

Nitric Oxide–Induced Motility in Aortic Smooth Muscle Cells

Role of Protein Tyrosine Phosphatase SHP-2 and GTP-Binding Protein Rho

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Abstract—We have previously reported that SHP-2 upregulation is necessary for NO-stimulated motility in differentiated rat aortic smooth muscle cells. We now test the hypothesis that upregulation of SHP-2 is necessary and sufficient to stimulate cell motility. Overexpression of SHP-2 via recombinant adenoviral vector stimulated motility to the same extent as NO, whereas the expression of C463S-SHP-2, the dominant-negative SHP-2 allele, blocked the motogenic effect of NO. On the basis of previous studies, we next tested the hypothesis that NO decreases RhoA activity and that this event is necessary and sufficient to explain NO-induced motogenesis. We found that NO decreased RhoA activity in a concentration-dependent manner. Moreover, a dominant-negative SHP-2 allele, DSH2, blocked the NO-induced inhibition of RhoA activity, indicating that upregulation of SHP-2 is necessary for this event. Expression of G14V-RhoA, the constitutively active RhoA allele, decreased cell motility and blocked the motogenic effect of NO, whereas the expression of T19N-RhoA, the dominant-negative RhoA allele, increased cell motility to an extent similar to that induced by NO. Dominant-negative RhoA reversed the effect of dominant-negative SHP-2, indicating that RhoA functions downstream from SHP-2. To investigate events downstream from RhoA, we treated cells with fasudil, a selective Rho kinase inhibitor, and found that it increased cell motility. These results indicate that upregulation of SHP-2, leading to downregulation of RhoA, which is followed by decreased Rho kinase activity, is a sequence of events necessary and sufficient to explain NO-induced cell motility in differentiated aortic smooth muscle cells. The results may be of relevance to in vivo events such as neointimal formation, angiogenesis, and vasculogenesis. (*Circ Res.* 2002; 91:390-397.)

Key Words: nitric oxide ■ SHP-2 ■ Rho ■ cell motility

Vascular smooth muscle cell motility is an important determinant of intimal enlargement, occurring in atherosclerosis, restenosis, or vascular transplantation.¹ Vasculogenesis/angiogenesis, a process associated with coronary vascular remodeling, also requires smooth muscle cell movement.²

The role of NO as a modulator of vascular smooth muscle cell motility in vitro is controversial. Several studies, including our own, have shown that NO has the capacity to decrease the motility of vascular smooth muscle cells in culture.³⁻⁵ These studies have used repetitively subcultured vascular smooth muscle cells or partially dedifferentiated cells isolated from newborn rats. In contrast, we have recently reported that in differentiated primary cultured cells originally obtained from adult rat aorta, NO induces cell motility.^{6,7} Moreover, we found that NO increased the expression of SHP-2, a nonreceptor protein tyrosine phosphatase that functions, for the most part, as a positive mediator of signal transduction.⁷ Attenuation of SHP-2 expression via the use of an antisense

oligonucleotide decreased the motility-stimulatory effect of NO. Moreover, NO-induced SHP-2 upregulation, and motogenesis could be demonstrated only in primary or early subcultures but not in dedifferentiated, repetitively subcultured cells. Taken together, these data indicate a potential causal linkage between NO-induced SHP-2 upregulation and increased cell motility.

SHP-2 is the mammalian homologue of the gene product of *Drosophila corkscrew* (*Csw*), which also encodes an N-terminal src homology-2 (SH₂)-containing protein tyrosine phosphatase. SHP-2 plays a critical role in embryonic development, as shown by the nonviability of homozygous SHP-2 mutant mouse embryos.⁸ SHP-2 is expressed ubiquitously, and predominantly, in vascular smooth muscle cells, consistent with the hypothesis that it may play an important role in vascular smooth muscle biology.⁹ Recent studies indicate that SHP-2 mediates the motogenic effects of fibronectin,¹⁰ platelet-derived growth factor,^{11,12} or insulin-like growth factor-1.¹³ SHP-2 also

Original received April 17, 2002; resubmission received June 21, 2002; revised resubmission received July 23, 2002; accepted July 31, 2002.

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DOI: 10.1161/01.RES.0000033524.92083.64

plays a role in the mitogenic effect of angiotensin II in vascular smooth muscle cells.¹⁴

The small GTP-binding protein RhoA can function downstream from SHP-2, and an inverse correlation between SHP-2 and RhoA activity in cultured cells has been demonstrated.^{15,16} Furthermore, NO has been reported to decrease RhoA activity via the stimulation of protein kinase G (PKG).¹⁷ Recent studies have found that NO can decrease RhoA in intact rat aortas¹⁸ and that a decrease in Rho is associated with increased cell motility in cultured fibroblasts.¹⁹

The purpose of the present study was to test the hypothesis that SHP-2 is both necessary and sufficient to explain the capacity of NO to stimulate cell motility in differentiated primary aortic smooth muscle cell cultures. The aforementioned inverse linkage between SHP-2 and RhoA further prompted us to test the hypothesis that the NO-elicited reduction of RhoA activity is both necessary and sufficient to explain NO-induced cell motility.

Materials and Methods

Materials

DMEM/F-12 and FBS were from GIBCO-BRL. Penicillin, streptomycin, *S*-nitroso-*N*-acetylpenicillamine (SNAP), and 1-(5-isoquinolinesulfonyl)homopiperazine (fasudil) were from Sigma-Aldrich Chemical Co. Insulin, transferrin, and selenium were from Collaborative Research. 2,2-(Hydroxynitrosohydrazino) bisethanamine (DETANO) was from Alexis Biochemicals. Antibodies directed against SHP-2 or phosphotyrosine were from Transduction Labs, and antibody directed against the hemagglutinin antigen (HA) epitope was from Santa Cruz Biotechnology.

Vascular Smooth Muscle Cell Isolation and Culture via Enzymatic Dissociation

Male Sprague-Dawley rats (100 to 150 g) were obtained from Charles River Laboratories (Bedford, Mass). Aortic smooth muscle cells were isolated and cultured as previously described.⁶ All experiments used primary cell culture, and each individual experiment was derived from a new isolate of primary cells. This study was performed via a protocol approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, in accordance with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, publication No. [NIH] 86-23).

Measurement of Cell Motility

Motility was measured by using two slightly different monolayer-wounding protocols. Details regarding the first method can be found in an earlier publication.⁶ During the performance of these studies, we developed a second improved cell motility protocol involving wounding the monolayers by making a ≈ 1 -mm gap with a 20- μ L pipette tip. In this protocol, cell motility, assessed using NIH Image software, was determined as the distance migrated in 24 hours.

Measurement of RhoA Activity

RhoA activity was measured via a kit purchased from Upstate Biotechnology. Briefly, active RhoA was captured via a pull-down assay using the RhoA binding domain of rhotekin, followed by Western blotting of RhoA. Results were expressed as the ratio of rhotekin-bound RhoA to total RhoA.

Preparation and Expression of Adenoviral Vectors

cDNA of human sequence wild-type (WT) and inactive SHP-2, containing a mutation of the catalytically essential cysteine 463 residue to serine (C463S-SHP-2), were generously donated by

Benjamin Neel (Harvard University, Cambridge, Mass). Recombinant replication-incompetent adenoviral vectors were generated with the use of a kit from Clontech. cDNA expressing the tandem domains of SHP-2, expressing the first 220 amino acids (DSH2), was prepared in our laboratory via polymerase chain reaction, using a template cDNA kindly donated by John Easton (St Jude Children's Research Hospital, Memphis, Tenn). cDNA for G14V-RhoA or T19N-RhoA was purchased from the Guthrie Institute. cDNA for RhoA alleles was flanked at the N-terminus by three copies of the HA epitope. Recombinant adenoviral vectors expressing RhoA were prepared according to a published procedure²⁰ with a kit generously provided by Beverly Davidson (University of Iowa, Iowa City). Adenoviral vectors were purified with use of a kit from Virapur and titrated via a conventional assay involving plaque formation in HEK293 cells.

Results

Overexpression of WT-SHP-2 Mimics, Whereas Expression of C463S-SHP-2 Blocks, Motogenic Effect of NO

We had previously found that NO increased the expression and activity of SHP-2 in primary aortic smooth muscle cells and that antisense oligodeoxynucleotide directed against SHP-2 blocked the motogenic effect of NO.⁷ To test the hypothesis that SHP-2 is both necessary and sufficient to transduce this effect, we treated cells with recombinant adenoviral vectors expressing either enhanced green fluorescent protein (EGFP), as control for viral infection per se, or with adenoviral vectors expressing WT-SHP-2 or C463S-SHP-2 in the absence or presence of the NO donor SNAP.

As shown in Figure 1B, SNAP stimulated cell motility, confirming our previous results.^{6,7} Treatment of the cells with adenoviral vectors expressing WT or mutant SHP-2 induced transgenic protein expression that was ≈ 3 -fold above background, as determined by Western blotting (Figure 1A). Treatment with control virus had no significant effect on smooth muscle cell motility (not shown). However, overexpression of WT-SHP-2 induced cell motility equivalent to that induced by treatment with NO donor. Expression of the catalytically inactive SHP-2 allele, C463S-SHP-2, had no significant effect on cell motility by itself, but it essentially blocked the capacity of the NO donor to induce cell motility (Figure 1B). Thus, WT-SHP-2 mimics, whereas C463S-SHP-2 blocks, the motility-stimulatory effect of NO by functioning as a dominant-negative allele. These results support the hypothesis that upregulation of SHP-2 is both necessary and sufficient to explain NO-stimulated cell motility. They also indicate that the catalytic activity of SHP-2 is necessary for transduction of the motility-stimulatory effect of NO.

DSH2 Blocks NO-Induced Cell Motility

In the next experiments, we tested the effect of expressing a dominant-negative polypeptide, which we termed DSH2 (double SH₂), encompassing the tandem SH₂ domains of SHP-2 but lacking the rest of the SHP-2 sequence. These experiments were performed to verify the aforementioned results by using the full-length dominant-negative C463S-SHP-2 and to investigate the importance of the tandem SH₂ domains in the interaction of SHP-2 with substrates involved in the transduction of its motility-stimulatory effect. As

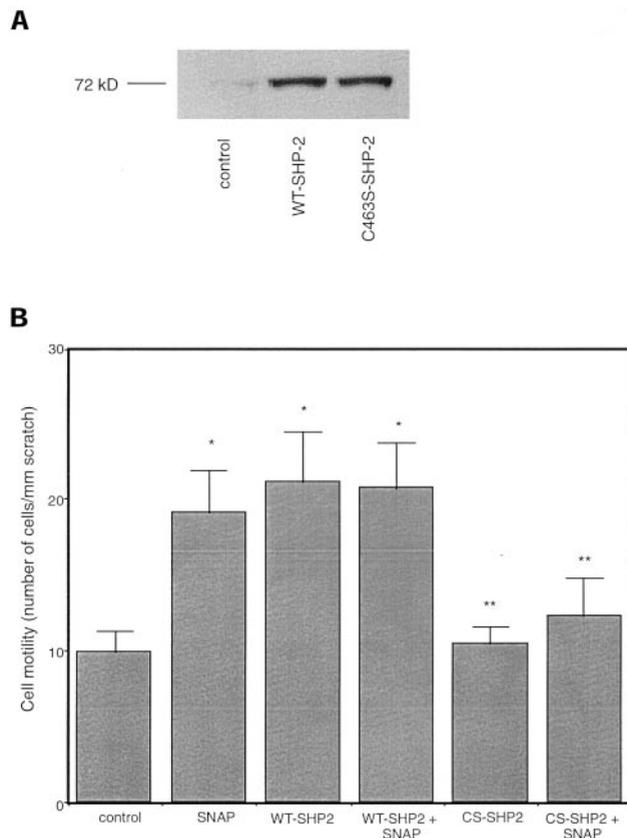


Figure 1. Effect of WT-SHP-2 or catalytically inactive SHP-2 on cell motility. Primary cultures of aortic smooth muscle cells were infected for 24 hours with recombinant adenovirus expressing EGFP (as control), WT-SHP-2, or C463S-SHP-2 (CS-SHP2) at a multiplicity of infection of ≈ 15 , followed by removal of virus-containing media and further incubation for 24 hours to allow time for protein expression. Cell migration was then measured in a wounded-culture model in cells treated for 24 hours without or with 50 $\mu\text{mol/L}$ NO donor SNAP, as described in detail earlier.⁶ A, Expression levels of transgenic protein, as determined by Western blotting. B, Result of transgenic protein expression on cell motility. Motility results are expressed as mean \pm SEM of 4 or 5 independent experiments. * $P < 0.05$ vs control and ** $P > 0.05$ vs control via ANOVA, followed by Fisher's post hoc test.

shown in Figure 2A, treatment of cells with adenovirus expressing DSH2 induced significant expression of this polypeptide; moreover, expression of DSH2 did not prevent the capacity of NO to increase the levels of endogenous SHP-2. Expression of DSH2 alone had no effect on motility, but the polypeptide induced complete inhibition of the motility-stimulatory effect of the NO donor DETANO, indicating that it also functions as a dominant-negative agent (Figure 2B).

NO Donor Increases SHP-2 Levels but Fails to Alter SHP-2 Phosphotyrosine Levels

Several agonists, including serum, platelet-derived growth factor, and thrombin, increase the phosphotyrosine levels of SHP-2 in cultured cells,²¹ and phosphorylation of key tyrosine residues has been reported to activate SHP-2.²² Moreover, SHP-2 has been reported to modulate its own phosphorylation levels.²³ These findings prompted us to investigate whether NO alters SHP-2 phosphotyrosine levels in cultured

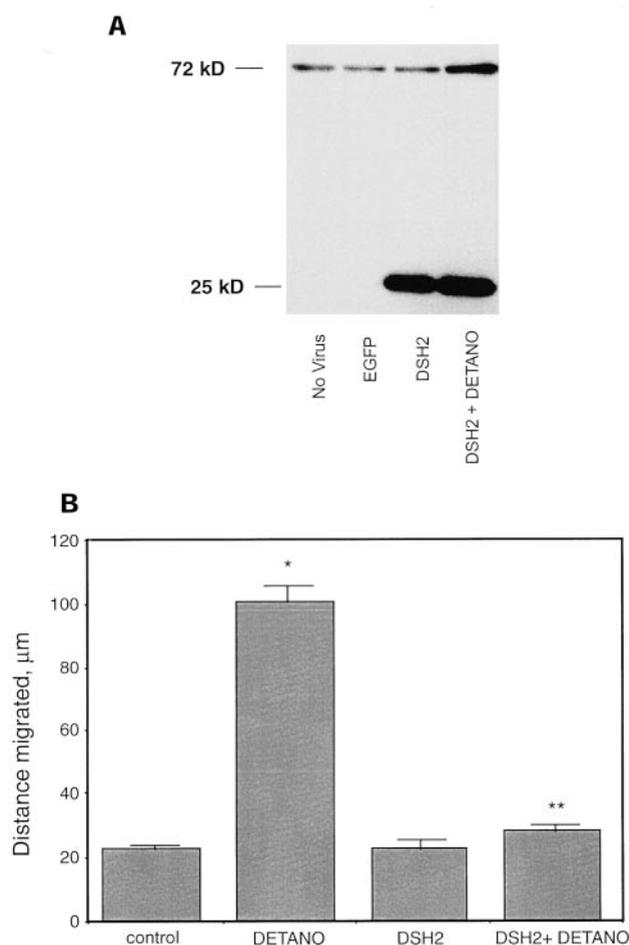


Figure 2. Effect of DSH2 on cell motility. Primary cultures of aortic smooth muscle cells were infected for 24 hours with recombinant adenovirus expressing EGFP (control) or DSH2 at a multiplicity of infection of 10, followed by removal of virus-containing media and further incubation for 24 hours to allow time for protein expression. Cell motility was then measured in a wounded-culture model in cells treated for 24 hours without or with 30 $\mu\text{mol/L}$ NO donor DETANO, as described in Materials and Methods. A, Expression levels of DSH2, as measured by Western blotting. It should be noted that antibody directed against SHP-2 specifically recognizes the N-terminal SH₂ domains and is therefore capable of recognizing DSH2. B, Effect of transgenic protein expression on cell motility. Motility results are expressed as mean \pm SEM of 3 independent experiments. * $P < 0.01$ relative to control; ** $P < 0.01$ relative to DETANO.

rat aortic smooth muscle cells. As shown in Figure 3, DETANO increased SHP-2 levels in a concentration-dependent manner, confirming our earlier results.⁷ Although the NO donor increased absolute phosphotyrosine levels, the levels normalized to protein (ie, phosphotyrosine per mole protein) remained constant. These results indicate that unlike several growth factors, NO upregulates SHP-2 by increasing SHP-2 protein levels rather than by inducing tyrosine phosphorylation.

NO Donor Decreases RhoA Activity

SHP-2 has the capacity to reduce RhoA activity in fibroblasts, in association with increased cell motility.¹⁵ Therefore, we were prompted to determine the effect of the NO donor on

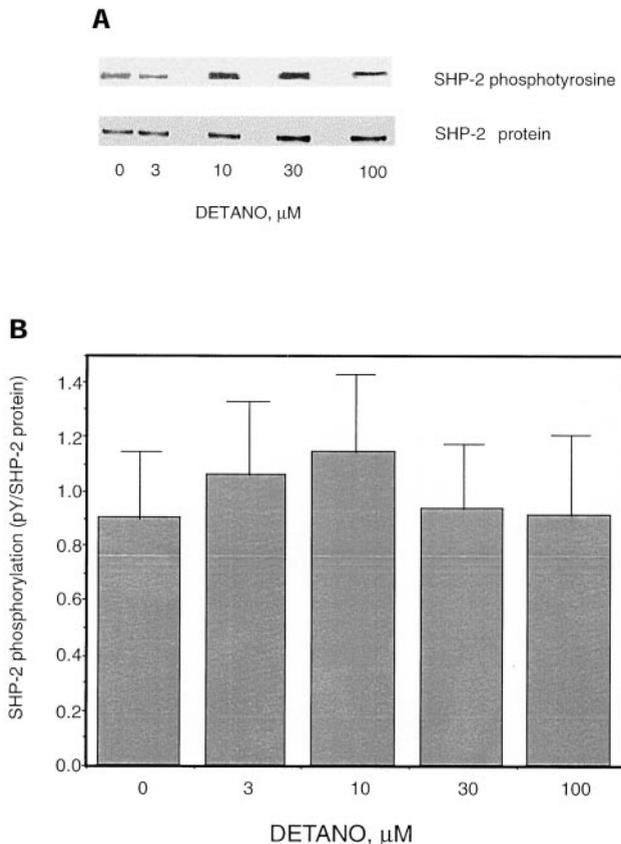


Figure 3. Effect of NO donor on SHP-2 phosphotyrosine levels. Cells were treated for 4 hours with DETANO (based on optimal time for upregulation of SHP-2, according to our previous study).⁷ Immunoprecipitated SHP-2 was measured via Western blotting by using an antibody directed against SHP-2. Blots were then stripped and reprobbed with antibody directed against phosphotyrosine. A, Results from a representative experiment. B, Summary of all experiments. Results are given as mean \pm SEM of the ratio of phosphotyrosine (pY), normalized to SHP-2 protein (for 3 independent experiments). None of the values from treated cells was significantly different from control.

RhoA activity in aortic smooth muscle cells. The results provided in Figure 4 indicate that DETANO decreased RhoA activity in a concentration-dependent manner, supporting the possibility that this effect could be causally related to NO-induced cell motility.

DSH2 Blocks Capacity of NO Donor to Decrease RhoA Activity

Previous studies have reported that inhibition of SHP-2 activity is associated with increased RhoA activity.¹⁵ To determine whether upregulation of SHP-2 is necessary to account for the NO-induced inhibition of RhoA activity, we treated cells with adenovirus expressing DSH2. As shown in Figure 5, DSH2 did not have a significant effect of its own. However, the dominant-negative polypeptide essentially blocked the DETANO-induced decrease of RhoA activity, consistent with the hypothesis that upregulation of SHP-2 is necessary for NO-induced downregulation of RhoA.

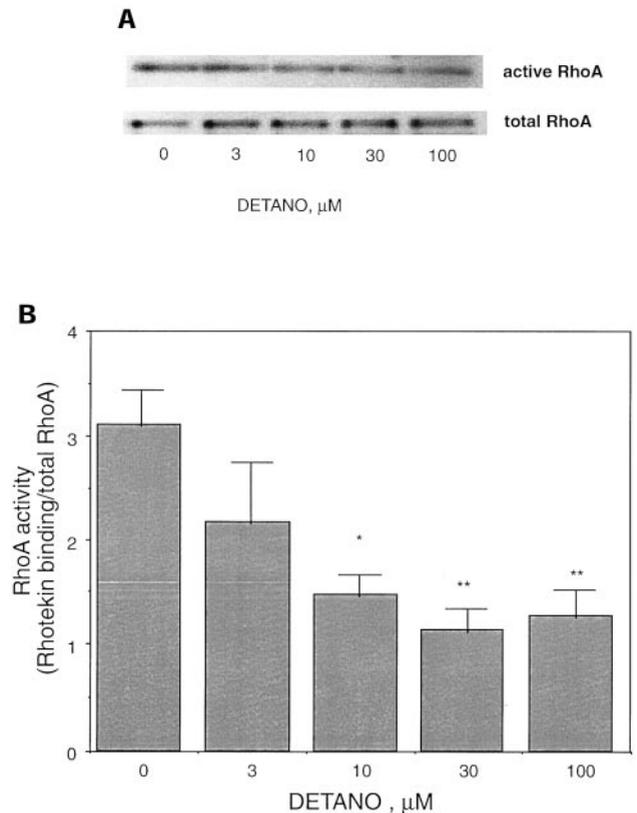


Figure 4. Effect of NO donor on RhoA activity. Cells were treated with DETANO for 4 hours, followed by measurement of active RhoA via a pull-down experiment, as described in Materials and Methods. A, Results of a representative experiment. B, Summary of all experiments. Values were normalized to total RhoA and expressed as mean \pm SEM of 5 independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs control.

DN-RhoA Mimics, Whereas Constitutively Active RhoA Blocks, NO-Induced Cell Motility

On the basis of the aforementioned results, we tested the hypothesis that a reduction of RhoA is both necessary and sufficient to explain NO-induced cell motility. Treatment of the cells with adenovirus expressing a dominant-negative RhoA allele, T19N-Rho (DN-RhoA), increased cell motility to the same extent as DETANO, as shown in Figure 7. Conversely, expression of a constitutively active RhoA allele, G14V-Rho (CA-RhoA), decreased cell motility and blocked the motogenic capacity of NO (Figure 6). These results support the hypothesis that an inhibition of RhoA is both necessary and sufficient to transduce the capacity of NO in stimulation of cell motility.

DN-RhoA Rescues the Inhibitory Effect of DSH2

If RhoA were to function downstream from SHP2, we would expect that DN-RhoA would rescue the inhibitory effect of DSH2 on NO-induced cell motility. To test this hypothesis, we treated cells without or with adenovirus expressing DN-RhoA and cells without or with adenovirus expressing DSH2. As shown in Figure 7, we found that DN-RhoA abrogated the inhibitory effect of DSH2, both in the absence or presence of NO donor. This finding supports the hypothesis that RhoA functions downstream from SHP2.

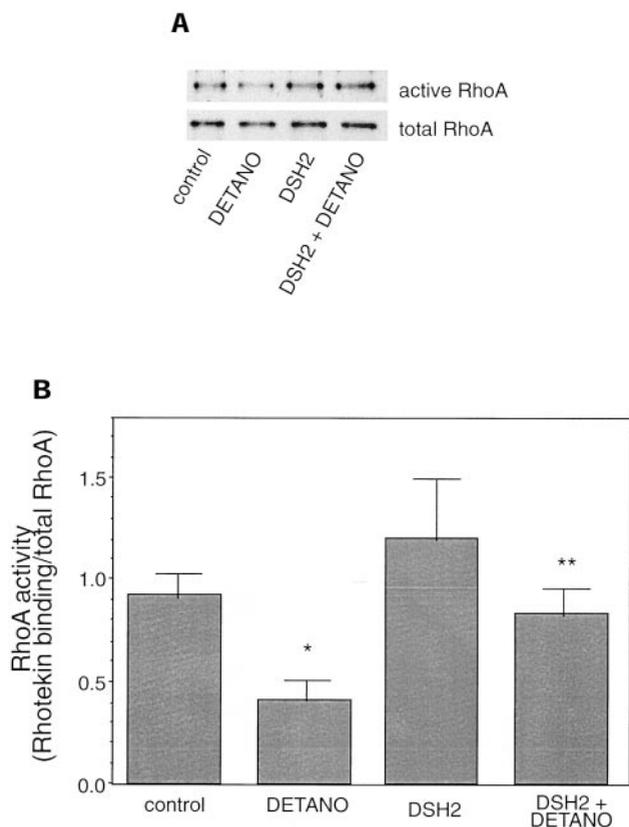


Figure 5. Effect of DSH2 on DETANO-induced decrease of RhoA activity. Cells were treated with recombinant adenovirus expressing EGFP (control) or DSH2 for 24 hours at a multiplicity of infection of ≈ 10 , followed by removal of virus-containing media and further incubation for 24 hours to allow for expression of transgenic protein. Infected cells were treated with DETANO for 4 hours, followed by measurement of active RhoA via a pull-down experiment, as described in Materials and Methods. A, Results of a representative experiment. B, Summary of all experiments. Values were normalized to total RhoA and expressed as mean \pm SEM of 4 independent experiments. * $P < 0.01$ relative to control; ** $P < 0.01$ relative to DETANO.

Rho Kinase Inhibitor Fasudil Increases Cell Motility

Rho kinase is a well-established Rho effector. To test the hypothesis that Rho kinase may be involved in NO-stimulated cell motility, we treated aortic smooth muscle cells with a selective Rho kinase inhibitor, fasudil.²⁴ As shown in Figure 8, fasudil increased cell motility to an extent similar to that of DETANO; moreover, the combined effect of fasudil and DETANO was not significantly different from that of either agent alone. These results are consistent with the view that decreased RhoA and Rho kinase activities are sufficient to account for the motility stimulatory effect of NO.

Discussion

We report the novel findings that the NO-induced upregulation of SHP-2, leading to decreased Rho activity, is necessary and sufficient to explain the motogenic effect of NO in differentiated primary cultures of aortic smooth muscle cells. The present results are consistent with our previous study showing that reduction of SHP-2 levels via the use of antisense oligonucleotides attenuates the motogenic effect of NO.⁷ Moreover, the finding that catalytically inactive SHP-2

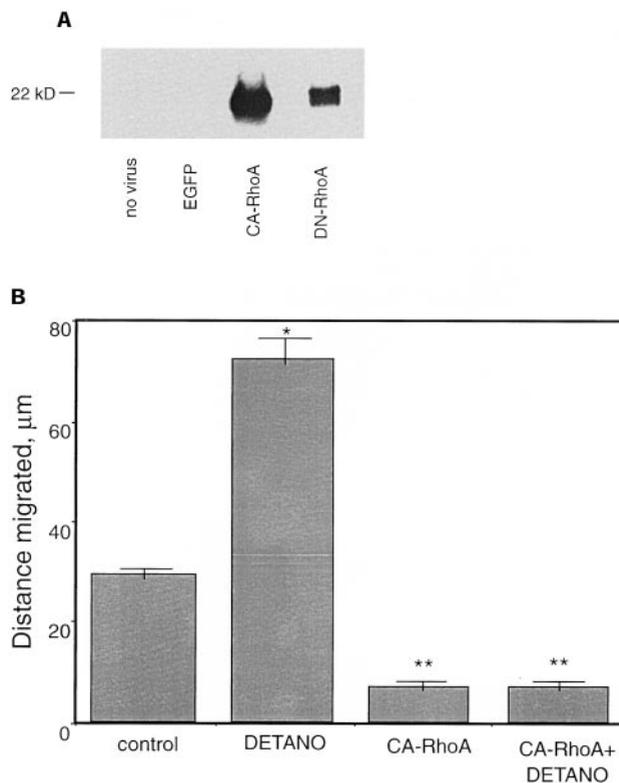


Figure 6. Effect of constitutively active RhoA on cell motility. Cells were treated with recombinant adenovirus expressing EGFP (control) or constitutively active RhoA (CA-RhoA, G14V-RhoA) for 24 hours at a multiplicity of infection of 10, followed by removal of virus-containing media and further incubation for 24 hours to allow time for protein expression. Cells were then treated for 24 hours without or with 30 $\mu\text{mol/L}$ DETANO, and cell motility was measured as described. A, RhoA expression as determined by Western blotting, using antibody directed against HA. B, Results of 4 to 7 independent cell motility experiments, expressed as mean \pm SEM. * $P < 0.01$ relative to control; ** $P < 0.01$ relative to treatment with DETANO.

fails to mediate the motility effect of NO but acts instead as a dominant-negative allele indicates that the activity of SHP-2 is necessary for inducing cell motility. This is in contrast to at least one previous publication, which reported that the catalytic activity of SHP-2 is dispensable for JAK tyrosine kinase-mediated signal transduction.²⁵

NO induces opposite effects on cell motility and proliferation in cultured cell models expressing different phenotypes. Thus, in relatively dedifferentiated aortic smooth muscle cells, including subcultured cells or cells isolated from newborn rats,²⁶ NO decreases cell motility as well as proliferation.^{3–5,27} We have recently reported that the antimotogenic effect of NO in cells from newborn rats requires upregulation of a protein tyrosine phosphatase, PTP1B, generally considered to be a negative regulator of signal transduction.^{4,5}

In contrast, in more differentiated aortic smooth muscle cells isolated from adult rat aorta, NO potentiates the effect of growth factors on cell proliferation and induces cell motility, as shown by the current and previous studies from our laboratory.^{6,7,28} It is also noteworthy that our present findings are consistent with the results of another group documenting

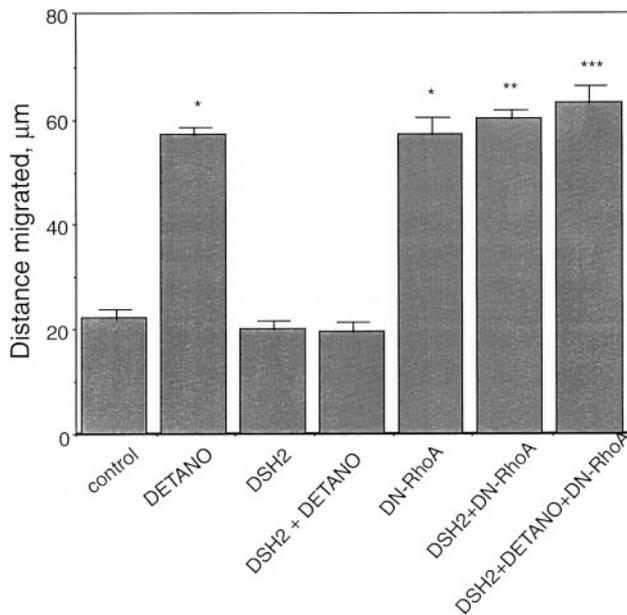


Figure 7. Effect of DN-RhoA on basal or DSH2-induced inhibition of cell motility. Cells were treated with adenovirus expressing EGFP (control), DSH2, or DN-RhoA for 24 hours at a multiplicity of infection of ≈ 10 , followed by removal of media and further incubation for 24 hours to allow time for protein expression. Cells were then treated for 24 hours without or with $30 \mu\text{mol/L}$ DETANO, and cell motility was measured as described. Results are mean \pm SEM of 3 experiments. * $P < 0.01$ relative to control; ** $P < 0.01$ relative to treatment with DSH2; and *** $P < 0.01$ relative to DSH2+DETANO.

that NO stimulates the mitogen-activated protein kinase pathway in differentiated aortic smooth muscle cells.²⁹ Moreover, a dual effect of the same agent on growth and movement of cells is not unique to the NO system, inasmuch as transforming growth factor- β and tumor necrosis factor ligands also induce inhibition or stimulation of proliferation in smooth muscle and other cell types.^{30,31}

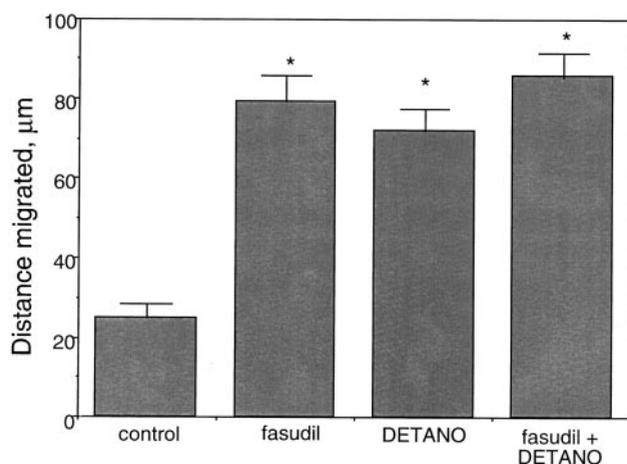


Figure 8. Effect of fasudil, a Rho kinase inhibitor, on cell motility. Cells were treated without or with $30 \mu\text{mol/L}$ fasudil or $30 \mu\text{mol/L}$ DETANO for 24 hours. Cell motility was measured in a wounded-culture model as described earlier. Results are expressed as mean \pm SEM of 6 independent experiments. * $P < 0.01$ vs control.

SHP-2 contains two SH₂ domains consisting of conserved sequences of ≈ 100 amino acids each and a C-terminal hydrophilic domain containing several tyrosine phosphorylation sites.³² The enzyme binds directly via its N-terminal SH₂ domains to phosphotyrosine residues of several activated tyrosine kinase-linked receptors, such as the platelet-derived growth factor and epidermal growth factor receptors.³³ SHP-2 then becomes tyrosine-phosphorylated, which creates binding sites for SH₂ domain-containing molecules such as the adapter protein Grb2.³⁴ These effects are associated with activation of the Ras/mitogen-activated protein kinase cascade, leading to increased cell proliferation or motility. Moreover, a recent study has indicated that phosphorylation of two specific tyrosine residues induces upregulation of SHP-2 activity.²² Thus, a potential mechanism of activation of SHP-2 by NO would be increased tyrosine phosphorylation. However, we found that NO-stimulated upregulation of SHP-2 failed to alter SHP-2 phosphotyrosine levels. Thus, increased SHP-2 protein levels induced by NO, rather than tyrosine phosphorylation, can explain the finding of increased SHP-2 activity, as reported previously.⁷ However, we cannot rule out the possibility that tyrosine phosphorylation in one domain of SHP-2 is masked by dephosphorylation occurring in a different region.

At least two potential mechanisms could account for the capacity of NO to reduce RhoA activity via upregulation of SHP-2. First, a recent study has found that SHP-2 decreases RhoA activity, possibly via dephosphorylation and inactivation of a Rho-guanine-nucleotide exchange factor.¹⁵ Second, cGMP causes translocation of Rho to the cytosol, indirectly indicative of Rho inactivation, together with phosphorylation of a serine residue in RhoA.³⁵ The relevance and relative importance of these mechanisms for NO-induced cell motility remain to be determined. However, we have previously shown that NO-induced SHP-2 upregulation and cell motility are mediated via a cGMP-dependent mechanism and that NO fails to upregulate SHP-2 and motility in subcultured aortic smooth muscle cells that are deficient in PKG.⁷ These observations and the present findings together support the view that NO induces cell motility via a mechanism requiring cGMP and SHP-2.

In contrast to the present findings, at least two studies have reported that increased Rho and Rho kinase activities are associated with increased smooth muscle cell motility.^{36,37} However, both of these studies were performed in subcultured vascular smooth muscle cells, which express a fundamentally different cytoskeletal phenotype than the differentiated cells used in the present study. Whether and how the difference in cytoskeletal phenotype explains the divergent effects of RhoA on cell motility remains to be determined.

The mechanism by which decreased RhoA activity contributes to increased cell motility is unclear, although recent studies have provided evidence that the capacity of cells to extend protrusions and polarize in the direction of migration is associated with reduced RhoA activity.^{19,38} Another potential mechanism that could lead to increased cell motility is altered cell adhesion. We and others have reported that NO decreases cell adhesion in several cell types, including smooth muscle cells,^{4,39,40} a finding that could explain in-

creased cell motility. Moreover, inhibition of SHP-2, which increases RhoA activity, has been shown to enhance cell adhesion in fibroblasts¹⁵; therefore, we speculate that reduction of RhoA activity may, reciprocally, decrease cell adhesion, resulting in increased motility.

Because of the capacity of a selective Rho kinase inhibitor, fasudil, to mimic the motility stimulatory effect of NO, we infer that the reduction of Rho kinase activity is a downstream effect mediating NO-stimulated cell motility. Proteins downstream from Rho kinase include LIM (Lin-11, Isl-1, Mec-3) kinase, phosphatidylinositol-4-phosphate 5-kinase, and myosin light chain phosphatase.^{41,42} Precisely how these potential pathways might regulate NO-induced cell motility is not known, but it has been reported that increased LIM kinase, phosphatidylinositol-4-phosphate 5-kinase, and/or myosin phosphorylation may contribute to the assembly of focal adhesions, leading to increased adhesion.⁴³ Reciprocally, it would be expected that decreased Rho kinase activity would contribute to the disassembly of focal adhesions, leading to decreased adhesion and increased motility.

An independent mechanism involved in the regulation of the cytoskeleton involves the binding of NO-stimulated PKG to myosin light chain phosphatase, inducing activation of the phosphatase.⁴⁴ This would then be expected to be associated with cytoskeletal disassembly and decreased cell adhesion. Although we cannot rule out a requirement for a direct association between PKG and myosin phosphatase, the present results show that a direct interaction is insufficient to elicit NO-stimulated cell motility, the basis of the requirement of SHP-2 as well as RhoA downregulation in this effect.

That vascular injury-induced neointimal formation is dependent on vascular smooth muscle proliferation and motility and is associated with the upregulation of inducible NO synthase (iNOS) is well established.⁴⁵ However, the effect of NO on vascular injury-induced neointimal formation is controversial. A preponderance of evidence indicates that NO attenuates neointimal enlargement after vascular injury. Paradoxically, iNOS knockout in mice attenuates injury-induced neointimal formation, indicating that some aspect related to iNOS activity, perhaps NO generation, may be necessary for the development of a neointima.⁴⁶ Moreover, neointimal enlargement is a complex process that is also associated with infiltration of injured tissues by inflammatory cells, the activity of which is independently suppressed by NO.⁴⁷ In the face of this complexity and in the absence of additional *in vivo* experiments, it is difficult to speculate on the direct pathophysiological relevance of our results to injury-induced neointimal formation.

A second pathophysiological process that may involve vascular smooth muscle motility under the influence of NO is vasculogenesis/angiogenesis. This process is of importance in coronary collateral generation and requires vascular smooth muscle motility as part of a process of remodeling, generally triggered by ischemia or an inflammatory response.^{2,48} An early event that occurs in remodeling is decreased smooth muscle cell adhesion.² Moreover, flow-induced remodeling has been reported to be NO dependent,⁴⁹ raising the possibility that NO is also an important mediator of vasculogenesis/angiogenesis. Indeed, at least two recent studies have re-

ported a requirement of NO in angiogenesis,^{50,51} supporting the possibility that the present results may be of relevance to new blood vessel formation.

In summary, we have shown that NO stimulates cell motility in differentiated aortic smooth muscle cells in culture via a mechanism requiring SHP-2 upregulation followed by RhoA inhibition. Moreover, either SHP-2 upregulation or RhoA inhibition is sufficient to mediate the mitogenic effect of NO. These findings may be of relevance to neointimal formation and vasculogenesis/angiogenesis.

Acknowledgments

This study was supported by US Public Health Service grant HL-63886; the Vascular Biology Center, University of Tennessee, Memphis; and a postdoctoral fellowship to Nair Sreejayan from the American Heart Association, Southeastern Affiliate.

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