Basic Polyamino Acids Rich in Arginine, Lysine, or Ornithine Cause Both Enhancement of and Refractoriness to Formation of Endothelium-Derived Nitric Oxide in Pulmonary Artery and Vein

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The objective of this study was to elucidate the mechanism by which polyamino acids containing L-arginine, L-lysine or L-ornithine cause endothelium-dependent relaxation of bovine intrapulmonary artery and vein. Basic but not acidic or neutral polypeptides ranging in average molecular weights from 17 to 225 kDa elicited time- and concentration-dependent relaxation and cyclic GMP accumulation in precontracted rings of artery and vein by endothelium-dependent mechanisms. Vascular responses were markedly inhibited by oxyhemoglobin, methylene blue, or potassium. The basic polyamino acids stimulated the formation and/or release of an endothelium-derived relaxing factor (EDRF) identified as nitric oxide (NO) in perfused segments of both artery and vein as assessed by bioassay. The polyamino acids and A23187 released a similar endothelium-derived NO (EDNO) from artery and vein, as assessed by the similar half-life (3-5 seconds), antagonism by superoxide anion or oxyhemoglobin, enhancement by superoxide dismutase, and lack of influence by indomethacin. The basic polyamino acids elicited potent relaxant responses with EC50 values ranging from 3×10^-9 to 2×10^-7 M, and a direct correlation was obtained between molecular weight and relaxation potency irrespective of the basic amino acid incorporated. Prolonged contact of arterial or venous rings with basic polyamino acids resulted in the rapid development of marked refractoriness to relaxation and cyclic GMP formation on addition of polyamino acid. Moreover, refractoriness developed to the vascular responses of other endothelium-dependent vasodilators but not to glyceryl trinitrate or isoproterenol. The mechanism of refractory responses was attributed to interference with EDNO formation and release as assessed by bioassay and chemical assay. The hypothesis is forwarded that the basic polyamino acids serve as partial substrates for the enzyme system that catalyzes the conversion of L-arginine to NO. Prolonged contact between substrate and enzyme results in enzyme desensitization and the development of refractoriness or a form of tolerance to vasodilators whose action is mediated by EDNO. (Circulation Research 1989;64:315-329)

Recent studies have indicated that small vasoactive polypeptides containing basic amino acids such as L-arginine or L-lysine cause relaxation of vascular smooth muscle by diverse mechanisms of action. Moreover, the mechanism of action of a given polypeptide may be different in one type of blood vessel or species than in another. Endothelium-dependent arterial relaxation has been demonstrated for numerous polypeptides including bradykinin, vasoactive intestinal polypeptide, substance P, thrombin, cholecystokinin, bombesin, neuropeptide, melitin, and others.1-12 Bradykinin was demonstrated also to elicit endothelium-dependent relaxation of venous smooth muscle.11,12 Where studied, such polypeptides have been shown to stimulate endothelium-dependent cyclic GMP accumulation in vascular smooth muscle.6,11,12 The direct formation and/or release of endothelium-derived nitric oxide (EdNO) has been shown to mediate the relaxation of arterial and venous smooth muscle by many of these endothelium-dependent vasodilators.6,11,12 Basic polypeptides containing basic amino acids such as L-arginine or L-lysine have been shown to relax arterial and venous smooth muscle by mechanisms and/or release of EdNO.6,11,12
relaxing factor (EDRF) from intact blood vessels or cultured endothelial cells has been shown to occur in response to bradykinin and substance P. 13-16 Some of these polypeptides relax vascular smooth muscle by mechanisms involving stimulation of prostacyclin formation. 12,17-19 In addition, some of the above polypeptides, as well as parathyroid hormone and several atriopeptins that contain basic amino acids, were found to elicit endothelium-independent arterial relaxation.4,7,10-22

Thus, there appears to be no unifying mechanism of action for such vasodilator polypeptides. In a more extensive study we reported that bradykinin and vasoactive intestinal polypeptide each elicit endothelium-dependent relaxation of bovine intrapulmonary artery by two independent mechanisms involving both cyclic GMP formation triggered by EDRF and cyclic AMP formation triggered by prostacyclin or a related prostaglandin. 12 On the other hand, bradykinin relaxed veins by endothelium-dependent mechanisms involving only EDRF release and cyclic GMP accumulation, whereas vasoactive intestinal polypeptide was inactive.12 A recent brief report indicated that two relatively large polypeptides, which can also be termed polypeptides, composed of the basic amino acids L-arginine or L-lysine caused endothelium-dependent relaxation of rat aorta that was inhibited by methylene blue, whereas acidic polypeptides, polypeptides, and amino acids were inactive.23 The objective of the present study was to elucidate the mechanism of vascular smooth muscle relaxation elicited by such basic polypeptides and to compare this with the vascular actions of bradykinin and endothelium-independent relaxants. Polyamino acids ranging in molecular weight from 17 to 225 kDa and composed of L-arginine, L-lysine, L-ornithine, or D-lysine were tested on unrubbed and rubbed rings of bovine intrapulmonary artery and vein in the absence and presence of inhibitors such as oxyhemoglobin, methylene blue, and indomethacin. Both vascular cyclic GMP and cyclic AMP levels were monitored, and the capacity of the basic polyamino acids to stimulate the formation and/or release of endothelium-derived nitric oxide (EDNO) from intact artery and vein was assessed by bioassay and chemical assay.

Materials and Methods
Preparation of Rings of Bovine Intrapulmonary Artery and Vein

Bovine lungs were obtained from freshly slaughtered cows under 5 years of age as described previously. 24 Intrapulmonary arterial and venous branches were rapidly isolated, gently cleaned of parenchyma, fat, and adhering connective tissue, and placed in cold preoxygenated Krebs-bicarbonate solution. Segments of the second arterial branch and underlying second venous branch extending into the larger lobe were isolated. Outside diameters were 4-6 mm (artery) and 6-8 mm (vein). Vessel segments were sliced into rings (4 mm wide) with a specially designed microtome. 25 Rings prepared in this manner possessed an intact or functional endothelium as assessed by 80–100% relaxation responses to 10^-7-10^-6 M acetylcholine (arteries) or 10^-8 M bradykinin (veins). These vascular rings are referred to in the text as unrubbed. Endothelial cells were largely removed from vascular rings by everting the rings (intimal surface outside), gently rubbing the intimal surface with moistened filter paper for 30–40 seconds, and again everting the rings (intimal surface inside). This procedure of endothelium denudation was very effective and did not result in damage to the medial layer due to stretching or tearing that could occur through the luminal insertion of a wooden stick or cotton swab. These endothelium-denuded rings sharply contracted in response to 10^-6 M acetylcholine (arteries) or 10^-8 M bradykinin (veins), and relaxed to 10^-6-10^-7 M NO (arteries and veins).

Mounting Rings and Recording of Muscle Tension

Arterial and venous rings were mounted by means of fine nichrome wires in jacketed, 25 ml capacity, drop-away chambers containing Krebs-bicarbonate solution (37° C) gassed with 95% O_2-5% CO_2 as described previously.24,25 Changes in isometric force were measured, and length-tension relations were determined initially for unrubbed and endothelium-denuded rings of artery and vein exactly as described previously for isolated rings of bovine pulmonary artery and vein.24 Arterial and venous rings were routinely depolarized by addition of 120 mM KCl following 2 hours of equilibration at optimal tension, and were subsequently washed and allowed to equilibrate for 45 minutes before initiating any given protocol. This procedure increases and stabilizes any subsequent submaximal precontractile responses to U46619, phenylephrine, and other contractile agents, presumably by loading the smooth muscle cells with calcium. This procedure has been employed routinely for bovine pulmonary vessels in this laboratory.24,25

Bioassay Cascade Superfusion Technique

A modification26,27 of the procedure developed by Vane28 was employed. Briefly, isolated segments (5–7 cm) of endothelium-intact, bovine intrapulmonary artery or vein were cleaned of adhering connective tissue and any small branches were ligated with titanium hemostatic clips (Pilling Co, Fort Washington, Pennsylvania) to prevent leakage during perfusion of the vessel. Vessel segments were fitted at either end with polyethylene tubing, were submerged in chambers containing oxygenated Krebs-bicarbonate solution at 37° C, and were perfused (3.5 ml/min) with oxygenated Krebs-bicarbonate solution at 37° C. The perfusate was allowed to superfuse three isolated, helically cut, precontracted strips of endothelium-denuded artery or vein arranged in a cascade at 37° C. Vascular strips were contracted by
10 μM phenylephrine (artery) or 0.01 μM U46619 (vein) delivered by superfusion from a separate line, and responses were monitored exactly as described previously. Arterial and venous segments as well as vascular strips were pretreated with 10 μM indomethacin as described to prevent the formation of prostaglandins, especially prostacyclin, whose formation could be provoked by various chemicals and manipulations. The time delay between each vascular strip in the cascade was 2.5 seconds, as was the delay between the perfused vessel and the first vascular strip, to observe a decrement in the relaxant responses to EDRF during the cascade superfusion. At the start and end of each experiment, glycercyl trinitrate, a stable endothelium-independent vasodilator, was superfused (0.1 μM) over the vascular strips to be certain that the magnitudes of relaxation did not deviate appreciably among the three strips during the experiment.

**Determination of Cyclic Nucleotide Levels**

Cyclic GMP and cyclic AMP determinations were made in arterial and venous rings that had been equilibrated under tension and depolarized with KCl. Tone was monitored until the time of freeze-clamping. The use of drop-away bath chambers, freeze-clamping of rings, preparation and extraction of tissues for cyclic nucleotide determinations, and radioimmunoassay procedures were described previously. Cyclic GMP and cyclic AMP levels were determined in aliquots from the same ring extract. None of the test agents added to bath chambers interfered directly with antigen-antibody binding in the radioimmunoassay procedures. Recoveries of standard amounts of added cyclic nucleotides were determined periodically and the values ranged from 92% to 104%. Therefore, no corrections for sample recoveries were made.

**Chemical Assay of Nitric Oxide**

The concentration of NO in the superfusion media collected after perfusion of intact bovine pulmonary artery with and without A23187 or basic polyamino acids was determined by spectrophotometric procedures based on the diazotization of sulfanilic acid by NO at acidic pH, exactly as described previously.

**Scanning Electron Microscopy**

Isolated arterial rings were prepared for scanning electron microscopy using standard techniques described previously. In brief, following myographic protocols, arterial rings were immediately fixed in 4% glutaraldehyde. Arterial rings were subsequently postfixed in osmium tetroxide and then coated with a gold-palladium film. Samples were examined and photographed in an ETEC Auto-Scan Electron Microscope (Haywood, California).

**Chemicals and Solutions**

Acetylcholine chloride, bradykinin triacetate, A23187, phenylephrine hydrochloride, methylene blue, hemoglobin, indomethacin, sodium dithionite, diethyldithiol, pyrogallol, superoxide dismutase (bovine liver), all polyamino acids, amino acids, bradykinin analogues, neurotensin, and other peptides were obtained from Sigma Chemical Co, St. Louis, Missouri. Glycerol trinitrate (nitroglycerin; 10% wt/wt triturated mixture in lactose) was a gift from ICI Americas, Inc (Wilmington, Delaware). U46619 ([15S]-hydroxy-11α, 9α [epoxymethano] prosta 5Z, 13E dienoic acid) was provided by The Upjohn Co (Kalamazoo, Michigan) and was dissolved in absolute ethanol at a concentration of 5 mg/
Relaxant Effects of Basic Polyamino Acids

Endothelium-Dependent Arterial and Venous responses in U46619- or produced potent, marked, and concentration-consisting of L-arginine, L-lysine, or L-ornithine are expressed as the mean±SEM and represent statistically significant difference was p<0.05. Student’s / test for unpaired values. The level of unpaired data. Comparisons were made using the unpaired tolerance. Values represent the mean±SEM from eight rings established for each ring due to the subsequent development of relaxation. Values represent the mean±SEM from eight rings isolated from four animals (two rings per animal). Values corresponding to a comparable molecular weight, irrespective of the polyamino acid, are significantly different (p<0.05) from values for the other molecular weights within the indicated column (artery or vein).

ml. Dilutions were prepared in cold distilled water just before use. Solutions of hygroscopic acetycholine chloride were prepared, aliquoted, and stored frozen as described previously. Bradykinin and its analogues, other small peptides, pyrogallol and superoxide dismutase are unstable and were prepared fresh in distilled water just before use. Polyamino acids were prepared in distilled water and stored frozen as described previously. After peak contracture responses developed, the indicated polyamino acids were added at cumulatively increasing concentrations at threefold increments until 100% relaxation was observed. Percent relaxation signifies the percent decrease in submaximal precontractile tone elicited by U46619. Only one concentration-response relation was established for each ring due to the subsequent development of relaxation. Values represent the mean±SEM from eight rings isolated from four animals (two rings per animal). Values corresponding to a comparable molecular weight, irrespective of the polyamino acid, are significantly different (p<0.05) from values for the other molecular weights within the indicated column (artery or vein).

Calculations and Statistical Analysis

Relaxation was measured as the decrease in tension below the elevated tension elicited by precontracting arterial or venous smooth muscle with U46619. Values in the tables and in Figures 4 and 5 are expressed as the mean±SEM and represent unpaired data. Comparisons were made using the Student’s t test for unpaired values. The level of statistically significant difference was p<0.05.

Results

Endothelium-Dependent Arterial and Venous Relaxant Effects of Basic Polyamino Acids

High molecular weight basic polyamino acids consisting of L-arginine, L-lysine, or L-ornithine produced potent, marked, and concentration-dependent relaxant responses in U46619- or phenylephrine- but not potassium-precontracted unrubbed rings of bovine intrapulmonary artery and vein (Figure 1). Relaxation elicited by the basic polyamino acids was generally slow in onset and development when compared with the relaxant responses to either acetylcholine in artery or bradykinin in vein. Prior endothelial denudation of the vascular rings abolished all relaxant responses. Table 1 indicates the eight basic polyamino acids studied together with their approximate average molecular weights and EC50 values as arterial and venous smooth muscle relaxants. Compounds are listed in order of decreasing potencies as arterial relaxants. There was a direct correlation between molecular weight and relaxation potency, different polyamino acids of comparable molecular weights showed similar potencies, and potencies as arterial relaxants were similar to potencies as venous relaxants for each polyamino acid tested. Moreover, the relaxant potencies of poly-D-lysine 40 and 225 kDa were similar to the potencies of the corresponding poly-L-amino acids, and the responses were endothelium-dependent (not shown). In contrast to the basic compounds, acidic polyamino acids such as poly-L-aspartic acid 40 kDa, poly-L-glutamic acid 75 kDa, and poly-gamma-benzyl-L-glutamic acid 115 kDa were completely inactive in arterial and venous rings at concentrations of up to 10^{-6} M. Similarly, neutral polyamino acids of comparable molecular weights (poly-L-glycine, poly-L-leucine, or poly-L-tyrosine) were completely inactive, as were the basic monoamino acids themselves such as L-arginine, L-lysine, and L-ornithine, and polyamines such as spermine, spermidine, and putrescine.

Effects of Inhibitors on Vascular Relaxant Responses to Basic Polyamino Acids

The above data suggest that the EDRF first described by Furchgott and Zawadzki mediates the relaxant effects of the basic polyamino acids. Oxyhemoglobin, which binds and inactivates EDRF and NO, and methylene blue, which inhibits the stimulatory action of EDRF and NO on soluble guanylate cyclase, each abolished or markedly inhibited the arterial and venous relaxant effects of basic polyamino acids. Data for four such polypeptides are illustrated in Figure 2. As observed previously in this and other laboratories, oxyhemoglobin and methylene blue rendered arterial and venous rings supersensitive to the contractile effects of agonists. Therefore, lower concentrations of U46619 were used to contract rings in the presence of either inhibitor. The following agents were tested by preincubating arterial or venous rings for 15–30 minutes before initiating relaxations and were found to be without appreciable effect in altering relaxant responses: 10^{-2} M indomethacin, 10^{-4} M atropine, 10^{-6} M propranolol, 10^{-5} M chlorpheniramine, 10^{-5} M quinacrine (mepacrine), 10^{-4} M nordihydroguaiaretic acid, and 10^{-4} M SKF-525A.

Table 1. Potencies of Basic Polyamino Acids as Relaxants of Bovine Intrapulmonary Artery and Vein

<table>
<thead>
<tr>
<th>Polyamino Acid</th>
<th>EC50 (M)</th>
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<tbody>
<tr>
<td></td>
<td>Artery</td>
</tr>
<tr>
<td>Poly-L-lysine 225 kDa</td>
<td>3.0±0.2x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-ornithine 120 kDa</td>
<td>6.0±0.5x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-lysine 115 kDa</td>
<td>6.2±0.5x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-arginine 115 kDa</td>
<td>6.8±0.5x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-ornithine 50 kDa</td>
<td>2.2±0.3x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-lysine 41 kDa</td>
<td>2.4±0.4x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-arginine 41 kDa</td>
<td>2.8±0.3x10^{-9}</td>
</tr>
<tr>
<td>Poly-L-lysine 17 kDa</td>
<td>2.2±0.1x10^{-9}</td>
</tr>
</tbody>
</table>
Artery

VEIN

Bioassay Cascade Superfusion

The release of EDNO from intact segments of perfused artery and vein by the basic polyamino acids is illustrated in Figure 3. Superfusion of glyceryl trinitrate over the three endothelium-denuded vascular strips caused relaxant responses that were of comparable magnitudes in all three strips. In contrast, perfusates from intact artery or vein in the absence or presence of A23187 evoked decremental relaxant responses down the cascade of strips, thus reflecting the very short half-life of arterial and venous EDNO (3-5 seconds as estimated from the tracings). Poly-L-arginine 41 kDa (artery and vein) and poly-L-lysine 41 kDa (vein), when perfused through intact vessels, relaxed bioassay strips similarly to perfused A23187 (Figure 3). The higher molecular weight basic polyamino acids were more potent than lower molecular weight analogues (not shown). In the presence of perfused superoxide dismutase, the relaxant effects and duration of relaxation as well as the chemical stability of EDNO were all enhanced in comparison to control responses. On the other hand, superfused pyrogallol, which generates superoxide anion in oxygen-rich aqueous media, nearly abolished the EDNO-mediated relaxant responses. Only the data obtained in artery with poly-L-arginine 41 kDa are illustrated in Figure 3. Superfused oxyhemoglobin abolished the venous relaxant responses of EDNO released by A23187, poly-L-arginine 41 kDa, and poly-L-lysine 41 kDa (Figure 3). Oxyhemoglobin similarly inhibited arterial and venous EDNO released by several different basic polyamino acids (not shown).

Stimulation of Arterial and Venous Cyclic GMP Accumulation Elicited by Basic Polyamino Acids

In view of the observations that the basic polyamino acids caused slowly developing relaxant responses, whereas other endothelium-dependent relaxants such as acetylcholine, bradykinin, and A23187 caused rapidly developing relaxant responses, a time-course analysis of cyclic GMP accumulation and relaxation in response to a relatively high concentration of poly-L-arginine 115 kDa was conducted in artery and vein (Figure 4). Peak cyclic GMP accumulation was observed at about 180 seconds after addition of the polyamino acid, and a good correlation was observed between cyclic GMP accumulation and relaxation in both artery and vein. A similar time-course pattern was observed for poly-L-arginine 41 kDa, poly-L-lysine 41 and 225 kDa, and poly-L-ornithine 50 kDa in artery and vein (not shown). The onset of cyclic GMP accumulation consistently preceded the onset of relaxation in both artery and vein. Relaxation continued for 2-3 minutes beyond the time of peak cyclic GMP accumulation. Thus, a contact time of 180 seconds was selected for subsequent experiments. Figure 5 illustrates that poly-L-arginine 115 kDa (artery) and poly-L-lysine 225 kDa (vein) produced concentration-dependent cyclic GMP accumulation and relaxation, and a good correlation was observed between cyclic GMP accumulation and relaxation in
FIGURE 3. Release of EDNO from perfused artery and vein by basic polyamino acids. Endothelium-denuded strips of bovine intrapulmonary artery or vein were precontracted submaximally (50–70% of maximum) by superfusion with U46619 (10^{-9}–10^{-8} M). The numbers 1, 2, and 3 signify the cascade arrangement of the strips. Glyceryl trinitrate (GTN, 10^{-7} M) was superfused over the strips for 1 minute. Perf signifies perfusate delivered from the unrubbed segment of artery or vein. Breaks in the tracings represent periods of tissue equilibration. A23 (3x10^{-7} M), poly-L-arginine 41 kDa (ARG, 3x10^{-7} M), and poly-L-lysine 41 kDa (LYS, 1.6x10^{-7} M) were perfused through artery and/or vein for 3 minutes as indicated. Superoxide dismutase (SOD, 100 units/ml) was perfused through the arterial segment during concomitant perfusion with A23 or ARG as indicated. Pyrogallol (PYR, 10^{-5} M) was superfused over the arterial and vein segment during concomitant perfusion of artery with A23 or ARG. Oxyhemoglobin (HbO2, 10^{-4} M) was superfused over the strips during concomitant perfusion of vein with A23, ARG, or LYS. Each panel of tracings is representative from a total of four separate experiments.

FIGURE 4. Time-dependent relaxation and cyclic GMP accumulation in artery and vein elicited by poly-L-arginine 115 kDa. Unrubbed arterial and venous rings were mounted under 4 g of tension, and were precontracted submaximally (65–80% of maximum) by addition of U46619 (10^{-9}–10^{-8} M). After peak contractile responses developed, 3x10^{-8} M poly-L-arginine 115 kDa (Poly-L-Arg) was added. Rings were quick-frozen at the indicated times after addition of the polyamino acid. Relaxation was monitored up until the time of tissue freezing. Percent relaxation signifies percent decrease in submaximal precontractile tone elicited by U46619. Values represent the mean±SEM from 12–16 rings isolated from three to four animals (four rings per animal).

Refractoriness to Endothelium-Dependent Arterial and Venous Relaxation Produced by Basic Polyamino Acids

Throughout the conduct of this study we recognized that relatively high concentrations of the basic polyamino acids which produced 100% relaxant responses were much less effective in causing relaxation in the same vascular preparations during second exposure to the relaxant. No such diminution in relaxant responses to acetylcholine, bradykinin, or A23187 was ever observed. Figure 6 illustrates that a second exposure to low concentrations of poly-L-ornithine 50 kDa produced relaxant responses that were similar in magnitude to the initial relaxant responses and contractile responses to U46619 were unaltered. Relaxant responses to a second exposure of higher concentrations of poly-L-ornithine 50 kDa, however, were markedly diminished but glyceryl trinitrate still produced maximal relaxation (Figure 6). Concentration-response curves to glyceryl trinitrate or NO were unaltered in the
presence of basic polyamino acids (not shown). Moreover, rings that were pretreated with poly-
amino acids were supersensitive to the contractile effects of U46619. Therefore, 10-fold lower concentrations of U46619 were used to precontract the refractory rings. Arterial rings were pretreated with high concentrations of poly-L-arginine 41 kDa and subsequently challenged with acetylcholine after tissue washing, equilibration and precontraction. The relaxant responses to acetylcholine were abolished, whereas maximal relaxation was still obtained with glyceryl trinitrate (Figure 6). In arterial rings cut from the same vessel used to prepare rings for the preceding experiment, acetylcholine failed to produce any impairment of relaxation elicited by itself (Figure 6) or by poly-L-arginine 41 kDa (not shown). Similarly, high concentrations of poly-L-lysine 225 kDa abolished relaxant responses to A23187, and rendered the rings supersensitive to the contractile effects of U46619, whereas maximal relaxation was obtained with glyceryl trinitrate (Figure 6). A23187 did not produce any impairment of relaxation to itself or to poly-L-lysine 225 kDa. Arterial rings pretreated with poly-L-lysine 225 kDa that showed refractoriness to acetylcholine did show partial relaxation in response to bradykinin (not shown in Figure 6 but is demonstrated by bioassay in Figure 7). Indomethacin abolished this partial relaxant response to bradykinin.

Experiments were conducted to determine whether refractoriness developed to cyclic GMP accumulation as well. Table 3 illustrates that pretreatment with poly-L-lysine 225 kDa nearly abolished cyclic GMP accumulation and relaxation in response to either the polyamino acid or acetylcholine, whereas no inhibition developed to the actions of glyceryl trinitrate. Similarly, refractoriness developed to the actions of poly-L-lysine 225 kDa and bradykinin but not glyceryl trinitrate in vein (Table 3). Resting levels of cyclic GMP in rings pretreated with the polyamino acid did not differ from control rings.

The bioassay cascade superfusion technique was used to ascertain whether poly-L-lysine 225 kDa (3 x 10^-7 M) could impair the release of EDNO from perfused arterial segments elicited by 10^-8 M poly-

<table>
<thead>
<tr>
<th>Test agents</th>
<th>Cyclic GMP (pnmol/g tissue)</th>
<th>Percent relaxation</th>
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<tbody>
<tr>
<td>None</td>
<td>34±3</td>
<td></td>
</tr>
<tr>
<td>Poly-L-arginine 115 kDa, 5 x 10^-8 M</td>
<td>212±31</td>
<td>72±9</td>
</tr>
<tr>
<td>+1 μM oxyhemoglobin</td>
<td>22±4*</td>
<td>0*</td>
</tr>
<tr>
<td>+10 μM methylene blue</td>
<td>81±11*</td>
<td>16±3*</td>
</tr>
<tr>
<td>Poly-L-lysine 41 kDa, 5 x 10^-8 M</td>
<td>168±24</td>
<td>58±7</td>
</tr>
<tr>
<td>+1 μM oxyhemoglobin</td>
<td>18±3*</td>
<td>0*</td>
</tr>
<tr>
<td>+10 μM methylene blue</td>
<td>44±6*</td>
<td>8±2*</td>
</tr>
<tr>
<td>Poly-L-lysine 225 kDa, 5 x 10^-4 M</td>
<td>232±40</td>
<td>80±11</td>
</tr>
<tr>
<td>+1 μM oxyhemoglobin</td>
<td>46±5*</td>
<td>4±1*</td>
</tr>
<tr>
<td>+10 μM methylene blue</td>
<td>74±9*</td>
<td>17±3*</td>
</tr>
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</table>

Unrubbed arterial rings were mounted under 4 g of tension and were precontracted submaximally (65–75% of maximum) by addition of U46619 (10^-8–10^-6 M). Oxyhemoglobin and methylene blue were added 10 minutes before initiation of precontraction. After peak contractile responses developed, the indicated polyamino acids were added (5 x 10^-3 M). Oxygenated rings (indicated as None) were quick-frozen at the time of peak contractile responses to U46619. Values represent the mean±SEM from 18 rings isolated from three animals (six rings per animal).

*Significantly different (p<0.01) from corresponding values obtained in the absence of inhibitor.

FIGURE 5. Concentration-dependent relaxation and cyclic GMP accumulation in artery and vein elicited by poly-L-arginine 115 kDa and poly-L-lysine 225 kDa. Unrubbed arterial and venous rings were mounted under 4 g of tension, and were precontracted submaximally (65–80% of maximum) by addition of U46619 (10^-8–10^-6 M). After peak contractile responses developed, poly-L-arginine 115 kDa (Poly-L-Arg) or poly-L-lysine 225 kDa (Poly-L-Lys) was added to artery or vein, respectively, at the indicated bath concentrations. Rings were quick-frozen at 180 seconds after addition of polyamino acid, or at the time of peak contraction to U46619 in the absence of added test agent (zero concentration). Relaxation was monitored up until the time of tissue freezing. Percent relaxation signifies percent decrease in submaximal precontractile tone elicited by U46619. Values represent the mean±SEM using 12–16 rings isolated from three to four animals (four rings per animal).
L-lysine 225 kDa and $10^{-6}$ M A23187. After obtaining control responses to perfused A23187 and the polyamino acid, and to superfused $10^{-7}$ M glyceryl trinitrate, a 30-fold higher concentration of polyamino acid was perfused through the artery (but not superfused over the bioassay strips) for 30 minutes. The arterial perfusate was then allowed to superfuse the strips, and A23187 and the polyamino acid were retested. No evidence of EDNO release from arterial segments pretreated with high concentrations of poly-L-lysine 225 kDa was seen, whereas the relaxant responses to superfused glyceryl trinitrate were comparable to control responses (Figure 7). Release of EDNO from perfused intact artery was quantified by chemical assay as described. Table 4 illustrates that arterial perfusates with or without A23187 or poly-L-lysine 225 kDa evoke the formation and/or release of EDNO that is associated with concomitant relaxation of the superfused arterial strips. After pretreatment of intact artery with the basic polyamino acid for 30 minutes, however, both relaxation and formation/release of EDNO in response to arterial perfusion are markedly impaired.

Perfusion of normal artery with A23187 or bradykinin in the absence of indomethacin caused the release of an EDRF that appeared to be much more stable chemically than that released in the presence of indomethacin (Figure 7). This was particularly true for bradykinin and is attributed to the generation of a prostanoid relaxing factor such as prostacyclin. Following treatment of the perfused artery with poly-L-lysine 225 kDa as described above, the release of EDRFs by A23187 in the presence of oxyhemoglobin was nearly abolished, whereas release by bradykinin was only slightly inhibited. Such relaxant responses to perfused bradykinin were nearly abolished, however, in the presence of indomethacin.

Scanning electron microscopy was employed to determine whether the basic polyamino acids caused morphologic damage to vascular endothelial cells. The photomicrographs illustrated in Figures 8 and 9 reveal that exposure of arterial rings to high concentrations of poly-L-ornithine 50 kDa, poly-L-arginine 41 kDa, or poly-L-lysine 225 kDa for 15 minutes did not cause overt endothelial cell damage. Rubbing the intimal surface as described in the methods section completely denuded the surface of endothelial cells (Figure 8). Mounting the arterial rings in organ baths followed by manipulations such as washing and contracting the tissues alters the morphological appearance of the endothelial cells when compared with that of freshly sliced rings.
polyamino acids produced endothelium-dependent, time-dependent, and concentration-dependent increases in arterial and venous cyclic GMP levels, and cyclic GMP accumulation correlated well with concomitant relaxant responses in the same vascular rings. Both cyclic GMP accumulation and relax-

**Vascular Actions of Bradykinin Analogues and Other Peptides**

Bradykinin, Lys-bradykinin, and Met-Lys-bradykinin relaxed arterial rings with comparable potencies (Figure 10). However, changing the L-proline to D-phenylalanine at position 7 or L-arginine to L-lysine at position 1 abolished relaxant activity (Figure 10). Similar observations were made with venous rings. Neurotensin and several analogues of neurotensin and bradykinin, each of which contains arginine and/or lysine residues, were completely inactive at concentrations up to $3 \times 10^{-7}$ M (not shown). The D-Phe analogue of bradykinin was found to antagonize the relaxant effect of bradykinin but not that of poly-L-arginine 115 kDa (Figure 10). The above observations indicate that the endothelial cell receptors involved with the relaxant action of bradykinin are different from those involved with the basic polyamino acids.

**Discussion**

The present observations indicate that high molecular weight polyamino acids composed of the basic amino acids L-arginine, L-lysine, or L-ornithine cause relaxation of isolated rings of bovine intrapulmonary artery and vein by endothelium-dependent mechanisms that are attributed to the formation and release of one type of EDRF. This particular EDRF is EDNO as demonstrated recently in bovine pulmonary artery and vein by this laboratory. The average molecular weights of the basic polyamino acids tested ranged from 17 to 225 kDa, and the EC$_{50}$ values ranged from $3 \times 10^{-9}$ to $2 \times 10^{-7}$ M in artery and vein. No significant relaxant responses were elicited by acidic or neutral polyamino acids of comparable molecular weights, polyanines, or basic amino acids. However, poly-D-lysine was just as potent a relaxant as poly-L-lysine. There was a close direct correlation between molecular weight and relaxant potency among the basic polyamino acids, and this relation was similar in artery and vein. The most potent compound tested was poly-L-lysine 225 kDa, which had an EC$_{50}$ of $3 \times 10^{-9}$ M and a minimal effective concentration of about $10^{-10}$ M. The present findings confirm and extend those of a recent report, which indicated that poly-L-arginine and poly-L-lysine, each 70 kDa, caused endothelium-dependent relaxation of rat aorta.

The general mechanism of endothelium-dependent vascular smooth muscle relaxation elicited by the basic polyamino acids is attributed to enhanced formation and release of EDNO. This view is supported by the following experimental evidence. The basic polyamino acids produced endothelium-dependent, time-dependent, and concentration-dependent increases in arterial and venous cyclic GMP levels, and cyclic GMP accumulation correlated well with concomitant relaxant responses in the same vascular rings. Both cyclic GMP accumulation and relax-

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**Figure 7. Development of refractoriness to formation and/or release of EDNO from perfused artery caused by poly-L-lysine 225 kDa.** Endothelium-denuded strips of bovine intrapulmonary artery were precontracted submaximally (50–70% of maximum) by superfusion with U46619 ($10^{-8}$–$10^{-9}$ M). The numbers 1, 2, and 3 signify the cascade arrangement of the strips. Glycerol trinitrate (GTN, $10^{-7}$ M) was superfused over the strips for 1 minute. Perf signifies perfusate delivered from the unrubbed segment of artery. Breaks in the tracings represent periods of tissue equilibration. A23187 (A23, $1.6 \times 10^{-7}$ M), bradykinin (BKN, $3 \times 10^{-8}$ M), and poly-L-lysine 225 kDa (LYS, $1.6 \times 10^{-8}$ M) were perfused through the artery for 3 minutes as indicated. Tol signifies tolerance (impaired EDNO formation/release) induced by perfusion of artery with $1.6 \times 10^{-7}$ M poly-L-lysine 225 kDa for 30 minutes, without allowing the perfusate to superfuse the strips. In the lower panel, $10^{-2}$ M oxyhemoglobin was added to the superfusion medium after induction of tolerance for the duration of the experiment. Ind signifies indomethacin ($5 \times 10^{-5}$ M), which was present in both the perfusion and superfusion media for the duration of the experiment. Each panel of tracings is representative from a total of three separate experiments.

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**Figure 8.** Tissue manipulation or prolonged exposure to bathing solutions caused retraction of endothelial cell borders, perforations, and detachments of cell edges without causing denudation or loss of responsiveness to endothelium-dependent relaxants (Figure 9A). Exposure to the basic polyamino acids as indicated did not exaggerate the above morphologic changes (Figure 9B–D).
TABLE 3. Impairment by High Concentrations of Poly-L-Lysine 225 kDa of Subsequent Relaxation and Cyclic GMP Accumulation in Artery and Vein

<table>
<thead>
<tr>
<th>Test agents</th>
<th>Cyclic GMP</th>
<th>Percent relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>34±3</td>
<td>···</td>
</tr>
<tr>
<td>Poly-L-lysine 225 kDa (5×10^{-4} M)</td>
<td>221±34</td>
<td>77±9</td>
</tr>
<tr>
<td>Acetylcholine (10^{-4} M)</td>
<td>332±46</td>
<td>82±11</td>
</tr>
<tr>
<td>Glycerol trinitrate (10^{-4} M)</td>
<td>298±37</td>
<td>85±14</td>
</tr>
<tr>
<td>Vein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>33±5</td>
<td>···</td>
</tr>
<tr>
<td>Poly-L-lysine 225 kDa (5×10^{-4} M)</td>
<td>254±37</td>
<td>79±14</td>
</tr>
<tr>
<td>Bradykinin (10^{-4} M)</td>
<td>286±41</td>
<td>71±12</td>
</tr>
<tr>
<td>Glycerol trinitrate (10^{-4} M)</td>
<td>263±36</td>
<td>78±10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pretreatment with poly-L-lysine 225 kDa</th>
<th>Cyclic GMP</th>
<th>Percent relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td>36±5</td>
<td>···</td>
</tr>
<tr>
<td>Poly-L-lysine 225 kDa (5×10^{-4} M)</td>
<td>60±5*</td>
<td>8±2*</td>
</tr>
<tr>
<td>Acetylcholine (10^{-4} M)</td>
<td>62±8*</td>
<td>0*</td>
</tr>
<tr>
<td>Glycerol trinitrate (10^{-4} M)</td>
<td>286±33</td>
<td>87±12</td>
</tr>
</tbody>
</table>

Unrubbed arterial and venous rings were mounted under 4 g of tension, and were precontracted submaximally (65–75% of maximum) by addition of U46619 (10^{-10}–10^{-4} M). The indicated test agent was added after peak contractile responses developed. Rings were quick-frozen either at 180 seconds after addition of poly-L-lysine 225 kDa or at 60 seconds after addition of acetylcholine, bradykinin or glycerol trinitrate as indicated. Control rings (indicated as None) were quick-frozen at the time of peak contractile responses to U46619. Pretreatment signifies rings that were initially relaxed by exposure to 5×10^{-7} M poly-L-lysine 225 kDa for 20 minutes following by tissue washing and 45 minutes of equilibration. Percent relaxation signifies percent decrease in submaximal precontractile tone elicited by U46619. Values represent the mean±SEM from 12 rings isolated from three animals (four rings per animal).

*Significantly different (p<0.01) from corresponding values obtained in the absence of tolerance.

GMP accumulation in vascular smooth muscle.35,38–41

Indomethacin failed to modify the vascular actions of the basic polyamino acids, thereby indicating that cyclooxygenase products of arachidonic acid such as prostacyclin are not involved. Moreover, no metabolite of arachidonic acid appears to be involved as neither quinacrine nor nortrihydroguaiaretic acid inhibited relaxant responses to the polyamino acids. The bioassay technique revealed that both the basic polyamino acids and the calcium ionophore A23187 caused the release of EDNO from perfused artery and vein. An approximate half-life of 3–5 seconds was observed for EDNO, and the apparent stability of EDNO was increased by superoxide dismutase and markedly decreased by superoxide anion (generated by pyrogallol). These observations are consistent with those reported for EDNO released from cultured aortic endothelial cells by bradykinin or A23187 as assessed by bioassay.36,42,43

Arterial rings that were precontracted by depolarizing potassium solution were markedly less sensitive than were U46619-precontracted rings to the relaxant effects of acetylcholine and basic amino acids. It is well appreciated, although not well understood, that EDRF-elicited vascular relaxant responses are greatly diminished in potassium-depolarized vascular preparations, whereas endothelium-independent vasodilators markedly relax such preparations.12,25,27,44

Finally, the release of EDNO from artery and vein caused by basic polyamino acids was verified by chemical assay, as was previously done for EDNO released by A23187.27

The precise mechanism by which the basic polyamino acids stimulate the formation and/or release of EDNO is presently unknown. Possible mecha-
FIGURE 8. Scanning electron microscopy of arterial rings before and after endothelial denudation. Intimal surfaces were photographed at ×600 magnification by scanning electron microscopy. Panel A: Arterial ring was fixed immediately after removal from the arterial vessel. Panel B: Arterial ring was prepared, rubbed along its intimal surface, and then fixed.

Mechanisms include interaction with 1) extracellular receptors or ion channels that are coupled to EDNO formation and/or release, 2) intracellular processes that govern the release of preformed or stored EDNO, and 3) intracellular enzyme systems associated with EDNO formation. The first possibility is unlikely because the data are not consistent with an interaction between polyamino acids and selective extracellular receptors. The basic polyamino acids elicit much slower rates of vascular smooth muscle relaxation than do acetylcholine, bradykinin, or A23187, as observed with isolated vascular rings and by bioassay. Clearly, the data indicate that the extracellular receptors with which bradykinin and its active analogues interact are not involved in the actions of the basic polyamino acids. Moreover, the finding that the polyamino acids impair or cause refractoriness to the relaxant effects of acetylcholine, bradykinin, and A23187 argue against a selective interaction between basic polyamino acids and extracellular receptors. Finally, the findings that basic polyamino acids rich in arginine, lysine, or ornithine are equally active and that the L- and D-isomers of polylysine are equipotent as relaxants further support the view that the basic polyamino acids do not interact with selective cell surface receptors. The second possibility appears to be unlikely at present because NO is highly unstable, possessing a half-life of only 3–5 seconds and is lipophilic, so that storage of preformed free NO may not occur to any appreciable extent.

The most likely mechanism of action of the basic polyamino acids is that they penetrate endothelial cells by diffusion or special transport systems and facilitate the formation of EDNO. These basic polyamino acids could serve as precursors for the synthesis of NO. More specifically, the basic amino groups could be cleaved and oxidized to NO or a more stable nitroso-derivative by enzyme systems that apparently catalyze the conversion of L-arginine to NO in cultured porcine aortic endothelial cells as reported recently. The component pathways of this enzyme system are presently unknown but may possess broad substrate specificity for polypeptides rich in various basic amino acids. L-Arginine may serve as an endogenous precursor to EDNO, but exogenous peptides or polyamino acids rich in arginine or other basic amino acids could behave as alternate or partial enzyme substrates. Consistent with this proposed mechanism of action are the observations that 1) vascular smooth muscle relaxation occurs in response only to basic and not to the corresponding acidic or neutral polyamino acids, 2) relaxant potencies increase with increasing molecular weights (greater number of moles of basic amino functions per mole of basic polyamino acid), 3) the rates of relaxation in response to these substances are much slower than those to agents that interact with membrane receptors or ion channels, and this may be attributed to a slower rate of conversion of the basic polyamino acids to NO; and 4) prolonged interaction between polyamino acid and vascular preparations.
FIGURE 9. Scanning electron microscopy of arterial rings after treatment with basic polyamino acids. Intimal surfaces were photographed at \( \times 600 \) magnification in Panels A, B, and C, and at \( \times 1,500 \) magnification in Panel D by scanning electron microscopy. Panel A: Unrubbed arterial ring was mounted in a bath chamber under tension, depolarized with KCl, contracted with \( 10^{-4} \) M U46619, rinsed with Krebs solution and then fixed. Panel B: Unrubbed arterial ring was mounted and precontracted with U46619 as described above, challenged with \( 10^{-7} \) M poly-L-ornithine 50 kDa for 15 minutes, rinsed and then fixed. Panel C: Unrubbed arterial ring was handled as in Panel B except that \( 10^{-7} \) M poly-L-lysine 225 kDa was tested. Panel D: Same as Panel C except at higher magnification.
causes refractoriness or a form of tolerance characterized by impairment of EDNO formation in response to any vasodilator whose action is mediated by EDNO.

The refractoriness to vascular smooth muscle relaxation that develops to higher concentrations of the basic polyamino acids or after prolonged tissue contact is attributed to a desensitization of one or more enzymes that may be involved in the conversion of the basic amino groups to NO. If arginine is indeed the natural substrate for the enzyme that initiates the reaction sequence leading to the formation of NO, 45 then the basic polyamino acids could conceivably act as alternate or partial substrates whereby they compete with arginine for enzyme binding sites and initially serve as substrates. As these binding sites become saturated with polyamino acid, however, enzyme desensitization occurs and the formation of EDNO is impaired. This hypothesis is supported by the observations that 1) refractoriness or tolerance occurs to the endothelium-dependent relaxant effects not only of all basic polyamino acids but also to acetylcholine, bradykinin, and A23187 but does not occur to endothelium-independent relaxants, and 2) impairment of NO formation/release from perfused polyamino acid-pretreated artery and vein, as assessed by bioassay and chemical assay, occurs in response to various endothelium-dependent vasodilators. Meaningful time course experiments to determine whether vascular preparations can recover from the refractoriness to endothelium-dependent relaxants were not conducted because the tissues begin to deteriorate after 7–8 hours in the tissue baths. Prolonged contact of the basic polyamino acids with the endothelium-denuded vascular strips in the bioassay cascade does not alter relaxation in response to other relaxants such as glyceryl trinitrate delivered by superfusion. Similarly, isolated arterial and venous rings rendered tolerant by pretreatment with basic polyamino acids undergo complete relaxation in response to glyceryl trinitrate.

The apparent tolerance and cross-tolerance observed with the basic polyamino acids cannot be attributed to 1) desensitization at the level of soluble guanylate cyclase or action of cyclic GMP on calcium-binding proteins because relaxant responses to glyceryl trinitrate are no different from control responses, 2) damage to the vascular endothelium as assessed by scanning electron microscopy and because bradykinin can still evoke the formation/release of a prostanoid relaxing factor, 3) desensitization of selective extracellular endothelial receptors because refractoriness to all tested endothelium-dependent relaxants occurs and because the basic polyamino acids do not interact with the receptors that interact with bradykinin, Lys-bradykinin, and Met-Lys-bradykinin; and 4) direct inactivation of EDNO because relaxant responses to authentic NO are no different from control. Thus, the mechanism of refractoriness induced by the basic polyamino acids may occur as a result of impairment of NO formation in vascular endothelial cells.

The small arginine-containing polypeptide, bradykinin, and two of its close structural analogues elicit endothelium-dependent relaxation by mechanisms that are unrelated to those for the arginine-rich polyamino acids. Bradykinin and vasoactive intestinal polypeptide, which contains both arginine and lysine, interact with selective extracellular receptors that are coupled to the biosynthesis and/or release of both EDNO, which stimulates cyclic GMP formation, and prostacyclin, which stimulates cyclic AMP formation. 12 Atriopeptins, which contain both arginine and lysine, interact with vascular smooth muscle receptors linked to particulate guanylate cyclase and cause endothelium-independent relaxation. 22 The actions of the arginine- or lysine-rich polyamino acids tested in this study are attrib-
ute to endothelium-dependent mechanisms that do not involve interactions with receptors for the smaller arginine- or lysine-containing polypeptides. Competitive antagonists of bradykinin do not alter relaxant responses to basic polyamino acids, and the latter do not elevate vascular levels of cyclic AMP. Thus, there is no common or unifying mechanism of vasodilator action for polypeptides containing arginine and/or lysine.

In conclusion, basic polyamino acids rich in arginine, lysine, or ornithine elicit endothelium- and cyclic GMP-dependent relaxation of artery and vein by a novel mechanism. The hypothesis offered is that basic polyamino acids act as partial or alternate substrates for the enzyme system(s) that convert L-arginine to NO, thereby causing increased formation of EDNO. The refractoriness to other endothelium-dependent vasodilators that develops in vascular preparations after prolonged contact between endothelium and basic polyamino acids is attributed to enzyme desensitization that results in decreased EDNO formation. The proposed mechanisms of action and refractoriness for the basic polyamino acids are mutually compatible. It appears that although both EDNO and prostacyclin are released concomitantly by diverse stimuli, the two events can be dissociated not only by the use of indomethacin but also by basic polyamino acids. The basic polyamino acids are useful pharmacological probes for future studies on the biosynthesis and physiological actions of EDNO.

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References


Basic polyamino acids rich in arginine, lysine, or ornithine cause both enhancement of and refractoriness to formation of endothelium-derived nitric oxide in pulmonary artery and vein.

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