Lipid Alterations in Renal Membrane of Stroke-Prone Spontaneously Hypertensive Rats

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Phospholipase A₂ activity, phospholipids, and phospholipid fatty acids were investigated in renal membrane of male stroke-prone spontaneously hypertensive rats (SHRSP) and age-matched Wistar-Kyoto rats. Renal phospholipase A₂ activity increased and membranous phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, decreased with age in SHRSP. Arachidonate in phospholipid also decreased with age in SHRSP. To determine the effect of pressure load on the lipid alterations in renal membrane, SHRSP that received antihypertensive treatment with hydralazine, enalapril, or nicardipine for 5 weeks were compared with those without treatment. Antihypertensive treatments prevented phospholipid degradation and increased arachidonate in phospholipid relative to the control group. Phospholipase A₂ activity in each group treated with antihypertensive drugs did not differ from that in the control group. These results suggest that the course of hypertension causes renal membranous phospholipid degradation and increases phospholipase A₂ activity. Antihypertensive treatments may prevent these lipid alterations in SHRSP. These renal membranous structural changes may provide an explanation not only for functional abnormalities such as decreased membrane fluidity but also for the progression of hypertension. (Hypertension 1989;13:456-462)

The physiological states of phospholipids and their fatty acids are directly relevant to several important properties of biomembranes in many ways because of their 1) effect on lipid fluidity, 2) effect on membrane thickness, 3) effect on the lipid phase, and 4) specific interaction with membrane proteins. Many functional abnormalities, such as decreased membrane fluidity, decreased sodium-potassium adenosine triphosphatase (Na,K-ATPase) activity, and increased Ca²⁺ permeability have been described in cell membrane in both human essential hypertension and spontaneously hypertensive rats (SHR).¹⁻⁸ These functional abnormalities may contribute to the elevation of blood pressure. However, little is known about the phospholipid metabolism in the course of hypertension. We have demonstrated that activities of the phospholipases A₂ and C, which hydrolyze arachidonic acid from phospholipids, are elevated with age in the renal membrane of stroke-prone spontaneously hypertensive rats (SHRSP).⁹,¹⁰ These enzymes may cause a structural change in the membrane leading to changes in renal phospholipids and phospholipid fatty acids. The purpose of this study is to clarify the relation between the phospholipid metabolism in renal membrane and the course of hypertension in SHRSP.

Materials and Methods

Chemicals

Phosphatidylycholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, lysophospholipid, arachidonic acid, phospholipase A₂, heptadecanoic acid, and arachidonic acid were obtained from Sigma Chemical Co. (St. Louis, Missouri), and 1-palmitoyl-1-[1-¹⁴C]arachidonylphosphatidylcholine (60.1 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, UK. Kiesel Gel 60 thin-layer plates were from Merck (Darmstadt, FRG). The sources of antihypertensive drugs used in this work were as follows: nicardipine from Yamanouchi Pharmaceutical Co. (Tokyo, Japan), enalapril from Banyu Pharmaceutical Co. (Tokyo, Japan), and hydralazine hydrochloride from Ciba-Geigy Co. (Tokyo, Japan). All others chemicals were of analytical grade.
Animals

Male SHRSP were used for experiment 1 at the age of 5, 10, 20, and 40 weeks with age-matched Wistar-Kyoto (WKY) rats as controls. Each age group comprised 6–13 rats. The rats were kept in a thermohygrostat room and provided with standard rat chow (CE-2, Japan Clea, Tokyo, Japan) and tap water ad libitum. The original breeding rats were kindly donated by Dr. Kozo Okamoto, Department of Pathology, Kinki University.

Male SHRSP at the age of 15 weeks were used for experiment 2. The rats were bred in a thermohygrostatic room for 5 weeks. At the age of 19 weeks and 20 weeks, after body weight and blood pressure measurements were taken, the rats were divided into four test groups with approximately equal mean body weights and blood pressures. The groups were then randomly assigned to administration of a vasodilator (hydralazine, 40 mg/kg/day, n=8), a calcium antagonist (nicardipine, 160 mg/kg/day, n=8), an angiotensin converting enzyme inhibitor (enalapril, 30 mg/kg/day, n=8), or to a control group without treatment (n=7). These agents were perorally given to the rats for 5 weeks, and the treatment groups were compared with the control group.

Blood Pressure

In each experiment, the rats were anesthetized with α-chloralose (50 mg/kg i.p.) and urethane (500 mg/kg i.p.) and a polyethylene cannula was inserted into a femoral artery and connected to a Statham P23Db transducer for direct measurement of blood pressure. In experiment 2, blood pressure was measured five times with tail-cuff plethysmography at the age of 19 weeks and 20 weeks.

Subcellular Fractions

After blood pressure measurement, the kidneys were excised from each rat and perfused with 0.25 mol/l sucrose at 0° C to wash out the blood. Then the kidneys were cut with a razor blade into strips 2–3 mm thick, and the cortex and medulla were separated microscopically. Cortical and medullary tissues were minced and homogenized for 60 seconds with a Polytron Homogenizer (Brinkmann Instrs. Inc., Westbury, New York) in 10 vol 0.25 mol/l sucrose. The homogenate was centrifuged for 900g for 20 minutes, and the supernatant was centrifuged at 10,000g for 30 minutes. Then the supernatant was centrifuged at 105,000g for 90 minutes. The resultant pellet was suspended in 0.25 mol/l sucrose and homogenized in a Potter-type homogenizer to obtain a microsomal fraction that denoted the cell membrane. All procedures were done at 4° C.

Phospholipase A2 Activity

Phospholipase A2 activity in the subcellular fraction was determined by following a method previously described as optimal for kidney tissue.11 Nonradioactive phosphatidylcholine obtained from egg yolk lecithin was added to radioactive phosphatidylcholine to adjust the specific activity of the substrate to 50,000 cpm/50 nmol. Each subcellular fraction from the cortex and medulla was incubated with 50 nmol 1-palmitoyl-2-[1-14C]arachidonylphosphatidylcholine and 5 nmol CaCl2 in 0.1 mol/l Tris HCl, pH 8.5 (total volume 500 μl) at 37° C for 60 minutes. The reaction was terminated by adding 4.0 ml chloroform/methanol (2:1, vol/vol). The chloroform phase was removed and dried under vacuum. The residues were applied to silica gel G plates and developed in a solvent system of ethyl acetate/acetone/acetic acid (99:1, vol/vol). The respective spots of phospholipids were scraped, counted with a scintillation spectrometer, and analyzed.

Lipid Determination

Lipids were extracted by the method of Folch et al.12 The chloroform phase was evaporated under a N2 stream, and the residues were applied to thin-layer chromatography. The thin-layer chromatography was developed first in a solvent system of ethyl acetate/acetone/acetic acid (90:10:1, vol/vol/vol) and then in a solvent system of chloroform/methanol/acetic acid/water (50:30:6:2, vol/vol/vol/vol). The respective spots of phospholipids were scraped and quantified by the method of Bartlett.13

Fatty Acid Determination

The extracts from subcellular fractions were developed in a solvent system of ethyl acetate/acetone/acetic acid (90:10:1, vol/vol/vol), and phospholipids at the origin were scraped. After evaporation, the phospholipids were methylated with boron-fluoride-3-methanol by the method of Morrison and Smith.14 The phospholipid methyl esters were then analyzed by gas liquid chromatography (model 663, Hitachi Factory Co., Tokyo, Japan) using a 3% EGSS-X column (0.3×200 cm) on chromosorb W. The column temperature was 190° C, and the nitrogen flow was 50 ml/min. Heptadecanoic acid and arachidonic acid methyl esters were added to serve as the internal standard. The peaks were identified from the retention times of known fatty acid methyl esters and quantitation was carried out by calculating the peak area.

Other Methods

Protein was determined by the method of Lowry et al.,15 with bovine serum albumin as the standard. Triplicate samples were analyzed in all experiments. Comparisons between two groups were analyzed by the unpaired t test, and comparisons of more than two groups were performed by analysis of variance (ANOVA) followed by multiple comparison tests according to the method of Bonferroni as used by Wallenstein et al.16

Results

Blood Pressure

As shown in Figure 1A, systolic blood pressure increased progressively with age in SHRSP in exper-
**Figure 1.** Line graphs showing systolic blood pressure in experiment 1 (Panel A) and systolic blood pressure in experiment 2 (Panel B). Values are expressed as mean±SD. *p<0.05; **p<0.005, compared with age-matched Wistar-Kyoto (WKY) rats in experiment 1 or control group in experiment 2. SHRSP, stroke-prone spontaneously hypertensive rats.

**Figure 2.** Line graph showing phospholipase A2 activity in renal medulla. Renal membranous fraction was incubated with 50 nmol 1-palmitoyl-2-[1-14C]arachidonyl-phosphatidylcholine, 5 mmol/l CaCl2, 0.1 mol/l Tris HCl, 0.5 mg microsomal protein, pH 8.5 at 37°C for 60 minutes. Results are given as mean±SEM. *p<0.05; **p<0.001, compared with age-matched Wistar-Kyoto (WKY) rats. SHRSP, stroke-prone spontaneously hypertensive rats.

**Phospholipase A2 Activity**

To study the subcellular distribution of phospholipase A2 activity in the renal cortex and medulla, each fraction was incubated with 5 mmol/l CaCl2 for 60 minutes at 37°C. All of the subcellular fractions analyzed had phospholipase A2 activity and the microsomal fraction had the highest such activity. The distribution of phospholipase A2 activity among subcellular fractions was the same for SHRSP and WKY rats. Therefore, the microsomal fraction was used as the source for enzymes in the following experiments.

Figure 2 shows the phospholipase A2 activity of the renomedullary microsomes in experiment 1. There was no significant difference in phospholipase A2 activity between SHRSP and WKY rats at the age of 5 weeks and 10 weeks. Phospholipase A2 activity was increased in SHRSP after the age of 20 weeks. This activity remained unchanged in WKY rats. The results in cortex were similar to those in medulla. In experiment 2, there were no differences in the renal phospholipase A2 activities of the four groups (data not shown).

**Total Phospholipid Contents**

WKY rats between 5 and 10 weeks of age showed an increase in total phospholipid content in the renal microsomal fraction in both cortex and medulla, but no change was observed later. SHRSP showed values similar to those in WKY rats at 5 weeks of age, but the decrease in total phospholipid content in cortex and medulla occurred from 10 weeks and from 5 weeks onward, respectively. Figure 3A shows the results in medulla in experiment 1, and the changes in cortex were similar to those in medulla.

In experiment 2, antihypertensive treatments caused an increase in total phospholipid content in both cortex and medulla, and the values went up to nearly those of WKY rats aged 20 weeks. Figure 3B shows the results in medulla. The results in cortex were similar to those in medulla.

**Individual Phospholipids and the Phosphatidylcholine/Sphingomyelin Ratio**

WKY rats showed an increase in phosphatidylethanolamine in cortex between 5 and 10 weeks of age, but no other change was observed in any individual phospholipid at any age. At the age of 5 weeks, SHRSP showed lower phosphatidylethanolamine values than WKY rats in both cortex and medulla. SHRSP showed a decrease in phosphatidylcholine from the age of 5 weeks onward in both cortex and medulla. Phosphatidylethanolamine showed a similar decrease in cortex after the age of 10 weeks in medulla after 5 weeks of age. In addition, the values of phosphatidylserine and phosphatidylinositol also decreased in cortex after the age of 5 weeks and in medulla after the age of 10 weeks. However, no other change was observed in any individual phospholipid, including sphingomyelin (data not shown).
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A. Experiment 1

Protein content in relation to age in experiment 1 (Panel A) and effect of antihypertensive treatment on total phospholipid content in renal medulla (Panel B). The results in cortex were similar to those in medulla. Results are given as mean±SEM. *p<0.05; **p<0.005 compared with age-matched Wistar-Kyoto (WKY) rats. SHRSP, stroke-prone spontaneously hypertensive rats. Panel B, control group without treatment.

B. Experiment 2

Phosphatidylethanolamine content in relation to age in experiment 2 (Panel A) and effect of antihypertensive treatment on phosphatidylcholine content in experiment 2. E, group treated with enalapril; H, group treated with hydralazine; N, group treated with nicardipine; C, control group without treatment.

In experiment 2, antihypertensive treatment caused an increase in phosphatidylcholine in each group. Furthermore, the values of phosphatidylethanolamine increased in cortex in the groups given nicardipine and enalapril, and in medulla in the group given hydralazine (Figure 4B).

The phosphatidylcholine/sphingomyelin ratio decreased in cortex of SHRSP after the age of 20 weeks and in medulla after the age of 10 weeks in experiment 1 (Figure 5A). Antihypertensive treatment increased the ratio significantly in each group (Figure 5B).

Renal Microsomal Fatty Acids in Phospholipid

There was little difference in the composition of fatty acid in renal cortex in WKY rats and SHRSP. In renal medulla, a high ratio of palmitate and a low ratio of arachidonate were observed in both WKY and SHRSP at the age of 5 weeks (palmitate, 32.0±0.8% and 29.2±0.6%; arachidonate, 16.2±1.8% and 17.9±2.3%, respectively); in contrast, a low ratio of palmitate and an increased ratio of arachidonate were observed in WKY rats and SHRSP after the age of 20 weeks (palmitate, 18.3±0.2% and 21.3±0.2%; arachidonate, 36.6±2.2% and 31.7±0.6%, respectively). The ratios for WKY rats and SHRSP at 40 weeks of age were: palmitate, 19.9±3.5% and 20.3±0.5%; arachidonate, 35.1±2.5% and 30.4±1.0%, respectively. In SHRSP, a high ratio of linoleate and a decreased ratio of arachidonate were observed after the age of 20 weeks. The respective ratios for WKY rats and SHRSP at 5 weeks of age were: linoleate, 9.8±0.8% and 12.9±0.9%; arachidonate, 16.2±1.8% and 17.9±2.3%. For WKY rats and SHRSP at the age of 20 weeks the ratios were: linoleate, 12.0±0.5% and 14.2±0.6%; arachidonate, 36.6±2.2% and 31.7±0.6%, and for WKY rats and SHRSP at the age of 40 weeks: linoleate, 11.8±3.5% and 14.2±0.6%.
FIGURE 5. Line graphs showing the phosphatidylcholine/sphingomyelin (PC/S) ratio. Panel A, PC/S ratio in relation to age in experiment 1. Panel B, effect of antihypertensive treatment of PC/S ratio. E, group treated with enalapril; N, group treated with nicardipine; H, group treated with hydralazine; C, control group without treatment. Values are expressed as mean±SEM. *p<0.05, **p<0.005, compared with age-matched Wistar-Kyoto (WKY) rats in experiment 1 or control group in experiment 2.

16.1±0.8%; arachidonate, 35.1±2.5% and 30.4±1.0%, respectively.

Figure 6A shows changes in arachidonate in medulla. There was no difference in arachidonate in phospholipid between WKY rats and SHRSP at the age of 5 weeks and 10 weeks. A decrease in arachidonate was observed in SHRSP after the age of 20 weeks, whereas in WKY rats arachidonate increased after the age of 20 weeks. These results were similar to those in cortex.

In experiment 2, there was little difference in the composition of fatty acid in renal cortex in each group. In renal medulla, a decreased ratio of linoleate and an increased ratio of arachidonate were observed in the groups given nicardipine and hydralazine (linoleate: control group, 13.0±0.6%; nicardipine group, 5.7±0.7%; hydralazine group, 13.1±2.1%; enalapril group, 6.4±2.0%; arachidonate: control group, 30.7±0.6%; nicardipine group, 39.9±0.1%; hydralazine group, 31.4±1.1%; enalapril group, 32.8±1.1%), although the composition of the group given hydralazine was almost the same as that of the control group.

Figure 6B shows arachidonic acid content in each group treated with antihypertensive drugs; arachidonic acid in phospholipid showed an increase compared with the control group.

Discussion

Cell membrane is composed of a phospholipid bilayer, and the lipid composition and architecture of the membrane play important roles in cation transport, some enzyme activities, some receptor functions, and other aspects of cell physiology. In studies on the cell membrane of erythrocytes of essential hypertension and SHR, Montanay-Garestier et al and Tsuda et al have found lower cell membrane fluidity and higher microviscosity. Postnov et al and Friedman showed enhanced permeability of the membrane to Na+ and Ca2+; Robinson demonstrated inhibited activities of Na,K-ATPase and calcium-magnesium (Ca,Mg) ATPase, and Devyuck et al pointed out the presence of disorders in Ca2+ handling, such as low Ca2+ binding capacity of the membrane or high intracellular Ca2+.
content. Furthermore, Yamori et al. and Whitmer et al. proposed that such functional abnormalities of cell membrane could be observed not only in erythrocytes, but also in a wide range of cells such as vascular smooth muscle, neurocytes, kidney, and heart. Although Bing et al. suggested that the functional abnormalities in cell membrane originate from a change in the lipid structure of the membrane, this has not been confirmed yet. The present study was pursued taking the above reports into account, and lipid alterations were examined in renal membrane of SHRSP. In consequence, phospholipase A2 activity was enhanced and phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, and arachidonate in phospholipid decreased with the progress of hypertension in SHRSP. Renal membranous phospholipid degradation in SHRSP seems to influence the membrane function. Borochov et al. and Untracht and Shipley have shown that a decrease in the phosphatidylcholine/sphingomyelin ratio causes a decrease in membrane fluidity. Schnitzky demonstrated that phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, act as membrane fluidizers, whereas proteins, cholesterol, and sphingomyelin act as rigidifiers. Therefore, it was estimated that the renal membrane of SHRSP became rigid with age due to the changes in phospholipids. It is known that a decrease in membrane fluidity causes a decrease in membrane cation permeability and a decrease in the activities of enzymes, such as Na,K-ATPase and Ca,Mg-ATPase. It has also been reported that SHRSP show high Na+ and Ca++ levels in renal tubular cells. Therefore, the changes in renal membranous phospholipid of the SHRSP observed in this study have an important meaning for the explanation of the development of hypertension. Since similar changes in phospholipid were reported in the ischemic kidney, phospholipid degradation may be relevant to the development of cell injury. Our experiment showed that phospholipase A2 was enhanced in the renal membrane of SHRSP after the age of 20 weeks. In our preliminary study, this phospholipase A2 had higher substrate specificity to phosphatidylcholine and phosphatidylethanolamine than any other class of phospholipids. This enhanced phospholipase A2 may cause decreases of phosphatidylcholine and phosphatidylethanolamine. It is quite interesting that phosphatidylcholine and phosphatidylethanolamine decreased gradually in the rats from the age of 5 weeks before the enhancement of phospholipase A2. This suggests that the reacylation process is impaired from the early stage of hypertension in SHRSP. Although the mechanism of enhancement in the activity of renal phospholipase A2 has not yet been clarified, this enhancement may be related to the fact that the intracellular Ca++ level is high because of abnormal Ca++ handling in SHR from the infantile period, and this enzyme is Ca++ dependent. Further investigations are under way to elucidate the mechanism of phospholipase A2 activation and the phospholipid metabolism.

In experiment 2, antihypertensive treatment inhibited the decreases of membranous phospholipids. Although the actions of antihypertensive agents differed, all of the drugs are known to increase renal blood flow. Therefore, it is suggested that the prevention of phospholipid degradation was attributable to an improvement in renal hemodynamic behavior. Our experiment showed that phospholipase A2 activity did not differ from group to group. This result demonstrates that phospholipid alterations might not merely be due to increased phospholipase A2 activity.

The composition of phospholipid fatty acids is considered to be related to the development of hypertension for two reasons. One is that membrane fluidity becomes higher when the ratio of cis-unsaturated to saturated fatty acid, especially the proportion of arachidonate, increases. The other is that cis-unsaturated fatty acids, particularly arachidonate, are precursors to prostaglandin synthesis. In experiment 2, hydralazine caused no change in the composition of membranous fatty acids, whereas quantitative analysis showed that arachidonate in phospholipid was decreased concomitant with phospholipid degradation and was reversed to a certain extent with antihypertensive treatment. We reported that fatty acid binding protein binding to arachidonate increased in cytosol in the kidney of SHRSP. This released arachidonate may be used as the substrate for prostaglandin synthesis. On the other hand, the changes in medulla after nicardipine and enalapril administration suggest a conversion of linoleate to arachidonate. It was found that a linoleate-rich diet had the same effect on the composition of fatty acids in SHR. Singer et al. suggested that fatty acid chain elongation and the desaturation system were inhibited in SHR. Our results show that the fatty acid composition varies with age. Thus, further work is needed to explain the chain elongation and desaturation system.

In conclusion, we have demonstrated that renal membranous phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, decrease concomitant with enhanced phospholipase A2 activation and that antihypertensive treatment prevents this phospholipid degradation. The mechanisms responsible for the phospholipid degradation and increased phospholipase A2 activity were not elucidated in the present study. These membranous structural changes may provide an explanation not only for functional abnormalities such as decreased membrane fluidity, but also for the progress of hypertension.

Acknowledgment

We thank Dr. Hiroko Togashi for her technical assistance.
References


KEY WORDS • phospholipase A2 • phospholipid • renal membrane • fatty acids • stroke-prone spontaneously hypertensive rats.
Lipid alterations in renal membrane of stoke-prone spontaneously hypertensive rats.
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doi: 10.1161/01.HYP.13.5.456

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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