Increased formation of lysophosphatidic acids by lysophospholipase D in serum of hypercholesterolemic rabbits

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Abstract **Lysophosphatidic acid (LPA) is a biologically active phospholipid that has been identified as a vasoactive principle in incubated plasma and serum of mammals. Previously, we found that mammalian plasma and serum contain a lysophospholipase D, which hydrolyzes lysophosphatidylcholines (LPCs) with different fatty acyl groups to the corresponding LPAs during its incubation at 37°C. In this study, we examined whether lysophospholipase D activity and levels of LPCs in rabbit serum were modulated by feeding rabbits a high cholesterol diet. Results showed that the serum levels of LPCs increased gradually in animals fed a high cholesterol diet for 12 weeks. We found that the levels of individual LPAs formed on incubation of serum for 24 h increased with an increase in the period of feeding of rabbits a high cholesterol diet. LPA with a linoleate residue was the most abundant LPA, followed in order by 16:0-, 18:1- and 18:0-LPAs. LPA was found to increase attachment of the monocytic cell line THP-1 to vascular endothelial cells pre-stimulated with tumor necrosis factor-α. These results indicated that increases in the levels of LPAs generated by lysophospholipase D in the blood of hypercholesterolemic rabbits may be relevant to attachment of monocytes to vascular walls, a key phenomenon observed at an early stage of atherosclerosis.**—Tokumura, A., Y. Kanaya, M. Kitahara, M. Miyake, Y. Yoshioka, and K. Fukuzawa. **Increased formation of lysophosphatidic acids by lysophospholipase D in serum of hypercholesterolemic rabbits.** ***J. Lipid Res.*** **2002.** 43: **307–315.**

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Patients suffering from chronic diseases relevant to atherosclerosis, as a basic vascular dysfunction, are still increasing in many countries. The formation of fatty streaks in atheromatic lesions has been thought to be related to increased levels of lipids including cholesterol in the blood circulation. The migration of blood monocytes into subendothelial spaces is known to be closely associated with the formation of fatty streaks (1). For evaluation of the relationship of hypercholesterolemia with atherosclerosis or vascular endothelial function, rabbits were fed a high cholesterol diet for several weeks, since atheroma formation rapidly advances in this animal model (2). In this model, high dietary cholesterol was found to promote vascular lesions such as the formation of fatty streaks (2, 3), and to impair dilatory responses of the blood vasculature due to nitric oxide generation (2, 4). Active identities inducing the vascular lesions such as fatty plaques and accompanying vascular dysfunction are still in debate. Oxidative modification of LDL penetrating through vascular endothelial cells has been recognized as a key phenomenon for induction of atherogenesis (1). Two bioactive phospholipids in the oxidized lipoproteins have been suggested as critical for the pathogenesis of atheroma formation. One is a family of phosphatidylcholines (PCs) having an oxidatively shortened fatty acyl chain, the structures of which resemble that of platelet-activating factor (5, 6). The other is lysophosphatidylcholines (LPCs) formed from platelet-activating factor-like PCs by the action of the platelet-activating factor acetylhydrolase in plasma (5). Both components in the oxidized LDL are known to activate blood monocytes (7, 8), which participate in the impairment of vascular cells at an early stage of atherosclerosis (1). However, altered levels of LPC in the blood and vascular tissues were reported in various pathophysiological situations without increased oxidative stress (9). Thus, unrecognized mechanisms are likely to be relevant to early events inducing atherogenesis.

Lysophosphatidic acid (LPA) is an important phospho-

Abbreviations: GC-MS, gas chromatography-mass spectrometry; HUVEC, human umbilical venous endothelial cell; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; TMS, trimethylsilyl.

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lipid mediator with diverse receptor-mediated biological activities (6, 10, 11). Three different genes encoding G protein-coupled receptors specific for LPA have been characterized so far: Edg-2 (12), Edg-4 (13), and Edg-7 (14). Recently, this bioactive lysophospholipid has been postulated to be responsible for the pathogenesis of atherosclerosis (15, 16), since it aggregates platelets (17, 18), promotes the de-differentiation (16) and proliferation (19) of vascular smooth muscle cells, and activates vascular endothelial cells (15), monocytes (20), and macrophages (21). Second, LPA was identified as a major platelet-aggregating substance in oxidized LDL, a key component for induction of atherogenesis (22).

LPA is known to be produced by sequential intracellular reactions of phospholipid remodeling, and released, in part, into an extracellular medium (6, 23). However, a more specialized mechanism, extracellular production of LPA, is also known. The latter mechanism involves a novel metal-requiring lysophospholipase D activity that was originally found in heparinized rat plasma (24); it hydrolyses different molecular species of LPC to the corresponding LPA (24–28). In order to gain information about the possible contribution of LPAs generated by lysophospholipase D to the induction of atherogenesis, in this study, we examined whether serum lysophospholipase D activity increased on feeding a high cholesterol diet for several weeks to rabbits, an animal model of atherosclerosis.

MATERIALS AND METHODS

Phospholipase D from cabbage, 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (17:0/17:0[2]-phosphatidylcholine; PC), 1-palmitoyl (16:0)- and heptadecanoyl (17:0)-2-lyso-*sn*-glycero-3-phosphocholines (16:0- and 17:0-LPCs), 1-oleoyl (18:1)-2-lyso-*sn*-glycerol-3-phosphate (18:1-LPA), and botulinum toxin C3 were obtained from Sigma Chemical (St. Louis, MO). 1-Linoleoyl (18:2)-2-lyso-*sn*-glycero-3-phosphocholine (18:2-LPC) was from Doosan Serdary Research Laboratories (London, Canada). 1-Arachidonoyl (20:4)-2-lyso-*sn*-glycero-3-phosphocholine (20:4-LPC) was prepared by hydrolysis of 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (Doosan Serdary Research Laboratories) as described previously (29). 1-Palmitoyl-, heptadecanoyl-, linoleoyl-, and arachidonoyl-2-lyso-*sn*-glycerol-3-phosphate (16:0-, 17:0-, 18:2-, and 20:4-LPAs) were prepared by the hydrolyses of 16:0-, 17:0-, 18:2-, and 20:4-LPCs with cabbage phospholipase D (19, 28). Trimethylchlorosilane, *N,O-bis*(trimethylsilyl)trifluoroacetamide and Sintisol EX-H were from Wako Pure Chemicals (Osaka, Japan). 2,7-*Bis*(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) was from Dojindo (Kumamoto, Japan).

Male New Zealand white rabbits weighing 2.4–2.9 kg were housed with a cycle of 12 h light and 12 h dark. Rabbits received a normal diet or a diet supplemented with 1% cholesterol (100 g/day) and 250 ml/day of water. Before receiving the diet, blood (5–10 ml) was withdrawn from an ear vein, and serum was prepared by centrifugation of the blood at 3,000 rpm for 15 min after standing it for 3 h at room temperature.

Serum preparations (0.15 ml) were mixed with 230 nmol of 17:0-LPC and 350 nmol of 17:0/17:0-PC per ml of serum, as internal standards. Phospholipids were extracted by the method of Bligh and Dyer (30), and fractionated by TLC on a Whatman Silica gel K6 TLC plate as described above. Both PC and LPC were extracted from silica gel by the method of Bligh and Dyer (30) after their mixing with 1–2 ml of distilled water. The recovered PC and LPC were heated in 0.5 ml of 5% methanolic hydrochloride solution at 100°C for 3 h to convert them to fatty acid methyl esters. The fatty acid methyl esters were extracted with *n*-hexane three times, and quantified by GLC, as described previously (26).

Aliquots of 0.1 ml of serum preparations from normal and hypercholesterolemic rabbits were incubated with 1-[^{14}C]16:0-LPC (0.15 mCi/mmol, 16.7 nmol/ml of serum) at 37°C for 2, 4, and 6 h. The radioactive LPC and its metabolites were extracted by the method of Bligh and Dyer (30) after the addition of 0.2 g KCl, dilution with distilled water to 1 ml, acidification of the aqueous phase to pH 2.5, and finally separated by TLC with chloroform-methanol-20% ammonium hydroxide (60:35:8, v/v/v) on a Merck Silica gel 60 TLC plate after addition of 18:1-LPA (0.1 μmol/sample) as a carrier. Lipids were localized under an ultraviolet lamp after spraying with 1% *p*-toluidino-2-naphthalene sulfonic acid in 50 mM Tris/HCl buffer. Silica gel from lipid zones containing PC, LPC, LPA, and free fatty acid were scraped off the plate. The radioactivity of the silica gels was counted in 5 ml of Sintisol EX-H in a liquid scintillation counter to calculate the rate of conversion of [^{14}C]16:0-LPC to LPA (%/h/ml of serum), as described previously (25).

LPAs purified by TLC on a Whatman K6 silica gel TLC plate were treated with 25 μl of pyridine and 25 μl of *N,O-bis*(trimethylsilyl)trifluoroacetamide containing 2% trimethylchlorosilane at 65°C for 45 min. Aliquots of 1 μl of the reaction mixture were directly injected at 280°C into a gas chromatograph equipped with a DB-1 [J & W Scientific (Foster City, CA); 30 m × 0.25 mm, 0.25 μm thickness] with a program of increase in the column temperature from 200°C to 320°C at a rate of 10°C/min. The temperature of the ion source was 180°C. Electron impact ionization-mass spectra were measured with a JEOL JMS FX102A mass spectrometer coupled to the gas chromatograph under the following conditions: 70 eV of ionizing voltage, 300 μA of ionizing current, 10 kV of accelerating voltage.

To examine whether LPA can impact functions of blood monocytes, we measured adhesion of BCECF-AM-labeled monocytic cell line THP-1 cells to human umbilical venous endothelial cells (HUVEC), essentially as described (31), since use of fluorescent dyes including BCECF-AM in the determination of adherence of human leukocytes to endothelial cells is known to be powerful tool (32). THP-1 cells were stimulated with 18:1-LPA and then co-cultured with HUVEC pre-activated with tumor necrosis factor-α.

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD), and maintained in RPMI-1640 medium supplemented with 20% FBS. HUVEC were obtained from Clontics Corporation (Walkersville, MD), and maintained in endothelial cell basic medium supplemented with 2% FBS, 10 ng/ml epidermal growth factor, 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, and 50 μg/ml gentamicin. THP-1 cells were suspended at a cell density of 5×10^5 in 1 ml of the culture medium supplemented with a 0.002 volume of a solution of 18:1-LPA at various concentrations in DMSO. Botulinum toxin C3 (ADP ribosyltransferase C3) was dissolved in the culture medium containing 5 μM digitonin and 50 μM 18:1-LPA. THP-1 cells were incubated in the medium with or without supplementation of 18:1-LPA for 48 h at 37°C under 5% CO_2. The cells were then incubated with a medium containing 10 μM BCECF-AM for 30 min. HUVEC cultured at confluence on 96 well culture plates

[2] Fatty acyl moieties of phospholipids are designated in terms of the number of carbon atoms: number of double bonds, e.g., 16:0 for palmitoyl.

were stimulated with 10 ng/ml tumor necrosis factor-α. After 8 h, a monolayer of HUVEC was loaded with BCECF-AM-labeled THP-1 cells (8×10^4 cells/well) at 37°C for 2 h under 5% CO_2. Nonadherent THP-1 cells were washed out, and fluorescence from the remaining adherent cells was measured at an excitation wavelength of 485 (half bandwidth: 20) nm, and emission wavelength of 530 (half band width: 25) nm. The number of adherent THP-1 cells was calculated from the intensities of fluorescence.

In another experiment, HUVEC were cultured in the presence of 3, 10, or 100 μM 18:1-LPA for 48 h, and then stimulated with 10 ng/ml tumor necrosis factor-α for 12 h. BCECF-AM-labeled THP-1 cells (1.5×10^5 cells/well) were loaded on the HUVEC culture and incubated for 2 h under 5% CO_2. Nonadherent THP-1 cells were washed out, and fluorescence was measured, as described above.

Differences of means were analyzed by Student's *t*-test, and considered significant at the 5% level.

RESULTS

Altered serum levels of LPCs by feeding rabbits with a high cholesterol diet affect activity of lysophospholipase D

Rabbits were placed on a diet containing 1% cholesterol for 12 weeks. Blood was collected at intervals, and fresh and incubated sera were prepared. First, we measured the concentration of different LPCs in the serum preparations before incubation. As shown in **Table 1**, the concentrations of total LPC and individual LPCs in the unincubated serum were greatly increased at an early stage of feeding of rabbits with a cholesterol-rich diet.

Previously, we reported that the concentrations of LPC in human serum and rat plasma were significantly increased within several hours after their incubation at 37°C (26–28). Hence, we examined whether the concentration of LPC was altered significantly during 6 h incubation of serum preparations of rabbits fed a normal or high cholesterol diet. Table 1 shows the levels of various molecular species of LPC in 6 h incubated serum preparations of normal and hypercholesterolemic rabbits. There were increases in the levels of 16:0- and 18:0-LPCs after 6 h incubation of sera from normal rabbits or rabbits fed a high cholesterol diet for 8–12 weeks, whereas the serum levels of other LPCs remain unchanged during the incubation. The changes in 16:0- and 18:0-LPCs were less with 6 h incubated sera from rabbits fed a high cholesterol diet for 1–4 weeks than those in incubated serum from rabbits fed a high cholesterol diet for 8–12 weeks. **Figure 1** represents the composition of molecular species of LPC in these serum preparations, expressed as percentages of total LPC. Results with unincubated and 6 h incubated sera showed that the percentage of 18:2-LPC increased at an early stage of high cholesterol-feeding (1–4 weeks), concomitant with decreases in the percentages of 16:0- and 18:0-LPCs. The percentage of 18:2-LPC, however, decreased slowly by prolonging high cholesterol-feeding up to 12 weeks, whereas those of 16:0- and 18:0-LPCs gradually increased to those of normal rabbits. There were no significant changes in the percentages of 18:1- and 20:4-LPCs throughout high cholesterol-feeding.

By multiplying the level of 16:0-LPC in the 6 h incubated sera from normal and high cholesterol-fed rabbits (Table 1, nmol/ml) by the fractional change of the conversion of [^{14}C]16:0-LPC to LPA (%/h), we estimated the initial production rate of 16:0-LPA (nmol/h) generated by lysophospholipase D activity. **Figure 2** shows that the corrected activity of lysophospholipase D was decreased after the high cholesterol-feeding for 1 week followed by a small increase in the activity at early stages of feeding with a high cholesterol diet. However, the corrected activity did not significantly change with further prolonged feeding of a high cholesterol diet.

Analysis of LPA generated by lysophospholipase D during incubation of serum

Because the effect of dietary high cholesterol on the serum lysophospholipase D activity toward the single mo-

TABLE 1. Serum levels of different molecular species of LPCs before and after 6 h incubation of sera from normal and hypercholesterolemic rabbits

Serum/Cholesterol Feeding	LPC					
	16:0	18:0	18:1	18:2	20:4	Total
weeks	*nmol/ml*					
Unincubated serum						
0	88 ± 14[a]	65 ± 7	29 ± 2	45 ± 13	2.0 ± 0.8	234 ± 14
1	167 ± 30	106 ± 13	46 ± 7	111 ± 19	3.3 ± 1.5	444 ± 66
2	280 ± 68	213 ± 38	90 ± 25	256 ± 41	13 ± 3.5	867 ± 172
4	336 ± 111	309 ± 96	146 ± 48	361 ± 101	20 ± 8	1,199 ± 314
8	425 ± 181	325 ± 57	200 ± 40	284 ± 58	35 ± 6	1,301 ± 324
10	577 ± 63	421 ± 90	178 ± 27	308 ± 74	34 ± 6	1,562 ± 221
12	482 ± 170	305 ± 68	98 ± 47	187 ± 23	11 ± 1.5	1,099 ± 194
6 h incubated serum						
0	125 ± 20	89 ± 10	30 ± 2	45 ± 11	2.5 ± 1	297 ± 22
1	156 ± 34	119 ± 17	47 ± 8	77 ± 15	3.5 ± 0.8	407 ± 70
2	292 ± 65	244 ± 56	98 ± 26	275 ± 78	14 ± 5	957 ± 212
4	400 ± 76	301 ± 55	150 ± 30	334 ± 60	22 ± 6	1,222 ± 199
8	586 ± 101	455 ± 93	211 ± 37	309 ± 54	30 ± 1.1	1,629 ± 253
10	535 ± 74	573 ± 35	225 ± 47	365 ± 126	52 ± 2.7	1,801 ± 285
12	557 ± 124	419 ± 94	145 ± 25	186 ± 35	11 ± 2.6	1,338 ± 234

[a] Values of are means ± SEM for 4–5 animals.

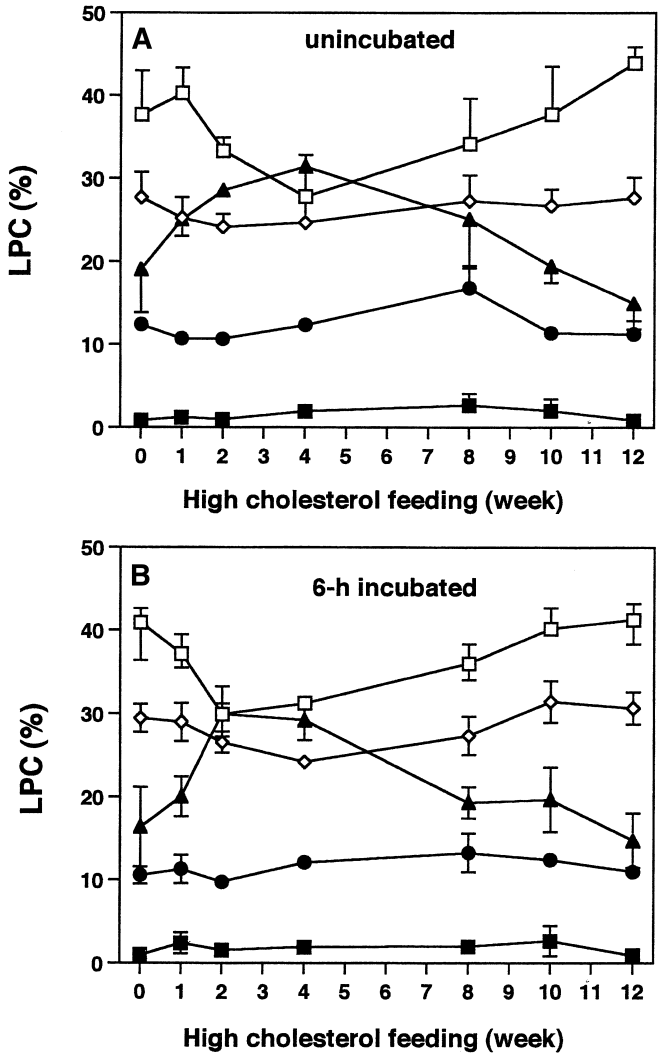

Fig. 1. Fatty acid compositions of lysophosphatidylcholine (LPC) before (A) and after (B) 6 h incubation of serum from normal and hypercholesterolemic rabbits. Values are means ± SEM for 4–5 animals. Symbols used are (open square) (16:0), (open diamond) (18:0), (closed circle) (18:1), (closed triangle) (18:2), and (closed square) (20:4).

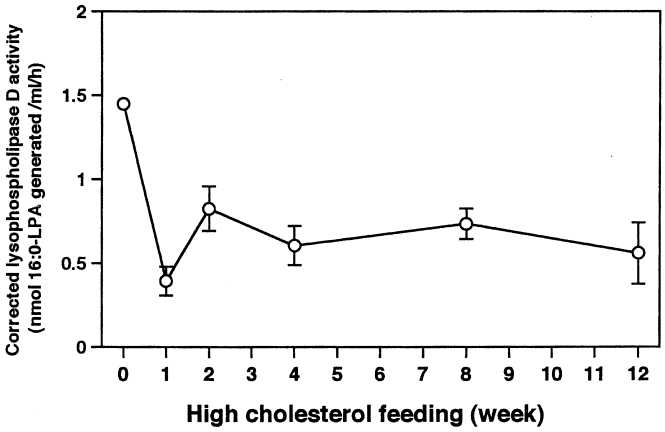

Fig. 2. Lysophospholipase D activity corrected for the level of endogenous substrate, 16:0-LPC, in incubated serum. The amount of 16:0-LPA produced by lysophospholipase D per h was estimated by multiplying the percentage conversion of [^{14}C]16:0-LPC by the level of endogenous 16:0-LPC in 6 h incubated serum (Table 1).

lecular species of LPC was rather inhibitory, and somewhat complicated, we attempted to examine this issue by adapting other approaches that can monitor the conversions of different LPCs to corresponding LPAs. For this purpose, we decided to utilize direct analysis of TLC-purified LPAs by GC-MS after their conversion to volatile derivatives having three trimethylsilyl (TMS) groups, without any hydrolytic pretreatment. **Figure 3B** shows typical results with LPAs purified from 24 h incubated serum of a rabbit fed a high cholesterol diet for 4 weeks, indicating the ion-current profiles of various ions at *m/z* 639, 637, 635, 625, 611 corresponding to $[M - CH_3]^+$ of TMS derivatives of 18:0-, 18:1-, 18:2-, 17:0-, and 16:0-LPA, respectively, with various magnifications for the 24 h incubated serum. These ion peaks were normalized to have the same peak height by magnifying them to different degrees, as shown in Fig. 3. Little signals of $[M - CH_3]^+$ of TMS derivatives of endogenous LPAs were observed for unincubated serum of a rabbit fed with a high cholesterol diet for 4 weeks (Fig. 3A). The amounts of LPAs in these serum preparations were determined on the basis of peak areas of $[M - CH_3]^+$ of TMS derivatives of endogenous LPAs relative to that of TMS-17:0-LPA, an internal standard, after corrections on the basis of variable heat-stability of various TMS derivatives of LPAs in the gas phase and their efficiencies of ionization upon electron impact. For this purpose, we measured ratios of $[M - CH_3]^+$ of TMS derivatives of standard LPAs to that of TMS derivative of 17:0-LPA with different molar ratios. Because the retention times of TMS derivatives of 18:1- and 18:2-LPAs were the same under our conditions, the $[M - CH_3]^+$ of TMS derivative of 18:1-LPA could not be distinguished from $[M - CH_3 + 2]^+$ of TMS-18:2-LPA. This was also corrected for quantification of 18:1-LPA as follows: the peak area of $[M - CH_3 + 2]^+$ due to 18:2-LPA-TMS was subtracted from the observed peak area of $[M - CH_3]^+$ of 18:1-LPA-TMS (*m/z* 637). The former was calculated by multiplying the observed peak area due to $[M - CH_3]^+$ of 18:2-LPA-TMS (*m/z* 635) by the ratio of $[M - CH_3 + 2]^+$ to $[M - CH_3]^+$ obtained by GC-MS of standard 18:2-LPA-TMS. **Figure 4** shows results of such quantitative experiments for LPAs in the 24 h incubated serum preparations of rabbits fed a normal diet or high cholesterol diet. The total amount of LPA accumulated during the 24 h incubation of serum was found to be remarkably elevated with increase in the feeding period for up to 8 weeks, and thereafter declined slightly. The most predominant molecular species of LPA was 18:2-LPA followed by 16:0-, 18:1-, and 18:0-LPAs in order. At a late stage of feeding of the high cholesterol diet, the production of 18:2-LPA declined, whereas the generation of 16:0-LPA increased slightly for up to 12 weeks. The levels of other LPAs reached maxima after 8–10 weeks feeding of the high cholesterol diet. However, the molecular species compositions of fresh serum LPA from hypercholesterolemic rabbits were not similar to those from normal animals; 18:2-LPA was the predominant species for the

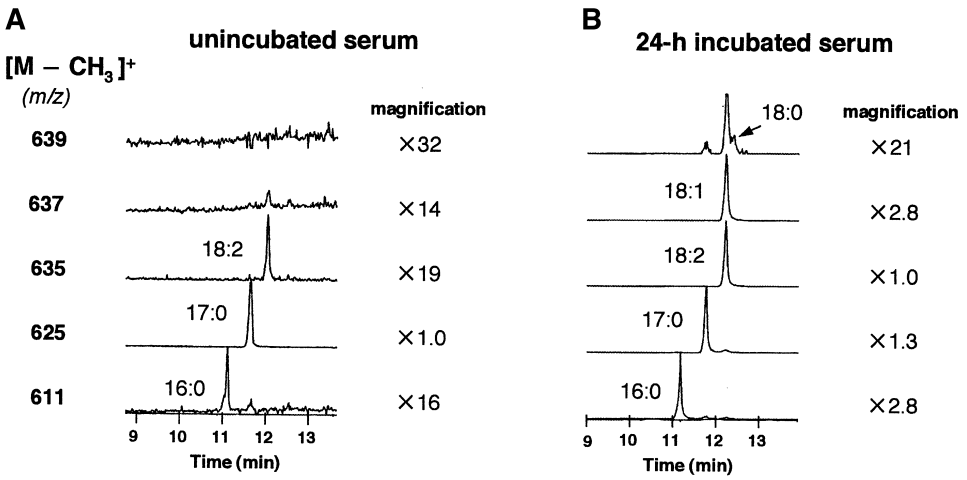

Fig. 3. Gas chromatography-mass spectrometry (GC-MS) of trimetylsilyl (TMS) derivatives of lysophosphatidic acids (LPAs) purified from lipid extracts of rabbit serum before and after incubation for 24 h. Serum from a rabbit fed a high cholesterol diet for 4 weeks was incubated at 37°C for 24 h. Lipids were extracted from the serum before (A) and after (B) incubation, and fractionated by TLC. Purified LPAs were recovered from the silica gel, and converted to volatile derivatives with three TMS groups. Ion-current profiles of five selected ions (*m/z* 639, 637, 635, 625, 611 for $[M - CH_3]^+$ of 18:0-, 18:1-, 18:2, 17:0-, and 16:0-LPAs, respectively) are shown at various magnifications where the major ions has the same peak height.

hypercholesterolemic groups, whereas 16:0-LPA was abundant in the serum of normal rabbits (**Fig. 5**).

LPA activates monocytes to attach to vascular endothelial cells

As described above, the increased formation of LPA by lysophospholipase D in the blood circulation in hypercholesterolemic rabbits may contribute to early events inducing atherosclerosis. In this study, we examined whether LPA was able to activate monocytic THP-1 cells to attach to vascular endothelial cells that is known to be a key phenomenon related to atherogenesis. After THP-1 cells were pre-activated with various concentrations of 18:1-LPA, the monocytic cells were incubated with HUVEC pre-stimulated with tumor necrosis factor-α. Under these conditions, LPA promoted the adhesion of THP-1 cells to the endothelial cells in a concentration-dependent manner (**Table 2**, experiment A). The enhancing effect of 50 μM 18:1-LPA was inhibited by pretreatment of THP-1 cells with 15 or 50 μg/ml ADP ribosyltransferase C3, indicating that LPA augments the monocyte/vascular endothelial cell interaction by a Rho-mediated mechanism (Table 2, experiment B). **Figure 6** shows a result of similar adherent experiment where HUVEC, but not THP-1 cells, were incubated with LPA, and then stimulated with tumor necrosis factor-α. The result showed that LPA failed to enhance the activating effect of tumor necrosis factor-α on HUVEC to bind THP-1 cells.

DISCUSSION

An animal model is useful in studies on LPA generation under pathophysiological conditions to evaluate the source of serum LPA and diseases to which the altered level of LPA may be relevant. Therefore, we conducted experiments with rabbits fed a high cholesterol diet as a model of atherosclerosis. We found great, gradual increases in PC content in the serum of the animals given 1% cholesterol in their diet for 12 weeks (data not shown), consistent with an earlier report of about nine times higher PC in the serum of rabbits fed a diet with 1% cholesterol than in normal rabbits (33). There are several reports that the level of LPC, the endogenous substrate of lysophospholipase D, is increased in hypercholesterolemic rabbits (34–36), and our results confirmed these previous findings. These results appear to be closely related to a large increase in lipoprotein concentration in the serum of hypercholesterolemic rabbits. Plasma LPC was proposed as a potential causal agent of atherosclerosis, based on the good correlation between atherosclerotic risk factors and LPC levels (9). In mammals other than rabbits, hypercholesterolemic and hyperlipidemic serum or plasma preparations were shown to contain more LPC than normal preparations (37–40).

In this study, we found that the total amount of LPA in unincubated serum of hypercholesterolemic (8–12 weeks) was higher than that of normal rabbits. The higher serum level of total LPA in the hypercholesterolemic rabbits may be due to the increased level of lipoproteins that are potential reservoirs for plasma LPA, while a predominant portion of LPA in rat plasma was reported to be bound to albumin, not to lipoprotein (26). The reason for the observed low level of total LPA in fresh serum from hypercholesterolemic rabbits (1–4 weeks) remains obscure. However, the increased level of 18:2-LPA was more likely to be attributed to elevated apparent activities of LPA-producing enzymes such as lysophospholipase D, as discussed below.

At the end of the 1970s, LPA was reported to be a vasoactive principle in incubated plasma and serum of mam-

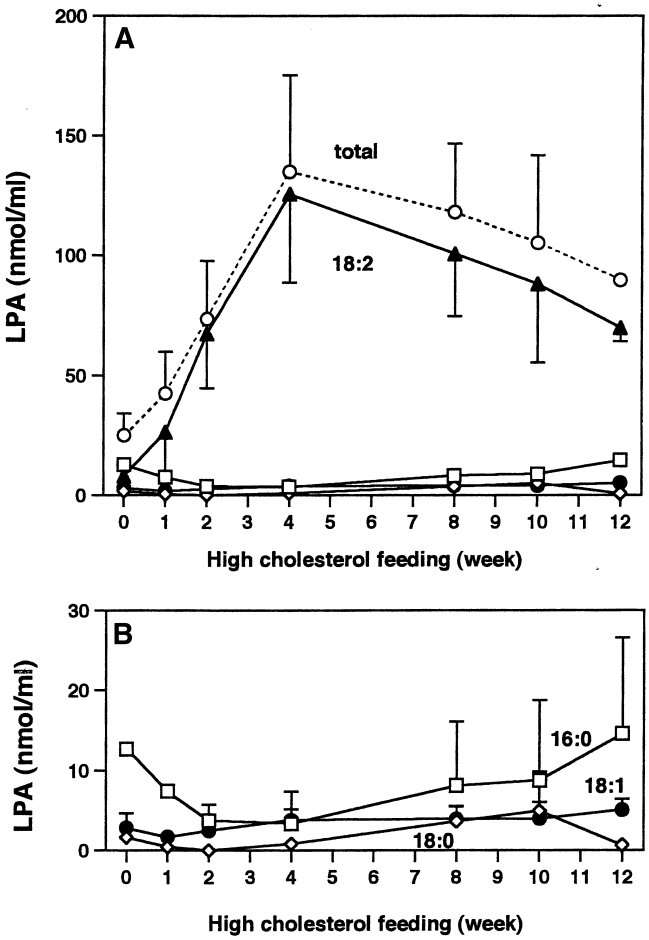

Fig. 4. Levels of various LPAs in 24 h incubated sera from rabbits fed a normal or high cholesterol diet. Serum was prepared from rabbits fed a normal diet or a high cholesterol diet for various periods. Levels of four major species of LPA were measured in these serum preparations after incubation at 37°C for 24 h (A). Data for 16:0-, 18:0-, and 18:1-LPAs were enlarged (B). Values are means ± S.E.M. for 4–5 animals. Symbols used are (open square) (16:0), (open diamond) (18:0), (closed circle) (18:1), (open triangle) (18:2), and total (open circle).

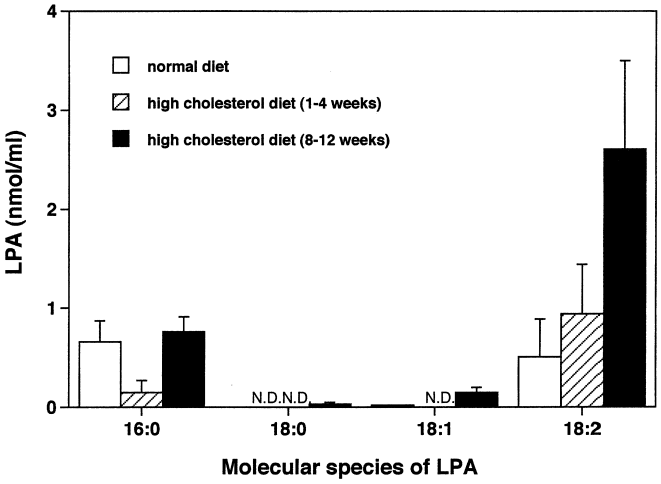

Fig. 5. Levels of various LPAs in unincubated sera from rabbits fed a normal or high cholesterol diet for various periods. Serum preparations were prepared from rabbits fed a normal diet or a high cholesterol diet for various periods. Levels of four major species of LPA were measured on these serum preparations. Values are means ± SEM for two or three animals. White, hatched, and black bars show results on rabbits fed a normal diet, high cholesterol diet for 1–4 weeks, and high cholesterol diet for 8–12 weeks, respectively. ND, not detected.

mals (17), but this finding attracted little attention. There has been more interest in the occurrence of LPA in animal serum since a report by van Corven et al. who identified LPA as an active component in animal serum, causing proliferation of fibroblasts (41). Although the origin of LPA in human serum was shown to be activated platelets (42), the serum preparation contains lysophospholipase D activity that may convert endogenous LPCs in serum to LPAs during its preparation (28). For preparing the serum preparation, the withdrawn blood was often stood for several hours at various temperatures. Therefore, caution would be needed to suppress potential formation of LPA by lysophospholipase D activity during the preparation of serum. For assessment of the LPA level in the blood circulation in vivo, plasma anticoagulated with EDTA would be suitable, since we previously found that lysophospholipase D activity in rat plasma was suppressed by pretreatment of the blood with EDTA, but not citrate or heparin (25). However, the absolute amounts of LPA in unincubated rabbit serum preparations, that seem to reflect in vivo situation, were much less than those in in vitro 24 h incubated serum preparations.

The present study showed that 18:2-LPC was an abun-

TABLE 2. Stimulatory effect of LPA on adhesion of the THP-1 monocytic cell line to HUVEC activated by tumor necrosis factor-α and its inhibition by treatment of monocytes with ADP ribosyltransferase C3

Experiment	Treatment	Number of Adherent Cells
A	Basal adhesion	655 ± 124
	Control adhesion	966 ± 85
	LPA	
	12.5 μM	1,197 ± 113
	25 μM	2,860 ± 854[a]
	50 μM	3,214 ± 65[a]
	100 μM	3,530 ± 280[a]
B	Digitonin control	3,888 ± 199
	LPA+C3	
	0 μg/ml	5,897 ± 1,389
	15 μg/ml	4,056 ± 144[b]
	50 μg/ml	3,652 ± 1014[b]

Experiment A: THP-1 cells were pre-stimulated with 12.5, 25, 50, or 100 μM 18:1-LPA for 48 h. After incubation of the LPA-stimulated THP-1 cells with BCECF-AM, the cells at a density of 8×10^4 were loaded on HUVEC cultured in a 96 well plate, and incubated for 2 h. Control adhesion and basal adhesion represent results of experiments with LPA-unstimulated THP-1 cells and HUVEC with or without pre-stimulation with tumor necrosis factor-α, respectively. The numbers of adherent THP-1 cells were measured as described in Materials and Methods. Experiment B: THP-1 cells were pre-stimulated with 50 μM 18:1-LPA for 48 h in the presence (C3+LPA) of ADP ribosyltransferase (15 or 50 μg/ml) or vehicle containing 5 μM digitonin alone (digitonin control). The BCECF-AM-treated THP-1 cells (8×10^4 cells) were loaded on HUVEC cultured in a 96 well plate, and incubated for 2 h. Values are means ± SD for six determinations.

[a] $P < 0.01$ versus control adhesion.

[b] $P < 0.05$ versus LPA+C3 (0 μg/ml).

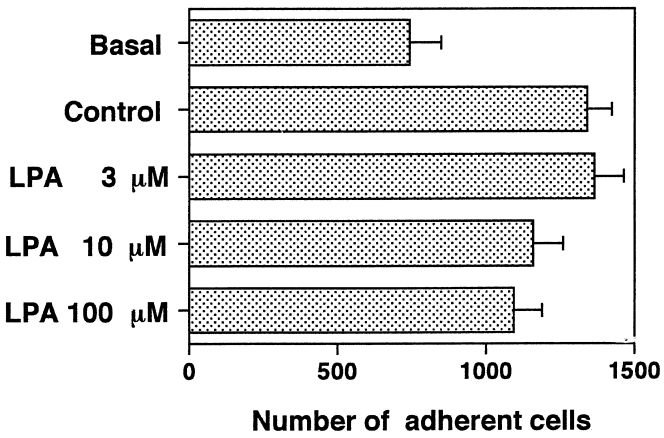

Fig. 6. No significant enhancing effect of LPA on capability of HUVEC stimulated with tumor necrosis factor-α-induced to bind THP-1 cells. Basal: experiment on HUVEC unstimulated with tumor necrosis factor-α. Control: experiment on HUVEC stimulated with tumor necrosis factor-α alone. LPA: experiments on HUVEC incubated with LPA and then stimulated with tumor necrosis factor-α.

dant molecular species of LPC as well as 16:0-LPC (Fig. 1), but the generation of 18:2-LPA was preferable to that of 16:0-LPA in rabbit serum incubated at 37°C for 24 h, suggesting that the substrate specificity of rabbit serum lysophospholipase D resembles that of lysophospholipase D in rat (24–27) and human (28) plasma; they prefer unsaturated LPCs over saturated LPCs. An additional novel observation is that percentage conversion of 18:2-LPC to 18:2-LPA in hypercholesterolemic rabbits after 2 weeks feeding with a high cholesterol diet was about 2-fold that in normal rabbits. Two explanations are likely for this observation: the first is that a lysophospholipase D with a distinct substrate specificity preferring more 18:2-LPC may be secreted into the blood circulation of rabbits after high cholesterol feeding, and cooperate with a constitutive lysophospholipiase D, giving rise to more 16:0-LPA. Alternatively, the relative enrichment of 18:2-LPC over other LPCs in the blood plasma may tend to preferential production of 18:2-LPA over other LPAs. The increased levels of lipoprotein in the blood after cholesterol feeding of rabbits may lead to not only the increases in serum levels of individual LPC, but also the alteration of the pattern of their distribution among proteins in the serum. The altered pattern of LPC distribution in hypercholesterolemic rabbits may be related to utilization of much more 18:2-LPC than other LPCs by lysophospholipase D. Taken together, we conclude that the feeding of rabbits with a high cholesterol diet not only induces elevation of the total amount of LPA in the serum, but also change its molecular species composition.

The position of the fatty acyl moiety attaching to the glycerol backbone of LPA in rabbit serum was not determined in this study. However, the determining the percentage of the *sn*-1 and 2-acyl LPA is of potential value for a better understanding of their physiological role, because Edg-7 has been characterized as a gene of an LPA receptor selective for LPAs having an *sn*-2-unsaturated fatty acyl group (14). We previously reported that unsaturated LPCs in fresh and incubated rat plasma existed as mainly an 1-acyl isomer, indicating that unsaturated LPAs generated from the LPCs by lysophospholipase D activity during the incubation of rat plasma would be predominantly 1-acyl isomers (26). Croset et al. (43) reported similar results on ratios of positional isomer of LPCs in human and rat plasma. They further found that about 50% of the unsaturated LPC was 2-acyl isomer when the blood was directly collected in organic solvent, indicating that the fast isomerization of LPC occurred during preparation of plasma. Perhaps both isomers of unsaturated LPCs circulate in the blood in vivo. These results suggest that both 1- and 2-unsaturated acyl LPAs can be produced from the corresponding LPCs in the blood circulation. In this context, it should be mentioned that a recent paper by Hayashi et al. (16) who found that unsaturated LPA, but not saturated LPAs, in human serum modulated a phenotype of vascular smooth muscle from the differentiated state to the dedifferentiated one, that is involved in the development of atherosclerosis, although they did not determine the position of the unsaturated fatty acyl moiety on these LPAs.

Activation of blood monocytes and their adhesion to injured vascular endothelium has been suggested as a participant in the early processes leading to atherosclerosis (1, 8). In this study, we first focused on the effect of LPA on monocytes, because LPA generated in blood circulation was expected to interact with blood monocytes, and little is known about LPA-induced monocyte activation except for a report showing that high concentrations (0.1–1 mM) of LPA induced directional migration of human monocytes by a haptotactic mechanism after binding to its receptor coupled to a pertussis toxin-sensitive G protein in a polycarbonate filter assay (20). Our results clearly showed that LPA at physiological concentrations activated the monocytic cell line THP-1 to attach to HUVEC prestimulated by tumor necrosis factor-α, and that the effect of 18:1-LPA was prevented by pretreatment of THP-1 cells with ADP ribosyltransferase C3 exoenzyme. These results suggest that LPA stimulates cell surface adhesion molecules via activation of Rho GTPase in THP-1 cells. Rho GTPase is well-known to be involved in the intracellular signaling pathways triggered by LPA in a variety of cells (44, 45). We also found that LPA failed to enhance the adhesion of HUVEC pre-stimulated with tumor necrosis factor-α to resting THP-1 cells. However, LPA was found to activate human aortic endothelial cells to bind monocytes and neutrophilic HL-60 cells, by the mediating of the pertussis toxin-sensitive induction of vascular cell adhesion molecule-1 and E-selectin on the endothelial cells (15). The reason for the inconsistencies between these studies remains unknown, however, might be due to different cell types and/or distinct experimental conditions with or without stimulation by tumor necrosis factor-α.

Taken altogether, we suggest that the elevated production of LPAs by lysophospholipase D in the blood plasma of hypercholesterolemic rabbits may play a role in induction of atherosclerosis by enhancing the penetration of blood monocytes into the subendothelial space of vascular

tissues. We are now conducting experiments on the structure-activity relationship and mechanism of LPA-induced activation of monocytes to attach vascular endothelial cells.

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