Protamine Releases Endothelium-Derived Relaxing Factor From Systemic Arteries

A Possible Mechanism of Hypotension During Heparin Neutralization

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**Background.** When used to reverse the anticoagulant effect of heparin, protamine sulfate often causes vasodilation that can lead to systemic hypotension. Protamine is rich in the basic amino acid arginine, which is the precursor of endothelial cell synthesis of nitric oxide, and nitric oxide is the active component of endothelium-derived relaxing factor (EDRF).

**Methods and Results.** To determine whether the hypotensive effect of protamine could be due to stimulated release of EDRF, we studied rings (4–5 mm) of canine coronary, femoral, and renal artery suspended in organ chambers containing physiological salt solution (37°C and 95% O₂-5% CO₂). Arterial rings with and without endothelium were contracted with prostaglandin F₂α (2×10⁻⁶ M) and exposed to increasing concentrations of protamine (final organ bath concentration, 40–400 µg/ml). In arterial segments without endothelium, protamine caused only a modest decrease in tension. However, protamine induced concentration-dependent relaxation in all arterial segments with endothelium, which was significantly greater than in segments without endothelium (p<0.05). The endothelium-dependent relaxation induced by protamine was inhibited by NG-monomethyl-L-arginine (L-NMMA) (10⁻⁵ M), but L-NMMA had no effect on rings without endothelium. The action of L-NMMA could be reversed by L-arginine (10⁻⁴ M) but not D-arginine (10⁻⁴ M).

**Conclusions.** This study demonstrates that protamine stimulates the release of EDRF from arterial endothelium, and that endothelium-dependent vasodilation may be an important cause of systemic hypotension during protamine infusion. (Circulation 1992;86:289–294)

**Key Words** • L-arginine • L-NMMA • NG-monomethyl-L-arginine • vasodilation

Protamine sulfate is a polycationic peptide that is commonly used to reverse the anticoagulant effects of heparin, and systemic hypotension is a frequently observed and unwanted side effect of protamine infusion. Sixty-seven percent of the amino acid composition of protamine is arginine. L-Arginine is the physiological precursor of nitric oxide, the active component of endothelium-derived relaxing factor (EDRF), which functions as an endogenous nitrovasodilator. Indeed, infusion of L-arginine in human subjects produces hypotension similar to that observed after protamine infusion. We hypothesized that protamine stimulates release of EDRF, which then leads to vasodilation.

In the present study, we quantified vascular responses to protamine and determined whether any vasoactive effects were modified by heparin.

**Methods**

**Animal Preparation**

Heartworm-free mongrel dogs (weight, 25–30 kg) of either sex were anesthetized with pentobarbital sodium (30 mg/kg i.v.; Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and exsanguinated via the carotid arteries. The chest, abdominal cavity, and both groin areas were quickly opened, and the heart, renal arteries, and femoral arteries were harvested and immersed in cool, oxygenated physiological salt solution of the following composition (mM): NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.22, CaCl₂ 2.5, NaHCO₃ 25.0, Ca-EDTA 0.016, and glucose 11.1 (control solution). The procedures and handling of the animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

**In Vitro Experiments**

The left circumflex coronary artery, the right or left renal artery, and the right or left femoral artery were carefully dissected free of connective tissue and placed in control solution. Segments (4–5 mm in length) of blood vessel were prepared from each artery, and great care was taken not to touch the intimal surface. Segments from a given artery in an animal were randomly assigned to the experimental conditions, so that at most

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one segment per artery (from each animal) was assigned to the same experimental condition. Because of the varied number of segments prepared from each artery, the experimental conditions were not replicated equally. In some segments, vascular smooth muscle function was tested without the influence of the endothelium; in these rings, the endothelium was removed by gently rubbing the intimal surface of the blood vessel with a pair of watchmaker’s forceps. This procedure removes endothelium but does not affect ability of vascular smooth muscle to contract or relax.14,15

Coronary, renal, and femoral arterial segments, with and without endothelium, were suspended in organ chambers (25 ml) filled with control solution maintained at 37°C and bubbled with 95% O2–5% CO2 (pH, 7.4). Each ring was suspended by two stainless steel clips passed through the lumen. One clip was anchored to the bottom of the organ chamber, and the other was connected to a strain gauge for measurement of isometric force (Grass FT03, Grass Instrument Co., Quincy, Mass.). The rings were placed at the optimal point of their length–tension relation by progressively stretching them until contraction to potassium ions (20 mM) at each level of distension was maximal.16 In all experiments, the presence or absence of endothelium was confirmed by determining the response to acetylcholine (10⁻⁵ M) in rings contracted with potassium ions (20 mM).14,15,17 After optimal tension was achieved, the arterial segments were allowed to equilibrate for 30–45 minutes before administration of drugs.

Drugs

The following drugs were used: acetylcholine chloride, indomethacin, prostaglandin F₂ₐ (Sigma Chemical Co., St. Louis, Mo.), L-arginine, D-arginine, and N⁰⁰-monomethyl-L-arginine (L-NMMA; Calbiochem, San Diego, Calif.), heparin sodium injection (bovine, 1,000 units/ml, Upjohn Co., Kalamazoo, Mich.), protamine sulfate injection (10 mg/ml), and protamine sulfate powder (100 IU/mg, lot 05925, Elkins-Sinn Inc., Cherry Hill, N.J.). All powdered drugs were prepared with distilled water except for indomethacin, which was dissolved in Na₂CO₃ (10⁻⁵ M). Protamine sulfate was added to the organ bath in a cumulative manner with 11 doses that corresponded to organ bath concentrations of protamine ranging from 40 to 400 μg/ml. Experiments were undertaken with purified protamine powder to ensure that any observed effects of the compound were due to the protein itself and not the vehicle used to suspend the commercially used product. Because of the buffering effect of the sodium bicarbonate in the organ bath solution and aeration with 5% CO₂, addition of either the protamine powder or solution, L-NMMA, L-arginine, or D-arginine did not alter organ bath pH. All drug concentrations are expressed as final molar concentration in the organ chambers. The effect of protamine on vascular reactivity of arterial segments with and without endothelium was studied on untreated arteries (control) or on arterial segments that were treated with L-NMMA, L-NMMA plus L-arginine, or L-NMMA plus D-arginine.

Data Analysis

In vascular segments contracted with prostaglandin F₂ₐ, responses are expressed as percent change from the contraction level to account for interanimal variability in the maximal contraction of the tissue to prostaglandin. In all experiments, n refers to the number of animals from which vascular segments were taken. Results are expressed as mean±SEM. All tests were two sided at an α level of 0.05.

Linear regression analysis was used to obtain an estimate of the relation (slope) between percent prostaglandin F₂ₐ contraction and the protamine sulfate concentration for each arterial segment. For a given arterial segment, the obtained value estimated the average change in the percent of prostaglandin F₂ₐ contraction per one dosage level increase in protamine sulfate. For a specific experimental condition, a test for change in tension with increasing concentrations of protamine was based on a one-sample t test using the estimated slopes.

Comparisons among the experimental conditions were made using the average percent change at a protamine concentration level of 400 μg/ml and the averaged slopes. Comparisons between two experimental conditions were based on a two-sample t test. Comparisons among more than two experimental conditions were based on a one-way ANOVA. Pairwise comparisons were made using the Student-Newman-Keuls multiple comparisons test, thereby controlling the overall type I error rate for all comparisons. Analysis of the average area under the dose–response curves rather than the slopes provided similar results.

Similar analyses were used to examine the effects of increasing concentrations of heparin, L-arginine, and D-arginine.

Results

Endothelium-Dependent Relaxation

In the canine renal arteries contracted with prostaglandin F₂ₐ, the progressive addition of protamine sulfate (to achieve final bath concentrations of 40, 120, 200, 300, and 400 μg/ml) produced sustained relaxation in segments with endothelium (to 400 μg/ml protamine, p<0.05) (Figure 1). However, the addition of increasing
concentrations of protamine sulfate caused no significant change in contracted renal artery segments without endothelium (Figure 1). The stepwise addition of protamine sulfate (40–400 μg/ml) in the organ bath produced progressive concentration-dependent relaxation in canine renal, femoral, and coronary artery segments with endothelium, reaching 15.85±9.11%, 5.90±10.01%, and 5.91±10.04% of the initial contraction to prostaglandin F2α, respectively (mean±SEM, p<0.05 compared with slope of concentration–response curves for arterial segments without endothelium) (Figures 2–4).

Increasing concentrations of protamine sulfate (40, 120, 200, 300, and 400 μg/ml, final organ bath concentrations) caused no significant change in tension of renal and femoral arterial segments without endothelium that had been contracted with prostaglandin F2α, reaching 92.38±4.13% and 90.37±7.64% of the initial contraction to prostaglandin F2α, respectively (mean±SEM). However, protamine sulfate induced a modest but significant decrease in tension of coronary arterial segments without endothelium, reaching 67.85±14.02% of the initial prostaglandin F2α contraction (mean±SEM, average slope of concentration–response curve compared with zero, p<0.05).

Addition of L-NMMA (10⁻⁵ M), the competitive inhibitor of nitric oxide production from L-arginine, to the organ bath 10 minutes before contraction with prostaglandin F2α did not induce any consistent change in tension in arterial segments with or without endothelium. However, pretreatment of arterial segments with L-NMMA completely blocked the endothelium-dependent relaxation to protamine in renal, femoral, and coronary artery segments (Figures 1–4). The inhibitory effect of L-NMMA could be overcome by the addition of L-arginine (10⁻⁴ M) but not by D-arginine (10⁻⁴ M) (Figure 5). Indomethacin (10⁻⁶ M) did not alter the endothelium-dependent relaxation to protamine in canine arteries (data not shown).

Effect of Heparin

Heparin (8 units/ml, final organ bath concentration) caused no significant change in tension in contracted arterial segments with or without endothelium. However, increasing concentrations of heparin (to 60 units/ml, final organ bath concentration) caused a significant decrease in tension in contracted femoral artery segments with and without endothelium (p<0.05). Com-
parable concentration-dependent relaxation was found in femoral artery segments with and without endothelium at a heparin concentration of 60 units/ml, reaching 38.7±8.6% and 33.9±4.9% of the initial contraction to prostaglandin $F_2\alpha$ (mean±SEM, n=6).

In organ chambers containing heparin (8 units/ml), the addition of protamine sulfate (40–400 μg/ml) caused the initially clear fluid to become turbid, possibly because of formation of protamine–heparin complexes. However, even with heparin, addition of protamine caused concentration-dependent relaxation in femoral artery segments with endothelium and no change in tension in segments without endothelium (Figure 6). This response was comparable to the femoral artery response to protamine sulfate in the absence of heparin. In preliminary experiments, heparin (8 units/ml, final organ bath concentration) did not modify endothelium-dependent relaxation induced by acetylcholine ($10^{-9}$–$10^{-4}$ M, data not shown).

**Effect of L-Arginine and D-Arginine**

L-Arginine ($10^{-6}$–$10^{-4}$ M) caused no significant change in tension in femoral artery segments with or without endothelium (maximal change, 109.7±6.9% and 93.4±2.1% of the initial prostaglandin $F_2\alpha$ contraction, respectively; n=6). No direct endothelium-dependent effect was observed, even though L-arginine was in contact with the tissue for over 40 minutes.

D-Arginine ($10^{-5}$–$10^{-4}$ M) induced a small increase in tension in femoral artery segments with endothelium (maximal change to 113.5±4.6% of initial prostaglandin contraction) and a slight decrease in tension in femoral artery segments without endothelium (maximal re-

**Discussion**

The purpose of this study was to determine whether protamine sulfate could release EDRF from systemic arteries in vitro, which could account for its hypotensive effect in vivo. The main findings of the study are 1) protamine sulfate induces the release of EDRF from systemic arteries, 2) endothelium-dependent relaxation to protamine is unaffected by the presence of heparin, and 3) protamine most likely induces the release of EDRF by interaction with endothelial cell membrane receptors.

For over 50 years, protamine sulfate has been known to induce transient, profound hypotension when infused into experimental animals. However, until the development of cardiac surgery, protamine-induced hypotension was an interesting experimental phenomenon with little clinical relevance. In current practice, protamine sulfate is widely used to reverse the anticoagulant effect of heparin after extracorporeal circulation and hemodilysis, and protamine reactions can lead to systemic hypotension, pulmonary hypertension, and shock. Such adverse reactions are especially dangerous in the period immediately after extracorporeal circulation when intravascular volume fluctuates and cardiac function may be impaired.

Protamine sulfate may also cause hemodynamic disturbance by anaphylactic reactions. Indeed, Horrow has classified protamine reactions as three separate and distinct entities: 1) systemic hypotension, which Harrow classifies as common; 2) anaphylactoid reactions; and 3) catastrophic pulmonary vasoconstriction. The hypotensive effect of protamine has been demonstrated in many animals and in human studies. Although the precise mechanism is unknown, it appears that protamine decreases peripheral vascular resistance rather than depressing myocardial function.
In a previous study from our institution, cardiac output, systemic blood pressure, and high-fidelity left ventricular pressure measurements were recorded continuously during protamine administration after cardiopulmonary bypass. Protamine infusion consistently lowered peripheral vascular resistance, and hypotension occurred when the increase in cardiac output was insufficient to offset the decline in arterial resistance. A small depression in left ventricular contractile element velocity ($V_{\text{pe}}$) was detected only in those patients who experienced a mean blood pressure fall greater than 10 mm Hg. Recent data from Wakefield et al. suggest that protamine causes coronary artery vasodilation. In their study, protamine (250 µg/ml) caused a significant increase in coronary flow in an isolated perfused rabbit heart; this increase was transient when the protamine was infused with heparin but sustained when the protamine was infused alone.

From the present study, we conclude that protamine causes a decrease in vascular resistance by stimulating the release of EDRF. EDRF is produced by the endothelium in a basal amount (basal release) and during stimulation by various agonists. In addition to inhibiting platelet adhesion and aggregation, EDRF serves as an endogenous nitrovasodilator to modulate vascular tone. Thus, stimulated release of EDRF has a peripheral vascular effect similar to that of a nitrovasodilator. In the present study, prostacyclin could not be implicated as a mediator of endothelium-dependent vasodilation to protamine because vascular relaxation was unaffected by the presence of indomethacin.

The active component of EDRF is the nitric oxide radical, which is also the active component of such compounds as molsidomine, sodium nitopruisside, and nitroglycerin. In the endothelial cell, the substrate for nitric oxide synthesis is the basic amino acid L-arginine. Nitric oxide synthesis can be competitively inhibited by L-NMMA, the methylated form of the amino acid. Indeed, in the present experiment, endothelium-dependent relaxation to protamine was inhibited by L-NMMA. The specificity of L-NMMA inhibition of L-arginine metabolism was proven by the finding that the effect of L-NMMA could be reversed by adding high concentrations of L-arginine but not D-arginine.

Protamine may stimulate EDRF production by enhanced provision of the substrate L-arginine and/or receptor-mediated EDRF release. We initially hypothesized that protamine sulfate, which is rich in L-arginine, augmented EDRF production by supplying exogenous L-arginine as a metabolic substrate. However, two findings in this experiment argue against this hypothesis. First, the addition of very high concentrations of L-arginine to the organ bath failed to produce endothelium-dependent relaxation. We have observed endothelium-dependent relaxation with poly-L-arginine (a high-molecular-weight polypeptide containing L-arginine), but this relaxation cannot be blocked by L-NMMA and appears to be mediated by a nitric oxide–independent EDRF.

Second, protamine induces endothelium-dependent relaxation in the presence of excess amounts of heparin sulfate. Heparin sulfate, which is polyanionic, forms strong ionic bonds with the polycationic protamine. In the present experiment, the formation of heparin–protamine complexes was signaled by clouding of the initially clear organ chamber fluid as increasing concentrations of protamine were added into solution. Even in the presence of heparin and with the formation of heparin–protamine complexes, protamine induced endothelium-dependent relaxation, which was comparable to that induced without heparin present. This argues against the “L-arginine precursor” hypothesis of protamine-induced EDRF release because the large heparin–protamine complex would not be expected to enter the endothelial cell.

Thus, we hypothesize that protamine (either free drug or bound to heparin) acts on endothelial cell receptors to stimulate the production of EDRF (Figure 7). Protamine-induced release of EDRF would promote vasodilation and could be the mechanism of systemic hypotension after infusion. In addition, compounds such as L-NMMA could be infused concomitantly with protamine to inhibit the endothelium-dependent vasodilation of the compound, as has been done with other endothelium-dependent vasodilators in humans.

References
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