Laboratory Investigations

Systemic hypotensive response to protamine following chronic inhibition of nitric oxide synthase in rats

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Purpose: The aims of the present studies were to determine whether the systemic hypotensive response to protamine was modified in rats pre-treated for two weeks with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME), and to evaluate the inhibitory effect of heparin on the systemic hypotensive response to protamine *in vivo*.

Methods: Male rats were randomly assigned into four groups. Normal saline 12 $\mu l \cdot day^{-1}$, D-NAME (an inactive enantiomer of L-NAME), 10 $mg \cdot kg^{-1} \cdot day^{-1}$, L-NAME, 1 or 10 $mg \cdot kg^{-1} \cdot day^{-1}$ *ip* was administered for two weeks and the haemodynamic changes were measured after protamine administration. In another experiment, male rats were assigned to two groups. In one, the heparin group, protamine was administered after heparin had been administered and in the other, protamine group, protamine alone was administered.

Results: L-NAME inhibited the decrease in systemic arterial pressure after protamine administration ($P < 0.05$), but D-NAME had no effect. Also, heparin reduced the decrease in systemic arterial pressure after protamine ($P < 0.05$).

Conclusion: Nitric oxide is mainly responsible for mediation of the systemic hypotensive response to protamine which is also reduced by heparin.

Objectif : L'objectif des présentes études était de déterminer si la réaction hypotensive généralisée à la protamine était modifiée chez les rats prétraités pendant deux semaines avec l'inhibiteur de l'oxyde nitrique synthase, N^G-nitro-L-arginine-methyl ester (L-NAME), et d'évaluer l'effet inhibiteur de l'héparine sur la réaction hypotensive généralisée à la protamine, *in vivo*.

Méthode : Des rats mâles ont été répartis au hasard en quatre groupes. Une solution salée de 12 $\mu l \cdot jour^{-1}$, D-NAME (un énantiomère inactif de L-NAME), 10 $mg \cdot kg^{-1} \cdot jour^{-1}$, L-NAME, 1 ou 10 $mg \cdot kg^{-1} \cdot jour^{-1}$ *ip* a été administré pendant deux semaines et les changements hémodynamiques ont été mesurés après l'administration de protamine. Lors d'une autre expérience, les rats ont été divisés en deux groupes. Dans l'un d'eux, le groupe héparine, l'administration de la protamine a suivi celle de l'héparine et, dans l'autre groupe, le groupe protamine, seule la protamine a été administrée.

Résultats : L-NAME a empêché la baisse de tension artérielle générale après l'administration de la protamine ($P < 0,05$), mais D-NAME n'a pas eu d'effet. De même, l'héparine a réduit la baisse de la tension artérielle générale après l'administration de protamine ($P < 0,05$).

Conclusion : L'oxyde nitrique est principalement responsable de la médiation de la réaction hypotensive généralisée à la protamine, laquelle est aussi réduite par l'héparine.

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PROTAMINE, a polycationic protein used for the reversal of heparin, induces systemic arterial hypotension, bradycardia, dyspnea, transient flushing, and a feeling of warmth.[1] These adverse responses may be especially dangerous in cardiac surgery. Systemic hypotension after protamine administration has been considered to be caused by type I or type II allergic reaction or by the activation of complement. Recently, the hypotensive response to protamine has been reported to be mediated by endothelium-derived nitric oxide (NO). We attempted to clarify the mechanism of the systemic hypotensive response to protamine in an *in vivo* study using N^G-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NO synthase. In an earlier study,[2] we showed that systemic hypotension after protamine was inhibited by L-NAME pretreatment. However, this result may have been indirectly influenced by a rapid increase in blood pressure after L-NAME administration. This rapid pressure change may activate several pressure control mechanisms, such as baroreceptor feedback mechanism, central nervous system ischaemic mechanisms, and the chemoreceptor mechanism.[3] Thus, in the present study, we studied the effect of L-NAME on the systemic hypotensive response to protamine using rats in which L-NAME was administered intraperitoneally for two weeks, to minimize the influences of the rapid increase in blood pressure after L-NAME administration.

Comparable concentrations of heparin (8 $U \cdot ml^{-1}$) have been reported to completely inhibit the vasodilation response to protamine (40 - 400 $\mu g \cdot ml^{-1}$) in the canine pulmonary arterial ring.[4] However, this finding has not been confirmed by *in vivo* study.

The aims of the present study were to determine whether nitric oxide mediated the systemic hypotensive response to protamine in chronic L-NAME pretreated rats and to confirm the inhibitory effect of heparin on the systemic hypotensive response to protamine *in vivo*.

Materials and methods

Animal preparation

The studies were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. The animal procedures and handling were approved by the Institutional Animal Care and Use Committee of the Akita University.

Agents and chemicals

L-NAME and D-NAME, which is the biologically inactive enantiomer of L-NAME were obtained from Sigma Chemical (St Louis, MO), heparin was obtained from NOVO (Copenhagen, Denmark) and protamine was obtained from Simizu (Tokyo, Japan). All chemicals and agents were freshly diluted in saline 0.9%.

Protocol

Chronic inhibition of nitric oxide synthase

Two weeks before the experiments, male Sprague-Dawley rats (404 ± 2g) were anaesthetized with 60 $mg \cdot kg^{-1}$ pentobarbital *ip*. An osmotic pump (Alzet model 2002, ALZA Co, CA, USA) was implanted in the peritoneal cavity of each animal. After implantation, 2 ml bupivacaine (0.25%) was infiltrated *sc* in the surgical area for relief of postsurgical pain, and 20 $mg \cdot kg^{-1}$ cefazolin sodium *ip* was injected. To verify the effect of L-NAME on systemic hypotensive response to protamine, 12 $\mu l \cdot day^{-1}$ saline (n = 6), 10 $mg \cdot kg^{-1} \cdot day^{-1}$ D-NAME (n = 4), 1 or 10 $mg \cdot kg^{-1} \cdot day^{-1}$ L-NAME (n = 6,6) was continuously delivered into the peritoneal cavity through the pump until the end of the experiment. On the day of the experiment, animals were anaesthetized with at 60 $mg \cdot kg^{-1}$ pentobarbital sodium *ip*, and then their tracheas were intubated after tracheostomy. The lungs were mechanically ventilated with a volume controlled ventilator (7025 Rodent ventilator, Comerio-Varese, Italy) to keep $P_{ET}CO_2$ at approximately 35 mmHg. Anaesthesia was maintained with isoflurane at an inspired concentration of 0.75%. The right jugular vein was cannulated for the administration of agents, and the left carotid artery was cannulated for the measurement of systemic arterial pressure. The systemic arterial pressure was continuously recorded using a calibrated transducer (TP200T, Nihon Kohden, Tokyo, Japan). Rectal temperature was kept in the range of 38 ± 0.5°C using a heating lamp. After systemic arterial pressure was stabilized for more than 15 min, baseline measurements were obtained. Then, protamine *iv* was injected to animals at 10 $mg \cdot kg^{-1}$. This dose of protamine was determined in a pilot study. After stabilization from the first dose of protamine, 300 $mg \cdot kg^{-1}$ L-arginine *iv* was given.

Heparin pretreatment

Male Sprague-Dawley rats (363 ± 12g) were anaesthetized with 60 $mg \cdot kg^{-1}$ pentobarbital sodium, their tracheas were intubated and the lungs were mechanically ventilated with a volume controlled ventilator (7025 Rodent ventilator, Comerio-Varese, Italy) to keep $P_{ET}CO_2$ approximately 35 mmHg. Anaesthesia was maintained with isoflurane 0.75%. The right jugular vein was cannulated for drug administration, and the left carotid artery was cannulated for measurement of systemic arterial pressure. Rectal temperature was kept at 38 ± 0.5°C using a heating lamp. After systemic arterial pressure had stabilized for 15 min, baseline measurement was obtained.

Six rats were given 10 mg·kg^{-1} heparin *iv* and the other six rats were given saline 0.9% as control. Ten minutes later, 10 mg·kg^{-1} protamine *iv* was given to both groups. Systemic arterial pressure was continuously recorded using a calibrated transducer (TP200T, Nihon Kohden, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SD. Repeated measures analysis of variance (ANOVA) was performed for serial measurements to assess change over time. The effects of treatment; among and within groups were made with the use of contrast. Differences were considered significant at $P < 0.05$.

Results

In the control rats systemic arterial pressure rapidly decreased to 41% of baseline within five minutes of administration of protamine and recovered almost to baseline within 30 min (Figure 1). In the L-NAME10 group, the decrease in systemic arterial pressure after protamine was limited. Systemic arterial pressure decreased by 12.3% of baseline. The decrease in systemic arterial pressure after protamine in the D-NAME group was similar to that in the control group. Systemic arterial pressures in all groups decreased rapidly to the same degree after L-arginine administration (Figure 2).

The maximal decrease in systemic arterial pressure after protamine was less than that in the heparin group (Figure 3), (–45.6 ± 7.9% *vs* –9.4 ± 6.7%, respectively).

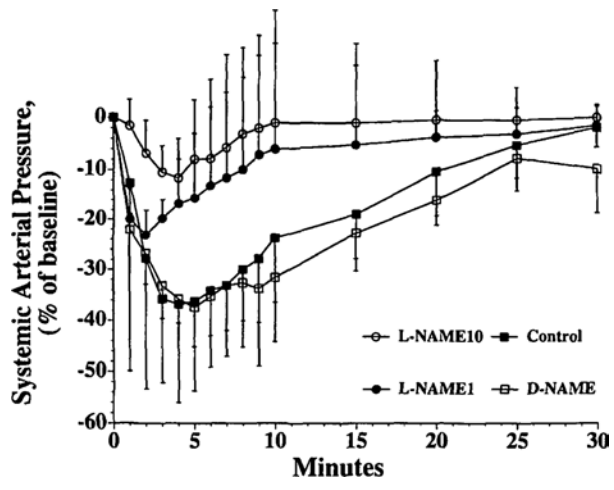

FIGURE 1 Effect of L-NAME on systemic arterial pressure after protamine administration in rats.
Mean ± SD.
L-NAME, 10 mg·kg^{-1}·day^{-1}, inhibited the decrease in arterial pressure after protamine compared with control group ($P < 0.05$).

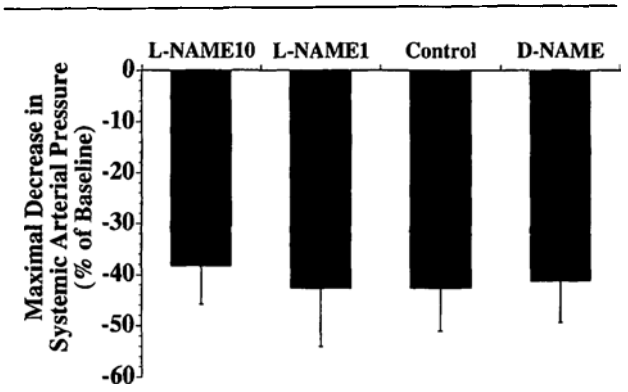

FIGURE 2 Changes in systemic arterial pressure after L-arginine administration after L-NAME or saline. *P*:NS among groups
Mean ± SD.

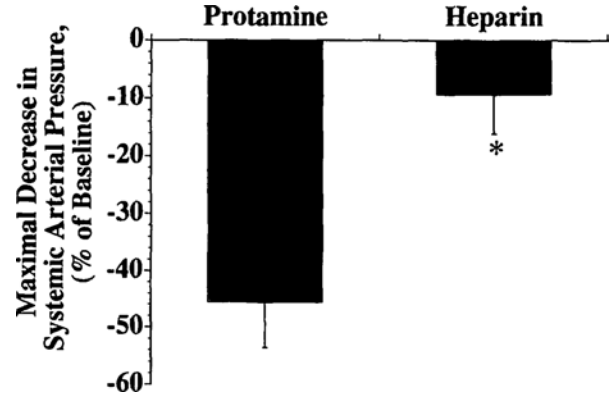

FIGURE 3 Effect of heparin on systemic arterial pressure after protamine.
Heparin inhibited the maximal decrease of arterial pressure (*; $P < 0.05$. unpaired T test).
Mean ± SD.

Discussion

This study demonstrated that L-NAME, a potent competitive inhibitor of nitric oxide synthase from L-arginine, attenuated the systemic hypotensive response to protamine, but that the inactive enantiomer, D-NAME, had no effect. Thus, it appears that nitric oxide mediates the systemic hypotension caused by protamine.

Preciously, it has been shown that the hypotensive response to protamine was decreased in dogs pretreated with N^G-monomethyl-L-arginine (L-NMMA).[5] Moreover, Pearson *et al.* reported that protamine induced concentration-dependent relaxation in canine arterial rings and that the protamine-induced relaxation is attenuated by L-NMMA.[4] Up to 70% of the decrease in systemic blood pressure after protamine may be

attributed to NO, because L-NAME pretreatment inhibited the maximal decrease of blood pressure after protamine administration by 70%.

The long-term administration of nitric oxide synthase inhibitor may affect these results, because chronic administration of NO synthase inhibitor causes vascular endothelial damages.[6,7] However, the vascular response to L-arginine, an antagonist of L-NAME was similar in all groups (Figure 2)

The hypotensive response to protamine in heparinized rats was less than that in unheparinized rats (Figure 3). This can be explained by an electrostatic interaction. Protamine binds easily to vascular endothelial receptors, inducing NO production, because protamine is a cation and the surface of the endothelial cell membrane is a anion. Similar electrostatic binding by protamine occurs at the endothelium in the pulmonary circulation.[8] When protamine is administered after heparin administration, protamine binds to heparin and a heparin-protamine complexes is formed. Thus, the binding capacity of the heparin-protamine complexes to the vascular endothelial receptor is weaker than that of protamine. We speculate that this difference between the binding capacities of protamine, and of the heparin-protamine complex causes the difference between the vascular responses to protamine and heparin-protamine complexes. The hypotensive response to a second administration of protamine is less than in our earlier study[9] perhaps because of diminished receptor binding. These results are also consistent with the clinical observation that the haemodynamic change after protamine are less when it is given slowly than quickly.[10,11] It is possible that with rapid administration, uncomplexed protamine is presented to the systemic circulation, resulting in hypotension. However, the inhibitory effect of heparin may be overcome by adding higher concentrations of protamine.

In summary, in rats, chronic pretreatment of L-NAME attenuated the systemic hypotensive response to protamine, but this was not affected by D-NAME. Also, heparin diminished the decrease in pressure. We conclude that nitric oxide is an important mediator in the hypotensive response to protamine and that heparin can reduce the systemic hypotensive response.

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