Changes in Glycerophospholipid Profile in Experimental Nephrotic Syndrome

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We investigated changes in the glycerophospholipids in kidney tissue and its various intracellular fractions in rats with nephrotic syndrome induced by puromycin aminonucleoside. The ethanolamine plasmalogen, 1-*O*-alk-1′-enyl-2-acyl-GPE (EP), was increased in kidney tissue obtained from the puromycin-treated animals. A similar increase was found in the mitochondria and endoplasmic reticulum (microsomes) of this tissue. These increases were not found in the liver. Since platelet-activating factor (PAF) is known to be produced in increased amounts in inflammatory disorders, it is suggested that the higher plasmalogen found in rat kidneys during experimental nephrotic syndrome might be derived from increased levels of this autacoid. The increase in PAF may also result in the elevation of plasma PAF-acetylhydrolase (AH) activity observed in these animals.

NEPHROTIC SYNDROME is a condition with proteinuria, hypoalbuminemia, edema, and hypercholesterolemia.[1] In addition, hyperlipidemia has long been recognized as a frequent metabolic abnormality in patients with this syndrome.[2] Moorhead et al[3] were the first to suggest that lipoproteins may play a role in the genesis of progressive kidney disease and suggested that plasma lipoproteins may contribute to the tissue damage. It was proposed that the lipoproteins may damage the glomerular basement membrane, either directly or after macrophage-activated peroxidation. Lipid mediators, including eicosanoids, platelet-activating factor (PAF), and other chemotactic factors, have also been proposed to contribute to the leukocyte infiltration, mesangial proliferation, extracellular matrix protein production, vasoreactivity, and coagulation.[1]

We[4] and others[5,6] have reported that PAF is converted into ethanolamine plasmalogen [1-*O*-alk-1′-enyl-2-acyl-GPE] (EP) in a variety of cell types. It was proposed that the enrichment in EP that occurs in amnion tissue late in gestation may be a reflection of the increased production of PAF at this time. It has previously been reported that phospholipids,[7] specifically the phosphatidylethanolamine (PE) fraction,[8] are increased in the urine of rats in which a nephrotic syndrome has been induced by administration of puromycin. PAF is one of the most potent proinflammatory agents known, and it has been suggested that an increase of PAF may play an important role in the altered glomerular permeability to proteins in this tissue.[9] Urinary excretion of PE in patients with chronic glomerular diseases has also been reported.[10] In consideration of these findings, we have investigated the alterations of various phospholipids in the kidney during puromycin-induced nephrotic syndrome. The phospholipid pattern was determined in the whole tissue and in various subcellular fractions of kidney and liver of control and puromycin-treated animals. The activity of both plasma and cytosolic forms of the enzyme responsible for PAF degradation, PAF-acetylhydrolase (PAF-AH), was also examined.

MATERIALS AND METHODS

Male Wistar rats (Simonsen, Gilroy, CA) with a mean weight of 270 g (10 weeks of age) were housed in individual cages on a rotating 12-hour light-dark cycle, and had free access to both standard rat chow and water. Nephrotic syndrome was induced by a single intraperitoneal injection of puromycin aminonucleoside (6-dimethyl-amino-9[3′-amino-3′-deoxyribosyl]-purine; Sigma Chemical, St Louis, MO) 100 mg/kg body weight, which was dissolved in saline at a concentration of 500 mg/mL. Control rats were injected with saline. The animals were housed in metabolic cages, and a 24-hour urine sample was collected on day 7. The induction of nephrotic syndrome was confirmed by marked albuminuria, hypoproteinemia, hypercholesterolemia, and ascites.

Phospholipid analyses and PAF-AH activity measurements were performed 8 days after puromycin injection. Food was withdrawn 18 hours before death. The rats were anesthetized with Nembutal (65 mg/kg; Abbot Laboratories, Chicago, IL). The abdomen was opened, and a blood sample was collected from the abdominal aorta. The kidney and liver were removed, rinsed with ice-cold saline, blotted on filter paper, and weighed.

Tissue Subcellular Fractionation

The kidney and liver were homogenized with a Teflon-glass homogenizer in 5 vol of a solution containing sucrose (0.25 mol/L), EDTA (1 mmol/L), and MOPS [3-(*N*-morpholino)propanesulfonic acid] (20 mmol/L), adjusted to pH 7.4. Homogenization and all subsequent procedures were performed at 0° to 4°C. An aliquot of the homogenate was removed for analysis of the whole tissue. Subcellular fractions were obtained by centrifugation. Cell debris and nuclei were removed following centrifugation at 700 × *g* for 10 minutes. Mitochondria were pelleted by centrifugation (15,000 × *g* for 10 minutes). Microsomes were separated from the remaining supernatant fraction by centrifuging at 105,000 × *g* for 60 minutes. The mitochondrial and microsomal pellets were suspended in sucrose (0.25 mol/L). All fractions were stored at −20°C until use. Protein concentration was determined by the method of Lowry et al.[11]

Lipid Extraction

Total lipids were extracted from whole tissue and subcellular fractions by the method of Bligh and Dyer.[12] Urinary lipids were extracted as follows. Four volumes of urine were mixed with 6 vol of

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methanol for 60 minutes at room temperature. Precipitated proteins were removed by centrifugation at 10,000 × *g* for 10 minutes. Six volumes of chloroform and 1.4 vol of water were added to the supernatant fraction, and the mixture was vigorously shaken. The pellet fraction was reextracted by the above method, and both chloroform fractions were combined. The resulting chloroform phase was dried under nitrogen.

Thin-Layer Chromatography

Prepared silica gel thin-layer plates (0.25-mm thickness, Silica Gel 60 without indicator; Merck, Darmstadt, Germany) were used. A mobile phase consisting of chloroform:methanol:water (65:35:6) was used for separation of phospholipids. Known standards were applied to separate lanes of each chromatographic plate, and their position was established by exposure to I_2 vapor. To confirm the identity of the PE spot, the chromatograms were sprayed with 0.2% ninhydrin in ethanol. For further analysis of the PE fraction, the desired area was scraped from the plate and lipids were extracted by the method of Bligh and Dyer. Glycerophospholipids were quantified by phosphorus analysis according to the procedure previously described.[13]

Cleavage of Plasmalogens (EP)

To cleave the 1′,2′-alkenyl bond in the *sn*-1 position of plasmalogens, the isolated PE samples were applied to the thin-layer chromatography plates. After drying, the plate was subjected to HCl fumes for 30 minutes and dried under N_2 for 30 minutes. The vinyl ether linkage of EP is labile to acid treatment and is cleaved, with the formation of 1-lyso-PE. Diacyl- and alkyl-acyl-PE are unaffected by acid treatment.[14] Thin-layer chromatography was used to separate lyso-PE from PE. Bovine brain PE (Sigma Chemical), which contains a high proportion of EP (60%), was used as control.

Albumin concentrations in urine and plasma were determined by the bromocresol green–binding method of Doumas et al.[15] Urine and plasma creatinine and plasma cholesterol were determined with reagent kits. All kits were obtained from Sigma Chemical. The glomerular filtration rate was determined by creatinine clearance.

PAF-AH activity was measured by the modified method of Miwa et al,[16] as described previously.[17] Statistical comparisons were performed using the Mann-Whitney test, and p less than .05 was regarded as significant. Results are presented as the mean ±SD. All values are expressed as the mean of two experiments.

RESULTS

Physical and Biochemical Parameters

Physical and biochemical characteristics of the puromycin-treated animals are listed in Table 1. The mean body weight of nephrotic rats was higher than that of controls; in all likelihood, this is due to the presence of ascites. Kidney wet weight in nephrotic rats was also higher than in controls. This may be due to tissue edema. Similarly, the mean weight of the liver in nephrotic rats was slightly higher than in control animals, although the difference was not significant.

Marked proteinuria (albuminuria) and ascites were observed in the nephrotic rats. As expected, creatinine clearance and plasma albumin decreased in nephrotic rats. Both total plasma cholesterol and plasma creatinine were significantly increased in these nephrotic animals.

Table 1. Physical and Biochemical Characteristics of Nephrotic Syndrome Rats and Controls

Characteristic	Controls (n = 6)	Nephrotic Syndrome (n = 5)	Significance (*P*)
Weight (g)	287 ± 22	320 ± 38	
Kidney weight (g)	1.12 ± 0.05	1.29 ± 0.08	<.01
Liver weight (g)	10.3 ± 0.5	11.2 ± 1.3	NS
Urine albumin (mg · d^{-1})	50.8 ± 30.8	537 ± 142	<.01
Creatinine clearance (L · d^{-1})	1.15 ± 0.37	0.63 ± 0.25	<.05
Plasma albumin (g · dL^{-1})	3.98 ± 0.15	1.88 ± 0.21	<.01
Plasma cholesterol (mg · dL^{-1})	63 ± 10	416 ± 97	<.01
Plasma creatinine	1.03 ± 0.12	1.30 ± 0.19	<.05

Protein, Glycerophospholipid, and PE Content of Whole Tissue

Total tissue levels of protein, phospholipids, and PE in kidney and liver are listed in Tables 2 and 3. The total phospholipid concentration in nephrotic rats was significantly reduced. However, no differences in the total phospholipids were observed between control and nephrotic kidney when expressed per milligram protein. Similarly, the proportion of PE in total cellular phospholipids was unchanged in both the kidney and liver of nephrotic rats.

Protein, Phospholipid, PE, and Phosphatidylcholine Content of Microsomal and Mitochondrial Fractions in Kidney and Liver

Microsomal protein was reduced significantly in nephrotic rats. However, no differences were found in total

Table 2. Kidney Tissue Composition of Nephrotic Syndrome Rats and Controls

Parameter	Controls (n = 6)	Nephrotic Syndrome (n = 5)	Significance (*P*)
Whole kidney			
Protein (mg · g^{-1} wet weight)	159 ± 15	137 ± 20	NS
Total phospholipids (μg phosphate · g^{-1} wet weight)	878 ± 45	764 ± 49	<.05
μg phospholipid phosphate/mg protein	5.55 ± 0.36	5.63 ± 0.64	NS
Ethanolamine phospholipids/total phospholipids	0.25 ± 0.017	0.24 ± 0.64	NS
Kidney mitochondria			
Protein (mg · g^{-1} kidney wet weight)	20.9 ± 2.9	18.0 ± 3.2	NS
μg phospholipid phosphate/mg protein	11.9 ± 0.7	11.5 ± 0.6	NS
Ethanolamine phospholipids/total phospholipids	0.21 ± 0.022	0.21 ± 0.01	NS
Kidney microsomes			
Protein (mg · g^{-1} kidney wet weight)	21.8 ± 1.0	17.3 ± 1.1	<.01
μg phospholipid phosphate/mg protein	12.0 ± 1.8	11.5 ± 2.3	NS
Ethanolamine phospholipids/total phospholipids	0.23 ± 0.025	0.22 ± 0.016	NS

kidney microsomal phospholipid or PE when expressed per milligram protein. Similarly, no differences were found in any of these parameters in the kidney mitochondrial fraction.

No differences between the control and nephrotic groups were found in the whole liver or any of its subfractions, with the exception that the phospholipid to protein ratio was decreased in the liver microsomal fraction ($P < .005$). No changes were observed in the ratio of choline glycerophospholipids and total phospholipids between control and nephrotic animals in either liver or kidney or their subcellular fractions (Table 3).

We also determined the choline glycerophospholipid content of kidney and liver and the corresponding mitochondrial and microsomal fractions of each tissue. No differences were found between the control and nephrotic animals in either tissue or the subcellular fractions (data not shown). Only trace quantities of choline plasmalogen were found in any of these fractions (<2% to 3%).

EP Content

The PE fraction of whole kidney tissue, microsomes, or mitochondria from rats with nephrotic syndrome contained a significantly higher proportion of EP as compared with control animals (Fig 1). The change in kidney microsomal EP was the greatest and most significant.

On the other hand, liver EP concentration in the whole tissue or subfractions was not different in nephrotic and control rats (Fig 1). These observations would suggest that there is a tissue specificity for increased EP.

Table 3. Liver Tissue Composition of Nephrotic Syndrome Rats and Controls

Parameter	Controls (n = 6)	Nephrotic Syndrome (n = 5)	Significance (P)
Whole liver			
Protein (mg · g^{-1} wet weight)	175 ± 18	181 ± 10	NS
Total phospholipids (μg phosphate · g^{-1} wet weight)	1,060 ± 112	1,121 ± 105	NS
μg phospholipid phosphate/mg protein	6.07 ± 0.21	6.30 ± 0.19	<.05
Ethanolamine phospholipids/total phospholipids	0.23 ± 0.01	0.22 ± 0.02	NS
Liver mitochondria			
Protein (mg · g^{-1} liver wet weight)	17.0 ± 3.7	15.1 ± 3.1	NS
μg phospholipid phosphate/mg protein	9.7 ± 1.2	10.9 ± 1.0	NS
Ethanolamine phospholipids/total phospholipids	0.21 ± 0.01	0.20 ± 0.01	NS
Liver microsomes			
Protein (mg · g^{-1} wet weight)	22 ± 1.5	23.7 ± 1.2	NS
μg phospholipid phosphate/mg protein	14.2 ± 1.2	11.5 ± 1.1	<.05
Ethanolamine phospholipids/total phospholipids	0.20 ± 0.016	0.18 ± 0.028	NS

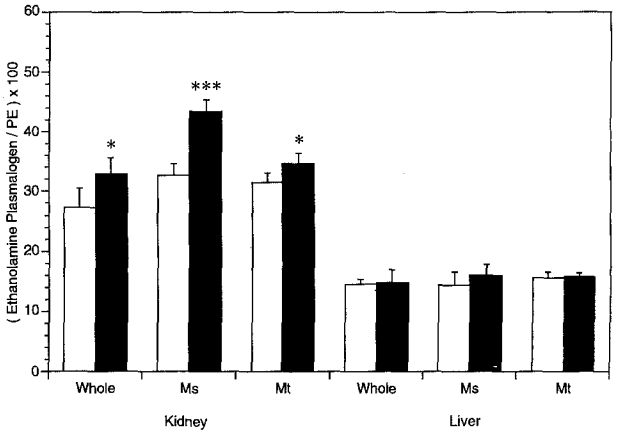

Fig 1. Ratio of EP to total PE in kidney and liver of control rats (□, n = 6) and nephrotic syndrome rats (■, n = 5). Ms, microsomes; Mt, mitochondria. *P < .05, **P < .01.

Urinary Phospholipids, PE, and EP

Urinary PE and EP were significantly higher in nephrotic rats than in control rats (Table 4). These findings of increased urinary content of PE are consistent with previous investigations in which this phospholipid was shown to be elevated both in the rat nephrotic syndrome model[8] and in patients with chronic glomerular disease.[10]

PAF-AH Activity in Plasma and Tissues

As previously discussed, we[4] and others[5,6] have suggested a close relationship between PAF metabolism and EPs. The activity of PAF-AH has been shown to modulate the concentration of PAF.[18] Therefore, we examined the activities of various isozymes of PAF-AH. Plasma PAF-AH and liver and kidney cytosolic PAF-AH activities of control and nephrotic rats were assayed.

Values for plasma and tissue PAF-AH activity in nephrotic and control groups are shown in Fig 2. Plasma PAF-AH activity in the nephrotic group was twice that in control animals. In contrast, no change in specific activity

Table 4. Urinary Phospholipid Composition of Nephrotic Syndrome Rats and Controls

Variable	Controls (n = 6)	Nephrotic Syndrome (n = 5)	Significance (P)
Phospholipids (μg phosphate · d^{-1})	8.0 ± 2.4	59.6 ± 19.9	<.01
Ethanolamine phospholipids (μg phosphate · d^{-1})	1.8 ± 0.6	3.6 ± 0.4	<.01
Ethanolamine plasmalogens (μg phosphate · d^{-1})	0.32 ± 0.11	1.03 ± 0.09	<.01
Urine ethanolamine plasmalogens/total ethanolamine phospholipids	0.17 ± 0.026	0.29 ± 0.033	<.01

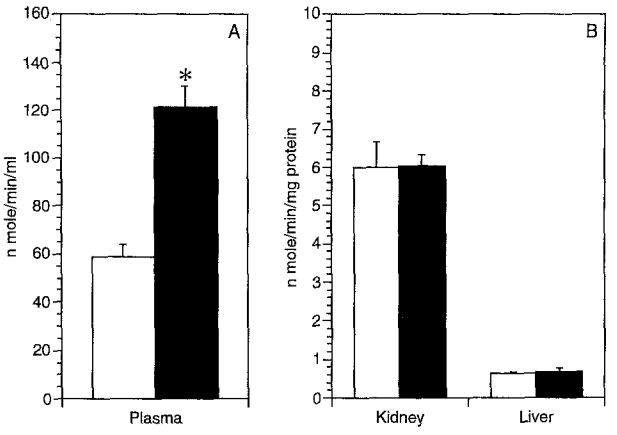

Fig 2. (A) Plasma PAF-AH of control rats (□, n = 6) and nephrotic syndrome rats (■, n = 5). (B) Cytosolic PAF-AH activity in kidney and liver of control rats (n = 6) and nephrotic syndrome rats (n = 5). *P < .01.

was observed in the cytosolic PAF-AH of either the kidney or liver. However, cytosolic PAF-AH activity in kidney was 10 times higher than in liver.

DISCUSSION

In the present investigation, we found a marked increase in EP concentration in kidneys obtained from rats with nephrotic syndrome. The urinary PE fraction also contained a significant amount of EP. It has been reported that the PE fraction is markedly elevated in rats with nephrotic syndrome,[8] as well as patients with chronic glomerular diseases.[10] In both of these studies, PE was not subfractionated into EP. In the present study, EP increased approximately twofold in the urine of nephrotic rats (Table 4).

Plasmalogens are present in significant amounts in a number of tissues, particularly as components of the PE fraction.[19] In most tissues, the *sn*-2 position is enriched with arachidonic acid.[20,21] The biochemical functions of this class of glycerophospholipids are not well understood. Zoeller et al[22] have proposed that the vinyl ether linkage of EP acts as a scavenger of reactive oxygen species and thereby protects against oxidation. In support of the role of these compounds as antioxidants, Vance[23] has suggested that the plasmalogens found in lipoprotein may have a protective role against oxidation and subsequent uptake by macrophages by the scavenger pathway. Others have proposed that the deficiency of plasmalogens might be responsible for at least some of the clinical symptoms of Zellweger's syndrome.[24-26]

One mechanism by which ether lipids can be metabolized is via conversion into plasmalogens.[27] Conversion of 1-alkyl-2-lyso-GPC (lysoPAF) into PE derivatives was first observed in Madin-Darby canine kidney cells,[5] and a similar conversion has subsequently been described in a human amnion-derived cell line.[4] PAF synthesis in kidney occurs in glomerular mesangial cells and medullary interstitial cells, and appears to be the main source of circulating PAF.[28-30] The enzymes responsible for the synthesis of PAF by both the de novo and remodeling pathways have been described in kidney tissue.[30] In the rat, glomerular PAF production has been shown to be elevated in nephrotic serum nephritis.[31] In a rabbit model of nephrotic serum nephritis, a PAF receptor antagonist ameliorated both histopathologic and physiologic signs of the disease.[32] In this model, the PAF receptor antagonist also decreased glomerular thromboxane synthesis, suggesting a relationship between PAF release and eicosanoid metabolism.

An additional relationship between PAF and kidney was shown by the demonstration that at least part of the hypotensive factor produced in dog kidney[33] was PAF.[34] Furthermore, the kidney has been reported to be the major source of circulating PAF.[35]

In the present study, we observed an increased EP in nephrotic kidney and its subcellular fractions and in the urine of these animals. It is suggested that the increase in plasmalogen may be a reflection of higher levels of PAF in the kidney as a consequence of the syndrome induced by puromycin, due to the fact that EP accumulates rather than being a metabolically active intermediate. To provide more direct evidence for the suggestion that increased EP levels are due to higher PAF production, determination of PAF and EP in the kidney as a function of time subsequent to the administration of puromycin aminonucleoside would be of importance. Whether the increase in EP is a consequence of the nephrotic syndrome or a specific effect of the puromycin aminonucleoside remains to be established in other experimental models of this disease. However, careful determination of tissue levels of this lipid mediator, which is active at concentrations of 10^{-12} mol/L, has been difficult.

The data presented in Table 4 clearly indicate that the increase of the total urinary phospholipids far exceeds that of the ethanolamine glycerophospholipids. It is well known that hyperlipidemia is a key feature of nephrotic syndrome, and that many plasma lipids, including cholesterol, sphingomyelin, and choline glycerophospholipids, are present in the urine. However, it is clear that there is a substantial increase in the proportion of plasmalogens in the ethanolamine phospholipids. Only trace quantities of choline plasmalogens were detected in the urine of nephrotic animals.

Further evidence of a relationship between PAF and the development of nephrotic syndrome is the marked elevation in plasma PAF-AH activity. This increase is at least twofold (Fig 2). A relationship between increased PAF and secretion of PAF-AH of the plasma type by macrophages has been reported.[36,37] A similar relationship has been described in HepG2 cells.[38] From the present study, we suggest that during the nephrotic syndrome, increased PAF results in the eventual elevation of plasma PAF-AH activity by a similar mechanism.

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