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The inhibitory action of protamine on human internal thoracic artery contractions: the effect of free hemoglobin

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Abstract

Objective: We investigated the mechanism of the protamine action and the effects of free hemoglobin on protamine-induced responses in endothelium-denuded and-intact human internal thoracic artery (ITA) rings precontracted with phenylephrine (PE) or high KCl. **Methods**: Samples of redundant ITA obtained from patients undergoing a coronary artery bypass graft surgery were cut into 3 mm wide rings and suspended in 20 ml organ baths. Isometric tension was continuously measured with an isometric force transducer connected to a computer-based data acquisition system. **Results**: Acetylcholine (Ach, 10^{-8}–10^{-5} M) caused a concentration-dependent relaxation of PE-precontracted ITA rings. Free hemoglobin (0.1 and 0.5 μM) produced a concentration-dependent and significant decrease in sensitivity (pD_2) and maximal contractility (E_{max}) in response to Ach in PE-precontracted ITA rings ($P < 0.0001$). Protamine (50–800 μg/ml), free hemoglobin (0.1 and 0.5 μM), nitric oxide (NO) blocker N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) or soluble guanylate cyclase inhibitor methylene blue (10 μM) administration did not cause a significant alteration on basal tonus of endothelium-intact or -denuded ITA rings. Protamine (50–800 μg/ml) induced concentration-dependent relaxation responses in ITA rings precontracted by either PE or high KCl. There was no difference in sensitivity or maximal response to protamine between the endothelium-intact and -denuded rings. Incubation of endothelium-intact or -denuded ITA rings with L-NAME or free hemoglobin or methylene blue did not cause a significant inhibition on relaxation responses to protamine. ITA ring contractions induced by stepwise addition of calcium to high KCl solution with no calcium were almost completely inhibited by protamine ($P < 0.0001$). **Conclusions**: It was suggested that protamine induced relaxation responses in human ITA rings is not NO- or endothelium-dependent but seems to depend on the interactions of protamine with calcium influxes and/or calcium release from intracellular stores in this tissue.
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Keywords: Protamine; Human internal thoracic artery; Hemoglobin; Nitric oxide

1. Introduction

Protamine sulfate administration is widely used in cardiovascular surgery practise to reverse the anticoagulant effect of heparin despite its well known side effects including alterations in hemodynamics [1]. Despite its extensive clinical application, the mechanism of protamine-induced hemodynamic changes is unclear. Endothelium-dependent relaxation was thought to be one of the mechanisms of protamine-induced systemic hypotension [2] Accordingly, protamine was shown to induce nitric oxide (NO)-dependent relaxation of arteries [2,3] including the human internal thoracic artery (ITA) [4] by activation of endothelial nitric oxide synthase (NOS) pathway. On the other hand, other mechanisms such as the toxic influence on vascular endothelium or reduction in adenosine triphosphate in the vascular wall or interactions with calcium [5–7] might also be involved in the vascular effects of protamine. In accordance, protamine was shown to cause vascular relaxations that are neither NO nor endothelium-dependent [2,3,4,6]. Some investigators have even reported that protamine itself could not cause any hemodynamic response in vivo in the absence of heparin; therefore by itself, may

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not have any significant action on vascular smooth muscles [8].

Hemolysis and postoperative hypertension are well-recognised complications of cardiopulmonary bypass (CPB). Catecholamine release, activation of the renin-angiotensin system, vasopressin release and aortic baroreceptor dysfunction are among the reasons for the transient postoperative hypertension following CPB [9]. Recently, the role of hemoglobin released by erythrocyte hemolysis has gained substantial importance [10]. Vascular exposure to free hemoglobin in vivo may alter local and systemic hemodynamics [11]. It is well known that the free hemoglobin binds and inactivates NO [12]. Accordingly, it was shown that free hemoglobin causes a significant inhibition in endothelium-dependent relaxation of human ITA rings as a consequence of NO binding [13]. Depending on this finding, it was suggested that such an interaction of circulating free hemoglobin with NO may have an important role in the pathogenesis of both ITA spasm and postbypass hypertension [13].

It is possible that the protamine administration and rise in free hemoglobin concentrations may occur coincidentally during CPB and the potential temporal relationship between these two agents may cause significant alterations in vasomotor responsiveness. To our knowledge, the effect of free hemoglobin on the vascular action of protamine in human ITA preparations has not been studied yet. Therefore, in the present study, we investigated both the mechanism of the protamine action and the effects of free hemoglobin on protamine-induced responses in this tissue.

2. Materials and methods

Samples of redundant ITA were obtained from patients, undergoing a coronary artery bypass graft surgery. None of the patients had diabetes mellitus or hypertension. The samples of the ITA were transported in cold (4°C) physiological salt solution (PSS) to the laboratory. They were cleaned of the connective tissue and cut into 3 mm wide rings. The rings were carefully suspended by two stainless steel clips passed through the vessel lumen in 20 ml organ baths filled with PSS (mM: NaCl 118, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.2) maintained at 37°C gassed with 95% O_2 and 5% CO_2 to obtain a pH of 7.4. The rings were placed at the optimal point of length-tension relation by gradually stretching them until contraction induced by 20 mM of KCl was maximal at each level of distention [14]. Isometric tension was continuously measured with an isometric force transducer (FDT10-A, Commat Ltd.), connected to a computer-based data acquisition system (TDA 97, Commat Ltd.).

Experiments were done on sets of two preparations, one containing ($n = 20$) and the other ($n = 20$) denuded of endothelium. After an equilibration period of 1 h, the presence of a functional endothelium was confirmed by the ability of acetylcholine (Ach, 10^{-6} M) to produce relaxation of tissues precontracted with phenylephrine (PE, 10^{-6} M). In order to denude the endothelium intimal surface of ITA rings were gently rubbed with a cotton swab. Successful removal of the endothelium was confirmed by the inability of Ach to induce relaxation in PE-precontracted rings.

The effect of free hemoglobin (0.1 and 0.5 μM) on Ach-induced relaxations (10^{-8}–10^{-5} M) was investigated in endothelium intact ITA rings that were precontracted with PE. After further rinsing, ITA rings precontracted with PE (3 μM) or KCl (80 mM) were exposed to protamine (50–800 μg/ml) in the presence of free hemoglobin (0.1 and 0.5 μM) or NOS blocker N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) or methylene blue (10 μM) and relaxation responses were recorded. The effects of protamine, free hemoglobin, L-NAME and methylene blue on basal tonus of ITA rings were also tested. Free hemoglobin, L-NAME and methylene blue were added to the bath 5, 20 and 20 min prior to the study, respectively. In another set of experiments, the effect of protamine on calcium-induced contractions in endothelium-intact ITA rings was studied in calcium-free solution containing high (80 mM) KCl ($n = 12$). After a 10 min period of depolarization, various concentrations of calcium were cumulatively applied in a step-wise manner to a final concentration of 2.5 mM. The high KCl solution was prepared by replacing NaCl with KCl, isoosmotically. In calcium-free solutions, $CaCl_2$ was replaced with $MgCl_2$ and 2 mM ethyleneglycol-bis-(β-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added.

In a seperate set of experiments, we investigated the effect of protamine, alone and following L-NAME or methylene blue incubation, on PE precontracted endothelium-intact ($n = 6$) and endothelium-denuded ($n = 6$) rat thoracic aortic rings using this tissue as the control vessel. Additionally, in order to test whether the relaxation responses to Ach were preserved following the exposure of tissues to protamine, we produced concentration-response curves for this agent in PE precontracted rat aortic rings with intact endothelium before and after the experiments with protamine. All the agents were used in concentrations mentioned above.

The pH of the bath solution was checked to ascertain if any changes occurred after the addition of protamine. All experiments were carried out in the presence of indomethacine (1 μM).

2.1. Materials

Ach, PE, L-NAME, indomethacine, methylene blue, free hemoglobin, EGTA and the salts for the PSS were purchased from Sigma Chemical (St. Louis, MO). Protamine (Protamin 1000) was obtained from ICN Pharmaceuticals (Istanbul).

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2.2. Statistical analysis

All values are expressed as mean ± SEM. Responses to protamine and Ach are expressed as percentages of the reversal of the tension developed in response to PE or KCl. The logarithm of the concentration of agonist which elicited a 50% maximal response (E_{max}) was designated as the EC_{50}. These values were determined by regression analysis of the linear portions of the log concentration-response curves. Sensitivity was expressed as pD_2 ($-\log EC_{50}$). Statistical analysis of the results was performed using one-way analysis of variance and Student's *t*-test where appropriate. *P* values lower than 0.05 were considered significant.

3. Results

Administration of Ach caused a concentration-dependent relaxation of PE precontracted ITA rings. Free hemoglobin caused a concentration-dependent and significant decrease in sensitivity (pD_2) and maximal contractility (E_{max}) in response to acetylcholine in PE precontracted ITA rings (Fig. 1, Table 1).

Protamine, free hemoglobin, L-NAME or methylene blue administration did not cause a statistically significant alteration on the basal tonus of endothelium-intact or endothelium-denuded ITA rings (data not shown). PE and KCl caused stable and sustained contractions of ITA preparations (Fig. 2). Protamine caused concentration-dependent relaxation responses in endothelium-intact as well as endothelium-denuded ITA rings precontracted by either PE or KCl (Fig. 2). Removal of endothelium did not significantly alter the relaxation responses to protamine (Fig. 2, Table 2). Incubation of endothelium-intact or endothelium-denuded ITA rings with L-NAME or free hemoglobin or methylene blue did not cause a significant inhibition on relaxation responses to protamine (Fig. 2, Table 2). ITA ring contractions induced by stepwise

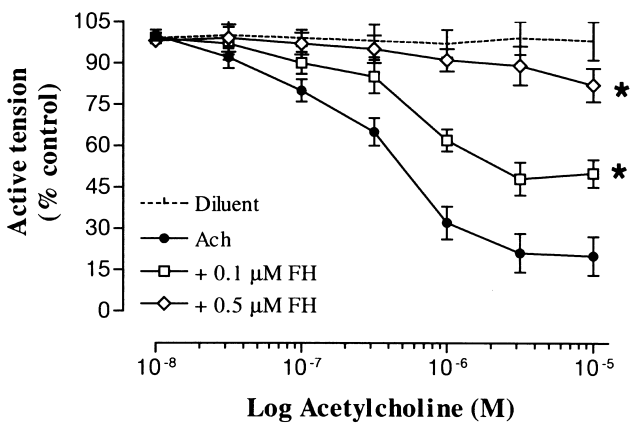


Fig. 1. Effect of free hemoglobin (FH) on acetylcholine (Ach)-induced relaxations in endothelium-intact human thoracic artery rings precontracted by phenylephrine. Each point represents the mean with SEM shown by vertical bars. $n = 20$ for all groups. *$P < 0.05$ as compared with Ach alone.

Table 1
pD_2 ($-\log EC_{50}$) and E_{max} (% of phenylephrine-induced contractions) values for acetylcholine (Ach) on phenylephrine (PE)-induced contractions of human internal thoracic artery rings with intact endothelium: effect of free hemoglobin (FH, 0.1 and 0.5 μM)[a]

	Ach alone	+0.1 μM FH	+0.5 μM FH
pD_2	6.46 ± 0.01	6.23 ± 0.02*	5.66 ± 0.21*
E_{max}	80 ± 7	52 ± 6*	18 ± 6*

[a] FH was added to the bath 5 min prior to response to PE was elicited. $n = 12$. *: $P < 0.0001$ as compared with Ach alone.

addition of calcium to high KCl solution with no calcium were significantly inhibited by protamine applied at the highest concentration. E_{max} values for calcium-induced contractions with and without protamine were 100 ± 3 and 17 ± 7, respectively ($P < 0.0001$, Fig. 3).

Administration of Ach caused concentration-dependent relaxations of PE precontracted rat aortic rings with intact endothelium. The sensitivity ($pD_2 = 6.86 \pm 0.11$ versus 6.91 ± 0.08) and maximal responses ($E_{max} = 81.5 \pm 8$ versus 79.8 ± 9) of rat aortic rings to Ach did not show a significant alteration following protamine experiments.

Protamine caused concentration-dependent relaxation responses in endothelium-intact and endothelium-denuded rat thoracic aortic rings precontracted by PE (Fig. 4). Removal of endothelium ($E_{max} = 72 \pm 11$ and 68 ± 9 for endothelium-intact and endothelium-denuded rings, respectively) or L-NAME ($E_{max} = 64 \pm 13$) or methylene blue ($E_{max} = 70 \pm 14$) incubation of endothelium-intact rat aortic rings did not produce a significant alteration in maximal relaxation responses to protamine (Fig. 4).

Protamine administration did not cause a significant change in pH of the bath solution.

4. Discussion

The free hemoglobin that normally complexes with haptoglobin starts to circulate when the binding becomes

Table 2
E_{max} (% of phenylephrine- or KCl-induced contractions) values for protamine on phenylephrine (PE)- or KCl-induced contractions of human internal thoracic artery rings with intact (E +) and denuded (E −) endothelium: effects of free hemoglobin (FH, 0.5 μM), methylene blue (MB, 10 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM)[a]

	Phenylephrine (E_{max})		KCl (E_{max})	
	E (+)	E (−)	E (+)	E (−)
Protamine alone	83 ± 3	85 ± 7	82 ± 4	88 ± 6
+FH	90 ± 4	88 ± 7	ND	ND
+MB	88 ± 3	81 ± 5	ND	ND
+L-NAME	82 ± 4	84 ± 5	ND	ND

[a] FH, MB and L-NAME were added to the bath 5, 20 and 20 min prior to response to PE or KCl was elicited. $n = 20$ for all groups. ND: not determined.

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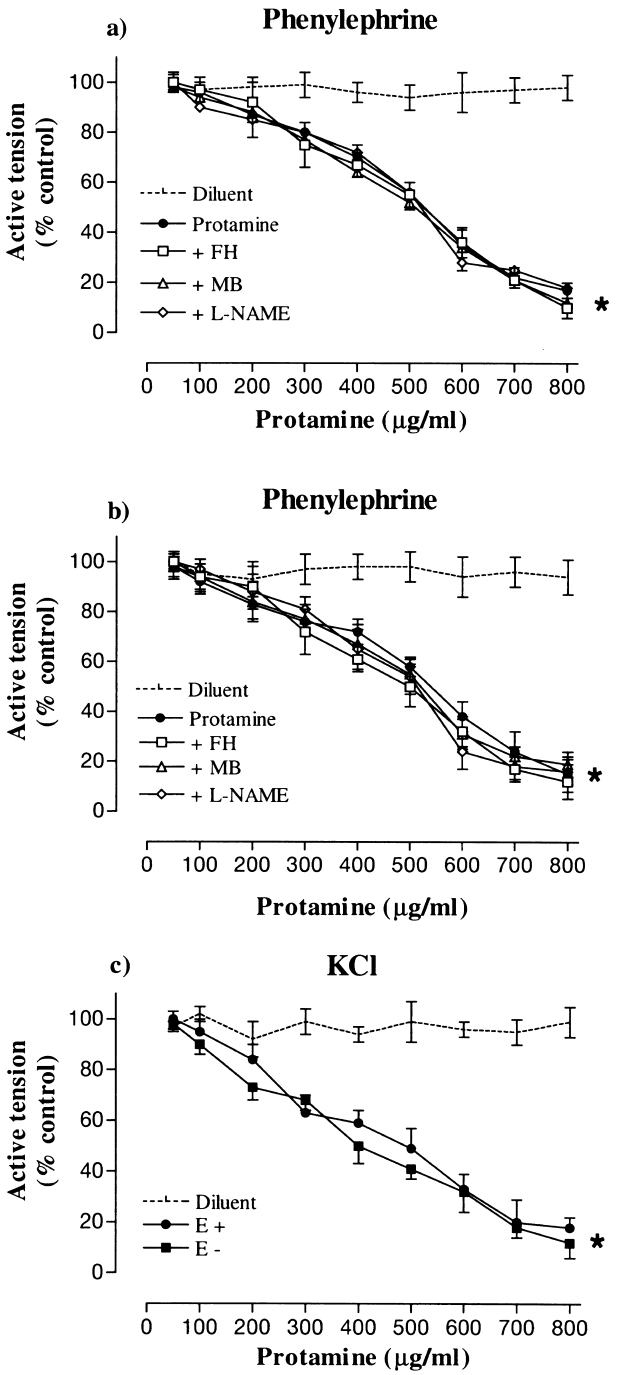


Fig. 2. Protamine-induced relaxations in endothelium-intact (E + , a and c) and endothelium-denuded (E − , b and c) human thoracic artery rings precontracted by phenylephrine (a, b) and KCl (c): effects of free hemoglobin (FH, 0.5 μM), methylene blue (MB, 10 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM). Each point represents the mean with SEM shown by vertical bars. $n = 20$ for all groups. FH, MB and L-NAME were added to the bath 5, 20 and 20 min prior to response to PE or KCl was elicited. $^*P < 0.05$ as compared with diluent.

saturated with increasing hemolysis during CPB [10]. It has been reported that the rise in concentration of free hemoglobin is directly related to the duration of CPB [10]. In the present study, in agreement with the other

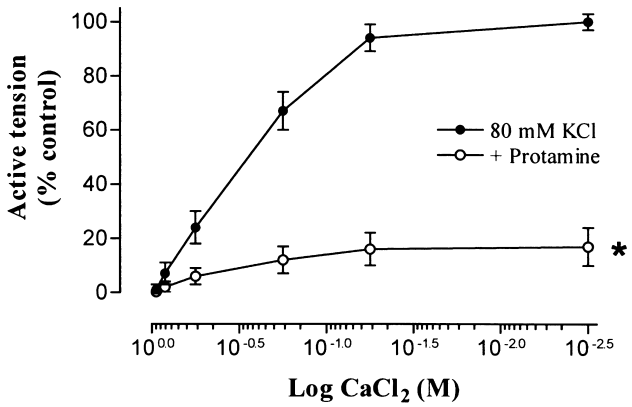


Fig. 3. Effect of protamine (800 μg/ml) on contractions induced by various concentrations of $CaCl_2$ in calcium-free solution containing 80 mM KCl in endothelium-intact human thoracic artery rings. Each point represents the mean with SEM shown by vertical bars. $n = 12$. $^*P < 0.0001$ as compared with 80 mM KCl alone.

studies [13] free hemoglobin in concentrations similar or less than the ones detected during CPB surgery [10] produced a significant decrement in Ach-induced endothelium-dependent relaxation of human ITA rings. Our results confirmed the findings of Spyt et al. [13] who suggested that the circulating free hemoglobin might have an important role in the pathogenesis of both ITA spasm and postbypass hypertension by preventing the NO-mediated modulation of vascular smooth muscle contraction.

Protamine caused a concentration-dependent inhibition of the PE-induced contractions of both ITA and rat thoracic aortic rings. The sensitivity to or the maximal effect of protamine in endothelium-denuded arteries was not significantly different than those in endothelium-intact arteries,

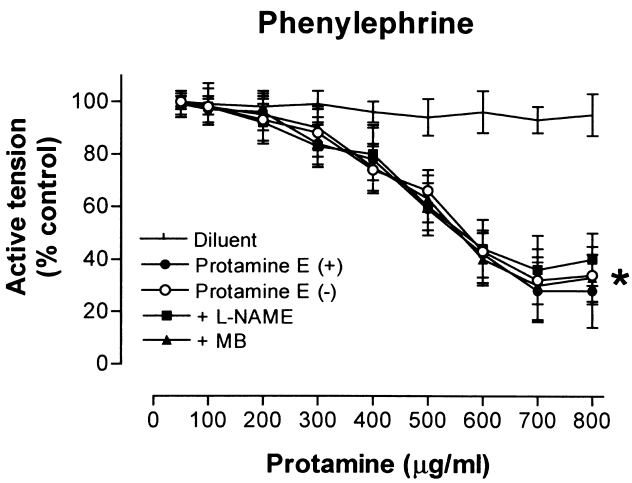


Fig. 4. Protamine-induced relaxations in endothelium-intact (E +) and endothelium-denuded (E −) rat thoracic aortic rings precontracted by phenylephrine: effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) and methylene blue (MB, 10 μM) on protamine-induced relaxations in endothelium-intact rings. Each point represents the mean with SEM shown by vertical bars. $n = 6$ for all groups. L-NAME and MB were added to the bath 20 min prior to response to PE was elicited. $^*P < 0.05$ as compared with diluent.

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which suggested that the inhibitory effect of protamine on PE-induced contractions is independent of endothelium. In contrast, it has been reported that endothelial removal abolished all vascular relaxations induced by arginine-rich basic polypeptides [15]. It is well known that the amino acid L-arginine is the physiological precursor of NO [16]. Protamine is rich in the amino acid L-arginine [17]. Accordingly, protamine was shown to induce NO-dependent relaxation of arteries [2,3] including the human ITA [4] by activation of endothelial NOS pathway. This view was further supported by the findings of Akata et al. [6] who showed that agents such as hemoglobin [18], methylene blue [18] and N^G-nitro L-arginine (L-NNA) [19] which binds and inactivates NO, inhibits soluble guanylate cyclase and blocks cytosolic NO synthesis from L-arginine, respectively, all significantly decreased the protamine-induced inhibition in endothelium-intact rabbit mesenteric artery strips. Contrastingly, in our study, incubation of endothelium-intact or endothelium-denuded ITA rings with L-NAME or free hemoglobin or methylene blue did not cause a significant inhibition on relaxation responses to protamine. Similarly, L-NAME or methylene blue incubation did not exert a significant inhibition on protamine-induced relaxations in PE-precontracted rat aortic rings. These findings further supported the view that the NO does not play a major role in vasodilatory effect of protamine in this tissue. On the other hand, Castresana et al. showed that protamine did not cause a significant effect on basal intracellular levels of cGMP, the second messenger for endothelium-derived relaxing factor, until a concentration of 250 μg/ml in pig vascular smooth muscle cells. However, at this concentration, protamine produced a significant increase in basal cGMP concentrations [20]. It was also reported that the effects of basic proteins rich in the amino acid L-arginine on NOS could be either stimulatory or inhibitory, depending on whether the basic proteins exert their effects extracellularly or intracellularly, respectively [21]. The reasons for the present contradictory results are not clear at the moment but, they might partly depend on the experimental setting and the tissues used and need further research.

Although the endothelium releases other vasodilators such as the prostacyclin, a possible role of this compound in the observed effects of protamine was excluded by the presence of cyclooxygenase inhibitor indomethacine in PSS throughout the experiments. Additionally it was also shown that indomethacine does not significantly inhibit the vasodilation in response to protamine in canine pulmonary artery segments [3].

The view that protamine may also cause endothelium-independent inhibition on vascular contractions was supported by several studies [2–4,6]. Mechanisms such as the toxic influence on vascular endothelium or on smooth muscle or reduction in adenosine triphosphate available for contraction in the vascular wall or interactions with calcium [4–7] were all proposed to play a role in the inhibitory effect of protamine in vascular tissues. Various studies reported that polycationic compounds, including protamine can cause endothelial injury [5,15,22]. Both impairment of Ach-induced relaxations [22] and the evidence of endothelial cell injury [5,22] have been reported following protamine treatment. However, the toxic effect of protamine on endothelial cells still seems to be controversial as Rapoport et al. noted that protamine exerted little effect on endothelial integrity and relaxation due to Ach [15]. In support of this later view, we found that prior exposure to protamine did not cause a significant alteration in sensitivity and maximal responses to Ach in rat thoracic aortic rings indicating that Ach relaxations were preserved following treatment with protamine.

In the present study, protamine relaxed both the endothelium-denuded and endothelium-intact ITA rings precontracted with high KCl. Smooth muscle contractions induced by depolarizing the cell membrane with high concentrations of KCl depend mainly upon calcium influx through the voltage-dependent channels. Beside that, protamine inhibited the calcium-induced contractions of endothelium-denuded and -intact ITA rings evoked in calcium-free solution containing high KCl. Akata et al. also showed that protamine significantly inhibited the high KCL-induced contractions and also the calcium-induced contractions evoked in calcium-free high KCl in endothelium-denuded rabbit mesenteric artery strips [6]. It was suggested that [6,7] positively charged protamine may inhibit the calcium influxes by replacing the superficially bound calcium known to be essential for calcium influx [23] at the anionic superficial calcium binding sites. Although there are controversies [6], protamine was reported to be able to enter the cells [24]. It was also proposed that protamine may bind with anionic phosphatdyl-inositol 4,5-biphosphate (PIP_2) preventing its breakdown with consequent inhibition of IP_3-induced calcium release from the intracellular stores [6]. Noradrenergic agents can induce a change in the membrane potential which elicits the opening of voltage-gated channels in the plasma membrane and thereby allows the entry of calcium into the cell [25]. Additionally, noradrenergic contractions of vascular tissues also partly depend on the IP_3-induced calcium release from intracellular stores [26] Therefore, it might be suggested that possible interactions of protamine with calcium influxes at the membrane level and with IP_3-induced calcium release from intracellular stores at cytosolic level may explain its inhibitory effects on both PE- and high KCl-induced contractions in endothelium-denuded and -intact ITA rings. It should also be noted that protamine may still have some endothelium- or NO-dependent inhibitory effects on PE- or high KCl-induced vascular contractions but they might have been masked by its greater inhibitory effects on PE- or high KCl-induced alterations in cellular calcium kinetics.

A major limitation of the present study is the usage of isolated blood vessels that are denervated. It is well known

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that both neural pathways and the other circulating hormones influence the vasoreactivity in vivo. However, as noted by Evora et al. [3], the direct vascular responses to protamine observed in the organ baths undoubtedly occur in vivo and may modulate the effects of these other factors.

The findings of the present study imply that protamine may cause inhibitory effects on vascular tissues and this inhibitory action may at least be partly responsible from its hypotensive effect during heparin reversal. The lowest concentration of protamine used in the present study was 50 μg/ml. A plasma concentration of 50 μg/ml was normally achieved after the intravenous administration (i.v.) of 0.75 mg/kg protamine [27]. Therefore, even in cases of adequate heparin reversal, protamine may exist in the blood in free form, i.e. uncomplexed with protamine, particularly when it is injected rapidly [6]. Therefore, it might be expected that slow protamine administration from a peripheral line for heparin reversal might be helpful in preventing protamine-induced circulatory collapse.

Free hemoglobin and free protamine (uncomplexed with heparin) concentrations may rise coincidentally during the CPB operations. We found that although free hemoglobin caused a significant decrement in Ach-induced endothelium-dependent relaxation of human ITA rings, it did not cause a significant alteration either in sensitivity or in maximal effect of protamine on PE- or high KCl-induced contractions. Depending on this finding, one can rule out the possibility of a significant alteration in vascular responsiveness of human ITA due to a temporal interaction between the effects of these two agents. However, such an interaction might operate in other vessels since the vascular effects of protamine may show differences in different vessels [28]. It should also be noted that the independent and the opposite effects of these two agents on vascular responsiveness may play important roles in pathogenesis of both hemodynamic alterations and of ITA spasm and postbypass hypertension following CPB operations.

In summary, the present findings suggested that protamine induced relaxation responses in human ITA rings did not seem to be NO- or endothelium-dependent. A possible interactions of protamine with calcium influxes and/or calcium release from intracellular stores might be involved in the observed relaxant effect of protamine in this tissue, but the other and yet undefined mechanisms might also take part.

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