Statin-mediated correction of STAT1 signaling and inducible nitric oxide synthase expression in cystic fibrosis epithelial cells

Norman E. Kreiselmeier, Nathan C. Kraynack, Deborah A. Corey, and Thomas J. Kelley

Departments of Pediatrics and Pharmacology, Case Western Reserve University and Rainbow Babies and Children’s Hospital, Cleveland, Ohio 44106

Submitted 29 April 2003; accepted in final form 22 August 2003

Kreiselmeier, Norman E., Nathan C. Kraynack, Deborah A. Corey, and Thomas J. Kelley. Statin-mediated correction of STAT1 signaling and inducible nitric oxide synthase expression in cystic fibrosis epithelial cells. Am J Physiol Lung Cell Mol Physiol 285: L1286–L1295, 2003.—The expression of the inducible form of nitric oxide synthase (NOS2) is reduced in cystic fibrosis (CF) epithelium despite the presence of aggressive inflammation. A potential mechanism for reduced NOS2 expression in CF is diminished signal transducer and activator of transcription-1 (STAT1) activity, possibly due to an increase in expression of protein inhibitor of activated STAT1 (PIAS1). Previous evidence also suggests that NOS2 expression can be negatively regulated by increased activation of the GTPase RhoA, leading to the hypothesis that CF-related increases in PIAS1 expression and altered STAT1 signaling may be mediated by Rho GTPase function. Consistent with this hypothesis, data demonstrate increased expression of RhoA in two models of CF epithelium with a proportional increase in the active GTP-bound RhoA. Mouse embryonic fibroblasts null for p190B Rho GTPase-activating protein exhibit increased RhoA protein content and activation, similar to what is observed in CF models, and also exhibit CF-like alterations in STAT1 regulation, including decreased STAT1 activation, increased PIAS1 protein expression, and reduced NOS2 induction, implicating RhoA-mediated signaling in CF-related STAT1 alterations. Inhibition of the Rho GTPase pathway at the level of isoprenoid/cholesterol synthesis with mevastatin reduces PIAS1 expression, increases STAT1 activation, and restores NOS2 expression in models of CF epithelium, suggesting that pharmacological inhibition of the isoprenoid synthesis/Rho GTPase pathway may represent a potential avenue for therapeutic intervention for CF.

RhoA, isoprenoid; mevastatin; protein inhibitor of activated signal transducer and activator of transcription-1; GTPase

THERE IS MOUNTING EVIDENCE that cystic fibrosis (CF) airway epithelial cells exhibit fundamental alterations in cell signaling regulation leading to increased inflammatory responses and susceptibility to bacterial infection. Among these altered cell signaling pathways is a CF-related reduction in signal transducer and activator of transcription-1 (STAT1)-mediated signaling stimulated by interferon-γ (IFN-γ) (12). Reduced STAT1-mediated signaling may contribute to the reduced expression of the inducible form of nitric oxide synthase (NOS2) (10, 17); interferon response-factor-1 (12, 31); and regulated upon activation, normal T cell expressed, and secreted (6, 22), in CF epithelium. One potential mechanism of reduced STAT1 signaling is an increase in the expression of the protein inhibitor of activated STAT1 (PIAS1) in CF epithelial cells (12). PIAS1 inhibits STAT1 function by binding to phosphorylated STAT1 and preventing its action in the nucleus (15). Other functions attributed to PIAS1 in addition to the inhibition of STAT1 activity include the regulation of Gqu RNA helicase II activity and influence of the regulation of p53 function and p53 sumoylation (9, 16, 20, 27). It is currently unknown by what mechanism PIAS1 expression and STAT1 signaling are altered in CF, and identifying such a mechanism may illuminate a root cause for cell-signaling alterations and the broad-ranging transcriptional irregularities that have been reported in CF models (3, 5, 11, 28, 29, 31).

Reduced NOS2 expression in the presence of increased inflammatory signaling is an unpredicted scenario that may represent a clue to fundamental signaling abnormalities inherent to CF. Recent evidence demonstrates that inhibition of Rho GTPase signaling in smooth muscle cells results in increased NOS2 expression, and we have shown that inhibition of the Rho GTPase pathway results in increased expression of NOS2 in airway epithelial cells (13, 19, 32). The regulation of Rho GTPase activity requires posttranslational modifications with either a 15-carbon farnesyl moiety or 20-carbon geranylgeranyl isoprene unit (1). These reactions are catalyzed by farnesyl transferase and two distinct geranylgeranyl transferases (GGTase I and II). The relationship between Rho GTPase signaling and the regulation of NOS2 expression suggests a possible link between STAT1-mediated signaling and Rho GTPase function, leading to the hypothesis that alterations in RhoA function in CF epithelial cells are influencing NOS2 expression through the regulation of STAT1 signaling. The goals of this study are to examine RhoA signaling in CF epithelial models, to explore the role of the Rho GTPase pathway in mediating regulation of STAT1 signaling, and to determine whether...
inhibition of Rho GTPase-dependent mechanisms can correct previously identified STAT1 and NOS2 regulatory abnormalities in a model of CF epithelium.

With respect to STAT1 signaling, the CF models we have examined are characterized by reduced STAT1 activation, increased expression of PIAS1 protein, and reduced NOS2 expression compared with respective controls. Consistent with the hypothesis above, our data demonstrate that two models of CF epithelium exhibit elevated expression of RhoA protein with a corresponding increase in the active GTP-bound RhoA. To determine whether elevated RhoA expression and activation could be responsible for altered STAT1 regulation, we examined a mouse fibroblast line that exhibits increased RhoA protein content and RhoA activation. Compared with controls, fibroblasts overexpressing RhoA exhibit the same STAT1 signaling alterations observed in CF cells: reduced STAT1 activation, increased expression of PIAS1 protein, and reduced NOS2 expression. These data demonstrate directly that RhoA-dependent mechanisms are capable of regulating STAT1 signaling independently of CPTR function, suggesting that altered RhoA signaling in CF cells is the direct mechanism leading to reduced NOS2 expression. Inhibition of the Rho GTPase pathway leads to a normalization of PIAS1 protein expression, correction of STAT1 signaling, and restoration of NOS2 expression in CF phenotype epithelial cells. These data are the first to identify a mechanism by which PIAS1 expression can be regulated and also offer insight into a possible source for CF-related cell signaling alterations.

**MATERIALS AND METHODS**

**Cell Culture**

The human alveolar type II epithelial adenocarcinoma cell line (A549) was grown at 37°C in 95% O₂-5% CO₂ on Falcon 10-cm-diameter tissue culture dishes in Ham’s F-12 Kaign’s Modification (Biofluids) with 10% fetal bovine serum, 2 mM L-glutamine, and 1 unit/ml penicillin and streptomycin. Wild-type (wt) and Rho GTPase-activating protein (GAP)/−/− mouse embryonic fibroblasts (MEFs) were provided by Dr. Jeffrey Settleman (Harvard University, Cambridge, MA). MEFs were grown at 37°C in 95% O₂-5% CO₂ on Falcon 10-cm-diameter tissue culture dishes in DMEM with 5% fetal calf serum, 2 mM L-glutamine, and 1 unit/ml penicillin and streptomycin.

**Real-Time RT-PCR Analysis of PIAS1 mRNA Expression**

PIAS1 mRNA expression was determined with a Rotogene (F. Hoffmann-La Roche) quantitative PCR machine. The PIAS1 primers were used 5′-GGA TAT TTC TGG GAC CAA-3′ and 5′-TAA AGA GTT GGC TCA AAA-3′. Total RNA was obtained from cells by the TRIzol method, and cDNA was produced using Moloney murine leukemia virus with random primers according to the manufacturer’s instructions (GIBCO-BRL, Gaithersburg, MD). NOS2 and GAPDH mRNA expression levels were determined with a LightCycler (F. Hoffmann-La Roche) quantitative PCR machine. Human NOS2 and GAPDH were amplified using primer mix obtained from Search LC (Heidelberg, Germany). LightCycler parameters were provided by Search LC.

**Transfection Protocol and Evaluation of Luciferase Expression**

We utilized a γ-interferon-activated site-luciferase (pGAS-luc) reporter construct available from Stratagene (La Jolla, CA) to test for STAT1 activation in 9/HTEo- pCEP and pCEP cells provided by Dr. Pam Davis (Case Western Reserve University (CWRU), Cleveland, OH). Cells were seeded at a density of 50,000 cells/well in 96-well tissue culture dishes 24 h before transfection. For each transfection, 0.1 μg of DNA was placed into 10 μl of serum-free culture medium with 1 μl of Lipofectamine Plus reagent (GIBCO-BRL) and incubated for 15 min at room temperature. Renilla luciferase was transfected simultaneously at a concentration of 0.004 μg/well as an internal control for transfection efficiency. Lipofectamine reagent, 0.5 μl/reaction, was placed into 10 μl of serum-free culture medium. These two solutions were gently mixed at room temperature and incubated for an additional 15 min. After the second incubation at room temperature, the transfection mix was diluted in 50 μl of serum-free medium per well. Seventy microliters of the diluted transfection mix were added to each well, and cells were allowed to incubate at 37°C in 95% O₂-5% CO₂ for 5 h before 70 μl of 20% serum medium were added to bring final serum concentration to 10%. IFN-γ (10 units/ml) was added, and the cells were incubated for an additional 4 h at 37°C in 95% O₂-5% CO₂. Cells are then lysed in 1× Passive Lysis Buffer (Promega, Madison, WI) at room temperature for 20–30 min and assayed for luciferase activity according to the manufacturer’s instructions. Results are expressed in relative light units and normalized to Renilla luciferase activity.

A549 cells were transfected with either empty vector (pUSEamp) or constitutively active (ca)-RhoA by the same Lipofectamine Plus protocol described above with the exception that 60-mm dishes were used, and cells were transfected with DNA at a concentration of 2 μg of DNA/dish and assayed for protein content 24–48 h later.

**Protein Isolation and Western Blot Analysis**

Cells were plated onto 6-cm-diameter cell culture dishes and grown to 90–95% confluence at 37°C in 95% O₂-5% CO₂. Cells were lysed in 250 μl of ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 200 μM Na₃VO₄, and 10 μg/ml pepstatin and leupeptin; all chemicals from Sigma, St. Louis, MO) for ~30 min at 4°C. Plates were scraped on ice to suspend cells, and the cell lysate suspension was microcentrifuged at 4°C (14,000 g for 10 min). The supernatant was removed and stored at −20°C. Proteins were separated by SDS-PAGE on a 7.5 or 12% acrylamide gel at 100 V. The samples were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Blots were blocked in phosphate-buffered saline (PBS: 138 mM NaCl, 15 mM Na₂HPO₄, 1.5 mM KCl, and 2.5 mM KH₂PO₄) containing 10% nonfat dried milk and 0.1% Tween 20 (Sigma) overnight at 4°C. Blots were incubated overnight at 4°C in PBS with anti-PIAS1 primary antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-RhoA antibody (Santa Cruz Biotechnology) and then washed three times in PBS with 0.1% Tween 20. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase (1:4,000 dilution; Sigma) for 1 h at room temperature and washed again as described above. Signal was visualized by incubation with Super Signal chemiluminescent substrate (Pierce, Rockford, IL) and exposure of the membrane to Kodak scientific imaging film (Kodak, Rochester, NY).
RhoA Activity Assay

Rho activity was determined with a Rho Activation Assay Kit (Upstate Biotechnology, Lake Placid, NY). This assay was performed according to the protocol supplied by the manufacturer. Briefly, mouse nasal epithelium (MNE) was extracted from Cfr−/− and wt mice as previously described (11, 12). MNE was placed in 300 μl of 1× Mg2+ lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 2% glycerol) on ice and homogenized for 60 s. Tissue homogenates were centrifuged at 14,000 g for 5 min at 4°C to clear cellular debris. To assay for guanosine 5′-O-(3-thiotriphosphate) (GTPγS)-dependent activation of RhoA, we either left samples untreated or loaded them with GTPγS. Five microliters of 10 mM GTPγS were added to each respective aliquot and incubated for 30 min at 30°C. We terminated the reaction by placing samples on ice and adding 32 μl of 1 M MgCl2. Active (GTP/GTPγS-bound) RhoA was isolated by pull-down assay: 50 μl (30 μg) of rhotekin Rho binding domain (RBD) agarose bead slurry (50 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM PMSF, and 1 μg/ml each of aprotinin and leupeptin) were added to each 500-μl aliquot and incubated for 45 min at 4°C with gentle agitation. The rhotoke-RBD agarose bead-Rho complex was pulled down by brief centrifugation (10 s, 14,000 g, 4°C). The supernatant was removed, and the beads were washed three times. The beads were resuspended in 40 μl of Laemmli buffer containing 5% 2-mercaptoethanol, and Western blot analysis was performed with anti-Rho (A,B,C) rabbit polyclonal IgG (1:1,000 dilution) incubated overnight at 4°C.

Mice

Mice lacking CFTR expression (CFTRtm1Unc) were obtained from Jackson Laboratories and backcrossed onto a C57BL/6J background in the CF Animal Core Facility at CWRU. CFTR wt and wt heterozygous mice were siblings of Cfr−/− mice. All mice used were between 6 to 8 wk of age. Mice were cared for in accordance with CWRU Institutional Animal Care and Use Committee guidelines by the CF Animal Core Facility. Mice were treated with mevastatin at a concentration of 20 mg/kg -1 · day -1 in 50% dimethyl sulfoxide with Alzet microosmotic pumps (model 1003D) purchased from DURECT (Cupertino, CA). Pumps were placed in the mice under sterile conditions following manufacturer’s instructions.

RESULTS

RhoA Expression in CF Epithelium

On the basis of our previous findings that RhoA signaling is capable of modulating NOS2 expression in airway epithelial cells, we hypothesized that RhoA signaling is altered in CF epithelial cells, leading to reduced STAT1 signaling and reduced NOS2 expression. To test this hypothesis, we examined two models of CF for alterations in RhoA expression and activation. The 9/HTEx- pCEPR (CF phenotype) and pCEP (wt phenotype) cells were also examined for alterations in GTPase expression. The pCEPR cells exhibit a lack of cAMP-mediated chloride transport, reduced STAT1-mediated signaling, increased PIAS1 expression, and excessive inflammatory responses compared with controls and represent a consistent model system for examining CF-related signaling changes (4, 14, 29). CF phenotype pCEPR cells exhibit a 4.1 ± 0.6-fold increase (n = 6, P = 0.002) in total RhoA protein expression compared with wt pCEP cells (Fig. 1, A and B). Excised nasal epithelium from wt and Cfr−/− mice on a congenic C57BL/6J background were also examined for expression of RhoA, since we have previously identified alterations in STAT1 regulation in this model system (12). Consistent with what is observed in pCEPR cells, RhoA protein content exhibited a 4.2 ± 0.3-fold increase (n = 7, P < 0.0001) in the nasal epithelium of Cfr−/− mice compared with wt mice (Fig. 1, A and B).

GTPγS-activated RhoA content was also examined in excised MNE from both wt and Cfr−/− mice. The assay utilizes the RBD of the protein rhotekin expressed as a fusion protein with glutathione-S-transferase and then coupled to agarose beads. Only RhoA bound with GTP will bind to the RBD; therefore, the RBD beads precipitate only the active RhoA-GTP complex. Cfr−/− MNE contained 2.0 ± 0.6-fold (n = 6, P = 0.01) more RhoA-GTP than respective wt controls (Fig. 1, C and D). Ratios of RhoA-GTPγS and total RhoA protein did not significantly differ between CF and non-CF samples (not shown).

PIAS1 Mediates Control of STAT1-Related Signaling

PIAS1 expression. On the basis of the above data demonstrating increased RhoA protein content and activation in CF epithelial cells and previous work demonstrating the negative regulatory role RhoA has on NOS2 expression, we examined the role of RhoA in modifying CF-like alterations in STAT1 signaling. To test RhoA regulation of STAT1 signaling independently of CFTR function, we used RhoGAP−/− MEF cells and wt controls. RhoGAP−/− MEF cells possess increased RhoA protein content and increased GTP-bound RhoA, RhoA-GTP (24), similar to what is observed in CF epithelium as shown above.

We have previously reported that CF epithelial cells exhibit alterations in STAT1 signaling compared with non-CF controls, consisting of increased PIAS1 protein expression, reduced STAT1 activation, and reduced NOS2 expression. Each of these CF-related alterations was examined in RhoGAP−/− cells and controls to determine whether increased RhoA signaling could similarly influence STAT1 regulation independently of CFTR function. RhoGAP−/− cells exhibit a 2.0 ± 0.1-fold increase (n = 10, P < 0.0001) in PIAS1 protein expression compared with wt control MEFs (Fig. 2, A and B). Although not a robust increase in protein content, this is a significant and reproducible increase in PIAS1 protein that is consistent with what we observe in CF epithelial cells. Because PIAS1 protein content was only increased twofold in RhoGAP−/− MEFs compared with controls, a second model system of increased RhoA activity was used to confirm these findings. To more directly test the role of RhoA in mediating control of PIAS1 expression, we transfected A549 cells with either a control empty vector or a construct expressing ca-RhoA. A549 cells expressing...
active RhoA displayed a 2.1 ± 0.2-fold increase (n = 5, P = 0.01) in PIAS1 protein expression compared with control cells (Fig. 2, C and D), consistent with what was observed in RhoGAP−/− cells. PIAS1 mRNA expression of the above model systems was also examined. Although there was a slight trend toward increased PIAS1 mRNA content in both RhoGAP−/− and control cells, increases did not achieve statistical significance (data not shown).

**STAT1** activation. Using a STAT1-specific GAS reporter construct (pGAS-luc), we examined STAT1 activation in response to IFN-γ (10 units/ml) in RhoGAP−/− and wt MEFs. We have previously shown that STAT1 activation is reduced in CF epithelial cells (12). Consistent with what is observed in CF model systems, RhoGAP−/− MEFs exhibit significantly reduced STAT1 activation compared with wt controls. RhoGAP−/− cells support a 1.9 ± 0.1-fold increase in STAT1 activity in response to IFN-γ compared with a 17.4 ± 2.0-fold increase in wt MEFs (n = 12, P < 0.0001; Fig. 3).

**NOS2** expression. Because RhoGAP−/− cells exhibit reduced STAT1 activity compared with controls just as is observed in CF epithelial cells, it is predicted that RhoGAP−/− cells will also display reduced NOS2 expression in response to induction by inflammatory cytokines. RhoGAP−/− and wt MEFs were treated with cytomix (0.5 ng/ml IL-1β, 1.0 ng/ml TNF-α, and 100 units/ml IFN-γ) for 24 h and assayed for NOS2 protein expression. Wt MEFs exhibited a 53.6 ± 7.9-fold increase in NOS2 protein expression compared with only a 13.0 ± 1.7-fold increase in RhoGAP−/− MEFs (n = 4, P = 0.01; Fig. 4, A and B).

The above data demonstrate that a cell model system of elevated RhoA protein expression and activation faithfully replicates each of the STAT1-related signaling alterations observed in CF epithelial cells. These findings, coupled with the observation of elevated RhoA protein expression and activation in CF epithelial cells, are strong support for the hypothesis that RhoA-dependent mechanisms are responsible for altered STAT1 and NOS2 regulation in CF.

**Increased STAT1-Mediated Signaling and Restoration of Induced NOS2 Expression in Response to Isoprenoid/Cholesterol Synthesis Inhibition in CF Epithelial Cells**

The above data demonstrate increased RhoA protein content in two models of CF epithelium along with evidence that RhoA-dependent mechanisms are capable of mediating CF-like alterations in STAT1 regulation and NOS2 expression. The goal of the following experiments is to determine whether manipulation of the RhoA signaling pathway can normalize STAT1...
regulation and NOS2 expression in CF models systems.

**PIAS1 expression.** We have previously demonstrated increased PIAS1 expression and reduced STAT1-mediated signaling in 9/HTETo- cells overexpressing the CFTR regulatory domain (pCEPR) compared with a control cell line (pCEP) as described above. To determine whether Rho-associated kinase (ROCK) activity is involved in elevated PIAS1 expression in this model system, we treated pCEPR cells with Y-27632 for 24 h. After Y-27632 treatment, PIAS1 protein expression was reduced to near-control amounts in pCEPR cells, suggesting the involvement of the RhoA/ROCK pathway in PIAS1 expression regulation (Fig. 5, A and B).

Again, since PIAS1 expression is not profoundly different between cell lines, the role of ROCK was examined in mediating control over PIAS1 protein expression. It was found that Y-27632 resulted in a significant reduction in PIAS1 expression in A549 cells expressing ca-RhoA, suggesting that ROCK activity is involved in RhoA-mediated control of PIAS1 protein expression (Fig. 5, C and D).

Because RhoA requires isoprenoid modification for full activation, the ability of the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor mevastatin (50 μM) to reduce PIAS1 content in CF phenotype pCEPR cells was examined. Treatment with mevasta-
Addition of mevalonate (50 μM) to the reaction mixture completely reversed mevastatin-induced correction of STAT1 signaling in both pCEP and pCEPR cells (Fig. 6).

**NOS2 expression.** We have previously demonstrated that pCEPR cells fail to increase NOS2 mRNA in response to stimulation (25) and that inhibition of tin restored PIAS1 expression to normal levels in pCEPR cells (Fig. 5, A and B).

**STAT1 activation.** On the basis of the signaling interactions proposed above, it is predicted that inhibition of isoprenoid/cholesterol synthesis should lead to an increase in STAT1-mediated IFN-γ signaling and a restoration of STAT1-mediated signaling in 9/HTEO-pCEPR cells. We measured activation of STAT1 signaling using the GAS reporter construct (pGAS-luc). CF-phenotype pCEPR cells exhibited a slight, but statistically insignificant response to IFN-γ (10 units/ml), whereas wt phenotype pCEP cells demonstrated a 4.5 ± 0.4-fold increase in STAT1-mediated signaling (n = 20, P < 0.0001; Fig. 6). Treatment with the HMG-CoA reductase inhibitor mevastatin alone (50 μM) for 24 h had no effect on STAT1 activation in either cell line. However, treatment with both IFN-γ (10 units/ml) and mevastatin (50 μM) augmented STAT1 activation in pCEP cells (10.4 ± 1.6-fold increase; n = 24, P < 0.0001) and restored signaling in CF phenotype pCEPR cells, leading to a 4.7 ± 0.6-fold increase in activity (n = 30, P < 0.0001; Fig. 6). To determine whether correction of STAT1-mediated signaling in pCEP and pCEPR cells by mevastatin was specific to HMG-CoA reductase inhibition, we tested the ability of mevalonate (the product of HMG-CoA reductase activity) to reverse the effects of mevastatin. Addition of mevalonate (50 μM) to the reaction mixture completely reversed mevastatin-induced correction of STAT1 signaling in both pCEP and pCEPR cells (Fig. 6).

**Fig. 4.** Reduced inducible nitric oxide synthase (NOS2) protein expression in RhoGAP−/− MEFs compared with wt controls in response to cytomix (CM) stimulation. A: Western blots demonstrating NOS2 protein induction by CM in RhoGAP−/− MEFs and wt controls (RhoGAP+/+). Blots shown are representative of 4 separate experiments. B: densitometry analysis of NOS2 protein expression relative to actin protein expression (NOS2/actin) normalized to RhoGAP+/+ MEFs. Number of replicates is n = 4 for each condition. Filled bars represent RhoGAP+/+ MEFs, and hatched bars represent RhoGAP−/− MEFs. Significance determined by t-test; *P = 0.01.

**Fig. 5.** RhoA-mediated signaling regulates PIAS1 protein expression. A: Western blot of PIAS1 and ERK1 expression in pCEP (wt phenotype), in pCEPR (CF phenotype) 9/HTEO- cell model system, in pCEPR cells treated with the Rho-associated kinase (ROCK) inhibitor Y-27632 (Y-27), and in pCEPR cells treated with the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor mevastatin (Mev). B: densitometry analysis of PIAS1 expression relative to ERK1 protein content (PIAS1/ERK1) normalized to respective wt phenotype controls. Number of replicates is shown in parentheses above each bar. Significance determined by ANOVA with post hoc comparisons between groups performed by Duncan's multiple-range test; *significant difference compared with untreated group; #significant difference compared with empty vector control with P = 0.0002; #significant difference compared with untreated pCEP CF phenotype samples with P = 0.002. C: PIAS1 and ERK1 expression in A549 cells transfected with empty control vector, transfected with ca-RhoA, and transfected with ca-RhoA treated with the ROCK inhibitor Y-27632. D: densitometry analysis of PIAS1 expression relative to ERK1 protein expression (PIAS1/ERK1) normalized to empty vector transfected cells. Number of replicates is shown in parentheses above each bar. Significance determined by ANOVA with post hoc comparisons between groups performed by Duncan's multiple-range test; †significant difference compared with empty vector control with P < 0.05; ‡significant difference compared with untreated ca-RhoA samples with P < 0.05.
isoprenoid-dependent signaling leads to increased NOS2 expression in A549 cells (13). Increased STAT1-mediated signaling in pCEP cells in response to mevastatin treatment suggests that NOS2 expression may also be improved. The treatment of wt phenotype pCEP cells with cytomix results in a 4.2 ± 1.4-fold increase (n = 7, P < 0.05) in NOS2 mRNA content normalized to GAPDH as measured by quantitative real-time RT-PCR. Cytomix stimulation of CF phenotype pCEPR cells had no effect on NOS2 mRNA concentration, consistent with previous results (25). However, the addition of mevastatin (50 μM) to cytomix in pCEPR cells resulted in a 5.5 ± 1.1-fold increase (n = 7, P = 0.004) in NOS2/GAPDH mRNA content (Fig. 7, A and B).

Reduced NOS2 expression has also been demonstrated in vivo in Cftr−/− mice compared with wt controls (10, 25). The restoration of NOS2 mRNA induction in pCEPR cells by mevastatin and the observation of increased RhoA expression in Cftr−/− MNE lead to the hypothesis that systemic administration of mevastatin to Cftr−/− mice should restore endogenous NOS2 protein expression. Because the observation of increased RhoA expression and activation was made in the nasal epithelium of Cftr−/− mice, NOS2 expression was examined in this tissue as well. Mice were treated with mevastatin (20 mg·kg⁻¹·day⁻¹) or carrier via 72-h Alzet osmotic pumps as described in MATERIALS AND METHODS. After ~60 h, the nasal epithelium was excised and examined for NOS2 expression. NOS2 expression was apparent in wt MNE but significantly reduced in MNE isolated from Cftr−/− mice. However, mice receiving mevastatin exhibited a significant correction of NOS2 protein expression (Fig. 8, A and B).

**DISCUSSION**

We have previously reported that CF epithelial cells exhibit diminished STAT1-mediated signaling and reduced NOS2 expression. However, it is currently unknown by what mechanism these CF-related STAT1 signaling alterations are regulated, and the goal of this study is to identify these regulatory mechanisms in CF epithelial cells.

Reduced NOS2 expression in CF epithelial cells has now been reported by several laboratories in both mouse and human tissues (4, 10, 17, 18). Reduced NOS2 expression is an unexpected finding due to the
excessive inflammation characteristic of the CF lung (30). We recently demonstrated that the Rho GTPase pathway can negatively regulate the expression of NOS2 in airway epithelial cells, while others have shown that this pathway can positively influence nuclear factor-κB activation and thus inflammation (7, 13, 23, 33). These dual roles of the Rho GTPase-mediated signaling suggest that this pathway can potentially account for the CF-related conditions of reduced NOS2 expression in the presence of enhanced inflammation. The potential importance of Rho-regulated pathways to CF pathogenesis leads to the hypothesis that Rho GTPase signaling influences STAT1 signaling and thus NOS2 expression in CF epithelium.

To test the above hypothesis, we examined both a mouse model and a human cultured cell model of CF epithelium for expression of Rho family GTPases. Both models of CF epithelium exhibited increased RhoA expression compared with wt controls. A proportional increase in GTP-bound RhoA (RhoA-GTP) was also observed in MNE compared with respective non-CF controls. Although the mechanisms by which RhoA expression is altered in CF epithelium are unknown and currently being investigated, the consequences of the Rho pathway in modulating a variety of signaling pathways and in influencing the expression of multiple genes in CF epithelium are significant.

In this study, the role of the Rho GTPase pathway in modulating STAT1 signaling was examined. Cells deficient in p190B RhoGAP, a negative regulator of RhoA, exhibit both increased RhoA content and activation, similar to what we observe in models of CF epithelium. RhoGAP−/− fibroblasts exhibit identical alterations in STAT1 regulation that are observed in CF cells, including reduced STAT1 activation, increased PIAS1 protein expression, and reduced NOS2 induction in response to cytomix. These data demonstrate that RhoA-dependent mechanisms are capable of modulating STAT1 signaling in a manner consistent with CF-related observations. The replication of CF-like cell signaling alterations in a system that is independent of CFTR function suggests that CFTR activity is not directly influencing STAT1 regulation but is influencing an upstream event, such as RhoA regulation that is subsequently altering STAT1-dependent processes.

The pathway being proposed to link CFTR function to NOS2 regulation is shown in Fig. 9. Whether these interactions are the only mechanisms by which statin-sensitive pathways can influence RhoA activation is unclear. Because mevastatin also augments STAT1 activation in wt phenotype pCEP 9/HTEo- cells, statin-sensitive pathways may be endogenous negative regulators of STAT1 signaling and may act through multiple points in the STAT1 pathway. However, the signaling cascade shown in Fig. 9 is proposed to be specifically responsible for linking CFTR function to NOS2 regulation. It is proposed that a loss of CFTR function leads to increased RhoA expression and activation, a condition mimicked by RhoGAP−/− fibroblasts, which results in elevated PIAS1 protein expression and reduced STAT1 activation, resulting in attenuated NOS2 induction in CF epithelial cells.

As mentioned above, it is not known how CFTR function may influence RhoA regulation. One report demonstrates that RhoA protein expression is elevated in cells from patients with Tangier disease (26), a disease caused by dysfunction of the cholesterol transport protein ATP-binding cassette protein A1 (ABCA1). ABCA1 and CFTR share considerable structural similarity (21), suggesting the possibility of shared functional abilities. The dependence of RhoA activation and stability on products of the cholesterol synthesis pathway and the role of ABCA1 in regulating cholesterol transport point to a potential role of lipid transport in

---

**Fig. 8.** Restoration of endogenous NOS2 protein expression in nasal epithelium of Cfr−/− mice. A: Western blot of NOS2 and ERK1 protein expression in wt MNE (lane 1), in MNE from mock-treated Cfr−/− mice (lane 2), and in mevastatin-treated Cfr−/− mice (lane 3). B: densitometry analysis of NOS2 protein expression relative to ERK1 protein content (NOS2/ERK1) normalized to wt controls. Number of replicates is shown in parentheses above each bar. Significance determined by t-test; *P < 0.05.

---

**Fig. 9.** Diagram of proposed signaling interactions linking CFTR function to NOS2 regulation; +, positive regulation; −, negative regulation.
CFTR-mediated RhoA regulation. CFTR has been reported to mediate transport of sphingosine-1-phosphate (2), but the relationship between CFTR function and lipid transport needs to be more clearly defined. The role of lipid transport regulation in mediating CF-related RhoA regulation, and thus STAT1 and NOS2 regulation, is currently being explored.

The first finding described in this paper is that total RhoA protein expression and GTP-bound RhoA content are increased in two models of CF epithelium. Secondly, it is demonstrated that elevated RhoA activity in RhoGAP−/− MEFs leads to CF-like alterations in STAT1 regulation including reduced STAT1 activation, increased PIAS1 protein expression, and reduced NOS2 induction in response to cytokinin. These data strongly implicate a role for RhoA-mediated signaling in the CF-related alterations in STAT1 and NOS2 regulation. Finally, it is shown that inhibition of Rho GTpase-mediated signaling can normalize STAT1 regulation and restore NOS2 expression in CF cells. This is the first report demonstrating RhoA-mediated regulation of STAT1 signaling and gives insight into fundamental cell signaling changes taking place in CF epithelial cells. These findings suggest that pharmacological inhibition of the Rho GTpase pathway may represent a potential avenue for therapeutic intervention for CF.

The authors thank Drs. P. Davis and J. Settleman for providing materials necessary for the completion of this study and P. Bead for technical assistance.

DISCLOSURES

This work was supported by National Heart, Lung, and Blood Institute Grant HL-64899 and a Leroy Matthews Physician Scientist Award from the Cystic Fibrosis Foundation to N. C. Kraynack.

REFERENCES


