Effects of Recombinant Nitrophorin-2 Nitric Oxide Complex on Vascular Smooth Muscle

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Received February 22, 1999; Accepted April 14, 1999

Nitrophorin-2, isolated from the salivary gland of the blood-sucking insect *Rhodnius prolixus*, is a nitric oxide (NO) binding protein. We investigated the effects of recombinant nitrophorin-2 NO complex on vascular smooth muscle. The course of relaxation was relative to released NO from recombinant nitrophorin-2 NO complex. Our data suggested nitrophorin-2 was tightly adhesive to the membranes to transport NO into the cell during the insect sting.

Key words: cGMP; hemoprotein; nitric oxide; *Rhodnius prolixus*; vasodilator

The blood-sucking bug, *Rhodnius prolixus*, is known to have a vector of Trypanosome cruzi, which causes American trypanosomiasis (Chagas’ disease). Nitrophorin-2 (NP-2) is a hemoprotein to regulate blood pressure, and mainly acts as vasodilator, which facilitates blood feeding while the insect suck the host blood. The effect of NO on smooth muscle relaxant is related to an increase in cGMP production. In this report, we show that recombinant NP-2 (rNP-2) NO complex produces a potent vasodilator effect associated with an increase in the cGMP level of the rabbit thoracic aorta from which the endothelium was completely removed.

The overexpression and purification of rNP-2 were done as described previously. The Soret band absorption was shifted from λmax at 400 nm to 420 nm, indicating the formation of rNP-2 NO complex, when NO was added to rNP-2 under anaerobic conditions (Fig. 1). By purging with He gas, the absorption of rNP-2 NO complex finally returned to that of the unliganded state (data not shown), which showed rNP-2 could bind NO reversibly as reported for salivary gland hemoprotein.

Free NO of rNP-2 NO complex solution was removed through Sephadex G-25 column equilibrated elution buffer (50 mM potassium phosphate buffer, pH 6.0). The cGMP concentration of the strips was measured by the binding assay described by Murad et al. As shown in Fig. 2, the intracellular cGMP levels increased by rNP-2 NO complex, as well as stimulation of free NO and FK409 (a chemical synthetic reagent, Dojindo Laboratories, Japan) which acts as an NO donor.

The kinetics of NO binding and the removal reaction of rNP-2 was previously reported. The reaction was a two-step consecutive NO binding process. The initial step of the reaction is the first NO binding to the heme site and it induces the slow conformational change of the heme site and/or whole conformational changes of the rNP-2 NO complex. Vasodilation activity of NP-2

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Abbreviations: He, helium; NO, nitric oxide; rNP-2, recombinant nitrophorin-2
Nitrophorin-2 Acts as the Vasodilator

A

90 mM KCl 1μM FK409
0.8 μM rNP-2 NO Complex
5 μM Free NO

B

Relaxation (%)

-Log [Stimulant] M

Fig. 3. The Vasodilatation Effects of rNP-2 NO Complex, FK409, and Free NO on the 90 mM K⁺ Induced Contraction in Rabbit Thoracic Aorta.

(A) Typical responses of FK409, rNP-2 NO complex, and free NO, respectively. (B) Dose-response curves of rNP-2 NO complex (○), FK409 (●), and free NO (▲). In contraction-relaxation curves were expressed as % inhibition of 90 mM K⁺ induced contraction, which defined as 100%, and computer fitted to a following logistics equation: Y = M×Dᵖ/(K⁰ + D). Where M is the maximum effect of each reagent, D is reagent concentration, K is EC₅₀ value of each reagent and p is the slope parameter.

depends on NO bound to the heme moiety in molecular NP-2. An isometric change in the strip tension was recorded and analyzed using a Mac-Lab (hardware with Chart version 3.2 software). In the strip contracted with 90 mM K⁺, a depolarization-induced contraction, rNP-2 NO complex induced relaxation in a concentration-dependent manner (Fig. 3). The course of relaxation induced by rNP-2 NO complex was rapidly and biphasic (Fig. 3(A)). The relaxation was significantly attenuated in the presence of methyl blue (data not shown). Figures 1, 2, and 3 indicated that rNP-2 produces its physiological action via spontaneously released NO from rNP-2 NO complex. The peak physiological concentration of NO for signaling purposes is believed to be 100-500 nM. Table 1 shows that the EC₅₀ value of rNP-2 NO complex is within the physiological range and close to the apparent NO dissociation constant of rNP-2, K₅ = 315 ± 56 nM at 37°C in pH 7.4. Interestingly, the contraction-relaxation curve of rNP-2 NO complex appeared to be conspicuously sigmoid (Fig. 3(B)). Although the rNP-2 NO complex-induced relaxation pattern is similar to that of free NO, the maximum relaxation continues (Fig. 3(A)). These data suggested that the properties of NP-2 might make it tightly adhesive to the vascular smooth muscle membranes to transport NO. The explanation of this conclusion is the following: before the stylo of R. prolixus injects the host vein, NP-2 NO complex is secreted from the salivary gland of the insect, and then it releases NO from the NP-2 NO complex, facilitating stinging and blood feeding by functioning as a vesicular smooth muscle relaxant. Furthermore, the insect injects the protein into the vessel during feeding. The efficiency of relaxation continues. On the other hand, relaxation activity induced by free NO is markedly attenuated in low concentrations. Because, free NO is very unstable and susceptible to oxidation, the half-life of free NO is 6.41 sec in O₂ saturated physiological buffer. The results have implications as regards the relaxation by the action of free NO, which require NO-donor in vivo such as hemoglobin.

The calcium dependent potassium channel is activated by NO, leading to hyperpolarization and reduction of membrane excitability. To clarify the relationship between rNP-2 NO complex activities and vascular relaxation more precisely, the interaction between ion channel(s) and NO should be investigated.

Acknowledgments

The authors are grateful to Dr. N. Miyake of Mic University for his excellent technical assistance.

References

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Table 1. Effects of FK409, rNP-2 NO Complex, and free NO on the Contractile Responses of Rabbit Aorta

<table>
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<tr>
<th>Stimulants</th>
<th>EC₅₀ (μM)</th>
<th>pseudo Hill coefficient</th>
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<tbody>
<tr>
<td>FK409</td>
<td>16±2.3</td>
<td>1.07±0.11</td>
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<tr>
<td>rNP-2 NO Complex</td>
<td>82±5.0</td>
<td>1.73±0.88</td>
</tr>
<tr>
<td>Free NO</td>
<td>250±20</td>
<td>0.67±0.16</td>
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Values indicated the means ± S.D.


