Nitric oxide attenuates IGF-I-induced aortic smooth muscle cell motility by decreasing Rac1 activity: essential role of PTP-PEST and p130cas

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Recent studies have provided broad support for a critical role for oxidative stress in the pathogenesis of vascular disease (23, 24). Several publications have reported that nitric oxide (NO) functions as an antioxidant in leukocytes (5, 10, 28). Interestingly, in human blood vessels, NO levels are inversely correlated with O2− levels, consistent with a potential antioxidant role for NO (13).

Exogenous and endogenous NO attenuate neointima formation in several models of vascular injury, consistent with the inhibition of vascular smooth muscle cell (VSMC) motility and proliferation by NO (29, 38). We reported previously (22) that NO decreases aortic SMC motility by increasing the activity of protein tyrosine phosphatase-proline, glutamate, serine, and threonine sequence protein (PTP-PEST). PTP-PEST is a ubiquitously expressed nonmembrane protein tyrosine phosphatase the upregulation of which is both necessary and sufficient for NO-induced inhibition of motility in cultured rat aortic SMCs (22). IGF-I is thought to play an important role in neointima formation (21, 42, 43). We reported previously (44) that IGF-I increases the motility of aortic SMCs and that H2O2 elevation induced by IGF-I is attenuated by NO, via decreased NAD(P)H oxidase activity. We also previously reported (44) that H2O2 functions as a second messenger in the regulation of cell motility, thus providing a potential explanation for the finding that antioxidants attenuate cell motility and neointima formation. A requirement of the small GTPase Rac1 in the activation of NAD(P)H oxidase (15) further suggests a potential interaction of NO with Rac1 in VSMCs.

The present study was performed to test the hypothesis that the mechanism by which NO decreases rat aortic SMC motility involves decreased levels of phosphotyrosine in adapter protein p130cas via increased PTP-PEST activity followed by decreased H2O2 levels and cell motility.

Despite the importance of Rac1 for vascular reactive oxygen species (ROS) generation, its regulation in the cardiovascular system is only partially established. Our results involving decreased Rac1 activity induced by PTP-PEST upregulation uncover a novel and important mechanism by which NO might interfere with oxidant production in VSMCs.

MATERIALS AND METHODS

Materials. Type I collagenase, soybean trypsin inhibitor, p-nitrophenyl phosphate, and protease inhibitor cocktail were obtained from Sigma (St. Louis, MO). Porcine pancreatic elastase was obtained from Collaborative Research (Lexington, MA). DMEM-Ham’s F-12 medium and FBS were purchased from Gibco-BRL (Grand Island, NY) or CellGro (Herndon, VA). (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonio-ethyl)amino]-diazen-1-ium-1,2-diolate (DETA-NO) was purchased from Alexis Biochemicals (Carlsbad, CA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR). Rabbit antisera against PTP-PEST was raised by Covance (Denver, PA) using an oligopeptide immunogen corresponding to PTP-PEST amino acid residues 444–457 in the mouse sequence or amino acid residues 445–458 in the human sequence. Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated antibodies, as well as mouse antibody against p130cas, were purchased from Transduction Laboratories (Lexington, KY). Antibody against phosphotyrosine (RC20) was obtained from BD Transduction Laboratories (San Jose, CA). Mouse antibody against Rac1 was purchased from Upstate Biotechnology (Lake Placid, NY).

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Cell isolation and culture. Lactating Sprague-Dawley rats and their pups were obtained from Charles River Laboratories (Wilmington, MA), or pups of the same strain were bred in the University of Tennessee vivarium. VSMCs from the thoracic aortas of 6- to 9-day-old Sprague-Dawley rats were isolated by performing enzymatic dispersion as described previously (4). Cells were grown in a 5% CO₂ atmosphere at 37°C in DMEM-Ham’s F-12 culture medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. Cells were maintained in quiescence medium lacking FBS for 48 h before treatment. Each experiment was performed using primary cultures from an independent isolate. The studies were performed according to protocols approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center and in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication no. 86-23).

Preparation and expression of adenoviral vectors. Replication-deficient (E1 deleted) recombinant type 5 adenoviruses expressing wild-type or mutant proteins were prepared using a kit provided by the University of Iowa Gene Transfer Vector Core Facility (Iowa City, IA; Ref. 2). PTP-PEST and p130cas viruses were generated as described previously (22). Plasmid pcDNA3.1-3xHA-Rac1 containing the wild-type human Rac1 cDNA sequence, as well as plasmid pcDNA3.1-3xHA-Rac1TN17 containing the Rac1 dominant-negative mutant, were purchased from the Guthrie Institute (Sayre, PA). Both cDNA were excised via the KpnI/HindIII restriction sites and then subcloned into equivalent restriction sites of the adenovirus pShuttle vector (pAd5CMV). Recombinant adenovirus was obtained by cotransfecting linearized recombinant pShuttle and adenoviral backbone DNA (pAd59.2-100) as described previously (2). Experimental cells were infected with recombinant adenovirus at a multiplicity of infection (MOI) index of 5–10. This MOI provides transduction efficiency of 80–100% (22, 33).

Rac1 activity assay. Confluent, serum-starved primary aortic SMCs were infected for 48 h with recombinant adenoviruses expressing enhanced green fluorescent protein (EGFP) or PTP-PEST. Cells were then treated without or with 3.3 nM IGF-I for 30 s, followed by measurement of GTP-bound Rac1 using a pull-down assay (30). Samples were loaded onto 4–12% SDS gradient gels. After being transferred onto polyvinylidene difluoride membrane, GTP-bound Rac1 was measured using Western blot analysis with antibody directed against Rac1 and then normalized to levels of total Rac1 in lysates.

Measurement of intracellular H₂O₂ levels. Intracellular H₂O₂ levels were estimated by measuring the fluorescence of oxidized dichlorodihydrofluorescein diacetate (DCF-DA) as described previously (44). Confluent, serum-starved primary aortic SMCs were infected for 48 h with recombinant adenoviruses expressing only adenoviral proteins (empty adenovirus), Rac1, p130cas, dominant-negative (dn)-p130cas, PTP-PEST, or dn-PTP-PEST. Infected cells were then treated with 10 µM DCF-DA for 10 min. These cells were then treated without or with 30 µM DETA-NO for 30 min, followed by treatment without or with 3.3 nM IGF-I for 30 s. Selected samples were treated with catalase as described previously (44) to verify that oxidized DCF-DA fluorescence levels detected only H₂O₂.

Immunoblot analysis and measurement of cell motility. Experiments were performed as described previously (4, 22, 44). Western blot band intensities were measured using densitometry. For measurement of motility, cells were treated for 24 h without or with IGF-I (3.3 nM) and/or DETA-NO (30 µM).

Statistical analysis. All values are expressed as means + SE. Statistical comparisons were performed using two-way ANOVA of raw data, followed by Fisher’s protected least-significant difference test. Differences were considered significant at \( P < 0.05 \).

RESULTS

IGF-I increases Rac1 activity, whereas treatment with the NO donor DETA-NO or overexpression of PTP-PEST blocks IGF-I-induced Rac1 activation. Several studies have implicated plasma membrane NAD(P)H oxidase as the principal enzyme generating ROS in VSMCs (3, 12). Furthermore, a recent study conducted at our laboratory indicated that IGF-I increases the levels of H₂O₂ in cultured rat aortic SMCs and that this effect is both necessary and sufficient to induce cell motility (44). The activation of small GTPase Rac1 is thought to be necessary for expression of NAD(P)H oxidase activity, the principal enzyme that generates agonist-induced ROS in VSMCs (26, 35). Therefore, we were prompted to test the hypothesis that IGF-I has the capacity to induce the activation of Rac1. As shown in Fig. 1, A and B, IGF-I increased Rac1 activity severalfold. Taken together with our previous findings (44) indicating that elevation of H₂O₂ is necessary for the stimulation of cell motility, the results of the present study are consistent with the hypothesis that the activation of Rac1 is necessary for the induction of cell motility.

A previous study conducted at our laboratory indicated that NO decreases cell motility by attenuating agonist-induced activation of NAD(P)H oxidase and H₂O₂ levels (44). Separately, we reported that NO induces activation of PTP-PEST and that this effect is necessary to explain the occurrence of the antimotogenic effect of NO (22). We were therefore interested in determining the effects of the NO donor DETA-NO and overexpression of PTP-PEST on IGF-I-induced Rac1 activation. The present study was performed using primary cultures that are highly responsive to the inhibitory effects of NO as described in several publications by investigators at our laboratory (22, 33, 44). As shown in Fig. 1D, PTP-PEST was expressed well in cells treated with recombinant adenovirus expressing PTP-PEST. Moreover, treatment of cells with the NO donor DETA-NO (Fig. 1, A and B) or with adenovirus expressing PTP-PEST (Fig. 1, A and C) blocked IGF-I-induced Rac1 activation, consistent with the hypothesis that the capacity of NO to attenuate Rac1 activation is dependent on PTP-PEST activity.

IGF-I increases p130cas phosphotyrosine levels, whereas overexpression of PTP-PEST blocks the effect of IGF-I. p130cas is an adapter protein thought to link agonist-induced receptor activation to Rac1 function (20). We reported previously that NO decreases p130cas phosphotyrosin levels via activation of PTP-PEST (22). Subsequent experiments were performed to test the hypothesis that IGF-I induces the elevation of phosphotyrosine levels in p130cas and that overexpression of PTP-PEST attenuates this effect. As shown in Fig. 2, treatment of cells with IGF-I increased the levels of p130cas phosphotyrosine, suggesting that this event could play an important role in transducing the capacity of IGF-I to increase Rac1 activity. Moreover, treatment with adenovirus expressing PTP-PEST blocked the capacity of IGF-I to increase p130cas phosphotyrosyl levels. These results are consistent with the hypothesis that the phosphorylation of p130cas plays a role in IGF-I-induced signaling.

Overexpression of p130cas mimics the stimulatory effect of IGF-I on Rac1 activation, whereas expression of dn-p130cas suppresses the effect of IGF-I. If p130cas were involved in signaling leading to Rac1 activation, we would expect overex-
expression of p130cas to increase Rac1 activity. To test this hypothesis, we infected cells with adenovirus expressing p130cas and found that it induced a significant increase in p130cas protein levels (Fig. 3 C). Moreover, as shown in Fig. 3, A and B, overexpression of p130cas induced marked Rac1 activation.

A previous study conducted by researchers at our laboratory indicated that treatment of aortic SMCs with NO decreased the levels of phosphotyrosine in adapter protein p130cas via up-regulation of PTP-PEST activity and that dephosphorylation of p130cas was sufficient to decrease cell motility (22). On the basis of these findings, we hypothesized that dn-p130cas lacking the so-called substrate domain encompassing at least 15 potential phosphotyrosine residues (19) would mimic the capacity of NO to block IGF-I-induced Rac1 activation. In accordance with this prediction, the use of an adenovirus expressing dn-p130cas resulted in significant expression of the truncated recombinant protein (Fig. 3 C). Moreover, as shown in Fig. 3, A and B, overexpression of p130cas induced marked Rac1 activation.

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mediate the stimulatory effect of IGF-I on Rac1 activity. Conversely, these data also support the concept that the dephosphorylation of p130cas via PTP-PEST activation is sufficient to explain the capacity of NO to suppress IGF-I-induced Rac1 activation.

Overexpression of Rac1 increases H$_2$O$_2$ levels and cell motility and rescues the stimulatory effect of IGF-I in cells treated with NO donor. The aforementioned results are consistent with the hypothesis that activation of Rac1 is sufficient to induce elevation of H$_2$O$_2$ and cell motility levels. To test this hypothesis directly, we treated cells with adenosine expressing Rac1 or with control virus expressing viral proteins only. These experiments were performed using “empty” adenovirus as a control because the control adenovirus expressing EGFP that we used in other experiments interfered with H$_2$O$_2$ measurement using fluorescence microscopy. As shown in Fig. 4, A and B, overexpression of Rac1 induced an increase in H$_2$O$_2$ and cell motility levels equivalent to the increases induced by IGF-I. Moreover, enhanced expression of Rac1 rescued the effects of IGF-I in cells treated with NO donor. It should also be noted that control virus had no significant effect compared with untreated cells (Fig. 1A). These results support the hy-
hypothesis that the activation of Rac1 is sufficient to account for the motogenic effect of IGF-I via increased H$_2$O$_2$ levels.

Expression of dn-Rac1 mimics the inhibitory effect of NO donor on IGF-I-induced H$_2$O$_2$ and cell motility elevation. The aforementioned findings are consistent with the hypothesis that NO and PTP-PEST attenuate cell motility by decreasing Rac1 activity. To test this hypothesis directly, we determined the effect of dn-Rac1 (T17N-Rac1) on IGF-I-induced H$_2$O$_2$ elevation and cell motility. As depicted in Fig. 4, A and B, expression of dn-Rac1 using an adenoviral vector had no effect of its own, but it attenuated IGF-I-induced H$_2$O$_2$ elevation and cell motility. These results indicate that Rac1 activation is necessary for the induction of H$_2$O$_2$ elevation and cell motility by IGF-I and that inhibition of Rac1 activity is sufficient to explain the antimotogenic effect of NO.

Overexpression of PTP-PEST attenuates IGF-I-induced H$_2$O$_2$ elevation and cell motility. We reported previously (22, 44) that PTP-PEST upregulation and decreased H$_2$O$_2$ levels are both necessary and sufficient to mediate the antimotogenic effect of NO. The results reported in the present study indicate that PTP-PEST overexpression suppresses IGF-I-induced phosphorylation of p130$^{a}$ and activation of Rac1. To test the hypothesis that these events could be linked by the reduction of H$_2$O$_2$ levels, we determined the effect of PTP-PEST overexpression on IGF-I-induced elevation of H$_2$O$_2$ and cell motility. As shown in Fig. 5, A and B, treatment of cells with adenovirus expressing PTP-PEST attenuated the capacity of IGF-I to increase H$_2$O$_2$ levels and induce cell motility.

Overexpression of Rac1 rescues the effect of IGF-I on H$_2$O$_2$ elevation and cell motility in cells overexpressing PTP-PEST. If Rac1 were downstream of PTP-PEST in the signal transduction pathway associated with the inhibitory effects of NO, we would expect that overexpression of Rac1 would rescue the stimulatory effect of IGF-I in cells overexpressing PTP-PEST. To test this hypothesis, we treated aortic SMCs with adenovirus expressing PTP-PEST or Rac1 or both. Figure 5C shows the expression of ectopic PTP-PEST or Rac1 in cells treated with corresponding adenoviruses. As shown in Fig. 5, A and B, overexpression of Rac1 increased H$_2$O$_2$ levels and reversed the inhibitory effects of PTP-PEST on H$_2$O$_2$ elevation and cell motility, consistent with the hypothesis linking PTP-PEST to downstream inhibition of Rac1 activity.

$dn$-PTP-PEST rescues the stimulatory effects of IGF-I in cells treated with NO donor. We previously reported (22) that a catalytically inactive PTP-PEST allele (C231S-PTP-PEST) functions as a dominant-negative agent and attenuates the inhibitory effect of NO donor on cell motility, indicating that PTP-PEST function is necessary to mediate NO-induced inhibition of cell motility. To test the hypothesis that the capacity of NO to attenuate IGF-I-induced elevation of H$_2$O$_2$ and cell motility requires PTP-PEST function, we used an adenovirus that expresses $dn$-PTP-PEST (C231S-PTP-PEST). As shown in Fig. 6C, $dn$-PTP-PEST was expressed well and its expression attenuated the capacity of NO to decrease IGF-I-induced H$_2$O$_2$ elevation (Fig. 6A) and cell motility (Fig. 6B).
**DISCUSSION**

The motility of VSMCs is thought to play a pivotal role in neointimal enlargement in a manner associated with the occurrence of vascular disease (27). IGF-I has been reported to play an important role in vascular injury as documented by findings that neointima formation is decreased in rats treated with an IGF-I antagonist and is reciprocally increased in transgenic mice in which IGF-I is overexpressed in blood vessels (21, 43). Moreover, significantly higher IGF-I levels have been observed in coronary VSMCs obtained from patients with atherosclerotic lesions (27) and in injured rat arteries (18).

NO is a well-established inhibitor of SMC motility and proliferation (11, 14), which may explain its capacity to attenuate various aspects of vascular disease in experimental models. Consistent with cell culture studies, NO attenuates neointima formation after vascular injury in most models as documented by numerous studies in rats, rabbits or mice (8, 17, 29, 37, 41). In a previous study, we reported (22) that NO increases PTP-PEST activity in cultured VSMCs and that this effect is both necessary and sufficient to account for the capacity of NO to decrease H2O2 levels occurs via the activation of PTP-PEST activity. Finally, we tested the hypothesis that the capacity of NO to decrease H2O2 levels is both necessary and sufficient to account for the antagonistic effect of NO.

Rac1 is a small GTPase with the capacity to modulate cell motility by altering the cytoskeleton (36). Rac1 also appears to be an essential component of the NAD(P)H oxidase complex in VSMCs via enzyme activity that generates O2− in response to various agonists followed by metabolism of O2− to H2O2 (44). Several studies have linked the generation of ROS to the pathogenesis of vascular disease (34, 39). dn-Rac1 has been reported to attenuate fibroblast motility (9), indicating that it is an essential regulator of cell motility in at least one cultured cell line.

In the present study, we tested several novel hypotheses related to mechanisms mediating the motogenic effect of IGF-I as well as the capacity of NO to block this effect. First, we tested the hypothesis that IGF-I activates Rac1 and that this effect is necessary and sufficient to account for increased cell motility. Second, we tested the hypothesis that NO attenuates the agonist-induced elevation of H2O2 levels and cell motility by suppressing agonist-induced Rac1 activity via increased PTP-PEST activity. Finally, we tested the hypothesis that the loss of phosphotyrosine in adapter protein p130cas is necessary and sufficient to account for the capacity of NO to decrease Rac1 activity.

We report that IGF-I has the capacity to activate Rac1. These findings add IGF-I to the list of cardiovascular agonists, such as ANG II (31), PDGF, thrombin (32), endothelin (6), or leukotriene B4 (40), that have been reported to induce O2− production via the activation of Rac1.

We also have found that treatment of cells with an NO donor or the overexpression of PTP-PEST attenuates IGF-I-induced Rac1 activation, which supports the hypothesis that the capacity of NO to decrease H2O2 levels occurs via the activation of...
PTP-PEST and the downregulation of Rac1 activity. This concept is further supported by the findings that dn-Rac1 mimics the inhibitory effect of NO in IGF-I-treated cells, whereas overexpression of Rac1 rescues the stimulatory effect of IGF-I in cells treated with NO or with adenovirus expressing PTP-PEST. It is interesting to note that overexpression of Rac1 alone induces motility equivalent to that induced by stimulation with IGF-I. These results are similar to those of previous reports indicating that overexpression or microinjection of Rac1 may activate downstream effectors in various cell lines (1, 25) inducing increased cell motility in a manner independent of direct receptor activation. Other studies have shown that cultured VSMCs from neonatal rats may be subject to autocrine and/or paracrine stimulation in the absence of added growth factors (16) and that increased levels of ROS may induce the synthesis of IGF-I (7), thus describing additional potential mechanisms that might explain the motogenic effect of overexpressed Rac1.

Adapter protein p130cas contains a sequence, termed the substrate domain, encompassing 15 tyrosine residues that are thought to be phosphorylated in response to various agonists. We have shown that p130cas is dephosphorylated in response to treatment with NO donor by upregulating PTP-PEST (22). We have also shown that p130cas associates with adapter protein CrkII and that the capacity of NO to decrease the p130cas-CrkII association is sufficient to explain decreased aortic SMC motility. In the current study, we found that treatment of cells with IGF-I increased the levels of phosphotyrosine in p130cas and that overexpression of p130cas mimicked the effect of IGF-I on Rac1 activation, which supports the view that increasing the levels of p130cas is sufficient to mediate the effects of IGF-I. This effect of p130cas can be explained by the finding that the overexpression of p130cas also leads to increased levels of tyrosyl-phosphorylated p130cas (data not shown), which in turn would increase Rac1 activity. We also infected cells with adenovirus expressing a truncated p130cas allele that lacks the substrate domain and found that it mimicked the inhibitory activity of NO, most likely via a dominant-negative effect directed against endogenous p130cas activity (22). These data indicate that reduction of p130cas phosphotyrosine levels can account for the capacity of NO to decrease the activity of Rac1. These findings are consistent with those of a previous study indicating the pivotal importance of Cas-Crk association in fibroblast motility (20).

Our results are summarized in the scheme shown in Fig. 7. We have found that NO decreases cell motility via a mechanism that targets the small GTPase Rac1. This effect occurs via increased PTP-PEST activity, which induces the dephosphorylation of its substrate, p130cas, followed by the downregulation of Rac1 activity. Decreased Rac1 activity then induces the reduction of H2O2 levels and ultimately decreases cell motility.

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Fig. 7. Scheme summarizing our results. Stimulatory events are indicated by thin arrows, whereas inhibitory events are indicated by thick arrows ending in perpendicular solid lines. CrkII-CT 10, chicken tumor virus 10 regulatory kinase; sGC, soluble guanylyl cyclase.


