Inhibition of Rho protein stimulates iNOS expression in rat vascular smooth muscle cells

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Am J Physiol Heart Circ Physiol 278: H1762–H1768, 2000.—Inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (VSMCs) is upregulated in arterial injury and plays a role in regulating VSMC proliferation and restenosis. Inflammatory cytokines [e.g., interleukin-1β (IL-1β)] released during vascular injury induce iNOS. Small GTP-binding proteins of the Ras superfamily play a major role in IL-1β-dependent signaling pathways. In this study, we examined the role of Rho GTPases in regulating iNOS expression in VSMCs. Treatment of VSMCs with mevastatin, which inhibits isoprenylation of Rho and other small GTP-binding proteins, produced significantly higher amounts of IL-1β-evoked NO and iNOS protein compared with control. Similarly, bacterial toxins [Toxin B from Clostridium difficile and C3 ADP-ribosyl transferase (C3) toxin from Clostridium botulinum] that specifically inactivate Rho proteins increased NOS products (NO and citrulline) and iNOS expression. Toxin B increased the activity of iNOS promoter-reporter construct in VSMCs. Both toxins enhanced IL-1β-stimulated iNOS expression and NO production. These data demonstrate for the first time that inhibition of Rho induces iNOS and suggest a role for Rho protein in IL-1β-stimulated NO production in VSMCs.

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METHODS

VSMC preparation and culture. VSMCs were isolated from Sprague-Dawley rat thoracic aorta by enzymatic dissociation as described previously (43). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Complete medium) in an incubator at 37°C in 95% humidified air and 5% CO2. Serial passages (through passage 7) of VSMCs were obtained by treating confluent cultures with 0.2% trypsin-EDTA in Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (trypsin-HBSS, Sigma Chemical, St. Louis, MO). Cells were characterized as smooth muscle cells by a hill-and-valley pattern displayed at confluence and by positive immunostaining with a monoclonal antibody to smooth muscle cell (SMC)-specific α-actin (Sigma). For experiments in which nitrite (NO release) was to be measured, cells were treated in phenol red-free media to avoid interference with the assay.

Treatment of VSMCs with mevastatin and toxins. Mevastatin was activated as described before (20). VSMCs (50–60% confluent) were treated with mevastatin (50 µmol/l, a dose with maximal response) for 24 h before the addition of IL-1β. Confluent VSMCs were treated with different concentrations of Clostridium difficile toxin B (TECHLAB, Chantilly, VA) for the indicated period. In some experiments toxin B was neutralized by a polyclonal anti-toxin B antibody (TECHLAB). To examine the effects of Clostridium botulinum C3 toxin (List Biological Laboratories, Campbell, CA), VSMCs were loaded with C3 toxin (5 µg/ml) during mechanical agitation similar to scrape loading (25).

Nitrite assay. The enzymatic production of NO by VSMCs was assayed in culture medium by measuring nitrite, a stable reaction product of NO and oxygen (12). Briefly, 100 µl of medium was allowed to react with an equal volume of Griess reagent (1 part of 0.1% naphthylethenediamine dihydrochloride and 1 part of 1% sulfanilamide in 0.1 N HCl) at room temperature for 15 min before colorimetric quantitation at 550 nm (Dynatech instruments). Nitrite concentrations were calculated from a sodium nitrite standard curve.

Citrulline determination. Citrulline, a coproduct of the NO biosynthetic reaction, was determined using a colorimetric assay (4). Four hundred microliters of the culture medium were incubated with urease (45 U/ml) for 30 min at 37°C. The mixture was deproteinized by the addition of ice-cold trichloroacetic acid to a final concentration of 5%. After centrifugation, 400 µl of the supernatant were mixed with 3 ml of chromogenic solution, 1 part 0.5% diacetylmonoxime/0.01% thiourea and 2 parts acid-ferric solution (0.025% of FeCl₃ in a solution containing 25% sulfuric acid and 20% phosphoric acid), and boiled at 96°C for 5 min. After the mixture was cooled to room temperature, the absorbance was measured at 570 nm in a plate reader. L-Citrulline concentrations in the sample were calculated from a citrulline standard curve.

Transfection and reporter assay. VSMCs were seeded at a density of 3 × 10⁴ cells/dish (35 mm) in Complete medium and cultured overnight. The medium was replaced by Opti-MEM (GIBCO BRL) for another 16 h before transfection. Cells (60–70% confluent) were transfected with 2 µg of an iNOS promoter-luciferase reporter plasmid DNA (−1,485 to +31) (34) by using lipofectamine (Life Technologies, Grand Island, NY). To control for variations in both cell numbers and transfection efficiency, VSMCs were cotransfected with 1 µg of pCMVSPORTβgal (GIBCO BRL). Twenty hours after transfection, cells were treated with IL-1β (20 ng/ml) for 24 h or with toxin B (1 ng/ml) for 6 h. The luciferase and β-galactosidase activities of cellular extracts were determined using the luciferase and β-galactosidase assay kits (Promega, Madison, WI) according to manufacturer's instructions. β-Galactosidase activity in each sample served as a measure of normalized luciferase activity.

F-actin labeling of VSMC. VSMCs were grown on glass coverslips in complete medium for 24–48 h. Treated and control monolayers were washed in PBS, permeabilized with 0.03% saponin in PBS for 10 min, and then fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min at ambient temperature. Fixed VSMCs were stained with FITC-labeled phalloidin (Sigma) for 30 min. The slides were examined with a fluorescent microscope and photographed on Kodak 400 ASA film.

Immunodetection of iNOS, Rho A, and α-actin. Equal amounts of whole cell lysate protein (40–50 µg) were separated on a 7.5% or 15% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell) in Tris-glycine transfer buffer with 20% methanol in a Trans-Blot Cell (Bio-Rad). Membranes were blocked overnight at 4°C with 9% instant nonfat dry milk (Carnation) in Tris-buffered saline (TBS in (mmol/l): 20 Tris and 137 NaCl, pH 7.6, containing 0.3% Tween 20), washed in TBS, and incubated with the appropriate primary antibody; a monoclonal antibody against iNOS, 1:2,500 (Transduction Laboratories, Lexington, KY) (31); polyclonal antibodies against Rho A, 1:800 (Santa Cruz Biotech, Santa Cruz, CA); or SMC-specific α-actin, 1:1,000 (Sigma) for 2 h. The membranes were washed thoroughly and incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG antibody (1:5,000, for mouse and 1:2,500 for rabbit, Amersham, Arlington Heights, IL) for 1 h. After thorough washings, the bound antibodies were visualized by enhanced chemiluminescence using the ECL system (Amersham) and exposure to Kodak X-Omat film. Multiple exposures of each blot were performed to ensure that signals were within the linear range of the film.

Statistics. All data are expressed as means ± SE; n represents the number of experiments. Statistical analyses were performed by Student's t-test for paired or unpaired observations when appropriate, and more than two treatments were compared by one-way ANOVA followed by Student-Newman-Keuls method for multiple comparisons. A value of P < 0.05 was considered statistically significant.

RESULTS

Mevastatin enhances iNOS expression. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme catalyzing the rate-limiting step in the cholesterol biosynthetic pathway, prevents isoprenoid synthesis and prenylation of small GTP-binding proteins (11). We examined the effect of HMG-CoA reductase inhibitors (mevastatin and lovastatin) on IL-1β-induced iNOS expression. VSMCs pretreated with mevastatin (50 µmol/l) for 24 h were stimulated with IL-1β (5 ng/ml). Nitrite accumulation in culture media was measured at the end of 24 h. NO production was increased twofold in cells exposed to mevastatin (Fig. 1A). Because iNOS is regulated mainly at the level of expression (28, 32), we measured levels of iNOS in VSMC lysates by Western blotting. As shown in Fig. 1B, mevastatin increased levels of IL-1β-induced iNOS compared with control, whereas the levels of SMC-specific α-actin was unchanged. These data suggest that mevastatin exerts its enhancing effect at the level
of expression of NOS protein, which is then reflected by the increased NO production. Similar results were observed with lovastatin (data not shown). Mevastatin induced VSMC rounding within 24 h of exposure, an effect that was reversible by replacement with mevastatin-free media.

C. difficile toxin B inhibits Rho and induces iNOS. Bacterial toxins A and B from C. difficile have been used recently as selective inhibitors of Rho proteins (2, 3). These cell-permeable toxins are glucosyl transferases catalyzing the transfer of glucose from UDP-glucose to Rho proteins (17). Monoglucosylation of Rho proteins on threonine-37 renders them inactive (38). Inhibition of Rho proteins causes disaggregation of actin cytoskeleton followed by cell rounding (15). As shown in Fig. 2A, toxin B-treated cells exhibited retraction, rounding of cell bodies, and loss of F-actin, and this morphological change was completely prevented by neutralization of toxin with an excess of antitoxin.

Covalent modification of Rho dramatically decreases its stability (6). As an indirect confirmation of such a modification, we measured levels of RhoA by Western blot in toxin B-treated cells. Immunoreactive RhoA was significantly decreased in toxin-treated cellular lysates (Fig. 2B).

Nitrite levels in culture media of toxin-treated VSMCs were measured at the end of 12 h. Toxin B increased the release of NO in a concentration-dependent fashion (Fig. 3A). Neutralization of the toxin abolished toxin-stimulated NO production. We also measured citrulline, a coproduct of the NOS reaction, using a colorimet-
ric assay. Toxin B (1 ng/ml) increased citrulline levels in culture media (n = 3, Fig. 3B) at the end of 12 h. VSMCs, coincubated with toxin B (1 ng/ml) and IL-1β (5 ng/ml), released higher amounts of NO (n = 4) and citrulline (n = 3) compared with treatment with either agent alone (Fig. 3). Immunoblotting for iNOS confirmed that the increase in release of NO and citrulline by toxin B-exposed VSMCs was accompanied by an increase of iNOS protein (Fig. 4). Toxin-evoked expression of iNOS was concentration dependent and reflected similar increases in NO production. Also, IL-1β induced a greater amount of iNOS in Rho-inactivated VSMCs. As seen in Fig. 4, levels of SMC-specific α-actin were relatively unchanged in these treatment groups. These results show that inactivation of Rho GTPases (Rho, Rac, and Cdc42) enhances the induction of iNOS.

Inactivation of Rho stimulates transcription of iNOS. To test whether the effect of toxin B might be mediated by increased transcription of the iNOS gene, we transfected VSMCs with a plasmid of the iNOS promoter (−2,1 to +31) containing the iNOS 5′-flanking region upstream from a luciferase reporter gene. Luciferase activity in extracts from toxin B (1 ng/ml)- and IL-1β (20 ng/ml)-treated cells was significantly higher compared with that of untreated VSMCs (n = 4, Fig. 5).

Inhibition of NF-κB activation blocks iNOS induction. NF-κB is a multisubunit transcription factor that can rapidly activate gene expression. NF-κB activation plays a major role in iNOS expression induced by IL-1β (33). Pyrrolidone dithiocarbamate (PDTC), a thiol compound, blocks the activation of NF-κB without affecting DNA binding of other transcription factors (37). In the present study, PDTC (150 µmol/l) decreased iNOS activity in response to both toxin B and IL-1β (Fig. 6). PDTC had a similar inhibitory effect on iNOS promoter activity in transfected VSMCs exposed to toxin B and IL-1β (data not shown). PDTC had no effect on VSMC morphology and did not affect cytoskeletal changes induced by toxin B.

C3 exoenzyme (C3 toxin) inhibits Rho and induces iNOS. To corroborate our previous findings, we used C3 toxin, a different Rho-modifying clostridial toxin, C3 toxin produced from C. botulinum. This toxin ADP-ribosylates (asparagine-41) Rho (A, B and C) but not Rac or Cdc42, resulting in its inactivation (3). VSMCs incubated with C3 toxin (10–30 µg/ml) for 48 h failed to show any change in cell morphology, suggesting a failure to incorporate C3 toxin into VSMCs. We therefore loaded VSMCs with C3 toxin (5 µg/ml) by a method known to introduce macromolecules into cells (27). After an overnight incubation, toxin-loaded VSMCs showed morphological changes similar to toxin B-treated cells. Immunoreactive RhoA was similarly decreased in C3 toxin-treated cells (Fig. 7B), suggesting a modification of Rho.

C3 toxin markedly enhanced both nitrite and citrulline content (Fig. 7A) in the supernatant of the cells. Also, Western blot analysis of VSMC lysates from C3-loaded cells showed the presence of iNOS, whereas control cells showed no iNOS immunoreactivity (Fig. 7C). In addition, IL-1β-stimulated levels of citrulline and iNOS protein were significantly higher in cells exposed to C3 toxin. These results agree with similar findings from toxin B experiments.
that Rho proteins act as components in signal-transducing kinase cascades (24). Activated Rho proteins are able to stimulate the activity of c-Fos and NH2-terminal kinases/stress-activated protein kinase (JNK/SAPK) and p38 kinase (24). These kinase cascades mediate the activation of transcription factors and gene expression. The availability of bacterial toxins that specifically inactivate Rho proteins has aided in elucidating the role of Rho proteins in various cellular processes. Both toxin B and C3 toxin covalently modify and inactivate Rho proteins. Modification of Rho by either toxin decreases Rho protein levels in intact cells (6, 25). The loss of Rho protein occurs after an hour of toxin exposure and precedes any significant morphological change (25), whereas the inhibition of Rho-GTPase activity and its membrane translocation is a rapid process (in minutes) (14, 38). Thus in our study a combination of these mechanisms may contribute to the cytotoxic effect of the toxins. The use of two different toxins in our study suggests 1) glucosylation or ADP-ribosylation leads to Rho inactivation and iNOS induction; 2) Rho modification by either mechanism leads to loss of RhoA; and 3) specific inactivation of the Rho protein by C3 toxin causes inactivation of Rho (A, B, or C) in the regulation of iNOS expression.

DNA binding of many transcription factors to the promoter region of the iNOS gene stimulates its transcription (28, 32). Activation of nuclear factor-κB (NF-κB) plays a crucial role in the expression of iNOS in VSMCs (47). Toxin B increased promoter activity of the iNOS gene in transfected VSMCs, suggesting a direct action on the transcription machinery. The inhibitory effect of PDTC, an inhibitor of NF-κB, on the promoter activity of both IL-1β and toxin B-treated cells indicates a role for NF-κB in mediating effects of both agents; however, the residual activity in the presence of PDTC indicates that other transcription factors may also play a role. These results agree with recent reports demonstrating regulation of NF-κB by Rho proteins (29) and highlights the role of Rho proteins in nuclear signaling and gene expression. This is in contrast to recent reports demonstrating negative regulation of endothelial NOS (eNOS) expression by decreasing eNOS mRNA stability by Rho proteins (23). Unlike the constitutively expressed eNOS, quiescent VSMCs do not express iNOS, and regulation of iNOS principally occurs at the level of transcription (28, 32). Because inhibition of Rho induces iNOS expression by activating the iNOS promoter activity, the predominant effect appears to occur at the level of transcription. Similarly, Rho protein has been shown to negatively regulate transforming growth factor (TGF)-β1 receptor expression, and inhibition of Rho by C3 toxin or HMG-CoA reductase inhibitors have reversed the effect by stimulating TGFβ1-promoter activity in cultured cardiac myocytes (33).

IL-1β stimulates various kinase cascades, which include the mitogen-activated protein kinase (p42/44 MAPK), SAPK, and p38 kinase (13). Rho proteins are known to mediate IL-1-induced activation of p38 kinase and SAPK, whereas p42/44 MAPK is activated by the...
Ras-Raf signal pathway. In fact, recruitment of Rho to
the activated IL-1-receptor complex is crucial for the
activation of kinases (40, 41). Interestingly, inhibition of
p38 kinase increases, whereas inhibition of the
Ras-Raf-MAPK pathway decreases iNOS expression
(13). It is conceivable that IL-1β activates two different
signaling pathways simultaneously that regulate iNOS
expression in opposite directions. This dynamic regula-
tion of iNOS gene concurs with the recurring cellular
theme of multiplex signaling by parallel MAPK cascades
to modulate expression of various genes. Hyper-
trophic gene expression in cardiac myocytes has been
shown to be modulated in opposite directions by simul-
taneous and parallel activation of MAPK and Rho-
dependent signaling cascade (45). In contrast, these
cascades can have positive and similar effects on gene
expression (46). Thus it appears that well-coordinated
signaling networks may act in concert or in opposition
to mediate various aspects of cellular function. Possible
downstream mediators of Rho could also include phos-
phatidylinositol (PI) 3-kinase, an enzyme reported to be
directly activated by Rho-GTPase (42). In fact IL-1 activates
PI3-kinase, and like Rho, physically associates
to the activated IL-1 receptor complex (40, 41). Interes-
tingly, inhibition of PI3-kinase by specific inhibi-
tors have shown to upregulate iNOS expression in
macrophages (5). It therefore may be conjectured that
the Rho-p38 kinase pathway and/or the Rho-PI3-
kinase pathway may mediate the inhibitory effect on
iNOS expression. Our study strongly supports the
notion that Rho-mediated pathways have a repressing
effect on iNOS expression in VSMCs, and relief of this
tonic repression by geranylgeranylation inhibitors or
Rho-inactivating toxins elicits an enhanced expression of
iNOS.

Cytoskeleton is known to regulate gene expression,
and because both Rho-inactivating toxins and meva-
statin affect the cytoskeleton in a negative manner, it
could be argued that depolymerization of the cytoskel-
eton affects iNOS expression. However, according to a
recent report (26), disruption of VSMC microfilaments
with either colchicine or nocodazole not only failed to
augment IL-1β-respose but actually inhibited it. This
is consistent with the emerging evidence of diverging
pathways controlling cytoskeletal changes and MAPK
cascades and is thought to be secondary to distinct
target proteins interacting with the activated Rho
protein (14). These findings indicate that inhibition of
Rho rather than cytoskeletal breakdown mediates iNOS
induction.

To conclude, we demonstrate for the first time that
inactivation of Rho induces iNOS in VSMC. Inhibition
of Rho in an injured vessel (postangioplasty/atherecto-
my) may help prevent restenosis by enhancing iNOS
expression. This might partly explain the beneficial
effect of HMG-CoA reductase inhibitors in reducing
neointimal proliferation and restenosis (36). Thus po-
tent inhibitors of Rho could be used as therapeutic
agents to increase iNOS expression locally for the
prevention of restenosis after vascular injury.

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