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Activation of particulate guanylyl cyclase by *Vibrio vulnificus* hemolysin

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Abstract

Recently we reported that *Vibrio vulnificus* hemolysin, an exotoxin produced by *V. vulnificus*, dilates rat thoracic aorta via elevated cGMP levels without affecting nitric oxide synthase. We investigated the mechanism further by observing the guanylyl cyclase activities in cytosolic, membrane, unfractionated, or reconstituted preparations. Hemolysin did not activate guanylyl cyclase in the membrane or cytosolic fraction, while it activated guanylyl cyclase in unfractionated or reconstituted preparation. The increased activity was not inhibited by the HS-142-1, a microbial polysaccharide which antagonizes atrial natriuretic peptide receptor, or 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase inhibitor. However, it was attenuated by 6-(phenylamino)-5,8-quinolinedione (LY 83,583), which inhibits the catalytic domain of both guanylyl cyclases, and by cholesterol, which blocks hemolysin-incorporation into the membrane. Removing ATP, a cofactor of particulate guanylyl cyclase, attenuated the activation and ATPγS, a non-phosphorylating analog, restored it. These results suggest that *V. vulnificus* hemolysin activates particulate guanylyl cyclase via hemolysin incorporation into the vascular smooth muscle cell membrane in cooperation with certain unidentified cytosolic component(s).

Keywords: (*Vibrio vulnificus*); Hemolysin; Vasodilatation; Guanylyl cyclase; (Rat)

1. Introduction

Vibrio vulnificus is a unique estuarine bacterium causing septicemia in susceptible subjects when they ingest contaminated seafood such as raw oysters. This Gram-negative bacterium leads the victims to hypotensive septic shock within 2 or 3 days after the onset, resulting in a high mortality, over 50% (Blake et al., 1979; Park et al., 1991). It is well known that lipopolysaccharide (endotoxin) of Gram-negative pathogenic bacteria stimulates various cells to produce a large amount of nitric oxide (NO) which serves as a primary mediator of hypotension in septic shock (Moncada et al., 1991). However, hemodynamic changes caused by bacterial proteinaceous exotoxins are not well documented.

Recently, we observed that *V. vulnificus* hemolysin, which is one of the most potent exotoxins produced by *V. vulnificus* (Kreger and Lockwood, 1981) and is known to mediate the acute cytotoxic effect (Kothary and Kreger, 1987), induces hypotension and vasodilatation. Previously we suggested that hemolysin directly elevates tissue cGMP, leading to vasodilatation, without any involvement of either NO/NO synthase or endothelium (Kook et al., 1996). However, it is not clear where the guanylyl cyclase ultimately responsible for the elevated cGMP is located. Therefore, in this present study we tried to locate the guanylyl cyclase by determining the effects of hemolysin on guanylyl cyclase activity in membrane and cytosolic fractions, as well as in unfractionated or reconstituted preparations.

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2. Methods

2.1. Aorta preparation

All procedures was carried out under approval of the Ethics Committee in the Research Institute of Medical Sciences of Chonnam University Medical School. Sprague Dawley rats (180–300 g) of either sex were killed by decapitation. The aorta was then excised, the lumen was opened longitudinally, and the aorta was cut into small pieces (approximately 5×7 mm).

2.2. Guanylyl cyclase activity

Aorta preparations were pooled and homogenized with a Polytron homogenizer (5 strokes, each stroke for 15 s with 20 s intervals) in an ice-cold homogenization buffer (50 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose). The homogenates were centrifuged at $1000 \times g$ for 15 min at 4°C and the supernatants were collected. In some experiments, this preparation was diluted to 1 μg/μl of protein and used as the *unfractionated preparation*. The preparation was fractionated further by centrifuging at $100{,}000 \times g$ for 1 h at 4°C. The resulting supernatant was collected and used as the *cytosolic fraction*. The pellet was resuspended by ultrasonication in membrane-resuspension buffer (50 mM Tris–HCl, pH 7.6 containing 1 mM EDTA) and used as the *membrane fraction*. In some experiments, the pellet was resuspended in the cytosolic fraction instead of membrane-resuspension buffer, and was used as the *reconstituted preparation*.

Particulate guanylyl cyclase activity was assayed by the method of Winquist et al. (1984) with slight modifications. Working solution, 90 μl (50 mM Tris–HCl, pH 7.6, containing 15 mM phosphocreatine, 20 μg/ml creatine phosphokinase, 2 mM 3-isobutyl-1-methylxanthine, and 1 mM ATP), was warmed to 37°C. The test agents and the membrane fraction containing 15–20 μg of protein were added in succession. The reaction was started by adding $MgCl_2$ and GTP to final concentrations of 4 and 1 mM, respectively. After 2.5 min, the reaction was stopped with cold stopping solution containing 50 mM sodium acetate, pH 4.0, and centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was collected and the cGMP contents were assayed as described previously (Steiner et al., 1972).

Soluble guanylyl cyclase activity was measured according to the method of Rapoport and Murad (1988) slightly modified, in that ATP was omitted from the working solution used for the membrane fraction. The amount of soluble protein was 3–6 μg.

In the experiments measuring guanylyl cyclase activity in unfractionated or reconstituted preparations, the working solution for particulate guanylyl cyclase activity was used except for the ATP-free experiment where ATP was omitted. To confirm whether ATP serves as a cofactor in hemolysin-induced guanylyl cyclase activation in the same manner as it does in the activation of atrial natriuretic peptide (ANP)-induced particulate guanylyl cyclase, 1 mM ATPγS was substituted for ATP in the working solution.

2.3. Hemolysin incorporation into cell membrane

To determine the optimal cholesterol concentration to inhibit membrane incorporation of hemolysin, hemolysin-induced hemolysis, known to be mediated by hemolysin incorporation into the red blood cell membrane (Kim et al., 1993), was assayed in the presence of different cholesterol concentrations as follows. Hemolysin, 10 HU (hemolytic units), was preincubated with 0 to 40 μg/ml cholesterol for 30 min at 37°C, and 1% human red blood cell suspension was added to the incubated mixtures and incubated further for 1 h. Hemolysis was determined by measuring the optical density at 540 nm. For the determination of optimal incubation time, 10 HU/ml hemolysin was mixed with 5 μg/ml cholesterol and aliquots of the mixture were taken successively at 5 min intervals to determine residual hemolytic activity. Cholesterol (≥ 5 μg/ml) almost completely inhibited ($> 95\%$) hemolysis by 10 HU/ml hemolysin. It took at least 30 min to reach over 90% of the hemolysis-inhibiting effect of 5 μg/ml cholesterol. Therefore, 1 h was adopted as the optimal incubation time. The cholesterol concentration was varied from 0.625 to 5 μg/ml. Simultaneous incubation of cholesterol and hemolysin with red blood cells produced no detectable inhibition of hemolysis. Possible effects of ethanol, the vehicle for cholesterol, were minimized by limiting the final concentration in the working solution to less than 0.1%. At this concentration, ethanol did not affect the guanylyl cyclase activation induced by ANP or sodium nitroprusside.

2.4. Materials

Hemolysin was obtained from the culture supernatant of *V. vulnificus* C7184 strain and purified by gel chromatography, as described previously (Gray and Kreger, 1985; Kook et al., 1996). Absence of lipopolysaccharide contamination in the hemolysin preparation was confirmed by the Limulus amebocyte lysate (E-TOXATE®) test. Sodium nitroprusside, ANP, EDTA, phenylmethylsulfonyl fluoride, creatine phosphate, creatine phosphokinase, 3-isobutyl-1-methylxanthine, cholesterol, ATP, ATPγS (Adenosine 5′-*O*-(3-thiotriphosphate), tetralithium salt), GTP, magnesium chloride ($MgCl_2$), E-TOXATE® were obtained from Sigma (St. Louis, MO), 6-(phenylamino)-5,8-quinolinedione (LY 83,583) from Eli Lilly (Indianapolis, IN), 1*H*-[1,2,4]oxadiazolo[4,3*a*]quinoxalin-1-one (ODQ) from BioMol (Plymouth, PA), and cGMP RIA kit from DuPont (NEN; Boston, MA). HS-142-1 was kindly supplied by Dr. S. Nakanishi, Tokyo Research Laboratories, Kyowa Hakko Kogyo, Japan.

2.5. Statistics

Statistical significance was evaluated with Student's unpaired *t*-test. Significance was accepted at the 0.05 level of probability.

3. Results

3.1. Effects of hemolysin on guanylyl cyclase activity in membrane, cytosolic, or unfractionated preparations

Hemolysin did not affect particulate or soluble guanylyl cyclase activity (Fig. 1). However, 0.3–30 HU/ml hemolysin concentration dependently increased the guanylyl cyclase activity in both unfractionated and reconstituted preparations (Fig. 2). As positive controls, the effects of ANP on particulate and soluble guanylyl cyclase activities were determined. ANP stimulated particulate guanylyl cyclase activity (Fig. 1), while it did not affect soluble guanylyl cyclase activity. Stimulation was dose-dependent with an EC_{50} of 3.1×10^{-8} M (data not shown). Contamination of soluble guanylyl cyclase in the membrane fraction was ruled out by the absence of a response to sodium nitroprusside. The EC_{50} value against sodium nitroprusside for soluble guanylyl cyclase activity was 9.6×10^{-5} M, and ANP did not increase soluble guanylyl cyclase activity (data not shown).

3.2. Effects of guanylyl cyclase inhibitors on hemolysin-induced activation of guanylyl cyclase activity

HS-142-1, a microbial polysaccharide which antagonizes ANP (Morishita et al., 1991), did not influence the increase in guanylyl cyclase activity induced by 30 HU/ml hemolysin, while it attenuated the increase in guanylyl cyclase activity induced by 10^{-6} M ANP (Fig. 3A). ODQ, a specific soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995; Sobey and Faraci, 1997), also did not affect the activation of guanylyl cyclase induced by either hemolysin or ANP, whereas it reduced the increase induced by 10^{-4} M sodium nitroprusside (Fig. 3B).

LY 83,583, a non-specific inhibitor of the catalytic domain of both particulate and soluble guanylyl cyclase (O'Donnel and Owen, 1986; Ijioma et al., 1995), abolished the increase in guanylyl cyclase activity induced by

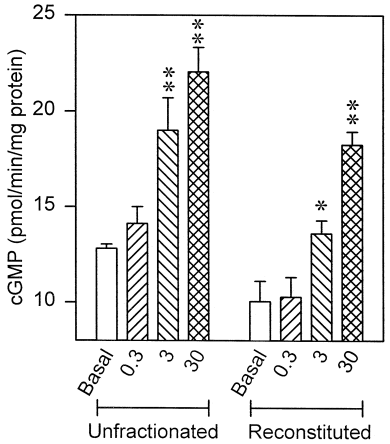

Fig. 2. Effects of hemolysin on guanylyl cyclase activity in unfractionated and reconstituted preparations. Each bar represents the mean ± SEM from 8 experiments. The hemolysin concentration (abscissa) varied from 0.3–30 HU/ml. Asterisks indicate significant differences from basal (* $P < 0.05$; ** $P < 0.01$).

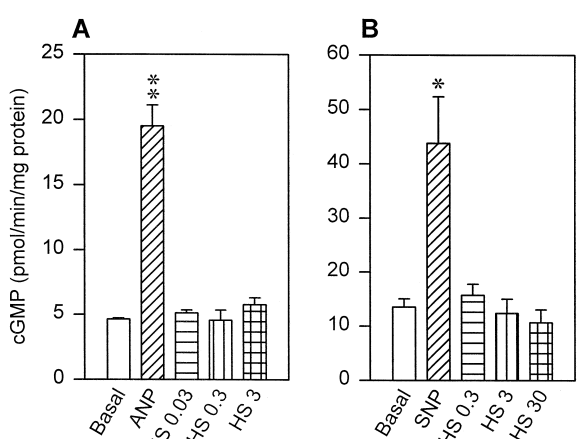

Fig. 1. Effects of hemolysin on particulate and soluble guanylyl cyclase activity. (A). 10^{-7} M atrial natriuretic peptide (ANP) or hemolysin (HS; 0.03–3 HU/ml) at the indicated concentrations was added to the membrane fraction. (B). 10^{-4} M sodium nitroprusside (SNP) or hemolysin (0.3–30 HU/ml) was added to the cytosolic fraction. Each bar represents the mean ± SEM from 4 experiments. Asterisks indicate a significant difference from basal (* $P < 0.05$; ** $P < 0.01$).

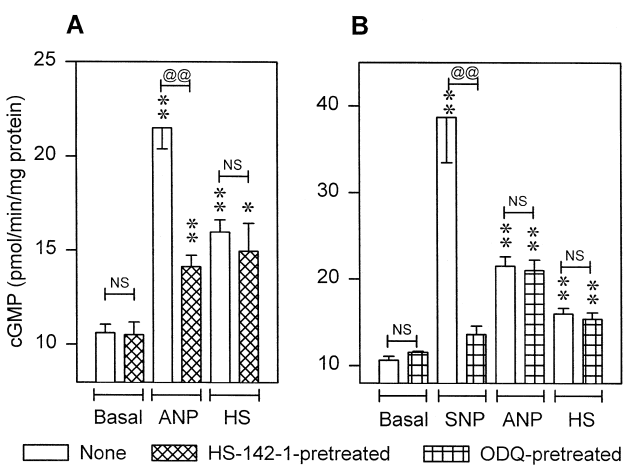

Fig. 3. Effects of HS-142-1 and ODQ on hemolysin-induced increases in guanylyl cyclase activity in unfractionated preparations. (A). 3 μg/ml HS-142-1, a non-peptide ANP receptor antagonist, was added prior to 10^{-6} M ANP or 30 HU/ml hemolysin (HS). (B). 10^{-5} M ODQ was added prior to 10^{-4} M sodium nitroprusside (SNP), 10^{-6} M ANP, or 30 HU/ml hemolysin. Each bar represents the mean ± SEM from 6–10 experiments. Asterisks and @ indicate significant differences (* $P < 0.05$; **, @@ $P < 0.01$). NS: not significant.

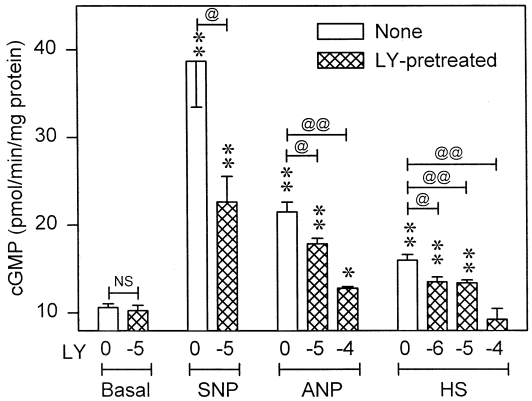

Fig. 4. Effects of LY 83,583 (LY) on hemolysin-induced increase in guanylyl cyclase activity in unfractionated preparations. 10^{-6} to 10^{-4} M LY was added prior to sodium nitroprusside (SNP, 10^{-4} M), ANP (10^{-6} M) or hemolysin (HS, 30 mHU/ml). Numerals below abscissa are the log molar concentrations of LY. Each bar represents the mean ± SEM from 6–14 experiments. Asterisks and @ indicate significant differences (∗, @ $P < 0.05$; ∗∗, @@ $P < 0.01$). NS: not significant.

hemolysin as well as that due to sodium nitroprusside or ANP (Fig. 4).

3.3. Effects of removing ATP and substituting ATPγS for ATP on hemolysin-induced increase in guanylyl cyclase activity

Removing ATP, a cofactor of particulate guanylyl cyclase (Kurose et al., 1987), attenuated the increase in guanylyl cyclase activity induced by hemolysin or ANP, whereas it did not affect the sodium nitroprusside-induced guanylyl cyclase activation (Fig. 5). Adding ATPγS, a non-phosphorylating ATP analog, to ATP-free working solution restored the activity induced by hemolysin or ANP (Fig. 5).

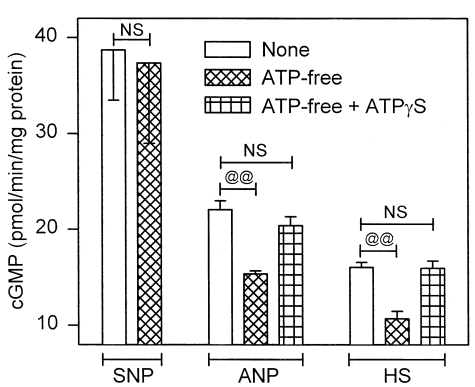

Fig. 5. Effects of elimination of ATP from working solution and substitution of ATPγS for ATP on hemolysin-induced increase in guanylyl cyclase activity. Sodium nitroprusside (SNP, 10^{-4} M), ANP (10^{-6} M) or hemolysin (HS, 30 mHU/ml) was added to normal or ATP-subtracted (ATP-free) or ATPγS-substituted (ATP-free + ATPγS) working solution. Each bar represents the mean ± SEM from 4–8 experiments. @@ indicates significant differences ($P < 0.01$). NS: not significant. See Section 2 for detailed explanation.

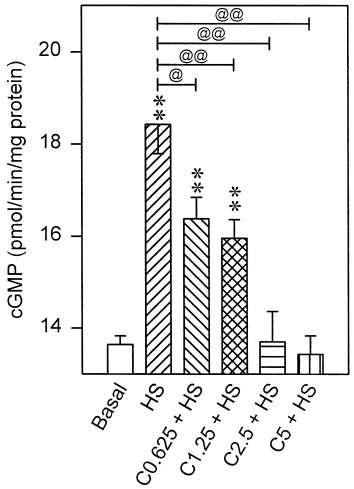

Fig. 6. Effects of cholesterol on hemolysin-induced increases in guanylyl cyclase activity. Hemolysin (30 mHU/ml) was preincubated with 0.625 to 5 μg/ml cholesterol (C) or vehicle and added to the working solution as described in methods. Each bar represents the mean ± SEM from 6 experiments. Asterisks and @ indicate significant differences (@ $P < 0.05$; ∗∗, @@ $P < 0.01$).

3.4. Effects of cholesterol-preincubated hemolysin on guanylyl cyclase activity

Preincubation of hemolysin with cholesterol attenuated the hemolysin-induced increase in guanylyl cyclase activity in a dose-dependent fashion (Fig. 6), while cholesterol itself did not significantly influence basal guanylyl cyclase activity (data not shown).

4. Discussion

We had observed that neither removing endothelium nor treating with NO synthase inhibitors, such as N^{ω}-nitro-L-arginine methyl ester or aminoguanidine, affected the hemolysin-induced vasodilatation, demonstrating that NO, which is an important hypotensive mediator in Gram-negative septic shock (Moncada and Higgs, 1993), is not involved in the *V. vulnificus* hemolysin-induced vasodilatation (Kook et al., 1996). In agreement with our previous findings, we observed that treatment with hemolysin, in contrast to endotoxin, did not affect the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (data not shown), confirming that NO synthase is not involved in the hemolysin-induced vasodilatation.

The present study demonstrated that hemolysin did not increase guanylyl cyclase activity in either membrane or cytosolic fraction, suggesting that hemolysin does not directly activate particulate or soluble guanylyl cyclase. However, since the activity in the unfractionated preparation, as well as the reconstituted preparation, was increased dose dependently by hemolysin treatment, we suggest that the co-existence of both fractions is necessary for guanylyl

cyclase activation and, thus, for the increase in cGMP. While the nature of the interaction between the membrane and cytosolic components is not known, the ANP binding site of particulate guanylyl cyclase does not appear to be involved in hemolysin-induced guanylyl cyclase activation, since the increase in guanylyl cyclase activity was not influenced by HS-142-1, an ANP receptor antagonist (Morishita et al., 1991). In addition, it appears that soluble guanylyl cyclase is not ultimately responsible for the increase in cGMP, since the increase in guanylyl cyclase activity was not affected by ODQ, a specific soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995; Sobey and Faraci, 1997).

In agreement with the previous reports that LY 83,583 inhibits particulate as well as soluble guanylyl cyclase (O'Donnel and Owen, 1986; Ijioma et al., 1995), we also observed that it inhibited the increase in guanylyl cyclase activation induced by ANP as well as by sodium nitroprusside, suggesting that LY 83,583 affects a common catalytic pathway of both enzymes, irrespective of the ANP binding site of particulate guanylyl cyclase. In the present study, the hemolysin-induced guanylyl cyclase activation was inhibited by the pretreatment with LY 83,583 in a dose-dependent fashion, raising the possibility of the involvement of catalytic domains of particulate guanylyl cyclase.

It is known that, in contrast to soluble guanylyl cyclase, ATP serves as cofactor of particulate guanylyl cyclase (Kurose et al., 1987) and that its binding to the kinase domain is necessary for full activation of the enzyme (Chinkers and Garbers, 1989). It is further suggested that ATP allosterically modulates particulate guanylyl cyclase, since its non-hydrolyzable analog, ATPγS, also activates the enzyme (Kurose et al., 1987; Shigematsu et al., 1994). As expected, we observed that removing ATP from the working solution did not influence the guanylyl cyclase activation induced by sodium nitroprusside. Interestingly, however, removing ATP inhibited the hemolysin-induced guanylyl cyclase activation as it did the ANP-induced activation. Using ATPγS in place of ATP restored the activity induced by HS as well as ANP. These results indicate that particulate guanylyl cyclase is the ultimate source of cGMP, for which ATP serves as a simple cofactor rather than a complex mediator in the same manner as was observed for ANP-induced activation.

Membrane perturbation by pore-forming cytolysins results in various pathophysiological effects on the circulatory system (Bhakdi et al., 1994). Thiol-activated cytolysins that form large pores, such as streptolysin O and cereolysin, were reported to activate rat lung particulate guanylyl cyclase (Lad, 1980), while staphylococcal α-toxin produces a vascular pressor response mediated by thromboxanes (Seeger et al., 1984). *Escherichia coli* α-hemolysin was reported to stimulate endothelial cells to produce NO that might be responsible for the *E. coli* septic shock (Grimminger et al., 1997). *V. vulnificus* hemolysin can also be classified as a pore-forming bacterial cytolysin (Bhakdi et al., 1996), since it is well known that the hemolysin molecule, like staphylococcal α-hemolysin (Fussle et al., 1981), oligomerizes in the cytoplasmic membrane to form small pores which allow the passage of monovalent cations such as K^+ and Na^+ rather than divalent cations, leading to colloid-osmotic hemolysis (Kim et al., 1993). Thus, we postulate that incorporation of the hemolysin into the cell membrane, resulting in alteration of the membrane, is a prerequisite for the activation of particulate guanylyl cyclase. This postulate is supported by our observations that cholesterol, which was previously shown to bind to hydrophobic sites of hemolysin and to inhibit hemolysis by interfering with the incorporation of hemolysin into the membrane (Kim et al., 1993), significantly attenuated the hemolysin-induced increase in guanylyl cyclase activity.

Schulkes et al. (1992) reported that particulate guanylyl cyclase of *Dictyostelium discoideum* requires a soluble factor, suggested to be a protein, because that the addition of the cytosolic fraction to washed membranes restored the lost activity of the enzyme. Although their suggestions could not satisfactorily account for the biochemical mechanism in our eukaryotic vascular smooth muscle cell, we speculate that a certain diffusible molecule(s) in the cytosol may carry a signal to particulate guanylyl cyclase, since the activation of guanylyl cyclase was observed only in the preparations where both membrane and cytosolic fractions exist together.

In summary, the present results demonstrate that *V. vulnificus* hemolysin activates particulate guanylyl cyclase via hemolysin incorporation into the vascular smooth muscle cell membrane in cooperation with certain unidentified cytosolic component(s). These findings present an alternative pathway resulting in vasodilatation induced by *V. vulnificus* hemolysin through its membrane-incorporating properties and may clarify the mechanism underlying the septic shock these dreadful bacteria bring about.

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