A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery

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Abstract In this report, we deal with the isolation of a novel vasorelaxing peptide from a chymotryptic digest of ovalbumin and its vasorelaxing activities. This peptide is composed of Arg-Ala-Asp-His-Pro-Phe (RADHPF) in its sequence, corresponding to residues 359–364 of ovalbumin. This peptide (30–300 μM) exerted a dose-dependent vasodilation in an isolated mesenteric artery from a spontaneously hypertensive rat which was pre-constricted by phenylephrine, besides the relaxation being endothelium-dependent. It is noteworthy that the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester inhibited this relaxation, implying involvement of nitric oxide in its mechanism of action. Following oral administration of RADHPF at a dose of 10 mg/kg, the systolic blood pressure in a spontaneously hypertensive rat was significantly lowered.
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Key words: Ovalbumin; Ovokinin; Nitric oxide; Vasorelaxation; Blood pressure; Spontaneously hypertensive rat

1. Introduction

Many kinds of biologically active peptides including opioid peptides and inhibitory peptides for angiotensin I-converting enzymes have been isolated from enzymatic digests of various food proteins [1–4].

In the previous study, we reported that ovokinin, an octapeptide with vasorelaxing activity in an isolated canine mesenteric artery, was obtained from the peptic digest of ovalbumin [5]. Onset of this relaxation was partly dependent on endothelium and attributable to the activation of bradykinin B_1 receptors. Moreover, the endothelium-dependent relaxing factor involved therein was identified as prostaglandin I_2 but not nitric oxide (NO).

In sharp contrast, however, the present study revealed that the novel peptide isolated from the chymotryptic digest of ovalbumin showed completely endothelium-dependent vasorelaxation in an isolated spontaneously hypertensive rat (SHR) mesenteric artery and the relaxing factor was evidenced to be NO.

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Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; ODS, octadecyl silica; i.p., intraperitoneal

2. Materials and methods

2.1. Chemicals and reagents

Ovalbumin (grade VI), bovine α-chymotrypsin (TLCK-treated, type VII) and indomethacin were purchased from Sigma Chemical. N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from Nacalai Tesque. Phenylephrine hydrochloride was obtained from Research Biochemicals, papaverine hydrochloride from Daiichi Seiyaku, des-Arg⁹-(Leu⁸)-bradykinin, HOE140 and charybdotoxin from Peptide Institute and apamin from American Peptide Company.

2.2. Chymotryptic digestion of ovalbumin

Ovalbumin (25 mg/ml) was adjusted to pH 7.8 with 0.5 N NaOH and digested two times by chymotrypsin (E/S = 1/100 (weight/weight)) for 12 h each at 37°C. The reaction was stopped by boiling for 10 min and then, the reaction mixture was centrifuged.

2.3. Purification of peptides

The digest was fractionated by reversed phase high performance liquid chromatography (HPLC) on an octadecyl silica (ODS) column (Cosmosil 5C18-AR, 20×250 mm, Nacalai Tesque). The column was eluted with a linear gradient of acetonitrile (1‰/min), containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 10 ml/min. The elution was monitored at 215 nm. Each fraction was dried with a centrifugal concentrator and vasorelaxing activity was measured in an isolated SHR mesenteric artery. The active fraction was further purified by a phenethyl silica column (Develosil PhA-T-5, 4.6×250 mm) developed by the same linear gradient of acetonitrile as described above at a flow rate of 1 ml/min.

2.4. Amino acid sequence analysis and peptide synthesis

The amino acid sequence of the purified peptide was determined by a 492 protein sequencer (Applied Biosystems), whereby PS3 (Rainin) peptide synthesizers were used to synthesize the peptide.

2.5. Vasorelaxation assay

Male SHRs (15–29 weeks old) and age-matched normotensive Wistar-Kyoto rats (WKYs) were exsanguinated from the common carotid artery under sodium pentobarbital anesthesia (25 mg/kg, intraperitoneal (i.p.)). The mesenteric arteries were then removed and cut into ring strips. The endothelium was removed mechanically by gently rubbing the intimal surface. The absence of functional endothelium was confirmed by the inability of acetylcholine to induce relaxation. Intact and endothelium-denuded arteries were mounted between two stainless steel hooks in an organ bath containing a Krebs-Henseleit solution (120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose) at 37°C and bubbled with a mixture of 95% O_2/5% CO_2. The end of the hook was attached to the lever of a force-displacement transducer (T7-8-240, Orientec), which was connected to a Recti-Horiz-8K recorder (NEC San-ei Instrument), thereby recording the isometric tension changes. The applied tension was adjusted to 1 g, followed by a 3 h equilibration period. The artery was constricted by phenylephrine (10^{-7}–10^{-6} M). The relaxing activity of samples was assayed in the presence or absence of inhibitors or antagonists applied 15 min before the addition of phenylephrine. To determine the complete relaxation point, 10^{-4} M papaverine was added at the end of each examination.

2.6. Measurement of the blood pressure

Male SHRs (15–29 weeks old) and age-matched normotensive WKYs were used. The peptide emulsified with 30% egg yolk in saline was administered orally in SHRs using a metal zonde in a volume of

0.5 ml. Following oral administration of the peptide, the blood pressure was measured by the tail cuff method using a UR-5000 (Ueda Seisakusho) to ascertain its anti-hypertensive activity, while only egg yolk was administered as a control.

2.7. Data analyses

All results are expressed as means and S.E.M. Comparisons of the results between the two groups were statistically made with Student's t-test.

3. Results

3.1. Isolation of the vasorelaxing peptide from the chymotryptic digest of ovalbumin

It was disclosed that the chymotryptic digest of ovalbumin exerted a vasorelaxing activity in an isolated SHR mesenteric artery. As shown in Fig. 1A, the chymotryptic digest relaxed a SHR mesenteric artery which was pre-constricted by phenylephrine. In order to isolate the vasorelaxing peptide, the digest was fractionated by reversed phase HPLC on an ODS column. Among all the fractions measured, two active peaks were eluted at about 25 and 28% of acetonitrile (Fig. 2A). The former peak showed a higher vasorelaxing activity than the latter one. Consequently, our research has focused on the former in this report. For further purification, fractionation of
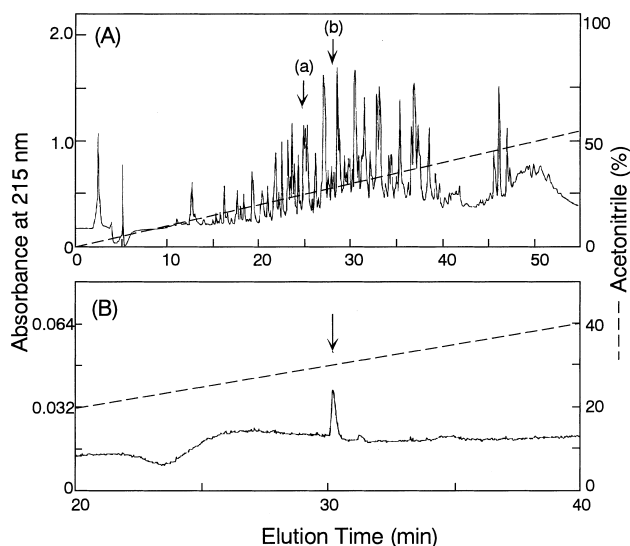

Fig. 2. Purification of vasorelaxing peptides from the chymotryptic digest of ovalbumin by reversed phase HPLC on an ODS column (A) and further purification of peak (a) on a phenethyl silica column (B). Active fractions are indicated by arrows.

the active peak was conducted on a phenethyl silica column, allowing isolation of an active peptide in a pure form (Fig. 2B). The structure of this peptide, determined by a protein sequencer, was Arg-Ala-Asp-His-Pro-Phe, being consistent with residues 359–364 of ovalbumin. Furthermore, this peptide was identical to the 2-7 fragment of ovokinin (FRADHPFL). Incidentally, ovokinin was previously isolated in our laboratory as a vasorelaxing peptide acting via the bradykinin B_1 receptors in an isolated canine mesenteric artery [5]. Although neither ovokinin (10^{-4} M) nor endogenous B_1 agonist des-Arg^9-bradykinin (10^{-7} M) exhibited a significant vasorelaxing activity in an isolated SHR mesenteric artery (data not shown), a synthetic 2-7 fragment of ovokinin (ovokinin(2-7)) showed a potent vasodilation at 10^{-4} M in the same artery specimens (Fig. 1B).

3.2. Relaxation mechanism of ovokinin(2-7)

As shown in Fig. 3A, ovokinin(2-7) induced a dose-dependent relaxation in a SHR mesenteric artery. The percentage of dilation of the phenylephrine-constricted mesenteric artery by 10^{-4} M ovokinin(2-7) accounted for 67.5%, with the relaxation saturated at 3×10^{-4} M. On the other hand, the vasorelaxation in a WKY mesenteric artery was significantly smaller than that in SHR's. As shown in Fig. 3B, ovokinin(2-7)-induced vasorelaxation was endothelium-dependent. To discern the features characteristic of this new endothelium-dependent relaxing factor, investigations were performed on the effect of inhibitors of NO synthase (L-NAME), cyclooxygenase (indomethacin) and Ca^{2+}-activated K^+ channels (apamin plus charybdotoxin) on the ovokinin(2-7)-induced vasorelaxation. As illustrated in Fig. 3B, L-NAME inhibited the relaxation, whereas indomethacin failed to show any interaction. Although the combination of apamin plus charybdotoxin slightly attenuated the relaxation (20.9%), the inhibitory effect was not significant ($P = 0.085$). In addition, neither des-Arg^9-(Leu^8)-bradykinin (B_1 antagonist) nor HOE140 (B_2 antagonist) were found to be effective.
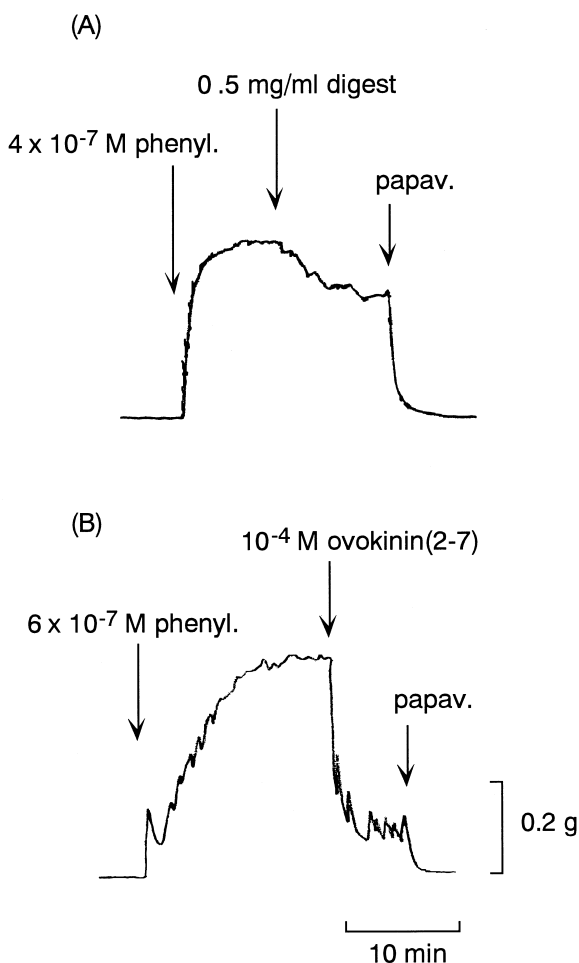

Fig. 1. Vasorelaxing activity of a chymotryptic digest of ovalbumin (A) and ovokinin(2-7) (B) in an isolated SHR mesenteric artery. phenyl.: phenylephrine. papav.: 10^{-4} M papaverine.
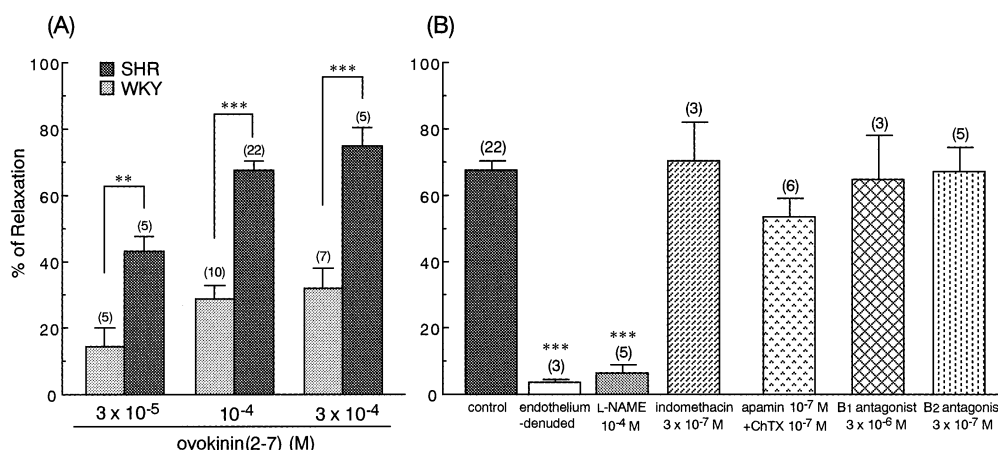
Fig. 3. (A) Dose-dependent vasorelaxing activity of ovokinin(2-7) in the mesenteric artery of SHRs and WKYs. (B) The effect of endothelium removal and various inhibitors on ovokinin(2-7)-induced vasorelaxation. Control: 10^{-4} M ovokinin(2-7). ChTX: charybdotoxin. B_1 antagonist: des-Arg^9-(Leu^8)-bradykinin. B_2 antagonist: HOE140. Each value is expressed as mean ± S.E.M. Parentheses represent the number of experiments. **, ***: Significant difference against the parallel control (**$P < 0.01$, ***$P < 0.001$).

3.3. Anti-hypertensive activity of ovokinin(2-7) after oral administration in SHRs

Vasorelaxing activity of ovokinin(2-7) in the isolated mesenteric artery specimen prompted our investigation on the hypotensive effect after its oral administration to SHR. Given the fact that the effect of various anti-hypertensive peptides was potentiated by emulsification in 30% egg yolk [6,7], we orally administered ovokinin(2-7) emulsified in 30% egg yolk to SHRs. As is obvious in Fig. 4A, 2 h after the administration of ovokinin(2-7) at a dose of 20 mg/kg, the systolic blood pressure of SHRs started to decrease, with the significant effect observed 4 and 6 h post-administration. The maximal effect was dose-dependently noted 6 h after administration (Fig. 4B). On the other hand, ovokinin(2-7) at a dose of 20 mg/kg did not show any significant hypotensive effect in WKYs (Fig. 4B).

4. Discussion

In this study, we isolated a novel vasorelaxing peptide, ovokinin(2-7), from a chymotryptic digest of ovalbumin and confirmed its vasorelaxing effect by using an isolated SHR mesenteric artery. Our view is that this assay system seems to be useful for the screening of new vasorelaxing peptides.

Ovokinin(2-7) showed a dose-dependent relaxation in an isolated SHR mesenteric artery. However, removal of the endothelium was associated with disappearance of the relaxation effect, demonstrating that this relaxation is endothelium-dependent. Moreover, the dilation was inhibited by L-NAME but not by indomethacin, indicating implication of the NO release from the endothelial cells upon onset of the vasorelaxation. Although the relaxation was slightly attenuated in the presence of apamin plus charybdotoxin, the inhibitory effect was not significant, suggesting that endothelium-derived hyperpolarizing factor (EDHF) does not play an essential role in the relaxation.

It is well known that production of the superoxide anion (O_2^-), which inactivates NO, is increased in the vessel of SHRs [8,9]. Nevertheless, pretreatment of superoxide dismutase (150 U/ml) did not blunt the ovokinin(2-7)-induced vasorelaxation in a SHR mesenteric artery (data not shown), suggesting that this relaxation is not caused by scavenging O_2^- but by stimulating the NO production.

Taken together, it is postulated that ovokinin(2-7) might act on receptors of some endogenous vasoactive peptide condu-
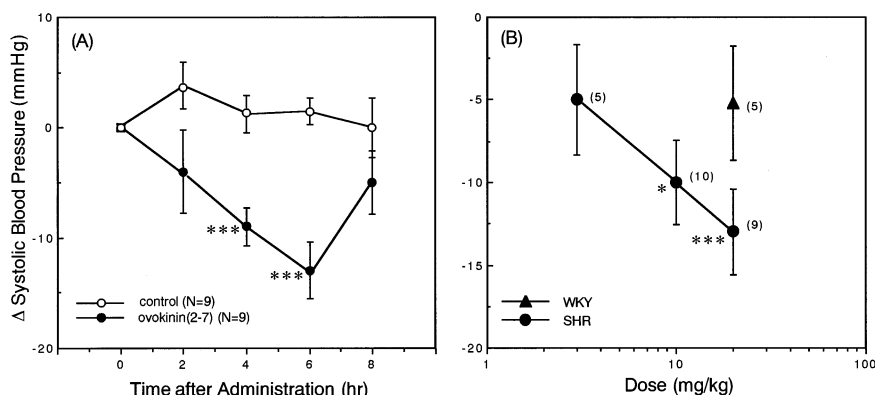

Fig. 4. (A) Time course of ovokinin(2-7)-induced anti-hypertensive activity (20 mg/kg) after oral administration in SHRs. (B) Dose-dependent anti-hypertensive effect of ovokinin(2-7) 6 h after administration. Changes in the systolic blood pressure from zero time are expressed as means ± S.E.M. Parentheses represent the number of experiments. *, ***: Significant difference against the parallel control (*$P < 0.05$, ***$P < 0.001$).

cive to mediating the activation of NO synthase in the endothelial cells. However, bradykinin B_2 receptor, a well known receptor contributing to the NO-mediated vasorelaxation, is not involved in the ovokinin(2-7)-induced vasorelaxation because B_2 antagonist HOE140 failed to block the relaxation (Fig. 3B). Moreover, atropine (10^{-6} M) had no effect on the dilation, indicating that this relaxation is not mediated via a muscarinic receptor (data not shown). Further investigation is required to identify the receptor for ovokinin(2-7).

Despite the structural similarity in its sequence to ovokinin(2-7), ovokinin itself hardly induced vasorelaxation in an isolated SHR mesenteric artery. In addition, the previous study evidenced that ovokinin-induced vasorelaxation was blocked by the bradykinin B_1 receptor antagonist des-Arg^9-(Leu^8)-bradykinin and inhibited by indomethacin but not by L-NAME in an isolated canine mesenteric artery. These results are quite contradictory to those shown in the present study on ovokinin(2-7), albeit that different animal species are used. Moreover, endogenous B_1 agonist des-Arg^9-bradykinin (10^{-7} M) failed to dilate the SHR mesenteric artery, suggesting that B_1 receptors are not involved in the vasorelaxation. Given all this, it is conceivable that ovokinin(2-7) is an obviously different type of peptide from ovokinin in terms of their modes of vasorelaxing activities. It is very interesting that these two types of vasorelaxing peptides are released from the same region of ovalbumin by different enzymes, i.e. chymotrypsin and pepsin.

Orally administered ovokinin(2-7) significantly lowered the blood pressure of SHR in a dose-dependent manner (Fig. 4B). Most of the orally active anti-hypertensive peptides derived from food proteins are known to be the inhibitory peptides for angiotensin I-converting enzymes [10–15]. However, ovokinin(2-7) was devoid of any inhibitory activity against these enzymes ($IC_{50} = >1$ mM, data not shown). Coupled with these facts, ovokinin(2-7) exerting direct actions on endothelial cells to stimulate the NO release responsible for vasodilation can be regarded as the first example of such a unique peptide derived from a natural food protein. While further investigation remains to be conducted to verify the possibility whether other mechanisms are involved in the ovokinin(2-7)-induced hypotensive activity, our results indicate that vasorelaxing peptides derived from food proteins could play a role as a new example of anti-hypertensive peptides.

It is noteworthy that ovokinin(2-7) showed a more potent vasorelaxant and anti-hypertensive effect in SHRs than in WKYs. These results reinforce the concept that the receptor for ovokinin(2-7), which is unidentified yet, might be significantly expressed in SHRs to compensate the hypertension. Consequently, ovokinin(2-7) could play an important role in the improvement of endothelial dysfunction as well as hypertension.

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