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Effects of Kanagawa Hemolysin on Blood Pressure and Arterial Tone in Rats

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Kanagawa hemolysin (KH), an exotoxin produced from Kanagawa phenomenon-positive *Vibrio parahemolyticus*, has been shown to possess various biological activities including hemolysis, enterotoxicity, cytotoxicity, and cardiotoxicity. The aim of this study was to investigate the effect of KH on the cardiovascular system and its mechanism, employing in vivo and in vitro experiments of the rat. Intracerebroventricular (icv) administration of 100 mHU KH produced a marked and continuous pressor effect (icv KH-pressor effect), and the icv pressor effect was not repeatable. However, intravenous (iv) injection of the same dose of KH induced a prominent depressor effect (iv KH-depressor effect). The icv KH-pressor effect was inhibited by acid-denaturation, while the iv KH-depressor effect was not. Simultaneous icv administration of the three agents (ouabain, diltiazem, or bumetanide: 10 μg/kg each) significantly reduced the pressor effect. The icv KH-pressor effect was inhibited by treatment with iv phentolamine or chlorisondamine, but was not affected by iv candesartan. The iv KH-depressor effect was repeatable and was attenuated by treatment with iv NAME or methylene blue. In vitro experiments using isolated thoracic aorta, 10^{-6} M phenylephrine (PE) and 50 mM KCl produced a sustained contraction. In rings contracted with either agents, KH showed relaxant responses in a concentration-dependent fashion and the relaxation (KH-vasorelaxation) was not dependent on the existence of the endothelium. The KH-vasorelaxation in the endothelium-intact rings contracted by PE was abolished by methylene blue treatment. In summary, the present findings suggest that in the icv KH-pressor effect the cation leak-inducing action of KH is implicated, which leads to the increased central sympathetic tone, that the iv KH-depressor effect results from the vasorelaxation via NO-guanylate cyclase system, and that the KH-vasorelaxation is independent of the endothelium and the guanylate cyclase system is involved in it. In conclusion, the mechanism of KH producing the icv pressor effect may not be identical to that of KH producing the iv depressor effect.

Key Words: Kanagawa hemolysin, Pressor effect, Depressor effect, Ion transport, Vasorelaxation, Intracerebroventricular, Aorta

INTRODUCTION

Vibrio parahemolyticus is an enteric pathogen that causes acute gastroenteritis primarily in humans, when consumed raw undercooked seafood (DePaola et al, 1990). The pathogenic effect on humans has been associated with thermostable direct hemolysin (TDH) produced by the strains (Miyamoto et al, 1969). TDH produced by a strain of the pathogen was referred to as Kanagawa phenomenon-positive and was identified by β-type hemolysis on Wagatsuma blood agar medium, and hence it is called Kanagawa hemolysin (KH) (Sakazaki et al, 1968; Takeda, 1982; Kaysner, et al, 1992; Honda & Iida, 1993). KH has shown various biological activities including enterotoxicity, cytotoxicity, cardiotoxicity and hemolytic properties (Cherwonogrodzky & Clark, 1982; Honda & Iida, 1993). Recently, TDH was shown to elicit cytotoxic effects, mainly morphological damages in various cultured cells, including mouse myocardial and melanoma cells (Goshima et al, 1978), FL cells (Sakurai et al, 1976), Rat-1 cells (Tang et al, 1997), as well as in erythrocytes (Huntley & Hall, 1996).

The cytotoxic effects of TDH have been observed with various cells. In studies using erythrocytes, TDH was suggested as a pore-forming protein that induces hemolysis by increasing the cation permeability of the cell membrane (Huntley et al, 1993; Huntley & Hall, 1994). In addition, KH increased extracellular calcium influx in a cultured cell line, Intestine 407 (Tang et al, 1995), and in human red cells (Huntley & Hall, 1996). Naim et al (2001a, b) recently found that monodansylcadaverine, an inhibitor of transglutaminase, could protect Rat-1 cells against cytotoxicity of TDH, and they suggested that TDH can exert its cytotoxity both from outside and inside the cells and kill the cells through apoptosis. However, in spite of a number of

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ABBREVIATIONS: KH, Kanagawa hemolysin; icv, intracerebroventricular; iv, intravenous; PE, phenylephrine; TDH, thermostable direct hemolysin.

studies on KH, the exact mechanism of the cytotoxic action and hemolysis are still poorly understood.

In contrast to numerous *in vitro* studies on KH, very few *in vivo* studies have been undertaken thus far. Early investigators observed only that intravenous TDH produced bradycardia or cessation of spontaneous beating of heart in mice (Takeda et al, 1978) and in rats (Honda et al, 1976), and the inhibitory action on the atrioventricular conduction system has been suggested as its mechanism. And as for the KH effect on the cardiovascular function, no extensive study has been undertaken thus far.

On the other hand, *Vibrio vulnificus* is another estuarine bacterium that produces cytotoxic hemolysin causing septicemia in susceptible patients when they ingest contaminated raw seafood (Blaker et al, 1979; Gray & Kreger, 1985). Recently, our department observed that the hemolysin (VH) produced by *Vibrio vulnificus* caused hypotension by intravenous injection in anesthetized rats and dilated isolated thoracic aorta of the rat, and they suggested that the relaxant response to VH results from activation of guanylate cyclase independent of NO synthase (Kook et al, 1996). Since VH and KH seem to be closely related, VH was used as a reference in elucidating the effect of KH in part of this study.

The aims of the present study were to investigate effects of KH on the cardiovascular function by observing changes of blood pressure elicited by intravenously as well as intracerebroventricularly given KH in the whole rat and influence on the tension in the isolated thoracic aorta.

METHODS

Blood pressure measurements

Male Sprague-Dawley rats weighing 200~300 g were used. The rat was anesthetized with intraperitoneal injection of 1.2 g/kg urethane and 50 mg/kg α-chloralose, and ventilated through a cannula inserted into the trachea. The arterial blood pressure (BP) was continuously measured with a pressure transducer (Gould) in the left carotid artery, and heart rate (HR) was counted with a biotachometer (Grass). All signals were recorded on the polygraph (Grass).

Drug administration

Intravenous (iv) injection was done through a polyethylene tube inserted into the right jugular vein. Intracerebroventricular (icv) administration was infused slowly in a volume less than 10 μl through a polyethylene cannula inserted into the right lateral cerebral ventricle. At the termination of each experiment, 10 μl methylene blue was injected into the ventricle and the correctness of administration was confirmed by dissecting the brain.

Arterial tension experiments

Rats were sacrificed by decapitation and their thoracic aorta was carefully excised. The isolated aorta was placed in cold (4°C) physiological salt solution (PSS) and cleaned of connective and adipose tissues under a stereoscope. Then, the aorta was cut into rings of ~5 mm in width. In some cases, the endothelium was removed by gentle rubbing with an angular metal rod inserted into the lumen of the ring. The completeness of functional removal of the endothelium was ascertained by absence of relaxant response to acetylcholine in the ring contracted with phenylephrine (PE). The ring preparations were mounted in a tissue bath by sliding them over two parallel stainless-steel hooks. The lower hook was fixed on the bottom of the bath and the upper one was suspended from the cantilever of the isometric transducer (Grass FT03), and changes of the tension were recorded on the polygraph. All ring preparations were equilibrated and maintained under the resting tension of 2 g for 2 hrs. The double jacketed tissue bath was connected to a circulator and filled with 5 ml PSS saturated with 95% O_2+5% CO_2 at 37°C (pH=7.4). The composition of PSS was as follows (in mM): 122 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2 KH_2PO_4, 1.18 $MgSO_4$, 15 $NaHCO_3$, 11.5 dextrose, 0.026 EDTA, and 0.12 ascorbic acid. To test tissue viability and reproducibility of contraction amplitude, at the end of the equilibration period the rings were challenged with 35 mM KCl 2~3 times in succession and rinsed with PSS after each challenge.

Heat-denaturation and acid-denaturation of KH

The KH denatured by exposure to heat or strong acid was used in some experiments. The heat-denaturation was done by exposing the KH solution to 100°C for 10 min (Douet et al, 1992). Acid-denatured KH was made as follows. 10 μl of 0.1 N HCl solution was added to 100 μl KH solution, and the solution was neutralized with 10 μl of 0.1 N NaOH solution after it was left at room temperature for 10 min.

Drugs

Kanagawa hemolysin (KH), phenylephrine HCl, diltiazem HCl, ouabain, bumetanide, N_ω-nitro-L-arginine methyl ester (NAME), and sodium nitroprusside were obtained from Sigma, phentolamine methanesulfonate and chlorisondamine from Ciba, and methylene blue from Merck. All drugs were dissolved and diluted in 0.9% saline.

One hemolytic unit (HU) of KH is defined by Sigma as the amount that causes 50% lysis of 1% suspension of human erythrocytes in phosphate-buffered saline, pH 7.0, after 2-hr incubation at 37°C followed by refrigeration for 12~24 hrs at 4°C. VH was obtained from the culture supernatant of Vibrio vulnificus C7184 strain and purified by gel chromatography, as described previously (Gray & Kreger, 1985; Kook et al, 1996). Aliquots of KH and VH prepared in concentration of 100 HU/ml were stored at −22°C until used. Doses of in vivo KH and VH were described as mHU/200g body weight of rat and the doses employed in th study did not produce significant hemolysis (data not shown). In vitro experiments, the final concentrations in tissue bath were described as mHU/ml.

Statistics

Statistical evaluation was done by Student's unpaired *t*-test, and the significance was accepted at the $p<0.05$ level of probability. All the data are presented as mean±SEM.

RESULTS

Pressor effects of icv KH

KH administered icv in doses less than 10 mHU did not

affect the arterial blood pressure (BP) in anesthetized rats, but 30 mHU caused slight increase of BP without any change in heart rate. However, increasing of KH dose to 100 mHU produced a marked and sustained increase of BP and a transient bradycardia. The pressor effect of icv 100 mHU (designated as "icv KH-pressor effect") reached maximal increase of 89±9.7 mmHg (n=16), 10~20 min after the icv injection and then the BP slowly returned to the original level with the lapse of 1~2 hrs (Fig. 1). The KH-induced bradycardia appeared at initial period of the pressor effect and disappeared after 10~20 min even though the marked pressor effect persisted. The bradycardia was abolished by bilateral vagotomy (Fig. 1, 3). When the pressor effect by the first dose of icv KH waned and BP returned to the original level, administration of the same dose of icv KH was repeated. But the pressor effect of the second dose was only less than half the first dose, and the bradycardia was not seen (Fig 1, 3). That is, both pressor and bradycardiac effects of icv KH were not repeatable, indicating that a rapid tachyphylaxis developed. Therefore, in this study, all results of icv KH injection were obtained from single dose of 100 mHU KH in bilaterally vagotomized rats and were statistically analyzed with unpaired *t*-test. On the contrary, hemolysin (VH) produced by *Vibrio vulnificus* did elicit no pressor effect but a marked depressor effect when 10~100 mHU was given icv (Fig. 1).

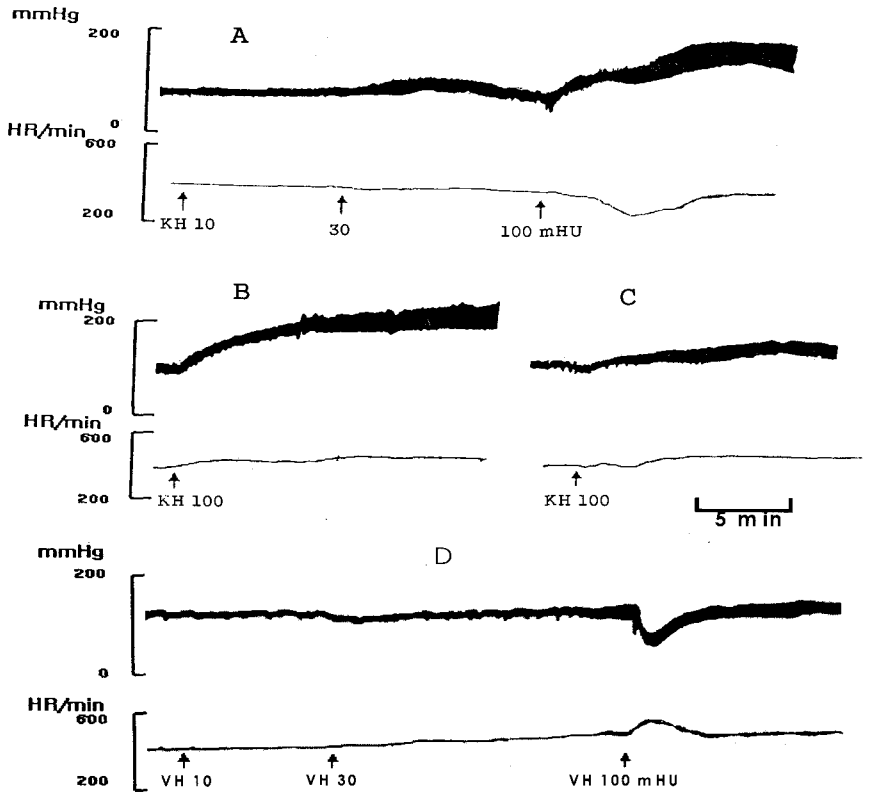

Fig. 1. Effects of icv administration of KH and VH on arterial blood pressure and heart rate in anesthetized rats. At arrows, the indicated doses of agents were icv administered. Upper and lower traces in each panel show changes of blood pressure and heart rate, respectively. A: Effects of icv 10~100 mHU KH in a control rat. B: Effect of 100 mHU KH in a bilaterally vagotomized rat. C: Effect of secondary icv infusion of the same dose of KH 2~3 hrs after the first icv 100 mHU KH. D: Effects of icv 10~100 mHU VH in a rat.

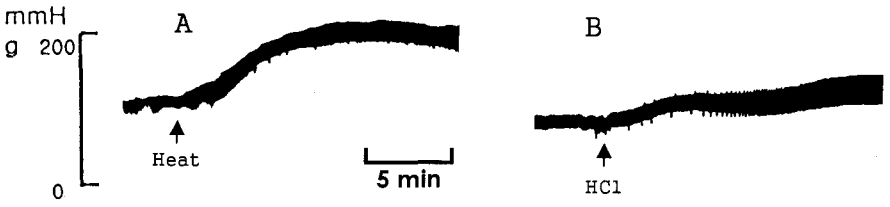

Fig. 2. Effects of icv administration of heat-denatured and HCl-denatured KH on arterial blood pressure in anesthetized rats. At arrows, the indicated KH was icv administered. A: Effect of icv 100 mHU KH denatured by heat. B: Effect of icv 100 mHU KH denatured by HCl.

Effects of heat-denatured and acid-denatured KH: The icv KH-pressor effect was not affected by heat-denaturation at all, whereas acid-denaturation reduced the pressor effect to less than half of the control (Fig. 2, 3).

Effects of ion transport inhibitors given icv on icv KH-pressor effect: The icv treatment with 10 μg/kg ouabain produced slight increase (23±4.7 mmHg, n=4) of BP, but did not affect the icv KH-pressor effect. The icv treatment with 10 μg/kg diltiazem or bumetanide did not produce any changes in both the basal BP and the icv KH-pressor effect (Fig. 4, 5). When the above three drugs were simultaneously administered into the ventricle, the basal BP was significantly increased (28±5.5 mmHg, n=5). Under this condition, the icv KH-pressor effect was markedly attenuated to about half of the control (Fig. 4, 5).

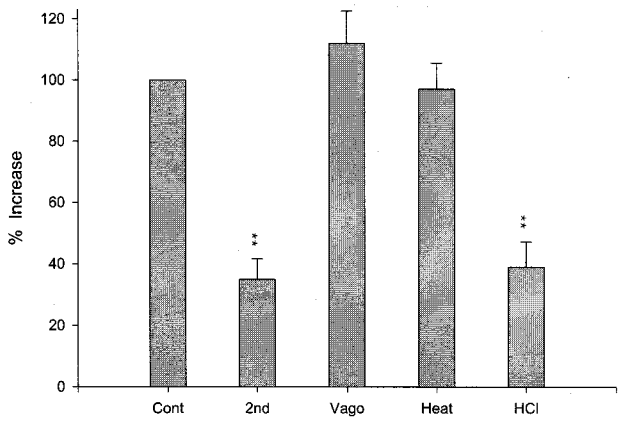

Fig. 3. Effects of icv 100 mHU KH on arterial blood pressure in various conditions. Each column shows mean±SEM from 4~16 rats. Cont: control (100%); 2nd: second injection of icv KH after the first icv KH; Vago: bilaterally vagotomized rat; Heat: icv administration of heat-denatured KH; HCl: icv administration of HCl-danatured KH. **: $p<0.01$ compared with the control.

Effects of iv blockers on icv KH-pressor effect: *Phentolamine*; Iv injection of 2 mg/kg phentolamine produced marked and consistent decrease of BP, and the icv KH-pressor effect was significantly inhibited by the iv phentolamine (Table 1).

Chlorisondamine; 1 mg/kg chlorisondamine, iv, also produced marked and consistent decrease of BP, and the icv KH-pressor effect was markedly inhibited under this condition, (Table 1).

Candesartan; When 10 mg/kg of candesartan was administered iv, the basal BP was slightly decreased, but the icv KH-pressor effect was not affected in its presence (Table 1).

Depressor Effects of iv KH

When KH was intravenously administered, it decreased the basal BP in a dose-dependent fashion in the dose range of 10~100 mHU (Fig. 6). The second dose of iv 100 mHU KH also produced depressor effect unabated, indicating that the depressor effect of iv 100 mHU KH (desinated as "iv KH-depressor effect") was repeatable, in contrast to the icv KH-pressor effect (Fig. 7).

Table 1. Effects of iv treatment with blockers on the icv KH-pressor effect in anesthetized rats

Blockers (Dose)	n	Decrease by blocker	icv KH-pressor effect	
			Before (treatment)	After
Phentolamine (2 mg/kg)	5	31±4.1	89±9.7	43±5.2*
Chlorisondamine (1 mg/kg)	5	39±5.6	89±9.7	55±6.5*
Candesartan (10 mg/kg)	4	12±3.1	89±9.7	83±8.1

Numerals show mean±SEM. *$P<0.01$ compared with before.

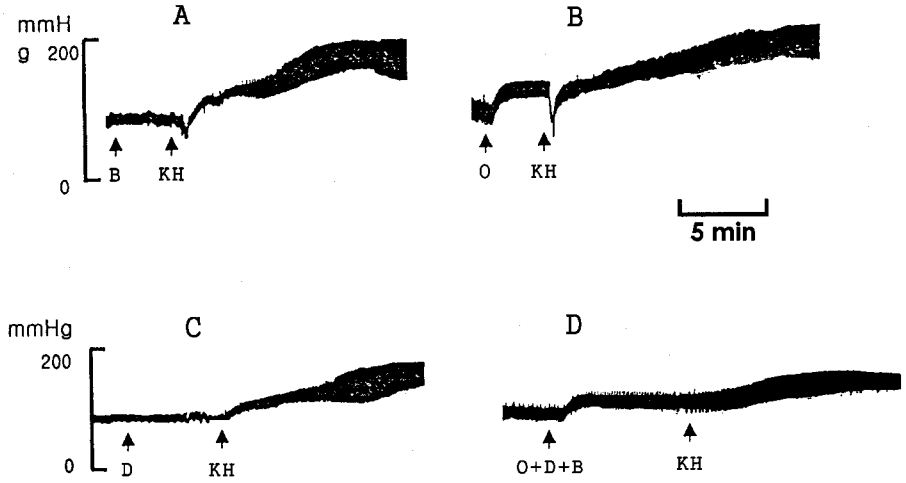

Fig. 4. Effects of ion transport inhibitors given icv on the icv KH pressor effect in anesthetized rats. At arrows, the indicated drugs were icv administered. A, B, C: Effects of icv 10 μg/kg injection of bumetanide (B), ouabain (O) and diltiazem (D), respectively. D: Effect of icv combined injection of three drugs. Other details are in the text.

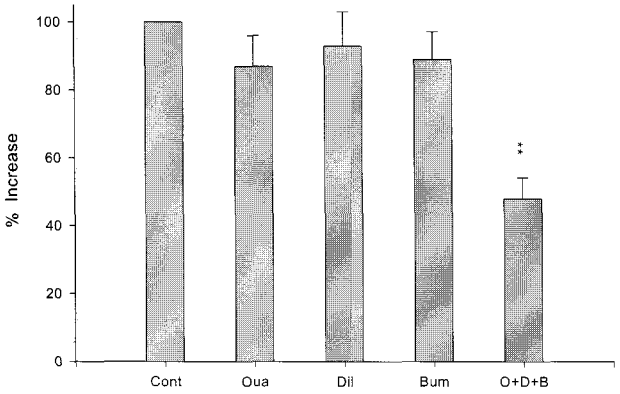

Fig. 5. Effects of ion transport inhibitors given icv on the icv KH-pressor effect in anesthetized rats. Each column shows mean± SEM from 4~16 rats. Cont: control (100%); Oua, Dil and Bum indicate ouabain, diltiazem and bumetanide, respectively, and the dose of each drug is 10 μg/kg each. O+D+B: This column was obtained in rats pretreated simultaneously with three drugs. **: $p<0.01$ compared with the control.

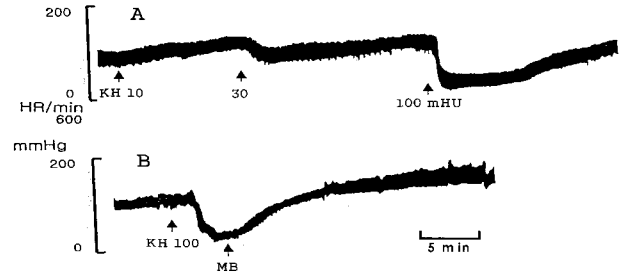

Fig. 6. Effects of iv KH on arterial blood pressure and iv methylene blue on iv KH-depressor effect in anesthetized rats. At arrows, the indicated doses and drugs were given intravenously. A: Effects of iv 10~100 mHU KH in a control rat. B: Effect of additional injection of 3 mg/kg methylene blue on the iv 100 mHU KH depressor effect.

Effects of iv injection of heat-denatured and acid-denatured KH: The iv injection of either heat-denatured or acid-denatured KH (100 mHU) produced depressor effects, not significantly different from the control (Fig. 7). This finding showed a definite discrepancy from the icv KH-pressor effect, which was markedly inhibited by the acid-denaturation (Fig. 2, 3).

Effects of iv blockers on iv KH-depressor effect:

NAME; The basal BP was slightly increased by 10 mg/kg NAME iv, and the iv KH-depressor effect was significantly attenuated by iv NAME pretreatment (Fig. 7).

Methylene blue; The basal BP was slightly increased by 3 mg/kg methylene blue iv, and the iv methylene blue pretreatment markedly inhibited the iv KH-depressor effect (Fig. 7). In another series of experiments, when the order of the administration was reversed, methylene blue immediately reversed the hypotension by iv KH to the hypertensive state (Fig. 6).

Inhibitors of ion transport; When 10 mg/kg each of ouabain, diltiazem, and bumetanide were simultaneously injected intravenously, the basal BP slightly decreased.

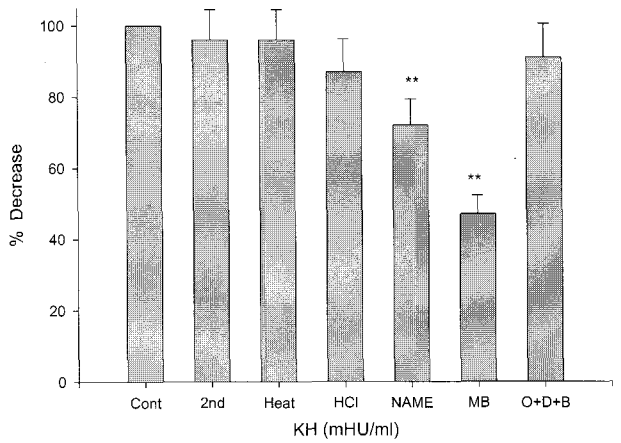

Fig. 7. Effects of various treatments on the iv KH-depressor effect in anesthetized rats. Each column shows mean±SEM from 4~7 rats. Cont: control (100%); 2nd: second iv KH after the first iv KH; Heat: iv injection of heat-denatured KH; HCl: iv injection of HCl-danatured KH. NAME: treatment with iv 10 mg/kg NAME; MB: treatment with iv 3 mg/kg methylene blue; O+D+M: simultaneous iv treatment with 10 μg/kg each of ouabain (O), diltiazem (D) and bumetanide (B). **: $p<0.01$ compared with the control.

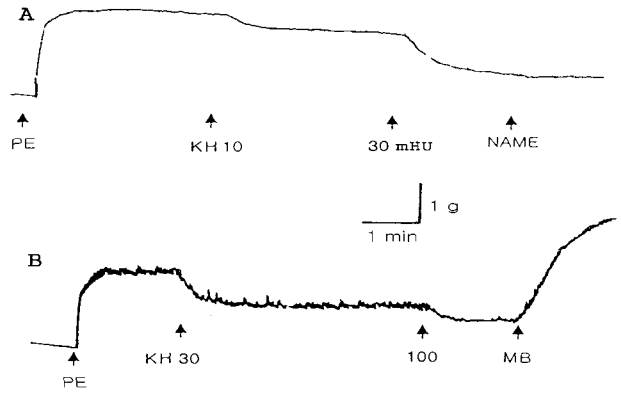

Fig. 8. Concentration-dependent relaxant responses to KH, and effects of additional NAME and methylene blue on the KH-vasorelaxation in the endothelium-intact rings contracted with phenylephrine. At arrows, the indicated drugs and concentrations were added to the bath. PE: 10^{-6} M phenylephrine; NAME: 10^{-4} M NAME; MB: 5×10^{-5} M methylene blue.

Even in the presence of the three drugs, the iv KH-depressor effect was not affected (Fig. 7).

Effects of KH on isolated thoracic aorta

Relaxant effect of KH: *Phenylephrine contraction;* In isolated aortic rings of rats, 10^{-6} M phenylephrine (PE) produced consistent contractile responses regardless of the presence or absence of endothelium, and PE-induced contraction (0.87±0.10 g, n=11) in endothelium-intact rings was smaller than in deendothelized rings (1.33±0.17 g, n=11). However, PE-induced contractions of both rings were relaxed by 3~100 mHU/ml KH in a concentration-dependent fashion (designated as "KH-vasorelaxation") and

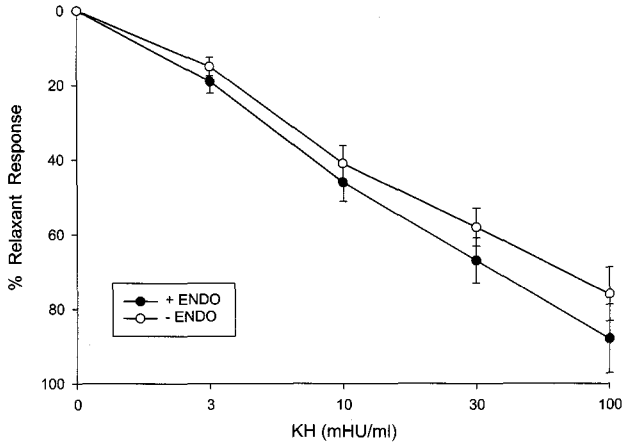

Fig. 9. Effects of removal of the endothelium on the KH- vasorelaxation in the rings contracted with 10^{-6} M phenylephrine. Each point shows mean±SEM from 11 rings. +ENDO and −ENDO curves were obtained from the endothelium-intact rings and endothelium-removed rings, respectively.

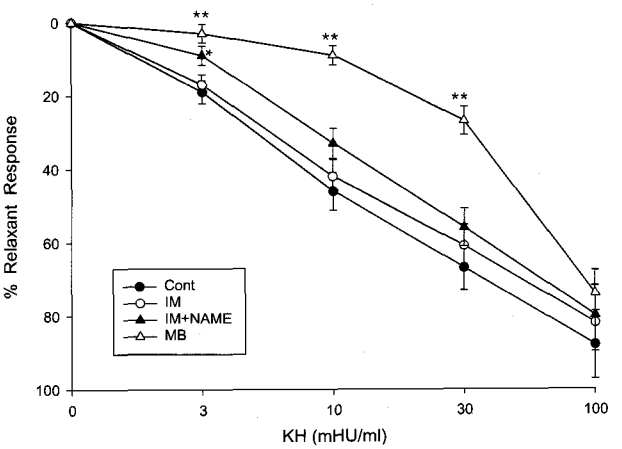

Fig. 11. Effects of various agents on the KH-vasorelaxations in the endothelium-intact rings contracted with 10^{-6} M phenylephrine. Each point shows mean±SEM from 7~9 rings. Cont: control (100%); IM: 10^{-5} M indomethacin; IM+NAME: 10^{-5} M indomethacin+10^{-4} M NAME; MB: 5×10^{-5} M methylene blue. *,**: $p<0.05$, $p<0.01$ compared with the control, respectively.

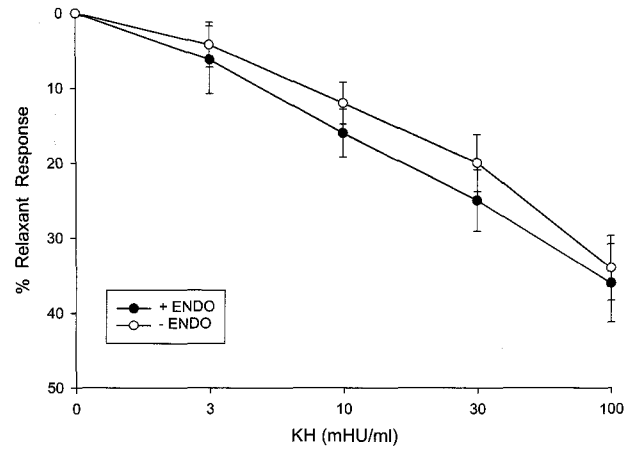

Fig. 10. Effects of removal of the endothelium on the KH-vasorelaxation in the rings contracted with 50 mM KCl. Each point shows mean±SEM from 9 rings. +ENDO and −ENDO curves were obtained from the endothelium-intact rings and endothelium-removed rings, respectively.

the maximal relaxation was no less than 80%. The magnitudes of relaxant responses in both types of the ring preparations were not different each other (Fig. 8, 9).

KCl contraction; 50 mM KCl elicited consistent contractile responses in both rings with (0.72±0.05 g, n=10) and without (0.69±0.07 g, n=9) endothelium, not different from each other. The KCl-induced contractions in both rings were concentration-dependently inhibited by 3~100 mHU/ml KH, and they were not significantly different in both rings (Fig. 10). The maximal inhibition by 100 mHU/ml KH was about 35%, about half of the maximum relaxation (80%), as seen in the PE contraction.

Effects of blockers on KH-vasorelaxation: *Indomethacin*; In the endothelium-intact rings contracted by 10^{-6} M PE, treatment with 10^{-5} M indomethacin did not affect the KH-vasorelaxation (Fig. 11). Neither did additional administration of 10^{-4} M NAME affect the KH-relaxant response (Fig. 8).

Indomethacin+NAME; In endothelium-intact rings contracted by 10^{-6} M PE, co-administration of 10^{-5} M indomethacin and 10^{-4} M NAME inhibited the KH-vasorelaxation slightly, but non-significantly (Fig. 11).

Methylene blue; Treatment with 5×10^{-5} M methylene blue significantly inhibited the relaxations induced by 3~30 mHU/ml KH in the endothelial ring contracted by PE, but did not affect the maximal relaxation by 100 mHU/ml KH (Fig. 11). Additional administration of 5×10^{-5} M methylene blue immediately reversed the relaxation induced by 100 mHU/ml KH to a contractile response (Fig. 8).

DISCUSSION

The icv KH-pressor effect

The icv administration of 100 mHU KH produced a marked and sustained increase of BP while the same dose, when given intravenously, induced a marked and consistent decrease of BP. To our best knowledge, the present finding that a bacterial exotoxin can elicit pressor effect when administered icv is the first report. It is unique and intriguing, because icv VH, an exotoxin from *Vibrio vulnificus* (Kreger & Lockwood, 1981; Kook et al, 1996), did not produce pressor effect at all but rather caused a marked depressor response, in contrast to the pressor effect of icv KH, another exotoxin produced by *Vibrio parahemolyticus* (Cherwonogrozky & Clark, 1982). The fact that KH and VH produce quite opposite effects when given icv suggests that they differ very much in their nature, although they seem to be closely related.

The icv injection of 10 and 30 mHU KH did not produce significant increase of BP, but 100 mHU produced a marked pressor effect. When the icv KH produced pressor effect, heart rate decreased accompanied with the pressor

effect. The bradycardia recovered after 10~20 min in spite of the continued pressor effect, but it was abolished by bilateral vagotomy. The second icv injection of the same dose of KH elicited pressor and bradycardiac effects that were either markedly attenuated or abolished. These findings suggest that: ① the icv KH-pressor effect is not graded but perhaps quantal in response, ② the pressor effect results from the central, but not peripheral action of KH, ③ the lack of repeatability in the pressor effect may be related to the degenerative change in the target action site, and ④ the icv KH-induced bradycardia appears to be a vagal reflex in response to the pressor effect. Supporting evidence for the interpretation ③ comes from observations, showing that TDH killed cells through apoptosis (Naim et al, 2001a), that KH lysed erythrocytes of various animal species (Douet et al, 1992), and that TDH destroyed the functional integrity of the intestinal epithelium (Yanagase et al, 1970). However, the precise mechanism of the degenerative change of brain cells induced by KH remains obscure and awaits further intensive investigations.

Heat-denaturation of KH did not influence the icv KH-pressor effect, but denaturation by acid inhibited the pressor effect. This suggests that the component producing the pressor effect of icv KH is acid-sensitive and heat-stable. Douet et al (1992) have already reported that KH is not inactivated by heating. The responses to the acid-denatured KH will be discussed in the next section which dealt the iv KH-depressor effect.

The icv KH-pressor effect was inhibited by iv treatment with phentolamine and chlorisondamine, but not affected by iv treatment with candesartan. Phentolamine is an antagonist of α-adrenergic receptor (Dismukes & Mulder, 1976), chlorisondamine is a blocker of autonomic ganglia (Ekstrom, 1978), and candesartan is an antagonist of AT_1-receptor (Morsing et al, 1999). On the bases of the present findings together with the described above reports, the icv KH-pressor effect might have been originated in the central sympathetic nervous system, but the angiotensin system was not involved in its manifestation.

Regulation of ionic balance is one of the critical processes in the central nervous system, involving a complex array of molecules for moving ions into and out of brain cells. Alterations in extracellular-to-intracellular ion gradients have both direct and indirect effects on neuronal discharge (Philip et al, 1998). Therefore, in order to determine the role of cellular ion transport in the icv KH-pressor effect, the effects of inhibitors of ion transport such as ouabain, diltiazem and bumetanide were investigated by simultaneous or individual icv administration of the three drugs. When the drugs were simultaneously administered, the icv KH-pressor effect was inhibited to less than half the control level. However, the pressor effect was not affected by treatment with one of the three drugs alone. In various tissues including neuronal tissues, ouabain is an inhibitor of Na^+/K^+ ATPase and blocks the membrane transport of Na^+ and K^+ ion (Bernhardt et al, 1991; Budzikowski et al, 1998). Diltiazem is a blocker of voltage-dependent Ca^{2+} channel and inhibits calcium entry through the channel (Popoli et al, 1991; Rabkin, 1992). In addition, bumetanide is a loop-diuretic related to furosemide, which inhibits activity of Na^+-K^+-$2Cl^-$ cotransporter as well as K-Cl cotransporter in several tissues including cardiac and brain cells (Philip et al, 1998; Kelso et al, 2000; Michea et al, 2001). Considering the present findings together with those reports, it is assumed that the icv KH-pressor effect may be closely related to the transcellular ion transport mechanism. Supporting evidences for this assumption are as follows: The endogenous cation transporters are blocked by ouabain, bumetanide and nitrendipine (Huntley et al, 1993); KH elevates cation (K^+, Ca^{2+}) permeability, which has both direct and indirect actions on the behaviour of a variety of cell types in vivo (Huntley & Hall, 1996); TDH is an important leak-inducing agent of KH (Huntley & Hall, 1994); and KH induces influx of extracellular Ca^{2+} (Tang et al, 1995) and a pore-forming toxin (Honda et al, 1992). The above studies suggest that KH induces a pore-like lesion on cell membrane and stimulates the membrane transport of Na^+, K^+, Cl^-, Ca^{2+} ions, and that combined treatment with ouabain, diltiazem and bumetanide, as used in the present study, might sufficiently inhibit the transport of those ions.

In summary, it is inferred that icv KH generates a pore-like lesion on central nervous system, increases membrane permeability of ions, and subsequently elevates the sympathetic tone in part and then the BP. However, the reasons of why the icv KH-pressor effect was only partially inhibited by adrenergic blockers and why it was not affected by treatment with each one of the ion transport inhibitors remain enigma to be clarified.

The iv KH-depressor effect

While icv KH administration produced a marked pressor effect, iv KH injection of the same dose caused a marked and continuous depressor effect, and the depressor effect was repeatable. Kook et al (1996) reported that iv VH produced hypotensive response similar to the iv KH-depressor effect observed in this study. In the icv experiments, the icv KH-pressor effect was inhibited by the acid-denaturation. In contrast, the iv KH-depressor effect was not affected by the acid-denaturation as well as by the heat-denaturation. These findings indicate that the component inducing the icv KH-pressor effect is acid-sensitive, while the component inducing the iv KH-depressor effect is acid-resistant. Thus, the results suggest that both components of KH are not at least identical, and/or, the acid-treatment causes a conformational change of KH molecules, thereby the altered molecules may lose a part of the activity. This hypothesis may be supported by following reports showing that TDH is the important leak-inducing agent of KH (Huntley & Hall, 1994); that the mechanism of cytotoxicity of TDH on Rat-1 cells was different from that of hemolytic activity of TDH on red blood cells (Naim et al, 2001a); that the N-terminal region in TDH may be involved in binding process while the region near C-terminal may be involved in postbinding process (Tang et al, 1997); and that the culture supernatants of *Vibrio parahemolyticus* contain several constituents including phospholipase A (Yanagase et al, 1970), lysophospholipase (Yanagase et al, 1970), TDH (Douet et al, 1992; Huntley et al, 1993), and TDH-related hemolysin (Nishibuchi et al, 1992).

On the other hand, the iv KH-depressor effect was repeatable in contrast to the icv KH-pressor effect, and it was inhibited by NAME, an inhibitor of NO synthase (Kline et al, 1997), and methylene blue, an inhibitor of guanylate cyclase (Avontuur et al, 1995). To be of note is the fact that additional injection of methylene blue reversed the iv KH-depressor effect to the original level or further. However, the iv KH-depressor effect was not affected by simul-

taneous treatment with the three inhibitors of ion transport, in contrast to the icv KH-pressor effect described above. These results suggest that the NO-guanylate cyclase system, not the ion transport system, is involved in the iv KH-depressor effect.

The KH-vasorelaxation

KH relaxed the rings of rat thoracic aorta contracted by PE in a concentration-dependent fashion. The KH-vasorelaxation was reproducible and was independent of the endothelium. KH relaxed concentration-dependently also the rings contracted by KCl, but the magnitudes of the relaxations were much smaller than those in the rings contracted with PE and the relaxations were also not affected by the removal of the endothelium. These data suggest that the vasorelaxant response to KH is not dependent on the endothelium.

The KH-vasorelaxation was not affected by treatment with indomethacin alone, and also combined treatment with indomethacin and NAME. However, treatment with methylene blue inhibited the KH-vasorelaxation. In addition, additive administration of NAME in the state relaxed by KH did not affect the relaxed state at all, while methylene blue recovered the relaxed state to the orignal level or further. Indomethacin inhibits synthesis of prostaglandins by inhibiting cyclooxygenase activity (Kristova et al, 2000). NAME is a NO synthase inhibitor and methylene blue is an inhibitor of guanylate cyclase as described previously. Atrial natriuretic peptide activates guanylate cyclase directly, and produces the endothelium-independent relaxation inhibited by guanylate cyclase inhibitors (Waldman et al, 1984). In the experiments using the isolated aortic rings of the same animals, Kook et al (1996) found that NAME and aminoguanidine, NO synthase inhibitors, did not affect the vasorelaxation induced by VH, whereas the VH-induced relaxation was inhibited by LY 83,583, a guanylate cyclase (Ijioma et al, 1995). Thus, they proposed that VH activated guanylate cyclase independently of NO synthase and subsequent increase of cGMP levels resulted in vasorelaxation and hypotension. The present findings together with those reports suggest that the KH-vasorelaxation does not involve the endothelium-NO synthase pathway, and that it results from direct activation of guanlyate cyclase in vascular smooth muscles. And reproducibility of the KH-vasorelaxation also suggests that KH does not at least cause a degenerative functional change of vascular smooth muscle.

In summary, the present findings suggest that in the icv KH-pressor effect the cation leak-inducing action of KH is implicated, which leads to the increased central sympathetic tone, and that the iv KH-depressor effect results from vasorelaxation via NO-guanylate cyclase system, and that the KH-vasorelaxation is independent of the endothelium and the guanylate cyclase system is involved. In conclusion, the nature of KH producing the icv pressor effect may not be identical to that of KH producing the iv depressor effect.

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