Decreased sensitivity to vasoconstrictors in aortic rings after acute exposure to nitric oxide

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Kanagy, Nancy L., John R. Charpie, Jamie Dananberg, and R. Clinton Webb. Decreased sensitivity to vasoconstrictors in aortic rings after acute exposure to nitric oxide. Am. J. Physiol. 271 (Heart Circ. Physiol. 40): H253–H260, 1996.—Nitric oxide (NO) has been postulated as a regulator of vascular reactivity, and the current study tested the hypothesis that NO-induced decreased sensitivity to vasoconstrictors persists following removal of NO. Endothelium-denuded segments of rat aorta were incubated 2–4 h at 37°C with the NO donor S-nitroso-N-acetylpenicillamine (SNAP). Incubation produced rightward shifts in concentration response curves for phenylephrine (i.e., half-maximum effective concentration (EC50); in μM): control = 0.016, NO = 0.141, aluminum fluoride (i.e., EC50 in mM: control = 1.66, NO = 2.29), and KCl (i.e., EC50 in mM: control = 5.9, NO = 23.9). Similar shifts were seen for two other NO donors. The SNAP-induced shift was not attenuated by a guanylyl cyclase inhibitor, LY-83583 (10 μM) and other NO donors. The SNAP-induced shift was not attenuated by 1,4-naphthoquinone (3.3 ± 1.0 pmol/mg protein), an increase unaffected by 1,4-naphthoquinone (3.3 ± 1.0 pmol/mg protein) but prevented by LY-83583 (1.6 ± 0.36 pmol/mg protein). ADP ribosylation of three proteins was observed in membranes from HEK 293 cells: 88, 66, and 38 kDa. ADP ribosylation of the 38-kDa protein was stimulated in a concentration-dependent manner by NO but was not decreased by 1,4-naphthoquinone. In conclusion, NO produces a long-lasting inhibition of vascular contractility by both a cGMP-dependent and -independent mechanism. Based on the observations of 1,4-naphthoquinone, we conclude that the cGMP-independent mechanism is not stimulation of endogenous ADP ribosylation but some other covalent modification in the pathway that mediates contraction.

vascular smooth muscle; guanosine 3',5'-cyclic monophosphate; adenosine 5'-monophosphate ribosylation; guanylyl cyclase

NITRIC OXIDE (NO) has been shown to be an important endogenous regulator of vascular tone. The major endogenous source of NO in the vasculature is from enzymatic synthesis in vascular endothelial cells. Diffusion of NO into the vascular smooth muscle cells leads to activation of soluble guanylyl cyclase and increased cGMP production (16). It has been demonstrated that inhibition of NO production can lead to altered vascular reactivity and increased blood pressure (24), and it has been observed that there is a decreased production of NO in some forms of hypertension and in atherosclerosis (19, 27). These observations have led to the hypothesis that a deficit in NO-mediated cGMP production limits vasodilatation and contributes to the elevated vascular tone characteristic of these disease states (6).

However, since its discovery as an endogenous modulator of vascular tone, NO has been demonstrated to mediate some of its physiological effects by mechanisms independent of guanylyl cyclase activation (23). One cGMP-independent mechanism is NO-induced covalent modification of intracellular enzymes, an event demonstrated to cause inactivation of enzyme function (14, 23). It is proposed that these covalent modifications are responsible for a portion of NO effects on long-term potentiation in the hippocampus (28) and for the cytotoxic effects of NO released from macrophages (16). We propose that in vascular smooth muscle, NO is also able to modulate function through a cGMP-independent modification of one or more proteins mediating contraction. We hypothesized that exposure of arterial segments to exogenous NO would cause a persistent inhibition of agonist-mediated vasoconstriction by uncoupling ligand-bound receptors from the contraction. We further predicted that this inhibition was mediated in part by NO actions independent of cGMP production (4).

METHODS

Animals

Adult male Wistar Kyoto (WKY) rats (250–350 g) from Harlan Sprague Dawley (Indianapolis, IN) were used for all procedures.

Drugs and Chemicals

8-Bromoguanosine 3',5'-cyclic monophosphate (8-Brc-GMP), S-nitroso-N-acetylpenicillamine (SNAP), and LY-83583 were purchased from BioMol Research Laboratories (Plymouth Meeting, PA), diethylamine-NONOate from Cayman Chemical (Ann Arbor, MI), S-nitroso-γ-glutathione from Alexis Biochemicals (San Diego, CA), and 32P-labeled β-nicotinamide adenine dinucleotide (32P-NAD) from New England Nuclear-DuPont (Boston, MA). Polyacrylamide gels, molecular weight standards, and running buffer reagents were purchased from BioRad (Hercules, CA), and the cGMP assay kit was from Amersham (Arlington Heights, IL). All other drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO).

General Protocols

On the day of the experiment, animals were anesthetized with pentobarbital sodium (50 mg/kg) and exsanguinated. The thoracic aorta was removed and placed in cold physiological saline solution (PSS) containing (in mM) 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4·7H2O, 14.9 NaHCO3, 5.5 dextrose, 0.026 CaNa2·EDTA, and 1.6 CaCl2. Vessels were cleaned and cut into four segments (4 mm each). The segments were denuded of endothelial cells by gently rubbing the lumen with closed forceps. The denuded rings were incubated in 1 ml of PSS for 2 h at 37°C with or without a NO donor. After incubation, rings were suspended in tissue baths filled with PSS maintained at 37°C and aerated with 95% O2/5% CO2.
CO₂. Rings were stretched with 3 g of passive force and rinsed every 15 min during a 1-h equilibration period. Indomethacin (10 μM) was added to the bath during the final 30 min of equilibration. After equilibration, tissues were challenged with phenylephrine (PE; 10⁻⁷ M), and the absence of endothelium was verified by failure of acetylcholine to cause relaxation (10⁻⁸−10⁻⁵ M). Tissues were rinsed for an additional 45 min before cumulative concentration-response curves were generated. Each ring was used for only one concentration-response curve per agonist and for a maximum of two concentration-response curves. The number of animals used is represented by n, and all data are expressed as the mean ± standard error (SE). Force was recorded with an FT03 transducer attached to a polygraph (Grass Instruments Quincy, MA).

Experimental Protocols

Protocol 1: NO and cGMP incubations. To determine if elevated NO could produce a lasting inhibition of vasconstriction, denuded aortic rings were incubated in 1 ml of PSS containing either a NO donor, a cGMP analogue, or a NO donor plus a guanylyl cyclase inhibitor before the 1-h equilibration period in PSS containing no additions. The four incubation solutions used were 1) PSS alone, 2) PSS + 10 μM SNAP, 3) PSS + 100 μM 8-BrcGMP, and 4) PSS + 10 μM SNAP + 10 μM LY-83583 (a guanylyl cyclase inhibitor). The incubation for this protocol was carried out for 4 h at 4°C. Rings were randomly paired (2/bath), equilibrated, and washed with plain PSS for 1 h, challenged with PE and acetylcholine as described above, and cumulative concentration-response curves to PE (10⁻⁵−10⁻⁴ M), AlF₃ (0.25–8 mM), and KCl (1.5–95 mM) were generated. These experiments verified the ability of short-term exposure to NO in vitro to modify the response to contractile agents after the NO was removed from the tissue.

Protocol 2: NO and 1,4-naphthoquinone incubations. Four incubation solutions were used: 1) PSS alone, 2) PSS + 10 μM SNAP, 3) PSS + 50 μM 1,4-naphthoquinone (a mono-ADP ribosyltransferase inhibitor; Ref. 1), and 4) PSS + 10 μM SNAP + 50 μM 1,4-naphthoquinone. Incubations were carried out for 2 h at 37°C. Rings were randomly paired (2/bath), and cumulative concentration-response curves were generated for PE (10⁻¹⁰−10⁻⁴ M) and AlF₃ (0.25–8 mM). Responses in the control and SNAP-treated rings were not different from those treated in protocol 1. These experiments evaluated the relative contribution of ADP ribosylation to the NO-induced changes in contractile responsiveness. Three additional sets of four rings were incubated with three different NO donors (10 μM) or vehicle (distilled water), and cumulative concentration-response curves were constructed to determine if the observed inhibition was independent of the carrier molecule for NO.

Protocol 3: cGMP assay. Rings were incubated in PSS according to protocol 2 and then suspended in tissue baths. After 1 h of equilibration in oxygenated PSS with rinsing every 15 min, 3-isobutyl-1-methylxanthine (IBMX; 100 μM) was added to the PSS and incubated for 30 min to prevent degradation of cGMP. Rings were rapidly removed from the bath, frozen in liquid nitrogen, and stored at −70°C until assayed. For the assay, each frozen ring was pulverized in liquid nitrogen, and the powdered tissue was acid-extracted for 1 h on ice (1 ml of 0.1 M IICl, 100 μM IBMX). The extract was homogenized in a ground glass homogenizer, spun at 6,000 g for 10 min, and the supernatant was removed and lyophilized. The lyophilized samples were reconstituted and assayed using a standard enzyme immunoassay and a microtiter plate reader (Amersham). The cGMP concentration was normalized for the protein content of the pellet, as determined by a Bradford assay. These experiments assessed the correlation between changes in cGMP content and changes in contractility. They also evaluated the degree of guanylyl cyclase inhibition during treatment with LY-83583.

Protocol 4: in vitro ADP ribosylation. Membranes were prepared from the human embryonic kidney cell line, HEK 293. This line is commonly used to study signal transduction mechanisms and stably expresses the G₉, G₁₉, and G₃ proteins (4). Cells in passages 7–10 were grown to confluence and then transferred into culture plates, suspended in lysis buffer (tris(hydroxyethyl)aminomethane (Tris)-HCl, pH = 7.4 with 0.2 mM phenylmethylsulfonyl fluoride and 1.0 mM EDTA) and disrupted with a ground glass homogenizer. The cell homogenates were centrifuged at 800 g for 10 min to remove cell debris, and the supernatants were aspirated and centrifuged at 120,000 g for 1 h in a refrigerated ultracentrifuge. The membrane pellet was suspended in lysis buffer, and the protein content was determined using the Bradford method, and aliquots were frozen at −20°C until used. Isolation procedures were carried out at 4°C.

ADP ribosylation was accomplished by incubating membrane proteins (150 μg protein/tube) in ribosylation buffer, using a modification of the protocol from Brune et al. (3). Ribosylation buffer contained (in mM) 50 Tris-HCl, 1 EDTA, 1 MgCl₂, 1 dithiothreitol, 1 ATP, 10 thymidine, 0.1 GTP, 0.1 NAHP, 10 L-arginine, and 0.01 [³²P]-labeled GTP. Pertussis toxin (200 nmol), SNAP (100 μM), 1,4-naphthoquinone (50 μM), or distilled water was added to individual incubation tubes containing membrane proteins in ribosylation buffer for a final volume of 100 μl. Tubes were incubated for 60 min at 30°C. The ADP-ribosylation reaction was terminated by adding 25 μl of 5× Laemmli buffer. Samples were separated in 10 or 12% sodium dodecyl sulfate-polyacrylamide gels with molecular weight standards included in each gel. Pertussis toxin-labeled Gi α-subunit proteins (0.3 ng) were also included to compare migration rates of unknown proteins with ADP-ribosylated G proteins. Autoradiograms were prepared to evaluate [³²P]ADP ribose incorporation. Bands on the developed film were analyzed by densitometry.

Statistical Analysis

All data are expressed as means ± SE. Comparisons between groups were made using paired (incubated rings) or unpaired (gels) Student's t-test. The Bonferroni correction was used when three or more individual comparisons were made, and a P value ≤ 0.05 was considered statistically significant.

RESULTS

Incubation of aortic rings with SNAP caused a rightward shift in the concentration-response curves to PE (Fig. 1A), aluminum fluoride (AlF₃; Fig. 1B), and KCl (Fig. 1C) compared with control. This was apparent as a significant increase in the half-maximum effective concentration (EC₅₀) values for the SNAP- versus control-treated tissues (Table 1). A similar shift was observed with three different NO donors for PE and AlF₃ (Fig. 2), indicating that the shift was dependent on NO generation and not an effect of the carrier molecule.

The decreased sensitivity to AlF₃ was attenuated in rings incubated with NO and the mono-ADP ribosyltransferase inhibitor 1,4-naphthoquinone (Fig. 3) but not in rings incubated with the guanylyl cyclase inhibitor LY-83583 (Table 1 and Fig. 4). In addition, the
rightward shift in the curves was not produced by incubation with the membrane-permeable cGMP analogue 8-BrcGMP (Table 1 and Fig. 4). These observations indicate that NO-induced desensitization is independent of guanylyl cyclase activation and suggest that ADP ribosylation might contribute to the persistent decrease in sensitivity to contractile agonists.

Calculated EC<sub>50</sub> values for the five different incubation protocols revealed a significant increase for both PE and AlF<sub>3</sub> in the NO-treated group and in the group treated with NO plus the guanylyl cyclase inhibitor. However, the EC<sub>50</sub> value for the groups treated with 8-BrcGMP and NO + 1,4-naphthoquinone were not different from control, again suggesting that the change in sensitivity to the agonists was dependent on ADP ribosylation but not on stimulation of guanylyl cyclase (Table 1).

The concentration of cGMP in rings was measured in each of the five groups, following a 1-h wash-out/equilibration period (see METHODS). Data revealed that cGMP content was still elevated in the SNAP-incubated rings (4.6 ± 0.8 vs. 1.5 ± 0.15 pg/mg protein in control tissue) even after the washing period. The SNAP-induced increase in cGMP was prevented by the addition of LY-83583 (1.6 ± 0.36 pg/mg protein) but was not affected by 1,4-naphthoquinone (3.38 ± 0.26 pg/mg protein; Fig. 5). This was in direct contrast to the changes in sensitivity observed in the contractile experiments, suggesting that the increased sensitivity in the 1,4-naphthoquinone-treated tissues was not dependent on decreased cGMP production.

The in vitro incubation of HEK 293 cell membranes with <sup>32</sup>P-NAD resulted in the incorporation of radiolabeled ADP ribose by three proteins; 38, 66, and 88 kDa (Fig. 6). However, only the incorporation at the 38-kDa band was stimulated by NO, and labeling of this band was enhanced by the addition of 1,4-naphthoquinone, suggesting that the NO-induced ADP ribosylation on this protein was independent of stimulation of an ADP ribosyltransferase. In contrast, labeling of the 88-kDa protein was attenuated by the addition of the naphthoquinone but was not dependent on the presence of NO.

Table 1. EC<sub>50</sub> values for aortic segments following NO incubation

<table>
<thead>
<tr>
<th>Condition</th>
<th>AlF&lt;sub&gt;3&lt;/sub&gt; EC&lt;sub&gt;50&lt;/sub&gt;, log mM</th>
<th>Maximal force, mg</th>
<th>Phenylephrine EC&lt;sub&gt;50&lt;/sub&gt;, log mM</th>
<th>Maximal force, mg</th>
<th>Potassium Chloride EC&lt;sub&gt;50&lt;/sub&gt;, log mM</th>
<th>Maximal force, mg</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.22 ± 0.03 (11)</td>
<td>2.18 ± 0.09</td>
<td>7.79 ± 0.19 (10)</td>
<td>1.59 ± 0.22</td>
<td>0.77 ± 0.07 (6)</td>
<td>1.48 ± 0.25</td>
</tr>
<tr>
<td>SNAP alone (10 µM)</td>
<td>0.36 ± 0.02* (11)</td>
<td>2.33 ± 0.16</td>
<td>6.84 ± 0.07* (10)</td>
<td>1.37 ± 0.30</td>
<td>1.38 ± 0.14* (6)</td>
<td>1.52 ± 0.12</td>
</tr>
<tr>
<td>SNAP + 1,4-naphthoquinone (50 µM)</td>
<td>0.19 ± 0.05 (5)</td>
<td>1.20 ± 0.43*</td>
<td>7.31 ± 0.13 (5)</td>
<td>1.38 ± 0.35</td>
<td>1.38 ± 0.14* (5)</td>
<td>1.52 ± 0.12</td>
</tr>
<tr>
<td>SNAP + LY-83583 (10 µM)</td>
<td>0.40 ± 0.03* (6)</td>
<td>2.23 ± 0.10</td>
<td>7.31 ± 0.13 (5)</td>
<td>1.38 ± 0.35</td>
<td>1.38 ± 0.14* (6)</td>
<td>1.52 ± 0.12</td>
</tr>
<tr>
<td>8-BrcGMP (100 µM)</td>
<td>0.31 ± 0.01 (6)</td>
<td>2.28 ± 0.22</td>
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Values are means ± SE; no. in parentheses (n), number of rings. EC<sub>50</sub>, half maximum effective concentration; SNAP, S-nitroso-N-acetylpenicillamine; 8-BrcGMP, 8-bromoguanosine 3',5'-cyclic monophosphate. *P ≤ 0.05.
to induce incorporation (Fig. 6). These results indicated that NO can influence the ADP ribosylation of at least three proteins at the concentration employed in the tissue incubations, but inhibition of this incorporation does not correlate with inhibition of changes in contractile sensitivity. These results indicate that NO and 1,4-naphthoquinone may have effects unrelated to ADP ribosylation that affect contractile sensitivity to PE and AlF₃.

**DISCUSSION**

The principal observation of this study was that exposure of isolated vascular smooth muscle segments to NO produces a long-lasting decrease in sensitivity to vasoconstrictors without decreasing the maximal response. This effect was independent of cGMP generation and indicates that NO can chronically regulate vascular tone in addition to modulation of moment-to-moment blood flow. This observation provides a mechanism for the observations in several previous reports that NO effects on vascular contractility persist after NO production is normalized. In one study, patients treated with NO donors had a decreased sensitivity to vasoconstrictors that persisted in the absence of the donor (8), whereas in another report, blood vessels removed from rats chronically treated with a NOS inhibitor exhibited increased sensitivity to vasoconstrictors (25).

Most previous studies examining NO regulation of vascular tone have focused on the ability of NO to rapidly relax tissues by stimulating soluble guanylyl cyclase (16). However, several recent reports have established that NO can also act in a cGMP-independent fashion. These cGMP-independent effects include direct stimulation of potassium channels (2), elevation of guanosine triphosphatase (GTPase) activity in heterotrimeric and small G proteins (14), activation of redox-sensitive kinases (10), and stimulation of ADP ribosylation (23). A recent review suggests that these cGMP-independent effects may all depend on NO initiation of redox reactions, leading to covalent modification of target proteins (23). NO inhibition of cell proliferation has also been shown to be independent of cGMP generation under certain conditions (9). Together these findings support the concept that NO can regulate intracellular enzymes in a cGMP-independent manner, profoundly affecting cellular function. The role of these NO-induced events has not been explored in vascular tissue, and the current study examined the novel concept that NO chronically regulates vascular contractility independent of cGMP generation.

One of the first non-cGMP effects of NO reported was the stimulation of endogenous ADP ribosylation in the cytosolic fraction of platelets (4). Subsequent reports identified the target of NO-induced ADP ribosylation in platelets as glyceraldehyde 3-phosphate dehydrogenase.
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Fig. 4. Role of cGMP in NO-induced inhibition of aluminum fluoride contractile response. ○, Response in control tissues; ●, response in tissues treated with 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP; 10 μM); ■, response in tissues incubated with SNAP alone (10 μM); and □, tissue incubated with SNAP and LY 83583 (50 μM). Each point illustrates the mean of 6 rats. *:P ≤ 0.05, significant
differences from control.

The concept of endogenous ADP ribosylation as a mechanism of enzyme regulation in eukaryotic cells was first proposed by Moss and Vaughan (17) in 1978. More recent measures of endogenous ADP ribosylation have provided substantial evidence that ADP ribosylation occurs in most cell types in vivo (15, 18) and that a large family of endogenous ADP ribosyltransferases modify a variety of intracellular proteins (11), including G proteins (18, 20). Endogenous ADP ribosylation of G proteins leads to increased adenylyl cyclase activity (17, 18), and ribosyltransferases have been proposed to function as endogenous regulators of G protein-mediated functions. These enzymes are very sensitive to several inhibitors, including novobiocin, aminobenzimidazoles, and the naphthoquinones (1). The 1,4-naphthoquinone used in the current study is 100-fold selective for the mono-ADP ribosyltransferases over poly-ADP ribosyltransferases (nuclear repair enzymes; 1). In the current study, this vitamin D analogue inhibited the functional effects of NO in vascular smooth muscle segments, providing functional support for the hypothesis that activation of ADP ribosyltransferases contributes to NO inhibition of vascular contractility. However, it has been difficult to document regulation pathways for endogenous ADP ribosylation, and the current study provides only weak support for the hypothesis that NO can act as an endogenous regulator. Contractile data support the concept that NO stimulates endogenous ADP ribosyltransferases, whereas the in vitro incubations indicate that NO stimulation of ADP ribosylation is not enzymatically driven. In combination with the evidence in the literature that NO labeling of GAPDH is nonenzymatic (3) and the paucity of data to indicate that NO can stimulate these enzymes, it appears that the non-cGMP effects of NO on contractility in the current study are also independent of this pathway.

The ability of NO incubation to alter the contractile responses to AlF₄⁻, PK, and KCl suggests that the incubation modifies a component in a pathway common to these three contractile agents. A possible site is a channel protein regulating membrane potential (a K⁺ channel or an L-type Ca²⁺ channel) or a protein involved in mediating excitation-contraction coupling. The decrease in contractile sensitivity following incubation with the NO donors was independent of the NO donor used and was not mimicked by incubation with a cGMP analogue. Measurement of vascular content of cGMP determined that the decreased agonist sensitivity was unrelated to changes in cGMP content. Thus the data support the hypothesis that NO can regulate vascular contractility through a cGMP-independent modification that persists for hours following NO exposure.

Fig. 5. cGMP content of vascular segments after incubation with NO donor SNAP. Tissue was treated and prepared as described in METHODS. cGMP content is normalized for protein content. 1,4-NQ, ADP-ribosyltransferase inhibitor 1,4-naphthoquinone (50 μM); LY 83583, guanylyl cyclase inhibitor (10 μM). Bars represent means of 6 rats and error bars show SE. *:P ≤ 0.05, significant differences from control.
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A

88 kDa

65 kDa

38 kDa

log [SNAP] (mol/L) -6 -5 -4 -4 -

1.4 N.O. (50 µmol/L) - - - + -

B

Fig. 6. NO-induced ADP ribosylation in whole cell extracts. ADP ribosylation was evaluated as incorporation of [32P]ADP ribose. Activity is expressed as densitometry units obtained using a computer imaging program. Concentration dependence of ADP ribosylation was evaluated by exposing cell extracts to indicated concentrations of SNAP in the presence of [32P]-labeled S-nicotinamide adenine dinucleotide (NAD) in the presence or absence of 1,4-NQ (50 µM; see Experimental Protocols for details). A: representative autoradiogram of ADP-ribosylated proteins. B: densitometry values of 38-kDa band in tissues treated with indicated amounts of SNAP. Error bars indicate SE, and n = 4 separate experiments. *P ≤ 0.05, significant differences from control.

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Fig. 6. NO-induced ADP ribosylation in whole cell extracts. ADP ribosylation was evaluated as incorporation of 32P-labeled ADP. Activity is expressed as densitometry units obtained using a computer imaging program. Concentration dependence of ADP ribosylation was evaluated by exposing cell extracts to indicated concentrations of SNAP in the presence of 32P-labeled S-nicotinamide adenine dinucleotide (NAD) in the presence or absence of 1,4-NQ (50 µM; see Experimental Protocols for details). A: representative autoradiogram of ADP-ribosylated proteins. B: densitometry values of 38-kDa band in tissues treated with indicated amounts of SNAP. Error bars indicate SE, and n = 4 separate experiments. *P ≤ 0.05, significant differences from control.

The reason for a discrepancy between the ability of 1,4-naphthoquinone to attenuate the NO effects in vascular segments, but not in the isolated membranes, is unclear but several possibilities exist. First, enzymatic incorporation of 32P-ADP ribose may have occurred in some proteins but at too low a level to be observed on the autoradiogram. If the decreased sensitivity to vasoconstrictors is caused by enzyme-mediated ADP ribosylation of a protein less abundant than GAPDH, such undetected labeling may have been inhibited by the 1,4-naphthoquinone. Alternatively, 1,4-naphthoquinone reversal of the NO-induced changes in sensitivity may have been due to a nonspecific effect of the inhibitor on another NADH-sensitive enzyme, independent of endogenous ADP ribosyltransferases.

Another possibility is that differences between the transformed kidney cell line used in the ADP-ribosylation studies and the intact vascular tissues used in the contractile studies may have contributed to the differences observed in the effects of the inhibitor. Perhaps there is a difference in the expression of the proteins responsible for the contractile inhibition (i.e., a channel protein, a protein kinase) that were modified in the smooth muscle cells but not in the kidney cells. The kidney cells were chosen because they express both the G and the G proteins at a relatively high level (4) and it was hypothesized that these were likely targets for ADP ribosylation. However, it is possible that the ribosylation pattern in vascular smooth muscle cells would have been different.

Alternatively, nonenzymatic modification of the glycolytic enzyme GAPDH may have caused the inhibition of vascular contraction, because GAPDH has been implicated in the regulation of many intracellular signaling events. These events include nuclear export of tRNA (22), phosphorylation of intracellular proteins (12), regulation of actin filaments and microtubules (9), regulation of Ca2+ release from the sarcoplasmic reticulum (13), and binding to small GTPases (7). Thus an NO-induced modification of GAPDH may have contributed to changes in vascular smooth muscle contractility. However, the data from the isolated membranes do not support this concept. In the current study, NO caused concentration-dependent ADP ribose incorporation on a protein of similar molecular weight to GAPDH but, unlike the changes in contractile sensitivity, the incorporation was not inhibited by 1,4-naphthoquinone. Therefore it appears that, like stimulation of guanylyl cyclase, NO-induced ADP ribosylation of GAPDH parallels but does not cause the changes in contractility. The conclusion most consistent with the data is that NO modified a naphthoquinone-sensitive protein that regulates excitation-contraction coupling in vascular smooth muscle.

It is unlikely that residual elevation in cGMP production following the 2-h NO incubation period persisted throughout the following 6 h of contractile experiments in view of the measured cGMP concentrations. In addition, the guanylyl cyclase inhibitor LY-83583 had no effect on the response to NO incubation, although it did prevent an increase in cGMP content. Secondly, intracellular cGMP was equally elevated in the SNAP-treated tissues with and without 1,4-naphthoquinone, although the contractile responses were very different. Finally, incubation with a concentration of the cell-permeable cGMP analogue 8-BrcGMP that causes maximal relaxation (100 µM) did not cause a similar shift in...
the concentration-response curves. Therefore, a portion of the persistent effect appears to be independent of cGMP production.

A possibility not directly addressed is that sustained NO-induced changes in sensitivity are independent of both ADP ribosylation and guanylyl cyclase stimulation. This is consistent with the inability of 1,4-naphthaquinone to completely reverse the NO-induced shift in the vascular responses and the lack of effect of the quinone on NO-induced ADP ribosylation in membranes. It is also supported by the shift in the KCl concentration-response curve, a contraction apparently independent of G protein pathways. The ability of NO to inhibit protein synthesis (9), stimulate calcium-sensitive K+ channels (2), or cause other as yet undefined effects may cause or at least contribute to the rightward shift in the concentration-response curves following incubation with NO donors. These are important avenues for future investigations of the mechanism of this effect. The current observation that NO incubation causes a profound desensitization of KCl-stimulated contraction implies that the mechanism is independent of receptor and G protein modification and is a modification of either a K+-channel (2), a Ca2+-channel, or a component downstream from these two proteins.

An attractive aspect to the hypothesis that NO can induce covalent modification of signaling proteins is the longevity of the effect. Although covalent modifications are reversible (26), the reversal is a gradual process and would account for the persistent decreased sensitivity to contractile agonists following removal of the NO stimulus: changes similar to those observed in physiological conditions with elevated NO production. This pathway would convert the transient signal of NO generation into a chronic modulator of total peripheral resistance and is consistent with the role of NO as an endogenous regulator of vascular tone. This is supported by the clinical observation that chronic nitrite treatment leads to a decreased contractile response in isolated internal mammary arteries (8) and by animal studies showing that chronic inhibition of NO production causes persistent changes in both endothelial and vascular function (25).

In summary, exposure of isolated aortic segments to NO donors decreased the sensitivity to vasoconstrictors. This inhibition was independent of guanylyl cyclase stimulation. These data suggest that NO-induced modification of signaling components inhibits vascular contractility and that NO-induced covalent modification may be an important mechanism for chronic regulation of vascular reactivity.

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