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PRODUCTION OF LYSOPHOSPHATIDIC ACID BY LYSOPHOSPHOLIPASE D IN INCUBATED PLASMA OF SPONTANEOUSLY HYPERTENSIVE RATS AND WISTAR KYOTO RATS

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Summary

Lysophosphatidic acid has been identified as a vasopressor principle in incubated mammalian plasma and sera, and shown to be generated extracellulary by lysophospholipase D-like activity. In this study, we monitored the time course of changes in the major phospholipid fractions during incubation of plasma, and found that polyunsaturated lysophosphatidic acids accumulate more rapidly than saturated lysophosphatidic acids at expense of the corresponding lysophosphatidylcholines. We compared the phospholipase activities for producing bioactive LPA in age-matched spontaeneously hypertensive rats and Wistar Kyoto rats. The lysophospholipase D activity in rat plasma was found to be independent of strain and age. We suggest that lysophospholipase D functions in rat for persistent production of bioactive LPA in the circulation throughout life. However, our finding that production of LPA in spontaenously hypertensive rats was not greater than that in Wistar Kyoto rats does not seem to support the idea that increased production of LPA is involved in the pathogenesis of hypertension.

Key Words: lysophosphatidic acid, lysophospholipase D, lysophosphatidylcholine, lecithin:cholesterol acyltransferase, spontaneously hypertensive rat, aging

It has long been known that a vasoactive lipid is produced during incubation of plasma and sera of various animals at 25-37°C. One research group named this lipid depressor active substance, because it promptly induced hypotension when

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injected i.v. into cats (1). Independently, other groups using rats as assay models, also found a vasopressor substance, which they named vasopressor phospholipid (2) and active pressor principle (3). In 1978, we reported that lysophosphatidic acid (LPA) identified in crude soybean lecithin as a vasopressor in rats (4) has species-specific vasoactivity, causing hypotension in cats and rabbits, and hypertension in rats and guinea pigs (5). Subsequently, Schumacher *et al.* (6) suggested from thin layer chromatographic analyses that both phosphatidic acid and LPA are active components of the depressor active substance from rat plasma. We have also identified a vasopressor phospholipid in incubated rat plasma as LPA by two-dimensional thin-layer chromatography (TLC) and gas chromatography-mass spectrometry, and shown that it is formed from circulating lysophosphatidylcholine (LPC), a predominant phospholipid in rat plasma, by lysophospholipase D (LPLD) (7). Maebashi and Yoshinaga (8) reported that the vasopressor activity of an unknown polar lipid, probably LPA, in incubated serum of patients with essential hypertension is significantly higher than that in incubated serum of healthy volunteers. Furthermore, they provided experimental evidence that the production of a pressor substance in incubated serum of spontaneously hypertensive rats (SHR) is more than that in serum from normotensive Wistar rats. Unfortunately, most of their results were dependent on the biological activity of the pressor substance, however, its chemical structure was not identified.

LPA has now been recognized as a biomodulator having diverse *in vitro* biological activities as well as *in vivo* vasoactivity: it causes contraction of smooth muscles, aggregations of platelets, proliferation of fibroblasts, activation of oocytes and inductions of neurite retraction (9-11). Moreover, there is evidence for the existence of LPA receptors (12,13), and LPA receptor genes have recently been cloned (14-16).

In this study, we measured the time-courese of accumulation of LPA by the action of LPLD together with the changes in amounts of phosphatidylcholine (PC) and LPC during incubation of plasma from SHR and Wistar Kyoto strain of rats (WKY), using a modified quantitative gas-liquid chromatographic method that we reported previously (17). Moreover, using a newly devised radioactive LPC method, we examined whether plasma from SHR of different ages accumulates more LPA than plasma from age-matched WKY.

Methods

Materials: Fatty acid-free bovine serum albumin (A6003), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (17:0/17:0-PC), 1-heptadecanoyl-2-lyso-*sn*-glycero-3-phosphocholine (17:0-LPC), 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (16:0-LPC), 1-oleoyl-2-lyso-*sn*-glycerol-3-phosphate (18:1-LPA) and phospholipase D from *Streptomyces chromofuscus* were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Heptadecanoyl-2-lyso-*sn*-glycerol-3-phosphate (17:0-LPA) was prepared by hydrolysis of 17:0-LPC with *Streptomyces chromofuscus* phospholipase D, as described previously (17). 1-[^{14}C]Palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (1480-2220 MBq/mmol) was purchased from Dupont-NEN Research Products.

Extraction of plasma lipids: Male SHR and WKY (8,15 and 30-weeks-old) were anesthetized with sodium pentobarbitol (40 mg/kg, i.p.) after starvation for 10-12h. Blood (6-8 ml) was withdrawn through a catheter inserted into an abdominal artery into a polyethylene tube containing sodium heparin (bovine intestine). The final concentration of heparin was adjusted to 2 I.U./ml. The blood was then promptly centrifuged at 4°C (1,500 x g, 30 min), and the plasma was incubated at 37°C for

various times. Aliquots (0.1-0.3 ml) of plasma were withdrawn after these times and diluted with 2% KCl to 2 ml. Lipids were extracted from the aqueous solution acidified to pH 2.5 with 1 N HCl by the method of Bligh and Dyer (18) after additions of 17:0/17:0-PC (160 μg/ml of plasma), 17:0-LPC (70 μg/ml of plasma) and 17:0-LPA (28 μg/ml of plasma) as internal standards. Lipid extracts in tubes were evaporated to dryness under a stream of nitrogen gas, and stored in the dark at -20°C.

TLC of plasma lipids: Lipid extracts from 0.1-0.3 ml of plasma were dissolved in a small volume of chloroform/methanol mixture (2:1, vol/vol), and the solution was applied to Merck Silica gel 60 plates as lines of 1-2 cm width. The plates were developed with chloroform/methanol/20% ammonium hydroxide (60:35:8, by vol). Lipid bands were located under a UV-lamp after spraying the plates with a solution of 6-*p*-toluidino-2-naphthalenesulfonic acid (1 mM) in 50 mM Tris-HCl (pH 7.4), using standard phospholipids as markers. The bands of PC, LPC and LPA were scraped off the silica gel plates and suspended in 2 ml of water. PC and LPC were extracted from the silica by the method of Bligh and Dyer (18). LPA was recovered by the same method after acidification of the aqueous phase to pH 2.5. Lipid-phosphate was measured with malachite green as described previously (19).

Fatty acid composition of PC, LPC and LPA: PC, LPC and LPA separated by TLC of lipid extracts from fresh and incubated rat plasma were dried in tubes with a glass stopper. Then 0.5 ml of 5% HCl in methanol was added. The mixtures were heated at 100°C for 3 h, and mixed vigorously with 3 ml each of *n*-hexane and 2% $KHCO_3$. After phase separation, the *n*-hexane layer was withdrawn, and the remaining aqueous solution was mixed with 3 ml of *n*-hexane. The two *n*-hexane layers were combined, dried on sodium nitrite, and then evaporated to dryness. The residue was dissolved in a small volume of *n*-hexane and aliquots were subjected to gas-liquid chromatography on a Hitachi 263-70 gas chromatograph with a capillary column (J & W Scientific, DB-225, 30 m x 0.24 mm I.D., 0.25 mm thickness). The column temperature was kept at 120°C for 1 min and then increased to rise to 220°C at 10°C/min. The detector and injector temperatures were 250°C.

Measurement of LPLD activity: In our standard method for assay of LPLD activity, we used [^{14}C]16:0-LPC as a substrate. Plasma (1.2 ml) from heparinized rat blood was preincubated for 5 min and mixed with 100 μl of saline containing 0.25% bovine serum albumin and [^{14}C]16:0-LPC (0.0074 MBq, 3.5 nmol). A 0.2-ml aliquot of plasma was withdrawn as a control immediately after the addition of [^{14}C]16:0-LPC. The remaining plasma was incubated at 37°C for up to 6 h, and 0.2 ml samples were withdrawn after 2, 4 and 6 h incubation. Lipids were extracted from the plasma after its dilution with 2% KCl and acidification with 1 N HCl, as described above. The lipid extracts were fractionated with 18:1-LPA as a carrier on Silica gel 60 TLC plates in a solvent system of chloroform/methanol/20% ammonium hydroxide (60:35:8, by vol.). Lipid bands were located under a UV-lamp after spraying with an ethanolic solution of 6-*p*-toluidino-2-naphthalenesulfonic acid, and were then scraped off, mixed with 5 ml of Sintisol EX-H, and counted in a liquid scintillation counter.

Statistical analysis: Experimental values are shown as means ± S.E.M. Differences between means were compared by Student's *t*-test. A *P* value of less than 0.05 was considered significant.

Results

We measured the PC, LPC and LPA contents of rat plasma as fatty acid methyl esters derived from TLC-purified phospholipids. Changes in PC, LPC and LPA contents during 48-h incubation of plasma from 8-week-old SHR are shown in Fig. 1-A. PC decreased rapidly in the first 6 h, and then more slowly. Conversely, LPC increased in the first 6 h of incubation, and slightly in the second 6 h of incubation. Figures 2-A and B show changes in the fatty acid compositions of PC and LPC, respectively, during incubation of plasma from 8-week-old SHR. The early increase in the total amount of LPC was found to be due to increases of palmitoyl (16:0)- and stearoyl (18:0)-LPCs, corresponding with decreased PCs with a palmitate and stearate residue, respectively. In contrast, the plasma concentrations of oleoyl (18:1)-, linoleoyl (18:2)- and arachidonoyl (20:4)-LPCs decreased continuously during incubation. These results suggest that an enzymatic reaction by lecithin:cholesterol acyltransferase (LCAT), which transfers an *sn*-2-unsaturated fatty acyl moiety of PC to free cholesterol (20), yielding 16:0- and 18:0-LPCs, was active in an early stage of incubation. But, the rate of this reaction was considerably less in the second 6 h of incubation than in the first 6 h, when the degradation of LPC by another enzyme, LPLD, which converts both saturated and unsaturated LPCs to the corresponding LPAs, was predominant. In fact, the accumulations of 18:0- and 16:0-LPAs were slow, and nearly linear during 24 h of incubation of plasma from SHR, as shown in Fig. 3-A and B. Therefore, the early phase of degradations of 18:0- and 16:0-LPC by LPLD would be masked by their larger increases caused by LCAT. The level of 18:1-LPC decreased slowly without an initial rise during incubation, unlike those of saturated LPCs (Fig. 2-B). Similarly, the accumulation of 18:1-LPA was slow, as shown in Fig. 3-C, but polyunsaturated LPAs accumulated more rapidly (Fig. 3-D and E) than saturated and mono-unsaturated LPAs (Fig. 3-A, B and C).

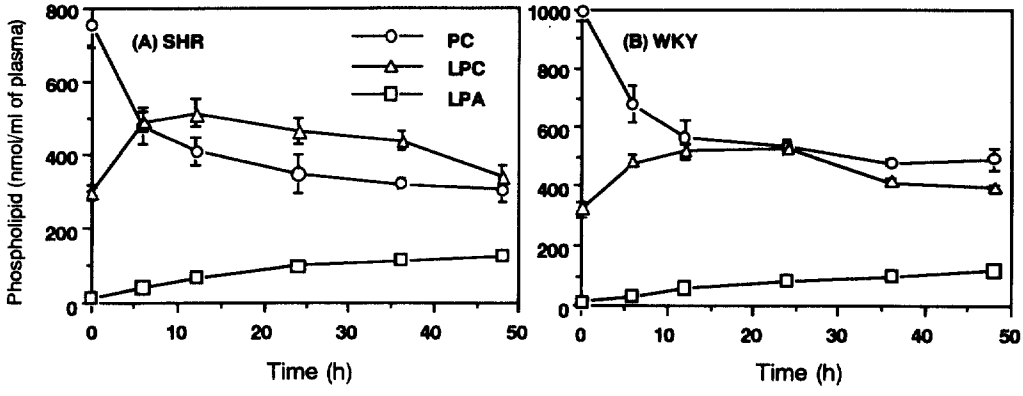

Fig. 1

Changes in total amounts of PC (circles), LPC (triangles) and LPA (squares) during 48-h incubation of plasma of 8-week-old SHR (A) and WKY (B).

Next, we analyzed phospholipids in plasma from WKY, before and after incubation at 37°C for up to 48 h. The total amount of LPC in fresh plasma from WKY (Fig. 1-B) was almost the same as that in SHR plasma (Fig. 1-A), whereas the total amount of PC in WKY was significantly higher than that in SHR. On incubation, various PCs in the plasma of WKY decreased markedly in the first 6 h and then gradually (Fig. 2-C), like the PCs in the plasma from SHR (Fig. 2-A). Changes in the plasma concentrations of

16:0- and 18:0-LPCs on incubation of WKY plasma were similar to those observed in SHR plasma, except that 18:0-LPC increased for up to 12 and 24 h in WKY and SHR, respectively (Fig. 2-D and B). As in the case of SHR, the levels of 18:1-, 18:2- and 20:4-LPCs in WKY plasma decreased slowly with time (Fig. 2-D). Furthermore, there were no significant differences in the apparent rates of production of major LPAs during incubation of plasma between WKY and SHR (Fig. 3).

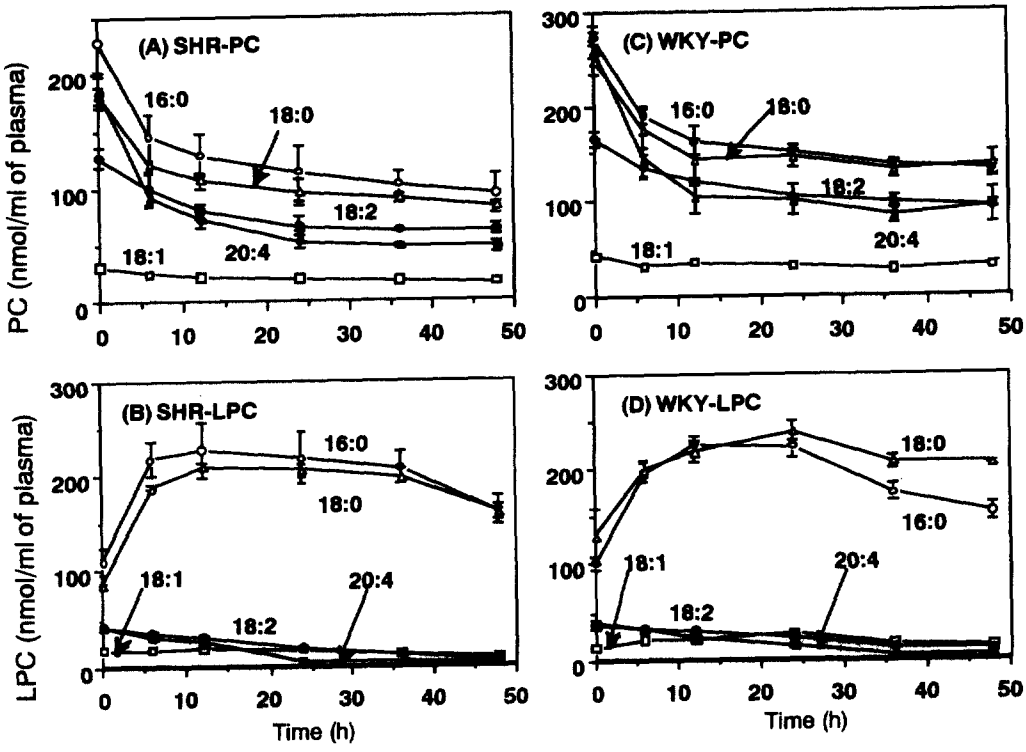

Fig. 2

Changes in fatty acid compositions of PC (A,C) and LPC (B,D) during incubation of plasma obtained from 8-week-old SHR (A, B) and WKY (C, D), respectively. Where no error bars are shown, the errors are within the symbol. 16:0 (○), 18:0 (△), 18:1 (□), 18:2 (▲), 20:4 (●)

Table I summarizes results with WKY on the percentage conversions of LPCs to LPAs in 12-h and 48-h incubations. Because the amounts of 16:0-LPC and 18:0-LPC increased maximally after 6-h incubation, their conversions (%) were calculated based on these values. Results showed that the percentage conversions of unsaturated LPCs to LPAs were higher than those of saturated LPCs. Although 18:1-LPC in the plasma was the lowest, the enzyme hydrolyzed 18:1-LPC as efficiently as it did 18:2- and 20:4-LPC.

For assessment of LPLD activity, rat plasma was incubated with [^{14}C]16:0-LPC for up to 6 h. The amount of radioactive LPA increased almost linearly throughout the incubation period, and thus, the rate of the reaction by LPLD was calculated from the slope of the straight line obtained. Using this convenient method, we examined the plasma LPLD activities of SHR and WKY of different ages. As shown in Table II, their

plasma LPLD activities were all independent of age. There was no significant difference between the activities of LPLD in age-matched SHR and WKY plasma.

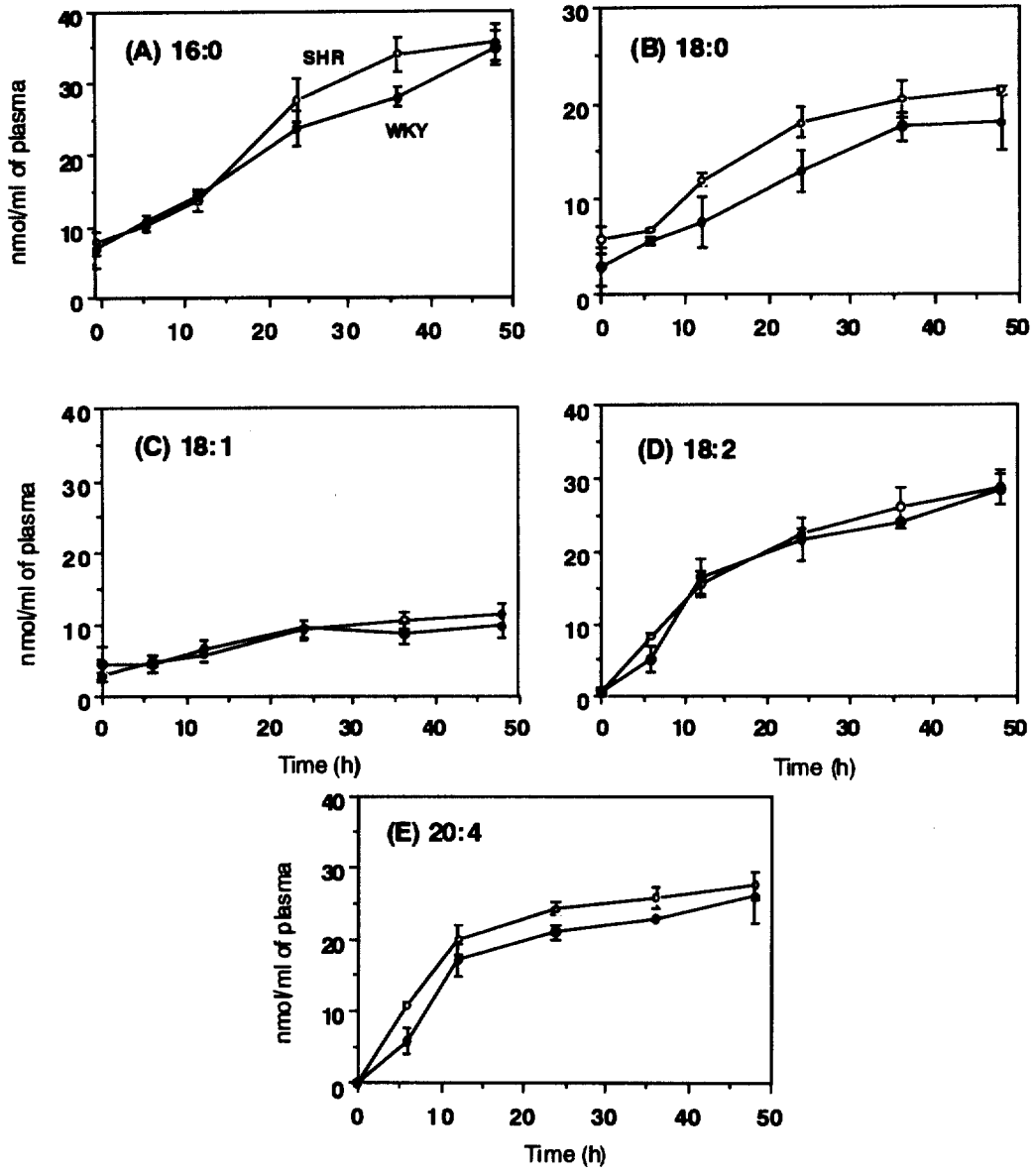

Fig. 3

Changes in levels of 16:0 (A)-, 18:0 (B)-, 18:1 (C)-, 18:2 (D)- and 20:4 (E)-LPA during incubation of plasma obtained from 8-week-old SHR (open circles) and WKY (closed circles), respectively. Values are means ± S.E.M. of 3-5 experiments. Where no error bars are shown, errors were lesser than the size of the symbols.

TABLE I

Metabolic Conversion of Unsaturated LPCs Rather Than Saturated LPCs to LPAs

Incubation time (h)	Conversion of LPC to LPA (%)				
	16:0	18:0	18:1	18:2	20:4
12	6.5[a]	3.5[a]	48.2[b]	44.5[b]	43.7[b]
48	15.5[a]	8.3[a]	69.4[b]	77.2[b]	66.9[b]

a) and *b)*

Values are molar percentages of LPAs produced during incubation of rat plasma, and calculated based on amounts of LPCs in 6h-incubated plasma (a) and fresh plasma (b).

TABLE II

Influences of Strain and Age of Rats on Plasma LPLD Activity

Strain	Age (weeks)	Number	LPLD (%/h)
SHR	8	3	0.66 ± 0.03
	15	4	0.56 ± 0.03
	30	3	0.64 ± 0.02
WKY	8	4	0.72 ± 0.03
	15	3	0.61 ± 0.01
	30	3	0.60 ± 0.01

Discussion

Many studies have established that LPA is a phospholipid acting as a first messenger with diverse biological activities (9-11), like platelet-activating factor. Furthermore, LPA is known to be an active component in the serum, aged plasma and ascites (6,7,17,21-23), and has been shown to be released from activated platelets (22,24) or generated enzymatically by LPLD-like activity in extracellular fluids such as plasma (7), and follicular fluid (A. Tokumura *et al.*, unpublished data). The present study focused on the LPLD reaction in rat plasma with two main findings.

First, we found that considerably high concentrations of LPA were accumulated by the action of LPLD, and that unsaturated LPCs in rat plasma were hydrolysed to LPAs more than saturated LPCs by LPLD as assesssed by percentage conversions of endogenous LPCs. However, the actual amounts of LPA accumulated during incubation decreased in the following order: 18:2=20:4>16:0>18:0>18:1, due to the various levels of endogenous LPCs. These results confirm our previous finding (7) showing that LPLD shows higher activity on unsaturated LPCs than on saturated LPCs.

Second, our results clearly showed that aging failed to affect plasma LPLD activity. Moreover, the LPLD activity in SHR plasma was found to be almost the same as that of age-matched WKY. This is not consistent with our expectation that accelerated generation of LPA in the circulation of SHR may be involved in the induction and maintenance of their hypertension, although their pressor response to i.v. injection of LPA is reported to be higher than that of WKY (25).

Intracellular LPLD activities that are activated by Ca^{2+} or Mg^{2+}, have been shown to generate LPA (26-29), but this type of enzyme activity in various tissues of rats was found to hydrolyze alkyl-ether type lysophospholipids, not acyl type lysophospholipids (27), and therefore should be limited to the control of biological activity of platelet-activating factor and local generation of alkyl-ether type LPA. As we reported previously (7), LPLD in rat plasma is responsible for the extracellular production of acyl type LPA, rather than alkyl ether type LPA, and it seems to require metal ions such as Zn^{2+} or Co^{2+}, but not Ca^{2+} or Mg^{2+} (30), indicating that it is different from intracellular LPLD. The regulatory mechanism of this novel type of lysophospholipase D in the circulation must be clarifed to understand the physiological and pathophysiological roles of bioactive LPA.

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