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Mechanisms of NOS2 regulation by Rho GTPase signaling in airway epithelial cells

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Kraynack, Nathan C., Deborah A. Corey, Heather L. Elmer, and Thomas J. Kelley. Mechanisms of NOS2 regulation by Rho GTPase signaling in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 283: L604–L611, 2002. First published April 12, 2002; 10.1152/ajplung.00459.2001.—The aberrant dysregulation of the inducible form of nitric oxide synthase (NOS2) is thought to play a role in many inflammatory disorders including cystic fibrosis (CF). The complex regulation of NOS2 expression is the subject of intense investigation, and one intriguing regulatory pathway known to influence NOS2 expression is the Rho GTPase cascade. We examined NOS2 regulation in response to inflammatory cytokines in a human alveolar epithelial cell line treated with inhibitors of different upstream and downstream components of the Rho GTPase pathway to better define potential signaling mechanisms. Statin-mediated 3-hydroxy-3-methylglutaryl-CoA reductase inhibition increased cytokine-dependent activation of the NOS2 promoter, reversible by the addition of geranylgeranyl pyrphosphate. However, inhibition of Rho-associated kinase (ROCK) with Y-27632 resulted in a decrease in NOS2 promoter activity, yet an increase in NOS2 mRNA and protein levels. Our results suggest that prenylation events influence NOS2 promoter activity independently of the Rho GTPase pathway and that Rho GTPase signaling mediated through ROCK suppresses NOS2 production downstream of promoter function at the message and protein level.

inflammation; prenylation; cystic fibrosis; nitric oxide synthase 2

NITRIC OXIDE (NO) is involved in a variety of vital biological functions in many tissues, including the lung (9, 13, 19, 22, 24, 30). These functions are protean and range from vascular smooth muscle relaxation and neurotransmission to inflammatory cell signaling and antimicrobial effects (30). Nitric oxide synthases (NOS) are the enzymes responsible for the synthesis of endogenous NO from L-arginine and occur in constitutive (NOS1 and NOS3) and inducible isoforms (NOS2) (19, 30). The constitutive isoforms generate relatively constant low levels of NO and are regulated by the intracellular calcium/calmodulin system. The inducible isoform of the enzyme generates much larger amounts of NO in response to stimuli such as bacteria, bacterial components, and cytokines, including interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α (9, 19, 30). The dysregulation of NO production, primarily from NOS2, is thought to play a role in many inflammatory disorders including autoimmune diseases (rheumatoid arthritis, ulcerative colitis, Crohn's disease), septic shock, and atherosclerotic vascular disease, and in lung diseases such as cystic fibrosis (CF) and diffuse panbronchiolitis (13, 22, 24, 30). The regulation of this enzyme is complex, with a wide variety of signal transduction pathways involved in inflammatory and epithelial cells (8, 9, 19, 25, 30). Elucidation of the regulatory pathways of NOS2, particularly in epithelial cells, is required to more fully understand the pathophysiology of such inflammatory diseases as asthma and CF. An intriguing pathway necessary for balancing inflammatory responses and NO production is the Rho GTPase cascade. Extracellular stimuli and cytokines that are known to induce NOS2 expression also activate specific G proteins of the Ras/Rho family (16), and there is recent evidence that Rho signaling negatively regulates NO production possibly as a feedback mechanism (23).

The Rho family of GTPases, including Rho, Rac, and Cdc42, are involved in the regulation of multiple cellular functions ranging from cell migration, proliferation, and adhesion, to ion transport, bacterial ingestion and inflammation (6, 12, 17, 23, 27, 29, 33, 34). In addition to GTP binding and each of its regulatory components, full Rho activation requires posttranslational prenylation with either a 15-carbon farnesyl moiety or 20-carbon geranylgeranyl isoprene unit (1, 2). These reactions are catalyzed by farnesyl transferase (FTase) and two distinct geranylgeranyl transferases, type I and II (GGTase I and II), respectively. Prenylation is initiated by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity that forms L-mevalonate, a precursor of isoprenoid lipids, and serves to properly localize the normally soluble protein to the plasma membrane (1, 2).

Several components of Rho GTPase signaling are available for pharmacological manipulation, including

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HMG-CoA reductase, GGTases, FTase, or the downstream effector Rho-associated kinase (ROCK), to examine Rho signaling mechanisms and function (18, 26, 31, 35). Focusing on NO, recent work has demonstrated that inhibition of Rho GTPase activity with mevastatin, an HMG-CoA reductase inhibitor, in vascular smooth muscle cells increases NOS2 expression, implying that Rho activation may decrease NOS2 expression (23). Other studies have demonstrated alterations of NOS2 expression in the presence of Rho GTPase pathway inhibitors such as *Clostridium difficile* toxin B, HMG-CoA reductase inhibitors, and prenyl transferase inhibitors in a variety of cell types, including fibroblasts, vascular smooth muscle, endothelial cells, neuronal cells, macrophages, cardiac myocytes, and human adenocarcinoma cell lines (5, 9, 10, 17, 23, 28).

The goal of this paper is to examine the relationship between components of the Rho GTPase pathway and NOS2 expression in airway epithelial cells. We utilized various compounds that affect different upstream and downstream components of the Rho GTPase cascade in a human alveolar epithelial cell line (A549 cells) and examined their effect on NOS2 promoter activity, mRNA levels, and protein expression in response to inflammatory cytokines. We determined that interruption of the Rho GTPase pathway at any point results in increased NOS2 production in airway epithelial cells. However, different mechanisms were utilized to bring about NOS2 regulation depending on the point of inhibition within the pathway.

MATERIALS AND METHODS

Cell culture and cytokine stimulation. The human alveolar type II epithelium-like adenocarcinoma cell line (A549) was grown at 37°C in 95% O2-5% CO_2 on Falcon 10-cm-diameter tissue culture dishes in Ham's F-12 Kaign's Modification (Biofluids) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 U/ml penicillin and streptomycin, and 250 ng/ml amphotericin B. Cells were transferred to 6-cm-diameter (protein assay/Western blot analysis) or six-well (luciferase assay) Falcon tissue culture plates for experiments. Cells were stimulated with a combination of cytokines (cytomix: 1 ng/ml TNF-α, 0.5 ng/ml IL-1β, 100 U/ml IFN-γ) for 12 (luciferase assay) or 24 h (Western blot analysis) 12–24 h after the addition of Rho pathway inhibitors as described in *Gene transfection and evaluation of NOS2 gene expression*.

RT-PCR analysis of NOS2 mRNA expression. NOS2 mRNA levels were determined on a LightCycler (F. Hoffmann-La Roche) quantitative PCR machine. Human NOS2 was amplified with primer mix obtained from Search-LC (Heidelberg, Germany). LightCycler parameters were provided by Search-LC. Total RNA was obtained from A549 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).

Gene transfection and evaluation of NOS2 gene expression. We utilized a NOS2-luciferase gene construct (NOS2-luc) provided by Dr. Joel Moss, National Institutes of Health, and nuclear factor (NF)-κB-luc purchased from Clontech (Palo Alto, CA). A549 cells were seeded at a density of 6.5×10^5 cells/well in six-well Falcon tissue culture dishes 24 h before transfection. For each transfection, 1 μg of DNA was placed into 100 μl of serum-free culture medium with 6 μl of Lipofectamine Plus reagent (GIBCO-BRL) and incubated for 15 min at room temperature. Lipofectamine reagent (5 μl/reaction) was placed into 100 μl of serum-free culture medium. These two solutions were gently mixed at room temperature and incubated for an additional 15 min. After being washed twice with serum-free culture medium, cells were placed in 0.8 ml of serum-free medium. The DNA-liposome mixture (0.2 ml) was added to each well and mixed gently. Cells were incubated at 37°C in 95% O_2-5% CO_2 with the complex for 3–5 h. After incubation, 1 ml of culture medium containing 20% FBS was added to each well (final concentration of FBS 10%), and the entire mixture was incubated overnight (24 h of total incubation). Cytomix was added, and the cells were incubated for an additional 12 h at 37°C in 95% O_2-5% CO_2. Cells were then lysed in 1× Cell Culture Lysis Reagent (Promega, Madison, WI) at room temperature for 20–30 min and assayed for luciferase activity according to manufacturer's instructions (Promega). Results are expressed in relative light units and normalized to luciferase activity of the cytomix-stimulated A549 cells in each experiment.

Protein isolation and Western blot analysis. A549 cells were plated on to 6-cm-diameter cell culture dishes and grown to 90–95% confluence at 37°C in 95% O_2-5% CO_2. Cells were lysed in 500 μl of ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 200 μM Na_3VO_4, 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 cell lysate suspension was microcentrifuged at 4°C (14,000 rpm for 10 min). The supernatant was removed and stored at −80°C. Proteins were separated using SDS-PAGE through a 6%–7.5% 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; in mM: 138 NaCl, 15 Na_2HPO_4, 1.5 KCl, and 2.5 KH_2PO_4) containing 5% nonfat dehydrated milk and 0.1% Tween 20 (Sigma) overnight at 4°C. Blots were incubated for 90–120 min at room temperature in PBS with primary antibody (1:1,000 dilution; Transduction Laboratories) 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).

RESULTS

RhoA activity influences NOS2 protein expression. Other reports have demonstrated the influence of the Rho GTPase pathway on NOS2 expression in various cell types (5, 9, 10, 17, 23, 28). To establish the role of this pathway on NOS2 regulation in A549 cells, we examined NOS2 protein expression in these cells in response to cytomix (1 ng/ml TNF-α, 0.5 ng/ml IL-1β, 100 U/ml IFN-γ) stimulation in the presence of the *Clostridium botulinum* toxin C3 ribosyl transferase, a RhoA-selective inhibitor. We found that NOS2 protein expression was increased in A549 cells in the presence of C3 toxin, indicating a role for the Rho GTPase pathway in NOS2 regulation (Fig. 1, *A* and *B*). Extracellular signal-regulated kinase (ERK) 1/2 levels were unchanged in the samples that were measured as a control for protein loading. Having established a basic

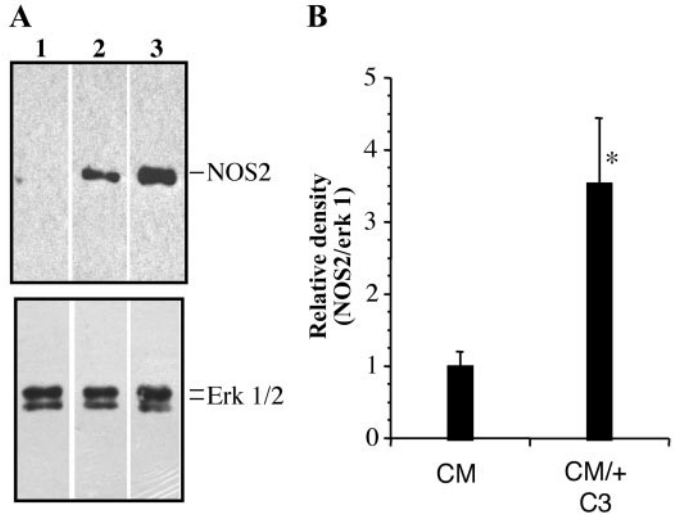

Fig. 1. Effect of *Clostridium botulinum* toxin C3 ribosyl transferase (C3) treatment on nitric oxide (NO) synthase (NOS) 2 protein levels in cytomix (CM)-treated A549 cells. *A*: A549 cells were treated with CM alone [1 ng/ml tumor necrosis factor (TNF)-α, 0.5 ng/ml interleukin (IL)-1β, 100 U/ml interferon (IFN)-γ; *lane 2*] or after treatment with C3 (62.5 ng/ml) for 24 h (*lane 3*) and examined for NOS2 (*top*) or extracellular signal-regulated kinase (ERK) 1/2 (*bottom*) by Western blot. Protein lysate from untreated cells is shown in *lane 1*. Cells were stimulated for 20 h with CM, and 50 μg of total protein were loaded in each lane. Immunoblotting for ERK 1/2 was performed to control for protein loading (*bottom*). *B*: densitometry analysis of NOS2 and ERK1 levels. Data are reported as NOS2/ERK1 ratio and are averages of 3 separate experiments. Error bars represent SE; $^*P < 0.05$ determined by *t*-test.

role for the Rho GTPase pathway in the regulation of NOS2 expression in A549 cells, we had as our goal to explore the roles of early and late events in the Rho GTPase cascade on this process.

Effect of prenyltransferase inhibitors on NOS2 promoter activity and protein expression. It has been previously reported that inhibition of protein prenylation in vascular smooth muscle cells with HMG-CoA reductase inhibitors potentiates the induction of NOS2 in response to cytokines (9). To assess the effect of HMG-CoA reductase activity on NOS2 promoter induction in human airway epithelial cells, we treated A549 cells transfected with NOS2-luc with mevastatin (Calbiochem, La Jolla, CA), an HMG-CoA reductase inhibitor, for 24 h before stimulation with cytomix. The addition of mevastatin to cell cultures at increasing concentrations resulted in a dose-dependent increase in NOS2 promoter activity. Maximum NOS2 promoter activity (277.0 ± 22.7%, $n = 8$) compared with cytomix alone was achieved with 50 μM mevastatin (Fig. 2*A*). To establish that mevastatin is acting directly on HMG-CoA reductase, we performed the same experiment in the presence of the product of HMG-CoA reductase mevalonate (100 μM). The addition of mevalonate effectively reversed the effects of mevastatin, indicating that inhibition of isoprenyl formation is being targeted (Fig. 2*B*). To address whether farnesyl or geranylgeranyl moieties are responsible for NOS2 promoter regulation, we added either farnesyl-pyrophosphate (pp) or geranygeranyl-pp to the assay system. Although reversal of the effect of mevastatin was achieved with both isoprenyl compounds, geranylgeranyl-pp was significantly more effective than farnesyl-pp, indicating that proteins involved in NOS2 promoter regulation are modified by geranylgeranyl isoprenylation (Fig. 2*B*).

Given the stimulation of the NOS2 promoter by mevastatin, we examined NOS2 protein production in A549 cells in response to HMG-CoA reductase inhibition. We treated cultured A549 cells with mevastatin

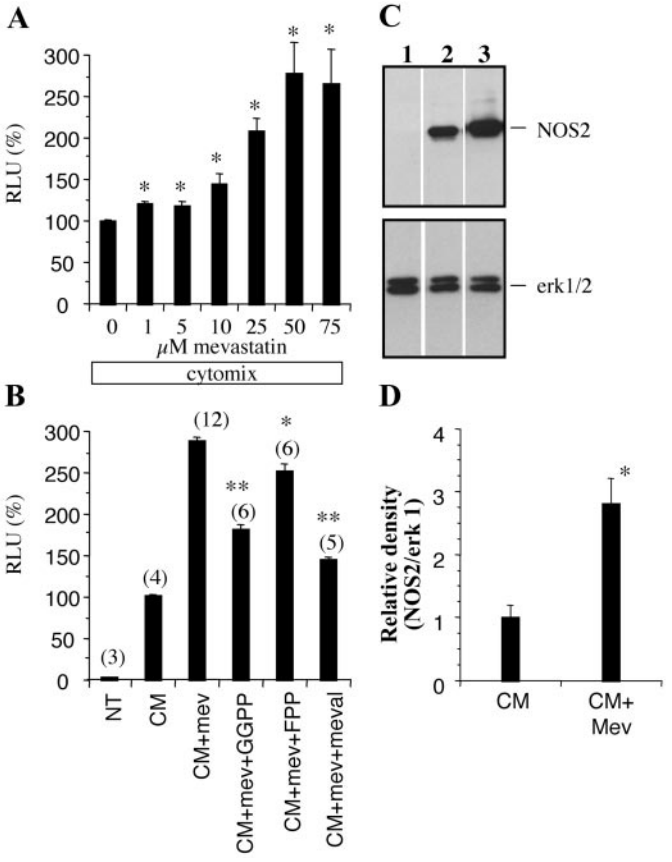

Fig. 2. Effect of mevastatin (Mev) on NOS2 promoter activity and protein levels in CM-treated A549 cells. *A*: NOS2 promoter activity, as measured by luciferase assay, in response to CM treatment for 12 h in the presence of increasing concentrations of Mev (0–75 μM). $^*P \leq 0.001$ determined by Duncan's multiple range test compared with zero Mev treatment. *B*: NOS2 promoter activity, as measured by luciferase assay, in response to either no treatment (NT) or CM treatment for 12 h in the presence of Mev (50 μM) alone, or in combination with farnesyl-pyrophosphate (FPP, 1.5 μg/ml), geranylgeranyl-pyrophosphate (GGPP, 132 ng/μl), or mevalonate (Meval, 100 μM). Results are given in relative light units (RLU) as a percentage of CM-treated cells. Numbers in parentheses above bars are number of experiments. Error bars represent SE. $^*P < 0.05$ and $^{**}P < 0.001$, determined by Duncan's multiple range test compared with CM + Mev treatment. *C*: effect of Mev (50 μM) treatment on NOS2 protein levels in CM-treated A549 cells. Cells were treated with CM alone (*lane 2*) or after treatment with Mev for 24 h (*lane 3*) and examined for NOS2 (*top*) or ERK 1/2 (*bottom*) by Western blot. Protein lysate from untreated cells is shown in *lane 1*. Cells were stimulated for 24 h with CM, and 50 μg of total protein were loaded in each lane. Immunoblotting for ERK 1/2 was performed to control for protein loading (*bottom*). *D*: densitometry analysis of NOS2 and ERK1 levels. Data are reported as NOS2/ERK1 ratio and are averages of 3 separate experiments. Error bars represent SE; $^*P < 0.005$ determined by *t*-test.

24 h before stimulation with cytomix. In the absence of cytokine stimulation, NOS2 protein is undetectable by Western blot analysis. Stimulation with cytomix for 24 h results in the appearance of a band at ~130 kDa, consistent with the size of NOS2. Pretreatment with mevastatin 24 h before stimulation resulted in elevated NOS2 protein levels above that produced by cytomix stimulation alone (Fig. 2*C*). These results are consistent with the previously reported results (5, 9, 10, 17, 23) and demonstrate that statin-mediated increases in NOS2 protein production are likely due to increased NOS2 promoter activity.

For optimal stimulation of NOS2 expression, two signaling components are necessary, NF-κB activation and IFN-γ-mediated signaling (20, 36). We have found that IL-1β and TNF-α stimulate the NOS2 promoter but only to ~10% of the efficiency obtained when IFN-γ is added to the mixture (Fig. 3*A*). To determine whether mevastatin influenced IL-1β/TNF-α response or the IFN-γ-mediated enhancement, we examined the effect of mevastatin on NOS2 promoter activity in response to both of these stimulatory mixtures. We found that mevastatin (50 μM) mediated a significant increase in promoter activity when A549 cells are stimulated with either IL-1β/TNF-α or cytomix and that fold stimulation is nearly identical. These data demonstrate that statin-mediated enhancement of NOS2 promoter activity is likely independent of the IFN-γ pathway.

Effect of the ROCK on NOS2 promoter activity and protein expression. Having established that inhibiting early events in the Rho GTPase cascade with mevastatin results in enhanced NOS2 promoter activity independent of IFN-γ signaling and in increased NOS2 protein expression, we chose to examine a later event in Rho signaling to better define the site within the Rho GTPase pathway involved in regulating NOS2 induction. We examined NOS2 promoter activity in response to cytokines in the presence of an inhibitor of ROCK, Y-27632 (11). Unexpectedly, we found that the addition of Y-27632 (100 μM) inhibited NOS2 promoter activity by ~32% whether cells were stimulated with cytomix

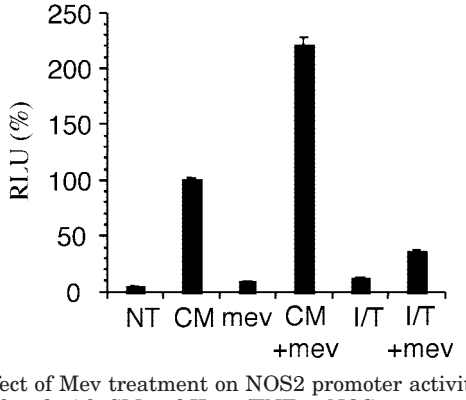

Fig. 3. Effect of Mev treatment on NOS2 promoter activity in A549 cells stimulated with CM and IL-1β/TNF-α. NOS2 promoter activity, as measured by luciferase assay, in response to CM or 0.5 ng/ml IL-1β and 1 ng/ml TNF-α (I/T) stimulation for 12 h in the presence or absence of Mev (50 μM). Results are given in RLU as a percentage of CM-treated cells. Error bars represent SE; $n = 6$.

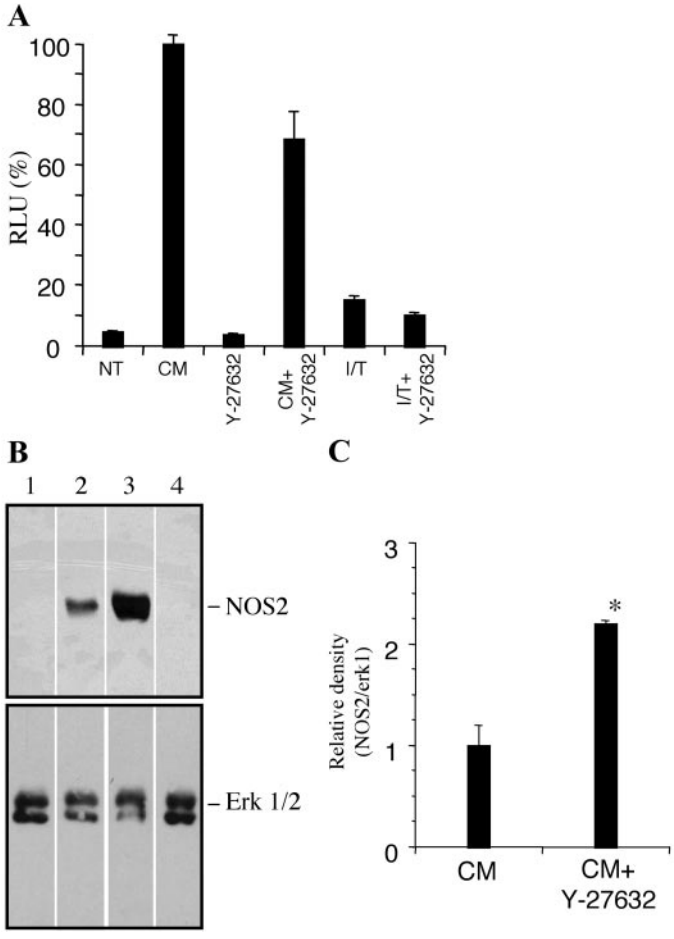

Fig. 4. Effect of Y-27632 treatment on NOS2 promoter activity and protein expression in A549 cells. *A*: NOS2 promoter activity, as measured by luciferase assay, in response to CM or I/T stimulation for 12 h in the presence or absence of 100 μM Y-27632 (+Y-27632). Results are given in RLU as a percentage of CM only-treated cells. Error bars represent SE; $n = 9$. *B*: effect of Y-27632 treatment on NOS2 protein levels in CM-treated A549 cells. Cells were treated with CM alone (*lane 2*) or after treatment with Y-27632 (CM + Y-27632) for 24 h (*lane 3*), and examined for NOS2 (*top*) or ERK 1/2 (*bottom*) by Western blot. Protein lysate from untreated cells is shown in *lane 1* and from unstimulated cells treated with Y-27632 (100 μM) in *lane 4*. Cells were stimulated for 24 h with CM, and 70 μg of total protein were loaded in each lane. Immunoblotting for ERK 1/2 was performed to control for protein loading (*bottom*). *C*: densitometry analysis of NOS2 and ERK1 levels. Data are reported as NOS2/ERK1 ratio and are averages of 6 separate experiments. Error bars represent SE; $^*P < 0.05$ determined by *t*-test.

or IL-1β/TNF-α (Fig. 4*A*). Despite the observed inhibition of promoter activity, protein production of NOS2 in A549 cells stimulated with cytomix in the presence of Y-27632 (100 μM) was increased (Fig. 4, *B* and *C*). These results suggest divergent mechanisms within the Rho GTPase cascade that can serve to negatively regulate NOS2 expression. Early events in the Rho pathway directly effect NOS2 promoter activity, whereas later events can influence NOS2 levels independently of the promoter.

Treatment of A549 cells with either Y-27632 or mevastatin increases NOS2 mRNA expression. Given the discrepancy between the decrease in NOS2 promoter

activity and increase in NOS2 protein expression in airway epithelial cells treated with an inhibitor of ROCK, we examined the effects of Y-27632 (100 μM) and mevastatin (50 μM) treatment on NOS2 mRNA expression using quantitative RT-PCR. NOS2 mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels with a NOS2/GAPDH ratio in unstimulated A549 cells arbitrarily set to a value of 1.0. Stimulation of A549 cells with cytomix increases NOS2 mRNA levels by 121.9 ± 30.0-fold (Fig. 5*A*). Treatment of cells with cytomix in the presence of Y-27632 or mevastatin, however, increased the NOS2/GAPDH ratio by 639.3 ± 90.9- and 694.9 ± 135.2-fold, respectively. Despite the opposite effects on promoter regulation, these data demonstrate that both mevastatin and Y-27632 have identical effects on NOS2 message levels. These findings are consistent with our data demonstrating increased NOS2 protein production in response to these compounds.

Regulation of NF-κB and activator protein-1 activity by early and late Rho GTPase signaling events. Our data indicate that both mevastatin and Y-27632 act independently of IFN-γ signaling in their opposite regulation of the NOS2 promoter. To identify a partial mechanism for this observation, we hypothesized that differential effects of mevastatin and Y-27632 on NF-κB activation were likely responsible for the observed effects on the NOS2 promoter. To test this hypothesis, we examined activation of a NF-κB-luc construct in response to IL-1β/TNF-α in the presence of either mevastatin (50 μM) or Y-27632 (100 μM). We found that mevastatin had no influence on NF-κB activation, whereas Y-27632 inhibited activation by 63.8 ± 1.4% (Fig. 6*A*). The examination of an activator protein (AP)-1-luc yielded identical results. Mevastatin had no effect on AP-1 stimulation, whereas Y-27632 inhibited activation by 57.0 + 1.7% (Fig. 6*B*).

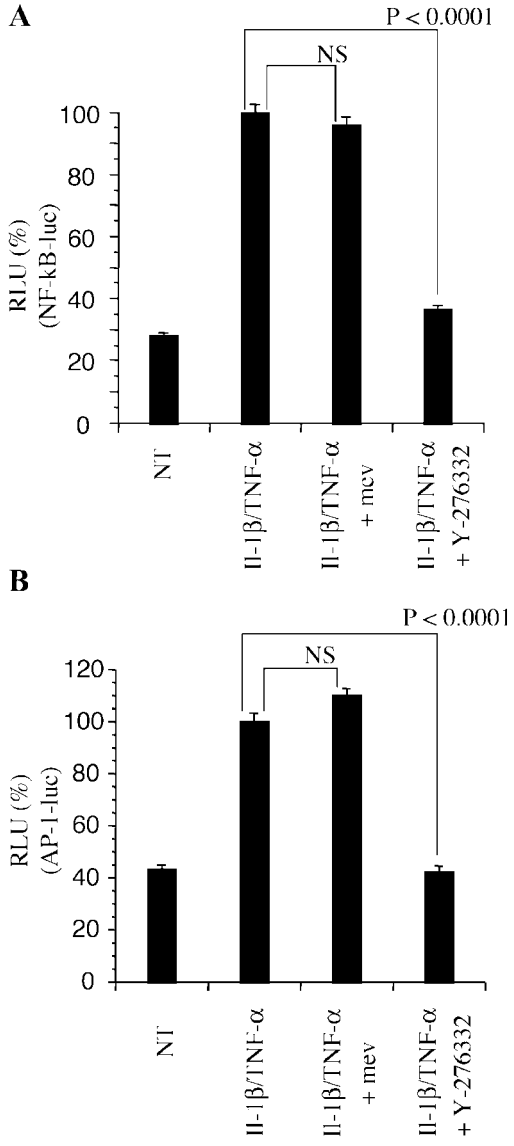

Fig. 6. Effect of Mev and Y-27632 on nuclear factor (NF)-κB activity in response to stimulation with I/T. *A*: NF-κB activity, as measured by luciferase assay, in response to I/T stimulation for 12 h in the presence or absence of 50 μM Mev and 100 μM Y-27632. Results are given in RLU as a percentage of I/T-treated cells. Error bars represent SE; $n = 6$. *B*: activator protein (AP)-1 activity, as measured by luciferase assay, in response to I/T stimulation for 12 h in the presence or absence of 50 μM Mev and Y-27632 (100 μM). Results are given in RLU as a percentage of I/T-treated cells. Error bars represent SE; $n = 8$. NS, not significant; luc, luciferase gene construct.

DISCUSSION

Previous studies have examined the regulation of NOS2 by Rho GTPases in a variety of cell types, including fibroblasts, vascular smooth muscle, cardiac myocytes, endothelial cells, neurons, macrophages, and human adenocarcinoma cell lines (5, 9, 10, 17, 23, 28). These studies conclude that inhibition of the Rho GTPase pathway enhances NO production in response to inflammatory stimuli (5, 9, 10, 17, 23). Hausding et al. (9) demonstrated increased NOS2 promoter activity and message accumulation in response to HMG-CoA reductase inhibitors, as well as increased NOS2 mRNA in response to direct Rho GTPase inhibition. Our data are consistent with these findings, and our goal was to

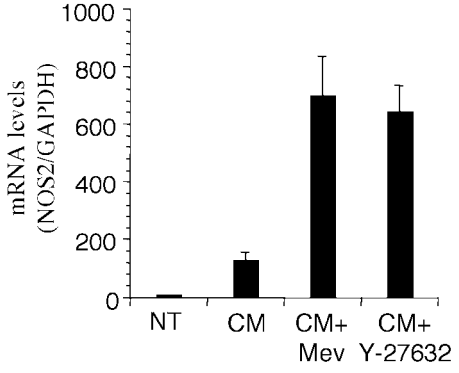

Fig. 5. Effect of Mev and Y-27632 on CM-stimulated NOS2 mRNA production. NOS2 mRNA levels as measured with quantitative PCR in A549 cells treated with CM in the presence of either 50 μM Mev (+Mev) or 100 μM Y-27632. NOS2 mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels with a NOS2/GAPDH ratio in unstimulated A549 cells (NT) arbitrarily set to a value of 1.0. Error bars represent SE; $n = 4$.

expand the studies to include downstream signaling events and to determine sites of interaction within the NOS2 regulatory pathway. In this study, we demonstrate that inhibition of HMG-CoA reductase augments NOS2 expression through a mechanism consistent with increasing NOS2 promoter activity. However, inhibition of the Rho GTPase downstream effector ROCK mediated an increase in NOS2 message and protein levels independently of promoter activation. The divergent mechanisms utilized by early and late events of the Rho GTPase cascade need to be considered when interpreting pharmacological interventions.

The fact that inhibition of mevalonate synthesis, the precursor of isoprenoids, with mevastatin and related compounds, increases NOS2 promoter activity has been clearly established (9, 23). These findings are taken as an implication that the Rho GTPase pathway is acting as a negative regulator of the NOS2 promoter. Rho GTPases require prenylation to be properly localized and to mediate proper function (1, 2). The effect of mevastatin appears to be specific to the inhibition of HMG-CoA reductase, as the addition of mevalonate abolishes the stimulatory effect of mevastatin. HMG-CoA reductase inhibitors, such as mevastatin, nonspecifically inhibit prenylation by removing isoprenoid precursors (4). Data presented here are consistent with previous findings in that NOS2 promoter activity is regulated by geranylgeranylation events (5, 10, 17). Our results, however, demonstrate that statin-induced NOS2 promoter activity is likely independent of the Rho GTPase pathway. The exact mechanism responsible for the role of prenylation in NOS2 promoter activation is unknown. Our data indicate that mevastatin-mediated enhancement of promoter stimulation is independent of IFN-γ signaling and mevastatin had no effect on either NF-κB or AP-1 activation. An examination of prenylation-dependent events in NOS2 promoter activation will be the focus of future studies.

We also explored the effects of modulating ROCK activity, a downstream effector of the Rho GTPase pathway involved in many Rho-dependent functions, on NOS2 induction (31). In contrast to results obtained with mevastatin, treating A549 cells with Y-27632 resulted in decreased NOS2 promoter activity. Despite this reduced NOS2 promoter activity in the presence of Y-27632, a significant increase in NOS2 mRNA and protein expression was observed in response to ROCK inhibition. These data suggest that a mechanism exists in the epithelial cell meant to balance inflammatory responses and NO production. Prenylation events negatively regulate NOS2 promoter activity, while simultaneously acting as positive mediators of Rho GTPase function. Increases in Rho GTPase function result in the activation of ROCK, which serves as another interesting branch point. ROCK activation in response to inflammatory stimuli has a positive effect on both NF-κB and AP-1 activity, as well as NOS2 promoter function but apparently has distinct negative influences on NOS2 mRNA levels and protein production (Fig. 7). The exact nature of message control by ROCK is unknown, but it is a key regulatory element in balancing NO production in response to inflammatory stimuli.

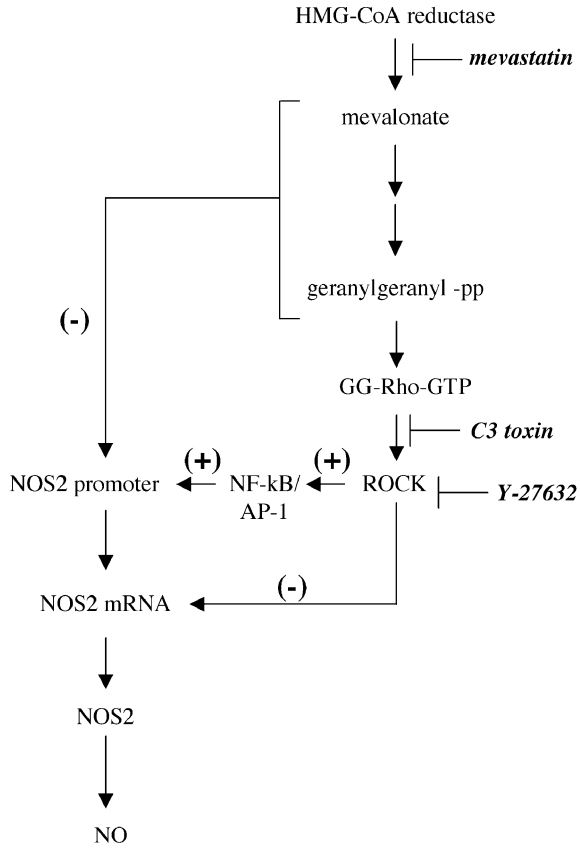

Fig. 7. Schematic of NOS2 and NF-κB regulation by Rho GTPase signaling pathways. The probable sites of activity of mevastatin, *C. botulinum* toxin C3 ribosyl transferase, and Y-27632 are shown. (+), Positive regulation of indicated signaling event; (−), negative regulation; HMG, 3-hydroxy-3-methylglutaryl; PP, pyrophosphate; ROCK, Rho-associated kinase.

We propose that Rho functions as a negative regulator of NOS2 production in response to inflammation and thus limits the synthesis of excessive NO that may lead to the overzealous production of reactive oxygen species and resultant tissue damage. This regulatory pathway balancing inflammation and NO production is relevant to pulmonary disorders such as diffuse panbronchiolitis, CF, and ciliary dyskinesia, which are thought to involve NO dysregulation in the presence of excessive inflammation (7, 13, 21, 22, 24). It has been previously reported that exhaled NO is depressed in patients with these disorders, in contrast to other inflammatory lung disorders such as asthma, bronchiectasis, and chronic bronchitis (3, 7, 14, 15, 21, 24), which may be partially due to reduced NOS2 expression in CF airway epithelial cells (13, 22). Because NO also acts as an anti-inflammatory, it is suggested that this lack of NO in CF airways contributes to the hyperinflammatory response to chronic bacterial infection characteristic of this disease (13, 22).

Our data indicate that prenylation events in conjunction with ROCK activity act to balance NO produc-

tion in the presence of inflammatory cytokines. An ongoing puzzle with regard to CF research is understanding how dysregulation between inflammatory responses and NO production develops. A previous report by Shen et al. (32) demonstrated inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) trafficking with lovastatin, another HMG-CoA reductase inhibitor, with a concurrent decrease in chloride transport. Prenylation-dependent events may represent a possible link between CFTR function and altered NOS2 expression characteristic of CF epithelium. The coordinated interactions between early and late signaling events within the Rho GTPase cascade in regulating NOS2 expression may be a indication of underlying defects related to pathology in CF and other similar airway inflammatory diseases. The inhibitors of Rho activation, such as the HMG-CoA reductase inhibitors and Y-27632, may represent novel therapeutic approaches to these inflammatory disorders. More research into the downstream effectors of Rho and ROCK and the interactions of these two complex inflammatory cascades is needed.

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REFERENCES

1. **Adamson P, Marshall CJ, Hall A, and Tilbrook PA.** Post-translational modifications of p21rho proteins. *J Biol Chem* 267: 20033–20038, 1992.
2. **Backlund PS.** Post-translational processing of RhoA. *J Biol Chem* 272: 33175–33180, 1997.
3. **Delen FM, Sippel JM, Osborne ML, Law S, Thukkani N, and Holden WE.** Increased exhaled nitric oxide in chronic bronchitis. *Chest* 117: 695–701, 2000.
4. **Essig M, Nguyen G, Prie D, Escoubet B, Sraer JD, and Friedlander G.** 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors increase fibrinolytic activity in rat aortic endothelial cells: role of geranylgeranylation and Rho proteins. *Circ Res* 83: 683–690, 1998.
5. **Finder JD, Litz JL, Blaskovich MA, McGuire TF, Qian Y, Hamilton AD, Davies P, and Sebti SM.** Inhibition of protein geranylgeranylation causes a superinduction of nitric-oxide synthase-2 by interleukin-1β in vascular smooth muscle cells. *J Biol Chem* 272: 13484–13488, 1997.
6. **Fiorentini C, Matarrese P, Straface E, Falzano L, Fabbri A, Donelli G, Cossarizza A, Bouquet P, and Malorni W.** Toxin-induced activation of Rho GTP-binding protein increases Bcl-2 expression and influences mitochondrial homeostasis. *Exp Cell Res* 242: 341–350, 1998.
7. **Grasemann H, Michler M, Wallot M, and Ratjen F.** Decreased concentration of exhaled nitric oxide in patients with cystic fibrosis. *Pediatr Pulmonol* 24: 173–177, 1997.
8. **Guo FH, DeRaeve HR, Rice TW, Stuehr DJ, Thunnissen FB, and Erzurum SC.** Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc Natl Acad Sci USA* 92: 7809–7813, 1995.
9. **Hausding M, Witteck A, Rodriguez-Pascual F, von Eichel-Streiber C, Forstermann U, and Kleinert H.** Inhibition of small G proteins of the Rho family by statins or *Clostridium difficile* toxin B enhances cytokine-mediated induction of NO synthase II. *Br J Pharmacol* 131: 553–561, 2001.
10. **Ikeda U, Shimpo M, Ikeda M, Minota S, and Shimada K.** Lipophilic statins augment inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes. *J Cardiovasc Pharmacol* 38: 69–77, 2001.
11. **Ishizaki T, Uehata M, Tamechika I, Keel J, Nomomura K, Maekawa M, and Narumiya S.** Pharmacological properties of Y-27632, a specific inhibitor of Rho-associated kinases. *Mol Pharmacol* 57: 976–983, 2000.
12. **Kazmierczak BI, Jou TS, Mostov K, and Engel JN.** Rho GTPase activity modulates *Pseudomonas aeruginosa* internalization by epithelial cells. *Cell Microbiol* 3: 85–98, 2001.
13. **Kelley TJ and Drumm ML.** Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. *J Clin Invest* 102: 1200–1207, 1998.
14. **Kharitonov SA, Wells AU, O'Connor BJ, Cole PJ, Hansell DM, Logan-Sinclair RB, and Barnes PJ.** Elevated levels of exhaled nitric oxide in bronchiectasis. *Am J Respir Crit Care Med* 151: 1889–1893, 1995.
15. **Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, and Barnes PJ.** Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 343: 133–135, 1994.
16. **Kjoller L and Hall A.** Signaling to Rho GTPases. *Exp Cell Res* 253: 166–179, 1999.
17. **Laufs U and Liao JK.** Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* 273: 24266–24271, 1998.
18. **Laufs U, Marra D, Node K, and Liao JK.** 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing Rho GTPase-induced down-regulation of p27(Kip1). *J Biol Chem* 274: 21926–21931, 1999.
19. **Liaudet L, Soriano FG, and Szabo C.** Biology of nitric oxide signaling. *Crit Care Med* 28: 37–52, 2000.
20. **Lorsbach RB, Murphy WJ, Lowenstein CJ, Snyder SH, and Russell SW.** Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. *J Biol Chem* 268: 1908–1913, 1993.
21. **Lundberg JO, Weitzberg E, Nordvall SL, Kuylenstierna R, Lundberg JM, and Alving K.** Primarily nasal origin of exhaled nitric oxide and absence in Kartegener's syndrome. *Eur Respir J* 7: 1501–1504, 1994.
22. **Meng QH, Springall DR, Bishop AE, Morgan K, Evans TJ, Habib S, Gruenert DC, Gyi KM, Hodson ME, Yacoub MH, and Polak JM.** Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *J Pathol* 184: 323–331, 1998.
23. **Muniyappa R, Xu R, Ram JL, and Sowers JR.** Inhibition of Rho protein stimulates iNOS expression in rat vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 278: H1762–H1768, 2000.
24. **Nakano H, Ide H, Imada M, Osanai S, Takahashi T, Kikuchi K, and Iwamoto J.** Reduced nasal nitric oxide in diffuse panbronchiolitis. *Am J Respir Crit Care Med* 162: 2218–2220, 2000.
25. **Nathan C and Xie QW.** Nitric oxide synthases: roles, tolls, and controls. *Cell* 78: 915–918, 1994.
26. **Negre-Aminou P, van Erck M, van Leeuwen RE, Collard JG, and Cohen LH.** Differential effect of simvastatin on various signal transduction intermediates in cultured human smooth muscle cells. *Biochem Pharmacol* 61: 991–998, 2001.
27. **Osada T, Watanabe S, Tanaka H, Hirose M, Miyazaki A, and Sato N.** Effect of mechanical strain on gastric cellular migration and proliferation during mucosal healing: role of Rho dependent and Rac dependent cytoskeletal reorganisation. *Gut* 45: 508–515, 1999.
28. **Pahan K, Faruk SG, Namboodiri AM, and Singh I.** Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J Clin Invest* 100: 2671–2679, 1997.
29. **Sander EE and Collard JG.** Rho-like GTPases: their role in epithelial cell-cell adhesion and invasion. *Eur J Cancer* 35: 1905–1911, 1999.
30. **Schmidt HW and Walter U.** NO at work. *Cell* 78: 919–925, 1994.

31. **Schmitz AA, Govek E, Bottner B, and van Aelst.** Rho GTPases: signaling L, migration, and invasion. *Exp Cell Res* 216: 1–12, 2000.
32. **Shen BQ, Widdicombe JH, and Mrsny RJ.** Effects of lovastatin on trafficking of cystic fibrosis transmembrane conductance regulator in human tracheal epithelium. *J Biol Chem* 270: 25102–25106, 1995.
33. **Singh R, Wang B, Shirvaikar A, Khan S, Kamat S, Schelling JR, Konieczkowski M, and Sedor JR.** The IL-1 receptor and Rho directly associate to drive cell activation in inflammation. *J Clin Invest* 103: 1561–1570, 1999.
34. **Szaszi K, Grinstein S, Orlowski J, and Kapus A.** Regulation of the epithelial Na(+)/H(+) exchanger isoform by the cytoskeleton. *Cell Physiol Biochem* 10: 265–272, 2000.
35. **Van Nieuw Amerongen GP, Vemeer MA, Negre-Aminou P, Lankelma J, Emeis JJ, and van Hinsbergh VW.** Simvastatin improves disturbed endothelial barrier function. *Circulation* 102: 2803–2809, 2000.
36. **Xie Q, Kashiwabara Y, and Nathan C.** Role of transcription factor NF-kappaB/Rel in induction of nitric oxide synthase. *J Biol Chem* 269: 4705–4708, 1994.