UltraRapid Communication

Treatment With Insulin Uncovers the Motogenic Capacity of Nitric Oxide in Aortic Smooth Muscle Cells

Dependence on Gab1 and Gab1-SHP2 Association

Madhulika Dixit, Daming Zhuang, Bogdan Ceacareanu, Aviv Hassid

Abstract—Contrary to the antimotogenic effect of NO in dedifferentiated vascular smooth muscle cells (VSMCs), we have reported that NO stimulates the motility of differentiated cultured VSMC isolated from adult rats. This process involves upregulation of protein tyrosine phosphatase SHP2, followed by downregulation of RhoA activity. In the present study, we tested the hypothesis that insulin alters the motogenic phenotype of cultured rat aortic smooth muscle cells exposed to NO from inhibition to stimulation of cell motility. We demonstrate for the first time that NO stimulates the motility of VSMCs cultured for several days in the presence but not the absence of insulin. Moreover, we show that NO blocks PDGF-induced cell motility in insulin-naive but not in insulin-treated cells. We also demonstrate that the scaffold adapter protein Gab1, considered a physiological activator of protein tyrosine phosphatase SHP2, increases cell motility in the presence but not the absence of insulin. In cells cultured in the presence of insulin, overexpression of Gab1 mimics, whereas a dominant-negative allele of Gab1 (Gab1YF) blocks, the motility-stimulatory effect of NO. Cotransfection experiments with dominant-negative Gab1 and wild-type SHP2 or wild-type Gab1 and dominant-negative SHP2 indicate that the two proteins work together as a functional unit to induce motility. Because chronic insulin can increase the levels of phosphatidylinositol 3 (PI3) kinase in several models of hyperinsulinemia, we also tested the potential involvement of this enzyme in mechanisms leading to increased cell motility. We found that the motogenic effect of NO, Gab1, and SHP2 was blocked by the selective PI3 kinase inhibitor LY294002, suggesting a requirement of PI3 kinase in mediating motogenesis. These observations may be relevant to molecular mechanisms related to the pathogenesis of vascular disease in hyperinsulinemic diabetes. The full text of this article is available online at http://www.circresaha.org. **(*Circ Res*. 2003;93:e113-e123.)**

Key Words: nitric oxide ■ insulin ■ Gab1 ■ SHP2 ■ cell motility

Type II diabetes is associated with increased vascular morbidity and mortality.[1] The major factor that induces enhanced neointima formation in diabetic pathology seems to be the presence of hyperinsulinemia rather than hyperglycemia.[2] Additionally, the development of vascular diseases such as atherosclerosis and restenosis involves migration of vascular smooth muscle cells (VSMCs) to the intima.[3,4] Angiogenesis, a process associated with vascular remodeling, also requires migration of smooth muscle cells to regions of emerging capillaries and arterioles.[5] However, the mechanisms underlying these effects are not well understood.

NO is an established modulator of VSMC motility.[6,7] Consistent with this observation, vascular injury–induced neointima formation is associated with upregulation of inducible NO synthase (iNOS).[8,9] However, the precise role of NO in smooth muscle cell motility is controversial. In repetitively subcultured cells or partially dedifferentiated cells isolated from newborn rats, NO decreases cell motility, whereas in differentiated primary aortic smooth muscle cells from adult rats, NO stimulates cell motility.[6,7,10–12] We have reported that NO stimulates the motility of primary cultures of VSMCs from adult rats via a cyclic GMP protein kinase G (PKG)-dependent pathway and that this effect requires upregulation of the protein tyrosine phosphatase SHP2, subsequently leading to decreased activity of the small GTPase Rho.[13] Moreover, the motogenic effect of NO is absent from subcultured cells, presumably because of the deficiency of PKG in such cells.[7]

Activation of SHP2 in response to growth factors is followed by its interaction with adapter protein Gab1,[14–16] an effect that is functionally related to downstream activation of the Ras/mitogen-activated protein kinase (MAPK) pathway thought to be involved in regulation of cell motility and proliferation.[17–19] Additionally, the NO cyclic GMP pathway has been shown to stimulate the MAPK pathway in differentiated VSMCs.[20] Taken together, these observations indicate

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that an interaction between Gab1 and SHP2 in differentiated primary aortic smooth muscle cells isolated from adult rat may be necessary to mediate the motogenic effect of NO.

SHP2 is an atypical protein tyrosine phosphatase that is mostly, but not exclusively, associated with a positive role in signal transduction pathways.[21,22] SHP2 contains two N-terminal SH2 domains, arranged in tandem, followed by a C-terminal catalytic domain.[22,23] Basally, the autoinhibitory influence of its SH2 domains renders the enzyme catalytically inefficient. However, binding of phosphotyrosines to these SH2 domains, such as phosphorylated tyrosine 627 and tyrosine 659 of Gab1, induces a conformational change that activates the enzyme.[18,23]

Gab1, the mammalian homologue of *Drosophila Daughter of Sevenless* (DOS) is involved in multiple signaling pathways mediated by receptor tyrosine kinases.[24] DOS itself was cloned as a potential substrate of Corkscrew (*Csw*), the Drosophila homologue of mammalian SHP2.[25] Gab1 is expressed ubiquitously during early development, and null mutants in mice are embryonically lethal.[26] These mice also display defective migration of precursor muscle cells. The Gab1 protein is broadly divided into a conserved N-terminal pleckstrin homology (PH) domain, a central proline-rich domain, and multiple tyrosine residues.[16] Phosphorylation of these tyrosine residues creates potential docking sites for SH2 domain containing proteins such as SHP2, p85 subunit of phosphatidylinositol 3 (PI3) kinase, PLCγ, or Crk.[16]

In the present study, we tested the hypothesis that insulin alters the motogenic phenotype of cultured rat aortic smooth muscle cells exposed to NO from inhibition to stimulation of motility. Using PKG-replete primary cultures of rat aortic smooth muscle cells as a model, we report the novel finding that NO stimulates motility only when cells are cultured for relatively long periods in the presence of insulin. Moreover, we show that in cells exposed to insulin, NO loses the capacity to decrease platelet-derived growth factor (PDGF)-induced motility. We additionally demonstrate that the functional interaction of Gab1 with protein tyrosine phosphatase SHP2 is necessary to explain the motogenic effect of NO. Finally, we show that the motogenic effects of adapter protein Gab1 and protein tyrosine phosphatase SHP2 also require exposure of cells to insulin and that these effects seem to depend on PI3 kinase activity.

Materials and Methods

Materials

DMEM/F12 and FBS were from CellGro and GIBCO-BRL, respectively. Penicillin, streptomycin, and protease inhibitor cocktail were from Sigma-Aldrich. Insulin was from Collaborative Research. Recombinant human PDGF-BB was from R&D Systems, 2,2-(hydroxynitrosohydrazino) *bis*-ethanamine (DETANO) was from Alexis Biochemicals, anti-Gab1 antibody was from Upstate Biotechnology, and anti-SHP2 antibody was from Transduction Laboratories.

Vascular Smooth Muscle Cell Isolation and Culture

Male Sprague-Dawley rats (weighing 100 to 150 g) were obtained from Charles River Laboratories (Wilmington, Mass). Aortic smooth muscle cells were isolated and cultured as described previously.[7] Cells were grown in DMEM/F12 medium supplemented with varying concentrations of insulin plus 10% FBS. The medium was changed every 48 hours until cells reached confluency. Each experiment used cells that were maintained in serum-free medium for the 24-hour period before induction of cell motility to elicit motogenic quiescence. All experiments used primary cell culture, and each isolate consisted of cells pooled from two to four individual rat aortas. 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, NIH publication No. 86-23).

Measurement of Cell Motility

Motility of primary cells alone or primary cells infected with adenoviral constructs in the presence or absence of NO was determined via a monolayer wounding assay, as described in a previous publication from our laboratory.[7] To prevent the effect of cell proliferation on cell migration, 5 mmol/L hydroxyurea was routinely added to culture media. We previously demonstrated that hydroxyurea has no significant effect on cell migration but blocks proliferation.[7] Motility was quantitated using NIH image software and expressed as distance migrated by cells in 24 hours.

Immunoprecipitation of Gab1

After experimental incubations, cells were lysed in RIPA buffer (150 mmol/L NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 50 mmol/L Tris, pH 7.2) containing 10 mmol/L sodium orthovanadate, 1 mmol/L phenylmethane sulfonyl fluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin. The lysates (1 mg of protein equivalent) were cleared for 30 minutes at 4°C with 5 μg normal rabbit IgG followed by immunoprecipitation with 10 μg Gab1 antibody overnight at 4°C and addition of protein G-Sepharose beads for 1.5 hours. After washing three times with RIPA buffer, samples were suspended in Laemmli buffer and boiled for 10 minutes and supernatants were loaded on Bis-Tris gels. Transfer of proteins to polyvinylidene difluoride membranes (Immobilon PVDF, Millipore) was followed by blocking in 4% BSA (in Tris-buffered saline supplemented with 0.5% Triton X-100) and probing with anti-SHP2 antibody (1:5000). Blots were then stripped and reprobed for Gab1. Immunoreactive bands were visualized via NEN Western Lightning chemiluminescence reagents (Perkin Elmer).

Preparation and Expression of Adenoviral Vectors

Plasmid pcDNA3HAGab1, containing the wild-type human Gab1 cDNA sequence, was a generous gift from Dr Keigo Nishida (Osaka, Japan). Wild-type Gab1 was excised from pcDNA3HAGab1 followed by subcloning into *Hind*III site of adenovirus pShuttle vector (pAd5CMV). Recombinant adenovirus was obtained by cotransfecting linearized recombinant pShuttle and adenoviral backbone DNA (pAd59.2-100, Gene Transfer Vector Core, University of Iowa) in HEK 293 cells.[27] Site-directed mutagenesis was performed to generate adenovirus expressing Gab1 containing an Y627F mutation (Gab1YF) via the use of polymerase chain reaction cocktail mix from Epicenter Technologies and adenovirus shuttle vector (pShuttle) carrying wild-type Gab1 sequence as template following a published protocol.[28] The following primers were used for site-directed mutagenesis: 5′-GGAGACAAACAGGTGGAATTCTTAGATCTCGACTTAGATTC-3′ (forward primer) and 5′-G$\underline{A}$ATCTAAGTCGAGATCTAAG$\underline{A}$ATTCCACCTGTTTGTCTCC-3′ (reverse primer) (the mutated nucleotide is underlined). The desired mutation was confirmed by sequencing before generation of recombinant adenovirus in HEK 293 cells. Recombinant adenoviral vectors were purified using virakit Adeno4 from Virapur and titrated via tissue culture infection dose assay in HEK293 cells. Confluent primary cells were infected with adenovirus at a multiplicity of infection of 10^{11} pfu/mL. After 24 hours of incubation, fresh medium was added and cells were incubated for an additional 24 hours before the start of experiments. Complementary DNAs of wild-type and inactive human SHP2, the latter of which contains a mutation of the catalytically essential cysteine at residue 463 to serine, were a generous gift from Dr Benjamin Neel (Harvard University, Cam-

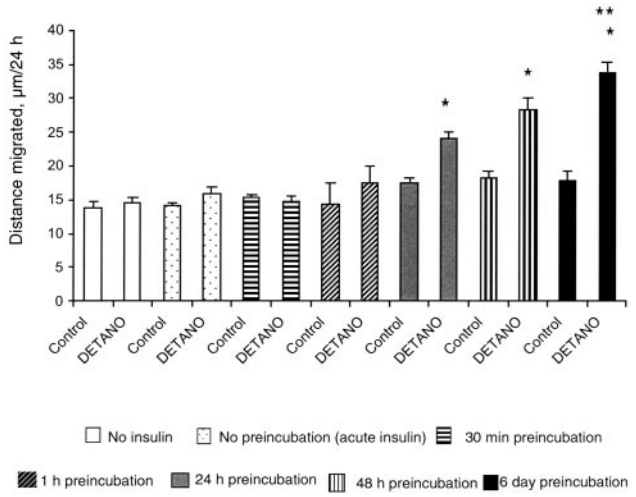

Figure 1. NO donor DETANO stimulates motility only in cells chronically treated with insulin. Aortic smooth muscle cells were preincubated in the presence or absence of 100 nmol/L insulin for periods ranging from 30 minutes to 6 days. Quiescent cells were then incubated for 24 hours in the presence or absence of 30 μmol/L DETANO and in the continued presence or absence of insulin, and cell motility was measured as described in Materials and Methods. Motility results are expressed as mean±SEM of 3 to 5 independent experiments. *$P<0.05$ vs respective controls; **$P<0.05$ vs DETANO (no insulin).

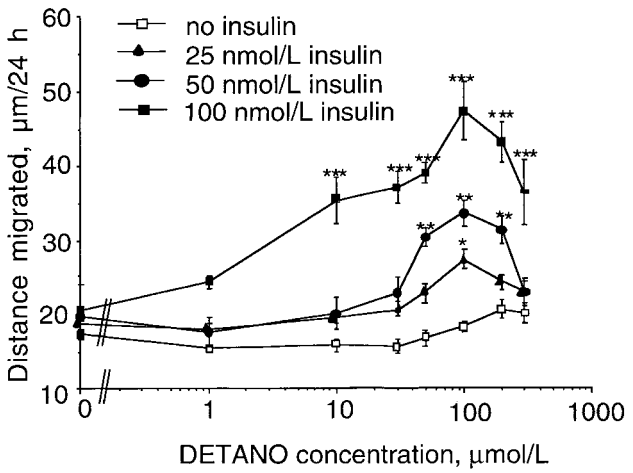

Figure 2. Concentration dependence of DETANO-induced motogenesis. Aortic smooth muscle cells were preincubated for 6 days in the presence of various concentrations of insulin (0 to 100 nmol/L). Quiescent cells were then treated with various concentrations of DETANO in the continued presence or absence of insulin, and cell motility was measured as described in Materials and Methods. Results are expressed as mean±SEM for 3 independent experiments. *$P<0.05$ vs 0 μmol/L DETANO plus 25 nmol/L insulin; **$P<0.05$ vs 0 μmol/L DETANO plus 50 nmol/L insulin; ***$P<0.05$ vs 0 μmol/L DETANO plus 100 nmol/L insulin.

bridge, Mass). Recombinant adenovirus expressing SHP2 was generated via a kit from Clontech. Complementary DNA expressing the tandem SH2 domains of SHP2, ie, the first 220 amino acids, termed DSH2, was prepared using template cDNA provided by John Easton (St Jude Children's Research Hospital, Memphis, Tenn). Adenovirus expressing DSH2 was prepared as described previously.[13]

Data Analysis

Data are expressed as mean±SEM and are statistically evaluated using two-way ANOVA, followed by Fisher's test. $P<0.05$ is considered statistically significant.

Results

NO-Induced Motility Requires Prolonged Preincubation With Insulin

Contrary to the role of NO as an antimotogenic agent, NO stimulates motility in primary cultures isolated from adult rats.[7,13] For technical reasons, our previous studies were performed in cells cultured in the presence of a commonly used culture supplement, namely insulin (5 μg/mL; 800 nmol/L), transferrin (5 μg/mL), and selenious acid (5 ng/mL). We were therefore prompted to test the hypothesis that the presence of insulin may have played a role in NO-induced motility. Thus, we cultured cells in the presence or absence of 100 nmol/L insulin, a concentration commonly used to simulate the effects of high insulin levels in culture.[29,30] As shown in Figure 1, NO donor DETANO stimulated motility in cells preincubated with insulin for 24 hours to 6 days but not in insulin-naive cells or cells preincubated for 30 minutes to 1 hour. Thus, the appearance of the comotogenic effect of insulin is time dependent. It is important to note that the comotogenic effect of insulin is independent of serum, inasmuch as 24-hour preincubation with insulin in the absence of serum is sufficient to uncover this effect (Figure 1).

Results shown in Figure 2 provide the concentration dependence of DETANO-induced motogenesis in the presence or absence of three different insulin concentrations. Thus, in the absence of insulin, NO donor was unable to induce cell motility at any concentration. Increasing insulin concentrations increased the potency and effectiveness of NO donor. At 100 nmol/L insulin, as little as 10 μmol/L DETANO induced significant motogenesis, whereas at lower insulin concentrations, only higher DETANO concentrations were effective. It is also worth noting that concentrations of NO donor >100 μmol/L tended to be less effective. This presumably reflects a cytotoxic effect of NO donor, which decreases protein synthesis at concentrations >100 μmol/L.[31]

NO Attenuates PDGF-Induced Cell Motility in the Absence but not the Presence of Insulin Pretreatment

Several studies have reported that NO decreases agonist-induced VSMC motility.[32–34] This prompted us to test the hypothesis that insulin determines the motogenic phenotype induced by NO in the presence of an established motogen such as PDGF, ie, that NO would reduce PDGF-induced cell motility but only in the absence of insulin treatment. Thus, we cultured primary aortic smooth muscle cells in the presence or absence of 100 nmol/L insulin and determined the capacity of NO donor to alter PDGF-induced motility. Figure 3 shows that DETANO significantly attenuated motility induced by PDGF in insulin-naive cells. In contrast, in cells that were exposed to 100 nmol/L insulin for a total of 7 days, the NO donor failed to decrease PDGF-induced motility. Thus, in the presence of prolonged insulin treatment, NO loses the capacity to decrease growth factor–induced cell motility.

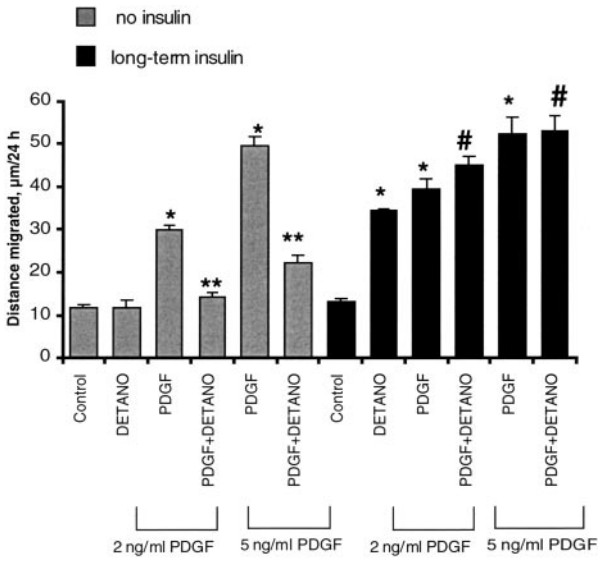

Figure 3. NO donor DETANO attenuates PDGF-induced motility in the absence but not presence of long-term insulin treatment. Aortic smooth muscle cells were preincubated for 6 days in the absence or presence of 100 nmol/L insulin. Quiescent cells were then incubated for 24 hours in the absence or presence of 30 μmol/L DETANO, PDGF (2 or 5 ng/mL), or PDGF plus DETANO in the continued absence or presence of insulin. Results are expressed as mean±SEM of 3 independent experiments. *$P<0.001$ vs respective controls; **$P<0.001$ vs respective PDGF alone (no insulin); #$P>0.05$ vs respective PDGF alone (long-term insulin treatment).

Overexpression of Gab1 Mimics the Motogenic Effect of NO

Because upregulation of SHP2 is both sufficient and necessary to account for the motogenic effect of NO, as shown by a previous study,[13] and because Gab1 can activate and recruit SHP2 to the plasma membrane,[16] we sought to determine whether overexpression of Gab1 could induce smooth muscle cell motility. A positive result would demonstrate that Gab1 overexpression is sufficient to induce increased motility. Thus, we treated cells with recombinant adenoviral vectors expressing either enhanced green fluorescent protein (EGFP) as control virus or with vector expressing wild-type Gab1 for 48 hours, followed by measurement of motility in the presence or absence of NO. As shown in Figure 4B, DETANO induced cell motility of primary aortic smooth muscle cells, confirming previous results.[7] Recombinant wild-type Gab1 adenovirus increased transgenic protein expression in aortic smooth muscle cells by ≈2-fold, as determined by Western blot analysis (Figure 4A). Control virus, expressing EGFP, did not interfere with the basal motility of primary cells or with the capacity of NO donor to stimulate motility. However, overexpression of wild-type Gab1 induced cell motility to the same extent as the NO donor DETANO (Figure 4B). Thus, Gab1 mimics the motility stimulatory effect of NO donor, presumably by directly interacting with and activating SHP2. It is also worth noting that treatment with DETANO plus Gab1 failed to increase motility beyond that induced by either agent alone, consistent with convergent mechanisms stimulated by these agents.

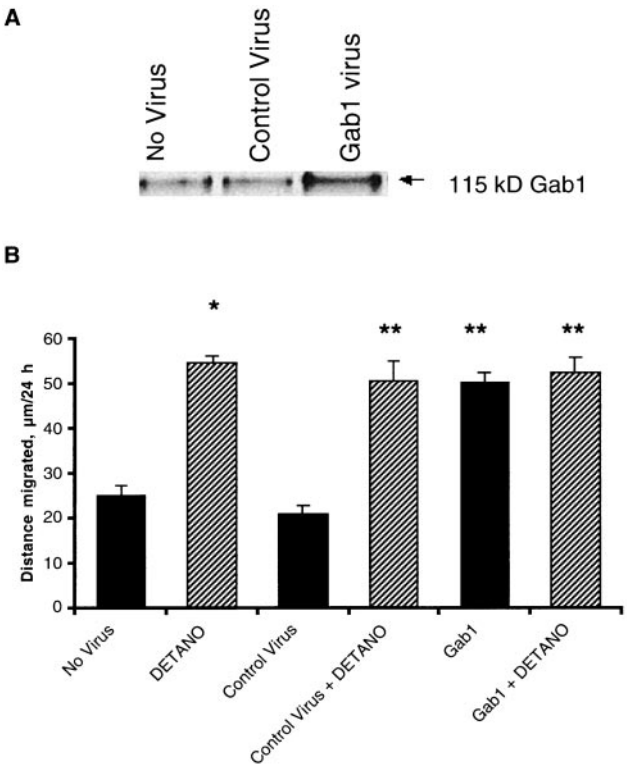

Figure 4. Gab1 mimics motogenic effect of NO donor. Aortic smooth muscle cells were infected with recombinant adenovirus expressing EGFP (as control virus) or wild-type Gab1 at a multiplicity of infection of ≈11, followed by measurement of cell motility as described in Materials and Methods. A, Levels of Gab1 as determined by Western blot analysis. B, Result of Gab1 overexpression on cell motility. Motility results are expressed as mean±SEM of 5 independent experiments. *$P<0.05$ vs control (no virus); **$P<0.05$ vs control virus.

Because SHP2 is upregulated in response to NO and Gab1 can independently mimic the effect of NO, there is a possibility that similar to NO, Gab1 overexpression may also upregulate SHP2 levels; this event could then provide a potential explanation for the motogenic effect of Gab1. However, we failed to detect a significant increase in SHP2 levels in cells overexpressing wild-type Gab1 (data not shown), indicating that the motogenic effect of Gab1 is unlikely to occur via an increase in SHP2 levels. It should also be noted that, unlike its effect on SHP2, NO donor failed to cause an increase in the protein levels of Gab1 (data not shown).

Prolonged Treatment With Insulin Is Necessary for SHP2-Induced Cell Motility

Previous studies from our laboratory showed that SHP2 upregulation is sufficient and necessary to explain NO-induced cell motility in insulin-treated cells.[7,13] To determine whether treatment with insulin is necessary to enable the motility-stimulatory effect of overexpressed SHP2, we infected aortic smooth muscle cells, antecedently treated with or without insulin, with adenovirus expressing SHP2 or control adenovirus expressing EGFP. As shown in Figure 5B, SHP2 overexpression induced motility only in cells treated with insulin. The lack of responsiveness to SHP2 in the

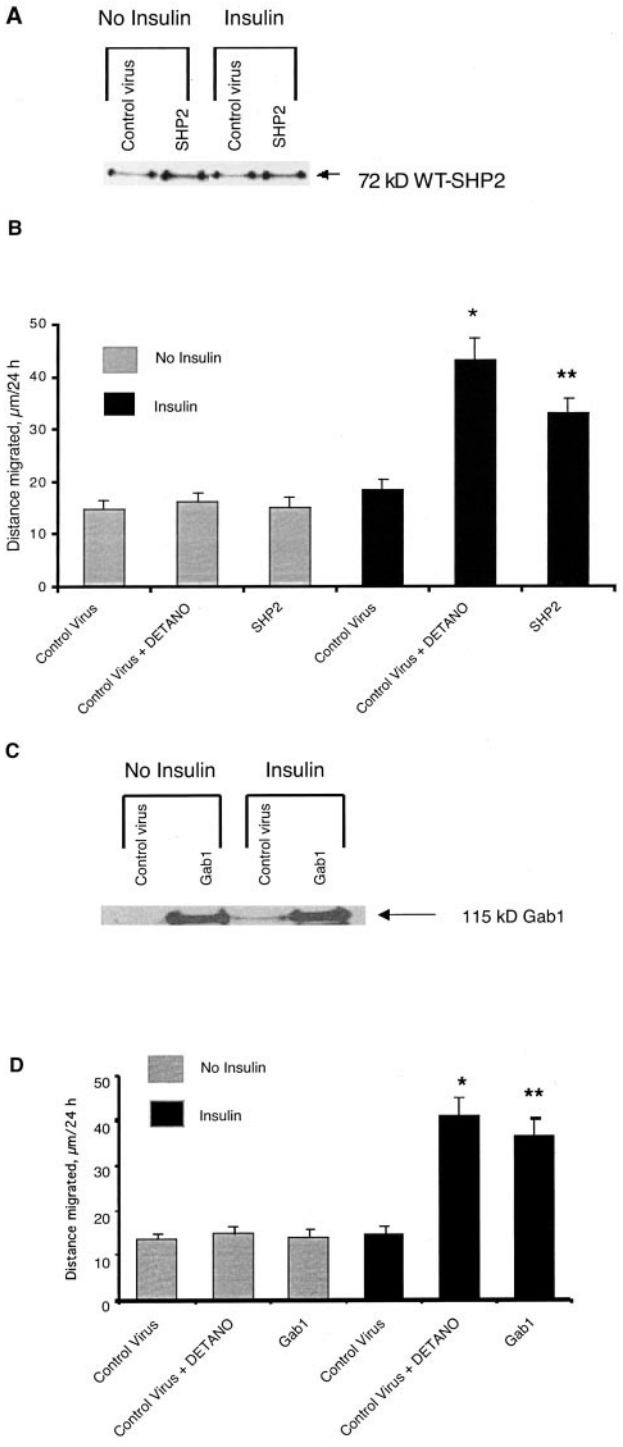

Figure 5. Gab1 and SHP2 stimulate motility in cells treated with insulin but not in insulin-naive cells. A, Levels of SHP2 in cells transfected with SHP2-expressing adenovirus in the presence or absence of insulin (7-day treatment) as measured by Western blot analysis. B, Effect of SHP2 overexpression on cell motility. Motility results are expressed as mean±SEM of 4 independent experiments. *$P<0.05$ vs control virus plus DETANO (no insulin); **$P<0.05$ vs SHP2 (no insulin). C, Levels of Gab1 in cells infected with Gab1-expressing adenovirus in the presence or absence of insulin, as measured by Western blot analysis. D, Effect of Gab1 overexpression on cell motility. Motility results are expressed as mean±SEM of 5 independent experiments. *$P<0.05$ vs control virus plus DETANO (no insulin); **$P<0.05$ vs Gab1 (no insulin).

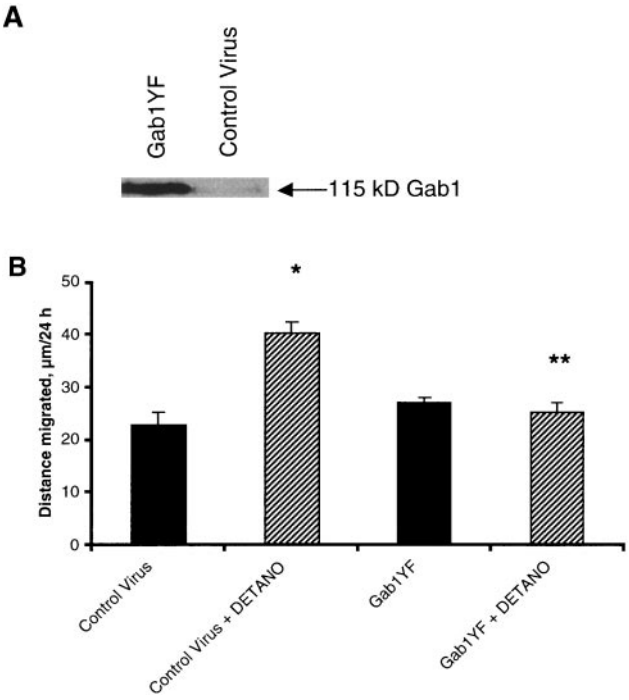

Figure 6. Gab1 is necessary for NO-induced cell motility. Aortic smooth muscle cells were infected with recombinant adenovirus expressing EGFP (as control virus) or Gab1YF at a multiplicity of infection of ≈11, followed by measurement of cell motility as described in Materials and Methods. A, Levels of Gab1YF protein, as determined by Western blot analysis. B, Effect of Gab1YF expression on cell motility. Motility results are expressed as mean±SEM of 3 independent experiments. *$P<0.05$ vs control virus; **$P<0.05$ vs control virus plus DETANO.

absence of insulin was not attributable to deficient expression of SHP2, because expression levels were similar in the presence or absence of insulin (Figure 5A).

Prolonged Treatment With Insulin Is Necessary for Gab1-Induced Cell Motility

These experiments were done to test the hypothesis that the motility-stimulatory effect of Gab1 also requires prolonged exposure to insulin, similar to the requirement of insulin in NO- or SHP2-induced motility. Cells cultured in the presence or absence of insulin were infected with adenovirus expressing Gab1. As shown in Figure 5D, Gab1 increased cell motility only in cells pretreated with insulin. The lack of motility in the absence of insulin was not attributable to deficient Gab1 protein expression, because the levels of expression for Gab1 induced by the recombinant adenovirus in the absence or presence of insulin were similar (Figure 5C).

Gab1 Function Is Necessary for NO-Induced Smooth Muscle Cell Motility

We next sought to determine whether, apart from being sufficient, Gab1 may be necessary for NO-mediated smooth muscle cell motility. Thus, we expressed a Gab1 allele that would be expected to function as a dominant-negative agent by blocking the activity of endogenous Gab1 in activation of SHP2. We therefore mutated Gab1 tyrosine residue 627 to

phenylalanine. Others have shown that this tyrosine residue is important for interaction of Gab1 with the N-terminal SH2 domain of SHP2, and mutation of this residue to phenylalanine markedly decreases the interaction between Gab1 and SHP2.[17–19] We reasoned that if interaction between Gab1 and SHP2 was necessary for NO-induced cell motility, we might block the motility-stimulatory effect of NO donor by hindering this interaction through expression of dominant-negative Gab1YF. Thus, we treated cells with recombinant adenoviral vectors expressing either EGFP, as a control for viral infection per se, or with adenovirus expressing Gab1YF for 48 hours. As shown in Figure 6B, DETANO increased cell motility of differentiated primary cells expressing control virus, thus confirming our earlier results.[7] Moreover, control adenovirus infection did not alter basal or NO-mediated motility of primary aortic smooth muscle cells (data not shown). Furthermore, expression of Gab1YF (4-fold over endogenous Gab1) (Figure 6A) did not have a significant effect on basal motility, but it blocked the motility stimulatory effect of NO (Figure 6B). Thus, Gab1YF acts as dominant-negative mutant and blocks NO-induced motility, suggesting that Gab1 function is necessary for motogenic signaling induced by NO.

Motogenic Effect of Gab1 Is Dependent on the Catalytic Activity of SHP2

In the next set of experiments, we were prompted to determine whether the effect of Gab1 on motility was dependent on the catalytic function of SHP2. We reasoned that if the effect of Gab1 requires SHP2 enzyme activity, then expression of catalytically inactive dominant-negative SHP2, together with Gab1, would block the motility-stimulatory effect of Gab1. In our previous study, we found that an allele expressing the tandem SH2 domains of SHP2 but lacking the rest of the molecule, termed DSH2, functioned as a dominant-negative agent in NO- and SHP2-induced cell motility.[13] As shown in Figure 7B, treatment of cells with adenovirus expressing DSH2 induced significant expression of this polypeptide. Furthermore coexpression of DSH2 did not affect the expression of transgenic Gab1 protein in cells infected with both vectors (Figure 7A). As reported previously,[13] expression of DSH2 alone had no effect on basal cell motility (Figure 7C), but the polypeptide blocked the motility-stimulatory effect of NO, confirming that SHP2 is necessary for NO-induced motility. Moreover, when DSH2 was expressed together with Gab1, it blocked the motility stimulatory effect of Gab1 (Figure 7C), thereby demonstrating that the effect of Gab1 is also dependent on SHP2.

It could be argued that the effect of truncated dominant-negative SHP2 allele DSH2 on Gab1 may be attributable to lack of the remaining protein sequence of SHP2, which may be required for SHP2 to act as an adapter protein.[22] Thus, to additionally investigate whether the catalytic activity of SHP2 is necessary for the effect of Gab1, we cotransduced primary aortic smooth muscle cells with Gab1 and a catalytically inactive SHP2 allele, C463S-SHP2, via adenoviral vectors. The latter allele of SHP2 contains the full sequence, except that the catalytically critical cysteine at residue 463 is mutated to serine, thereby rendering the protein enzymatically inactive.[35] As shown in Figure 8B, 2-fold overexpression of C463S-SHP2 (relative to endogenous SHP2) had no effect on basal motility, although it blocked NO- or Gab1-mediated cell motility (Figure 8C). Figure 8A verifies increased expression of transgenic wild-type Gab1. Thus, these experiments demonstrate that the motility-stimulatory effect of Gab1 requires functional SHP2.

Based on the independent motogenic effect of Gab1 and SHP2, we were prompted to determine if coexpression of Gab1 and SHP2 would lead to additionally increased cell motility. We observed that coexpression of Gab1 and SHP2 did not increase cell motility compared with the motility

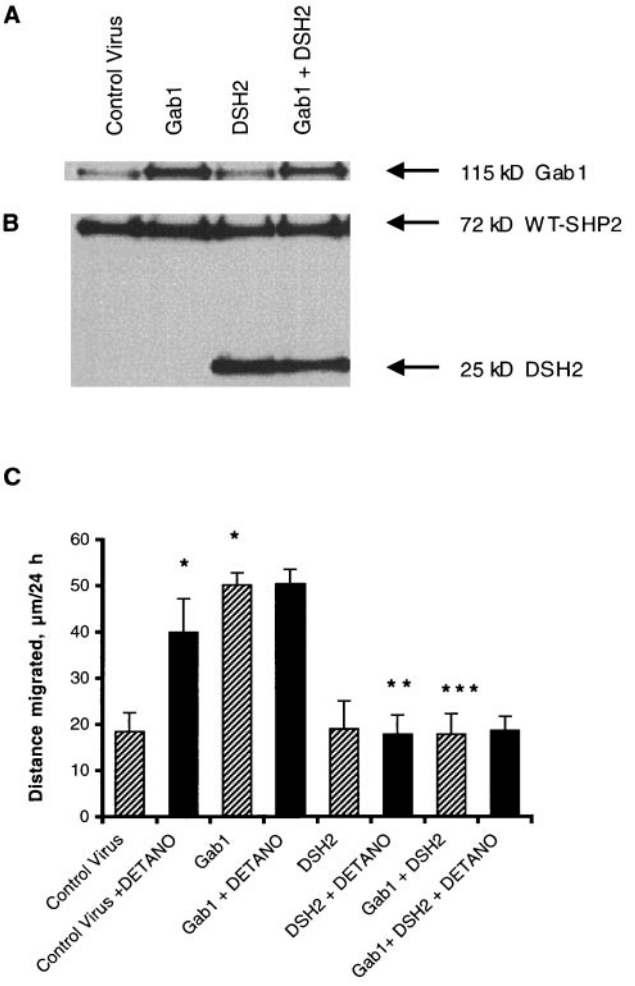

Figure 7. Dominant-negative truncated SHP2 polypeptide blocks Gab1-stimulated cell motility. Aortic smooth muscle cells were infected for 24 hours with recombinant adenoviruses for EGFP (control virus), Gab1, DSH2, or Gab1 plus DSH2 for coexpression at multiplicity of infection of 11, followed by 24 hours in the absence of virus and measurement of cell motility, as described in Materials and Methods. A, Levels of Gab1, as measured by Western blot analysis. B, Same blot reprobed with antibody directed against SHP2. It should be noted that this antibody recognizes specifically the N-terminal SH2 domain and is therefore capable of detecting both endogenous SHP2 and ectopic, truncated DSH2 polypeptide. C, Effect of transgenic protein expression on cell motility. Motility results are expressed as mean±SEM of 3 independent experiments. *$P<0.05$ vs control virus; **$P<0.05$ vs control virus plus DETANO; ***$P<0.05$ vs Gab1.

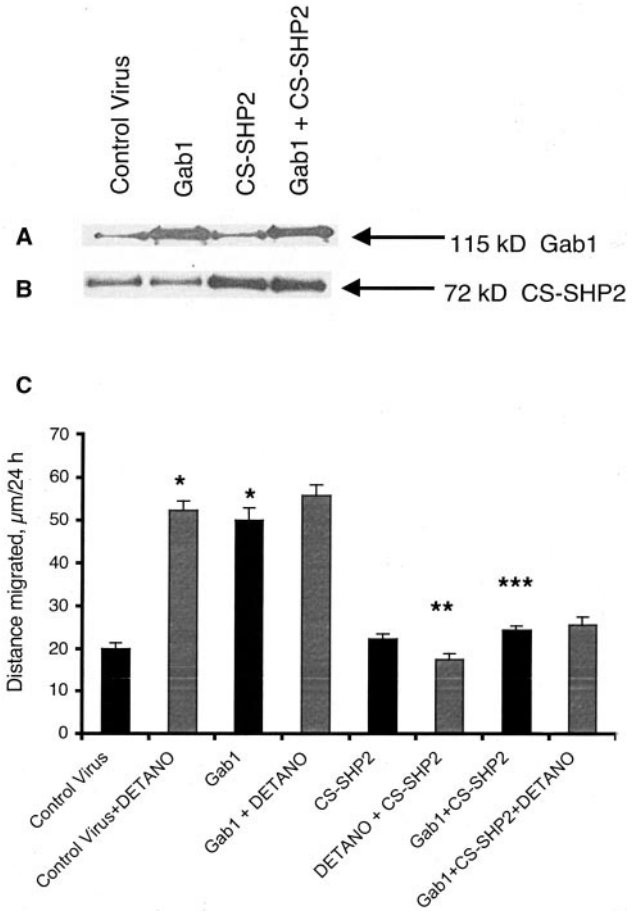

Figure 8. Catalytically inactive SHP2 (CS-SHP2) blocks Gab1-stimulated cell motility. Aortic smooth muscle cells were infected with control adenovirus expressing EGFP (control virus), Gab1, CS-SHP2, or CS-SHP2 plus Gab1 at a multiplicity of infection of 11, followed by measurement of motility, as described in Materials and Methods. A, Levels of Gab1 as measured by Western blot analysis. B, Same blot reprobed for SHP2. C, Effect of transgenic protein expression on cell motility. Motility results are expressed as mean±SEM of 3 independent experiments. *$P<0.05$ vs control virus; **$P<0.05$ vs control virus plus DETANO; ***$P<0.05$ vs Gab1.

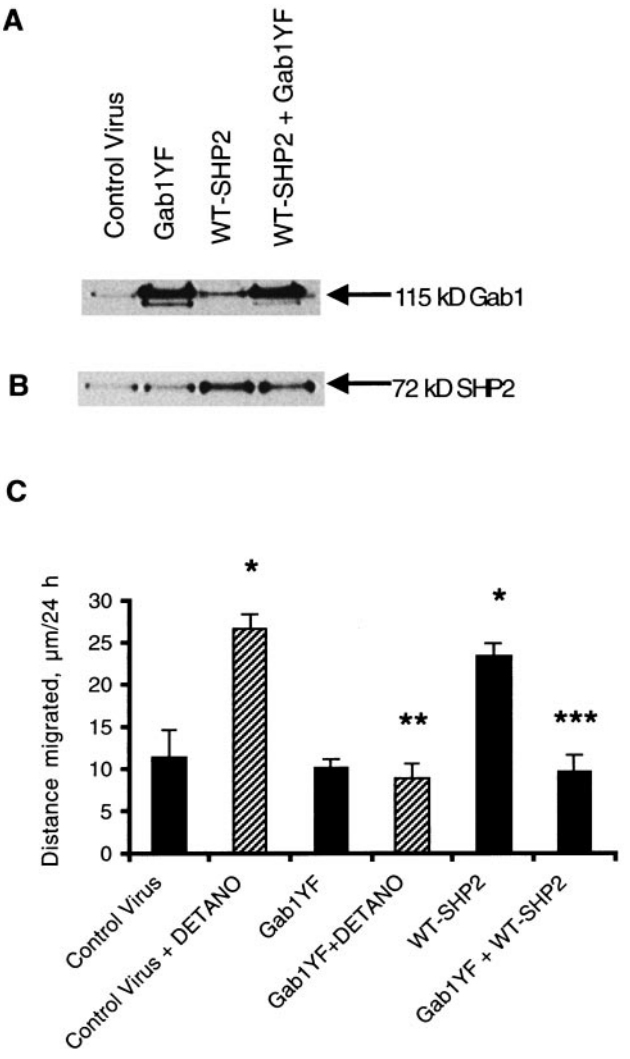

Figure 9. Dominant-negative Gab1 (Gab1YF) blocks SHP2-stimulated cell motility. Primary cultures of aortic smooth muscle cells were infected with control adenovirus expressing EGFP (control virus), wild-type SHP2, or Gab1YF at a multiplicity of infection of ≈11, followed by measurement of cell motility, as described in Materials and Methods. A, Levels of Gab1YF were determined by Western blot analysis. B, Levels of WT-SHP2. C, Effect of transgenic protein expression on cell motility. Motility results are expressed as mean±SEM of 3 independent experiments. *$P<0.05$ vs control virus; **$P<0.05$ vs control virus plus DETANO; ***$P<0.05$ vs WT-SHP2.

when either of the two adenoviruses was expressed independently (data not shown).

Stimulation of Cell Motility by SHP2 Is Blocked by Expression of Dominant-Negative Gab1

Having shown that the function of Gab1 is necessary to mediate NO-stimulated motility of smooth muscle cells and that the motility-stimulatory effect of Gab1 requires the catalytic activity of SHP2, we wanted to determine whether the reverse would also be true, ie, whether the motility-stimulatory effect of SHP2 would require functional Gab1. We reasoned that if the motility-stimulatory effect of SHP2 were to depend on Gab1 and vice versa, this would indicate that the two proteins work as a unit, probably in the form of a Gab1-SHP2 complex. It is known that such an interaction between Gab1 and SHP2 is indispensable for EGF-induced activation of ERK2 pathway.[36] Furthermore, apart from activating SHP2, Gab1 can also be a substrate for the phosphatase activity of SHP2.[16,18,22] Coinfection of mutant Gab1YF with WT-SHP2–expressing adenovirus in aortic smooth muscle cells was carried out. As is evident from Figure 9C, overexpression of WT-SHP2 mimicked the motility stimulatory effect of NO donor, in agreement with our previous publication.[13] However, the motogenic effect of SHP2 was blocked when Gab1YF was coexpressed with WT-SHP2. It should be noted that neither of the two adenoviruses affected the expression of the second protein when coinfected in primary cells, as shown by Western blot analysis (Figures 9A and 9B). Because the Gab1YF mutant has been repeatedly shown to have a reduced interaction with SHP2,[18,19,36] the results presented in Figure 9C demonstrate

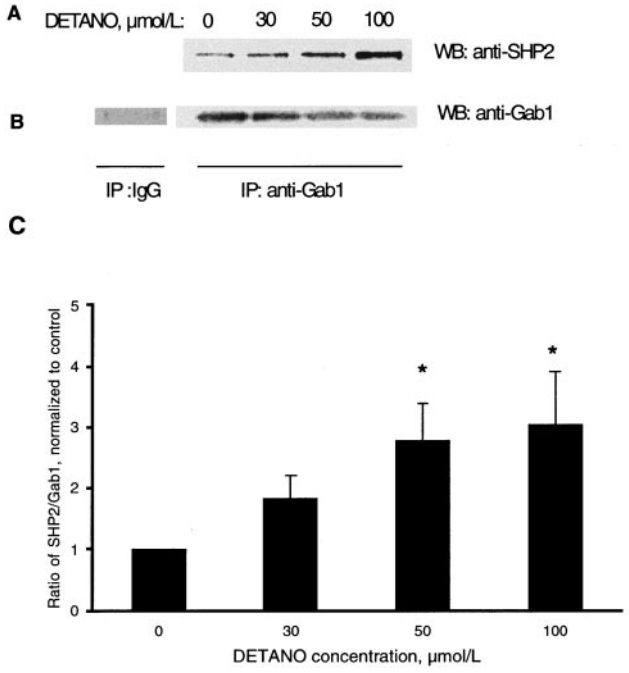

Figure 10. NO donor induces increased Gab1 coimmunoprecipitation with SHP2. A, Blot representative of coimmunoprecipitation of SHP2 with Gab1. Cells were treated with or without increasing concentrations of DETANO for 4 hours, followed by cell lysis and immunoprecipitation with antibody directed against Gab1, followed by Western blot analysis of SHP2, as described in Materials and Methods. A previous study indicated that 4 hours is optimal for upregulation of SHP2.[37] B, Same blot was reprobed for Gab1 to normalize for differential immunoprecipitation of Gab1. The specific coimmunoprecipitation of Gab1 via this method was confirmed by performing control immunoprecipitation with irrelevant control antibody (IgG control). C, Results represent the ratio of SHP2 to Gab1, normalized to control (no DETANO), and are the mean±SEM of 6 independent experiments. *$P<0.05$ vs 0 μmol/L DETANO. IP indicates immunoprecipitation; WB, Western blot.

that a functional interaction between Gab1 and SHP2 is necessary to explain NO signaling related to increased motility. Moreover, this finding emphasizes that a requirement of Gab1 for SHP2 and of SHP2 for Gab1 is mutual.

Gab1 Interacts With SHP2 in Aortic Smooth Muscle Cells

The tandem SH2 domains in SHP2 regulate the catalytic activity of SHP2.[18,23] Basally, SHP2 is maintained in an autoinhibited state, and binding of a specific phosphotyrosine to the N-terminal SH2 domain induces an allosteric change to release autoinhibition and activate the enzyme. Phosphotyrosines at positions 627 and 659 of Gab1 constitute a biphosphoryl tyrosine–based activation motif involved in activation of SHP2.[18] Given these findings, it is likely that a functional interaction between Gab1 and SHP2 exists in primary aortic smooth muscle cells to mediate signaling events. Because SHP2 protein levels are increased in response to NO,[37] it is also likely that NO donor may amplify a potential Gab1-SHP2 interaction.

To establish an interaction between Gab1 and SHP2 proteins, we performed coimmunoprecipitation experiments. For this purpose, lysates were prepared from cells treated without or with increasing concentrations of DETANO. These lysates were then subjected to immunoprecipitation with Gab1-specific antibodies, followed by SDS-PAGE and Western blot analysis of SHP2 and Gab1. As shown in Figure 10, SHP2 and Gab1 coimmunoprecipitated even in untreated cells, suggesting constitutive interaction of these proteins in differentiated aortic smooth muscle cells. It should also be noted that specific immunoprecipitation of Gab1 was confirmed via the use of nonspecific rabbit IgG control antibody (Figure 10B). Furthermore, the interaction between Gab1 and SHP2 increased significantly in response to NO donor (Figure 10C). In this complex, Gab1 may stimulate downstream signaling by either recruiting SHP2 to an appropriate subcellular location or by maintaining the enzyme in an activated state in response to NO. Hence, the Gab1-SHP2 interaction seems to be functionally important for NO-induced cell motility.

DETANO-, SHP2-, or Gab1-Induced Smooth Muscle Cell Motility Is Blocked by Selective PI3 Kinase Inhibitor

The finding that DETANO-induced as well as Gab1- or SHP2-induced motility of primary aortic smooth muscle cells requires preincubation with insulin indicates that these agonists most likely function via a common signaling pathway. PI3 kinase is likely to be important for recruitment of Gab1 to the plasma membrane via an increase of phosphatidylinositol-3,4,5-trisphosphate levels.[38,39] Moreover, PI3 kinase activity has been reported to be necessary for VSMC motility, and it has been recently shown that chronic insulin exposure is associated with increased protein levels and enzyme activity of PI3 kinase in primary 3T3-L1 adipocytes or intact rats.[29,40,41] These findings prompted us to test the hypothesis that NO-induced motility requires PI3 kinase activity. As shown in Figure 11A, the selective PI3 kinase inhibitor LY294002 significantly attenuated DETANO-induced motility. LY294002 also attenuated Gab1- and SHP2-induced motility (Figure 11B). These findings support the notion that PI3 kinase activity is necessary for NO-, Gab1-, or SHP2-induced VSMCs.

Discussion

We provide novel evidence that exposure of aortic smooth muscle cells to insulin alters the effect of NO from that of an inhibitor of cell motility to that of stimulator. Insulin induces comotogenesis in the absence of its own motogenic effect. Moreover, the comotogenic effect of insulin is independent of serum, because it is manifested in cultures maintained in serum-free medium.

It may be argued that the comotogenic effect of insulin is attributable to cross-activation of the insulin-like growth factor 1 (IGF1) receptor. However, at the concentration of insulin used for most of this study (100 nmol/L), insulin failed to phosphorylate the IGF1 receptor in our cells (data not shown). Another group has also reported that insulin fails to cross-activate the IGF1 receptor in VSMCs at concentrations up to 100 nmol/L.[30] It is also theoretically possible that prolonged insulin treatment may have led to an increase in total cell number, an effect that may have been indirectly

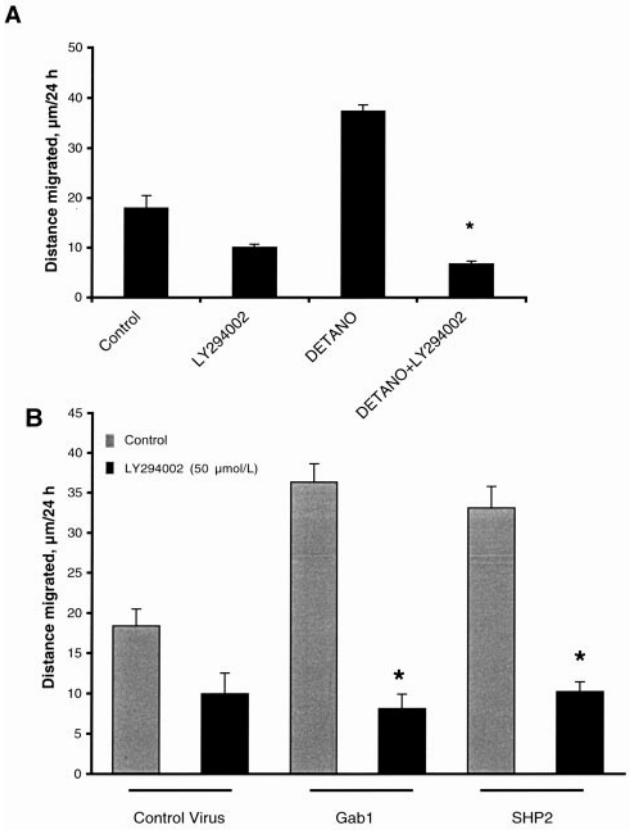

Figure 11. NO-, Gab1-, or SHP2-mediated motility is blocked by the selective PI3 kinase inhibitor LY294002. Aortic smooth muscle cells were grown to confluency in the presence of 100 nmol/L insulin. After 24 hours of culture in serum-free medium, motility was assessed as described in Materials and Methods. A, Effects of DETANO (30 μmol/L), LY294002 (50 μmol/L), or DETANO plus LY294002 on VSMC motility. Results are expressed as mean±SEM for 3 independent experiments. *$P<0.05$ vs DETANO. B, Effect of LY294002 on Gab1- or SHP2-mediated motility. Results are expressed as mean±SEM for 4 independent experiments. *$P<0.05$ vs respective controls. Overexpression of Gab1 and SHP2 was confirmed by Western blot analysis (data not shown).

responsible for increased cell motility. However, we did not observe a significant difference in the number of cells grown in the presence or absence of insulin (data not shown). Moreover, the total protein yields from cells grown in the presence or absence of insulin were not significantly different (data not shown). The capacity of insulin to uncover the motogenic effect of NO is therefore independent of altered cell number and overall cellular protein levels. These findings also additionally support the notion that insulin-induced comotogenesis is independent of IGF1 receptor cross-activation, because activation of the latter receptor would have increased cell number.

In the present study, we also report the novel finding of an obligatory involvement of the adapter protein Gab1 and its interaction with SHP2 in NO-induced cell motility in differentiated aortic smooth muscle cells. Additionally, we demonstrate that, similar to NO, the motogenic effect of Gab1 and SHP2 on VSMCs requires sustained preexposure to insulin. This suggests that the requirement of preexposure to insulin for NO-induced motility may be to drive Gab1-SHP2–dependent molecular events in the direction of increased motility. The fact that cell motility induced by NO, Gab1, or SHP2 was similarly attenuated by the PI3 kinase inhibitor LY294002 supports the notion that these agonists function via a common pathway, likely involving PI3 kinase. It has been demonstrated that membrane localization of Gab1 is dependent on the binding of its PH domain to PIP_3, the product of PI3 kinase activity.[38,39] Independently, it is known that diet-induced models of type II diabetes, which are characterized by chronic hyperinsulinemia, are associated with elevation of basal PI3 kinase activity.[29,41,42] Thus, it is possible that sustained exposure of VSMCs will lead to an overall increase in PI3 kinase activity, resulting in increased levels of PIP_3 for appropriate membrane localization of the Gab1-SHP2 complex. Whether chronic insulin treatment in our experimental system leads to an increase in PI3 kinase activity is the subject of present investigation in our laboratory.

The finding that catalytically inactive SHP2 blocks the motility stimulatory effect of Gab1 indicates that, similar to NO, Gab1 requires association with enzymatically active SHP2 to stimulate motility. Conversely, the motility-stimulatory effect of SHP2 depends on the presence of functional Gab1, thereby indicating that the two proteins function as a unit. It should be noted that Gab1 mimics the NO effect on cell motility but fails to upregulate SHP2 levels, suggesting that the motogenic capacity of Gab1 is independent of SHP2 upregulation. It should also be noted that NO fails to increase the protein levels of Gab1, indicating that endogenous Gab1 levels are sufficient to mediate increased motility and that the role of NO is to upregulate SHP2 or to functionally activate Gab1. Moreover, coexpression of Gab1 and SHP2 fails to increase motility beyond the use of either agent alone. The interpretation of this finding is 2-fold; either both Gab1 and SHP2 serve mechanistically equivalent functions, as suggested by our hypothesis, or, equally likely, treatment with Gab1 or SHP2 alone induces maximal cell motility that cannot be additionally increased by combining these agents.

Gab1 is an adapter protein that links SHP2 to Sos and thus to the Ras/MAPK pathway.[19] The fact that increased SHP2 activity correlates with enhanced MAPK activity suggests that in our experimental system, Gab1-SHP2 interaction may be involved in downstream activation of the MAPK pathway. Tyrosine phosphorylation of SHP2 has been reported to induce an increase in its enzyme activity.[43] However, we previously reported that although NO increased protein levels and activity of SHP2, it failed to increase overall SHP2 phosphotyrosine levels.[13] The present results are supportive of the existence of a mechanism formally independent of SHP2 tyrosine phosphorylation. Thus, it seems that after NO treatment, an increase of Gab1-SHP2 binding may activate the enzyme, thereby leading to dephosphorylation of downstream proteins. However, the identity of such SHP2 substrates remains to be determined. It is likely that Gab1 recruits SHP2 to the plasma membrane and thus to the vicinity of its potential substrates via its PH domain. Such localization of SHP2 may be necessary to decrease the activity of Rho or to

induce dephosphorylation of adhesion molecules, thereby decreasing cell adhesion and leading to cell motility.[7] Although the substrates for SHP2 largely remain unidentified, a candidate protein is $pp60^{src}$, the activity of which is increased via dephosphorylation of a negative regulatory phosphotyrosine residue.[44] Additionally, there are reports that Src may be a substrate for the phosphatase activity of SHP2.[45] Experiments in progress aim to test the hypothesis that NO has the capacity to activate Src via SHP2. Although we have shown that a functional interaction between Gab1 and SHP2 is necessary to explain the motogenic effect of NO, additional studies are required to determine the exact role of Gab1 in this pathway. The insulin-dependent molecular events that govern the motogenic capacity of NO and the exact biochemical role of Gab1 in this process are the subjects of present investigation in our laboratory.

Endogenous NO is thought to play an important role in modulating neointima formation, based on the findings that iNOS level is upregulated in vascular injury and that overexpression of NO synthase attenuates, whereas inhibition of NO synthase enhances, neointima formation.[8,9,46,47] However, the latter findings are somewhat controversial, inasmuch as neointima formation is actually attenuated in iNOS knockout mice.[48] Moreover, treatment of angioplasty patients with NO donors failed to show a significant benefit relative to neointimal expansion.[49,50] Given our present findings and the increased occurrence of cardiovascular morbidity in hyperinsulinemia and overt type II diabetes mellitus, it is reasonable to speculate that under conditions of hyperinsulinemia and the presence of vascular injury, increased levels of NO may contribute to neointimal enlargement, rather than the reverse. It is also possible that hyperinsulinemia may contribute to loss of the inhibitory effect of NO against growth factor–induced cell motility and neointima formation. However, to our knowledge, there are no studies of the effect of NO or endogenous NO synthase inhibition on neointima formation in hyperinsulinemic animal models or in humans with type II diabetes. Therefore, until such studies are completed, we cannot rule out the possibility that our results represent a tissue culture artifact unrelated to in vivo events. However, it should also be pointed out that previous experiments using tissue culture have accurately predicted many if not most in vivo findings related to the effects of NO.

In summary, we have reported several novel findings. First, we have shown that NO increases the motility of differentiated primary cultures of aortic smooth muscle cells continuously exposed to relatively high levels of insulin through a mechanism requiring Gab1. Second, we have shown that the motility-stimulatory effect of Gab1 is manifested in the presence but not the absence of insulin, consistent with its involvement in NO-induced motility. Third, we have reported that the motility-stimulatory effect of NO is dependent on a functional interaction between adapter protein Gab1 and protein tyrosine phosphatase SHP2. Finally, we have shown that the stimulation of cell motility by Gab1 or SHP2 requires prolonged exposure to insulin. Inasmuch as SHP2 has been reported to transduce the effects of several important cardiovascular agonists,[51] our results could have relevance potentially wider than just mechanisms related to the role of NO in cardiovascular remodeling.

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