell Workstation running the Windows XP Professional operating system. Images were analyzed with virtual instrumentation software for CBF analysis and recordings were made at 200 $\times$ magnification.

Experiments were all performed at ambient temperature (23°C). Prior to administration of test solution, a baseline recording of CBF was conducted for each cell monolayer, as apical fluid addition alone can increase CBF. Additional fluid depth overlying the respiratory epithelial cell surface stimulates CBF due to improved hydration and enhanced fluid dynamics. Thus, comparison of hesperidin to a PBS control solution was necessary. Whole field analysis was performed with each point measured representing one cilia. The reported frequencies (Hertz – Hz) describe arithmetic means of these values, followed by standard deviations. Each analysis was normalized to fold change of CBF Hz (treatment/baseline) and the average fold-change tested for significance using paired or unpaired t tests as appropriate. A p value < 0.05 was considered statistically significant.

Statistical Analysis

Statistical analysis was performed using two-tailed unpaired *t*-test and analysis of variance as indicated.

Results

Hesperidin is a Cl⁻ secretagogue

Hesperidin exhibited robust stimulation of CFTR-mediated anion transport in a dose-dependent fashion in MNSE cultures (Fig 2). The highest response was noted at 2.0 mM (ΔI_{SC} , 16.67 $\pm$ 0.43 μ A/cm²), but precipitation was noted with concentrations greater than 1.0 mM, so this dose was considered optimal for the remainder of the studies. Cl⁻ transport stimulated by hesperidin (1.0 mM) (ΔI_{SC} , 13.51 $\pm$ 0.77 vs. 4.4 $\pm$ 0.66 (control); *p*=0.000002) was not dependent on pretreatment with the cAMP agonist forskolin, although some baseline activity in the cAMP/PKA dependent pathway is required as the PKA

inhibitor H-89 blocks all hesperidin CFTR activation (data not shown). Total stimulated I_{SC} with the addition of 100 nM forskolin (ΔI_{SC} , 24.93 $\pm$ 1.96 vs. 18.8 $\pm$ 1.96; $p=0.01$) and CFTR blockade with INH-172 (ΔI_{SC} , -18.57 $\pm$ 1.40 vs. -13.27 $\pm$ 1.55; $p=0.03$) were also significantly different between groups.

Hesperidin increases transepithelial Cl^- transport in HSNE

We next sought to determine whether hesperidin activated Cl^- secretion in HSNE. (Figure 3) Hesperidin had a consistent effect in HSNE significantly increasing CFTR dependent Cl^- transport when compared to DMSO control (ΔI_{SC} , 12.28 $\pm$ 1.08 vs. 0.69 $\pm$ 0.32 (control); $p=0.000001$). (Figure 3) In addition, total stimulated I_{SC} (ΔI_{SC} , 15.29 $\pm$ 1.16 vs. 10.62 $\pm$ 0.74 (control); $p=0.007$) and CFTR blockade with INH-172 (ΔI_{SC} , -10.52 $\pm$ 0.37 vs. -8.86 $\pm$ 0.40 (control); $p=0.01$) were also significantly different from controls. This model has an overall decreased ion transport phenotype when compared to MNSE.¹⁷ However, hesperidin accounted for a large proportion of the total stimulated CFTR-mediated I_{SC} (80%), because of the minimal additive contribution of forskolin to the ΔI_{SC} post treatment.

Hesperidin activates CFTR-dependent Cl^- conductance across murine nasal epithelium in vivo

Transepithelial Cl^- transport measured by the murine NPD assay was also significantly enhanced with hesperidin (-2.3 $\pm$ 1.0mV) when compared to controls (-0.8 $\pm$ 0.8mV, $p=0.04$). Hesperidin activation of Cl^- transport was not significantly different than forskolin (-1.9 $\pm$ 1.4mV, $p=0.65$) (Fig 4).

Hesperidin activates CFTR despite no measurable effects on cAMP and R-domain phosphorylation

PKA/cAMP-dependent phosphorylation of the CFTR R-Domain is a critical step in channel activation,²¹ but also (through an independent mechanism) activates ciliary axonemes to drive stimulated CBF.²² To examine the basis of CFTR activation and CBF stimulation, we measured the effects of hesperidin on cAMP levels and on R-domain phosphorylation. Hesperidin had no detectable effects on cellular cAMP concentrations (2 mM, 0.60 $\pm$ 0.04 pmol/ml) when compared to DMSO control (0.54 $\pm$ 0.09 pmol/ml), or the marked cAMP elevations demonstrated with forskolin (377.76 $\pm$ 19.65 pmol/ml, positive control) (Fig 5A). As opposed to the cAMP agonist forskolin, hesperidin also had no detectable effect on recombinant CFTR R-D phosphorylation-dependent mobility shift, findings that together suggest independence from either CFTR or ciliary axoneme phosphorylation (Fig 5B). Thus, activation of CFTR and CBF by hesperidin differs from classic cAMP-signaling agonists, and is consistent with a mechanism of action that does not depend upon R-D phosphorylation, as reported with other flavonoid agents including genistein.²³

Hesperidin increases CBF

CBF (measured as fold-change over baseline) in MNSE was significantly increased when hesperidin (1 mM) was applied to the basal media (1.31 $\pm$ 0.07 vs. 0.93 $\pm$ 0.06 (control); $p=0.003$) (Fig 6). Delivery of the agent to the apical surface also resulted in a significant stimulation of CBF when compared to the control vehicle. Because CBF was also elevated following hesperidin (1.72 $\pm$ 0.09 vs. 1.40 $\pm$ 0.07, control; $p=0.01$), augmentation of CBF could not solely be attributed to electrolyte and fluid secretion across the apical surface mediated by CFTR. Apical and basal activation by hesperidin was additive in magnitude (2.26 $\pm$ 0.18 vs. 1.60 $\pm$ 0.21, respectively; $p=0.043$). When cultures were incubated with the PKA inhibitor, H-89 (50 μ M), there was a significant decrease in CBF compared to hesperidin alone (apical, 1.49 $\pm$ 0.15; basal, 1.1 $\pm$ 0.18; apical+basal, 1.61 $\pm$ 0.20; $p=0.04$).

Discussion

Flavonoids are responsible for the autumn colors in tree leaves and the numerous shades of red, yellow, and orange in flowers and food.²⁴ Fruits, vegetables, stems, flowers, nuts, seeds, herbs, spices, tea, and red wine all contain abundant concentrations of bioflavonoids with over 4000 structurally unique molecules identified.²⁵ The compounds display a variety of biological activities, including suppression of inflammation (via inhibition of the NF- κ B pathway), cancer chemoprevention, and protection from vascular disease and have had a tremendous safety profile when administered through oral or intravenous means.^{26,27}

In the current study, we demonstrated that the bioflavonoid, hesperidin (found in citrus fruits), is a robust activator of CFTR-dependent Cl^- transport in both murine and human primary cell culture models of sinonasal epithelium. Other flavonoids, such as quercetin and genistein, are established activators of CFTR-mediated Cl^- transport *in vitro* and *in vivo*.² CFTR, the major Cl^- channel in the apical membrane of sinonasal respiratory epithelia, mediates fluid and electrolyte transport and is critical to normal ASL homeostasis. While the absence or dysfunction of CFTR results in defective transport and the clinical manifestations of CF, recent evidence indicates that wild type CFTR processing, endocytic recycling, and function can also be markedly repressed by various environmental insults, including cigarette smoke exposure, high altitude/hypoxemia, inflammation, and infectious agents.^{18,28-30} Thus, CFTR represents a rational therapeutic target for improving MCC in CRS.

Hesperidin exhibited several unique properties in comparison to other flavonoids. While flavonoids have characteristic, inhibitory effects at higher concentrations,² we did not observe this feature with hesperidin (likely due to decreased solubility at higher concentrations). Furthermore, hesperidin did not maximally activate CFTR in MNSE cultures, but did account for nearly all of the stimulated CFTR-mediated anion transport in HSNE. In contrast to our prior measurements with the flavonoid quercetin (unpublished data), CFTR activation was weaker in MNSE, but more active in HSNE, implicating species dependent effects. While *in vivo* CFTR stimulation appears comparable to forskolin (according to murine NPD measurements), our *in vitro* data suggesting hesperidin can stimulate nearly all available CFTR-mediated Cl^- transport in HSNE will have important implications for future clinical trials.

Experiments with the specific CFTR inhibitor INH-172 demonstrated significant reduction in I_{SC} compared to controls, and indicates that hesperidin exerts its effects through interaction with a CFTR dependent pathway. Administration of hesperidin (without forskolin) established that pre-treatment with an adenylate cyclase activator is not necessary for function of the compound as a Cl^- secretagogue. However, some basal PKA activity is required for activation of CFTR by hesperidin, since I_{SC} was completely suppressed by the PKA inhibitor, H-89. Notably, hesperidin does not increase cellular cAMP and stimulates CFTR-mediated Cl^- transport independent of R-D phosphorylation, indicating the mechanistic effects of this compound may act through more direct opening of CFTR pore forming elements. Alternatively, an increased driving force for anion secretion through stimulation of basolateral ion transport by hesperidin cannot be excluded. Based on results with other flavonoid agents, our data suggest that the activity of hesperidin may be secondary, at least in part, to direct interaction with CFTR nucleotide binding domains.²³ Channel potentiation measured with membrane patch clamp analysis will be required to confirm this mechanism of action.

The present studies demonstrate that hesperidin also stimulates CBF, a critical component of the mucociliary apparatus. Activation of CBF and Cl^- secretion are linked by several common cell signaling mechanisms, including fluctuations in cellular cAMP and calcium.²²

While our data indicate that the administration of hesperidin to the basal membrane results in augmented CBF, increased fluid and electrolyte secretion via enhancement of CFTR-mediated Cl^- transport, resulting in improved ciliary fluid dynamics are also likely to contribute to the findings described here. The significant increase in CBF may not be attributable strictly to CFTR-dependent fluid secretion, since the fluid meniscus in the epithelial cell culture model is present at a far greater depth than the ASL (up to several mm). Thus, hesperidin also appears to stimulate CBF independent of its ability to increase bulk fluid and electrolyte secretion perhaps through modulation of periciliary viscosity, itself. However, CBF was decreased when incubated with the PKA inhibitor, H-89, indicating the majority of effects are secondary to CFTR-mediated hydration of the ASL. While effects on calcium signaling are unlikely, future investigation, including intracellular calcium imaging, are also warranted.

Conclusion

The *in vitro* and *in vivo* findings presented here indicate hesperidin is a robust Cl^- secretagogue and CBF activator in sinonasal epithelium. Our results, coupled with extensive preclinical and clinical experience with this flavonoid reported in the literature¹⁰⁻¹² provide strong support for further investigation of this molecule in human clinical trials for CRS.

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Figure 1. Pharmacologic Manipulation of Ion Transport and Ciliary Activation Pathways

Activators (forskolin, hesperidin) are indicated by the blue arrows; inhibitors by the red arrows (amiloride, INH-172). The structure of hesperidin is noted in the inset; the (2,3-dihydro-2-phenylchromen-4-one) nucleus of a flavone is shown in black. CBF and CFTR are activated through the PKA-dependent pathway. Hesperidin is thought to act as a CFTR channel potentiator through its interactions with the nucleotide binding domains (NBD) rather than activation (phosphorylation) of the Regulatory Domain (RD).⁸ Hesperidin also stimulates CBF through either localized changes in the composition of the periciliary fluid or an unknown mechanism that confers direct activation.

Figure 2. Hesperidin Stimulates Transepithelial Cl^- Transport in MNSE in a dose dependent fashion

(A) Representative Ussing chamber tracing demonstrating pharmacologic manipulation of ion transport. By convention, a positive deflection in the tracing (ΔI_{SC}) represents movement of an anion (i.e. Cl^-) from the serosal to mucosal direction. Hesperidin (1.0 mM) increases Cl^- transport when compared to DMSO control. (B) Hesperidin (1.0 mM) ($13.51 \pm 0.77 \mu\text{A}/\text{cm}^2$) and total stimulated ΔI_{SC} [hesperidin (1.0 mM) + forskolin (100 nm), $24.93 \pm 1.96 \mu\text{A}/\text{cm}^2$] significantly increased Cl^- transport over the corresponding controls. ($p=0.000002$ and 0.01 , respectively) There was also significant inhibition of CFTR-mediated I_{SC} following application of INH-172 ($-18.57 \pm 1.40 \mu\text{A}/\text{cm}^2$ vs. $-13.27 \pm 1.55 \mu\text{A}/\text{cm}^2$; $p=0.03$).

Figure 3. Hesperidin (1.0 mM) stimulates Cl^- transport in HSNE *in vitro*

(A) Ussing chamber tracing demonstrating hesperidin (1.0 mM) activation of Cl^- transport in HSNE culture and the corresponding DMSO control. (B) The total stimulation of ion transport in HSNE cultures is of smaller magnitude compared to MNSE.¹⁷ Hesperidin (1.0 mM) significantly stimulated Cl^- transport in comparison to DMSO controls (12.28 $\pm$ 1.09 vs. -0.69 $\pm$ 0.32, $p=0.000001$), but also accounted for a larger proportion of total stimulated ISC (15.29 $\pm$ 1.16). Total stimulation and CFTR blockade with INH-172 were also significantly different from controls.

Figure 4. Hesperidin activates CFTR-dependent ion transport across the murine nasal mucosa *in vivo*

(A) Mice underwent a standardized NPD protocol with the addition of 1.0 mM Hesperidin (or vehicle control) in the final perfusion solution. This tracing demonstrates a -4 mV polarization (positive deflection by convention) with 1.0 mM hesperidin. (B) Hesperidin perfusion resulted in a -2.3 ± 1.0 mV mean NPD polarization that was significant when compared to mice receiving vehicle alone (-0.8 ± 0.8 mV, $p=0.04$) Hesperidin activation of Cl^- transport was similar to forskolin in this *in vivo* assay (-1.9 ± 1.4 mV, $p=0.65$). ($n = 5$ for each condition)

Figure 5. Hesperidin does not increase cellular cAMP or phosphorylate the CFTR R-domain
 (A) Cellular cAMP was barely detectable in the presence of hesperidin at all concentrations and not significantly different when compared to DMSO controls. The adenylate cyclase agonist forskolin was used as positive control. (B) Polyclonal NIH-3T3 cells expressing HA-tagged R-domain were treated with hesperidin for 20 minutes, and compared to forskolin (100 nM) and DMSO control in an assay of R-D phosphorylation. Phosphorylation results in a 2-4 kD shift in migration (white arrow).

Figure 6. Hesperidin (1.0 mM) Activates CBF following Basal or Apical Exposure

CBF was significantly increased when compared to the control vehicle (PBS) when hesperidin was applied to the basal media (1.31 ± 0.07 vs. 0.93 ± 0.06 (control); $p=0.003$) and apical membrane (1.72 ± 0.09 vs. 1.40 ± 0.07 , control; $p=0.01$). Hesperidin delivered to the apical membrane and basal media at the same time had an additive outcome in magnitude (2.26 ± 0.16 vs. 1.60 ± 0.21 , respectively; $p=0.04$). The PKA inhibitor, H-89, significantly decreased hesperidin stimulated CBF under all conditions (apical, 1.49 ± 0.15 ; basal, 1.1 ± 0.18 ; apical+basal, 1.61 ± 0.20 ; $p=0.04$).