Activation of the Cystic Fibrosis Transmembrane Conductance Regulator by the Flavonoid Quercetin

Potential Use as a Biomarker of $\Delta F508$ Cystic Fibrosis Transmembrane Conductance Regulator Rescue

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Therapies to correct the $\Delta F508$ cystic fibrosis transmembrane conductance regulator (CFTR) folding defect require sensitive methods to detect channel activity *in vivo*. The β_2 adrenergic receptor agonists, which provide the CFTR stimuli commonly used in nasal potential difference assays, may not overcome the channel gating defects seen in $\Delta F508$ CFTR after plasma membrane localization. In this study, we identify an agent, quercetin, that enhances the detection of surface $\Delta F508$ CFTR, and is suitable for nasal perfusion. A screen of flavonoids in CFBE41o⁻ cells stably transduced with $\Delta F508$ CFTR, corrected to the cell surface with low temperature growth, revealed that quercetin stimulated an increase in the short-circuit current. This increase was dose-dependent in both Fisher rat thyroid and CFBE41o⁻ cells. High concentrations inhibited Cl⁻ conductance. In CFBE41o⁻ airway cells, quercetin (20 μ g/ml) activated $\Delta F508$ CFTR, whereas the β_2 adrenergic receptor agonist isoproterenol did not. Quercetin had limited effects on cAMP levels, but did not produce detectable phosphorylation of the isolated CFTR R-domain, suggesting an activation independent of channel phosphorylation. When perfused in the nares of Cftr⁺ mice, quercetin (20 μ g/ml) produced a hyperpolarization of the potential difference that was absent in Cftr^{-/-} mice. Finally, quercetin-induced, dose-dependent hyperpolarization of the nasal potential difference was also seen in normal human subjects. Quercetin activates CFTR-mediated anion transport in respiratory epithelia *in vitro* and *in vivo*, and may be useful in studies intended to detect the rescue of $\Delta F508$ CFTR by nasal potential difference.

Keywords: cystic fibrosis; short-circuit current; airway epithelia; nasal potential difference

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CLINICAL RELEVANCE

This article describes the use of the flavonoid quercetin as an important adjunct for detecting surface-localized $\Delta F508$ CFTR by nasal potential difference, a topic of considerable interest in the field of cystic fibrosis (CF) therapeutics, and relevant to emerging therapies in clinical testing. Our findings elucidate important mechanistic features of quercetin-mediated CFTR activation *in vitro*, and provide data in mice and humans that support the use of the agent in subjects with CF homozygous for $\Delta F508$ CFTR, to identify individuals with residual surface protein, and in the evaluation of correctors of $\Delta F508$ CFTR processing.

The most common cause of cystic fibrosis (CF) is attributable to the deletion of phenylalanine at position 508 of the cystic fibrosis transmembrane conductance regulator ($\Delta F508$ CFTR), which causes misfolding of the protein product in the endoplasmic reticulum (ER) and accelerated degradation in the proteosome. At least one copy of the $\Delta F508$ mutation is found in approximately 90% of patients with CF, and is therefore a logical target for treating the disease. The absence of CFTR at the cell plasma membrane results in defective ion transport and subsequent clinical manifestations of CF (1). Several maneuvers were identified that correct $\Delta F508$ CFTR misprocessing, including cell growth at low temperature (2), treatment with chemical chaperones that promote ER escape (3-5), and exposure to small molecules (identified by high-throughput screening programs and other means) that promote protein localization to the plasma cell membrane (6-9).

Numerous laboratories showed that the $\Delta F508$ CFTR demonstrates defective channel gating (10-12). These findings are evident in airway cell monolayers, because $\Delta F508$ CFTR rescue using low temperatures or small molecule correctors of misprocessing do not consistently restore the cAMP-dependent activation of Cl⁻ transport, a finding that is also germane to measures of ion transport across the human nasal membrane (13, 14). Thus, proof-of-concept studies testing agents that correct $\Delta F508$ CFTR processing may be hampered if assays of CFTR activity, such as nasal potential difference (NPD), fail to stimulate membrane-localized $\Delta F508$ CFTR adequately. Recently, an evaluation of the $\Delta F508$ CFTR processing corrector (VX-809) has entered Phase 2 testing in human subjects, and includes assessment via NPD, further emphasizing the importance of an adequate stimulus within the measurement of NPD (NCT00865904).

The standard stimulus of CFTR-dependent Cl⁻ transport is perfusion with β_2 adrenergic receptor (β_2 AR) agonists (e.g.,

isoproterenol) in the presence of a Cl^- secretory gradient. If cAMP-dependent activation is defective in human airway cells expressing surface-localized ΔF508 CFTR, the identification of a molecule capable of activating the mutant channel that is also suitable for use in NPD protocols may improve the sensitivity of the assay to detect ΔF508 CFTR at the cell surface. Because flavonoids are among the best-described activators of CFTR activity (15–17), we sought to determine the most suitable agent that could be applied to the measurement of NPD. Some results of these studies were previously reported in abstracts (18, 19).

MATERIALS AND METHODS

Voltage Clamp Studies in Ussing Chambers on Cultured Monolayers

The CFBE41o[−] and Fisher rat thyroid (FRT) cells stably transduced with ΔF508 or WT CFTR cDNA were grown on Costar permeable inserts and mounted in Ussing chambers, and the short-circuit current (I_{sc}) was measured under voltage clamp conditions, as previously described (13, 20). Briefly, cells were seeded on 0.4- μm permeable supports (5×10^5 cells/filter, 6.5-mm diameter; Costar, Bethesda, MD) after coating with fibronectin. Cells were grown to confluence, and then switched to at an air–liquid interface 48 hours before mounting in modified Ussing chambers (Jim's Instruments, Iowa City, IA), and initially bathed on both sides with identical Ringer solution. Measurements of I_{sc} were obtained by using an epithelial voltage clamp (University of Iowa Bioengineering, Iowa City, IA). A 3-mV pulse of 1-second duration was imposed every 100 seconds to monitor resistance, which was calculated using Ohm's law. To measure stimulated I_{sc} , the mucosal bathing solution was changed to a low Cl^- sodium gluconate solution plus 100 μM amiloride. Agonists (flavonoids, isoproterenol, and forskolin) were added to the bathing solutions as indicated (for a minimum 5 min of observation under each condition). Glibenclamide (200 μM) or CFTR_{inh}-172 (10 μM) was added to the mucosal bathing solution at the end of the experiments to block CFTR-dependent I_{sc} . All chambers were maintained at 37°C, vigorously stirred, and gassed with 95% O_2 /5% CO_2 .

Detection of Regulatory-Domain Phosphorylation and cAMP Concentrations

Polyclonal NIH-3T3 cells expressing the hemagglutinin (HA)-tagged R-domain were treated with quercetin for 20 minutes, and compared with forskolin (20 μM) $\times$ 5 minutes as a positive control. After lysis, equal amounts (50 μg) of total cell lysate were electrophoresed through a 12% SDS-PAGE gel, and immunoblotted with antibody to the HA tag (Covance, Cumberland, VA). Phosphorylation of the R-domain was visualized as a 2- to 4-kD shift in migration, as previously described (21). Cellular cAMP was measured using an ELISA-based detection kit (Cayman Chemicals, Ann Arbor, MI) according to published methods (13).

Mouse NPD Measurements

Mice were anesthetized with ketamine, and each mouse's tail was gently abraded, placed in lactated Ringer solution, and connected through a calomel cell to a high-impedance voltmeter (VF-1; World Precision Instruments, Sarasota, FL), as previously described (22, 23). Perfusion solutions included Ringer lactate plus amiloride (100 μM), a low Cl^- concentration solution with amiloride (100 μM), and an agonist in low Cl^- solution. Each condition was studied for 5 to 10 minutes until a stable signal was achieved before changing to the next solution (23). Additional details are described in the online supplement.

Human NPD Measurements

All subjects provided written, informed consent, and the study was approved by the Institutional Review Board of the University of Alabama at Birmingham. The NPD studies were performed in accordance with the report by Knowles and colleagues (24) and the standard operating procedure of the CF-Therapeutics Development Network (perfusion method) using electronic data capture (Biopac, San Francisco, CA), KCl calomel electrodes (Fischer Scientific, Pitts-

burgh, PA), and 3% agar bridges. Additional details are described in the online supplement.

Reagents

Forskolin was obtained from Calbiochem (San Diego, CA), and genistein from Sigma-Aldrich (St. Louis, MO). Quercetin (Jarrow Formulas, Los Angeles, CA) was freshly reconstituted for each experiment in DMSO at a 20 mg/ml stock concentration, and passed through a 0.2- μm filter to remove particulates. Quercetin from an alternative source (Sigma-Aldrich) was used for confirmation.

Statistics

For I_{sc} , cAMP, and Western blot measurements, descriptive statistics (mean, SD, and SEM) were compared using the Student *t* test or ANOVA, as appropriate. All statistical tests were two-sided, and were performed at a 5% significance level (i.e., $\alpha = 0.05$), using SPSS software (Chicago, IL) and Microsoft Excel (Seattle, WA).

RESULTS

Screen of Flavonoids Identifies Activators of Surface-Localized ΔF508 CFTR

To identify compounds that potentiate ΔF508 CFTR activity, 20 flavonoid agents were screened in ΔF508 CFTR-transduced CFBE41o[−] cells after temperature correction of aberrant protein processing (growth at 27°C $\times$ 48 h). As shown in Figure 1, cell monolayers were evaluated in modified Ussing chambers after prestimulation with forskolin (20 μM) and exposure to incrementally increasing doses (1, 10, and 50–100 μM) of the respective flavonoid agents. The concentrations of flavonoids tested were based on previous experience with genistein and other flavonoids on CF processing and maturation (13, 17, 25, 26). Several agents stimulated Cl^- conductance above that produced by forskolin and other cAMP agonists, which are poor stimuli of ΔF508 CFTR activity in this airway cell type (13). Genistein, a well-characterized flavonoid activator of ΔF508 CFTR, was found to be the most potent stimulus in our screen, but was not considered further because of potential human safety concerns with repeated nasal administration and off-target effects (27). Equol and apigenin were avoided because of their observed effects on ΔF508 CFTR processing. Of the agents that activated ΔF508 CFTR residing at the cell surface, quercetin was selected for additional evaluation because of its well-established safety profile for human use, including clinical trials in a variety of disorders encompassing cancer and heart disease, and because of its usefulness as an anti-inflammatory agent (28–32).

Quercetin Activates ΔF508 CFTR despite Minimal Effects on cAMP and R-Domain Phosphorylation

The protein kinase A (PKA)/cAMP-dependent phosphorylation of the CFTR R-domain is a critical step in channel activation, but this stimulus was shown to be insufficient to activate ΔF508 CFTR fully at the plasma membrane of airway epithelial cells (13, 33). To examine the mechanistic basis of CFTR activation by quercetin, we measured the effects of quercetin on cAMP levels and on R-domain phosphorylation. Quercetin had small but detectable effects on cell cAMP concentrations (126 pmol over vehicle control) that were less than 10% of that observed with forskolin treatment but greater than those seen with genistein (Figure 2A). As opposed to the potent cAMP agonist forskolin, quercetin had no detectable effect on the phosphorylation-dependent mobility shift of the recombinant CFTR R-domain (Figure 2B), suggesting that its effects were independent of CFTR phosphorylation, despite

Figure 1. Flavonoid activation of $\Delta F508$ CFTR-dependent Cl^- transport. Screen of flavonoid compounds for activation of temperature-corrected $\Delta F508$ CFTR in CFBE41o⁻ monolayers. Dose-response experiments in modified Ussing chambers were performed with flavonoid compounds. In each case, the concentration of compound eliciting maximal activation is indicated. Concentrations tested include 1, 10, 50–100 μM . * $P < 0.05$ versus control, $n = 4$ –6 filters in each condition, $\pm$ SEM. 100 μM quercetin = 33 $\mu\text{g}/\text{mL}$.

reports of quercetin as a phosphatase inhibitor and/or PKA activator in other cell types (34, 35). Together, the results of Figure 2 provide evidence that the quercetin-induced activation of $\Delta F508$ CFTR differs from classic cAMP-signaling agonists, and is consistent with a mechanism of action that imparts direct effects on CFTR that are independent of R-domain phosphorylation, as reported with other flavonoid agents including genistein (36–38).

Dose-Response Relationships between Quercetin and $\Delta F508$ CFTR Activity

To define the dose-response relationship between quercetin and $\Delta F508$ CFTR activation, and to confirm its effects in a distinct $\Delta F508$ CFTR-expressing cell model, additional studies were performed in $\Delta F508$ CFTR-transduced FRT monolayers after the temperature correction of $\Delta F508$ misprocessing ($27^\circ\text{C} \times 48$ h). This cell type is frequently used to examine $\Delta F508$ CFTR function and processing, including high-throughput screens of CFTR potentiators and $\Delta F508$ CFTR correctors

(7, 39). Quercetin activated CFTR-dependent Cl^- conductance at 1 and 10 $\mu\text{g}/\text{mL}$, but inhibited I_{sc} after the addition of higher concentrations (>20 $\mu\text{g}/\text{mL}$; Figures 3A and 3B). The Cl^- transport stimulated by quercetin (10 $\mu\text{g}/\text{mL}$) was not affected by forskolin (20 μM) pretreatment, insofar as quercetin stimulated I_{sc} by 57.6 ± 2.7 $\mu\text{A}/\text{cm}^2$ in the presence of forskolin, compared with 53.4 ± 3.8 $\mu\text{A}/\text{cm}^2$ produced by quercetin alone ($P = \text{NS}$, $n = 4$, data not shown). In monolayers without temperature correction of $\Delta F508$ CFTR processing (i.e., grown at 37°C), the effect of quercetin on Cl^- conductance was minimal, establishing the specificity of its effect on $\Delta F508$ CFTR residing at the cell surface. Experiments in wild-type CFTR-expressing FRT monolayers suggested a peak effect at 10 $\mu\text{g}/\text{mL}$ (Figure 3C). Confirmatory experiments performed in $\Delta F508$ CFTR-transduced CFBE41o⁻ cells after low temperature treatment showed a similar dose-response relationship (Figure 3D) and specificity for surface-residing $\Delta F508$ CFTR (no activation seen with growth at 37°C). As seen in FRT cells, the quercetin-dependent activation of Cl^- transport did not require forskolin preactivation in tem-

Figure 2. Quercetin activates CFTR through a mechanism independent of PKA and R-domain phosphorylation. (A) Concentrations of cAMP after forskolin, genistein, or quercetin exposure. CFBE41o⁻ cells were exposed to quercetin (50 $\mu\text{g}/\text{mL}$), genistein (50 μM), forskolin (20 μM), or vehicle (DMSO, 1:1,000 dilution) for 10 minutes before the assay. Quercetin induced a small but statistically significant increase in cAMP compared with genistein and vehicle controls, whereas forskolin-stimulated cAMP was statistically increased above all tested conditions. * $P < 0.05$, ** $P < 0.001$ compared with control, $n = 4$ per condition $\pm$ SEM. (B) Quercetin does not induce R-domain phosphorylation. Recombinant CFTR R-domain was expressed in stably transfected NIH-3T3 cells and detected by

Western blotting against an HA tag (solid arrowhead). A 2- to 4-kD mobility shift is seen, indicative of R-domain phosphorylation (open arrowhead) after a 5-minute treatment with forskolin (20 μM , positive control). Quercetin had no detectable effect on R-domain phosphorylation at any concentration tested. Results are representative of three experiments.

Figure 3. Dose-response effects of quercetin on I_{sc} in ΔF508 CFTR-transduced FRT and CFBE41o⁻ monolayers. Cells were studied in modified Ussing chambers, and stimulated I_{sc} was determined after treatment with a low chloride gradient + amiloride (100 μM), followed by the sequential addition of increasing concentrations of quercetin (1 μM , 10 μM , and 100 μM) or DMSO vehicle at the concentrations shown. Forskolin (20 μM), genistein (50 μM), and CFTR_{inh}-172 (10 μM) were included at the end of the experiments to confirm the ion transport response of the monolayer. The data reflect stimulated I_{sc} over the baseline current (before the first application of quercetin or vehicle). (A) Representative I_{sc} tracing in ΔF508 CFTR-transduced FRT cells grown at 27°C. (B) Quercetin stimulates a dose-dependent increase in I_{sc} in ΔF508 CFTR-transduced FRT cells grown at 27°C (right), with smaller effects seen in cells grown at 37°C (left). High doses of quercetin (100 $\mu\text{g/mL}$) were inhibitory in temperature-corrected cells. * $P < 0.05$ versus vehicle, ** $P < 0.005$ versus vehicle and $P < 0.05$ versus quercetin-treated cells without temperature correction (37°C growth), $n = 4$ –8 filters per condition, $\pm$ SEM. (C) Dose-response studies demonstrating that peak stimulation in CFTR-dependent I_{sc} occurs at 10 and 20 $\mu\text{g/mL}$ of quercetin in wild-type CFTR FRT cells. * $P < 0.05$ versus vehicle, $n = 4$, $\pm$ SEM. (D) Complementary dose-response experiments in ΔF508 CFTR-transduced CFBE41o⁻ cells grown at 27°C to correct ΔF508 CFTR misprocessing, using the same protocol as in A. No stimulation of I_{sc} was seen in cells without temperature correction of ΔF508 CFTR processing ($0 \pm 0 \mu\text{A}/\text{cm}^2$, data not shown). * $P < 0.005$ versus vehicle, $n = 4$ /condition, $\pm$ SEM. (E) In CFBE41o⁻ cells transduced with wild-type CFTR, quercetin exhibited similar effects on ion transport. ** $P = 0.10$, $n = 4$ /condition, $\pm$ SEM.

perature-corrected ΔF508 CFBE41o⁻ cells. The dose-response curve in CFBE41o⁻ cells transduced with wild-type CFTR was similar to that of ΔF508 CFTR-transduced cells (Figure 3E). Experiments using parental CFBE41o⁻ cells (without ΔF508 or wild-type CFTR transduction and undetectable expression of endogenous ΔF508 CFTR) (13) showed no activation by quercetin ($0 \pm 0 \mu\text{A}/\text{cm}^2$, data not shown), confirming the specificity of the agonist for CFTR.

Quercetin Enhances ΔF508 CFTR Activity Above That Produced by β_2 Adrenergic Receptor Stimulation

We next examined whether quercetin increased ΔF508 CFTR Cl^- conductance above that produced by the β_2 AR agonist isoproterenol, a well-established regulatory pathway to activate CFTR *in vivo* and a standard agonist for NPD studies. These experiments were designed to mirror CFTR detection in humans using NPD protocols (40), and used isoproterenol (10 μM) and a Cl^- secretory gradient in the presence of amiloride. These studies were limited to ΔF508 CFTR-transduced CFBE41o⁻

cells after low temperature growth (Figure 4), because FRT cells do not express β_2 ARs. Quercetin (20 $\mu\text{g/mL}$) significantly increased the Cl^- conductance above isoproterenol stimulation, increasing I_{sc} 12.3 $\mu\text{A}/\text{cm}^2$ above that produced by isoproterenol alone. To confirm that pre-exposure to quercetin did not induce tachyphylaxis (an effect that would impede its use in human NPD protocols requiring repeated measurements), monolayers were treated with 20 $\mu\text{g/mL}$ quercetin for two 30-minute intervals, 24 and 48 hours before evaluation in Ussing chambers. Pre-exposure to quercetin had no measurable effect on the subsequent activation of corrected ΔF508 CFTR by the compound (Figure 4D). The activation of CFTR-dependent ion transport was confirmed in primary human airway cells from a normal (non-CF) donor (Figures 4E and 4F).

Quercetin Activates CFTR-Dependent Cl^- Conductance across the Nasal Epithelium

We next examined the effects of quercetin on anion transport *in vivo* via standardized murine NPD measurements. As

Figure 4. Quercetin stimulates CFTR-dependent I_{sc} in conditions simulating human NPD protocols. A representative short-circuit current tracing in temperature-corrected $\Delta F508$ CFTR CFBE41o⁻ monolayers shows a poor response to isoproterenol perfusion, followed by a robust response to quercetin (A) compared with DMSO vehicle (B). After the establishment of a basolateral to mucosal Cl⁻ gradient and the addition of amiloride (100 μ M) to the apical compartment (LoCl⁻, Amil), cell monolayers were stimulated in the apical membrane by isoproterenol (10 μ M), followed by quercetin (20 μ g/ml) or vehicle. Glybenclamide (Glyb, 200 μ M, apical addition) was used to confirm the CFTR dependence of the stimulated current. (C) Summary data. The $\Delta F508$ CFTR-transduced CFBE41o⁻ monolayers grown at 27°C $\times$ 48 hours exhibited activation of I_{sc} after quercetin (20 μ g/ml). * P < 0.001, n = 6 filters per condition, $\pm$ SEM. (D) Quercetin pretreatment does not affect ion transport response. The $\Delta F508$ CFTR-transduced CFBE41o⁻ monolayers were exposed to either quercetin (20 μ g/ml) or vehicle for 30 minutes daily for 2 consecutive days before Ussing chamber analysis. Cells were grown at 27°C $\times$ 48 hours. Stimulation with DMSO vehicle is included as a negative control (gray). * P < 0.05 versus vehicle, n = 5–6/condition, $\pm$ SEM. (E) Representative short-circuit current tracing of fully differentiated primary human airway cells treated with quercetin (20 μ g/ml) or vehicle control. Cells were harvested from a single normal donor, expanded in culture, grown at the air-liquid interface until terminally differentiated, and then loaded into modified Ussing chambers under voltage clamp conditions. Cells were sequentially treated with amiloride (100 μ M), low Cl⁻ secretory gradient, quercetin or vehicle control, forskolin (20 μ M; to confirm that CFTR-dependent response was intact), and finally CFTR_{inh}-172. (F) Summary data of experiments shown in E. In these cells, the response to forskolin (20 μ M) under the exact same conditions is approximately 25 μ A/cm². * P < 0.05, n = 4 filters/condition, $\pm$ SEM.

shown in Figure 5, mice expressing wild-type CFTR (*Cftr*^{+/+} and *Cftr*^{tm1Kth} C57BL/6/J genetic background) demonstrated significant hyperpolarization after perfusion with quercetin (20 μ g/ml) in the presence of amiloride (100 μ M) and a Cl⁻ secretory gradient (-2.9 ± 0.7 mV), compared with the continued depolarization seen in mice perfused with vehicle and otherwise identical conditions ($+0.9 \pm 0.5$ mV). The *Cftr*^{-/-} mice did not demonstrate repolarization after quercetin perfusion, confirming the specificity of quercetin's effects in CFTR-dependent anion transport *in vivo* (Figure 5C). Consistent with the effects of quercetin on Δ F508 CFTR cells without temperature correction, quercetin perfusion did not hyperpolarize the nasal epithelial membrane of mice homozygous for murine Δ F508 CFTR, compared with isoproterenol or vehicle control (Figure 5D). In normal (non-CF) human subjects, sequential perfusion with quercetin at 5, 10, and 20 μ g/ml was well tolerated, and demonstrated a sequential dose-dependent polarization of the nasal membrane, indicative of CFTR-dependent anion transport (Figure 6). No adverse events were reported, and no changes were seen in the nasal examination rating or total nasal symptom score before and after the NPD procedure. The results confirm findings observed in polarized epithelial model systems, and demonstrate that quercetin retains bioactivity to stimulate CFTR Cl⁻ transport when topically applied to the nasal mucosa in an NPD protocol.

DISCUSSION

The detection of plasma membrane-localized Δ F508 CFTR is a vital step in the development of therapies designed to treat the underlying cause of CF in the majority of patients with CF. In this study, we evaluated several flavonoid compounds for the activation of surface Δ F508 CFTR, and identified a number of agents that robustly activate Δ F508 CFTR-dependent Cl⁻ transport in airway monolayers after temperature correction of aberrant processing. One agent in particular, quercetin, was selected for further investigation because it demonstrated efficient activation of Δ F508 CFTR-dependent I_{sc} , and has a well-established safety profile in human subjects. Two other flavonoids, equol and apigenin, also demonstrated potent Δ F508 CFTR activation, but were not studied further because of their inferior safety profile in the literature. Furthermore, additional studies indicated that these agents affect Δ F508 CFTR misprocessing, a characteristic that could confound studies intended to detect Δ F508 CFTR correction. Using two polarized epithelial models, we showed that quercetin produces a dose-dependent activation of Δ F508 CFTR after cell surface localization (low temperature rescue) (Figure 3). In both Δ F508 CFTR expressing FRT and CFBE41o⁻ cells, the effect was specific for CFTR at concentrations below 100 μ g/mL, as indicated by the lack of effect on anion transport in Δ F508 CFTR-transduced cells

Figure 5. Quercetin activates CFTR-dependent ion transport across the murine nasal mucosa *in vivo*. Mice underwent a standardized NPD protocol with the addition of quercetin (or vehicle control) in the final perfusion solution, that is, 20 μ g/ml in Cl⁻-free gluconate solution and amiloride (amil) (100 μ M). Representative tracings are from *Cftr*⁺ mice stimulated with quercetin (A) or DMSO vehicle (B) in the final perfusate. (C) Summary data of studies described above. Quercetin perfusion resulted in a -2.9 -mV mean PD polarization that was significantly different from that seen in *Cftr*⁺ mice receiving vehicle alone, or *Cftr*^{-/-} mice exposed to quercetin. * $P < 0.005$ versus vehicle or *Cftr*^{-/-} mice, $n = 6-8$ per condition, $\pm$ SEM. wt, wild-type. (D) The Δ F508 homozygous mice demonstrated no hyperpolarization after perfusion with isoproterenol (Iso, 10 μ M) or quercetin with isoproterenol in the final perfusate. $n = 6-8$ per condition, $\pm$ SEM.

Figure 6. Quercetin activates CFTR in humans by nasal potential difference. (A) Representative NPD tracing after sequential perfusion of Ringer solution, Ringer solution with amiloride (Amil), Cl⁻-free solution (Zero Cl⁻), Cl⁻-free solution + quercetin (5, 10, and 20 µg/ml), and finally quercetin + isoproterenol (Iso, 10 µM). (B) Summary data of NPD studies on 12 normal human subjects (mean of each two nostril tracings for each subject). **P* < 0.05 for within tracing change, ± SEM.

without temperature correction (or in cells lacking the $\Delta F508$ CFTR transgene). When repeated in a sequence analogous to a standard NPD protocol, quercetin stimulated CFTR-dependent I_{sc} without evidence of tachyphylaxis, indicating that the compound may be suitable for clinical protocols requiring repeated exposure (Figure 4D). Together with results demonstrating the stimulation of CFTR-dependent anion transport across the nasal epithelium *in vivo* (Figures 5 and 6), the findings provide support for further studies of this agent in human NPD protocols as a means to enhance the detection of $\Delta F508$ CFTR at the plasma membrane. Based on these findings, a multicenter study is being conducted to examine the response of $\Delta F508$ homozygotes to quercetin perfusion compared with isoproterenol, and is intended to determine whether the response to quercetin is associated with a more mild pulmonary phenotype.

In the Calu-3 serous glandular cell line, Illek and Fischer showed that quercetin (at concentrations >40 µM, equivalent to 12.1 µg/ml) stimulated Cl⁻ secretion (half-maximal doses were 22.1 ± 4.5 µM) *in vitro*, whereas higher doses were inhibitory (26). Our findings indicate that quercetin produced maximal I_{sc} at approximately 10 µg/ml (33 µM) *in vitro*, and hyperpolarized the NPD response between 5 to 20 µg/ml. These results concerning the effects of quercetin in the murine airway extend earlier studies, and firmly establish the CFTR dependence of observations at the concentrations tested. Interestingly, Illek and Fischer (26) suggested that pretreatment of Calu-3 cells with forskolin may sensitize to subsequent quercetin stimulation. Our results in $\Delta F508$ CFTR-expressing FRT cells did not show this effect, insofar as quercetin stimulated I_{sc} to a similar degree, regardless of forskolin pretreatment. This could indicate differences in the response of wild-type CFTR versus the $\Delta F508$ protein, or other variables attributable to the cell types being tested (26). The inhibition of anion conductance at higher concentrations observed in our studies was reported

with other flavonoids (26) and in studies of colonic epithelia (41). These results emphasize the importance of using the appropriate concentration for studies intended to activate CFTR. Moreover, tissue-specific differences may mediate anion conductance in response to quercetin. These concerns may be abrogated through the use of the NPD assay, which requires topical administration directly to the nasal epithelia, as opposed to systemic treatment in which pharmacokinetics are more difficult to control, and unintended consequences of CFTR inhibition may be more likely in off-target tissues.

In other studies, Lim and colleagues reported no activation of chloride efflux with the addition of quercetin (1 or 5 µM) to forskolin (13 µM) in IB3-1 bronchial epithelial cells after the correction of endogenous $\Delta F508$ CFTR with 4-phenylbuturate (42). This disparity may be attributable to the low concentrations of quercetin tested, because our experiments also showed that no $\Delta F508$ CFTR activation occurred with 3.3 µM quercetin. Plasma membrane $\Delta F508$ CFTR levels can also be markedly influenced by cell polarity and by growth at an air-liquid interface (13, 43, 44). Because the studies with IB3-1 cells were conducted under nonpolarizing conditions, this fundamental difference may have contributed to the previously reported negative results. These effects may also represent additional cell model-specific responses to flavonoid exposure, as reported by Hansen and colleagues regarding the quercetin modulation of heat shock protein-70 synthesis (45).

Although previous work with quercetin in Calu-3 cells demonstrated a cAMP/PKA-dependent activation of CFTR (26), our results provide evidence that the quercetin signaling that promotes corrected $\Delta F508$ CFTR activation may differ from that produced by classic PKA-dependent stimuli, and this effect may be unique to flavonoids. Quercetin produced a small increase in cAMP relative to forskolin, despite potent stimulation of ion transport compared with cAMP agonist (Figure

2). The cAMP levels resulting from quercetin treatment, however, were greater than those produced by genistein. Previous work from our laboratory has shown that $\Delta F508$ CFTR channel activation (by genistein) can be partially blocked by inhibiting PKA in CFBE41o⁻ cells (13). This implies that $\Delta F508$ CFTR requires some level of PKA activity (and thus PKA dependent RD phosphorylation) for genistein to confer Cl⁻ transport. While no RD phosphorylation was detected following quercetin treatment (further distinguishing the mechanistic basis of flavonoid versus PKA activation pathways; see Figure 2B), we speculate that quercetin may provide two stimuli that together optimize $\Delta F508$ -CFTR activation (including both cAMP-dependent and -independent effects), particularly in cells where endogenous cAMP levels could be limiting.

Several laboratories showed that $\Delta F508$ CFTR, in addition to a well-described cellular processing defect, also exhibits significant abnormalities in channel gating at the cell surface. For example, $\Delta F508$ CFTR exhibits reduced sensitivity to phosphorylation dependent activation in membrane patch and whole-cell studies (46). More recent findings by Wang and colleagues using a series of molecules (including genistein, NPPB-AM, and curcumin), indicate that $\Delta F508$ CFTR activation required stimuli independent of cAMP and PKA to rescue mutant channel function fully (47). Studies from our laboratory and from others using intact airway monolayers established that low temperature or pharmacologically corrected $\Delta F508$ CFTR is not fully activated by cAMP stimuli, including the β_2 agonists albuterol and isoproterenol or the A2B adenosine receptor agonists adenosine or 5'-N-ethyl-carboxamide adenosine. This defect appears to be more pronounced in pulmonary epithelia, including human alveolar and airway cells (13, 33). These findings provide a compelling rationale to investigate more sensitive biomarkers of $\Delta F508$ CFTR activity after processing correction, including agents such as quercetin. For example, current methods may not detect effective biochemical corrections of $\Delta F508$ CFTR, insofar as chemically corrected $\Delta F508$ CFTR may not be activated by the cAMP pathway because of persistent gating defects. As such, improved surface activation of CFTR by an agent such as quercetin would be expected to decrease the number of subjects required to observe a relevant improvement in CFTR-dependent NPD response, simplifying the designs of clinical trials.

In addition, agents such as quercetin could help detect and discriminate the $\Delta F508$ CFTR that resides in the plasma membrane, but that is unresponsive to endogenous activation because of a persistent gating defect, thus helping define the mechanistic basis underlying a $\Delta F508$ CFTR processing corrector under study. Although the mechanism by which quercetin and other flavonoids activate CFTR is unknown, our findings support a direct effect on the CFTR Cl⁻ channel that can overcome the defective gating of cell surface-localized $\Delta F508$ CFTR. These results are further supported by observations from whole-cell studies with quercetin (26) and single-channel studies with other flavonoids (47).

Because the endoplasmic reticulum-associated degradation of $\Delta F508$ CFTR was postulated to be less than 100% efficient *in vitro* (45), and because small amounts of $\Delta F508$ CFTR at the cell membrane were speculated to account for phenotypic differences among $\Delta F508$ CFTR homozygous individuals (48), the evaluation of CFTR-dependent Cl⁻ conductance by NPD using quercetin (or other potentiating stimuli) could be used as an approach to detect individuals with small but relevant amounts of $\Delta F508$ CFTR at the plasma membrane. Such an approach could identify $\Delta F508$ CFTR homozygotes who are potential candidates for treatment with other CFTR potentiators, including the agent VX-770, currently under investigation in

patients with CF harboring the G551D CFTR mutation (49). The relative safety of the approach, as confirmed during the human NPD studies described here, makes this assessment feasible.

Flavonoids are ubiquitous in the human diet, are highly bioavailable, and have been extensively studied with regard to their pharmacology and toxicology (50). Multiple laboratory clinical trials point to an excellent safety profile of quercetin, including studies using systemic, oral, and intravenous administration at levels far exceeding those found to activate $\Delta F508$ CFTR (51–53). For example, Shoskes and colleagues reported that oral quercetin (500 mg, twice daily) was well tolerated and significantly improved symptoms in men with chronic prostatitis (54). The intravenous administration of high doses of quercetin was well tolerated over a 6- to 7-month period in a study examining quercetin as a tyrosine kinase inhibitor (51). More recently, oral quercetin (730 mg daily) for 28 days produced no significant adverse events in a hypertension trial (55). Carcinogenic testing in hamsters by Morino and colleagues (56), and additional studies involving systemic exposure in rats (57, 58), further indicate the safety of the agent in animal models. This extensive preclinical and clinical experience, coupled with our results *in vitro* and *in vivo*, provide strong support for further studies to examine quercetin in subjects with CF as a means to activate $\Delta F508$ CFTR.

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