Essential role of protein kinase G and decreased cytoplasmic Ca\(^{2+}\) levels in NO-induced inhibition of rat aortic smooth muscle cell motility

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Zhuang, Daming, Alice-Corina Ceacareanu, Bogdan Ceacareanu, and Aviv Hassid. Essential role of protein kinase G and decreased cytoplasmic Ca\(^{2+}\) levels in NO-induced inhibition of rat aortic smooth muscle cell motility. Am J Physiol Heart Circ Physiol 288: H1859–H1866, 2005. First published December 2, 2004; doi: 10.1152/ajpheart.01031.2004.—Hyperinsulinemia is a major risk factor for the development of vascular disease. We have reported that insulin increases the motility of vascular smooth muscle cells via a hydrogen peroxide-mediated mechanism and that nitric oxide (NO) attenuates insulin-induced motility via a cGMP-mediated mechanism. Events downstream of cGMP elevation have not yet been investigated. The aim of our study was to test the hypothesis that antimitogenic effects of NO and cGMP in cultured rat aortic smooth muscle cells are mediated via PKG, followed by reduction of cytoplasmic Ca\(^{2+}\) levels and increased protein tyrosine phosphatase-proline, glutamate, serine, and threonine activity, leading to suppression of agonist-induced elevation of hydrogen peroxide levels and cell motility. Treatment of primary cultures with adenosine expressing PKG-Ia mimicked NO-induced inhibition of insulin-elicited hydrogen peroxide elevation and cell motility, whereas treatment with the pharmacological PKG inhibitor Rp-8-bromo-3',5'-cyclic monophosphorothioate (Rp-8-Br-cGMPS) rescued the stimulatory effects of insulin that were suppressed by NO donor. Treatment of cells with insulin failed to increase cytoplasmic Ca\(^{2+}\) levels, whereas NO donor decreased cytoplasmic Ca\(^{2+}\) levels in the presence or absence of insulin. Treatment of cells with the Ca\(^{2+}\) chelator BAPTA mimicked the effects of PKG and the NO donor and increased the activity of PTP-PEST. Finally, treatment with a dominant negative allele of PTP-PEST reversed the inhibitory effect of BAPTA on cell motility and hydrogen peroxide elevation. We conclude that NO-induced inhibition of cell motility occurs via PKG-mediated reduction of basal cytoplasmic Ca\(^{2+}\) levels, followed by increased PTP-PEST activity, leading to decreased hydrogen peroxide levels and reduced cell motility.

calcium chelator; protein tyrosine phosphatase-proline; glutamate; serine; and threonine; insulin; hydrogen peroxide

Hyperinsulinemia is a major risk factor for the development of vascular disease. Type 2 diabetes, involving hyperinsulinemia, is becoming an epidemic of major proportions in the United States (17, 25, 46). Although Type 2 diabetes generally manifests both hyperglycemia and hyperinsulinemia, the latter effect is thought to be the more important factor in vascular injury-induced neointima formation (15).

Many agents implicated in cardiovascular remodeling, including polypeptide growth factors, induce the generation of reactive oxygen species such as superoxide and/or hydrogen peroxide (24, 35). Reactive oxygen species are thought to be important in the pathogenesis of vascular disease based on reports indicating that antioxidants have the capacity to attenuate neointima formation (34, 49). Hydrogen peroxide has been reported to induce an increase of phosphotyrosine levels in growth factor receptor tyrosine kinases and to activate downstream effectors such as mitogen-activated protein (MAP) kinase, consistent with a putative role of this oxidant in regulation of mechanisms thought to be associated with vascular remodeling (1, 10, 30). Others (40) have reported that catalase attenuates platelet-derived growth factor (PDGF)-induced hydrogen peroxide generation, MAP kinase activation, and proliferation of cultured vascular smooth muscle cells, consistent with an essential role of hydrogen peroxide in signaling and cell proliferation. The inhibition of vascular smooth muscle cell proliferation and vascular remodeling by catalase further supports the view that hydrogen peroxide plays an important role in the regulation of vascular remodeling (4, 37).

Nitric oxide (NO) is an important modulator of vascular remodeling. Zhuang and colleagues (51) have shown that insulin increases cell motility in primary cultured rat aortic smooth muscle cells isolated from newborn rats via an effect mediated by the insulin receptor and that NO opposes the motogenic effect of insulin. NO also attenuates vascular remodeling in vivo, at least in some models of vascular injury. For instance, administration of exogenous NO (19, 23) or overexpression of endothelial NO synthase (eNOS) (44) or inducible NO synthase (36) attenuates injury-induced neointima formation.

Mechanisms mediating the antimitogenic effect of NO are not completely understood. Others have reported that NO attenuates signaling involving MAP kinases or the cell cycle (12, 18). Lin and colleagues (20) have reported that NO increases the activity of protein tyrosine phosphatase-proline, glutamate, serine, and threonine (PTP-PEST) and that this effect is necessary and sufficient to explain NO-induced attenuation of rat aortic smooth muscle cell motility. Hassid’s laboratory (51) has also reported that NO attenuates the motility of cultured rat aortic smooth muscle cells by decreasing agonist-induced hydrogen peroxide elevation via a cGMP-mediated mechanism that targets NAD(P)H oxidase. However, our previous studies did not identify events downstream of cGMP. cGMP increases the activity of PKG, which in turn decreases cytoplasmic Ca\(^{2+}\) levels (14, 31). The aim of our study was to test the hypothesis that the antimitogenic effect of NO and cGMP in cultured aortic smooth muscle cells is
mediated via PKG, followed by reduction of cytoplasmic Ca\(^{2+}\) levels and increased PTP-PEST activity, ultimately leading to decreased hydrogen peroxide levels and decreased cell motility.

**MATERIALS AND METHODS**

*Materials.* Lactating female rats of the Sprague-Dawley strain and their pups were purchased from Charles River Laboratories (Wilmington, MA), or pups of the same strain were bred in the University of Tennessee vivarium. Primaria tissue culture plates were from Falcon/Becton Dickinson (Oxnard, CA). Type I collagenase, soybean trypsin inhibitor, fetal bovine serum, and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO). Tissue culture medium DMEM plus Ham’s F-12 (1/1) was obtained from GIBCO (Grand Island, NY). Porcine pancreatic elastase and insulin were purchased from Collaborative Research (Lexington, MA). 2,2-(Hydroxynitrosohydrazino)-bis-ethanamine (DETANO) and S-nitroso-N-acetylpenicillamine (SNAP) were from Alexis Biochemicals (Carlsbad, CA). 2’,7’-Dichlorodihydrofluorescein diacetate (DCF-DA) and BAPTA-AM were from Molecular Probes (Eugene, OR). Rp-8-bromo-guanosine 3’,5’-cyclic monophosphorothioate (Rp-8-Br-cGMPS) was purchased from BioMol (Plymouth Meeting, PA). Rabbit antisera directed against PKG or PTP-PEST were raised by Covance (Denver, PA) via the use of oligopeptide immunogens corresponding to residues 648 to 669 in the bovine PKG-1α sequence or residues 445 to 458 in the human PTP-PEST sequence, with keyhole limpet hemocyanin via an NH\(_2\)-terminal cysteine residue. Goat anti-rabbit horseradish peroxidase-conjugated antibodies were purchased from Transduction Laboratories (Lexington, KY). All other reagents were of the highest quality available and were obtained from Sigma or Baxter (Edison, NJ) unless stated otherwise.

*Cell culture.* Smooth muscle cells were isolated from thoracic aortas of Sprague-Dawley rats (6–9 days old) and cultured as described (39). All experiments were performed using primary near-confluent or confluent cultures that were serum starved for 24–48 h before the experiments, and each individual experiment represents results from one cell isolate. The studies were performed via a protocol approved by the Animal Care and Use Committee, University of Tennessee Health Sciences Center, in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, NIH Publication No. 86-23).

*Preparation and expression of adenoviral vectors.* Replication-deficient (E-1-deleted) recombinant adenoviruses, expressing wild-type PTP-PEST or dominant negative PTP-PEST (C231S-PTP-PEST), were generated as previously described (20). Complementary DNA encoding the bovine PKG-1α isoform was obtained from F. Hofmann (Munich, Germany) and used to prepare recombinant adenovirus as previously described (20). The cDNA sequence of bovine PKG-1α was verified to conform to published data (47). Adenoviral titers were determined via a standard procedure by measurement of adenovirus-induced cytopathic effects in HEK-293 cells. Confluent aortic smooth muscle cells were infected with recombinant adenovirus at a multiplicity of infection index of 5–10. After the initial 24-h incubation, culture media were replaced and cells were subjected to a second 24-h incubation period in the absence of extracellular adenovirus before use in experiments.

*Measurement of intracellular hydrogen peroxide levels.* Intracellular hydrogen peroxide levels were measured by quantitation of the fluorescence of oxidized DCF, as described (51). A few of the cultures were

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**Fig. 1.** Overexpression of protein kinase G (PKG) attenuates insulin-induced cell motility, whereas selective PKG inhibitor 8-bromoguanosine-3’, 5’-cyclic monophosphorothioate (Rp-8-Br-cGMPS) reverses inhibition of cell motility induced by nitric oxide (NO) donor in concentration-dependent manner. A: effect of PKG-1α overexpression on cell motility. Cells were infected, at equivalent multiplicity of infection values, with adenovirus expressing bovine PKG-1α or enhanced green fluorescent protein (EGFP), with the latter used as control for the effects of viral infection per se. Cells were then incubated with or without insulin (100 nM, 24 h). Cell motility was measured via a wounded culture assay. Results are the means ± SE of 3 experiments. *P < 0.05 compared with control virus. B: representative Western blot analysis depicting expression of bovine PKG-1α. The faint second band appearing on the blot presumably represents endogenous rat PKG. C: concentration-dependent effect of PKG inhibitor on cell motility. Cells were preincubated with or without Rp-8-Br-cGMPS (0 to 100 μM, 30 min) and/or 2,2-(hydroxynitrosohydrazino)-bis-ethanamine (DETANO, 30 μM, 30 min). They were then incubated with or without insulin (100 nM, 24 h) in the continued absence or presence of Rp-8-Br-cGMPS and/or DETANO. Hatched columns indicate treatment with Rp-8-Br-cGMPS at micromolar concentrations shown as embedded values. Rp-8-Br-cGMPS alone had no significant effect (not shown). Cell motility was measured via a wounded culture assay. Control indicates the “no treatment” category. Results are the means ± SE of 3 experiments. *P < 0.05 compared with insulin + DETANO. **P < 0.05 compared with control.
treated with catalase to verify that oxidized DCF fluorescence accurately reflected intracellular hydrogen peroxide levels (not shown).

Measurement of PTP-PEST activity via immunoprecipitation assay. Confluent cells were infected with recombinant adenosine expressing wild-type PTP-PEST or “empty virus” encoding viral proteins only for 48 h. Expression of ectopic PTP-PEST increases the precision and accuracy of the measurements of enzyme activity levels, although a previous study from our laboratory (20) indicated that NO donors also increase the levels of endogenous PTP-PEST activity. After the experimental treatment, cells were subjected to lysis in ice-cold HEPES buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM EDTA, and 1% Triton X-100; pH 7.2) supplemented with a cocktail of protease inhibitors [1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, and 14 μM E-64]. Immunoprecipitation was performed by preclearing lysates with normal rabbit serum for 30 min at 4°C, followed by treatment with protein G-Sepharose beads (50%) for 30 min. Supernatants were incubated with PTP-PEST antiserum for 1 h at 4°C, followed by treatment with G-Sepharose beads for 1 h. Immunocomplexes were washed twice with the above-mentioned HEPES buffer and twice with ice-cold HEPES-DTT buffer (in mM: 25 HEPES, 50 NaCl, 2.5 EDTA, 10 DTT, and 5 β-mercaptoethanol; pH 7.3). Immunoprecipitated PTP-PEST was then incubated with 10 mM p-nitrophenyl phosphate for 15 min at 37°C. Enzyme activity was determined by measuring the increase of absorbance at 405 nm. The reaction was terminated by the addition of 10 M NaOH. Care was taken not to exceed 25% of substrate hydrolysis. Values found in the absence of enzyme (never >28% of experimental values) were taken as background and were always subtracted.

Measurement of cell motility. Cell motility was measured as described in publications from our laboratory via a wounded culture assay (39, 51).

Measurement of cytoplasmic Ca2+ levels via dual-wavelength fluorescence spectrometry. Cytoplasmic Ca2+ levels were measured via the use of fluorescent Ca2+ indicator fura-2. Brieﬂy, primary rat aortic smooth muscle cells were seeded on glass coverslips (Bellco) at a density of 2–3 × 104 cells/cm2 and cultured in DMEM-F12 supplemented with 10% fetal bovine serum. Several days later, serum-starved cells (at 70–90% confluency) were treated for 6 h in the dark with a mixture of fura-2-acetoxymethyl ester (5 μM) and pluronic acid F-127 (0.01%). Experimental coverslips were then perfused with Krebs buffer, supplemented with 5% BSA containing or lacking experimental agents. Measurements of Ca2+ levels were performed using a calibrated dual-wavelength Perkin-Elmer LS-50B spectrophotometer containing a water-jacketed cuvette holder to maintain the cells at 37°C. Cells were alternately illuminated with light of wavelength 340 nm and 380 nm, and fluorescence emission was measured at 510 nm, once per minute. Data were processed via with the ICBC (PE) software package and are expressed as the ratio of fluorescence emission of cells exposed to light at 340 and 380 nm.

Statistical analysis. Data are expressed as means ± SE (or means ± SE) and were statistically evaluated using ANOVA, followed by Fisher’s protected least-significant difference test or by paired Student’s t-test. P < 0.05 was considered to be significant.

RESULTS

Increase of PKG activity is both necessary and sufficient to mediate NO-induced inhibition of cell motility. NO is a well-established enhancer of cGMP levels in cultured smooth muscle cells and in vivo (26, 45). PKG is a major effector of cGMP (21), and we have reported that the antimitogenic effect of NO in cultured rat aortic smooth muscle cells is mimicked by cGMP analogs (51). From these findings, we tested the hypothesis that increased PKG activity is both sufficient and necessary to mediate the antimitogenic effect of NO. To determine whether an increase of PKG levels is sufficient to induce inhibition of cell motility, we treated cells with an adenosine expressing bovine PKG-1a or enhanced green fluorescent protein (eGFP) alone or together. Overexpression of PKG attenuates insulin-induced increase of hydrogen peroxide levels, whereas selective PKG inhibitor Rp-8-Br-cGMPS reverses NO-induced suppression of hydrogen peroxide levels in concentration-dependent manner. A: effect of PKG overexpression on hydrogen peroxide levels. Cells were infected, at equivalent multiplicity of infection values, with adenovirus expressing PKG-1a or with control virus expressing viral proteins only (“empty virus”). Cells were then treated without or with insulin (100 nM, 5 min). Hydrogen peroxide levels were determined via measurement of oxidized DCF fluorescence (DCF) levels. Results are the means ± SE of 3 experiments. *P < 0.05 compared with control virus; **P < 0.05 compared with control virus plus insulin.

B: effect of PKG inhibitor Rp-8-Br-cGMPS on intracellular hydrogen peroxide levels. Cells were pretreated with or without DETANO (30 μM, 30 min) and/or Rp-8-Br-cGMPS (0 to 100 μM, 30 min) before treatment without or with insulin (100 nM, 5 min) in the continued absence or presence of DETANO or Rp-8-Br-cGMPS, and hydrogen peroxide levels were determined via measurement of oxidized DCF levels. Hatched columns indicate treatment with Rp-8-Br-cGMPS at micromolar concentrations shown as embedded values. Rp-8-Br-cGMPS alone had no significant effect (not shown). Control indicates the no treatment category. Results are the means ± SE of 3 experiments. *P < 0.05 compared with insulin + DETANO; **P < 0.05 compared with control.
fluorescent protein (EGFP) to control for the effects of viral infection per se. As shown in Fig. 1B, ectopic recombinant bovine PKG-1α was well expressed in cultured cells. Data given in Fig. 1A show that overexpression of PKG essentially abrogated insulin-induced cell motility, without having a significant effect on basal motility. The use of “empty virus” expressing viral proteins only, as a control virus, provided similar results (data not shown).

We then tested the hypothesis that PKG activity is necessary for NO-induced inhibition of cell motility by using a selective, well-established pharmacological PKG inhibitor Rp-8-Br-cGMPS (27). The inhibitor had no effect of its own (data not shown), but it attenuated the antimotogenic capacity of NO donor in a concentration-dependent fashion (Fig. 1C), with a significant effect occurring at 3 μM inhibitor. At 30 μM, a concentration reported to inhibit PKG but not PKA activity in vascular smooth muscle cells (27), Rp-8-Br-cGMPS induced ~60% reversal of the antimotogenic effect of NO. Taken together, these findings indicate that PKG is both sufficient and necessary to transduce at least a major part, if not all, of the antimotogenic effect of NO.

Increase of PKG levels is both necessary and sufficient to mediate the inhibitory effect of NO on insulin-induced elevation of hydrogen peroxide levels. Our laboratory (51) has reported that an increase of intracellular hydrogen peroxide levels is sufficient to induce increased motility in cultured rat aortic smooth muscle cells and that NO induces inhibition of agonist-induced cell motility by decreasing intracellular hydrogen peroxide levels. On the basis of these findings, we tested the hypothesis that an increase of PKG levels is both sufficient and necessary to mediate NO-induced attenuation of the elevation of intracellular hydrogen peroxide induced by insulin. As shown in Fig. 2A, overexpression of PKG, via adenoviral infection, markedly attenuated insulin-induced hydrogen peroxide elevation, whereas the control virus expressing viral proteins only (empty virus) was ineffective. The intrinsic fluorescence of EGFP interfered with the measurement of DCF fluorescence and therefore prevented the use of EGFP-expressing adenovirus as control virus in these experiments; hence, the sole use of empty virus as a control. To test the hypothesis that PKG activity is necessary to transduce the capacity of NO to oppose hydrogen peroxide elevation, we treated cells with the PKG antagonist Rp-8-Br-cGMPS. As shown in Fig. 2B, Rp-8-Br-cGMPS attenuated the capacity of NO to reduce hydrogen peroxide levels elevated by insulin in a concentration-dependent manner. Additionally, comparison of Figs. 1C and 2B indicated similar concentration dependency for both the reversal of NO-induced antimotogenic effect and suppression of hydrogen peroxide levels. Taken together, these results support the hypothesis that activation of PKG is both necessary and sufficient to transduce the capacity of NO to suppress the increase of hydrogen peroxide levels induced by insulin.

Insulin fails to increase cytoplasmic Ca²⁺ levels, although NO induces reduction of basal cytoplasmic Ca²⁺ levels in absence or presence of insulin. Activation of PKG has been shown to decrease cytoplasmic Ca²⁺ levels in vascular smooth muscle cells (14, 31). Huang and colleagues (51) reported that NO induces reduction of NADPH oxidase activity in these cells, whereas others (16) found that NADPH oxidase activity is regulated by cytoplastic Ca²⁺ levels in neutrophils. From these findings, we tested the hypothesis that insulin induces an increase of cytoplasmic Ca²⁺ levels and that NO attenuates the effect of insulin. As shown in Fig. 3, insulin failed to increase cytoplasmic Ca²⁺ levels, although the NO donor SNAP induced a decrease of Ca²⁺ levels in the presence or absence of insulin. Similar results were obtained via the use of the alter-

![Fig. 3. Treatment of cells with insulin fails to increase cytoplasmic Ca²⁺ levels, whereas treatment with NO donor decreases basal Ca²⁺ levels in absence or presence of insulin. A: untreated cells. B: cells treated with NO donor S-nitroso-N-acetylpenicillamine (SNAP) followed by treatment with angiotensin II (ANG II) as the positive control. C: cells treated with insulin. D: cells treated with insulin plus SNAP, followed by treatment with ANG II as the positive control. Aortic smooth muscle cells were pretreated with fura-2 AM (5 μM) plus pluronic acid (0.01%), as described in MATERIALS AND METHODS. Cells were then treated with or without insulin (100 nM) and with or without SNAP (50 μM). Cytoplasmic Ca²⁺ levels were determined by dual-wavelength fluorescence spectroscopy via alternate excitation at 340 nm and 380 nm, and measurement of fluorescence emission was set at 510 nm. Data are expressed as means ± SE of 3–4 independent experiments. Effects of SNAP or ANG II were statistically significant compared with Ca²⁺ levels observed before treatment, as determined by analysis of variance, followed by Fisher’s post hoc test.](http://ajpheart.physiology.org/10.1152/ajpheart.00414.2004)
nate NO donor DETANO (data not shown). Angiotensin II, used as a positive control treatment, induced a marked increase of cytoplasmic Ca\(^{2+}\) levels, indicating that cultures used in our experiments were intrinsically responsive to appropriate agonists. These data are consistent with the view that NO-induced reduction of basal cytoplasmic Ca\(^{2+}\) levels elicits lower hydrogen peroxide levels and that basal Ca\(^{2+}\) levels play a permissive role in allowing insulin-induced hydrogen peroxide elevation. Parenthetically, the data also indicate that the capacity of insulin to induce hydrogen peroxide elevation is independent of increased cytoplasmic Ca\(^{2+}\) levels.

Intracellular Ca\(^{2+}\) chelator BAPTA attenuates insulin-induced cell motility and hydrogen peroxide elevation. The next experiments were designed to test the hypothesis that reduction of cytoplasmic Ca\(^{2+}\) levels is sufficient to transduce the antimotogenic effect of NO and PKG via suppression of hydrogen peroxide levels. As shown in Fig. 4A, treatment of vascular smooth muscle cells with intracellular Ca\(^{2+}\) chelator BAPTA mimicked the capacity of NO to attenuate insulin-induced cell motility. Data shown in Fig. 4B indicate that BAPTA attenuated insulin-induced elevation of hydrogen peroxide levels. Taken together, these results indicate that reduction of cytoplasmic Ca\(^{2+}\) levels, elicited by NO or PKG, is sufficient to transduce their antimotogenic effect.

Treatment with cytoplasmic Ca\(^{2+}\) chelator BAPTA increases PTP-PEST activity. We have reported that treatment of cultured rat aortic smooth muscle cells with the NO donor increases the activity of PTP-PEST and that this event is both necessary and sufficient to transduce the antimotogenic effect of NO (20). On the basis of these findings, we tested the hypothesis that reduction of cytoplasmic Ca\(^{2+}\), induced by the treatment of cultured cells with BAPTA, increases the enzyme activity of PTP-PEST. Accordingly, we found that a 30-min treatment with BAPTA increased PTP-PEST activity from 238 ± 36 to 501 ± 7 arbitrary units (means ± SE; result of 3 independent experiments, \(P < 0.05\) via paired Student’s \(t\)-test), indicating that reduction of cytoplasmic Ca\(^{2+}\) is sufficient to transduce the capacity of NO as stimulator of PTP-PEST activity.

PTP-PEST activity is necessary to mediate reduction of hydrogen peroxide levels and transduction of antimotogenic effect induced by lowering of cytoplasmic Ca\(^{2+}\) levels. Based on published and current findings indicating that reduction of cytoplasmic Ca\(^{2+}\) levels and elevation of PTP-PEST activity are both necessary and sufficient to transduce the antimotogenic effect of NO, and in view of the increase of PTP-PEST activity induced by NO and reduced cytoplasmic Ca\(^{2+}\) levels, we tested the hypothesis that the increase of PTP-PEST activity induced by reduced cytoplasmic Ca\(^{2+}\) levels is necessary to mediate inhibition of cell motility. We used dominant negative PTP-PEST (C231S-PTP-PEST), which antagonizes the effects of endogenous PTP-PEST in rat aortic smooth muscle cells, as reported by us (20). Treatment of cells with adenovirus expressing dominant negative PTP-PEST induced robust and equivalent expression of PTP-PEST protein in all treatment categories, as shown by Western blot analysis (Fig. 5C). Adenovirus expressing dominant negative PTP-PEST, but not control protein EGFP, rescued the motility stimulatory effect of insulin in the presence of the Ca\(^{2+}\) chelator (Fig. 5A). These results are consistent with the hypothesis that an increase of PTP-PEST activity mediates the antimotogenic effect induced by reduction of cytoplasmic Ca\(^{2+}\) levels.

On the basis of the aforementioned results, we tested the hypothesis that elevated PTP-PEST activity mediates the attenuation of hydrogen peroxide levels induced by BAPTA. Accordingly, we measured hydrogen peroxide levels in the absence or presence of dominant negative PTP-PEST expression. As shown in Fig. 5B, treatment of cells with insulin, plus adenovirus expressing dominant negative PTP-PEST, induced reversal of the attenuation of hydrogen peroxide levels elicited by BAPTA. Taken together, these results are consistent with the hypothesis that PTP-PEST is situated downstream of Ca\(^{2+}\)
but upstream of hydrogen peroxide in the signaling cascade regulating cell motility, as depicted in the scheme shown in Fig. 6.

**DISCUSSION**

Coronary restenosis is a significant problem for a subpopulation of patients undergoing angioplasty (22). Restenosis is especially prevalent in Type 2 diabetic patients manifesting hyperinsulinemia (42). Neointimal enlargement frequently occurs after vascular injury and is thought to be an important component of restenosis. Neointimal enlargement critically depends on vascular smooth muscle cell motility, as shown by experiments in animal models (5, 50).

Inducible NO synthase levels are significantly increased in vivo in medial cells, almost immediately after vascular injury and subsequently, in neointimal cells (13, 48). Inducible NO levels are also markedly increased in experimental diabetes (2). Many in vivo studies have shown that exogenous or endogenous NO attenuates neointima formation induced by vascular injury, consistent with inhibition of vascular smooth muscle

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**Fig. 5.** Dominant negative protein tyrosine phosphatase-proline, glutamate, serine, and threonine (C231S-PTP-PEST) attenuates the capacity of Ca$^{2+}$ chelator BAPTA to inhibit insulin-induced cell motility and hydrogen peroxide elevation.

**A**: effect of dominant negative PTP-PEST on inhibition of cell motility induced by BAPTA. Cells infected with virus expressing dominant negative PTP-PEST or with control virus expressing EGFP were preincubated with or without BAPTA-AM (50 μM, 30 min). They were then incubated with or without insulin (100 nM, 24 h) in the continued absence or presence of BAPTA-AM. Results are expressed as means ± SE of 3 independent experiments. *P < 0.05 compared with control virus.

**B**: effect of dominant negative PTP-PEST on decrease of hydrogen peroxide levels induced by BAPTA. Cells infected with virus expressing dominant negative PTP-PEST or with control virus (“empty virus”) were preincubated with or without BAPTA-AM (50 μM, 30 min). They were then incubated with or without insulin (100 nM, 5 min) in the continued absence or presence of BAPTA-AM. Results are expressed as means ± SE of 3 independent experiments. *P < 0.05 compared with control virus.

**C**: representative Western blot analysis depicting expression of dominant negative PTP-PEST levels. Cells were infected, at equivalent multiplicity of infection values, with adenovirus expressing dominant negative C231S-PTP-PEST or EGFP, with the latter as control for the effects of viral infection per se.
cell motility and proliferation in models of vascular injury. For instance, administration of exogenous NO (19, 23), or overexpression of eNOS (44) or inducible NO synthase (36), decreases vascular injury-induced neointima formation. Injury-induced neointima formation is enhanced in mice genetically lacking eNOS (33), whereas administration of arginine, the substrate of NO synthase, markedly attenuates neointima formation in the rat after vascular injury via balloon catheter (43). The aforementioned studies are consistent with the hypothesis that NO decreases cell motility in vivo.

We have reported that elevation of cGMP is both necessary and sufficient to transduce the antimotogenic effect of NO in cultured rat aortic smooth muscle cells (51). Separately, we have shown that treatment of cultured smooth muscle cells with NO increases PTP-PEST enzyme activity and that this effect is necessary and sufficient to transduce the antimotogenic effect of NO (20).

The main purpose of this work was to test the hypothesis that PKG is both necessary and sufficient to transduce the antimotogenic effect of NO in cultured rat aortic smooth muscle cells treated with NO. We have reported that PTP-PEST induces reduction of phosphorytrosine levels in adapter protein p130cas (20) and that this effect appears to be causally linked to reduction of small GTPase Rac1 activity (6), leading to inhibition of NADPH oxidase activity (51). Because treatment of cells with hydrogen peroxide is both sufficient and necessary to induce motility in cultured rat aortic smooth muscle cells, as shown by the previous study of Huang and colleagues (51), these findings, taken together, uncover a novel signaling pathway that describes how NO decreases the motility of cultured rat aortic smooth muscle cells.

NAD(P)H oxidase is thought to be the predominant system that generates superoxide, and subsequently hydrogen peroxide, in vascular smooth muscle (11, 28, 29). NADPH oxidase activity is upregulated in balloon injury or atherosclerosis, and increased activity of the enzyme has been linked to growth factor-induced cell proliferation (38, 40, 41). Consistent with these data are findings that antioxidants have the capacity to attenuate neointima formation (34).

NO has been reported to decrease superoxide generation in neutrophils via a direct interaction with NADPH oxidase (8, 9, 32). NO also reduces superoxide levels by interaction with superoxide, forming peroxynitrite (3). In contrast, the current studies support a novel mechanism involving PKG, reduced cytoplasmic Ca2+ levels, and increased PTP-PEST activity, although they do not rule out a potential involvement of peroxynitrite.

Our study shows that reduction of hydrogen peroxide levels and the antimotogenic effect induced by reduction of cytoplasmic Ca2+ levels are dependent on PTP-PEST activity, based on the finding that both processes are blocked by treatment with dominant negative PTP-PEST. These data are consistent with the view that PTP-PEST is situated downstream of cytoplasmic Ca2+ but upstream of NADPH oxidase in the signaling cascade that regulates vascular smooth muscle cell motility, as depicted in the scheme shown in Fig. 6. Activation of PTP-PEST induces phosphotyrosine dephosphorylation of p130cas, and this event then leads to reduced Rac1 activity, as reported by Hassid’s laboratory (6, 20). Finally, it is worth mentioning that a recent study from our laboratory (7) found that PTP-PEST protein levels are markedly increased in injured rat carotid arteries, supporting a potential counterregulatory role of the phosphatase in modulation of neointimal enlargement. On the basis of these findings, we suggest that the pathophysiological role of PTP-PEST in blood vessels and the biochemical details of the regulation of its activity by cytoplasmic Ca2+ deserve to be the subject of future investigation.

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