Enlarged Phospholipase C Activity in the Vascular Wall of Spontaneously Hypertensive Rats

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SUMMARY To explore the roles of vascular phospholipase C activity in the development of hypertension, phospholipase C activity was examined in the aortic wall of spontaneously hypertensive rats (SHR). Phospholipase C activity was significantly enhanced (+87%, p<0.005) in 14-week-old SHR as compared with normotensive Wistar-Kyoto rats (WKY). The enzymatic activities were positively correlated with the levels of blood pressure in both of the rat strains (r = 0.62, p < 0.003). Vascular phospholipase C was also significantly activated (+62%; p<0.006) in the aortic wall of 4-week-old prehypertensive SHR, as compared with age-matched WKY. In contrast, vascular phospholipase A2 activity was unaffected in the aortic wall of either adult or very young SHR. There was no difference in the cardiac phospholipase C activity between adult SHR and WKY. The vascular phospholipase C of SHR had a lower Michaelis constant (Km) value than that of WKY. Moreover, its pH profile and calcium requirement differed in part from those of WKY. These results indicate that the activation of vascular phospholipase C precedes the development of hypertension and that the enhancement may be induced by both quantitative and qualitative changes in phospholipase C in SHR.

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KEY WORDS - phospholipase C - phospholipase A2 - aortic wall - spontaneously hypertensive rats - hypertension

SPONTANEOUSLY hypertensive rats (SHR) have been studied extensively as an animal model for human hypertension. Recently, much evidence has been provided for the involvement of intracellular calcium metabolism in the development of hypertension. Indeed, Postnov et al. demonstrated that calcium binding by red blood cell membranes was decreased in SHR. However, both no increases and increases in cytoplasmic free calcium concentration have been reported in the circulating platelets or lymphocytes of SHR.

In addition to cyclic adenosine 3',5'-monophosphate and the protein kinase A system, phosphoinositide metabolism recently has been emphasized as an intracellular calcium messenger system that modulates cytoplasmic free calcium concentration and mediates the subsequent transmission of intracellular signals. Thus, the increase in cytoplasmic free calcium concentration in SHR may be accounted for by alterations of phosphoinositide metabolism. In this context, Koutouzov et al. demonstrated a rapid turnover rate of phosphoinositides in the red blood cell membranes of SHR, as compared with those of normotensive Wistar-Kyoto rats (WKY).

Phospholipase C, which is a key enzyme for phosphoinositide metabolism, degrades phosphoinositides, producing diacylglycerol and inositol polyphosphate, which are the integral components of the intracellular calcium messenger system. Thus, phospholipase C activity may play an important role in the alterations of cytoplasmic free calcium concentration in SHR. In this study, we attempted to define phospholipase C activity in the aortic wall of SHR and assess its relation to the development of genetic hypertension.
Materials and Methods

Preparation of Enzymatic Fraction

Nine 14-week-old SHR and ten 14-week-old WKY (from the University of Tokyo breeding colony) were fed a 0.6% NaCl chow after weaning. The blood pressure was measured in conscious rats by the tail-cuff method on two different days and averaged for each rat. On the following day, they underwent laparotomy while under pentobarbital sodium (25 mg/kg) anesthesia and the descending aortas were removed. The aortas were immediately immersed in ice-cold physiological saline, and the surrounding connective tissues were removed. Each of the prepared aortic vessels was minced by scissors and then homogenized in 1 ml of physiological saline at 0°C by a Polytron blender (Kinematica GmbH, Littau, Switzerland). The homogenate was spun at 10,000 g at 4°C for 1 hour, and the supernatant was used as an enzymatic solution for phospholipase C and phospholipase A2. The enzymatic fraction was stored at −70°C until the assay. The precipitate was dried at 70°C for 48 hours and weighted.

Phospholipase C and Phospholipase A2

of 4-Week-Old SHR and WKY

Seven 4-week-old SHR and ten 4-week-old WKY were fed 0.6% NaCl chow after weaning. The mean blood pressure was measured through a catheter inserted in the left carotid artery with a Nihon Kohden electronic manometer (Model RM-6000, Tokyo, Japan) with the rats under Inactin (80 mg/kg) anesthesia. After blood pressure was measured, they were immediately laparotomized and the descending aortas were removed and prepared as just described. The 104 g supernatant of the aortic wall homogenate was used to measure phospholipase C and A2 activities.

Assay for Phospholipase C Activity

Phospholipase C activity was measured based on a modified method of Rittenhouse.10 Our assay system used N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) buffer and a lower concentration of deoxycholate sodium. Briefly, 50 µl of the supernatant was preincubated at 4°C for 15 minutes in 200 µl of 50 mM HEPES buffer (pH 7.0) containing 0.1 M NaCl, 5 mM CaCl2, and 1.25 mM deoxycholate sodium. The reaction was started by adding the substrate, 50 µl of 1-stearoyl-2-[1-14C]arachidonyl L-3-phosphatidylglycerol (5-6 mCi/mmol; 3 × 106 dpm), and terminated using Folch's solvent after a 30-minute incubation. The [14C]arachidonate that was produced from the radioactive phosphatidylcholine was extracted using Folch's solvent and separated with a silicic acid TLC. The separation pattern was analyzed by an Aloca TLC scanner system. The [14C]arachidonate was scraped off, and its radioactivity was determined by an automatic liquid scintillation counter. The enzymatic activity was related to its dry tissue weight.

Phospholipase A2 Activity

Phospholipase A2 activity was measured with a modification of the method of Billah et al.12 Briefly, 50 µl of the supernatant was preincubated at 37°C for 30 minutes in 200 µl of 0.1 M glycine buffer (pH 8.0) containing 0.2 M NaCl, 2 mM CaCl2, and 1.25 mM deoxycholate sodium. The reaction was initiated by adding 50 µl of 1-stearoyl-2-[1-14C]arachidonyl L-3-phosphatidylcholine (5-6 mCi/mmol; 3 × 106 dpm) and terminated using Folch's solvent after a 30-minute incubation. The [14C]arachidonate that was produced from the radioactive phosphatidylcholine was extracted using Folch's solvent and separated with a silicic acid TLC. The separation pattern was analyzed by an Aloca TLC scanner system. The [14C]arachidonate was scraped off, and its radioactivity was determined by an automatic liquid scintillation counter. The enzymatic activity was related to its dry tissue weight.

Analysis of Enzymatic Properties of Vascular Phospholipase C of SHR

To disclose the activation mechanisms of vascular phospholipase C of SHR, we investigated the properties of phospholipase C in the aortic wall of SHR and WKY. Ten 14-week-old SHR and ten 14-week-old WKY fed 0.6% NaCl chow after weaning were anesthetized with pentobarbital sodium (25 mg/kg), and the descending aortas were removed and processed as already described. The pooled supernatant from the aortic wall homogenate of SHR and WKY was prepared separately for each strain. The Michaelis constant (Km) value, pH profile, and calcium requirement were determined for the vascular phospholipase C of SHR, and these values were compared with those of control WKY.

Reagents and Statistical Analysis

The reagents were all of analytical grade. The radioactive materials were purchased from Amersham, Buckinghamshire, England.

The values were expressed as means ± SE. The difference was analyzed by Student's t test or two-way analysis of variance.

Results

Figure 1 shows the radiochromatograms of phospholipase products in the vascular wall. As presented in the upper panel of the figure, the supernatant of the aortic wall preparation degraded 1-stearoyl-2[1-14C]arachidonyl L-3-phosphatidylglycerol and mainly produced [14C]diacylglycerol when the assay was run for phospholipase C. [14C]Arachidonate could be produced from either the further cleavage of [14C]diacylglycerol by diacylglycerol lipase or the direct uncoupling of the sn-2-position of [14C]phosphatidylglycerol by phospholipase A2. However, its radioactivity was...
FIGURE 1. Radiochromatograms of phospholipase C (upper panel) and phospholipase A2 (lower panel) products. The ordinate presents the radioactivity, and the abscissa shows the distance of migration. PI = phosphatidylinositol; PC = phosphatidylcholine; DAG = diacylglycerol; AA = arachidonate.

much less than that of [14C]diacylglycerol. On the other hand, in the assay condition for phospholipase A2, the radioactivity was detected only at the migration zone of arachidonate, and not around that of diacylglycerol (see Figure 1, lower panel).

The average systolic blood pressure was significantly higher in 14-week-old SHR than in age-matched WKY (164 ± 3 vs 110 ± 2 mm Hg; p < 0.001). As shown in Figure 2, vascular phospholipase C was significantly activated and almost doubled in SHR, as compared with that in normotensive WKY (112 ± 12 vs 60 ± 7 µg/min/g protein; p < 0.001). In contrast, the activity of vascular phospholipase A2, which is another membrane-bound phosphodiesterase, was not altered in SHR (see Figure 2).

Figure 3 shows the interrelation between vascular phospholipase activities and the blood pressure levels in 14-week-old SHR and WKY. Vascular phospholipase C activity was positively correlated with the blood pressure level (r = 0.62, p < 0.003), whereas vascular phospholipase A2 activity was not.

Phospholipase activities were also examined in the aortic wall of 4-week-old SHR and WKY. Although still in the prehypertensive stage, SHR had 12 mm Hg higher mean arterial blood pressure than WKY (95 ± 1 vs 83 ± 1 mm Hg; p < 0.001). As shown in Figure 4, vascular phospholipase C activity was significantly enhanced (+62%) in 4-week-old SHR, as compared with age-matched WKY (144 ± 17 vs 88 ± 6 µg/min/g protein; p < 0.006). There was no difference in vascular phospholipase A2 activity between the two strains.

We also investigated phospholipase C activity in the heart of 14-week-old SHR and WKY. Cardiac phospholipase C activities were 233 ± 20 µg/min/g protein for SHR (n = 6) and 274 ± 10 µg/min/g protein for control WKY (n = 7). In contrast, to vascular phospholipase C, cardiac phospholipase C in SHR tended to be decreased, but the difference was not statistically significant.

To unveil the activation mechanisms of phospholipase C in the aortic wall of SHR, we assessed the K_m values of vascular phospholipase C in 14-week-old SHR and control WKY according to the Lineweaver and Burk plots. As depicted in Figure 5, the K_m value of phospholipase C in SHR was about one fourth of that of WKY. The affinity of phospholipase C to the substrate was apparently four times greater in SHR than in WKY. Maximum velocity (V_max) values were 0.268 for WKY and 0.200 nmol/min for SHR.

Figure 6 shows the difference in the pH profile of vascular phospholipase C between the two strains. The pH profile for vascular phospholipase C of WKY presented two peaks of optimum pH at 5.5 and about 7.2, which is consistent with the results of Hirasawa et al. In contrast, only one peak at pH 5.5 occurred in SHR.

Next, we compared the calcium requirement of vascular phospholipase C of SHR with that of WKY (Figure 7). Whereas the vascular phospholipase C of control WKY was activated dose-dependently by calcium at concentrations of 0 to 5 × 10^{-3} M, the vascular phospholipase C of SHR was maximally stimulated at 5 × 10^{-4} M calcium.

Discussion

We used 10^4 g supernatant of aortic wall homogenate to measure phospholipase C and phospholipase A2 activities. Although this supernatant contained both enzymes, the substrate specificity and the employed assay condition made it feasible to assess each of the phospholipase activities separately (see Figure 1).

We demonstrated that the phospholipase C activity in vascular wall in 14-week-old SHR almost doubled, as compared with that in normotensive WKY. Moreover, the vascular phospholipase C activities bore a positive relation to the blood pressure levels in SHR and WKY. The cause and consequence of these findings remain obscure; however, 4-week-old SHR, which were still in the prehypertensive stage, also possessed enhanced vascular phospholipase C activity as compared with age-matched WKY. In addition, vascular phospholipase A2 activity, which is another type...
AORTIC PHOSPHOLIPASE IN GENETIC HYPERTENSION/Uehara et al.

Figure 2. Phospholipase C and phospholipase A2 activity in the aortic wall of SHR and WKY.

Figure 3. Relationship between vascular phospholipase C or phospholipase A2 activity and blood pressure level in 14-week-old SHR (*) and WKY (○).

Figure 4. Phospholipase C and phospholipase A2 activity in the aortic wall of 4-week-old SHR and WKY.
Calcium Requirement

![Graph showing Calcium Requirement](image)

**Figure 5.** Estimation of the $K_m^*$ value of the vascular phospholipase C of SHR and WKY. The constant was calculated according to Lineweaver and Burk plots. $K_m^*$ is the $K_m$ value of phospholipase C in WKY and $K_m^*$ is the value in SHR. Each plot was the average of four studies. Pooled materials from 20 SHR and 20 WKY were used. The same experiment was repeated three times, using a different batch of pooled materials each time.

**Figure 6.** The pH profile of the vascular phospholipase C of SHR and WKY. 1-Stearoyl-2-arachidonyl-[1-14C]phosphatidylinositol was used as substrate. The substrate concentration was 25 mM, and the assay was performed under $V_{max}$ conditions. The value was the average of five studies. Pooled materials from 15 SHR and 15 WKY were used. The same experiment was repeated three times, using a different batch of pooled materials each time. The pH profiles were analyzed by two-way analysis of variance. F values were 7.30 for strain effect ($p<0.018$) and 26.42 for pH effect ($p<0.0001$).

**Figure 7.** Calcium requirement of vascular phospholipase C in SHR and WKY. The value was the average of five studies. Pooled materials from 15 SHR and 20 WKY were used. The same experiment was repeated three times, using a different batch of pooled materials each time. Single (p<0.001) and double (p<0.05) asterisks indicate significant difference compared with each of the maximum values. Calcium requirement patterns were also analyzed by two-way analysis of variance. F values were 5.70 for strain effect ($p<0.077$) and 91.97 for calcium concentration effect ($p<0.0003$).

results indicate a causative role of enhanced vascular phospholipase C activity in the development of hypertension in SHR rather than a consequential event.

In this study we investigated hydrolysis of phosphatidylinositol by phospholipase C. Our results are not necessarily applicable to the breakdown of phosphatidylinositol polyphosphates, which is connected more directly to the intracellular calcium messenger system. However, there is some evidence that phospholipase C is able to cleave phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate as well. Ritpenhouse also found that the substrate affinity of platelet phospholipase C to phosphatidylinositol was greater than to phosphatidylinositol polyphosphates. Thus, alterations of vascular phospholipase C in SHR could affect the hydrolysis of phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-bisphosphate.

The enhanced phospholipase C activity might bring about a rapid turnover rate of phosphoinositides, thereby increasing the cytoplasmic free calcium through the production of inositol 1,4,5-trisphosphate. Indeed, Koutouzov et al. have reported that the turnover rate of phosphoinositides was increased in the membranes of red blood corpuscles of SHR. Although Murakawa et al. reported no increase, Furspan and Bohr found...
that intracellular free calcium was significantly higher in the lymphocytes of SHR as compared with control WKY. Similarly, Bruschi et al.⁵ have provided evidence for increased free calcium in the platelets of SHR. Thus, the rapid breakdown of phosphoinositides by enhanced vascular phospholipase C in SHR could have contributed at least partly to the elevation of cytoplasmic free calcium and the subsequent increase in vascular tonus or vasoconstrictor responses to vasoactive stimuli in SHR.¹⁷,¹⁸

The mechanisms of activation of vascular phospholipase C in SHR are uncertain. We found that the Kᵣ values of vascular phospholipase C were different between SHR and control WKY. The Kᵣ value of vascular phospholipase C in SHR was about one fourth of that of WKY. These results suggest that the enhanced phospholipase C activity in SHR could be accounted for not only by a quantitative increase in the enzyme molecule but also by structural changes in the phospholipase C, which are likely to strengthen the enzymatic activity. Structural alterations in vascular phospholipase C in SHR were also indicated by the finding that the pH profile of vascular phospholipase C in SHR was distinct from that in control WKY.

Guanosine 5'-triphosphate binding protein, which is activated by angiotensin I⁶ or vasopressin,²⁰ potentiates phospholipase C activity directly or through a decrease in its calcium requirement to as low as 10⁻⁵ M.²¹ Moreover, Hirasawa et al.¹³ recently reported that the phospholipase C in the brain comprised several isoenzymes. Thus, receptor-mediated activation or isoenzymatic alterations might elicit enhanced vascular phospholipase C activity in SHR. As shown in Figure 4, however, very young SHR, which were still in the prehypertensive stage, also possessed increased vascular phospholipase C activity. There was a discrepancy between vascular and cardiac phospholipase C activities in adult SHR. That is, cardiac phospholipase C activity in SHR almost equaled that in WKY. Vascular phospholipase C was also activated at the calcium concentration of 5 x 10⁻⁶ M by up to 64 or 67% of the maximum stimulation for SHR or WKY, respectively. Seemingly, all of these results are more suggestive of involvements of an activation mechanism other than receptor-mediated stimulation. Moreover, the changes in calcium sensitivity at the higher concentrations (e.g., 10⁻³ or 10⁻² M) may partly mediate the activation of vascular phospholipase C in SHR. To clarify the activation mechanism in SHR, we need to purify vascular phospholipase C of SHR and WKY, using isoelectric chromatofocusing, and further explore the differences in their structures and properties. Further experiments are now under way to address these questions more directly.
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