Requirement of protein tyrosine phosphatase SHP2 for NO-stimulated vascular smooth muscle cell motility

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Brown, Claire, Yi Lin, and Aviv Hassid. Requirement of protein tyrosine phosphatase SHP2 for NO-stimulated vascular smooth muscle cell motility. Am J Physiol Heart Circ Physiol 281: H1598–H1605, 2001.—We have previously reported that nitric oxide (NO) increases the motility of differentiated cultured primary aortic smooth muscle cells from adult rats. There is little information on the role of protein tyrosine phosphatases in vascular biology. One such phosphatase, Src homology 2 phosphatase 2 (SHP2), is essential for motility. We tested the hypothesis that NO increases SHP2 levels via a cGMP-mediated mechanism and that this effect is necessary for NO-stimulated cell motility. Here we report that two different NO donors increased SHP2 protein and enzyme activity. This effect was mimicked by several cGMP agonists and blocked by an inhibitor of guanylly cyclase. Specific decrease of SHP2 protein levels via the use of antisense oligodeoxynucleotides (ODNs), but not several control ODNs attenuated the motogenic effect of NO, which indicates the involvement of SHP2 in NO-elicited motogenesis. S-nitroso-N-acetylpenicillamine failed to increase SHP2 protein levels in subcultured aortic smooth muscle cells. This provides a potential explanation for the lack of effect of NO on cell motility in dedifferentiated cultured subcultured cells. These results support the hypothesis that NO-elicited upregulation of SHP2 via a cGMP-mediated pathway is necessary for NO-induced motogenesis in differentiated aortic smooth muscle cells.

S-nitroso-N-acetylpenicillamine; motogenesis; cell motility; signal transduction; oligodeoxynucleotides; nitric oxide; Src homology 2 phosphatase 2

VASCULAR SMOOTH MUSCLE cell motility is of critical importance in the development of atherosclerosis and restenosis (6, 28). Angiogenesis also requires cell motility for the recruitment of vascular smooth muscle cells to emerging capillaries and arterioles (28). However, little is known about the molecular events that regulate vascular smooth muscle cell motility.

Considerable evidence supports the notion that nitric oxide (NO) plays an important role in vascular remodeling. First, it is well established that inductive NO synthase levels are increased early after vascular injury (7, 12). Second, several recent reports (7, 18, 27) have suggested that NO plays a significant role in neointima formation and angiogenesis via processes that are thought to depend on increased vascular smooth muscle cell motility. NO has also been reported to be involved in the increase of vascular diameter, which includes remodeling in response to a chronic increase of blood flow (30).

We have previously reported that NO increases the motility of differentiated primary aortic vascular smooth muscle cell cultures isolated from adult rats (4); this is consistent with the notion that NO may contribute to neointima formation in vivo. In the current study, we targeted the involvement of the protein tyrosine phosphatase (PTP) Src homology 2 (SH2) phosphatase 2 (SHP2) as a mediator of this effect.

Whereas the role of protein tyrosine kinases (PTKs) in cell biology is relatively well established, that of the counterregulatory agents, namely PTPs, is just beginning to be investigated in detail. Most PTPs serve as negative modulators of signal transduction; however, SHP2 mainly subserves a positive signal transduction role. Recent studies have shown that SHP2 mediates the mitogenic effects of fibronectin (32), platelet-derived growth factor (22, 23), or insulinlike growth factor 1 (16) in various cultured cell lines. SHP2 has also been reported to be essential in signaling that is related to the mitogenic effect of angiotensin II in vascular smooth muscle cells (17). SHP2 is the mammalian homologue of the gene product of Drosophila corkscrew (csw) that also encodes an SH2-containing PTP. Genetic analysis in Drosophila (21) has revealed that csw participates in signaling downstream of the torso and sevenless receptor PTKs. SHP2 is critical for embryonic development as was shown by the nonviability of homozygous SHP2 mutant mice embryos that manifested multiple defects in mesodermal patterning after 8.5–10.5 days of gestation (26). Interestingly, SHP2 is expressed ubiquitously but predominantly in vascular smooth muscle cells; this is consistent with the hypothesis that it may play an important role in vascular smooth muscle biology (1). In the current study, we report that NO increases the levels of SHP2 protein and enzyme activity in differentiated primary but not dedifferentiated subcultured smooth muscle cells from adult rat aorta, and that the motogenic effect of NO is...
blocked by antisense oligonucleotide directed against SHP2 mRNA.

EXPERIMENTAL PROCEDURES

Materials. DMEM-Ham’s F-12 medium and fetal bovine serum (FBS) were from GIBCO-BRL (Gaithersburg, MD). 8-Bromo-cGMP, penicillin, streptomycin, S-nitroso-N-acetyl-penicillamine (SNAP), and N-acetyl-d1-penicillamine (NAP) were from Sigma-Aldrich (St. Louis, MO). Insulin, transferrin, and selenium were from Collaborative Research (Bedford, MA). Guanosine-3′,5′-cyclic monophosphate 8-(4-chlorophenylthio)triethylammonium salt (8-pCPT-cGMP) was from Calbiochem (San Diego, CA). 2,2-(Hydroxynitrosodimethylamino)bis-ethanethione (DETANO) and 1H-[1,2,4]oxadiazo[4,3-a]quinazolin-1-one (ODQ) were purchased from Alexis Biochemicals (San Diego, CA). The transfection reagent N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) was supplied by Boehringer Mannheim (Indianapolis, IN). Phosphorothioate oligodeoxynucleotides (ODNs) were synthesized and purified by Biosynthesis (Lewisville, TX). Antibody directed against SHP2 was purchased from Transduction Labs (Lexington, KY). [γ-32P]ATP was obtained from Amersham (Arlington Heights, IL).

Vascular smooth muscle cell isolation and culture via enzymatic dissociation. Male Sprague-Dawley rats (weight 100–150 g) were obtained from Charles River Laboratories. Male Sprague-Dawley rats (weight 250–300 g) were obtained from Charles River Laboratories. Male Sprague-Dawley rats (weight 100–150 g) were obtained from Charles River Laboratories. Subcultured cells were derived from primary cultures via detachment of cells with trypsin, replating at lower densities, and growth to confluence as described using collagenase and elastase (4). Each isolate consisted of cells pooled from 2–4 individual rat aortas. All cultures were maintained in a humidified atmosphere of 5% CO2-95% air. Isolated cells were seeded on Primaria tissue culture plates in DMEM/F-12 media supplemented with penicillin, streptomycin, and 10% FBS. Cultures were treated with serum-free medium for 1 day before experiments were initiated. Most experiments were performed using 100–150 μM SNAP (10 or 100 μM) or DETANO (100 μM) for varying time periods ranging from 0 to 24 h. After incubation, cells were lysed and SHP2 protein levels were measured via Western blotting. Both SNAP (see Fig. 1, A and B) and DETANO (see Fig. 1C) increased SHP2 protein levels two- to threefold. A lower concentration of SNAP (10 μM) elicited a sustained increase, whereas the effect of 100 μM SNAP (see Fig. 1B) was more

RESULTS

NO donors increased SHP2 protein and activity levels in primary cultured rat aortic smooth muscle cells. Confluent primary cells from adult rats were incubated without or with one of two NO donors [SNAP (10 or 100 μM) or DETANO (100 μM)] for varying time periods ranging from 0 to 24 h. After incubation, cells were lysed and SHP2 protein levels were measured via Western blotting. Both SNAP (see Fig. 1, A and B) and DETANO (see Fig. 1C) increased SHP2 protein levels two- to threefold. A lower concentration of SNAP (10 μM) elicited a sustained increase, whereas the effect of 100 μM SNAP (see Fig. 1B) was more
transient. NAP, a substance structurally identical to SNAP but lacking the NO moiety, failed to have a significant effect on SHP2 protein levels (data not shown). The increase of SHP2 protein levels by two chemically dissimilar NO donors and the lack of effect of NAP indicates that the increase of SHP2 was likely mediated by NO.

SHP2 may stimulate signal transduction mechanisms independently of its enzymatic activity via a capacity to function as an adapter protein (17). Therefore increased protein levels of SHP2 may not necessarily reflect increased enzyme activity. To measure the effect of a NO donor on SHP2 enzyme activity, we immunoprecipitated SHP2 from cells that had been treated without or with SNAP (100 μM) or the control substance NAP (100 μM) for 3 h and measured phosphatase activity in the immunoprecipitates. SNAP elicited a statistically significant 2.3-fold increase in SHP2 enzyme activity, whereas NAP failed to increase SHP2 activity (see Fig. 2A). The phosphatase activity of lysates subjected to mock immunoprecipitation with a nonspecific antibody was low and comparable to that of lysates subjected to mock immunoprecipitation in the absence of added antibody (see Fig. 2A). Additionally, the reduction of enzyme activity by vanadate to levels found in immunoprecipitations done with nonimmune antibody provided further verification of specificity (see Fig. 2A). As shown in Fig. 2B, a second NO donor,
DETANO, also increased SHP2 activity approximately twofold, and this activity was also blocked by the phosphatase inhibitor vanadate.

cGMP analogs and C-type natriuretic peptide mimicked capacity of NO donor to increase SHP2 protein levels. If the capacity of NO to increase SHP2 protein levels were mediated by cGMP as a second messenger, a similar effect by other cGMP agonists would be anticipated. Accordingly, we measured the effect of two cGMP analogs (8-bromo-cGMP and 8-pCPT-cGMP, both 100 μM) on SHP2 protein levels. Both agents mimicked the increase of SHP2 protein levels induced by a NO donor (see Fig. 3), which supports the view that the effect of NO was mediated via cGMP. To further test this hypothesis, we measured the effect of a C-type natriuretic peptide (CNP-22) on SHP2 protein levels, because CNP-22 is well known to increase cGMP levels in vascular smooth muscle cells (11). As shown in Fig. 4, CNP-22 (1 μM) increased SHP2 protein levels by approximately threefold, which provides further support for this hypothesis.

ODQ blocked SNAP-induced increase in SHP2 protein levels. To confirm the involvement of the cGMP pathway in the NO-elicited increase of SHP2 protein, we treated primary cultures without or with SNAP in the absence or presence of ODQ, a selective inhibitor of soluble guanylyl cyclase. As shown in Fig. 5, ODQ attenuated the SNAP-induced increase in SHP2 protein levels, whereas ODQ alone had no significant effect. These results further support the notion that cGMP is an important component of the NO-elicited increase in SHP2 protein levels.

Antisense ODN directed against SHP2 but not control ODN decreased SHP2 protein levels and attenuated NO-elicited cell motility. On the basis of our previous report that SNAPP increases the motility of differentiated cultured aortic smooth muscle cells (4), we were prompted to determine whether SHP2 was necessary for NO-induced motility. A conventional pharmacological inhibitor that selectively targets SHP2 is currently not available. However, a previous study (20) has reported the use of an antisense ODN that selectively downregulates the levels of SHP2 protein. Thus we treated cells with antisense ODN directed against SHP2 mRNA and measured protein levels of SHP2 and the capacity of aortic smooth muscle cells to migrate in response to SNAP. Primary aortic smooth muscle cell cultures at ~80% confluency were preincubated without or with antisense ODN or control ODNs for 24 h before measurement of SHP2 protein levels or cell motility. Control cells were treated only with the lipid transfection agent DOTAP. As shown in Fig. 6, antisense ODN but not the control scrambled antisense ODN sequence decreased the SHP2 protein levels. For cell-motility experiments, cell

Fig. 3. cGMP analogs increase SHP2 protein levels. A: cells were treated for 4 h without or with 100 μM guanosine-3’5’-cyclic monophosphate 8-(4-chlorophenythio)triethylammonium salt (8-pCPT-cGMP). B: cells were treated for 4 h without or with 100 μM 8-bromo-cGMP. SHP2 levels were measured via Western blotting. Blots show results from a single representative experiment; graphs represent the summary of all experiments normalized to control values (means ± SE of 5 experiments). *P < 0.05 compared with control values via Wilcoxon signed-rank test.

Fig. 4. C-type natriuretic peptide (CNP-22) increases SHP2 protein levels. Cells were incubated for 0–24 h in the presence of 1 μM CNP-22, and SHP2 protein levels were measured by Western blotting. Blot provides results from a single representative experiment; graph represents the summary of all experiments normalized to control values (means ± SE of 3 experiments). *P < 0.01 compared with control via randomized complete-block ANOVA and subsequent Dunnett’s test.
monolayers were wounded, ODN-supplemented medium was replenished, and cells were exposed to SNAP (100 μM) for 24 h, after which cells were fixed and stained and the number of migrating cells was counted. As shown in Fig. 7, SNAP alone increased cell motility, which confirms previous results (4). Furthermore, treatment with antisense ODN (10 μM) decreased the motogenic effect of SNAP by 50%, whereas neither three-nucleotide antisense mismatch ODN, sense ODN, nor scrambled ODN (all 10 μM) had a significant effect. The combined use of antisense and sense ODNs was also ineffective in blocking the effects of SNAP (results not shown).

NO failed to upregulate SHP2 protein levels in subcultured aortic smooth muscle cells. The data given suggest that the motogenic effect of NO in primary aortic smooth muscle cells may be dependent on the upregulation of SHP2. Moreover, we and others (4, 9, 25) have previously found that in contrast to primary cells, NO either has no effect or decreases motility in subcultured aortic smooth muscle cells. We were therefore prompted to test the hypothesis that the lack of NO-stimulated motogenesis in subcultured cells may be attributable to the lack of NO-elicited upregulation of SHP2 in subcultured cells. Indeed, as shown in Fig. 8, SNAP failed to increase SHP2 protein levels in three independent subcultured cell lines.

DISCUSSION

We have recently reported that NO increases the motility of differentiated cultured primary rat aortic smooth muscle cells isolated from young adult rats (4). Because SHP2 is expressed at relatively high levels in vascular smooth muscle cells (1) and appears to be involved in mediating cell motility in various cultured cell lines (16, 22, 32), we were prompted to investigate the role of SHP2 as a transducer of the motogenic capacity of NO in primary cultures of rat aortic smooth muscle cells. The current study identifies SHP2 as a pivotal signal transduction element in this effect, based on the findings that NO increased SHP2 protein and activity levels and that attenuation of NO-induced expression of SHP2 via antisense ODN decreased cell motility.

SHP2 contains two NH2-terminal SH2 domains consisting of a conserved sequence of ~100 amino acids and a COOH-terminal hydrophilic domain containing several tyrosine phosphorylation sites (19). The enzyme binds directly via its NH2-terminal SH2 domain to phosphotyrosine residues of several activated tyrosine kinase-linked receptors such as the platelet-derived growth factor and epidermal growth factor receptors (10). SHP2 then becomes tyrosine phosphorylated, which creates binding sites for SH2 domain-containing molecules such as the adapter protein Grb2.
These effects are associated with activation of the Ras/MAPK cascade and lead to increased cell proliferation or motility.

In our experiments, increased SHP2 expression in response to a relatively high concentration of NO donor was transient. This is not uncommon for signal transduction mechanisms and may reflect downregulation of the cGMP signal transduction pathways in the presence of relatively high levels of activation (29). When a lower concentration of NO donor was used, transiency was no longer evident; furthermore, CNP-22 also elicited a sustained increase in SHP2. The transiency of the effect with higher concentrations of the NO donor coupled with the significantly increased motility after 24 h in cells treated with high levels of the NO donor indicate that a sustained increase of SHP2 is not necessary to mediate robust cell motility.

Downstream of NO, the mechanisms of increased SHP2 expression (this study) and motility (4) both appear to involve the cGMP pathway. This conclusion is based on three distinct lines of evidence. First, two cyclic nucleotide analogs increased SHP2 protein levels and motility. Second, the effect of NO was blocked by ODQ (a selective inhibitor of cytoplasmic guanylyl cyclase). Third, CNP-22, which increases cGMP levels via a cell-surface receptor linked to guanylyl cyclase, also increased SHP2 protein levels. Taken together, these findings strongly support the involvement of cGMP.

To test whether SHP2 was necessary for NO-stimulated motility, we decreased SHP2 protein levels via the use of antisense methodology. We found that antisense ODN but not several control ODNs (including a stringent control involving the use of a three-nucleotide mismatch antisense ODN) significantly attenuated NO-stimulated cell motility.

As reported previously, NO-elicited migration of rat aortic smooth muscle cells is specific for differentiated primary cultures from adult rats, because NO fails to increase cell motility in dedifferentiated subcultured cells (4). To investigate the potential involvement of SHP2 in this divergence, we measured the effect of an NO donor on SHP2 levels in subcultured cells. We found that an NO donor lacked the capacity to increase SHP2 levels in these cells, which provides a potential explanation for the lack of a stimulatory effect of NO on motility in subcultured cells and indirectly strengthens the hypothesis that SHP2 is of critical importance to NO-stimulated cell motility.

The effect of NO on tissue remodeling and neointima formation is controversial. Several early studies indicated that administration of exogenous NO (14) or overexpression of NO synthase (31) inhibited neointima formation after vascular injury, whereas endothelial NO synthase knockout amplified injury-induced neointima formation (24). In contrast, other studies demonstrated that inducible NO synthase knockout attenuated injury-induced neointima formation (7) and vascular remodeling (30). Our laboratory has provided evidence that the effect of NO on motility is opposite in primary cultures from adult versus newborn rats via a difference that correlates with the phenotype of these vascular smooth muscle cells (3, 4, 13). We have also found that the antimotogenic effect of NO in cells from newborn rats is likely to be mediated via increased activity of PTP-1B (3, 4, 13), an enzyme that is considered to be a negative modulator of cell motility (15). This is in contrast to the current study, which shows that the motogenic effect of NO is likely to be mediated...
by SHP2, a phosphatase considered to be a positive transducer of cell motility (16). It seems plausible that the divergent results reported in vivo could be explained by differences in experimental detail between laboratories that are targeting different phenotypes of vascular smooth muscle cells. However, we also suggest that caution be exercised in directly extrapolating the current results obtained in cultured cells to the in vivo situation, which is likely to represent a much more complex environment.

In summary, we have shown that the increase in cell motility in response to NO is attributable to upregulation of the PTP SHP2, and that SHP2 expression is necessary for NO-elicted motility. Moreover, subculturing and dedifferentiation of aortic smooth muscle cells is associated with the unresponsiveness of these cells to the SHP2 stimulatory effect of NO, which may explain the lack of cell motility in subcultured cells in response to NO.

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