NO Attenuates Insulin Signaling and Motility in Aortic Smooth Muscle Cells via Protein Tyrosine Phosphatase 1B–Mediated Mechanism

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Objective—Hyperinsulinemia is a significant risk factor for the pathogenesis of vascular disease. Protein tyrosine phosphatase 1B (PTP1B) has been recognized as a modulator of insulin signaling in nonvascular cells, and we have recently reported that NO increases the activity of PTP1B in rat vascular smooth muscle cells. In the present study, we tested the hypothesis that NO attenuates insulin-stimulated cell motility via a PTP1B-mediated mechanism involving downregulation of insulin signal transduction.

Methods and Results—Treatment of primary aortic smooth muscle cells from newborn rats with the NO donor S-nitroso-N-acetylpenicillamine reduced cell motility, tyrosine phosphorylation levels of insulin receptor (subunit and insulin receptor substrate-1), and extracellular signal–regulated kinase activity. Overexpression of wild-type PTP1B via an adenoviral vector blocked the capacity of insulin to stimulate cell motility and insulin receptor phosphorylation, whereas expression of a dominant-negative mutant of PTP1B attenuated the capacity of NO to decrease cell motility.

Conclusions—Our findings indicate that activation of PTP1B is necessary and sufficient to account for the capacity of NO to decrease insulin-stimulated signal transduction and cell motility in cultured aortic smooth muscle cells. The results could explain the capacity of NO to oppose neointima formation in states of hyperinsulinemia. (Arterioscler Thromb Vasc Biol. 2002;22:●●●.●●●.)

Key Words: PLEASE ■ SUPPLY ■ KEYWORDS

Type II diabetes is a major risk factor for the pathogenesis of vascular disease, including that associated with hypertension and atherosclerosis. That diabetes increases the frequency of coronary vessel restenosis occurring after angioplasty is also well established. Most forms of vascular disease in conduit arteries manifest neointimal enlargement, and although type II diabetes is associated with hyperglycemia as well as hyperinsulinemia, recent studies have suggested that elevated insulin levels may be the more important factor in the pathogenesis of neointimal enlargement.

The proliferation and motility of vascular smooth muscle cells is regulated in reciprocal fashion by stimulators (e.g., fibroblast growth factor, platelet-derived growth factor, and heparin-binding epidermal growth factor) and inhibitors of mitogenesis or motility (e.g., prostanooids, heparin, atrial natriuretic factor, and transforming growth factor-β). Similar to many other growth factors, insulin has the capacity to stimulate cell motility in vitro, which could explain the pathogenesis of diabetic neointimal growth. Early studies from several laboratories, including ours, have reported that NO decreases vascular smooth muscle cell proliferation in subcultured cells from adult rats or in primary cultures from newborn rats. More recent studies have reported that NO also inhibits aortic smooth muscle cell motility. Furthermore, several studies in vivo have reported that NO, or its precursor arginine, attenuates neointima formation after experimental vascular injury. These findings are consistent with the capacity of NO to decrease smooth muscle cell motility in culture, at least in cells expressing a relatively dedifferentiated cytoskeletal phenotype, as found in cultured cells from newborn rats. Of further relevance is the finding that type II diabetes may be associated with a deficit of NO; moreover, amelioration of the consequences of hyperinsulinemia by NO has also been reported.

Potential biochemical mechanisms that could explain the inhibitory effect of NO on cell motility include an inhibition of Raf activity, leading to an inhibition of mitogen-activated protein kinase (MAPK) activity. More recently, we have reported that NO increases the activity of protein tyrosine phosphatase 1B (PTP1B), a tyrosine phosphatase that targets several focal adhesion proteins, and using antisense oligodeoxynucleotides, we have found that the capacity of NO to decrease baseline cell motility may be mediated via increased PTP1B activity.
Several publications have reported that tyrosine-phosphorylated insulin receptor β (IRβ) is a substrate for PTP1B. Moreover, a recent study has shown that a related enzyme, PTP-PEST, has the capacity to dephosphorylate a tyrosine-phosphorylated oligo peptide sequence based on the insulin receptor.

**Methods**

**Materials**

Lactating female rats of the Sprague-Dawley strain and their pups were purchased from Charles River Labs (Wilmington, Mass). Pups of the same strain were bred in the University of Tennessee (Memphis) vivarium. Primary tissue culture plates or dishes were from Falcon/Becton Dickinson. Type I collagenase, soybean trypsin inhibitor, FBS, BSA (fraction V), and N-acetylpenicillamine (SNAP) were from Sigma Chemical Co. The MAPK/Erk kinase (MEK) inhibitor 1,4-diamino-2,3-dicyano-1,4-dihydronicotinamide (SNAP) were from Sigma Chemical Co. The PTP-PEST–mediated dephosphorylation of the insulin receptor, followed by dephosphorylation of insulin receptor substrate-1 (IRS-1) and attenuation of extracellular signal–regulated kinase (Erk) activity.

**Immunoprecipitation and Immunoblotting**

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 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin. Protein concentration in lysates was determined by the bicinchoninic acid method. Immunoprecipitation was performed by incubation of cell lysates (~1 mg protein) with antibodies directed against the IRβ subunit or IRS-1 (4 μg) overnight at 4°C, followed by the addition of protein G–Sepharose beads (Pharmacia) and further incubation for 1.5 hours. Sepharose beads were then washed twice with the above-mentioned buffer and once with 50 mmol/L Tris buffer (pH 7.2) containing 150 mmol/L NaCl, 1 mmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonyl fluoride, followed by boiling of the beads in Laemmli sample buffer and loading of the supernatant onto polyvinylidene difluoride (PVDF) membranes (Immobilon PVDF, Millipore), followed by probing with anti-phosphotyrosine (PY20) antibodies (1:2500). Blots were then stripped and reprobed with primary antibodies directed against IRβ (1:500) or IRS-1 (1:500), followed by HRP-coupled secondary antibodies with appropriate specificity. Immunoreactive bands were visualized by using Renaissance chemiluminescence reagents (DuPont/NEN).

**Preparation of Recombinant Adenovirus Expressing Wild-Type or Mutant PTP Proteins**

Replication-deficient (E1–deleted) recombinant type 5 adenovirus expressing wild-type PTP1B or PTP-PEST (WT-PTP1B or WT-PTP-PEST, respectively) or mutant PTP1B or PTP-PEST was prepared with the use of a commercial kit (Adeno-X) purchased from Clontech; the manufacturer’s instructions were followed. Plasmids containing HA-tagged wild-type human sequence PTP1B (p3JH-PTP345), catalytically inactive PTP1B (p3JH-PTP345-C215S), or PTP1B mutated at the proline-rich domain (p3JH-PTP345-P309A, P310A) were kindly donated by Jonathan Chernoff (Fox Chase Cancer Institute, Pa). Plasmid (pGEM3Z-PTPPEST) containing mouse sequence WT-PTP-PEST was kindly provided by Dr Andre Veillette (Montreal, Canada). All sequences of PTP1B and PTP-PEST cDNAs were verified by nucleotide sequencing and confirmed to published data.

Adenoviral vector expressing WT-PTP-PEST was prepared as follows: Plasmid (pGEM3Z-PTPPEST) containing mouse sequence PTP-PEST was kindly provided by Dr Andre Veillette (Montreal, Canada). A 9–aminohydrol acid HA epitope at the N-terminus and BsgI and BstXI restriction sites was introduced by polymerase chain reaction. The polymerase chain reaction product was subcloned into pShuttle vector by ligation into the Xbal/BstXI site. The cDNA for PTP-PEST in the pShuttle vector was sequenced and conforms to published data. Recombinant adenoviral DNA expressing PTP-PEST was then generated as described above for PTP1B.

Adenoviral vector expressing constitutively activated S218D/S222D-MEK1 was prepared via subcloning of cDNA obtained from Upstate Biotechnology, with the use of an adenoviral preparation kit developed by the University of Iowa. Briefly, plasmid cDNA was digested with KpnI and XhoI and ligated into the KpnI/XhoI site of the pShuttle vector. Recombinant homogeneous virus was then generated in HEK 293 cells by homologous recombination, as described.

Adenoviral titers were determined via a standard procedure by measurement of their cytopathic effect in HEK 293 cells.

**Western Blot Analysis of Erk1/Erk2 Phosphotyrosine Levels**

After treatment with SNAP (50 μmol/L) in the absence or presence of insulin (100 mmol/L), cells were lysed in denaturing buffer of the following composition: 88 mmol/L Tris-HCl (pH 6.8), 10 mmol/L sodium pyrophosphate, 15% glycerol, 3% SDS, 1 mmol/L sodium vanadate, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 mmol/L EDTA. Cultures were subjected to vortexing for 15 minutes, and collected lysates were immediately boiled for 5 minutes and cleared by precipitation in a microcentrifuge at 16 000g for 20 minutes. Equivalent amounts of proteins were separated on 12% SDS-PAGE and electrophoretically transferred to PVDF membrane. Membranes were blocked and probed with polyclonal rabbit anti–phospho-Erk1/Erk2 antibodies (Promega, 1:20 000), followed by incubation with goat anti-rabbit IgG conjugated with HRP (Sigma, 1:10 000). To verify equivalent loading of lysates, membranes were stripped and reprobed with anti–MAPK (p44/42)-HRP conjugates (ZYMED Laboratories, 1:2000).

**Measurement of Erk Activity**

Erk activity was determined by use of a commercial kit (New England Biolabs), according to the manufacturer’s suggestions.

**Measurement of Cell Motility**

Cell motility was measured via a monolayer woundng assay, as described in a previous publication. The number of migrating cells was expressed as cell number per millimeter scratch.
Data Analysis

Data are expressed as mean±SEM and were statistically evaluated by using the Student paired t test or ANOVA, followed by the Fisher test. A value of P<0.05 was considered to be significant.

Results

NO Blocks Insulin-Stimulated Cell Motility

NO has the capacity to decrease cell motility in cultured aortic smooth muscle cells. In the present study, we investigated the effect of NO on insulin-stimulated cell motility. To this end, we used a standard monolayer-wounding assay in which cells migrate to fill the gap created by removal of a narrow strip of cells. Insulin stimulated the motility of aortic smooth muscle cells by ~3-fold, confirming a recent report. Conversely, the NO donor SNAP attenuated the capacity of insulin to stimulate cell motility by ~80% (please see online Figure I, which can be accessed at http://atvb.ahajournals.org).

WT-PTP1B Blocks Insulin-Stimulated Cell Motility

We have previously shown that NO has the capacity to increase PTP1B activity in vascular smooth muscle cells. Other studies have reported that the tyrosine-phosphorylated insulin receptor is a target for PTP1B. These findings prompted us to test the hypothesis that NO-induced elevation of PTP1B is sufficient to account for the motility-inhibitory effect of NO.

For these experiments, we used adenoviral vectors to transduce cells at high efficiency (>90% efficiency, as shown by enhanced green fluorescent protein expression; data not shown). Primary cultures of aortic smooth muscle cells were treated for 2 hours with adenovirus expressing enhanced green fluorescent protein (Ad-EGFP) as a control for viral infection, per se, and virus expressing WT-PTP1B (Ad-WT-PTP1B), catalytically inactive C215S-PTP1B (Ad-C215S-PTP1B, catalytically essential cysteine mutated to serine), or P309A/P310A-PTP1B (Ad-P309/P310A-PTP1B, mutated in the proline-rich region that serves as a ligand for Src homology-3 [SH3] domain–containing proteins). As shown in online Figure II (http://atvb.ahajournals.org), all 3 transgenic PTP1B proteins were expressed at equivalent levels that were ~5-fold greater than basal, as determined by Western blotting. In separate experiments, we measured the activity of PTP1B in immunoprecipitates of lysates from cells overexpressing WT-PTP1B and observed a ~4-fold increase in immunoprecipitated PTP activity compared with mock immunoprecipitation using an irrelevant antibody (data not shown). We also verified that phosphatase activity could be decreased by ~90% by the PTP inhibitor vanadate (10 mmol/L, data not shown).

To provide formal demonstration of transgenic protein expression, we also measured the levels of HA-tagged WT-PTP1B or mutant PTP1B via Western blotting using an antibody directed against HA and, as shown in online Figure II, we observed increased expression of HA-tagged protein. Cells overexpressing WT-PTP1B or mutant PTP1B proteins were then stimulated with insulin, and cell motility was measured as described in Methods. Overexpression of WT-PTP1B completely blocked the capacity of insulin to stimulate cell motility (Figure 1). In contrast, neither C215S-PTP1B nor P309A/P310A-PTP1B elicited a significant effect (Figure 1). These results support the view that the capacity of NO to increase PTP1B levels is sufficient to account for NO-induced inhibition of cell motility.

Dominant-Negative PTP1B Mutant, C215S-PTP1B, Blocks Motility Inhibitory Effect of NO

To test the hypothesis that NO-induced PTP1B is necessary for the inhibition of insulin-stimulated cell motility, we treated cells without or with Ad-C215S-PTP1B and measured the effect of expression of C215S-PTP1B on insulin-stimulated cell motility. As shown in Figure 2, expression of C215S-PTP1B antagonized the capacity of NO to decrease insulin-stimulated cell motility, indicating that endogenous PTP1B is necessary for the NO-induced inhibition of cell motility. Interestingly, treatment with Ad-C215S-PTP1B alone increased cell motility, suggesting that constitutively active PTP1B attenuates cell motility. This finding is in...
agreement with a previous study from our laboratory reporting that antagonism of endogenous PTP1B via the use of antisense oligonucleotides also increases basal cell motility.27

NO Decreases Insulin Receptor Phosphotyrosine Levels

The next experiments were performed to investigate the biochemical mechanisms underlying the capacity of NO to decrease insulin-stimulated cell motility. Insulin is thought to initiate its signaling cascade via activation and autophosphorylation of IRβ.28 Consequently, primary aortic smooth muscle cells were treated without or with the NO donor SNAP and/or insulin, followed by preparation of cell lysates for measurement of IRβ phosphotyrosine levels by Western blotting. As shown in Figure 3, treatment with insulin increased IRβ phosphotyrosine levels by ∼4-fold, as expected, whereas treatment with the NO donor blocked this effect (summary of all experiments is provided in online Figure III, http://atvb.ahajournals.org). Moreover, SNAP (1 to 100 μmol/L) elicited dephosphorylation of IRβ in concentration-dependent manner (data not shown).

WT-PTP1B but Not Catalytically Inactive PTP1B Decreases Insulin Receptor Phosphotyrosine Levels

The goal of our next experiments was to test the hypothesis that increased PTP1B is sufficient to account for NO-elicited dephosphorylation of IRβ. Thus, we measured the effect of (over)expression of WT-PTP1B or mutant PTP1B proteins on insulin-induced tyrosine phosphorylation of IRβ. Accordingly, aortic smooth muscle cells were treated with Ad-EGFP (control), Ad-WT-PTP1B, Ad-C215S-PTP1B, or Ad-P309/P310A-PTP1B. The latter virus expresses a catalytically active PTP1B allele that is mutated at proline residues 309 and 310 and is, therefore, unable to interact with important substrates such as p130cas, containing the cognate SH3 domain ligand. As shown in Figure 4 (summary of results is provided in online Figure IV, http://atvb.ahajournals.org), overexpression of WT-PTP1B blocked insulin-induced IRβ tyrosine phosphorylation. In contrast, neither catalytically inactive mutant C215S-PTP1B nor catalytically active but proline-deficient mutant P309A/P310A-PTP1B was able to decrease insulin receptor phosphotyrosine levels. Indeed, the C215S mutant actually enhanced insulin-stimulated phosphotyrosine levels, presumably by functioning as a dominant antagonist of endogenous PTP1B. These results support the view that elevation of PTP1B is sufficient to explain NO-induced dephosphorylation of the insulin receptor.

PTP-PEST Fails to Decrease Insulin Receptor Phosphotyrosine Levels

PTP-PEST targets many of the proteins also targeted by PTP1B,29 potentially including the insulin receptor.30 More-
over, our recent unpublished studies indicate that NO increases the activity of PTP-PEST (data not shown). Therefore, we investigated whether PTP-PEST may be involved in NO-elicited dephosphorylation of IRβ by measuring IRβ phosphotyrosine levels in insulin-treated cells overexpressing WT-PTP-PEST. However, overexpression of PTP-PEST failed to affect the phosphotyrosine levels of IRβ (data not shown), indicating that this enzyme is unlikely to mediate the capacity of NO to antagonize insulin-stimulated signal transduction pathways.

**NO Decreases IRS-1 Phosphotyrosine Levels**

IRS-1 has been identified as an important substrate of IRβ kinase and is a protein necessary for insulin activity.30 Because NO decreases phosphotyrosine levels of IRβ, we investigated whether NO might alter phosphotyrosine levels of IRS-1. Thus, cells were treated with SNAP, in the presence or absence of insulin, followed by measurement of IRS-1 phosphotyrosine levels. As shown in online Figure V (http://atvb.ahajournals.org), treatment with SNAP significantly reduced IRS-1 phosphotyrosine levels. Moreover, SNAP also attenuated the capacity of insulin to increase IRS-1 phosphotyrosine levels.

**NO Induces Loss of Phosphotyrosine and Inactivation of Erk1/Erk2**

Erk is a downstream effector in the insulin-signaling pathway that directs cell motility in vascular smooth muscle cells.31 Erk1/Erk2 activity is regulated, in part, via alteration of the phosphotyrosine levels of amino acid residues Thr183 and Tyr185.32 Therefore, we treated vascular smooth muscle cells without or with SNAP in the absence or presence of insulin and measured the levels of phospho-Erk via an antibody that specifically recognizes a 16–amino acid epitope containing phospho-Thr183 and phospho-Tyr185 residues. As shown in Figure 5 (summary of results shown in online Figure VI, http://atvb.ahajournals.org), insulin induced Erk1/Erk2 phosphorylation, whereas SNAP elicited the opposite effect. Moreover, SNAP blocked the capacity of insulin to induce the phosphorylation of Erk1/Erk2.

To confirm the effect of NO on phosphorylation levels of Erk1/Erk2, we also measured Erk1/Erk2 enzyme activity. Thus, cells were treated without or with SNAP in the absence or presence of insulin, followed by measurement of Erk activity via a specific immunokinase assay. As depicted in online Figure VII (http://atvb.ahajournals.org), Erk activity was reciprocally increased by insulin and decreased by SNAP. Moreover, treatment with SNAP blocked the stimulatory effect of insulin.

**Dominant-Negative PTP1B Mutant, C215S-PTP1B, Blocks Capacity of SNAP to Decrease Erk Phosphotyrosine Levels**

If PTP1B were necessary for NO-induced attenuation of insulin-stimulated Erk phosphorylation, we would expect a dominant-negative allele of PTP1B to block this effect. To test this hypothesis, we treated cells with Ad-C215S-PTP1B or Ad-EGFP in the absence or presence of insulin or SNAP.

**Figure 4.** WT-PTP1B, but not mutant PTP1B, blocks the capacity of insulin to increase IRβ phosphotyrosine levels. Cells (over)expressing WT-PTP1B or EGFP were treated without or with insulin (100 nmol/L) for 1 hour. After treatment, cells were lysed, and lysates were immunoprecipitated with antibody directed against IRβ. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were first immunoblotted with anti-phosphotyrosine (A). Loading of equivalent amounts of protein was confirmed by stripping membranes and reprobing with antibody directed against IRβ (B). Panels A and B show results from a single representative experiment.

**Figure 5.** Treatment with NO donor SNAP decreases levels of phospho-Erk1/Erk2 and attenuates the capacity of insulin to induce Erk phosphorylation. Cells were treated for 1 hour without or with SNAP (50 μmol/L) in the presence or absence of insulin (100 nmol/L), followed by lysis and immunoblotting using antibody directed against phospho-T183 and phospho-Y185 (A). Membranes were then stripped and reprobed with anti-Erk1/Erk2 to verify the presence of equivalent amounts of Erk protein (B).
As shown in Figure 6 (with summary of experiments given in online Figure VIII, http://atvb.ahajournals.org), Ad-C215S-PTP1B blocked the capacity of NO to decrease insulin-stimulated dephosphorylation of Erk1/Erk2, thus confirming the pivotal role of PTP1B in mediating the Erk-inhibitory effect of NO.

**Constitutively Active MEK Induces Cell Motility Whereas Selective MEK Inhibitor, U0126, Attenuates Insulin-Stimulated Cell Motility**

As shown above, NO decreases Erk activity and cell motility. To test the hypothesis that the decrease of Erk activity is necessary and sufficient to explain NO-induced inhibition of cell motility, we treated cells with an adenovirus encoding constitutively active S218D/S222D-MEK1, an upstream activator of Erk. Treatment of the cells with MEK1 adenovirus increased its expression levels by ~3-fold, as determined by Western blotting (data not shown). As shown in online Figure IX (http://atvb.ahajournals.org), overexpression of MEK1 increased the motility of aortic smooth muscle cells; moreover, the NO donor SNAP failed to attenuate this effect, consistent with the notion that NO targets a process upstream from MEK. In other experiments, a selective inhibitor of MEK, U0126, blocked the motility-stimulatory effect of insulin, further supporting the view that the NO-induced inhibition of Erk activity is causally related to the NO-induced decrease of cell motility (online Figure X, http://atvb.ahajournals.org).

**Discussion**

In the present study, we have used primary cultures isolated from newborn rats to investigate the role of NO as a modulator of insulin-stimulated cell motility in aortic smooth muscle cells.

We have used this particular model for 2 reasons: (1) Many of the biological effects of NO are most potently manifested in cells expressing relatively high levels of protein kinase G activity. Moreover, it is well established that subculture of the aortic smooth muscle cells reduces protein kinase G levels after a few subcultures, thus necessitating the use of primary or early subculture cells. (2) Neointimal cells that are generated in vascular disease manifest a partially dedifferentiated cytoskeletal phenotype that is closely mimicked in culture by cells isolated from newborn rats.

We have previously reported that NO increases PTP1B activity induced by NO. On the other hand, studies from other laboratories have found that PTP1B has the capacity to dephosphorylate the insulin receptor, followed by reduced phosphorylation of IRS-1 and attenuation of the MAPK pathway. This conclusion is based on multiple observations:

1. Overexpression of WT-PTP1B mimics the capacity of NO to decrease insulin-stimulated cell motility, indicating that PTP1B is sufficient to mediate the motility inhibitory effect of NO. It should be noted that the levels of PTP1B overexpression for protein and activity are 4- to 5-fold above baseline, similar to the previously reported induction of ~3-fold by NO.

2. Expression of the dominant-negative PTP1B mutant, C215S-PTP1B, blocks the capacity of NO to decrease insulin-stimulated cell motility, indicating that PTP1B is necessary for the inhibition of cell motility by NO. Interestingly, a second PTP1B mutant, P309A/P310A-PTP1B, lacking the SH2 domain binding site but expressing full enzyme activity, is ineffective as an inhibitor of cell motility, indicating the importance of the proline-rich region in modulating insulin signal transduction pathways.

3. NO decreases insulin receptor phosphotyrosine levels. Tyrosine phosphorylation of IR is necessary for insulin-induced activation of the Ras-Raf-MEK-MAPK pathway;
therefore, its dephosphorylation would be expected to block insulin-stimulated effects.

4. NO decreases IRS-1 phosphotyrosine levels, in association with decreased MAPK activity. IRS-1 contains 22 potential sites of tyrosine phosphorylation, providing multiple binding sites for Src homology-2 domain-containing proteins. The mechanism leading to the activation of p21ras by insulin is thought to involve binding of the phosphotyrosine-binding domain of IRS-1 to tyrosine-phosphorylated IRβ. IRS-1 is then tyrosine-phosphorylated by insulin receptor kinase, generating ligand sites for binding to the Src homology-2 domain of Grb2, an adapter protein that directly associates with IRS-1. Grb2 is constitutively associated with the GTP-GDP exchange factor msSOS (the mammalian homologue of Drosophila son-of-sevenless protein) that, in turn, activates small GTPase p21ras, leading to activation of the MAPK cascade. In our experiments, independent inhibition of MAPK via the use of a selective pharmacological inhibitor, U0126, also blocked cell motility. Conversely, expression of a well-established activator of Erk, constitutively active MEK1, stimulated cell motility. Taken together, these observations support the notion that the inhibition of Erk is sufficient to explain the inhibition of cell motility by NO.

5. Overexpression of PTP1B reduces IRβ phosphotyrosine levels, indicating that PTP1B is sufficient to mediate the effect of NO as an inhibitor of insulin-mediated signal transduction mechanisms.

6. Expression of the dominant-negative PTP1B mutant, C215S-PTP1B, attenuates the capacity of NO to elicit insulin receptor dephosphorylation and Erk dephosphorylation, most likely by blocking the effect of endogenous PTP1B. This indicates that PTP1B is necessary to mediate the effect of NO as an inhibitor of insulin-mediated signal transduction. Thus, taken together, these observations provide a biochemical explanation for the motility inhibitory effect of NO via a mechanism involving PTP1B-induced dephosphorylation of the IRβ subunit.

It is interesting to note that the present results are in contrast to recent publications from our laboratory in which we reported that NO has the capacity to stimulate the motility of primary cultured cells from adult rats. This difference appears to be correlated with the different cytoskeletal phenotypes of the 2 cell types, inasmuch as cells isolated from newborn rats express a phenotype less differentiated than that of cells isolated from adult rats. Moreover, the motility stimulatory effect of NO appears to require another nonmembrane protein tyrosine phosphatase, SHP2, as opposed to the present results, which implicate PTP1B as an inhibitor of cell motility. Experiments currently in progress aim to uncover mechanisms that could explain the differential involvement of PTP1B versus SHP2 in NO-induced changes in vascular smooth muscle cell motility.

The pathophysiological relevance of the present results is based on the findings that hyperinsulinemia appears to play an important role in the pathogenesis of atherosclerotic vascular disease or postangioplasty restenosis in diabetes. This notion is based on recent studies indicating that neointimal enlargement may be dependent on elevated insulin rather than elevated glucose levels. Interestingly, one of these studies found that hyperinsulinemia without diabetes also resulted in enhanced neointima formation after vascular injury, a finding that underscores the pivotal role of elevated insulin as a vascular pathogen. Other studies have reported that diabetes may be associated with NO deficiency, a finding that could explain the tendency of insulin to enhance neointimal formation in vascular disease.

In summary, the present study shows that NO decreases phosphotyrosine levels of the proximal downstream signaling pathway of insulin, including that of IRβ and IRS-1, via activation of PTP1B and that these effects then lead to reduced Erk1/Erk2 activity and cell motility.

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References


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Figure I: Treatment with NO donor SNAP attenuates insulin-stimulated cell motility. Aortic smooth muscle cells were treated without or with SNAP (50 µM) for 24 h in the absence or presence of insulin (100 nM). Cell motility was determined as described in Methods. Results are the mean ± SEM of 4 experiments. * Indicates p<0.001 relative to control whereas ** indicates p<0.001 relative to insulin, by analysis of variance, followed by Fisher’s post-hoc test.
A

B

CONTROL  EGFP  wt-PTP1B  CS-PTP1B  PA-ptp1B

C

PTP1B protein levels (fold stimulation)

control  EGFP  wt-ptp1b  cs-ptp1b  pa-ptp1b
Figure II: Adenoviral vector-mediated expression of wild type or mutant PTP1B in aortic smooth muscle cells. Cells were incubated for 2 h with Ad-EGFP (as control for viral infection), Ad-WT-PTP1B, Ad-C215S-PTP1B or Ad-P309A/P310A-PTP1B, at a multiplicity of infection (m.o.i.) of 10-15. Virus containing media were then removed and further culture was carried out for 48 h to allow time for expression of transgenic proteins. Cells were then lysed and proteins analyzed by SDS-PAGE, followed by Western blotting. Panel A shows a representative blot using anti-PTP1B, followed by reprobing with anti-HA (Panel B). Panel C provides the summary of all experiments for Western blotting with anti-PTP1B. Data are expressed as the mean ± SEM of fold-stimulation relative to control (untreated) for 3 experiments.
Figure III. Summary of data for effect of NO donor on basal or insulin-elevated insulin receptor beta subunit (IRβ) phosphotyrosine levels. Cells were treated without or with SNAP (50 μM), in the absence or presence of insulin (100 nM) for 1 h, followed by cell lysis and immunoprecipitation with antibody directed against IRβ. Immunoprecipitated proteins were separated by SDS/PAGE and transferred to PVDF membranes. Membranes were then immunoblotted with anti-phosphotyrosine. Loading of equivalent amounts of protein in each treatment category was confirmed by stripping membranes and reprobing with antibody.
directed against IRβ. Data are the mean ± SEM of 3 experiments and are expressed as fold stimulation relative to control (Ad-EGFP) for IRβ phosphotyrosine levels divided by IRβ protein levels. * indicates p<0.05, relative to control. ** Indicates p<0.05 relative to insulin via Student’s paired t-test.

Abbreviation: pY, phosphotyrosine
Figure IV. Summary of data for effect of overexpression of wild-type or mutant PTP1B on the capacity of insulin to increase IRβ phosphotyrosine levels. Cells (over)expressing WT-PTP1B, C215S-PTP1B, P309A/P310A-PTP1B or EGFP were treated without or with insulin (100 nM) for 1 h. Following treatment, cells were lysed and lysates immunoprecipitated with antibody
directed against IRβ. Immunoprecipitated proteins were separated by SDS/PAGE and transferred to PVDF membranes. Membranes were first immunoblotted with anti-phosphotyrosine. Loading of equivalent amounts of protein was confirmed by stripping membranes and reprobing with antibody directed against IRβ. Data are from 3 experiments representing IRβ phosphotyrosine levels divided by IRβ protein levels (mean ± SEM). Abbreviation: pY, phosphotyrosine.
Figure IX. Constitutively active MEK1 increases cell motility but NO fails to attenuate this effect. Cells were infected with recombinant Ad-EGFP or Ad-S218D/S222D-MEK1, at an m.o.i. of 10 for 2 h. Virus containing media were then removed and cells were cultured for an additional 48 h to allow for expression of transgenic proteins. This was followed by measurement of cell motility in the absence or presence of SNAP (50 µM), as described under Methods. Results are the mean ± SEM of three experiments. Abbreviation: CA-MEK1, constitutively active S218D/S222D-MEK1.
Figure V. NO donor SNAP attenuates the capacity of insulin to increase IRS-1 phosphotyrosine levels. Cells were treated without or with SNAP (50 µM), in the absence or presence of insulin (100 nM) for 1 h, followed by lysis and immunoprecipitation of lysates with antibodies directed against IRS-1. Immunoprecipitated proteins were separated by SDS/PAGE and transferred to PVDF membranes. Membranes were first immunoblotted with antiphosphotyrosine (panel A), followed by stripping and reblotting with antibodies directed against IRS-1 (panel B). Panels A and B show results from a single representative experiment whereas panel C gives the summary of 3 experiments. Results are expressed as fold stimulation relative to control (no treatment) of IRS-1 phosphotyrosine levels divided by IRS-1 protein levels (mean ± SEM). Abbreviation: pY, phosphotyrosine.
Figure VI. Summary of data for effect of treatment with NO donor SNAP on levels of phospho-Erk1/Erk2. Cells were treated for 1 h without or with SNAP (50 µM), in the presence or absence of insulin (100 nM), followed by lysis and immunoblotting using antibody directed against phospho-T183 and phospho-Y185. Membranes were then stripped and reprobed with anti-Erk1/Erk2 to verify the presence of equivalent amounts of Erk protein. Data are from 3 experiments (mean ± SEM) and are expressed as fold stimulation relative to control (no treatment), of phospho-Erk levels divided by Erk protein levels (mean ± SEM).
Figure VII: Treatment with NO donor SNAP decreases basal and insulin-enhanced Erk1/Erk2 activity. Cells were treated without or with SNAP (50 µM),
in the absence or presence of insulin (100 nM) for 1 h, followed by lysis and immunoprecipitation with immobilized anti-phospho Erk1/Erk2. Enzyme activity was determined by incubation of immunoprecipitated Erk1/Erk2 with Elk-1 as substrate in the presence of ATP, followed by Western blot analysis using anti-phospho-Elk-1. Panel B shows the summary of 3 experiments, expressed as mean ± SEM of fold increase relative to control (no treatment). * Indicates p<0.001 relative to control whereas ** indicates p<0.001 relative to insulin, via paired Student’s t-test.
Figure VIII. Summary of data for effect of treatment with dominant negative PTP1B mutant, C215S-PTP1B, on the capacity of NO donor SNAP to decrease insulin-stimulated Erk phosphorylation. Primary aortic smooth muscle cells were incubated with Ad-EGFP (control) or Ad-C215S-PTP1B for 2 h at an m.o.i. of 10. Virus containing media were then removed and cells were cultured for an additional 48 h to allow for transgenic protein expression. This
was followed by measurement of cell motility in the absence or presence of insulin (100 nM) or SNAP (50 µM), as described under Methods. Following treatment, cells were lysed and immunoblotted using antibody directed against phospho-T183 and phospho-Y185. Membranes were then stripped and reprobed with anti-Erk1/Erk2 to verify the presence of equivalent amounts of Erk protein. Figure shows the data from 3 experiments (mean ± SEM), expressed as fold stimulation relative to control (no treatment) of phospho-Erk levels divided by Erk protein levels (mean ± SEM).
Figure X: Treatment of vascular smooth muscle cells with MEK inhibitor U0126 attenuates insulin-stimulated cell motility. Cells were treated with 1 µM U0126 in the presence or absence of 100 nM insulin and cell motility was measured as described in Methods. Results are the mean ± SEM of 3 experiments.