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EFFECT OF HUMAN KIDNEY LIPIDS ON HUMAN KIDNEY RENIN ACTIVITY*

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Abstract—The major lipids of human kidney tissue were isolated by solvent extraction, and the lipid composition was determined by thin-layer chromatographic techniques. The positional distribution of fatty acyl groups in ethanolamine and choline phosphatides was determined after enzymatic hydrolysis. Major phosphatides were assayed for plasmalogen content. Triglycerides were characterized by argentation chromatography. The fatty acyl composition of these lipids was also determined. The effect of intact triglycerides, phospholipids, 1- and 2-monoacyl phosphatides and ether lipids on renin activity *in vitro* was determined by incubations with 3-[U^{14}C]valyl tetradecapeptide renin substrate. Kidney triglycerides, 1-monoacyl and 2-monoacyl phosphatidylethanolamines and phosphatidylcholines significantly inhibited renin activity. The renin-inhibitory effect of these lipids was comparable to inhibition by hog kidney phospholipid inhibitor. The intact phospholipids and cholesterol potentiated human kidney renin activity. Phosphatidylserines and synthetic glyceryl ether lipids have no significant effect. These results indicate that lipid-induced inhibition of human renin activity does not require the ethanolamine moiety, acyl group unsaturation, or the presence of a hydroxyl group at the 2-position. Additionally, no specific structure–activity relationships can describe lipid–renin interactions.

The concept of endogenous mediators of the renin–angiotensin system has existed for several years. The antihypertensive function of the kidney [1, 2] was proposed in relation to the experiment of Goldblatt *et al.* [3] but Tigerstedt and Bergman [4] were the first to show an increase in the pressor response to renin after nephrectomy. This observation, and reports of increased angiotensin generation in plasma from nephrectomized animals [5–7] implied that a renin inhibitor was present in normal renal tissue. Some investigators have also shown a potentiation in the pressor response to angiotensin in nephrectomized dogs [8, 9] and unaltered [10, 11] and increased [12, 13] responses to angiotensin in nephrectomized rats. Pickens *et al.* [14] added a constant amount of renin to different human plasma samples and found that the angiotensin generated in a 4-hr incubation varied significantly. Other similar observations [15–17] suggested the presence of renin activators or inhibitors in plasma.

Antihypertensive lipids from extracts of animal renomedullary tissue were first identified by Muirhead *et al.* [18]. Some of these medullary lipids were found to be prostaglandins [19, 20] and others neutral lipids [19, 21, 22]. These and other relations between the renal medulla and the hypertensive state in experimental animals [23] supported the view that the renal medulla acts as an antihypertensive organ [24].

Naturally occurring phospholipids are among the recently proposed antihypertensive substances. Phospholipid material isolated from acetone extracts of dog kidney has been shown to inhibit the action of dog renin on dog renin substrate *in vitro*, and reduce the blood pressure of chronic and renal hypertensive rats and dogs *in vivo* [25, 26]. The lysophosphatide product of the phospholipid was shown to be much more potent in terms of its ability to decrease blood pressure than the parent phospholipid [26]. The parent substance was termed preinhibitor [27] because the "active" inhibitor was derived from the phospholipid by phospholipase hydrolysis and provisionally identified as a lysophosphatidyl amino acid derivative [25]. Preinhibitors were also found in dog kidney [25], dog [28, 29] and human plasma [29, 30], human erythrocytes [28], blood of anephric humans [31], rat liver, rat heart and erythrocytes [29, 32, 33] and shark kidney [34].

Initially, the preinhibitor was reported to be nearly identical to bovine phosphatidylserine [25], but several reports favored the view that the preinhibitor was a phosphatidylethanolamine and the "active" inhibitor its lysophosphatidyl derivative [29, 33, 35, 36]. The active inhibitor from shark kidney was also identified as a polyunsaturated lysophosphatidylethanolamine [34]. Synthetic lysophosphatidylethanolamines [37], dilenolenyl phosphatidylethanolamine [38], and ethanolamine derivatives esterified with 1-adamantyl moieties [39] have been shown to inhibit renin *in vitro*.

Several recent reports have questioned the phospholipid theory of renin inhibition. Tinker *et al.* [29] stated that "inhibitory activity could not be assigned rigorously to a lysophosphatide structure, or to a specific class of phosphatide, or linked to the kidney, or attributed to a high content of polyunsaturated fatty acids." They were also unable to demonstrate renin inhibition *in vivo* by phospholipids from dog kidney.

The failure to observe a decrease in the circulating level of preinhibitor from blood of anephric rats [32], dogs [28] and humans [31] led some workers to

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believe that the kidney may not be the only source of preinhibitor. Consequently, an attempt to correlate phospholipase activity in nephrectomized animals with availability of the active renin inhibitor was studied to reconcile these observations and reports of increased renin activity after nephrectomy. Osmond *et al.* [40] found no change in rat plasma phospholipase activity after nephrectomy, suggesting that nephrectomized rats should have no lack of renin inhibitor. However, results of a recent study on the kinetics of phospholipase activity in serum from anephric rats indicated a decrease in enzyme activity [41] and theoretically a lack of circulating inhibitor.

Conflicting data, and the lack of definitive information regarding either the structure or the effect of purified human lipids on human renin activity provided the impetus for this study. Kotchen *et al.* [42] used exogenous human kidney renin to demonstrate the existence of an acetone-soluble renin inhibitor in normal human plasma; the plasma factor was not identified. Other workers [30] have reported the presence of an unidentified human plasma phospholipid inhibitor of human kidney activity on human substrate. Earlier, the effects of phospholipid inhibitors from human tissue, other than kidney, were studied with dog renin and dog substrate [31, 32].

In this study, human kidney lipids were separated into major lipid classes, and the fatty acid composition of each was determined. Positional analysis was performed on triglycerides, phosphatidylcholines and phosphatidylethanolamines. The effect of these purified lipids on the activity *in vitro* of human kidney renin was then determined.

MATERIAL AND METHODS

Tissue. Human kidneys were obtained at autopsy from adult individuals who died from trauma, quick-frozen, and stored (−85°). All kidneys were removed within 3 hr postmortem. Cryostat sections of each kidney were examined and judged normal. At the end of the collection period, the frozen kidneys were partially thawed, bisected laterally, freed as completely as possible of superficial fat and connective tissue, weighed and extracted immediately.

Preparation of human renin. Human renin was extracted from ground kidney tissue as described in detail previously [43]. The crude renin extract was purified at 5° by ascending gel filtration chromatography with Pharmacia K26/100 columns packed with Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The columns were standardized with Pharmacia Blue Dextran 2000 and protein molecular weight standards described earlier [43]. A crude renin sample (20 mg protein) was applied to the column and renin Fraction B (mol. wt = 39,500) was eluted with 0.05 M sodium phosphate–0.1 M NaCl buffer, pH 6.9 (phosphate–saline buffer). The buffer flow was maintained at 15 ml/hr with an LKB 1200 pump (LKB Instruments, Inc., Rockville, Md.). The A_{280} of the effluent was monitored (LKB Uvicord II) and 3-ml fractions were collected. Protein concentrations of samples were determined using crystalline human albumin as standard [44].

Extraction of tissue lipids. While semi frozen, five human kidneys were cut into small pieces and homogenized in 2:1, v/v, chloroform–methanol (30 ml solvent/g of tissue) containing 10 mg of butylated hydroxytoluene/100 ml of solvent. A Potter–Elvehjem homogenizer was used to obtain a final slurry and the lipids were extracted by the method of Folch *et al.* [45]. The extract was concentrated with a rotary evaporator and treated with methanol to remove traces of residual water. Non-lipid contaminants were removed on a Sephadex G-25 column by the method of Wuthier [46]. The purified total lipid extract was concentrated *in vacuo*, dissolved in chloroform, and separated into neutral and phospholipids by silicic acid column chromatography [47]. After the two fractions were brought to near dryness, the neutral lipids were dissolved in chloroform and the phospholipids in chloroform–methanol–benzene (1:1:1, by vol). The volumes of each were recorded and aliquots were taken for gravimetric analysis and phosphorus determinations [48]. The neutral and phospholipid fractions were stored under nitrogen at −85° prior to further analysis.

Separation of neutral lipid classes. The neutral lipid fraction was qualitatively separated into classes on 0.25-mm Silica gel H plates (Brinkmann Instruments, Inc., Westbury, N.Y.) using a two-dimensional thin-layer chromatography (t.l.c.) system [49]. Preparative t.l.c. class separation of neutral lipids was performed on 0.50-mm-thick Silica gel H plates developed in hexane–ether–acetic acid (90:10:1, by vol). The bands containing individual classes were scraped from the plate and eluted from the gel with chloroform–methanol (2:1, v/v). The pooled eluates were checked for class homogeneity; if found to be contaminated, they were chromatographed until homogeneity was obtained. The purified classes were filtered through solvent-washed hard filter paper (Whatman No. 50), brought to a volume, and stored under nitrogen (−85°).

Separation of phospholipid classes. Qualitative t.l.c. of phospholipid classes was performed on 0.25-mm-thick Silica gel G plates using two-dimensional solvent pairs. These solvents included: (1) development with chloroform–methanol–28% aqueous ammonia (65:25:5, by vol.) followed by chloroform–methanol–acetic acid–water (3:4:1:0.5, by vol.), and (2) chloroform–methanol–water (65:25:4, by vol.) followed by l-butanol–acetic acid–water (3:1:1, by vol.) according to Rouser *et al.* [50]. Preparative separation of phospholipids was performed on 0.50-mm-thick Silica gel G plates developed in chloroform–methanol–benzene (1:1:1, by vol.), checked for homogeneity, purified if necessary, and stored under nitrogen (−85°). Phospholipid classes were quantitated by lipid phosphorous analyses [48].

Isolation of triglyceride species. Argentation t.l.c. was used to fractionate the total trigylyceride class into its major component species according to the number of ethylenic linkages per triglyceride molecules. Triglyceride species were resolved on 0.50-mm-thick Silica gel H-silver nitrate (5%, w/w) plates with a solvent system of isopropanol–chloroform (1.5:98.5, v/v) [51]. The separated species were visualized with 0.1% dichlorofluorescein in 95% methanol (w/v), extracted from the gel with 2:1 (v/v) chloroform–

methanol and transesterified with methanolic–HCl prior to gas–liquid chromatography (g.l.c.).

Stereospecific analyses of triglycerides. The fatty acids esterified in each of the three positions of glycerol and the differentiation of the fatty acids in positions 1, 2 and 3 of the triglycerides were determined by the method of Brockerhoff [52]. The formation of 1,2- and 2,3-diglycerides was accomplished by deacylation of the triglycerides with ethyl magnesium bromide. The products were isolated by t.l.c. on Silica gel H layers containing 5% (w/w) boric acid developed in chloroform–acetone (96:4, v/v) according to Thomas *et al.* [53]. They were then converted to phosphatidylphenol derivatives by reacting them with phenyldichlorophosphate, and incubated with *Ophiophagus hannah* phospholipase A_2 (EC 3.1.1.4). The fatty acids were separated from the corresponding lysophospholipid and unreacted phosphatidylphenols by t.l.c. on Silica gel G developed in chloroform–methanol–water (80:25:3, by vol.). The bands were scraped from the plates, eluted, and transesterified for g.l.c.

The accuracy of the stereospecific analyses was confirmed by determining the 2-position fatty acids independently by hog pancreatic lipase (EC 3.1.1.3) hydrolysis [54]. The fatty acid composition of the resulting monoglyceride which represents that of position 2 of the intact triglyceride was determined by t.l.c., transesterification and g.l.c.

Plasmalogen content of human kidney lipids. Plasmalogens (1-alk-1′-enyl-2-acyl phospholipids) were detected in the purified phospholipid fraction by several procedures [55–57]. Prior to phosphorous determinations, the 2-acyl lysophosphatides derived from the plasmalogens were separated from diacylphospholipids and glycerol ether phospholipids by mercuric chloride hydrolysis [55]. A phospholipid fraction aliquot equal to 26 μg phosphorous was applied to a t.l.c. plate. At the conclusion of the chromatography, the separated components were sprayed with ninhydrin, followed by sulfuric acid spray, and charred. Ninhydrin reagent was necessary to clearly distinguish between phosphatidylserines and sphingomyelins. The charred spots were removed from the plates for phosphorous analysis [48]. The plasmalogen percentages were calculated on the quantities of the derived 2-acyl lysophosphatide phosphorous content. Plasmalogen control t.l.c. plates were developed as described, except that distilled water was substituted for the mercuric chloride spray reagent. Blank areas of Silica gel from the plates were analyzed to determine background phosphorous.

Positional analysis of phosphatidylcholine and phosphatidylethanolamine. The distribution of fatty acids esterified at the 1 and 2 positions of phosphatidylcholine was determined by t.l.c. and g.l.c. analysis after enzymatic hydrolysis. Phosphatidylcholines (5 mg) were subjected to *O. hannah* phospholipase A_2 hydrolysis by the procedure of Robertson and Lands [58]. After a 6-hr incubation in 4 mM calcium chloride–0.5 M Tris buffer (pH 7.5), the mixture was washed into a conical flask with 10 ml methanol and 20 ml chloroform, dried over sodium sulfate, and concentrated *in vacuo*. The products, free fatty acids and lysophosphatides containing the fatty acids of position 1 (l-monoacyl-*sn*-phosphatidylcholines) were separated by preparative t.l.c. on layers of Silica gel G developed in chloroform–methanol–water (80:25:3, by vol.) and visualized with dichlorofluorescein. The fatty acids of position 2 and the lysophosphatidylcholines were eluted from the adsorbent with chloroform–methanol (2:1, v/v) and chloroform–methanol–benzene (1:1:1, by vol.) respectively. The hydrolysis products and intact phosphatidylcholines were transesterified prior to g.l.c. analysis.

Phosphatidylethanolamines (5 mg) were hydrolyzed by incubation with *O. hannah* phospholipase A_2 according to the procedure of van Golde and van Deenen [59]. The lysophosphatidylethanolamines (l-monoacyl-*sn*-phosphatidylethanolamines) were isolated by the t.l.c. method described above.

Gas–liquid chromatography. Fatty acid methyl esters were prepared by a modification of the method outlined by Mason and Waller [60]. Each lipid class (4 mg) was taken to dryness with nitrogen and dissolved in 0.7 ml of dry benzene. Methanolic–HCl (0.25 ml) and 2,2-dimethoxypropane (0.50 ml) were added and transesterification proceeded for 12 hr at room temperature. The methyl esters were purified as previously described prior to g.l.c. [48]. The esters (2–5 μl) were analyzed isothermally at 190° with a Beckman model GC 65 gas chromatograph (Beckman Instruments, Fullerton, Calif.) on glass columns (6 ft × 0.25 in.) packed with either 15% DEGS on 80/100 mesh Chromosorb W (AW) or 10% EGSS-x on 100/120 mesh Gas Chrom P (Supelco, Inc., Bellefonte, Pa.). The instrument was equipped with a flame ionization detector; a nitrogen carrier flow rate of 50 cm^3/min was used. Methyl esters were identified by their retention times relative to standards. The amount of each ester present was determined by an Infotronics model CRS-208 automatic digital integrator (Infotronics Corp., Austin, TX.). Internal standardization techniques and calibrated methyl ester standards obtained from the National Institute of Health (Standards D and F) were used for quantitation.

Lipid standards, lipolytic enzymes and reagents. Lipid standards were obtained from Applied Science Laboratories, Inc., State College, Pa.; Supelco, Inc., Bellefonte, Pa.; and the Hormel Institute, Austin, Minn. Glyceryl ethers were obtained from Supelco, Inc. *O. hannah* phosphalipase A_2 and hog pancreatic lipase were obtained from Sigma Chemical Co., St. Louis, MO. Pig kidney phospholipid renin preinhibitor was obtained from Miles Laboratories, Inc., Kankakee, Ill. Thin-layer chromatography indicated that the preinhibitor was phosphatidylethanolamine. The "active" inhibitor, lysophosphatidylethanolamine, and the 2-acyl fatty acids of the preinhibitor were isolated on 0.50-mm-thick Silica gel G plates developed in chloroform–methanol–water (80:25:3, by vol.) after hydrolysis by *O. hannah* phospholipase A_2 [59]. The 2-monoacyl derivatives of choline and ethanolamine phosphatides were prepared with pancreatic lipase [54] and isolated from t.l.c. plates after development in chloroform–methanol–water (80:25:3, by vol.). Unless indicated, all other chemicals used were of reagent grade, obtained from commercial sources.

Specific spray reagents used to identify the lipids included: hydroxylamine for esters, and Schiff's reagent for plasmalogens [61]; molybdenum blue for phospholipids [62]; ninhydrin for free amino groups,

Dragendorff reagent for choline and antimony trichloride for cholesterol [63], and chlorine-benzidine for sphingolipids [64]. Iodine vapor was used as a general non-destructive t.l.c. detection reagent [65] as was 2′-7′ dichlorofluorescein in methanol [63]. Sulfuric acid spray was used to detect organic material present [66].

Labeled renin substrate (TDP) and angiotensin I. The 5-L-isoleucine, 3-L-[U^{14}C]valine tetradecapeptide (TDP) [67, 68] with a sp. act. of 40 mCi/m-mole was obtained from Schwarz BioResearch, Orangeburg, N.Y. High-voltage electrophoresis followed by radioactive strip scanning and quantitation indicated that this material was greater than 98 per cent homogeneous. For the stock solution, TDP was dissolved in 5 ml of distilled water (0.51 nmole/10 μl). Angiotensin I, 5-L-isoleucine, 10-L-[4,5-^{3}H(N)]leucine with a sp. act. of 250 mCi/m-mole, was obtained from New England Nuclear, Boston, Mass., and dissolved in 5 ml of distilled water (0.40 nmole/10 μl). Nonlabeled angiotensin I was purchased from Schwarz/Mann and was used as an electrophoretic carrier (15 nmoles/10 μl of distilled water).

Estimation of renin activity. A renin activity incubation was comprised of 10 μl phosphate-saline buffer (pH 6.9), 10 μl [^{14}C]TDP, and 10 μl of human kidney renin Fraction B [43]. Most incubations were for 30 min at 37°. The angiotensin I formed in the incubation media and the residual [^{14}C]TDP were separated by high-voltage electrophoresis and detected with a radiochromatogram scanner. The electrophoretogram was quantitatively assayed by liquid scintillation methods. These methods were described in detail previously [43]. The counting solution consisted of 7 g of 2,5-diphenyloxazole, 0.7 g of 1,4-bis-[2(4-methyl-5-phenyloxazole)]benzene and toluene to make 1000 ml. Radioactivity in each zone was expressed as absolute activity (dis./min) by the channels ratio method of quench correction [69] and calculated as a percentage of total dis./min on the electrophoretogram. The amount of [^{14}C]angiotensin I (product) formed was calculated from the percentage of activity in the product and the amount of substrate incubated, assuming a 1:1 molar relationship, and used as a basis for the calculation of enzyme activity.

Estimation of the effect of lipids on renin activity. The hydrolysis of [^{14}C]TDP by human kidney renin in the presence of purified human kidney lipids, their 1- and 2-monoacyl phosphoglyceride derivatives (lysophosphatides), and commercially available lipids was assayed using the system *in vitro* described above. Lipids were added as suspensions with phosphate-saline buffer. Lipid solutions were prepared immediately before incubation by exposure to ultrasonic vibrations at 0° for not more than 30 sec using a Biosonik IIA ultrasonic probe (Brownwill Scientific, Rochester, N.Y.). Several incubations were conducted with ethylene glycol monomethyl ether (EGMME) as a lipid vehicle. Heat-inactivated renin was included in each experiment as a negative control. Each lipid suspension and labeled angiotensin I standard were electrophoresed together to detect any changes in electrophoretic mobility of the product. The change in [^{14}C]angiotensin I product relative to renin activity controls served as a measure of the lipid effect. Inhibition was expressed as a percentage of the amount of [^{14}C]angiotensin I generated in the control incubation less the amount generated in the presence of added lipid, divided by the control level. When the amount of angiotensin I generated in the presence of lipid exceeded control levels, activation was expressed as a fold increase above the per cent of angiotensin I formed in the control.

RESULTS AND DISCUSSION

The total lipids isolated from human kidney by solvent extraction and partitioning comprised 2.7 per cent of the mean kidney wet weight (85.5 $\pm$ 12.1 g). Water accounted for 80.1 per cent of the kidney weight. The human kidney lipid extract contained 58.5% phospholipids, which were made up of about 27% choline and 23% ethanolamine phosphatides with smaller amounts of phosphatidylserines, phosphatidylinositols, sphingomyelins and diphosphatidylglycerols. The polar lipid content of human kidney (Table 1) generally compares well with previous studies of human kidney lipids [70, 71], and the kidney lipids reported for bovine and mouse [71], rat [72], pig [73, 74], and sheep [75]. However, we consistently found fewer sphingomyelins than have been reported for human or other mammalian species. Significant proportions of the total phospholipid content of human kidney were comprised of phosphatidylcholine (2.3%), phosphatidylethanolamine (6.6%), and phosphatidylserine plasmalogens (3.2%). The plasmalogen content of ethanolamine and choline phosphatides was 29 and 11 per cent, respectively, somewhat different from those reported for pig [76, 77] and dog [76] kidney. No previous data are available regarding the plasmalogen content of human kidney phospholipids. Nonpolar lipids comprised 41.6 per cent of the human kidney extract, nearly one half of which were triglycerides (47.6%). An abundance of free fatty acids (22.8%) was found, but it seems unlikely that the high free fatty acid content was due to lipolysis, since the cadaveric tissue was obtained less than 3 hr post mortem. The percentage distributions of polar and nonpolar lipids (Table 1) are in close agreement with those reported by Hagen [77] for pig kidney, and Yeung and Kuksis [76] for dog and pig kidney. None of the previous workers quantitated kidney neutral lipid classes.

Structure of human kidney triglycerides. Table 2 shows the major fatty acid composition of intact human kidney triglycerides. Oleic acid (18:1) accounted for about 50 per cent of the total fatty acid composition. More than 70 per cent of the triglyceride fatty acids were unsaturated. Oleic acid and palmitic acid (16:0) were the two most abundant triglyceride fatty acids of human [77], pig [77] and dog [78] kidney. The ratio of saturated to unsaturated fatty acids of human, dog, and pig kidney triglycerides were 31:69, 36:64 and 49:51 respectively.

Intact triglycerides were separated into subclasses according to the degree of unsaturation by silver nitrate t.l.c. Initially the separations were performed qualitatively to determine the major triglyceride types and then preparatively to isolate larger quantities for subsequent g.l.c. analysis. Seven bands were resolved and each was well separated except for band E. The percentage distribution of the seven bands is shown

Table 1. Lipid composition of human kidney

Lipid Class	Amount (% total phosphorus)†	Amount* (wt %)
Total phospholipids		58.52
Phosphatidylcholines	27.2 ± 1.7	
Lysophosphatidylcholines	3.1 ± 0.9	
Phosphatidylcholine plasmalogens	2.3 ± 0.1	
Phosphatidylethanolamines	22.7 ± 1.3	
Phosphatidylethanolamine plasmalogens	6.6 ± 1.5	
Phosphatidylserines	8.2 ± 0.5	
Phosphatidylserine plasmalogens	3.2 ± 0.3	
Phosphatidylinositols	6.9 ± 3.7	
Sphingomyelins	6.8 ± 2.6	
Diphosphatidyl glycerols	4.1 ± 1.0	
Origin	3.9 ± 0.5	
Total neutral lipids		41.61
Triglycerides		47.6 ± 4.3
Free fatty acids		22.8 ± 1.5
Cholesterol		22.3 ± 1.8
Cholesterol esters		5.3 ± 2.0

* Minor quantities of mono- and diglycerides were resolved on overloaded, double-developed plates [64].

† Duplicate determinations of five kidneys, mean ± S.D.

at the top of Table 3. Band A was the largest (49 per cent by weight) and represented the combination of three fatty acids (16:0, 16:1, 18:1). Nearly 50 per cent of human kidney triglycerides (band A) had the class characteristic 011 (saturated, monoene and monoene) and 19 per cent (band B) were of class 111. The major triglyceride subclasses reported for dog kidney [78] were 011, 012 and 001, while those of hog kidney were 011 and 001 [79]. Band B consisted primarily of 16:0, 16:1, 18:1 and 18:2. Each of the remaining bands, with the exception of band E (12.6 per cent), was present in amounts ranging between 2 and 7 per cent. The fatty acid composition of each band is also shown. The distribution of the major

Table 2. Fatty acid composition (mole %) of human triglyceride bands separated by argentation chromatography

Component	Band A	B	C	D	E	F	G	Intact triglyceride	Calculated triglyceride
Per cent total triglyceride	49.0	19.2	6.6	7.2	12.6	3.0	2.4	100	
14:0	6.5	2.7	4.3	6.5	3.9	12.4	13.8	3.6	5.7
15:0		2.9	3.2		7.3			2.3	1.5
16:0	21.3	12.2	16.4	5.5	15.0	17.4	19.8	16.4	17.2
16:1	11.9	10.2	11.7	8.1	10.1	8.1	10.5	9.2	10.9
17:0		1.2	1.8	2.1	5.5	1.6	2.5	1.8	1.3
18:0	3.3	2.7	3.2	1.9	9.4	4.1	6.3	4.4	4.0
18:1	55.7	56.2	37.4	48.7	17.3	23.7	32.5	44.9	47.7
18:2		12.3	22.0	27.0	12.2	32.6	14.6	10.8	8.6
18:3	1.3				4.1			2.6	1.2
20:4					5.2			1.6	0.7
20:5					10.0			2.4	1.2
Saturated	31.1	20.7	28.9	16.1	41.1	35.6	42.4	28.5	
Monoenes	67.6	66.4	49.1	56.9	27.4	31.8	43.0	54.1	
Diene		12.3	22.1	27.0	12.2	32.6	14.6	10.8	
Triene					4.1			2.6	
>Triene					15.2			4.0	

Table 3. Stereospecific analysis of human triglycerides.

	Triglyceride position	Fatty acid 14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:4
Amount of each fatty acid (mole%)	1	7.8	4.1	19.5	9.6	1.8	8.2	25.1	13.7	2.2	8.0
	2	8.4	3.4	22.6	11.6	2.2	7.5	34.8	9.4		
	2*	9.8	3.6	15.4	13.1	T†	3.4	47.1	7.3	T	T
	3	0.3	0.4	9.0	16.3			58.0	17.9	1.9	
Percent distribution of each fatty acid	1	47	52	38	26	44	52	21	33	54	100
	2	51	43	44	31	56	48	30	23		
	3	2	5	18	43			49	44	46	

* From pancreatic lipase hydrolysis
† T = trace, less than 0.5% compositions.

saturated and monoenoic fatty acids in the seven bands and the intact triglycerides can be readily calculated from Table 2.

The results of the stereospecific analysis are shown in Table 3. Data from the Grignard reagent and the pancreatic lipase hydrolysis were within close agreement for each fatty acid at position 2. Saturated fatty acids were represented in nearly equal proportions at positions 1 and 2 of glycerol; comprising 41.4 and 44.1 per cent of positions 1 and 2 respectively. Only 9.7 per cent of the fatty acids esterified to position 3 of glycerol were saturated. Greater percentages of saturated fatty acids have been reported at all three sites for hog kidney [80]. The triacylglycerols from human kidney did not show the common "2-unsaturated" structure. The two most abundant fatty acids at the 3 position were oleic and linoleic acid in both human and hog kidney.

The fatty acid composition of other nonpolar human kidney lipids has been reported elsewhere [81].

Effect of triglycerides on human kidney renin activity. Intact human kidney triglycerides were incubated for 30 min at 37° with 3-L-val[U^{14}C]tetradecapeptide ([^{14}C]TDP) and angiotensinase-free human kidney renin (mol. wt = 39,500) in pH 6.9 phosphate-saline buffer. Renin activity decreased 26.8 per cent when only 100 μg triglyceride was added to the incubation medium (Table 4). Increasing the added triglycerides to 1.0 mg resulted in 76 per cent inhibition. The R_f of angiotensin I, relative to L-histidine, remained 0.60 in the presence or absence of triglycerides and no angiotensin I was formed with heat-inactivated renin.

The molecular weight of human kidney triglycerides, based on the composite molecular weight of the mean fatty acid composition (Table 2) and the gly-

Table 4. Inhibition of human kidney renin activity by human kidney triglycerides

Triglyceride added	% ^{14}C-angiotensin I formed: control	triglyceride	% Inhibition
1.0 mg	24.4	5.8	76.2
0.1 mg	30.6	22.4	26.8
491 ng*	25.7	16.9	34.2
245 ng	25.7	25.3†	1.6
123 ng	25.7	30.0†	

Incubation of human kidney renin (10 μl) mol wt = 39,500, for 30 min at 37° in a medium containing: 10 μl (0.55 nmoles) [^{14}C]TDP, and 10 μl of various concentrations of human triglyceride in phosphate–saline buffer suspension (pH 6.9).
* Amount of lipid equivalent to twice renin substrate concentration.
† No significant change in renin activity at 95% confidence level.

cerol backbone, was estimated to be 446.5. The triglyceride was suspended in phosphate-saline buffer by sonication (30 sec, 0°) so that 5 μl was equivalent to the renin substrate concentration (0.55 nmole). When the added triglyceride was twice substrate concentration (1.10 nmoles), 34 per cent inhibition was obtained. Lower concentrations of triglyceride did not significantly alter renin activity. These decisions were based on the 95 per cent confidence interval about the mean control values 26.45 ± 2.9% angiotensin I, using the one-sided Student *t*-test ($t_{0.05}$, N − 1 = 4).

Structure of choline and ethanolamine phosphatides. Table 5 shows the composition and positional distribution of the major human kidney phosphatides. We considered the structural data of the phosphatidylcholine fraction to be valid since this fraction contained less than 10% phosphatidylcholine plasmalogens. The validity of the results was indicated by the close agreement (1–3 per cent absolute) of the experimentally determined and calculated fatty acid composition. The predominant fatty acids esterified to position 1 of human kidney phosphatidylcholine were 16:0, 18:0, 18:1 and 20:4. Unlike the positional analysis results for phosphatidylcholine of pig kidney [77], rat kidney [82] and other mammalian tissue, the ratio of saturated to unsaturated fatty acid esterified at both the 1 and 2 positions of glycerol was nearly 1:1. The common "2-unsaturated" structure of animal phosphoglycerides was not found in human kidney phosphatidylcholines.

Table 5 also gives the total and positional distribution of fatty acids in the ethanolamine phosphatides of human kidney. These data are presented for purposes of comparison with lysophosphatidylcholine, since both of the human kidney lysophosphatides were studied as potential renin inhibitors. Anderson and Sperling [83] reported that consistently more saturated acids and fewer polyunsaturated acids were recovered after phospholipase A_2 hydrolysis of bovine phosphatides than the original phosphoglyceride composition predicted. The same trend was observed for human kidney phosphatidylethanolamines, and attempts to resolve this apparent discrepancy by published methods [59, 66, 84] failed. The high plasmalogen content (29%) of human kidney phosphatidylethanolamines is probably responsible for the variation in original and reconstituted fatty acid content.

Effect of human kidney phospholipids on renin activity. The calculated molecular weight of choline and ethanolamine phosphatides of human kidney was 584.2 and 575.8 respectively. An absolute increase in renin activity was observed when either of these phosphatides was added to incubations *in vitro* of kidney enzyme and [^{14}C]TDP (Tables 6 and 7). Slight increases in the amount of [^{14}C]angiotensin I formed, relative to control values, were seen with the lipid concentrations equivalent to 1.1 nmoles of renin substrate, 633 ng of ethanolamine and 643 ng of choline phosphatides. Although these increases may be within the limits of experimental error, significant increases in renin activity were detected with larger phosphatide concentrations (1.0 and 0.1 mg).

Several workers have observed renin inhibition in the presence of lysophosphatidylethanolamines extracted from animal tissues and we found this to be true of human renin and human kidney lysophosphatidylethanolamines. However, the renin inhibitory effect of l-monoacyl-ethanolamine and l-monoacylcholine phosphatides was nearly identical, and the effect of both phosphatide derivatives appeared to be concentration dependent. These results are shown in Tables 6 and 7. Differences in renin inhibition by these l-monoacyl-phosphatides may be due to the high plasmalogen content of phosphatidylethanolamines. Because of the recently ascribed physiological and clinical importance of plasmalogens [76], additional investigations of human and other mammalian plasmalogens are being conducted. Tables 6 and 7 also show the effect of 2-monoacyl-ethanolamine and choline phosphatides on human kidney renin. Surprisingly, these pancreatic lipase-derived phosphatides of the intact kidney phospholipids were effective inhibitors of human renin. The action of the 2-monoacyl derivatives does not appear to be concentration dependent, at least within the range of concentrations studied.

Table 5. Composition and positional distribution of fatty acids of human kidney phosphoglycerides (mole%)*

	Phosphatidylcholine				Phosphatidylethanolamine			
Fatty acids	Total	1	2	Reconst.†	Total	1	2	Reconst.
14:0	4.7	4.9	5.9	5.4	3.4	4.6	5.9	5.2
15:0	4.6	3.8	5.6	4.7	5.6	1.6	9.5	5.5
16:0	26.4	29.0	23.4	26.2	14.0	32.1	13.0	22.6
16:1	2.5	5.8	2.8	4.3	2.8	5.3	6.3	5.8
17:0	5.4	0.9	13.9	7.4	1.6		1.9	0.9
18:0	9.8	14.7	5.5	10.1	20.0	27.6	7.4	17.5
18:1	20.4	18.5	21.0	19.7	19.2	27.8	25.1	26.4
18:2	11.0	5.6	17.4	11.5	8.6	1.0	10.2	5.6
18:3	T	0.8		0.4				
20:0	T	0.6		0.3	0.2			
20:1	T	1.0		0.5				
20:4	10.9	14.1	2.1	8.1	23.6		16.7	8.4
20:5	4.3		2.4	1.2	1.0		3.7	1.9

* Mean values of lysophosphatides (1) and fatty acids (2) liberated by three phospholipase A_2 hydrolysis reactions.
† Reconstituted composition, (1 + 2)/2.

Table 6. Effect of human kidney phosphatidylethanolamines and 1- and 2-monoacyl phosphatidylethanolamines on human kidney renin activity

	Intact phosphatidylethanolamines (PE)			1-monoacyl-PE*			2-monoacyl-PE*		
Conc.	%^{14}C-Angio I (control)	%^{14}C-Angio I (PE)	Fold increase† in Angio I	Conc. (mg)	%^{14}C-Angio I	% Inh.	Conc. (mg)	%^{14}C-Angio I	% Inh.
				—	52.8 ± 4.5 (3)‡		—	52.8 ± 4.5 (3)	
1.0 mg	26.8	43.7	1.63	1.0	21.1	60.8	1.0	14.6	72.9
0.1 mg	30.6	39.8	1.30	0.5	27.6	48.7	0.5	19.6	63.6
633 ng	26.5	28.6	1.08	0.1	39.5	26.6	0.1	27.5	48.7
316 ng	26.5	27.3	1.03						
158 ng	26.5	25.2							

Incubation of human kidney renin, mol. wt = 39,500, (10 μl) at 37° for 30 min with 10 μl of [^{14}C]-TDP (0.55 nmoles) and 10 μl of ethanolamine phosphatides sonicated (0°, 30 sec) with phosphate-saline buffer, pH 6.9.

* Lysophosphatidylethanolamines (1-monoacyl-PE) and 2-monoacylphosphatidylethanolamines are products of intact phosphatidylethanolamines from *O. hannah* phospholipase A_2 and pancreatic lipase, respectively.

† Increase in renin activity relative to the control value.

‡ Per cent [^{14}C]Angiotensin I formed in the absence of lipid, mean ± S.D. of three incubations.

Effect of pig kidney phosphatidylethanolamines on human kidney renin. Pig kidney phosphatidylethanolamines, phospholipid renin preinhibitor from Miles Laboratories, Inc., was incubated with phospholipase A_2 to produce the "active" lysophosphatidylethanolamine inhibitor and the 2-position fatty acids. The preinhibitor and the two hydrolysis products were incubated with human kidney renin and [^{14}C]TDP (Table 8). Renin activity decreased 13.8 per cent when 100 μg preinhibitor was added to the incubation mixture and decreased 33 per cent in the presence of the "active" inhibitor (100 μg). The 2-position fatty acids of pig kidney phosphatidylethanolamines had no effect on the percentage of [^{14}C]angiotensin I formed by human kidney renin.

Effect of other lipids on human kidney renin activity. The various biological activities of glyceryl ethers (*O*-alkyl lipids) have been reviewed recently [85, 86], but no reference was made to the effect of this lipid class on renin activity. Because of their similarity to plasmalogens (*O*-alk-1-enyl glycerols), we studied the effect of several representative glyceryl ethers on human kidney renin activity (Table 9). At the concentrations used, glyceryl ethers appear to have no significant effect on the activity *in vitro* of human renin. The alkoxylipids may stimulate renin activity, but the degree of stimulation is not pronounced and does not seem to be related to the structure of a specific glyceryl ether type.

Human kidney renin activity was not affected by

Table 7. Effect of human kidney phosphatidylcholines and 1- and 2-monoacyl phosphatidylcholines on human kidney renin activity

	Intact Phosphatidylcholines (PC)			1-monoacyl-PC*			2-monoacyl-PC*		
Conc.	%^{14}C-Angio I (control	%^{14}C-Angio I (PC)	Fold increase† in Angio I	Conc. (mg)	%^{14}C-Angio I	% Inh.	Conc. (mg)	%^{14}C-Angio I	% Inh.
					47.2 ± 1.6 (3)‡			55.3 ± 2.1 (3)‡	
1.0 mg	26.8	65.0	2.42	1.0	21.9	53.6	1.0	12.8	76.9
0.1 mg	30.6	60.0	1.96	0.5	28.9	38.8	0.5	14.6	73.6
643 ng	26.5	30.9	1.17	0.1	39.3	16.7	0.1	21.7	60.8
321 ng	26.5	30.2	1.14						
161 ng	26.4	27.3	1.03						

Incubation of human kidney renin (10 μl) at 37° for 30 min with 10 μl [^{14}C]TDP (0.55 nmoles) and 10 μl of choline phosphosphatides sonicated with phosphate-saline buffer, pH 6.9.

* 1- and 2-monoacylphosphatidylcholines are the products of *O. hannah* phospholipase A_2 and pancreatic lipase, respectively.

† Increase in renin activity relative to the control value.

‡ Per cent [^{14}C]Angiotensin I formed in absence of lipid, mean ± S.D. of three incubations.

Table 8. Effect of pig kidney phosphatidylethanolamines* on human kidney renin

Description	Amount (μg)	% ^{14}C-angiotensin I formed	% Inhibition
Control		52.8 ± 4.5 (3)	
Preinhibitor*	200	44.9	14.9
	100	45.5	13.8
Inhibitor†	100	35.2	33.3
Fatty acyl†	50	54.8	§

Human kidney renin (10 μl) incubated (30 min, 37°) with 10 μl [^{14}C]TDP (0.55 nmoles) and 10 μl of lipid–buffer suspension, pH 6.9.

* Miles phospholipid renin preinhibitor (Miles Laboratories, Inc., Kankakee, Ill.).

† Lysophosphatide derivative of preinhibitor produced by *O. hannah* phospholipase A_2 hydrolysis.

‡ The 2-position fatty acids of pig kidney phosphatidylethanolamines recovered by T.L.C. after phospholipase A_2 hydrolysis.

§ No significant change in renin activity at 95% confidence level.

human kidney phosphatidylserines when the phosphatide was added in 1.0-, 0.5-, 0.25- or 0.1-mg amounts. The same amounts of cholesterol resulted in a 1.27 ± 0.04 (n = 4)-fold increase in renin activity relative to control incubations. The 2-position free fatty acids of choline and ethanolamine phosphatides (40–50 μg) had no effect on renin activity.

Several renin–lipid incubations were made with EGMME or sodium deoxycholate as the lipid vehicle, but control incubations showed both EGMME and the bile salt to be potent human kidney renin inhibitors. Sen *et al.* [27] studied the action *in vivo* of dog kidney phospholipids on rat renin with the lipid dissolved in a 3 mg/ml aqueous solution of sodium deoxycholate. Their results may be misleading since Hiwada *et al.* [87] found sodium deoxycholate to be a competitive inhibitor of rabbit renin ($K_i = 4.7 \times 10^{-4}$ M). Sen *et al.* [25] also used ethylene glycol as a lipid carrier for inhibition studies *in vitro* of dog renin. Others [29] have used EGMME to dissolve possible lipid inhibitors of dog renin.

In conclusion, previous reports concerning lipid renin inhibitors have attempted to relate lipid structure and composition to changes in renin activity. Renin preinhibitor from dog and hog kidney was characterized by a high percentage of esterified polyunsaturated fatty acids [29, 37, 38]. However, renin-inhibitory activity of synthetic lysophosphatidylethanolamines apparently did not depend on acyl group unsaturation or hydroxyl groups at the 2 position [37]. The specific hydrophobic head of these synthetic inhibitors was not as important as the gross lipid hydrophobicity [37, 39]. Renin-inhibitory activity of dog kidney phospholipid was observed after treatment

Table 9. Effect of synthetic glyceryl ethers on human kidney renin activity

Alkoxylipids*	^{14}C-Angiotensin I formed
None	31.4 ± 2.7 (5)
Glyceryl-1-tetradecyl ether	39.5
Glyceryl-1-hexadecyl ether	40.2
Glyceryl-1-cis-9-octadecenyl ether	37.8
Glyceryl-2-cis-9-octadecenyl ether	36.7
Glyceryl-1-octadecyl ether	37.8
Glyceryl-1,2-cis-9-octadecenyl ether	39.3
Glyceryl-1,2-dipalmitoyl-3-hexadecyl ether	34.7
Glyceryl-1,2-dipalmitoyl-3-octadecyl ether	37.4

Human kidney renin (10 μl) was incubated with 10 μl of [^{14}C]TDP (0.55 nmoles) and 10 μl of each glyceryl ether at concentrations equivalent to twice the TDP concentration (1.1 nmoles). The glyceryl ether suspensions were prepared by sonication with phosphate–saline buffer (pH 6.9) for 30 sec at 0°.

* Glyceryl ether kit No. 04-6160, Supelco, Inc., Bellefonte, Pa.

with phospholipase A, but hydrolysis with either phospholipase B, C or D destroyed all inhibitory activity [26]. Smeby *et al.* [26] reported that phospholipase A cleaved saturated fatty acids from dog kidney phospholipids, while phospholipase B removed unsaturated fatty acids. This misconception lead them to relate unsaturation with renin-inhibitory activity.

Most interpretations concerning the actions of natural lipid inhibitors of renin failed to consider that the phosphoglycerides isolated from animal organs do not represent substances containing homogeneous acyl groups. Naturally occurring phospholipids which have previously been designated as renin preinhibitors were most probably complex classes of phosphoglycerides, indistinguishable by the thin-layer techniques utilized. Additionally, no mention was made of the plasmalogen content of the phosphatidylethanolamine preinhibitors. These factors, together with numerous contradictions in the literature, and the crude methods used by most investigators to isolate potential renin inhibitors, leave serious doubts about the reported structure–activity relationships of lipid renin inhibitors.

Our study indicates no clear structure–activity relationship between purified human kidney phospholipids and human kidney renin. The effects of ethanolamine and choline phosphatides and their monoacyl derivatives on renin activity were surprisingly similar. When present in relatively high concentration, the intact phosphatides, which are complex class mixtures of phosphatidylethanolamines or phosphatidylcholines, both containing plasmalogens, increased renin activity. These observations were expected, since a potentiation of renin activity in the rat by intact kidney phospholipids was reported by others [27, 29]. Lysophosphatidyl derivatives of choline and ethanolamine phosphatides significantly inhibited human kidney renin activity, and the degree of inhibition was concentration dependent. No arachidonic acid (20:4) was detected in l-monoacyl-phosphatidylethanolamines, while approximately 14 per cent was found in the phosphatidylcholine derivative. The plasmalogen content of choline and ethanolamine phosphatides was 11.4 and 29 per cent respectively. Although antioxidant was present, these values may not represent the percentage of plasmalogen in lysophosphatide derivatives, since the labile plasmalogens may have been oxidized during the phospholipase hydrolysis and subsequent isolation procedures. However, synthetic glyceryl ethers, a lipid class similar to plasmalogens, had no significant effect on human kidney renin activity. The 2-monoacyl derivatives of choline and ethanolamine phosphatides were also potent inhibitors of human renin. In contrast to the lysophosphatides, the effect of 2-monoacyl-phosphatides does not seem to be dependent upon lipid concentration, at least within the concentration range studied.

These data indicate that the ethanolamine moiety is not an absolute requirement for inhibition of human kidney renin; like the synthetic renin inhibitors [37], unsaturation in the acyl groups or the presence of hydroxyl groups at position 2 is not necessary for renin-inhibitory activity.

The inhibition of human kidney renin by intact triglycerides (0.1 mg) was comparable to the inhibitory effect seen with pig kidney phospholipid inhibitor. Significant renin-inhibitory activity was also seen when the triglycerides were present at a molar concentration equivalent to twice the renin substrate concentration. This is the first report of human kidney renin inhibition by a class of neutral lipids. The kidney triglycerides were composed of seven major classes identified mainly as class 011. The intact triglycerides had a saturated to unsaturated fatty acyl ratio of 28.5:71.5, and the major constituents were palmitic, palmitoleic and oleic acids. However, the common "2-unsaturated" structure of mammalian triacylglycerols was not a characteristic of the human kidney triglycerides.

Present evidence indicates that no specific structure–activity relationships can describe lipid–renin interactions. The mechanism of lipid-mediated enzyme activity is considered to involve either: (a) lipid interaction with and activation of the substrate, or (b) a direct lipid–enzyme hydrophobic group interaction resulting in a conformational change of the protein. Because the structural configuration of human kidney renin or the natural renin substrate is unknown, and effective analytical methods are not available for complete separation of intact individual molecules within classes of naturally occurring lipids [88], a true mechanism cannot be described for the observed effect of lipids on renin activity. Additionally, recent information indicates that human renin is not a single enzyme but a varied molecular weight class of complex peptidases [43, 89]. If this is true of animal renin, multiple renin forms may explain some of the literature contradictions related to lipid–renin inhibition.

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