Renal Phospholipase C and Diglyceride Lipase Activity in Spontaneously Hypertensive Rats

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SUMMARY Phospholipase C activity and diglyceride lipase activity were studied in the renal cortex and medulla of 10- and 40-week-old stroke-prone spontaneously hypertensive rats (SHRSP) and age-matched normotensive Wistar-Kyoto rats (WKY). Enhanced phospholipase C activity was found in the cortical and medullary cytosol of kidney from SHRSP, and microsomal diglyceride lipase in SHRSP also increased. In SHRSP, phospholipase C and diglyceride lipase activities increased with age, but this increase was not evident in WKY. Phospholipase C had high substrate specificity for phosphatidylinositol in renal cytosol of both WKY and SHRSP. The increased activities were accompanied by prostaglandin E₂ synthesis in renal medullary microsomes of 10-week-old SHRSP and were also present in the kidney of 40-week-old SHRSP. Total phospholipid and arachidonic acid contents in kidney were markedly high in the medulla of 10-week-old SHRSP, but these lipids were decreased in 40-week-old SHRSP. These results suggest that phospholipids and arachidonic acid in SHRSP may be genetically high and that the activated phospholipase C and diglyceride lipase hydrolyze phospholipids, providing arachidonic acid for prostaglandin synthesis, which results in a decrease of phospholipids and arachidonic acid in the kidney of 40-week-old SHRSP. These studies demonstrate that a phosphatidylinositol-specific phospholipase C-prostaglandin synthetic system may play an important role in the course of hypertension in SHRSP. (Hypertension 10: 100–106, 1987)

KEY WORDS • phospholipase C • phosphatidylinositol • prostaglandin E₂ • arachidonic acid • diglyceride lipase • phospholipids

PROSTAGLANDINS participate in blood pressure regulation in hypertensive states.²⁻⁴ The contributions of these substances to hypertension are of interest and might depend on changes in their local synthesis and release.³ The precursor of prostaglandins, arachidonic acid, is esterified intracellularly into membrane phospholipids.⁵ Phospholipases A₂ and C hydrolyze arachidonic acid from the phospholipids. Some studies have suggested that phospholipase A₂ and prostaglandins are important in both normal and pathological kidney function.⁷⁻⁹ We¹⁰ reported that phospholipase A₂ increases with age in the kidney of stroke-prone spontaneously hypertensive rats (SHRSP) and suggested that activated phospholipase A₂ is a rate-limiting step for prostaglandin synthesis in SHRSP. Recently, however, phospholipase C was reported to be an important enzyme for prostaglandin synthesis in platelets.¹¹ Phospholipase C activity in rat kidney was reported in 1980 by Hostetler and Hall.¹² We reported that phospholipase C existed in cytosol in the rat kidney.¹³ But renal phospholipase C activity in SHR has not been determined, and the relationship between phospholipid metabolism and phospholipase C in kidney of SHR is still not clear. This study examined the relationship between phospholipid metabolism and renal phospholipase C in the kidneys of SHRSP.

Materials and Methods

Chemicals

[1-¹⁴C]Arachidonic acid (60.2 mCi/mmol), and 1-stearyl-2-[1-¹⁴C]arachidonoylphosphatidylcholine (60.1 mCi/mmol), 1-palmitoyl-2-[1-¹⁴C]arachidonoylphosphatidylethanolamine (56 mCi/mmol), 1,2-dioleoylphosphatidyl[U-¹⁴C]serine (60 mCi/mmol), and 1-stearyl-2-[1-¹⁴C]arachidonoylphosphatidylinositol (57 mCi/mmol) were purchased from Radiochemical Centre, Amersham, England. Phosphatidylcholine, phosphatidylinositol (PI), phosphatidylserine, phosphati-
dylethanolamine, monoacylglycerol, diacylglycerol, triacylglycerol, arachidonic acid, prostaglandin E\(_2\) (PGE\(_2\)), prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)), thromboxane B\(_2\) (TXB\(_2\)), and 6-keto-prostaglandin F\(_{1\alpha}\) (PGF\(_{1\alpha}\)) were obtained from Sigma Chemical (St. Louis, MO, USA). Kiesel Gel 60 F-254 thin-layer plates were from Merck, Darmstadt, Federal Republic of Germany. All other chemicals were of analytical grade.

Animals

Experiments were performed on male SHRS, 10 (weight, 194 ± 37 g) and 40 (weight, 314 ± 30 g) weeks old, and age-matched normotensive male Wistar-Kyoto rats (WKY) weighing 250 ± 27 g (10 weeks) and 412 ± 44 g (40 weeks). The rats were kindly donated by Dr. Kozo Okamoto, Department of Pathology, Kinki University. Each age group consisted of six animals.

Blood Pressure

Blood pressure was measured with the rats under anesthesia (α-chloralose, 50 mg/kg, and urethane, 500 mg/kg i.p.) through a femoral artery.

Subcellular Fractions

Kidneys were excised from rats and placed in ice-cold 0.25 M sucrose; the cortex was separated from the medulla with a razor blade. Tissue was processed identically for hypertensive and normotensive rats. Cortical and medullary tissues were minced and homogenized for 60 seconds by a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) in 5 volumes of 0.1 M Tris HCl (pH 7.4). The homogenate was centrifuged at 700 g for 10 minutes, and the resultant supernatant was centrifuged at 9000 g for 20 minutes. The resultant pellet was used as the mitochondrial fraction, and the supernatant was centrifuged at 105,000 g for 60 minutes. The resultant pellet was suspended in 0.1 M Tris HCl buffer (pH 8.0) and used for each assay. All procedures were done at 4°C.

Prostaglandin Biosynthesis

The assay mixture contained, in a total volume of 200 μl, 0.1 M Tris HCl (pH 8.0), 0.2 mM L-tryptophan, 0.1 μM hemoglobin, 20 μM [1-\(^{14}\)C]arachidonic acid, and 500 μg of microsomal protein. Assays were performed at 37°C for 10 minutes, and the reactions were terminated with 30 μl of 1 N HCl. The reaction mixture was then extracted twice with 2 ml of ethyl acetate, and the combined extracts were evaporated under a vacuum. The residues, taken up in a small volume of acetone, were quantitatively spotted on plastic silica gel G plates along with authentic unlabeled prostaglandins; arachidonic acid was used as the control. Plates were developed with a solvent system containing 1% acetic acid in ethyl acetate. To separate PGE\(_2\) and PGF\(_{2\alpha}\) from TXB\(_2\), the plates were first allowed to develop up to 15 cm from the origin and, after drying, were again allowed to develop 17 cm from the origin in a solvent system. The plates were placed in iodine vapor for color development. Each plate was cut into small pieces, and these were placed in counting vials. The radioactivity was determined in a liquid scintillation counter. Further identification of the reaction products was done using a method involving high performance liquid chromatography. The retention times of 6-keto-PGF\(_{1\alpha}\), TXB\(_2\), PGF\(_{2\alpha}\), and PGE\(_2\) were 3.5, 5, 7, and 12 minutes, respectively.

Phospholipase C Activity

The preliminary study determined that optimal PH for phospholipase C was 7.0. Each subcellular fraction was incubated with \(^{14}\)C]arachidonic acid–labeled phospholipids (50,000 dpm/20 nmol) in 0.1 M Tris HCl, pH 7.0, for 2 minutes at 37°C. The released diglyceride and free arachidonic acid were extracted by the method of Folch et al. The chloroform phase was pooled and evaporated under vacuum. The residues were applied to thin-layer chromatography (TLC). TLC was developed first in isopropyl ether/acetonic acid (96:4, vol/vol) and then in a solvent system containing petroleum ether/diethyl ether/acetic acid (90:10:1, vol/vol/vol). The respective spots (monoacylglycerol, diacylglycerol, triacylglycerol, and arachidonic acid) were scraped, counted with a scintillation spectrometer, and analyzed.

Diglyceride Lipase Activity

2-[1-\(^{14}\)C]Arachidonoyldiglyceride was prepared by treating 2-[1-\(^{14}\)C]arachidonoylphosphatidylcholine with Bacillus cereus phospholipase C. Phospholipase C (20 μg/mol of phosphatidylcholine) was incubated in 50 mM Tris HCl, pH 7.4, for 20 minutes at 37°C. Diacylglycerol was extracted from residual phospholipid with diethyl ether and purified by TLC. TLC was developed first in isopropyl ether/acetonic acid (96:4, vol/vol) and then in a solvent system containing petroleum ether/diethyl ether/acetic acid (90:10:1, vol/vol/vol). The spot of diacylglycerol was scraped and extracted with chloroform/methanol (2:1, vol/vol). Diglyceride lipase assays were done in a 0.1 ml solution containing 50 mM HEPES-NaOH, pH 7.0, 5 mM CaCl\(_2\), 12 mM reduced glutathione, and 2-[1-\(^{14}\)C]arachidonoyldiglyceride (50,000 cpm/20 nmol). Renal microsomes (0.5 mg of protein) were incubated in this incubation mixture for 20 minutes at 37°C, and the assay was terminated by the addition of 1.5 ml of chloroform/methanol/heptane (1.25:1.4:1, vol/vol/vol) and 0.5 ml of K\(_2\)CO\(_3\). Portions of the upper aqueous layer were used for scintillation counting.

Other Methods

Protein was determined by the method of Lowry et al., with bovine serum albumin as the standard. Phospholipids in renal homogenates were separated by TLC, which was developed in chloroform/methanol/acetic acid/water (50:30:8:4, vol/vol/vol/vol), then determined as described earlier. Fatty acids in renal homogenates were determined by gas-liquid chromatography, as described earlier. TriPLICATE samples were analyzed in all experiments. Student's t test was used to analyze the results.
Results

As shown in Figure 1, systolic and diastolic blood pressure increased progressively with age in SHRSP. Phospholipids levels in renal homogenate of SHRSP were very high in medulla at 10 weeks of age but had markedly decreased by 40 weeks of age (Figure 2). They also decreased in the cortex of SHRSP, but the levels were not as high at 10 weeks as in the medulla. The phospholipid changes in WKY were smaller than those in SHRSP. The changes of arachidonic acid in renal homogenates were almost parallel to the decrease of phospholipids (see Figure 2). Figure 3 demonstrates that medullary arachidonic acid in SHRSP was significantly higher than that of controls at 10 weeks and that it dramatically decreased between 10 and 40 weeks of age. Similar but smaller changes occurred in the cortex of SHRSP. There was no significant change in medullary or cortical arachidonic acid in controls between 10 and 40 weeks.

PGE\(_2\) Synthesis

Medullary PGE\(_2\) synthesis was higher than that of cortex. There were no differences in PGE\(_2\) synthesis in renal cortical microsomes at 10 weeks of age in WKY and SHRSP. PGE\(_2\) synthesis increased in medulla and cortex of SHRSP at 40 weeks of age; however, no increase of PGE\(_2\) was seen with age in WKY (Figure 4).

Phospholipase C Activity

To study the subcellular distribution of phospholipase C activity in the renal cortex and medulla (Figure 5), each fraction from 10-week-old rats was incubated with 2-[\(^{14}\)C]arachidonylphosphatidylinositol, 5 mM CaCl\(_2\) for 2 minutes at 37°C. All of the subcellular fractions analyzed had phospholipase C activity, and cytosol had the highest activity for phospholipase C. The distribution of phospholipase C activity among fractions was the same for SHRSP and WKY, but phospholipase C activity was markedly enhanced in the cytosolic fraction of the cortex and medulla of SHRSP as compared with those of WKY (see Figure 5). Therefore, the cytosolic fraction was used as the source for enzymes in the next experiment.

As shown in Figure 6, phospholipase C had high substrate specificity toward PI. Substrate specificity to PI was the same in WKY and SHRSP, but its activity in SHRSP was higher than that in WKY in both medulla and cortex. Phospholipase C activity was completed within 5 minutes (Figure 7). There was no difference in time course between SHRSP and WKY. At 10 weeks of age, phospholipase activity in SHRSP was slightly higher than in WKY, and at 40 weeks of age this activity markedly increased in SHRSP, but not in WKY (Figure 8).

Diglyceride Lipase Activity

Increased diglyceride lipase activity was observed in 40-week-old SHRSP as compared with WKY (Figure 9). This activity was similar in both strains at 10 weeks of age, but it had increased about twofold in medulla and cortex of 40-week-old SHRSP.

Discussion

The changes in phospholipids and arachidonic acid in renal homogenates of SHRSP differ from those in...
WKY. These phospholipid changes affect the membrane fluidity, which may in turn alter the membrane transport of substances such as Ca^{2+}. The reason why the concentrations of phospholipids and arachidonic acid are remarkably high in younger SHRSP is not still clear, but our experiments suggest that the marked decrease of phospholipids may be caused by enhanced phospholipase activity, and the released arachidonic acid may be used for prostaglandin synthesis. Renal prostaglandins are thought to participate in the regulation of blood pressure by a variety of mechanisms.

**Figure 2.** Effect of age on total phospholipids in kidneys of SHRSP and WKY. All conditions were described in Materials and Methods. Double (p<0.001) asterisks indicate significant difference compared with WKY.

**Figure 3.** Effect of age on arachidonic acid in kidneys of SHRSP and WKY. All conditions were described in Materials and Methods. Single (p<0.05) and double (p<0.001) asterisks indicate significant difference compared with WKY.

**Figure 4.** Effect of age on PGE2 synthesis in medulla and cortical microsomes of SHRSP (•) and WKY (○). Enzymatic activity was determined with 0.5 mM L-tryptophan, 0.1 μM hemoglobin, 0.5 mg of microsomal protein, and 20 μM arachidonic acid at 37°C for 10 minutes. Results are given as means ± SE/min/mg protein. Single (p<0.05) and double (p<0.001) asterisks indicate significant difference compared with WKY.

**Figure 5.** Subcellular distribution of phospholipase C in medulla and cortex of SHRSP and WKY. Each fraction was incubated with 20 nmol 2-[1-3H]arachidonoylphosphatidylcholine, 5 mM CaCl2, and 0.5 mg of subcellular protein at 37°C for 2 minutes. Results are given as means ± SE/hr/mg protein. Single (p<0.05) and double (p<0.001) asterisks indicate significant difference compared with WKY.
Changes in the net synthesis of prostaglandins by the kidneys are thought to affect the course and severity of hypertension. We reported that the ratios of PGE/TXB, and 6-keto-PGF/TXB were lower in the cortex and medulla of SHRSP than of WKY. The lower ratios in SHRSP may produce vasoconstriction and increase vascular resistance.

Recently, Shibouta et al. reported that thromboxane A (TXA,) had an important role in development of hypertension. They showed that TXA synthetase inhibitor delayed the initiation of hypertension. But other investigators reported that TXA is not essential.
for the development of hypertension. Further work is needed to explain the discrepancy between these results.

The production of prostaglandins probably is a primary response to their effect on tissue because circulating prostaglandins are rapidly inactivated in the lungs and plasma. The availability of substrate probably is a rate-limiting step for prostaglandin synthesis. Arachidonic acid is incorporated into cell membrane phospholipids, and prostaglandin synthesis may depend on membrane phospholipase for substrate release. We reported earlier that there was an age-related increase in phospholipase A2 activity in cortical and medullary microsomes in hypertensive rats but not in normotensive rats. As changes in microsomal phospholipase A2 activity provide a substrate for prostaglandin synthesis, increased phospholipase A2 activity could result in a high rate of prostaglandin synthesis. Thus, changes in phospholipase A2 activity with age may play an important role in maintaining high blood pressure in SHRSP. We have attempted to obtain further information on the mechanism involved in the enhancement of prostaglandin synthesis in SHRSP and have tried to elucidate the relationship between phospholipid metabolism and hypertension in this report.

We determined that phospholipase C in rat kidney has substrate specificity toward PI. In this experiment, phospholipase C in the kidney of SHRSP also had high substrate specificity toward PI, and it was highly activated at 40 weeks of age. Recently, PI-specific phospholipase C has been observed in the relationship between prostaglandin synthesis and PI response. Several different agonists are known to alter the metabolism of PI while stimulating tissue responses as varied as secretion, muscle contraction, and mitogenesis. Both increased degradation and increased synthesis of PI have been observed, sometimes in a single response system. The concept has emerged that some agonists act through a common mechanism involving stimulation of a PI-specific phospholipase C and subsequent conversion of released diacylglycerol, first to phosphatidic acid and then to cytidine 5'-di-phosphate-diacylglycerol and PI. This diacylglycerol regulates protein kinase C, and phosphatidic acid stimulates the influx of Ca2+. These phenomena may alter the kidney metabolism. Our results showed that phospholipase C increased in the kidney of older SHRSP. This increase may lead to increased Ca2+ influx into kidney tissue. The increased Ca2+ could further activate phospholipase A2 and C.

The course of hypertension, regardless of its cause, can be influenced by the rate of synthesis and degradation of endogenous vasoactive substances. Previous investigators have postulated that the synthesis and degradation of PGE2 may affect the development of hypertension. They also reported that the decreased 15-hydroxy-dehydrogenase activity is an important factor in the development of hypertension. Prostaglandins synthesized within the kidney have the potential to modify the severity of blood pressure elevation by affecting smooth muscle tone and salt-water balance. But the capacity for prostaglandin synthesis may be limited by the availability of endogenous substrate. Our data suggest that renal prostaglandin synthesis and phospholipase C may play an important role in regulating hypertension. Further experiments on phospholipase C activity and prostaglandin synthesis in blood pressure-controlled SHRSP, using several antihypertensive agents, are underway in our laboratory to elucidate the involvement of phospholipase C and prostaglandin synthesis in blood pressure regulation in SHRSP.

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