NO decreases phosphorylation of focal adhesion proteins via reduction of Ca in rat aortic smooth muscle cells

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THE PROLIFERATION of vascular smooth muscle cells is thought to be important in the development of blood vessel disease (26). Acute injury to blood vessels induces proliferation of vascular smooth muscle cells and promotes the formation of neointimal regions in rodent models of vascular injury. Clinically, an enlarged neointima is known to occur in atherosclerosis and after angioplasty, coronary bypass surgery, or cardiac transplantation (17). Although the etiology of these conditions is likely to be different, they all exhibit, to varying degrees, enhanced vascular smooth muscle cell proliferation.

Numerous polypeptide growth factors stimulate the proliferation of vascular smooth muscle cells in vitro (26). Moreover, there is some evidence that fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) can modulate vascular smooth muscle cell proliferation in vivo (20). Whereas polypeptide growth factors play an important role in stimulating the proliferation of vascular smooth muscle cells, other agents appear to inhibit this process. Thus prostacyclin, heparin, and transforming growth factor-β decrease the proliferation of vascular smooth muscle cells in vitro and/or in vivo (1, 2, 23). Another important class of inhibitors of vascular smooth muscle cell proliferation comprises agents that increase the levels of cGMP. Included in this group are nitric oxide (NO) and atrial natriuretic peptides, which inhibit DNA synthesis and proliferation of certain types of vascular smooth muscle cells in vitro and attenuate the formation of neointimal regions in models of vascular injury (10, 18, 28).

We have recently reported that NO and cGMP decrease phosphorysotyrosine levels in specific proteins of primary aortic smooth muscle cells isolated from newborn rats, perhaps via activation of one or more protein tyrosine phosphatases (PTPases) (7). We also found that NO-induced antimitogenesis was inhibited by a selective PTPase inhibitor, pervanadate, suggesting a link between the antimitogenic effect of cGMP agonists and its capacity to activate PTPase and decrease phosphorysotyrosine levels.

The notion that intracellular Ca modulates mitogenic events in vascular smooth muscle cells finds considerable support in the literature (4, 13, 19), although the precise role played by Ca is complex and unclear. We and others (11, 25) have reported that elevation of cGMP levels reduces cytoplasmic Ca levels in vascular smooth muscle cells. Other studies have reported an inverse correlation between cellular Ca levels and PTPase activity. For example, a hematopoietic PTPase termed CD45 and an unidentified PTPase found in fibroblasts were reported to have decreased activity when intracellular Ca levels were increased (8, 22). These findings are compatible with the hypothesis that protein tyrosine dephosphorylation induced by cGMP agonists may be mediated via decreased cytoplasmic Ca levels. The purpose of the current study was to test this hypothesis and to identify some of the proteins that serve as substrate for NO-induced dephosphorylation of phosphorysotyrosine in cultured aortic smooth muscle cells from newborn rats.

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). Nifedipine, type I collagenase, soybean trypsin inhibitor, fetal bovine serum, and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO). DMEM-Ham’s F-12 (1:1) medium was from Gibco (Grand Island, NY). Porcine pancreatic elastase, insulin, transferrin, and selenium acid were from Collaborative Research (Lexington, MA).
S-nitroso-N-acetylpenicillamine (SNAP) was synthesized as described previously (7). All other reagents were of the highest quality available and were generally obtained from Sigma or Baxter (Edison, NJ). Antibodies against phosphotyrosine and paxillin were purchased from Transduction Laboratories (Lexington, KY), whereas those against cortactin were purchased from Upstate Biotechnology. Fura 2-AM, fura-2 free acid, and 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-AM were purchased from Molecular Probes (Eugene, OR).

Cell culture. Smooth muscle cells were obtained from the thoracic aortas of newborn Sprague-Dawley rats (6–9 days old) as described previously (7). The cells were seeded into Primaria culture dishes at a density of 1.8–2.3 × 10^4 cells/cm^2 and were cultured for the first 2 days in serum-free DMEM-Ham’s F-12 (1:1) medium supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), and selenium (5 ng/ml), plus 50 µU/ml penicillin and 50 µg/ml streptomycin, in a humidified atmosphere of 5% CO_2-95% air. Most cells (~95%) attached to the culture surface within the first few hours after seeding and were observed to spread after overnight incubation. After the initial 2-day culture in serum-free medium, fetal bovine serum was added to a final concentration of 10%. Cells were cultured for an additional 3–5 days in serum-containing medium. Cells were identified as smooth muscle in origin by positive immunostaining for α-smooth muscle actin. All experiments in this study were performed using primary cultures; moreover, each individual experiment represents results from one such cell isolate, generally obtained from two rat litters.

Measurement of cytoplasmic Ca via dual wavelength fluorescence spectroscopy. Cytoplasmic Ca was measured via the use of fura-2 according to a previously published method (11). Briefly, cells seeded on glass coverslips were treated with 4 µM fura-2-AM for 1 h at 37°C, on a rotary shaker. Coverslips were then perfused with Ca-supplemented HEPES-buffered, glucose-supplemented balanced salt solution, containing or lacking experimental agents, and cytoplasmic Ca levels were measured in a calibrated dual-wavelength Shimadzu spectrophotometer, using the excitation wavelengths of 340 nm and 380 nm and the emission wavelength of 505 nm.

Measurement of ^[3H]thymidine incorporation. DNA synthesis was measured via ^[3H]thymidine incorporation in mitogenically relatively quiescent aortic smooth muscle cells, as described previously (7). To induce relative mitogenic quiescence, cultures in 24-well plates, having densities of 1 × 10^5 to 2 × 10^5 cells/cm^2, were washed three times with serum-free DMEM-Ham’s F-12 medium plus penicillin and streptomycin and cultured for 2 days in the same medium lacking insulin, transferrin, and selenium. To investigate the effects of experimental agents on mitogenesis, cells previously cultured in serum-free medium for 2 days were incubated for 22 h in the absence or presence of these agents. After 20 h, 3 µCi ^[3H]thymidine were added to each well, and the culture was incubated for an additional 2 h. After washing of cells and solubilization of DNA, incorporated ^[3H]thymidine was measured by scintillation spectrophotometry, whereas a second aliquot was used for the determination of protein content via the bicinchoninic acid method (Pierce, Rockford, IL), using bovine serum albumin as standard.

Western immunoblot analysis of protein phosphotyrosine. Cells maintained in six-well culture dishes in serum-free medium were treated with various experimental agents at 37°C. After incubations, cells were lysed directly in the culture plates with 300 µl denaturing lysis buffer of the following composition: 250 mM Tris·HCl (pH 6.8), 10 mM sodium pyrophosphate, 10% glycerol, 4% SDS, 2 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 2 mM EDTA. Cells were agitated on a vortexer for 12 min. The supernatants were collected and were immediately boiled for 3 min. After microcentrifugation at 16,000 g for 10 min, samples were treated with 2-mercaptoethanol, to a final concentration of 5%, and equivalent amounts of protein were separated on 7.5% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked with phosphate-buffered saline, supplemented with 0.1% Tween 20 plus 3% bovine serum albumin, and probed for phosphotyrosine using recombinant antiphosphotyrosine antibodies conjugated to horseradish peroxidase, diluted 1:2,500 in blocking buffer. Immunoreactive bands were visualized using Renaissance chemiluminescence reagents (NEN). Densitometric analysis was done via the use of the National Institutes of Health Image software operating on a Macintosh 8600/200 computer.

Immunoprecipitation of cortactin or paxillin. Cells were incubated with experimental agents, followed by lysis at 4°C for 30 min, using buffer of the following composition: 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM sodium vanadate, 50 mM sodium fluoride, and 30 mM sodium pyrophosphate. Samples were cleared by preincubating with either protein A-agarose or protein G-Sepharose beads. Immunoprecipitation was initiated by incubation of cell extract overnight at 4°C with anticortactin antibody (1:150-fold dilution) or antipaxillin antibody (1:250-fold dilution), followed by addition of protein A-agarose or protein G-Sepharose beads and further incubation for 1.5 h. Agarose-Sepharose beads were then washed several times with the above-mentioned buffer, followed by boiling of beads in gel-loading buffer containing SDS and loading of supernatant onto gels for SDS-PAGE.

RESULTS

NO donor, SNAP, reduces cytoplasmic Ca levels in primary aortic smooth muscle cells from newborn rats. Previous studies from our laboratory have shown that cGMP agonists reduce basal cytoplasmic Ca levels in cultured aortic smooth muscle cells isolated from adult rats (11). To verify that a similar response occurs in cells isolated from newborn rats, we treated fura-2-loaded cells with the NO donor agent SNAP and measured cytoplasmic Ca levels by dual-wavelength fluorescence spectrometry. As shown in Fig. 1, SNAP decreased basal cytoplasmic Ca levels, thus verifying and extending the results of earlier studies from our
laboratory and that of others. 8-Bromoguanosine 3',5'-cyclic monophosphate elicited a similar result (not shown), suggesting that this effect is, at least in part, mediated by cGMP.

EGTA, BAPTA, or a Ca entry blocker, nifedipine, decreases cytoplasmic Ca and protein phosphoryosine levels. To test the hypothesis that a reduction of cytoplasmic Ca levels is associated with NO-induced dephosphorylation of proteins, we treated cells with a nominally Ca-free medium further supplemented with 1 mM EGTA and found, as expected, that this treatment reduced cytoplasmic Ca levels in primary aortic smooth muscle cell cultures (not shown). We then determined the effect of treatment of these cells with extracellular EGTA on phosphoryosine levels. As shown in Fig. 2, EGTA reduced phosphoryosine levels in proteins of 70- to 85-kDa molecular mass. Figure 2 also shows that similar-size proteins had reduced phosphoryosine levels after exposure of cells to SNAP, thus verifying published results (7).

In addition to the cell-impermeant Ca chelator EGTA, we also tested the effect of the Ca chelator BAPTA, which is cell permeant as the acetoxyethyl ester derivative. BAPTA was previously found to decrease cytoplasmic Ca levels and was thus used as an alternate method to lower cytoplasmic Ca levels in our experiments. Similar to the effect of EGTA, BAPTA decreased protein phosphoryosine levels, as shown in Fig. 3. Taken together, these findings clearly indicate that lowering of cytoplasmic Ca is associated with reduction of protein phosphoryosine levels.

The capacity of Ca entry blockers to decrease cytoplasmic Ca levels in vascular smooth muscle cells via inhibition of Ltype Ca channels is well established (21). Moreover, Ca entry blockers have been reported to inhibit vascular smooth muscle cell proliferation, consistent with the hypothesis that Ca modulates DNA synthesis (4, 19). We were therefore interested in investigating the effect of a prototypic Ca entry blocker, nifedipine, on phosphoryosine levels in aortic smooth muscle cells. We first verified that nifedipine had the capacity to decrease cytoplasmic Ca levels in aortic smooth muscle cells from newborn rats, as shown in Fig. 4A. Second, we verified the capacity of nifedipine to decrease DNA synthesis, as shown in Fig. 4B. Finally, we measured the effect of nifedipine on protein phosphoryosine levels. As shown in Fig. 4C, nifedipine decreased the levels of phosphoryosine in the 70- to 85-kDa protein cluster.

To further verify the functional consequences of lowered Ca and phosphoryosine dephosphorylation, we measured the effect of 100 µM BAPTA on DNA synthesis, as determined by thymidine incorporation. BAPTA decreased thymidine incorporation by 28–44% in three separate experiments.

Agents that increase cytoplasmic Ca levels attenuate or block dephosphorylation of protein phosphoryosine induced by SNAP. If reduction of cytoplasmic Ca levels were to be causally associated with phosphoryosine dephosphorylation, agents that oppose the decrease of Ca would be expected to attenuate or block the capacity of SNAP to induce dephosphorylation. Consistent with this expectation, we found that the Ca ionophore A-23187 blocked the decrease of phosphoryosine levels induced by SNAP (Fig. 2). Interestingly, A-23187 alone did not increase phosphoryosine levels, indicating that these levels may already have been maximal at basal Ca values. The notion that an increase of cytoplasmic Ca blocks SNAP-induced dephosphorylation of phosphoryosine was further tested via the use of elevated extracellular K⁺, a treatment that is known to increase cytoplasmic Ca levels in vascular smooth muscle cells via depolarization and activation of voltage-sensitive Ca channels. As shown in Fig. 5, high K⁺ attenuated the decrease of phosphoryosine induced by SNAP, consistent with the results using the Ca ionophore.

SNAP and EGTA induce dephosphorylation of phosphoryosine in two cytoskeleton-associated focal adhesion proteins, Paxillin and cortactin. Several cytoskeletal-associated focal adhesion proteins, including
cortactin and paxillin, are phosphorylated on tyrosine residues under the influence of growth factors and vasoactive agents such as PDGF, FGF, or angiotensin II (15, 24, 31). Similar findings have also been made in cells treated with agents that increase cytoplasmic Ca levels, such as the Ca ionophore A-23187 or elevated extracellular KCl (14). Because the apparent molecular mass of dephosphorylated proteins in our previous study (7) corresponded with that of focal adhesion proteins, paxillin (68–75 kDa) and cortactin (80–85 kDa), we tested the hypothesis that NO elicits dephosphorylation of these proteins. To this end, we treated cells with or without SNAP and immunoprecipitated paxillin or cortactin using specific antibodies, followed by Western blotting using either anti-phosphotyrosine or anticortactin/anti-paxillin. As shown in Fig. 6A, SNAP decreased the levels of phosphotyrosine in paxillin in a concentration-dependent fashion. To test the hypothesis that this effect was mediated by decreased intracellular Ca, we treated cells with Ca-replete or Ca-deficient medium, and, as shown in Fig. 6B, Ca-deficient medium elicited virtually complete dephosphorylation of paxillin.

Fig. 4. A: decrease of cytoplasmic Ca by nifedipine, as measured by dual-wavelength fura 2 fluorescence spectrometry. Similar results were obtained in 2 additional experiments. B: decrease of thymidine incorporation by nifedipine. Similar results were obtained in 2 additional experiments. C: decrease of phosphotyrosine levels in 70- to 85-kDa proteins by nifedipine, as determined by Western blotting for phosphotyrosine. Cells were incubated for 30 min in the presence or absence of 10 µM nifedipine before extraction of proteins for Western blotting. Densitometric values (in arbitrary units) were as follows: control: 100; nifedipine: 59.5. Similar results were obtained in 2 additional experiments.

Fig. 5. Attenuation of SNAP-induced decrease of phosphotyrosine levels in 70- to 85-kDa proteins by high extracellular K+*, as determined by Western blotting. Cells were incubated for 30 min before extraction of protein. Similar results were obtained in 2 additional experiments. Densitometric values (in arbitrary units) were as follows: control: 100; SNAP: 32.9; SNAP + 20 mM K+: 68.1; 20 mM K+: 106.5.

Fig. 6. A: treatment of cells with SNAP elicits dephosphorylation of phosphotyrosine in paxillin. Cells were treated without or with varying concentrations of SNAP for 30 min, protein was solubilized, and paxillin was immunoprecipitated as described in MATERIALS AND METHODS. After SDS-PAGE and transfer to polyvinylidene difluoride (PVDF), blots were probed with either anti-phosphotyrosine antibody (right) or stripped and reprobed with anti-paxillin antibody (left) to verify the presence of equal amounts of protein on the blot. Densitometric values (with values at 0 µM SNAP set to 100) were as follows: right, 0 µM: 100; 10 µM: 73.0; 50 µM: 30.9; left, 0 µM: 100; 10 µM: 151.8; 50 µM: 99.5; 100 µM: 86.1. B: treatment of cells with Ca-deficient medium elicits dephosphorylation of phosphotyrosine in paxillin. Cells were treated with PBS supplemented with 1 mM CaCl2 or 1 mM EGTA for 10 min, followed by immunoprecipitation with anti-paxillin and Western blotting, as described above. Blots were probed with either anti-phosphotyrosine (right) or stripped and reprobed with anti-paxillin (left). Densitometric values, with CaCl2 values set to 100, were as follows: right, CaCl2: 100; EGTA: 2.8; left, CaCl2: 100; EGTA: 93.1.
It is interesting to note that paxillin was found to constitute the majority of phosphoproteins in the 70- to 85-kDa range, as determined by quantitative immunoprecipitation and probing of the supernatant via antiphosphotyrosine Western blotting, which indicated relatively little remaining protein phosphotyrosine in the supernatant (results not shown). The diffuse nature of the bands for paxillin is similar to that found in previous studies (15, 27) and is attributable to variable and extensive serine/threonine and tyrosine phosphorylation, resulting in differential migration during SDS-PAGE (3).

Our next experiments were done to investigate the effect of NO on a second focal adhesion protein, cortactin. Results given in Fig. 7A indicate that SNAP elicited phosphotyrosine dephosphorylation of cortactin. As shown in Fig. 7B, this effect was also mimicked by incubation of cells in Ca-deficient medium, thus providing further support for the hypothesis that the effect of NO was mediated via reduction of intracellular Ca.

**DISCUSSION**

In a previous study from our laboratory, we reported that cGMP agonists, including NO, elicit dephosphorylation of protein phosphotyrosine and increase cellular PTPase activity in primary cultures of aortic smooth muscle cells from newborn rats (7). An earlier study from another group found that an unidentified 120-kDa protein was dephosphorylated on treatment of bovine pulmonary artery smooth muscle cells with sodium nitroprusside or cGMP (16). In the current study, we report data supporting a mechanism that explains these effects, and we identify paxillin and cortactin as two proteins that manifest decreased levels of phosphotyrosine in response to NO or reduction of cytoplasmic Ca. It should also be noted that our study is consistent with very recent work showing that overexpression of NO synthase attenuates PDGF-induced paxillin phosphorylation in vascular smooth muscle cells (9). A summary of the proposed mechanistic scheme based on the current study is given in Fig. 8.

Of note, the current studies were done in cells from newborn rat aorta that were not treated with exogenous growth factors. This is because a previous study from this laboratory had indicated that these cells are not responsive to exogenous PDGF, epidermal growth factor, or FGF-2 (7). These findings are in accord with previous studies from another laboratory that have reported the self-activation of cells from newborn rat aorta (12). Although these cells were responsive to insulin-like growth factor I (IGF-I), responses to NO in the presence of exogenous IGF-I could be attributed to events occurring in the basal condition and did not appear to represent specific interactions between signal transduction mechanisms generated by IGF-I and NO.

The results presented here are consistent with the hypothesis that NO induces dephosphorylation of protein phosphotyrosine via a mechanism involving reduction of cytoplasmic Ca levels. Moreover, on the basis of data indicating that the reduction of cytoplasmic Ca induced by NO is at least in part mediated via activation of cGMP-dependent protein kinase (6), we infer that the capacity of NO to tyrosine dephosphorylation is mediated via a cGMP-dependent protein kinase pathway (Fig. 8).

It should be noted that the mechanism linking lowered Ca with phosphotyrosine dephosphorylation is currently unknown, but we have evidence that it does not involve calmodulin or calmodulin kinase, based on the lack of effect of the calmodulin antagonist W-7 or the calmodulin kinase II inhibitor KN-62 on phospho-
The extracellular matrix and by FGF during the G1 phase of the cell cycle (5, 29, 31). Thus we speculate that the lack of responsiveness to such agonists requires an underlying change in cell physiology resulting in reduced cytoplasmic Ca to the cell. Moreover, a recent study indicates that intracellular signaling by several publications have reported reduction of cytosolic free Ca levels even in vascular smooth muscle cells. Regulation of intracellular Ca levels in cultured vascular smooth muscle cells. Reduction of Ca to by atriopeptin and 8-bromo-cyclic GMP is mediated by cyclic GMP-dependent protein kinase. J. Biol. Chem. 264: 1146–1155, 1989.


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