Hypotensive Compounds Isolated from Alcohol Extract of the Unossified Horn of Cervus elaphus L. var. xanthopygus MILNE-EDWARG (Rokujo). I.\(^1\) Isolation of Lysophosphatidyl Choline as a Hypotensive Principle and Structure–Activity Study of Related Compounds

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By the use of spontaneously hypertensive rats (SH rats) as a screening system, two hypotensive compounds were isolated from alcohol extract of the unossified horn of Cervus elaphus L. var. xanthopygus (Rokujo). One of the two compounds was identified as lysophosphatidyl choline (LPC). It was shown by gas chromatography that palmitic acid (C\(_{16:0}\)) accounted for about half of the fatty acid content. Next, the effects of various LPCs on SH rats were examined. Eleven LPCs with C\(_{10:0}\) to C\(_{20:0}\) fatty acids were examined, and all except for those with C\(_{10:0}\) and C\(_{20:0}\) fatty acids showed the hypotensive effect (3 mg/kg, i.v.). In particular, C\(_{14:0}\) and C\(_{16:0}\)-LPC showed rather potent activity. On the other hand, of the six LPCs with unsaturated fatty acids, only C\(_{16:1}\)-LPC showed the hypotensive effect (3 mg/kg). It was concluded that at least a part of the hypotensive action of alcohol extract of Rokujo is due to LPC.

Keywords—Rokujo; Cervus elaphus L. var. xanthopygus; lysophosphatidyl choline; spontaneously hypertensive rat; hypotensive compound; hypotensive action; Pantocrin; fatty acid composition; pharmacological action

Cervus elaphus L. var. xanthopygus and related animals which belong to Cervidae in Artiodactyla are widely distributed in Siberia and China. The unossified horn of these animals, so-called “Rokujo” has been used as a valuable drug in Chinese medicine from ancient times. The pharmacological actions of Pantocrin, alcohol extract of Rokujo, on the neuro-muscular and endocrine systems were examined by Pavlenko et al.\(^2\) In Japan, there have been studies\(^3,4\) on the effect of Pantocrin on experimental whiplash injury and on unidentified clinical syndrome. Pantocrin is clinically used as an injection. Takigawa et al.\(^4\) also reported that Pantocrin had a hypotensive effect as one of its pharmacological actions. However, no detailed work has yet been done on the active principles of alcohol extract of Rokujo.

In the present study, two hypotensive compounds were isolated from alcohol extract of the unossified horn of Cervus elaphus L. var. xanthopygus. One of the two compounds was identified as lysophosphatidyl choline (LPC), and its fatty acid composition was clarified. Furthermore, the hypotensive effects of LPC and related compounds were examined in spontaneously hypertensive rats (SH rats).

Materials and Methods

Chemicals—1-\(\alpha\)-Monocapryl lysophosphatidyl choline (C\(_{10:0}\)-LPC), 1-\(\alpha\)-monolauroyl lysophosphatidyl cho-
line (C12:0-LPC), L-\(\omega\)-monomyristoyl lysophosphatidyl choline (C14:0-LPC), L-\(\omega\)-monopalmityloyl lysophosphatidyl choline (C16:0-LPC), L-\(\omega\)-monostearoyl lysophosphatidyl choline (C18:0-LPC) and L-\(\omega\)-monooeyl lysophosphatidyl choline (C18:1-LPC) were obtained from Avanti Polar Lipid, Inc. L-\(\omega\)-Diundecanoyl phosphatidyl choline (C11:0-PC), L-\(\omega\)-ditridecanoyl phosphatidyl choline (C13:0-PC), L-\(\omega\)-dipentadecanoyl phosphatidyl choline (C15:0-PC), L-\(\omega\)-dihexadecanoyl phosphatidyl choline (C17:0-PC), L-\(\omega\)-dienoanoyl phosphatidyl choline (C19:0-PC), L-\(\omega\)-diarachidoyl phosphatidyl choline (C20:0-PC), L-\(\omega\)-dimyristoleoyl phosphatidyl choline (C14:1-PC), L-\(\omega\)-dilinoleoyl phosphatidyl choline (C18:2-PC), L-\(\omega\)-dilinolenoyl phosphatidyl choline (C18:3-PC) and L-\(\omega\)-dieicosanoyl phosphatidyl choline (C20:1-PC) were also obtained from Avanti Polar Lipid, Inc.

CI, C13:0-LPC, C15:0-LPC, C17:0-LPC, C19:0-LPC, C20:0-LPC, C14:1-LPC, C18:2-LPC, C18:3-LPC and C20:1-LPC were obtained by the hydrolysis of the respective phosphatidyl choline with phospholipase A2 according to the method of Wells and Hanahan.6) L-\(\omega\)-Palmitelaidoyl lysophosphatidyl choline (C16:1-LPC) was kindly supplied by Nippon Shoji Kaisha, Ltd.

Animals Male SH rats weighing about 250—300 g (12—14 weeks old) from the colony of the Department of Pharmacology, Jichi Medical School, were used.

Isolation of the Hypotensive Principles from Alcohol Extract of Cervus elaphus L. var. xanthopygus MILNE-EDWARG

The unossified horn of C. elaphus L. var. xanthopygus (1000 g) was finely cut and extracted three times with 50% EtOH (10 l) at room temperature for 18 h. The extracted solution was filtered and the filtrate (7 l) was evaporated to dryness under reduced pressure. The residue (50 g) was extracted with H2O (500 ml) and lyophilized. The lyophilized powder was extracted with various organic solvents. Among them, CHCl3 extract showed the hypotensive effect (−30 mmHg, 5 mg/kg, i.v.) on SH rats. The CHCl3 extract (5 g) was applied to a column of silica gel (200 g). Stepwise elusion of the column was carried out with CHCl3 (a), CHCl3—MeOH (8 : 2, b), CHCl3—MeOH (6 : 4, c), CHCl3—MeOH (4 : 6, d), CHCl3—MeOH (2 : 8, e) and MeOH (f) to give Fr-a (150 mg), Fr-b (1500 mg), Fr-c (300 mg), Fr-d (400 mg), Fr-e (300 mg) and Fr-f (500 mg). The Fr-f, eluted with MeOH, showed the hypotensive effect. The active fraction (f, 500 mg) was subjected to gel filtration on a Sephadex LH-20 column (2.0 × 135 cm) using MeOH as an eluent to give Fr-1 (300 mg), Fr-2 (100 mg) and Fr-3 (100 mg). According to the method of Rouser et al.,7) the active fraction (Fr-1, 300 mg) was chromatographed on a diethylaminoethyl (DEAE)-cellulose column (2.5 × 30 cm) with CHCl3—MeOH (9 : 1, 700 ml, A), CHCl3—MeOH (9 : 1, 300 ml, B), CHCl3—MeOH (7 : 3, 1000 ml, C), MeOH (500 ml, D), CHCl3—AcOH (3 : 1, 500 ml, E), AcOH (1000 ml, F) and MeOH (300 ml, G) as eluents to give Fr-1-A (150 mg), Fr-1-B (100 mg), Fr-1-C (10 mg), Fr-1-D (10 mg), Fr-1-E (10 mg), Fr-1-F (10 mg) and Fr-1-G (10 mg). Two active fractions (Fr-1-A and Fr-1-B) were further purified by preparative high-performance liquid chromatography (HPLC). Apparatus, high-performance liquid chromatograph (Toyo Soda HLC 803 Series A); column, YMC-Pack A-312 ODS (6 × 150 mm); detector, ultraviolet (UV) 215 nm; mobile phase, 95% MeOH; flow rate, 1 ml/min; temperature, ambient. Two active principles with hypotensive activity were designated as Fr-1-A-III (100 mg) and Fr-1-B-IV (5 mg). The Rf value of Fr-1-A-III on thin layer chromatogram was similar to that of lysophosphatidyl choline, and infrared (IR) and proton nuclear magnetic resonance (\(^{1}\)H-NMR) spectral data of Fr-1-A-III were identical with those of an authentic sample of lysophosphatidyl choline (Serdary Research Laboratories) isolated from bovine brain.

Hypotensive 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. SH rats, which showed the blood pressure above 160 mmHg, were used for the assay of the hypotensive activity.

Analysis of Fatty Acid Composition

Fatty acid methyl esters were prepared by methanolysis,8) and determined on a Shimadzu GC-7A gas chromatograph. The inlet system was equipped with a glass column (3.0 mm × 3.0 m) packed with 5% Advance DS on 60—80 mesh Chromosorb W. Column temperature, 200°C; injection temperature, 250°C; nitrogen flow rate, 28 ml/min.

Results

Isolation of Hypotensive Principles from Alcohol Extract of Cervus elaphus L. var. xanthopygus MILNE-EDWARG (Rokuju)

Two hypotensive principles were purified by extraction with 50% ethanol, water and chloroform in that order, followed by the combination of column chromatography and preparative HPLC as shown in Chart I. The effect of each fraction on the blood pressure in SH rats is summarized in Table I.

Identification of Fr-1-A-III

The physico-chemical properties of Fr-1-A-III were as follows: Dittmer—Lester and
Dragendorff reagents gave bluish and yellow colors on the thin layer chromatogram (TLC), respectively. These findings indicate that Fr-I-III may be a phospholipid containing choline. Furthermore, the $R_f$ values of Fr-I-III (0.15 in solvent system A, CHCl₃ : MeOH : AcOH : H₂O = 50 : 30 : 8 : 4; 0.13 in solvent system B, CHCl₃ : MeOH : 28% NH₄OH = 65 : 35 : 5; 0.08 in solvent system C, CHCl₃ : MeOH : H₂O = 65 : 25 : 4) on TLC were similar to those of LPC. Thus, the $^1$H-NMR and IR spectra of Fr-I-III were measured. The $^1$H-NMR and IR spectra of Fr-I-III were identical with those of an authentic sample of LPC. Thus, Fr-I-III was identified as LPC.

**Fatty Acid Composition of the Hypotensive Compound, Fr-I-III**

As shown in Table II, the methyl esters of fatty acids were separated into 8 components by gas chromatography. The fatty acid composition consisted of myristic, pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic and linoleic acids. Among them, the predominant fatty acid was palmitic acid and its content was 46.1%. The contents of the unsaturated fatty acids, palmitoleic, oleic and linoleic acids, were 6.8, 31.5 and 5.6%, respectively. The ratio of saturated and unsaturated fatty acids was approximately 1 : 1.
Effects of Various Lysophosphatidyl Cholines (LPCs) on the Blood Pressure in Anesthetized SH Rats

Table III shows the correlation between dose and hypotensive activity of LPC in SH rats. Hypotensive activity was seen at dose levels higher than 1.0 mg/kg, and this activity increased strongly with increasing dose of LPC. LPC showed a transient hypotensive effect (-53 mmHg, 3 mg/kg, i.v.) and the blood pressure recovered to the original level within one minute. LPC also caused pronounced hypotension in normotensive rats (data not shown).

Next, the relationship between fatty acid composition and hypotensive activity was examined in SH rats. The results are summarized in Tables IV and V. Of eleven LPCs with C10:0 to C20:0 fatty acids, all the LPCs except for C10:0 and C20:0 fatty acids showed the hypotensive effect, as shown in Table IV. Among them, C14:0-LPC showed the strongest hypotensive activity, followed by C16:0-LPC and then by C13:0-LPC.

On the other hand, of the six LPCs with unsaturated fatty acids, only C16:1-LPC showed the hypotensive effect, as shown in Table V. Its activity tended to be weaker than those of LPCs with saturated fatty acids.

Discussion

Two hypotensive compounds were isolated from alcohol extract of Rokojo by the use of
SH rats as a screening system (Table I). One of the two compounds was identified as LPC. D-1,9) which occurred in acetone extract of bovine brain and might be LPC or closely related compound, was already reported to have hypotensive activity. In addition to the hypotensive effect,10,11) LPC has antitumor activity,12) causes activation of various enzymes13) and has hemolytic activity.14) However, the isolation of LPC as a hypotensive factor from alcohol extract of Rokujo has been reported for the first time in this paper. It was found that at least a part of the hypotensive action of alcohol extract of Rokujo is due to LPC. Next, the fatty acid composition of LPC isolated from alcohol extract of Rokujo was examined (Table II). The predominant fatty acid was the C_{16:0} acid and its content was 46.1%. The contents of unsaturated fatty acids such as C_{18:1}, C_{16:1} and C_{18:2} were 31.5, 6.8 and 5.6%, respectively. The effects of various LPCs on blood pressure in anesthetized SH rats were next examined (Tables IV and V). Among the sample tested, C_{14:0}-LPC showed the strongest hypotensive activity (−81.7 ± 1.6 mmHg, 3 mg/kg, i.v.), followed by C_{16:0}-LPC (−76.7 ± 7.3 mmHg, 3 mg/kg, i.v.). Among LPCs with unsaturated fatty acids, only C_{16:1}-LPC showed the hypotensive effect (−37.5 ± 2.5 mmHg, 3 mg/kg, i.v.). LPC isolated from alcohol extract of Rokujo is rich in C_{16:0} and C_{16:1} acids, which showed rather strong hypotensive effect. The relationship between the fatty acid composition in LPC isolated from alcohol extract of Rokujo and the hypotensive activity is of considerable interest, since it may cast light on the mechanism of the hypotensive action of LPC. As mentioned above, it is well known that LPC has hemolytic activity. Thus, the relationship between the hemolytic and the hypotensive activities of LPC was investigated. First, the hemolytic activity of various LPCs was examined with the red blood cells of rats. C_{18:0}-LPC, C_{18:1}-LPC and C_{16:0}-LPC showed the strongest hemolytic activity among LPCs tested. On the other hand, C_{14:0}-LPC, which showed the highest hypotensive activity, had moderate hemolytic activity, and C_{18:1}-LPC completely lacked hypotensive activity (data not shown). Further, the hypotensive activity of LPC was decreased by the addition of bovine serum albumin or rat plasma, which completely inhibited the hemolytic action of LPC. However, LPC still retained weak hypotensive activity (data not shown). Therefore, it is unlikely that the hypotensive effect of LPC depends only on the hemolytic activity. The mechanism of the hypotensive activity of LPC and the chemical structure of Fr-l-B-IV will be reported in a subsequent paper.

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References and Notes

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