Prevention of arterial graft spasm by botulinum toxin: an in-vitro experiment

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Abstract

In coronary artery bypass surgery, arterial grafts result in improved patency rates. However, these grafts frequently fail due to spasm. Papaverine has been used to prevent graft spasm, but its effect is short-lived. Botulinum toxin inhibits muscle contraction for about three months. We investigated the usefulness of botulinum toxin in preventing arterial grafts spasm in vitro. Samples of abdominal aorta from male Wistar rats were cut into 2 mm rings and treated with various doses of botulinum toxin or papaverine for 30 min. All rings were stimulated with KC1 and noradrenaline. Tension was recorded using myography. We compared constriction caused by noradrenaline or KC1 in rings treated with botulinum toxin, or papaverine, or physiological salt solution (PSS) (control). In the presence of KC1 and noradrenaline, almost all concentrations of botulinum toxin completely inhibited arterial contraction when compared with controls. Spasm prevention was lost after 60 min in rings with papaverine but persisted for 120 min in rings with botulinum toxin. In the histological examination, arterial wall structure was not destroyed by botulinum toxin. Botulinum toxin prevented arterial graft spasm in vitro and had a longer lasting effect than papaverine, with no toxic effect on the artery.

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1. Introduction

There is increasing evidence that the use of arterial grafts in coronary artery bypass surgery improves patency rates. The radial artery was first suggested by Carpentier and colleagues in 1973 [1], but its use was soon abandoned because of unacceptably high early graft failure attributed to spontaneous contraction or spasm. Arterial grafts have a high incidence of spasm, which can lead to the serious complication of myocardial infarction.

Recently, papaverine has been used to prevent arterial graft spasm, but its effect is short-lived. The ideal pharmacologic agent to prevent arterial graft spasm is long acting and is not toxic to the graft. However, such agents are not readily available.

The bacterium Clostridium botulinum produces the most potent human biological toxins known and is responsible for the life-threatening paralytic illness, botulism. Botulinum toxin, a 150-kDa protein, binds irreversibly to presynaptic cholinergic nerve terminals, and once internalized, blocks the exocytosis of the neurotransmitter acetylcholine, thereby inhibiting muscle contraction for about three months. This toxin is used in minute doses both to treat painful muscle spasms and as a cosmetic treatment [2].

The present study investigated the usefulness of botulinum toxin in the inhibition of muscle contraction to assess whether it might be effective in preventing arterial grafts spasm.

2. Materials and methods

2.1. Animal handling and tissue samples

This study was approved by the Experimental Animal Ethics Committee of Gifu University. Male Wistar rats (~250 g) were anesthetized by ether inhalation and pentobarbital injection, and then sacrificed. All samples of abdominal aorta were taken from different rats in each of three groups described below.

2.2. Preparation of aortic rings

After laparotomy, the abdominal aorta was dissected free and placed in physiological salt solution (PSS) of the following composition in (mM): NaCl 119, NaHCO3 25, KCl 4.7, KH2PO4 1.18, MgSO4 × 7H2O 1.17, CaCl2 × 2H2O 2.5, ethylene diamine tetraacetic acid (EDTA) 0.027, and glucose 5.5 with pH adjusted to 7.4. Each artery was cleaned of adherent fatty tissue and cut into rings of ~2 mm width. Each experiment was conducted on rings prepared from different rats. Six aortic rings from different rats were included in each group.

2.3. Chemical treatment

2.3.1. Botulinum toxin treatment

Each group of six rings was soaked in PSS with various doses (20, 10, 5, 2.5, and 1 U/ml) of botulinum toxin,
maintained at 37 °C and continuously bubbled with a gas mixture of 95% O₂, 5% CO₂. Treatment time was 30 min for each dose.

2.3.2. Papaverine treatment
We used the same method to treat the six rings with 1.3 mmol/l of papaverine for 30 min. This dose was obtained from a previously published experiment [3].

2.3.3. Control (non-treated) group
Six rings in the control group were soaked in PSS only, maintained at 37 °C and continuously bubbled with a gas mixture of 95% O₂, 5% CO₂ for 30 min.

2.4. Measurement of isometric contractile responses
After chemical treatment, the rings were suspended in a Single Myograph System (Danish Myo Technology A/S, Denmark) to record changes in isometric force [4]. Briefly, two tungsten wires (each in 40 μm diameter) were passed through the segment’s lumen and each wire was fixed to the jaws of the myograph. The bathing chamber was filled with 10 ml PSS that was oxygenated with a mixture of 95% O₂ and 5% CO₂ (pH 7.3–7.5), and maintained at 37 °C using a built-in heat-exchanger device. After mounting, the rings were set to a ‘normalized’ internal circumference estimated at 90% of that they would maintain if relaxed and exposed to 100 mmHg transmural pressure (0.9 Lint). This was calculated for each individual ring on the basis of the passive length–tension characteristics of the artery and the Laplace relation [5].

After ‘normalization’ in PSS, each ring was first constricted with 60 mmol/l of KCl, and ring viability was assessed. After rinsing in purified warm water and restoring or readjusting baseline tone, rings were left for another 30 min. Only rings that responded to KCl were used for experiments with 2 μmol/l noradrenaline. The concentrations of vasoconstrictors used in the present study were obtained from previously published experiments [6].

2.5. Chemicals
The following suppliers were used: botulinum toxin (type C solution) and papaverine solution, Wako Pure Chemical Industries, Ltd (Japan); KCl, Maruishi Pharmaceutical Co. Ltd (Japan); and noradrenaline, Daiichi Sankyo Co, Ltd.

2.6. Data analysis
Results are expressed as median values. Non-parametric statistics were used for comparisons and P<0.05 was considered statistically significant.

2.7. Histological examination
Ten samples in each group (10 U/ml botulinum toxin treatment group, 1.3 mmol/l papaverine treatment group, and control group) were examined histologically by Elastica van Gieson (EVG) stain.

3. Results
3.1. Response to KCl in rings treated with various doses of botulinum toxin
The rings started to constrict immediately after 60 mmol/l KCl dropping, and both treated and non-treated rings reached a plateau in approximately 3 min. However, botulinum toxin diminished arterial ring constriction in response to KCl (Fig. 1a).

Maximum tension stimulated with KCl was 6.3 mN in non-treated rings, compared to 3.45 mN in 5 U/ml of botulinum toxin (P>0.016). There was complete inhibition of arterial graft spasm in the presence of KCl in the group treated with 5 U/ml of botulinum toxin (Fig. 1b).

3.2. Response to noradrenaline in rings treated with various doses of botulinum toxin
After exposing to KCl, we examined the effectiveness of botulinum toxin in the presence of noradrenaline. In all groups, aortic rings started to constrict just after 2 μmol/l noradrenaline exposure and reached a plateau in approximately 3 min. Botulinum toxin inhibited the constriction of the arterial rings in response to noradrenaline (Fig. 2a).

Maximum tension was 7.85 mN for non-treated rings. All groups except the 1 U/ml of botulinum toxin group had a significant difference when compared to the non-treated group. The most effective dose was 5 U/ml and the minimum dose with effectiveness was 2.5 U/ml. From 0 to 5 U/ml of botulinum toxin, we demonstrated dose-dependent inhibition of arterial graft spasm in the presence of noradrenaline (Fig. 2b).

3.3. Duration of prevention of noradrenaline-mediated constriction with botulinum toxin and papaverine
In the rings treated with papaverine, maximum tension was 1.3 mN after 30 min. However, after 120 min, maximum tension had increased to 4.3 mN. This differed significantly from the 30 min group, but did not differ from the non-treated group. The preventive effect of papaverine seemed to be lost after 60 min.

In contrast, in the rings treated with botulinum toxin, maximum tension was 2.0 mN after 30 min. At 120 min, maximum tension was 2.3 mN; this did not differ signifi-
The effect of botulinum toxin on noradrenaline-mediated constriction was maintained until 120 min, the effectiveness of botulinum toxin appeared to last longer than that of papaverine. However, in the rings treated with botulinum toxin, maximum tension recovered after 180 min, suggesting that botulinum toxin is not toxic to the artery (Fig. 3).

3.4. Histological examination of ring wall structure

Each of the 10 samples in each group were examined histologically. No significant difference was apparent among the three groups. The rat aorta wall structure was not destroyed by botulinum toxin (Fig. 4).

4. Discussion

We used noradrenaline and KCl to induce spasm in the arterial rings. In vascular smooth muscle cells, these molecules activate phospholipase C, thereby increasing cellular levels of inositol trisphosphate and 1,2-diacylglycerol. They induce mobilization of Ca from both intra- and extracellular pools to produce a biphasic increase in cytoplasmic free Ca concentration. A rise in Ca activates calmodulin and protein kinase C, both of which phosphorylate myosin light chains. These events lead to constriction in aortic smooth muscle [7]. Vasoconstrictors such as vasopressin, angiotensin II, endothelin-1, and Ca all work in the same way to constrict smooth muscle [8].

Fig. 2. The effectiveness of botulinum toxin in the presence of noradrenaline. Average maximum tension over time in each group of six rings treated with various doses of botulinum toxin is shown on the left (a). Average maximum tension is shown on the right (b).

Phenoxybenzamine irreversibly antagonizes the intracellular protein calmodulin. However, phenoxybenzamine failed to respond to noradrenaline but did respond to vasopressin, angiotensin II, endothelin-1, and KCl [6]. Glyceryl trinitrate increases smooth muscle nitric oxide, which inhibits receptor-stimulated calcium release from intracellular stores and reduces calcium influx by hyperpolarizing the cell membrane or inhibiting the voltage-gated calcium channel [9]. In addition, nitric oxide promotes calcium reuptake into the stores and calcium extrusion, as well as accelerating myosin light chain phosphorylation [9]. This effectively means that glyceryl trinitrate opposes both agonist-mediated contraction and subsequent sensitization caused by the activation of Rho kinase. Papaverine, acting predominantly as a type III phosphodiesterase inhibitor, raises intracellular cyclic adenosine monophosphate, leading to activation of protein kinase A and inhibiting many of the processes antagonized by nitric oxide [9]. Phenoxybenzamine, glyceryl trinitrate, papaverine, phosphodiesterase inhibitors, and calcium channel blockers inhibit the above-mentioned signaling pathways, causing a rise in intracellular calcium, which leads to contraction.

Botulinum toxin has seven serologically distinct types, designated A through G. The mechanism of botulinum toxin type C is well understood. Guanosine 5'-O-thiotriphosphate (GTPγS) dose-dependently enhances Ca(2+) -induced, wortmannin-sensitive phosphorylation of 20 kDa myosin light chain (MLC20). GTPγS does not potentiate thiphosphorylation of MLC20 but does inhibit its dephosphorylation. Pretreatment with botulinum toxin type C, which specifically ADP-ribosylates and inactivates the Rho family of the small molecular weight G proteins, completely abolishes the effects of GTPγS. These results indicate that botulinum toxin type C prevents phosphorylation of myosin light chains in aortic smooth muscle cells, thereby inhibiting aortic smooth muscle constriction [10].

Bordatella pertussis and cholera vibrio are other microbes known to cause ADP ribosylation [11, 12]. The vasodilata-
tion mechanism of botulinum toxin type C by ADP ribosylation not only prevents the phosphorylation of the myosin light chain by inhibiting Ca\(^{2+}\) rise in the cell, but also directly promotes dephosphorylation of the myosin light chain. The mechanism of action differs from that of the other vasodilators. In addition, by promoting dephosphorylation of the myosin light chain directly, botulinum toxin type C allows relaxation of vascular smooth muscle even after it has already constricted. Hence, botulinum toxin type C may help expand constricted arterial grafts as well as preventing arterial graft spasm.

Botulinum toxin type C had a longer-lasting effect than papaverine. Therefore, we would recommend that papaverine should not be used as the sole agent in the prevention of arterial graft spasm after coronary artery bypass graft surgery. Similarly, vasodepressors that reduce Ca\(^{2+}\) density in the cell (such as TNG and phenoxybenzamine) will be inadequate when used alone for the prevention of arterial graft spasm. Spasm has been recorded in 4–10% of radial artery grafts immediately after operation and the rate may even be higher as non-acute cases of spasm may go undetected [13].

It seems that arterial graft spasm can be effectively prevented with the concurrent use of conventional vasodepressors and those with totally different mechanisms such as botulinum toxin type C. In other words, by transiently reducing Ca\(^{2+}\) density in the cell using short-acting agents such as phenoxybenzamine, glyceryl trinitrate, papaverine, phosphodiesterase inhibitor, or calcium channel blockers, phosphorylation of myosin light chains is controlled, and dephosphorylation of myosin light chain by long-lived botulinum toxin type C is promoted. Together, these two mechanisms can produce long lasting and effective prevention of arterial graft spasm.

We used botulinum toxin type C in this experiment. Botulinum toxin types C and D are toxic for animals and birds, but not toxic for humans [14]. Only one case of infant human botulism caused by botulinum toxin type C has been reported [15]. Rings treated with botulinum toxin type C maintained viability of the vascular smooth muscle and retained their wall structure on microscopy. Therefore, botulinum toxin type C appears likely to be safe.

Botulinum toxin type C differs from papaverine in its action mechanism, having a longer preventative effect on arterial graft spasm. It may be effective for the prevention of myocardial infarction secondary to arterial graft occlusion after coronary artery bypass surgery. Next time we plan to perform this study in human radial arteries instead of rat aorta.

5. Conclusion

Botulinum toxin type C could prevent arterial graft spasm in vitro, and had a longer preventative effect than papaverine. Moreover, botulinum toxin type C had no toxic effect on the arterial structure.

References