Cardiothoracic Anesthesia, Respiration and Airway

Protamine sulfate causes endothelium-independent vasorelaxation via inducible nitric oxide synthase pathway

[Le sulfate de protamine cause un vasorelâchement indépendant de l'endothélium par la voie de l'oxyde nitrique synthase inductible]

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Purpose: The precise mechanism of systemic hypotension frequently observed with the use of protamine is unclear. Although it has been reported that protamine stimulates the release of nitric oxide (NO) from endothelium NO synthase (eNOS), the association with inducible NOS (iNOS) remains unknown, despite the induction of iNOS by lipopolysaccharides (LPS) and/or inflammatory cytokines during cardiopulmonary bypass (CPB). The purpose of this study was to determine whether protamine stimulates the release of NO from iNOS induced by LPS.

Methods: We performed prospective and controlled functional examinations with isolated endothelium-denuded thoracic aortas from 21 male Wister rats. Aortic strips were mounted in Krebs solution and treated with LPS (1 $\mu g \cdot mL^{-1}$) for six hours to induce iNOS. Changes in tension caused by L-arginine (a substrate of NOS), protamine or a heparin-protamine complex (heparin: protamine = 1 unit: 10 μg) were measured in strips pre-contracted by phenylephrine.

Results: No drug relaxed the strips before LPS-treatment, but each drug relaxed the strips in a dose-dependent manner after LPS-treatment ($P < 0.05$). Aminoguanidine (an iNOS inhibitor) and methylene blue (a guanylyl cyclase inhibitor) inhibited the relaxations.

Conclusion: These results indicate that protamine and the heparin-protamine complex stimulated the release of NO from iNOS. As iNOS is induced during CPB, protamine or a heparin-protamine complex might cause systemic hypotension, at least in part, by stimulating iNOS.

***Objectif** : Le mécanisme précis de l'hypotension générale souvent observée avec l'usage de protamine n'est pas clair. Il a été démontré que la protamine stimule la libération d'oxyde nitrique (NO) à partir de la NO synthase (NOS) de l'endothélium, mais l'association avec la NOS inductible (NOSi) est inconnue malgré l'induction de NOSi par les lipopolysaccharides (LPS) et/ou les cytokines inflammatoires lors de la circulation extracorporelle (CEC). Notre étude veut déterminer si la protamine stimule la libération de NO à partir de la NOSi induite par les LPS.*

***Méthode** : Nous avons réalisé des examens fonctionnels prospectifs et contrôlés d'aortes thoraciques sans endothélium prélevées chez 21 rats mâles Wister. Les bandes aortiques ont été montées dans des solutions de Krebs et traitées avec des LPS (1 $\mu g \cdot mL^{-1}$) pendant six heures pour induire la NOSi. Les modifications de la tension causées par la L-arginine (un substrat de la NOS), la protamine ou un complexe d'héparine-protamine (héparine : protamine = 1 unité : 10 μg) ont été mesurées dans les bandes précontractées par la phényléphrine.*

***Résultats** : Aucun médicament n'a détendu les bandes avant le traitement aux LPS, mais chaque médicament les a détendues en fonction de la dose après le traitement aux LPS ($P < 0,05$). L'aminoguanidine (un inhibiteur de NOSi) et le bleu de méthylène (un inhibiteur de la guanylyl cyclase) ont inhibé les relâchements.*

***Conclusion** : La protamine et le complexe d'héparine-protamine ont stimulé la libération de NO à partir de NOSi. La NOSi étant induite pendant la CEC, la protamine ou un complexe d'héparine-protamine peuvent, en partie, causer une hypotension générale en stimulant la NOSi.*

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ALTHOUGH protamine sulfate (protamine) is commonly used to reverse the anticoagulant effect of heparin in cardiovascular surgery, the doses required are associated with clinically significant hemodynamic adverse reactions. Horrow classified the protamine-induced adverse reactions into the following three categories:[1] systemic hypotension, anaphylactoid reactions, and catastrophic pulmonary vasoconstriction. Systemic hypotension, in varying degrees, occurs most frequently and is considered to result from decreasing peripheral vascular resistance rather than depression of myocardial function in humans.[2] Although the mechanisms of the decreased peripheral vascular resistance are still being investigated, three major hypotheses have been proposed: an immunologic reaction,[3] a direct toxic action,[4] and an endothelium-dependent relaxation.[5] The latter mechanism occurs via nitric oxide (NO) release from endothelium NO synthase (eNOS), stimulated by protamine or a heparin-protamine complex.[5]

Numerous studies have extensively evaluated protamine-induced vasorelaxation via the eNOS pathway since it was first reported.[5] However, the interaction between protamine and inducible NO synthase (iNOS) has never been investigated despite its induction by lipopolysaccharide (LPS) and/or inflammatory cytokines during cardiopulmonary bypass (CPB)[6,7] and demonstration of its existence at sites of atherosclerosis.[8] The present study was undertaken in order to determine whether protamine stimulates iNOS, by evaluating vascular responses to protamine and a heparin-protamine complex in endothelium-denuded rat aorta strips treated with LPS to induce iNOS.

Materials and methods

Animals

The experimental protocol was approved by the Institutional Animal Care Committee of the Fukui Medical University. Twenty-one male Wistar rats were purchased from Charles River Japan Inc. (8–10 weeks of age, 250–300 g). Rats were used for experiments with protamine, a heparin-protamine complex and L-arginine (eight rats), experiments with aminoguanidine and methylene blue (eight rats), and experiments with cycloheximide (five rats) mentioned below, respectively.

LPS treatment

Thoracic aortas isolated from rats were mounted in the organ chambers mentioned below and treated with LPS (1 $\mu g \cdot mL^{-1}$) for six hours, during which time the bath medium was changed with fresh medium containing the same concentration of LPS every hour.[9] The six-hour-treatment with LPS induces almost maximal iNOS activity and relaxation of vascular smooth muscle when L-arginine (100 $\mu mol \cdot L^{-1}$, a substrate for iNOS) is applied.[9,10] As a control, 10 $\mu mol \cdot L^{-1}$ cycloheximide (an inhibitor of protein synthesis) was added during LPS-treatment to inhibit synthesis of iNOS.[9] All responses to test drugs were obtained in an LPS-free medium.

Functional examinations

Rats were killed by decapitation under isoflurane anesthesia, and the thoracic aortas were isolated and removed.[9] The thoracic aortas were placed in Krebs Henseleit solution ([$mmol \cdot L^{-1}$]; NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 10; pH 7.4). Helical strips were carefully prepared under a dissecting microscope. To avoid the possible involvement of eNOS on the mechanical response, the endothelium was rubbed off with filter paper.[9] The absence of endothelium was verified by the lack of a relaxation response to the application of 10 $\mu mol \cdot L^{-1}$ acetylcholine. Each strip was carefully mounted in an organ chamber containing 20 mL of Krebs Henseleit solution bubbled with 95% O_2 – 5% CO_2 at 37°C. After strips were allowed to equilibrate over a one-hour period with a resting tension of 0.5 g (determined to be the optimal resting tension in preliminary length-tension experiments), changes in tension were recorded isometrically.

Before and after LPS (or plus 10 $\mu mol \cdot L^{-1}$ cycloheximide) – treatment for six hours, changes in the tension caused by L-arginine, protamine, or the heparin-protamine complex (heparin: protamine = 1 unit: 10 µg,[5] i.e., 100 $units \cdot mL^{-1}$, 1 $mg \cdot mL^{-1}$, respectively) in strips precontracted with 0.1 $\mu mol \cdot L^{-1}$ phenylephrine (this induced approximately 80% of the maximal contraction) were measured. Protamine and the heparin-protamine complex were applied cumulatively. The formation of the heparin-protamine complex was confirmed by the clouding of the initially clear protamine fluid while adding heparin.[5] To investigate the NO / cyclic guanylate monophosphate pathway, 1 $mmol \cdot L^{-1}$ aminoguanidine (an iNOS inhibitor)[11,12] or 30 $\mu mol \cdot L^{-1}$ methylene blue (a guanylyl cyclase inhibitor)[13] was applied five minutes before phenylephrine, and responses to test drugs were obtained in the presence of aminoguanidine or methylene blue.

Contractions were estimated as contractile force developed (mg) per wet tissue weight (mg). Relaxations were expressed as a percentage of the phenylephrine-induced contraction, i.e., a contraction after each drug's treatment per 0.1 $\mu mol \cdot L^{-1}$ phenylephrine-induced contraction × 100 (%).

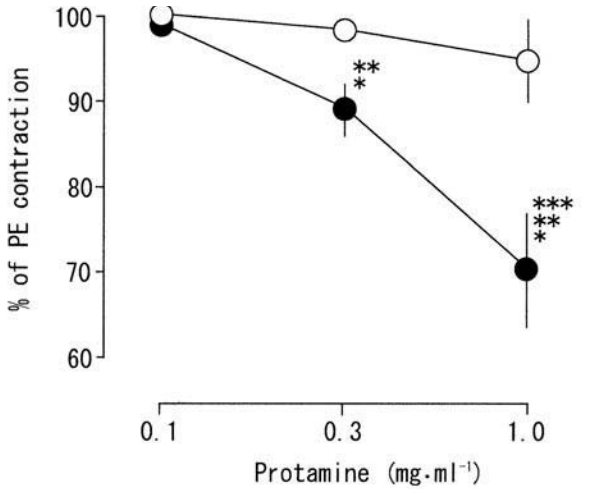

FIGURE 1 Concentration-response curves of protamine in endothelium-rubbed aorta strips before (open circles) and after (closed circles) lipopolysaccharide (LPS)-treatment. Strips were precontracted with 0.1 μmol·L^{-1} phenylephrine. Data are expressed as mean ± standard deviation of eight preparations. *P < 0.05 compared with before LPS-treatment at the same concentration of protamine. **P < 0.05 compared with after LPS-treatment at 0.1 mg·mL^{-1} protamine. ***P < 0.05 compared with after LPS-treatment at 0.3 mg·mL^{-1} protamine.

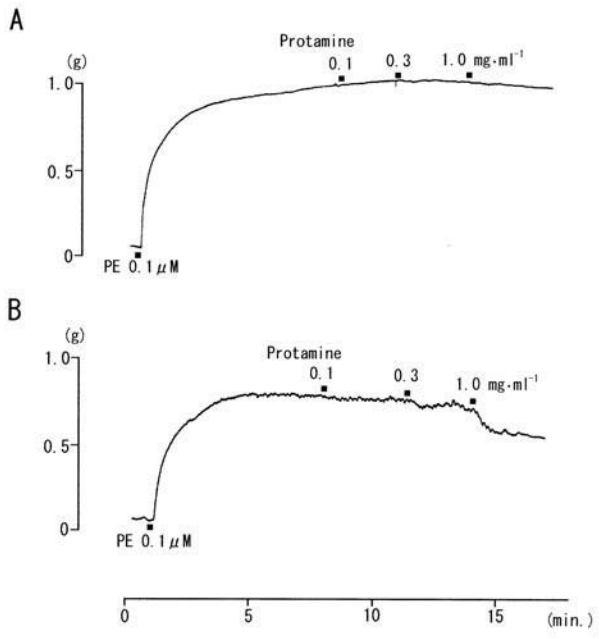

FIGURE 2 A: A representative trace of a phenylephrine-induced contraction to cumulative addition of protamine in an endothelium-rubbed aorta strip before lipopolysaccharide (LPS)-treatment. B: A representative trace of a phenylephrine-induced contraction to the cumulative addition of protamine in an endothelium-rubbed aorta strip after LPS-treatment. PE = phenylephrine.

Measurement of L-arginine concentration

After administration of protamine or a heparin-protamine complex in an organ chamber to perform functional experiments, Krebs Henseleit solution was drawn from the chamber to measure L-arginine concentration by high-performance liquid chromatography (lower limit: 1 nmol·L^{-1}) at a commercial laboratory (Mitsubishi Kagaku Bio-Clinical Labs, Inc., Tokyo, Japan).

The following drugs were used in the experiment: protamine sulfate, L-arginine, aminoguanidine, cycloheximide, phenylephrine, methylene blue (Sigma, St. Louis, MO, USA), lipopolysaccharide B (from E. coli 026; B6 Difco Laboratories, Detroit, MI, USA), and acetylcholine (Daiichi Seiyaku, Tokyo, Japan).

Statistical analysis

Results are expressed as mean ± standard deviation. To evaluate differences between groups, one-way analysis of variance (ANOVA) was used. When significant differences were detected by ANOVA, Scheffé's F test was applied for post hoc comparisons. Statistical significance was assumed at a P value < 0.05. Analyses were performed on a personal computer using Stat View II 4.0 software (Abacus Concepts, Berkeley, CA, USA).

Results

Responses to protamine and a heparin-protamine complex

The cumulative addition of protamine (to achieve final chamber concentration of 0.1, 0.3 and 1.0 mg·mL^{-1}) did not change the contraction level induced by phenylephrine in the endothelium-denuded aorta strips (Figure 1; open circles). However, the addition of protamine produced significant sustained relaxation in the strips after LPS-treatment (P < 0.05 at 0.3 and 1.0 mg·mL^{-1} protamine, Figure 1; closed circles). Representative traces of the cumulative addition of protamine in the strips precontracted with phenylephrine before or after LPS-treatment are shown in Figure 2. The heparin-protamine complex also relaxed the strips after LPS-treatment (64 ± 10%, P < 0.05 compared to LPS before treatment, 88 ± 7%).

Effects of aminoguanidine and methylene blue to protamine-induced relaxation

The addition of aminoguanidine (1 mmol·L^{-1}), as an iNOS inhibitor, to the organ chamber five minutes

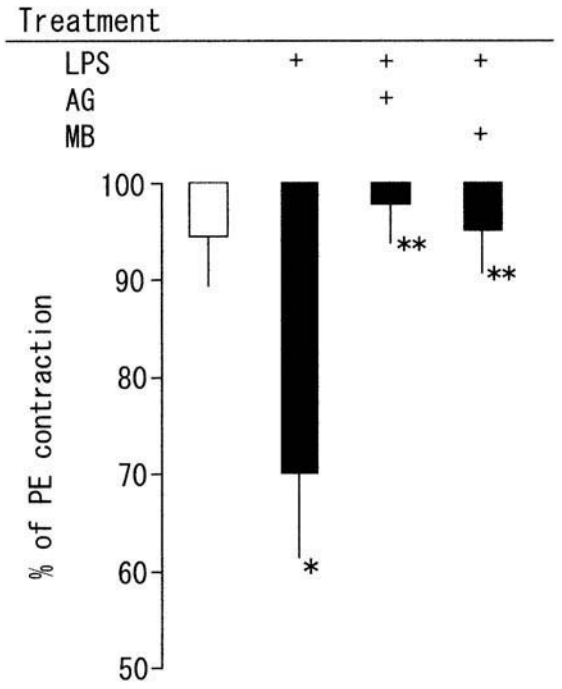

FIGURE 3 Effects of lipopolysaccharide (LPS; 1 μg·mL^{-1}), aminoguanidine (AG; 1 mmol·L^{-1}) and methylene blue (MB; 30 μmol·L^{-1}) on protamine (1.0 mg·mL^{-1}) in endothelium-rubbed aorta strips precontracted with phenylephrine (0.1 μmol·L^{-1}). Data are expressed as mean ± standard deviation of eight preparations. *$P < 0.05$ compared with no LPS-treatment. **$P < 0.05$ compared with LPS-treatment alone.

before contraction with phenylephrine significantly inhibited the protamine-induced relaxation in the endothelium-denuded aorta strips after LPS-treatment ($P < 0.05$, $n = 8$), (Figure 3). As with aminoguanidine, the addition of methylene blue (30 μmol·L^{-1}), a guanylyl cyclase inhibitor, significantly inhibited the protamine-induced relaxation in the strips treated with LPS ($P < 0.05$, $n = 8$), (Figure 3).

Effects of L-arginine

L-arginine (100 μmol·L^{-1}), a substrate for NOS, did not induce any consistent change in tension in the endothelium-denuded aorta strips before LPS-treatment (100 ± 2%, $n = 8$). However, L-arginine relaxed the strips after LPS-treatment (30 ± 8%, $n = 8$, $P < 0.05$ *vs* before LPS-treatment). This relaxation was inhibited by 1 mmol·L^{-1} aminoguanidine (108 ± 10%, $n = 8$).

LPS-treatment in the presence of cycloheximide led to different results after LPS-treatment, i.e., protamine (1 mmol·L^{-1}) and L-arginine (100 μmol·L^{-1}) were not associated with any relaxation of the strips, 98 ± 5% and 94 ± 7%, respectively ($n = 5$).

L-arginine concentration

L-arginine was not detected in the solution from the chamber after protamine or a heparin-protamine complex administration with or without LPS-treatment ($n = 5$).

Discussion

The results of our study show that protamine and a heparin-protamine complex relax endothelium-denuded rat aorta strips treated with LPS. This suggests that protamine-induced hypotension after CPB might, at least in part, be caused by vasorelaxation via the iNOS pathway.

Induction of iNOS has been demonstrated after CPB.[6,7] Although iNOS does not exist under physiological conditions, it is induced by inflammatory cytokines and/or LPS in immune cells, such as macrophages, and other cells, such as vascular smooth muscle cells[14] and myocardium.[15] Production of NO by iNOS occurs in much higher quantities than by eNOS, with peak concentrations occurring at four to eight hours.[16] Prolonged CPB is required in certain cardiac operations, and systemic inflammatory cytokines including interleukin-6, -8 and 1ß, and tumour necrosis factor α[17,18] and LPS[19] increase progressively during CPB. Cytokine-induced myocardial dysfunction[20] and sepsis-associated vasorelaxation[9,16] appear to be related to increases in iNOS activity. Suppression of increased iNOS activity prevented hemodynamic aggravation after CPB.[21] Therefore, it is obvious that iNOS is induced and activated in response to CPB, and plays an important hemodynamic role after CPB,[22] even if its activity is temporal. It is well-known that protamine stimulates the release of NO via the eNOS pathway and causes vasorelaxation,[5] but the interaction between protamine and iNOS has never been investigated despite the importance of iNOS after CPB.

Exogenous L-arginine (a substrate for NOS) induces vasorelaxation via a Ca^{2+}-independent iNOS pathway, but not via a Ca^{2+}-dependent eNOS pathway.[16] Exogenous L-arginine-induced vasorelaxation did not occur before LPS-treatment in the present study, which suggests that iNOS was absent. After LPS-treatment, L-arginine induced vasorelaxation and the relaxation was inhibited by aminoguanidine, an inhibitor of iNOS. These changes associated with LPS-treatment were not observed in the aortic strips treated with LPS and cycloheximide, a protein (iNOS

in this case) synthesis inhibitor. These results imply that iNOS was induced by a six-hour treatment with LPS, as previously reported.[9,10]

Exogenous protamine induces vasorelaxation via a Ca^{2+}-dependent eNOS pathway.[5] As eNOS is localized to endothelium, protamine can relax only intact vascular strips.[5] Once endothelium is rubbed according to the preparation in this study, protamine cannot relax vascular strips. However, after the induction of iNOS with LPS-treatment, protamine significantly relaxed the endothelium-rubbed strip pre-contracted with phenylephrine (Figure 1). As with L-arginine, protamine-induced vasorelaxation was inhibited by aminoguanidine and methylene blue (a guanylyl cyclase inhibitor), (Figure 2). Therefore, it is believed that protamine stimulated the iNOS pathway and relaxed vascular strips. Heparin-binding with protamine did not affect the interaction between protamine and iNOS.

Pearson *et al.* hypothesized that both protamine and a heparin-protamine complex act on endothelial cell receptors to stimulate the production of NO via eNOS, and do not act on eNOS directly, because of the large molecular weight of protamine, i.e., this drug would not be expected to enter the endothelial cells.[5] Furthermore, iNOS exists in the smooth muscle cells and protamine and a heparin-protamine complex are too large to enter them. Although almost all of the amino acid composition of protamine is L-arginine, protamine does not appear to decompose to L-arginine as an extracellular substrate of iNOS because L-arginine was not detected in the Krebs solution after protamine administration in the present study. Unfortunately, it remains unexplained how protamine stimulates the iNOS pathway.

There have been several *in vitro* functional studies using animal or human vascular strips on protamine and eNOS.[5,23,24] In these studies, the concentrations needed to relax each vascular strip were much greater than those used clinically. On the other hand, *in vivo* studies show that hypotension was induced by clinically feasible concentrations of protamine and was reversed by NOS- or guanylyl cyclase-inhibitors.[25,26] Accordingly, there is little doubt that eNOS plays an important role in protamine-induced hypotension, although high concentrations of protamine were needed to relax arteries via eNOS in the *in vitro* studies mentioned above. Also, in the present study, a high concentration of protamine was needed to relax the vascular strips via iNOS. Further *in vivo* studies with iNOS-inhibitors are required to elucidate whether clinically feasible concentrations of protamine induce hypotension via iNOS pathway.

It has been well documented that protamine administration after CPB is occasionally associated with clinically significant adverse hemodynamic reactions including systemic hypotension, pulmonary hypertension, and left ventricular dysfunction. The multiple cardiovascular responses are most likely mediated via several mechanisms such as complement activation, histamine release, antibody formation, thromboxane production, and NO release from eNOS.[27] This study supports NO release from iNOS as an additional mechanism to further our understanding of these complex cardiovascular responses.

In conclusion, protamine or the heparin-protamine complex causes vasorelaxation via NO release from iNOS. This implies that *in vivo* vasorelaxation and subsequent hypotension will ensue in the presence of serious inflammation with an iNOS. Inducible NO synthase induced by LPS and/or inflammatory cytokines during CPB,[6,7] often exists at sites of atherosclerosis in patients undergoing CPB,[8] and releases much higher quantities of NO than eNOS.[16] Under such situations, protamine after CPB might cause hypotension via NO release from iNOS, and the hypotension could be a marker of the level of inflammation.

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