Occurrence of Short-Duration Hypotensive Phospholipid from Dog Peritoneal Dialysate

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Abstract ◻ From the total lipid fraction of dog peritoneal dialysate after freeze drying and extraction, a hypotensive phospholipid was isolated through silicic acid, cellulose, and Sephadex LH-20 column chromatography in a pure grade; it showed a single spot on TLC. The purified hypotensive factor, designated as Peritoneal Dialysate Depressor-I, elicited potent depressor responses in anesthetized rats, and its threshold dose was ~35 μg/kg. The material resisted proteases and 15-hydroxyprostanoate oxidoreductase. In mobility on TLC, the hypotensive factor was distinguishable from water-soluble hypotensive substances and also from depressor lipids. Judging from its behavior on TLC and column chromatography during the purification procedure, the hypotensive factor seems to be a choline-containing phospholipid and shows the general characteristics of lysolecithin, except for its potent hypotensive activity, instability, and positive reactions against 2,4-dinitrophenylhydrazine and 2′,7′-dichlorofluorescein on TLC. The molar ratio of phosphorus–glycerol–choline–ester was ~1:1:1:0.8. Most of the fatty acids were saturated ones such as stearic and palmitic acids.

Keyphrases ◻ Antihypertensive agents—endogenous lipids, phospholipid from dog peritoneal dialysate, TLC, IR, NMR analysis ◻ Phospholipids—antihypertensive activity, endogenous phospholipid from dog peritoneal dialysate, TLC, IR, NMR analysis

Reports concerning the cardiovascular activity of endogenous lipids other than prostaglandins, especially phospholipids, have presented only limited information. Certain papers reported the renin inhibitory activity of lysophosphatidylethanolamines (1-acyl-*sn*-glycero-3-phosphoethanolamine) (1–4). Martini and coworkers (5–12) extracted vasoactive lipids from various sources, but the compounds remain unpurified. Previous papers reported purification of the vasoactive lipids in the tissues and fluids and isolation of "Depressor-I" (13) and 2,2,6,6-tetramethylpiperidone-(4) (14) from normal and protease-treated bovine brains, respectively.

The hypotensive activity of synthetic L-α-lysolecithins (1-acyl-*sn*-glycero-3-phosphocholine), carrying various fatty acid moieties on anesthetized rats also was reported (15). Moreover, a strong and unique vasoactivity was observed in L-α-lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate) obtained from crude soybean lecithin (16–18).

The present paper reports the chromatographic purification of a potent hypotensive factor from dog peritoneal dialysate. The substance is considered to be a phospholipid and tentatively designated as Peritoneal Dialysate Depressor-I.

EXPERIMENTAL

Chemicals—Prostaglandins were used as received[1]. L-α-Lysolecithin (palmitoyl), DL-α-lecithin (dipalmitoyl) (1,2-dipalmitoylglycero-3-phosphocholine), and sphingomyelin[2] (from bovine brain) were used without further purification. Also used as received were urethan[3] and pentobarbital sodium[4]. Chloroform, methanol[5], and other solvents were redistilled before use.

Bioassay—For routine assay, male Wistar rats, 240–260 g, were used under urethan anesthesia (1.8 g/kg ip) or pentobarbital anesthesia (50 mg/kg ip). The trachea and left carotid artery or left femoral artery were cannulated, and the latter was connected to a direct-writing mercury manometer. The arterial blood pressure response was recorded on a slow-moving smoked drum. The pressure response also was recorded by a pressure transducer[6] connected to a multipurpose polygraph[7].

The right femoral vein was cannulated and connected to a polyethylene tube for sample application. Samples were dissolved in 0.15 ml of saline (0.9% w/v NaCl), injected, and flushed with 0.1 ml of saline.

Purification of Peritoneal Dialysate Depressor-I—Conscious adult dogs were injected with 50–100 ml of 5.5% glucose solution/kg ip, and peritoneal dialysate was withdrawn after 3 hr according to a literature method (9). The dialysate was frozen immediately, lyophilized, extracted with chloroform–methanol (2:1 v/v), and washed with one-fifth of its volume of 0.7% NaCl solution (19). The chloroform-rich layer was separated and dried *in vacuo*. Approximately 300 mg of the total lipid fraction/liter of peritoneal dialysate was obtained. Because the crude extract (I) thus obtained was soluble in ethanol and partially soluble in acetone, it appeared that the lipid fraction consisted of simple lipids and lecithin.

For the purification of the crude extract (I), columns were prepared as follows:

1. Silicic acid[8] was activated at 110° overnight and partially deactivated (activity II B) before use. Silicic acid (50 g) and magnesium trisilicate[9] (25 g) were washed twice with 500 ml of chloroform and hexane and poured into a 2.8 × 60-cm glass column.
2. Fourteen grams of cellulose[10] was suspended in 40 ml of 95% methanol and poured into a 1.8 × 35-cm glass column. The column was washed with methanol and chloroform.
3. One hundred grams of Sephadex LH-20[11] was washed with 500 ml each of methanol, chloroform, and chloroform–methanol (1:1 v/v) or acetone–ethanol (1:1 v/v). The mixture was poured into a 2.7 × 90-cm glass column.

The crude extract (I) was applied to the top of the silicic acid column. Discontinuous gradient elution was carried out with hexane, hexane–chloroform (1:1 v/v), chloroform, and chloroform–methanol (98:2, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, and 40:60 v/v). Finally, methanol was applied. Five hundred-milliliter portions of the eluates were collected. An aliquot of each fraction was used for assay or phosphorus determination. The depressor activity was located in the chloroform–methanol 60:40, 50:50, and 40:60 (v/v) eluates.

From 1.29 g of I equivalent to 4200 ml of peritoneal dialysate, 596 mg of the active fraction (II) was obtained. Fraction II was applied to a cellulose column, and III was obtained. Fraction III was further subjected to a Sephadex LH-20 column, eluting with chloroform–methanol (1:1 v/v). The depressor activity (IV) was eluted in the fractions of the first 150–180 ml.

Finally, IV was chromatographed on another Sephadex LH-20 column with acetone–ethanol (1:1 v/v). The depressor factor was eluted in the first 190–210 ml and purified to an almost pure grade chromatographically (active fraction V).

TLC—In the routine work, the lipids in the chromatographed fractions

[1] Gift from Ono Pharmaceutical Industries Co. Ltd., Japan.
[2] Sigma.
[3] Wako, Japan.
[4] Dainippon, Japan.
[5] Nakarai, Japan.
[6] Nihon Kohden MPU-0.5.
[7] Nihon Kohden RM-45.
[8] Mallinckrodt AR 100 mesh.
[9] Hyflosuper-cel.
[10] Toyoroshi 300 mesh.
[11] Pharmacia.

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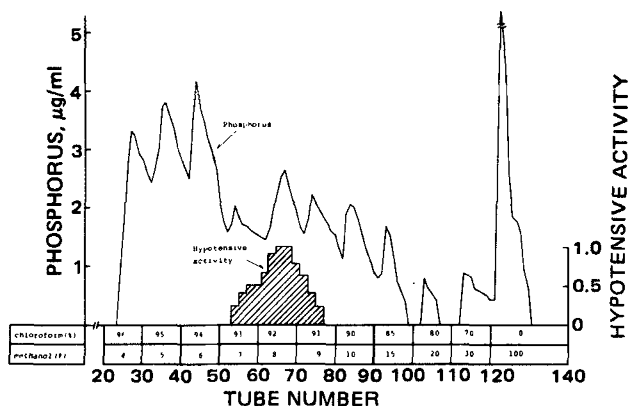

Figure 1—*Elution profile of Peritoneal Dialysate Depressor-I on a cellulose column. Active fraction II obtained through silicic acid chromatography (284 mg) of 2000 ml of original peritoneal dialysate was subjected to a cellulose column and developed with chloroform–methanol with stepwise polarity increases. Ten milliliters of eluate was collected. An aliquot of each tube was taken, dried, dissolved in saline, and bioassayed on an anesthetized rat. Phosphorus was determined according to the method described by Chalvardjian and Rudnicki (21).*

were monitored by TLC on a silica gel plate[12] with acidic, neutral, and basic solvent systems. The lipids were detected by charring with ethanolic sulfuric acid reagent or exposure in iodine vapor. Antimony trichloride, ethanolic phosphoric acid, phosphomolybdate, potassium permanganate, and 2′,7′-dichlorofluorescein spray reagents were used. The following specific spray reagents were used also: Dittmer's for phospholipids, Dragendorff's for choline bases, ferrous hydroxamate for ester groups, osmic acid for unsaturated bonds, ninhydrin for amino groups, ammoniacal silver nitrate and diphenylamine (acid free) for sugar moieties, benzidine for amide groups, mercuric chloride–Schiff's for plasmal, ferrous rhodanide for peroxide groups, periodate–Schiff's for α-glycol groups, and blue tetrazolium for corticoid moieties.

To identify the active material, preparative TLC was performed on a silica gel plate[12]. The active fraction (V) was spotted as a streak in the central part of a plate, and the plate was developed with chloroform–methanol–water (70:30:5 v/v) at −20° in the dark. Each desired zone of the central plate was scraped off horizontally and extracted with chloroform–methanol (1:1 v/v) and with methanol containing acetic acid (0.2 ml in 100 ml). The extracts were decanted off, filtered through a glass filter packed with a small amount of magnesium trisilicate[9], combined, and evaporated to dryness for bioassay. Most extraction procedures were carried out at 4° and completed within 6 hr.

For the partition experiment, aliquots of fraction V, dissolved in n-butanol, were added with equal volumes of distilled water, which were first adjusted to the desired pH. After vigorous shaking for 10 min at room temperature, n-butanol layers were separated from aqueous layers by centrifugation.

Both layers were neutralized and evaporated *in vacuo* for bioassay. The stability of the active material (V) was examined. Aliquots of V were dissolved in distilled water, adjusted to the desired pH, and incubated at 37° for 8 hr. The aqueous solutions were dried *in vacuo* after neutralization for bioassay. Fraction V was incubated with subtilisin or trypsin in phosphate buffer at pH 7.0 at 37° for 30 min.

Each reaction mixture was extracted with chloroform–methanol (2:1 v/v) three times and washed (19). The combined organic layers were dried over anhydrous sodium sulfate and evaporated *in vacuo.* Fraction V was tested with 15-hydroxyprostanoate oxidoreductase (20). To estimate the functional groups in V (194 µg containing 9.2 µg of phosphorus), various chemical reactions were tested and the recoveries of depressor activity were examined as follows:

1. With 5 ml of 0.1% hydrogen peroxide solution, the sample was kept at 25° for 30 min. An aliquot was assayed after drying *in vacuo*.
2. In 2 ml of chloroform sample solution, 10 ml of diluted bromine solution in chloroform was added, and the mixture stood at room temperature overnight.
3. To the sample dissolved in 5 ml of chloroform, diazomethane was added; the solution was kept at 0° for 12 hr in the dark.

[12] Merck silica gel plate 60, 9.25-mm thickness.

Table I—Mobilities of a Peritoneal Dialysate Depressor-I on TLC [a]

Substance	Solvent 1 [b]	Solvent 2 [c]	Solvent 3 [d]
Peritoneal Dialysate Depressor-I	0.18	0.29	0.13
Prostaglandin A_2	0.89	0.97	0.83
Dinoprostone	0.82	0.93	0.73
DL-Lecithin	0.52	0.86	0.53
L-α-Lysolecithin	0.16	0.28	0.11
Sphingomyelin (from bovine brain)	0.29	0.61	0.35

[a] Silica gel 60 TLC plate and iodine vapor detection. [b] Chloroform–methanol–acetic acid–water (260:100:4:14 v/v). [c] Chloroform–methanol–water (70:30:5 v/v). [d] Chloroform–methanol–0.3 *N* ammonia (230:90:15 v/v).

4. A chloroform solution of the sample and triphenylphosphine (500 µg) was allowed to stand for 5 hr.
5. A mixture of the sample dissolved in chloroform and phenyl isocyanate (5 mg) dissolved in dry benzene (2 ml) was left for 24 hr, treated with ethanol, dried, extracted with chloroform, and centrifuged. Both extract and sediment were bioassayed suspended in saline containing 2% polysorbate 80[13].

Analytical Procedure—Phosphorus was determined by the Chalvardjian–Rudnicki method (21). The glycerol (22) and choline (23, 24) contents were also determined. Fatty acid methyl esters prepared by methanolysis (25) were determined by GLC[14] and confirmed by combined GLC–mass spectrometry[15]. The inlet system was equipped with a glass column (3 mm i.d. × 2 m) packed with 1.5% SE-30 on 60–80-mesh Gas Chrom Q[3].

The column temperature was increased from 150 to 260° at a rate of 5°/min; the injection temperature was 260°; the carrier gas was helium at 40 ml/min. The molecular separator was maintained at 260°, and the mass spectrometer ion source was at 270°. Accelerating voltage was 3.2 kv. Spectra were recorded at ionizing energies of 20 and 70 ev. The IR spectrum[16] was recorded by liquid film. NMR[17] was measured by dissolving V (15 mg) in 0.35 ml of deuterochloroform.

RESULTS

Purification of Peritoneal Dialysate Depressor-I—By the described extraction procedure, the hypotensive factor in dog peritoneal dialysate was extracted with high efficiency, and the procedure was more advantageous than previously reported methods. Previous investigators (7–9, 11) reported that the crude extract obtained by their methods elicited hyper- or hypotensive responses, depending on the oxidation–reduction state of the extract. However, the total lipid fraction of dog peritoneal dialysate obtained in the present investigation elicited only depressor responses by intraperitoneal injections into anesthesized rats.

The behavior of the lipid fraction toward solvent fractionation suggests that the crude lipid contains no cephalins or mucolipids and that the hypotensive factor possibly belongs to the lecithin fraction. On silicic acid chromatography, a depressor factor was located on the eluates obtained at the near chloroform–methanol (50:50 v/v) mixture; no detectable hyper- or hypotensive activity was observed in other fractions. In subsequent cellulose chromatography, the depressor activity was located in the chloroform–methanol eluates (93:7, 92:8, and 91:9 v/v) (active fraction III) (Fig. 1). This cellulose chromatography was followed by gel filtration.

The elution pattern on a Sephadex LH-20 column eluting with chloroform–methanol (1:1 v/v) is depicted in Fig. 2. The chemical analysis showed the association of detectable organophosphorus with the hypotensive activity, suggesting that the active factor was a phospholipid resembling lysolecithin and apparently distinguishable from primary prostaglandin family compounds (active fraction IV). In the last chromatography on the second Sephadex LH-20 column, eluting with acetone–ethanol (1:1 v/v), the remaining minor contaminants such as sphingomyelin and lecithin were eliminated (active fraction V). The active depressor factor showed a single spot on TLC with three different solvent systems (Table I). Through purification procedures, the depressor

[13] Tween 80, Nakarai, Japan.
[14] Shimadzu gas chromatograph GC-5A.
[15] Hitachi GC–mass spectrometer RMU-6M equipped with datalyzer system.
[16] Hitachi grating IR spectrophotometer EPI-GII.
[17] Jeol NMR spectrometer JNM-PS-100.

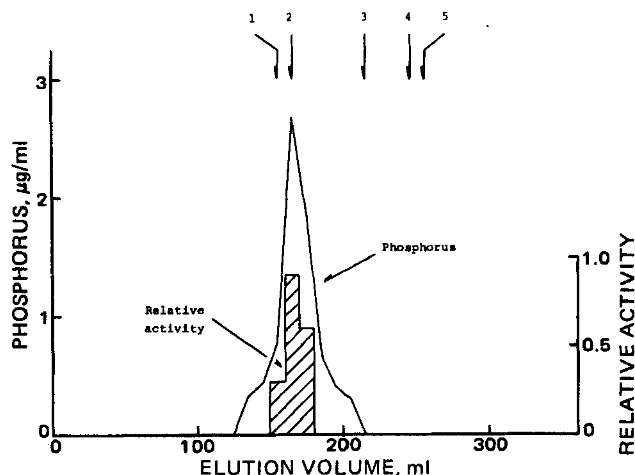

Figure 2—*Elution profile of Peritoneal Dialysate Depressor-I on a Sephadex LH-20 column. Active fraction (III) obtained through silicic acid and cellulose chromatography (3.50 mg), equivalent to 400 ml of original peritoneal dialysate, was subjected to a Sephadex LH-20 column and eluted with chloroform–methanol (1:1 v/v). Ten milliliters of the eluate was collected. Bioassay or phosphorus determination was carried out in the same manner as in Fig. 1. Arrows show locations of reference compounds: 1, tripalmitin; 2, L-α-palmitoyl-lysolecithin (1-palmitoyl-*sn*-glycero-3-phosphocholine); 3, palmitic acid; 4, prostaglandin A_2; and 5, cholesterol.*

activity was recovered almost quantitatively at each stage and the hypotensive factor Peritoneal Dialysate Depressor-I was purified ~150-fold over the crude extract (I). Per liter of original peritoneal dialysate, 1.94 mg of purified hypotensive factor (V) was obtained, which corresponded to ~0.65% of the total dog peritoneal dialysate lipid and contained ~92 µg of phosphorus.

The blood pressure responses of an anesthetized rat to the intravenous injections of the purified Peritoneal Dialysate Depressor-I preparation and to acetylcholine are shown in Fig. 3. A dose of 117.3 µg of the preparation/kg (4 U/kg) evoked a brief hypotensive response (~25 mm Hg) similar to that from acetylcholine. The purified preparation depressor activity was represented with a unit (U) value utilizing acetylcholine as a standard agent. The threshold dose was ~35.2 µg/kg (1.2 U/kg).

Chemical Properties of Peritoneal Dialysate Depressor-I—Although the purified preparation of Peritoneal Dialysate Depressor-I shows a single spot on TLC, whether the spot was responsible for the hypotensive activity was not certain. To solve that question, preparative TLC was carried out at −20°. The zone where the depressor activity was

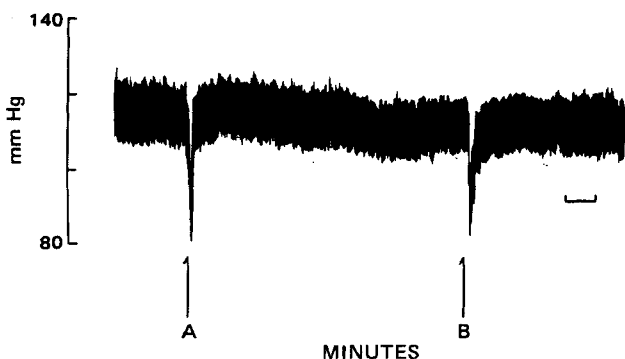

Figure 3—*Arterial blood pressure of an anesthetized rat following intravenous injections of acetylcholine and purified Peritoneal Dialysate Depressor-I. A male Wistar rat, 250 g, was anesthetized with pentobarbital (50 mg/kg ip). Arrows show intravenous sample injections: A, acetylcholine, 5 × 10^{-8} g/kg; and B, purified Peritoneal Dialysate Depressor-I, 4 U/kg (117.3 µg/kg containing 5.57 µg of phosphorus/kg or 0.18 µmole of phosphate/kg). The hypotensive activity of the purified factor was represented with a unit value. One unit (U) equals the response produced by injection of 5 × 10^{-8} g of acetylcholine/kg into the femoral vein of an anesthetized rat weighing 250 g; horizontal scale = 2 min.*

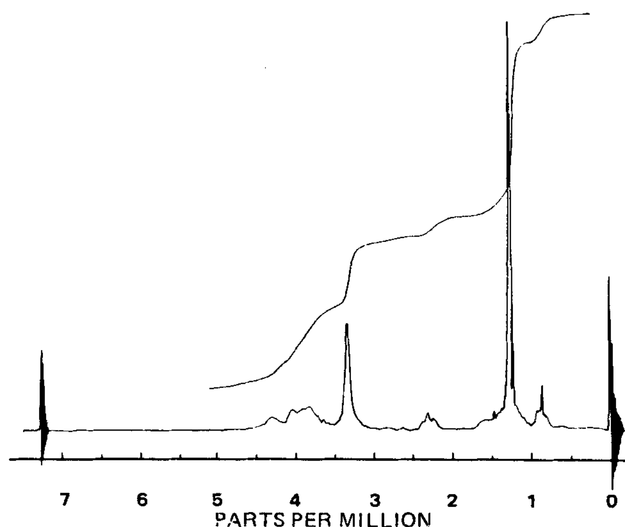

Figure 4—*NMR spectrum of Peritoneal Dialysate Depressor-I.*

recovered corresponded to the spot identified by exposure in iodine vapor; the recovery of the hypotensive activity was excellent (~90%).

The spot also was detectable by charring with ethanolic sulfuric acid reagent or by staining with antimony trichloride, ethanolic phosphoric acid, phosphomolybdate, 2′,7′-dichlorofluorescein, or potassium permanganate reagent. It gave positive tests against the sprays of Dittmer's, Dragendorff's, ferrous hydroxamate, and osmic acid reagents but negative tests against the sprays of ninhydrin, ammoniacal silver nitrate, diphenylamine (acid free), benzidine, mercuric chloride–Schiff's, ferrous rhodanide, periodate–Schiff's, and blue tetrazolium reagents.

A comparison of R_f values of Peritoneal Dialysate Depressor-I with other compounds is shown in Table I. In three different solvent systems, the depressor factor was distinguishable from water-soluble known hypotensive substances such as acetylcholine, histamine, serotonin, and adenosine derivatives and from known depressor active lipids such as prostaglandins and arachidonic acid, although all data were not listed. In a comparison with polar lipids, Peritoneal Dialysate Depressor-I strongly resembled lysolecithin. A purified preparation of the depressor active factor was readily soluble in polar solvents (methanol, ethanol, and *n*-butanol) but sparingly soluble in nonpolar solvents (*n*-hexane, benzene, ether, and ethyl acetate). In partitioning behavior between *n*-butanol and aqueous media at pH 3.0, 7.0, and 10.0, most depressor activity (~95%) was recovered from the organic layer regardless of the pH.

The preparation was relatively unstable and tended to lose activity when exposed to room temperature for a long time, especially when alkaline. The material in a syrup form, however, could be stored for months below −20° under a nitrogen atmosphere without loss of activity. When the preparation stood in aqueous media at 37° for 8 hr at pH 3.0 and 7.0, the depressor activity recoveries were ~80 and 100%, respectively, whereas the recovery was only ~40% at pH 11.0. Since the depressor activity of Peritoneal Dialysate Depressor-I was unaffected by incubations with proteolytic enzymes such as subtilisin and trypsin, it is not a peptide like substance P or bradykinin.

Neither prostaglandin E nor F was found in the purified preparation by a fluorometric determination based on an enzymic cycling system utilizing resazurin, nicotinamide adenine dinucleotide (nadide), and 15-hydroxyprostanoate oxidoreductase. No prostaglandins A, B, E, nor F were detected by GLC in the preparation.

For assessment of functional groups, examinations were performed for estimating the depressor activity recovery after treatment with various chemical reagents. After oxidation with 0.1% hydrogen peroxide, the hypotensive activity disappeared completely. The activity was substantially (~50%) damaged by bromine absorption; after treatment with cold diazomethane or triphenylphosphine, the depressor activity was recovered. Phenyl isocyanate destroyed the hypotensive activity.

No special IR or NMR absorption or signal was observable in the biologically active substance; only those corresponding to common lysolecithin were found, suggesting that the active factor may be a minor component in the lysolecithin fraction. The following absorptions were observed in the IR spectrum of the hypotensive factor: 3400, 2920, 2840, 1730, 1470, 1380, 1230, 1085, 1050, and 970 cm^{-1}. The NMR spectrum (Fig. 4) showed the following signals (δ): 0.88, 1.3, 2.26, 3.30, and 3.6–4.6 ppm.

In the chemical analysis of the purified Peritoneal Dialysate Depres-

sor-I preparation, the molar ratio of phosphorus–glycerol–choline was 1.00:0.99:0.97. The ratio of phosphorus–ester was 1.00:0.81 for the sample obtained by methanolysis of the active fraction. In the fatty acid analysis with GLC–mass spectrometry, stearic (54.0%) and palmitic (39.4%) acids were found most abundantly, similar to lysolecithins in mammalian organs and fluids. Oleic (3.1%) and myristic (1.8%) acids were the next most abundant. No other acids were detectable. No methylal of long chain aldehyde due to plasmal was detected.

DISCUSSION

In the present investigation, the hypotensive factor Peritoneal Dialysate Depressor-I was highly purified to show a single spot on TLC by extraction and repeated column chromatography. This active factor showed brief, potent hypotensive effects in anesthetized animals in dosages of some 10 μg/kg. Peritoneal Dialysate Depressor-I was a principal vasoactive factor in the total lipid fraction of the dog peritoneal dialysate, but its content was very minor.

Judging from the mobilities and findings obtained by TLC, the depressor factor resembles lysolecithin, and its effect was not due to known water-soluble compounds such as acetylcholine, histamine, serotonin, adenosine derivatives, lactic acid, or 2,2,6,6-tetramethylpiperidone-(4). The active peptides may be eliminated by the study with proteolysis. The possibility that the depressor effect is ascribable to prostaglandin compounds may be eliminated because Peritoneal Dialysate Depressor-I was more polar than the primary prostaglandin family compounds on column chromatography and TLC. On gel filtrations, the depressor active factor also was distinguished from prostaglandins. No report with phospholipid derivative of prostaglandins has been published. Lands and Samuelsson (26) were unsuccessful in isolating a phospholipid prostaglandin derivative from 1-palmitoyl-2-([2′-^{14}C]eicosatrienoyl)-glycero-3-phosphorylcholine by incubation with the seminal vesicle microsomal fraction.

No primary prostaglandin was detected in the hypotensive preparation by GLC. The presence of prostaglandins E or F was not detected enzymatically by assaying with 15-hydroxyprostanoate oxidoreductase. The hypotensive factor does not belong to a peroxide compound such as the prostaglandin endoperoxide intermediate since no detectable color was developed by the ferrous rhodanide reagent on TLC. Furthermore, its activity was recovered after treatment with triphenylphosphine.

Other known hypotensive lipids can be eliminated also. Peritoneal Dialysate Depressor-I resembles lysolecithin, but apparent differences were observed between two. The depressor active factor was detectable with 2,4-dinitrophenylhydrazine and by 2′,7′-dichlorofluorescein on TLC. In a comparison of hypotensive potency, the depressor factor showed a much stronger effect than the usual lysolecithin occurring in mammalian tissues. On the basis of phosphorus content, the hypotensive factor is ~60 times more potent than L-α-stearoyl-lysolecithin (15). The hypotensive factor was more unstable than lysolecithin, especially in alkaline conditions. In the active factor, the molar ratio of phosphorus–glycerol–choline was ~1:1:1; its fatty acid composition was mostly stearic and palmitic acids, like ordinary lysolecithin.

However, the molar ratio of phosphorus–ester was ~1.0:0.8, and this molar ratio gap differentiates the active factor from lysolecithin. The possibility remains that the active factor is new and different from the usual known lysolecithin, although its concentration may be very minor.

Several reports have been published on the hypotensive effects of lysophosphatidylethanolamines extracted from various animal kidneys (1–4). The effects of lysophosphatidylethanolamines were only observed in rats under physiologically abnormal conditions (acute or chronic renal hypertension) and were considered to be due to inhibition of the renin–angiotensin–aldosterone system. On the other hand, L-α-lysophosphatidic acid had strong hypertensive effects in rats and strong hypotensive effects in cats (18), although L-α-lysophosphatidic acid was first obtained from crude soybean lecithin (16, 17). The lyso-type phospholipid structure appears to have a close relation to the cardiovascular effect. Peritoneal Dialysate Depressor-I is considered to be a phospholipid closely resembling lysolecithin and possibly an analog, although its chemical structure remains undefined. Further pharmacological properties of the hypotensive factor will be reported.

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