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Hypotensive Compounds Isolated from the Dried Body of *Naja naja Kaouthia* LESSON. I.[1] Isolation of Inosine as a Hypotensive Principle and Structure–Activity Study of Related Compounds

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From the dried body of *Naja naja Kaouthia* from which the internal organs had been removed, three hypotensive compounds were isolated and one of them was identified as inosine. Inosine produced a transient increase in blood pressure followed by a prolonged fall lasting for 30 min in spontaneously hypertensive rats. Next, the relationship between the chemical structure and the hypotensive effect was examined by studying ribose-modified derivatives of inosine. 2′-Deoxyinosine and 3′-deoxyinosine completely lacked hypotensive effect. Phosphorylation at the 3′ or 5′-position except for inosine 5′-diphosphoribose and 5′-chloro-5′-deoxyinosine slightly decreased the hypotensive effect. The results indicate that the ribose configuration is important in relation to the hypotensive effect of inosine.

Keywords——*Naja naja Kaouthia*; Elapidae; hypotensive effect; ribose configuration; inosine; inosine-related compound; hypotensive compound; spontaneously hypertensive rat

The animals which belong to Elapidae are divided into fortythree genera and are widely distributed throughout the world. Among them, the genera of *Naja, Bungarus* and *Ophiophagus* were especially abundant in Asia. There have already been many reports about the toxic components of these animals.[2–5] Interestingly, the dried bodies of these animals have been used as an antihypertensive agent in traditional medicine from ancient times. However, there is no report about hypotensive agents contained in the dried bodies of these animals. In our study, three hypotensive compounds were isolated from the dried body of *Naja naja Kaouthia* mainly found in Asia. One of them was identified as inosine, and the hypotensive effects of inosine and related compounds were examined in spontaneously hypertensive rats.

Materials and Methods

Chemicals——Chemicals used were as follows: inosine (Yamasa Shoyu Co., Ltd.); inosine 3′-monophosphate, inosine 5′-monophosphate, inosine 5′-triphosphate, inosine 3′,5′-cyclic monophosphate, 2′-deoxyinosine, 2′-deoxyinosine 5′-monophosphate, 2′-deoxyinosine 5′-triphosphate, 5′-chloro-5′-deoxyinosine and inosine 5′-diphosphoribose (Sigma Chemical Co.); 3′-deoxyinosine (kindly supplied by Dr. Yusuke Wataya, Faculty of Pharmaceutical Science, Okayama University); anserine, carnosine and spermine (Sigma Chemical Co.); serotonin and histamine (Wako Pure Chemical Industries Ltd.).

Animals——Male spontaneously hypertensive rats (SH rats) weighing about 260—300 g from the colony of the Department of Pharmacology, Jichi Medical School, were used.

Isolation of the Hypotensive Compounds from the Dried Body of *Naja naja Kaouthia*——The dried body of *N. naja* from which the internal organs had been removed was finely cut and powdered. The powder (20 g) thus obtained, after being defatted with *n*-hexane (100 ml) and subsequently ether (100 ml), was extracted with 1 N AcOH containing 20 mM HCl (100 ml) at 100 °C for 10 min according to the method of Matsuo *et al.*[6] The same extraction was carried out three times. Each supernatant was collected and lyophilized to give an amorphous yellow powder (8 g). The

powder (0.2 g) was dissolved in 0.1 N AcOH and applied to a column (1.8 × 96 cm) of Sephadex G-25, using the same buffer as an eluent at a flow rate of 0.3 ml/min. The eluate was separated into Fr-I (20—30), Fr-II (45—60), Fr-III (61—75), Fr-IV (76—85) and Fr-V (86—95) based on the OD at 280 nm. Among these, Fr-III (elution volume: 280 ml) showed the strongest hypotensive effect (−90 mmHg, 10 mg/kg, *i.v.*) on SH rats. The yields were: Fr-I (60 mg), Fr-II (130 mg), Fr-III (3.0 mg), Fr-IV (0.7 mg) and Fr-V (0.2 mg). Fr-III was further purified by preparative high-performance liquid chromatography (HPLC). Apparatus, high-performance liquid chromatograph (Toyo Soda HLC-803 Series A); column, YMC-Pack S-343 ODS (20 × 250 mm); detector, UV 215 nm; mobile phase, H_2O–0.05% trifluoroacetic acid; flow rate, 3 ml/min; temperature, ambient. Sixteen peaks were obtained. Among these, four major peaks, Fr-A (t_R: 67 min 37 s), Fr-B (t_R: 85 min 12 s), Fr-C (t_R: 99 min 32 s) and Fr-D (t_R: 133 min 36 s) were isolated and lyophilized: Fr-A (0.36 mg), Fr-B (0.2 mg), Fr-C (0.7 mg) and Fr-D (0.25 mg). All fractions except for Fr-A showed potent hypotensive activity. Fr-C was recrystallized from 80% EtOH as colorless plates, mp 218 °C (dec.). *Anal.* Calcd for $C_{10}H_{12}N_4O_5$: C, 44.79, H, 4.46, N, 21.02. Found: C, 44.78, H, 4.51, N, 20.89. The ultraviolet (UV), infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectral data of Fr-C were identical with those of an authentic sample of inosine.

Antihypertensive Activity in SH Rats——SH rats were anesthetized with sodium pentobarbital, 40 mg/kg *i.p.* with supplemental doses as necessary. The trachea was isolated and cannulated with a short piece of polyethylene tubing. The systemic arterial blood pressure was measured *via* a carotid catheter connected to a pressure transducer (Nihon Kohden P 23 ID, with WI-621 G chart recorder). Samples were dissolved in 0.9% saline and administered through a cannula in the femoral vein.

Results

Isolation of Hypotensive Principles from the Dried Body of *Naja naja Kaouthia*

The acidic extract of the dried body of *N. naja* was found to show a hypotensive effect on SH rats. Therefore, isolation of the hypotensive principles was attempted. The lyophilized powder, after being extracted with 1 N AcOH containing 20 mM HCl, was applied to a column of Sephadex G-25, yielding five elution peaks as shown in Fig. 1. Fr-I—V were each lyophilized and their hypotensive effects were examined in SH rats. The results are summarized in Table I. Fr-II and Fr-III showed hypotensive effects on SH rats and Fr-III had the strongest hypotensive effect. (The yields of Fr-IV and Fr-V were too small to permit examination of the biological activity.)

A thin layer chromatogram of Fr-III is shown in Fig. 2. Fr-III gave two ninhydrin-positive spots and two fluorescent spots on UV light. The *Rf*-values of Fr-III were higher than those of carnosine[7] and anserine[8] found in the muscle of vertebrates. Fr-III also did not contain serotonin or histamine. The molecular weight of Fr-III was presumed to be about 200—300 from the elution pattern on Sephadex G-25 column chromatography.

Further purification of Fr-III was carried out by preparative HPLC. As shown in Fig. 3, Fr-III gave sixteen peaks. Among these, four major peaks, Fr-A, B, C and D, were isolated

TABLE I. Effect of Each Fraction on the Blood Pressure in Anesthetized Spontaneously Hypertensive Rats

Sample	Dose (mg/kg, *i.v.*)	Mean arterial blood pressure (mmHg)
Acidic extract	10	−18
Fr-I	10	0
Fr-II	10	−15
Fr-III	10	−90
Carnosine	10	−15
Anserine	10	−10

Body weight: 260 g. Anesthetic: Pentobarbital-Na (40 mg/kg, *i.p.*). Each value represents the mean of 3 rats.

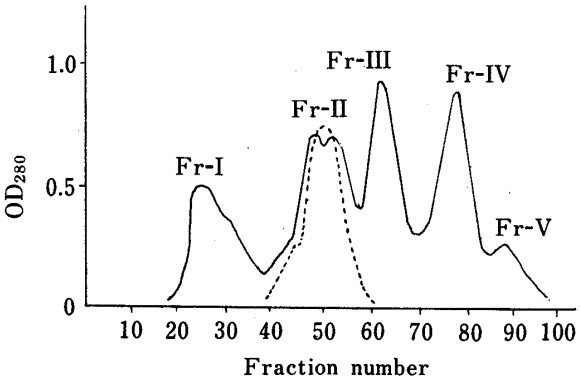

Fig. 1. Gel Filtration of the Acidic Extract on Sephadex G-25

Column size: 1.8 × 96 cm. Eluate: 0.1 N AcOH. Flow rate: 0.3 ml/min. Fraction size: 4 ml. ----: Peaks of carnosine and anserine.

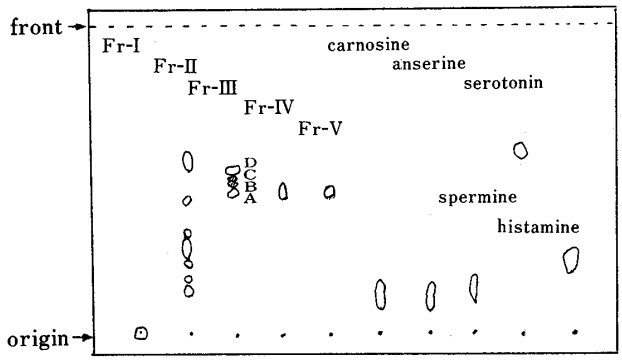

Fig. 2. Thin-Layer Chromatogram of Each Fraction and Standard Samples

Solvent system: *n*-BuOH–Pyridine–AcOH–H_2O (15:10:3:12 v/v).
Detection: ninhydrin ○, UV light ▨.
Spot C in Fr-III is inosine.

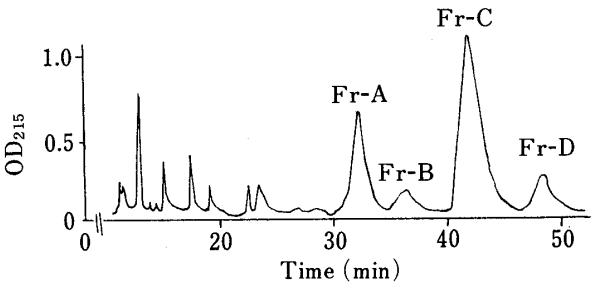

Fig. 3. Preparative High-Performance Liquid Chromatogram of Fraction-III

HPLC was performed on a 20×250 mm YMC-Pack S-343 ODS column.
The elution buffer was H_2O–0.05% TFA.
Flow rate: 3 ml/min.

TABLE II. Effect of Each Fraction on the Blood Pressure in Anesthetized Spontaneously Hypertensive Rats

Sample	Dose (mg/kg, *i.v.*)	Mean arterial blood pressure (mmHg)
Fr-A	3.00	0
Fr-B	3.00	−75
Fr-C	0.10	+10
	0.25	+18
	0.50	−62
	1.00	−65
	3.00	−102
Fr-D	3.00	−50

Body weight: 260 g. Anesthetic: Pentobarbital-Na (40 mg/kg, *i.p.*). Each value represents the mean of 3 rats.

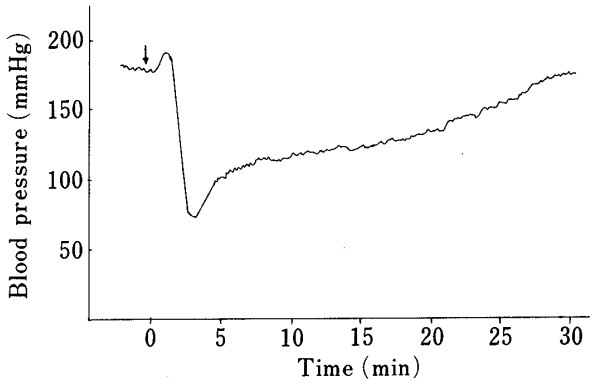

Fig. 4. Effect of Active Fraction on the Blood Pressure of an Anesthetized Spontaneously Hypertensive Rat

Body weight: 260 g. Arrow: injection of 3 mg/kg of active fraction.

and lyophilized. The hypotensive effects of these fractions were examined, and the results are summarized in Table II. All fractions except for Fr-A showed a hypotensive effect in SH rats. In particular, Fr-C produced a transient increase in blood pressure followed by a prolonged fall lasting for about 30 min as shown in Fig. 4 (−102 mmHg, 3 mg/kg, *i.v.*). In contrast, at doses 0.1 mg/kg and 0.25 mg/kg, Fr-C caused only a transient increase (+10—+18 mmHg) in blood pressure in SH rats.

Identification of Fr-C

Fr-C was obtained as colorless plates, mp 218 °C (dec.). The UV, IR and ^{1}H-NMR spectral data of Fr-C were very similar to those of inosine, and direct comparison of the spectral data with those of an authentic sample of inosine confirmed that Fr-C was inosine. The authentic sample of inosine had the same hypotensive effect as Fr-C. On the other hand, the hypotensive effects of Fr-B and D, unlike that of Fr-C, were transient and blood pressure recovered to the original level within one min.

The Hypotensive Effects of Inosine and Related Compounds

Table III also shows the correlation between dose and hypotensive activity of inosine in

TABLE III. Effects of Inosine and Related Compounds on the Blood Pressure in Anesthetized Spontaneously Hypertensive Rats

Compound	Dose (mg/kg, *i.v.*)	Mean arterial blood pressure (mmHg)
Inosine	0.10	+10
	0.25	+18
	0.50	−62
	1.00	−65
	3.00	−102
Inosine 3′-monophosphate	3.00	−65
Inosine 5′-monophosphate	3.00	−75
Inosine 5′-diphosphate	3.00	−67
Inosine 5′-triphosphate	3.00	−60
Inosine 3′,5′-cyclic monophosphate	3.00	+23
2′-Deoxyinosine	3.00	+28
2′-Deoxyinosine 5′-monophosphate	3.00	+27
2′-Deoxyinosine 5′-triphosphate	3.00	+10
3′-Deoxyinosine	3.00	+10
Inosine 5′-diphosphoribose	3.00	−120
5′-Chloro-5′-deoxyinosine	3.00	−110

Body weight: 260 g. Anesthetic: Pentobarbital-Na (40 mg/kg, *i.p.*). Each value represents the mean of 3 rats.

SH rats. Hypotensive activity was seen at dose levels higher than 0.5 mg/kg, and this activity increased strongly with increasing dose of inosine. However, at the doses of 0.1 and 0.25 mg/kg, inosine showed only a transient hypertensive effect (+10—+18 mmHg) and the blood pressure recovered to the original level within one min. Phosphorylation at 3′- or 5′-position of the ribose moiety slightly decreased the hypotensive effect. However, inosine 5′-diphosphoribose showed the most potent hypotensive effect among compounds tested.

Discussion

It was found that the acidic extract of the dried body of *Naja naja* from which the internal organs had been removed had a hypotensive effect on SH rats (Table I). Three hypotensive compounds were isolated from the dried body of *N. naja*, and one of them was identified as inosine (Table II). Various biologically active peptides, such as carnosine and anserine, with hypotensive effect have already been reported from the muscle of vertebrates. However, the isolation of inosine as a hypotensive principle from *N. naja* is reported for the first time in this paper. Inosine is used clinically as a treatment for leukopenia. However, it has not yet been used as an antihypertensive agent. There have already been several reports about the effect of inosine on the blood pressure, but its effect is controversial: namely, it was reported to have a hypotensive effect,[9,10] while it was also reported to show a hypertensive effect.[11] Our observation showed that inosine caused a transient small increase of blood pressure followed by a prolonged fall lasting for about 30 min (Table II and Fig. 4). Our results are in accordance with the reports of Stebbing *et al.*[9] and Macdonald *et al.*[10] The fact that inosine caused pronounced hypotension in normotensive and SH rats indicates that the effect is fundamental and not peculiar to hypertensive rats (data not shown).

Next, the relationship between chemical structure and the hypotensive activity was examined by modifying the ribose moiety of inosine (Table III). It was found that 2′-deoxyinosine and 3′-deoxyinosine completely lacked hypotensive activity, indicating that the

hydroxyl groups attached to C-2′ and C-3′ are necessary for inosine to show the hypotensive effect. Phosphorylation at C-3′ or C-5′ slightly decreased the hypotensive effects. However, the hypotensive effect of inosine 5′-diphosphoribose and 5′-chloro-5′-deoxyinosine were comparable with that of inosine. These results demonstrate the importance of the ribose configuration of the sugar moiety of inosine for the hypotensive activity. The mechanism of hypotensive activity of inosine and the chemical structures of Fr-B and D will be reported in the following paper.

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