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Evaluation of Inhibitory Actions of Flavonols and Related Substances on Lysophospholipase D Activity of Serum Autotaxin by a Convenient Assay Using a Chromogenic Substrate

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Overproduction of lysophosphatidic acid (LPA) by lysophospholipase D/autotaxin (lysoPLD/ATX) is postulated to be involved in the promotion of cancer and atherosclerosis. A lysoPLD inhibitor may be utilized to ameliorate the LPA-related pathological conditions. In this study, a new assay was devised to quantify *p*-nitrophenol from hydrolysis of chromogenic substrate by serum lysoPLD without tedious lipid extraction procedures. Flavonols, phenolic acids, free fatty acids, and *N*-acyltyrosines inhibited lysoPLD activity in a micromolar range. They were classified into competitive, noncompetitive, or mixed type inhibitors. The results show that the low hydrophobicity of an inhibitor is a critical factor in its preference for the binding to a noncatalytic binding site over a catalytic binding site. Considering its reported bioavailability and the low dependency of its inhibitory activity on serum dilution, flavonol is likely to be a more effective lysoPLD inhibitor in human blood circulation in vivo than the other inhibitors including LPA.

KEYWORDS: Autotaxin; lysophosphatidic acid; lysophospholipase D; flavonol; phenolic acid; free fatty acid

INTRODUCTION

Lysophosphatidic acid (LPA) is an important intercellular mediator that plays a physiological role in a large array of animal cells through its specific G-protein-coupled receptors. This lysophospholipid mediator is known to participate in several pathological dysfunctions such as cancer metastasis and atherosclerosis. In fact, plasma lysophospholipase D (lysoPLD), an LPA-producing enzyme, was originally identified as autotaxin (ATX), which was first isolated as a tumor cell motility-stimulating protein in the culture medium of human melanoma cells (*1*−*3*). The lysoPLD activity of ATX has been detected in the following human body fluids in addition to plasma and serum: urine (*4*), ascitic fluid (*5*,*6*), peritoneal washing fluid (*6*), seminal fluid (*7*), follicular fluid (*8*), and cerebrospinal fluid (*9*). Overexpression of ATX was found in malignant tumor cells such as melanoma (*1*), teratocarcinoma (*10*), lung cancer cells (*11*), breast cancer cells (*12*), thyroid carcinoma cells (*13*), prostate cancer cells (*14*), neuroblastoma (*15*), glioblastoma multiforme (*16*), and EBV-positive Hodgkin's lymphoma (*17*). Considered altogether, ATX, if expressed at an unusually high level, exerts tumor-aggressive and angiogenetic effects. Thus, development of inhibitors of ATX should be explored as potential candidates for clinical use.

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Several assays have been used to screen inhibitors of the lysoPLD activity of ATX. An assay with radioactive LPC was first used to determine lysoPLD activity in animal body fluids (*8*, *18*, *19*) and recently was applied to screen inhibitors of lysoPLD activity of ATX (*20*). Although the assay, which is accompanied by a tedious lipid extraction procedure, needs great care to avoid radioactive contamination throughout its performance, it identified the known phosphodiesterase inhibitors calmidazolium and vinpocetine, the protein kinase inhibitors damnacanthal and hypericin, and the tyrosine protein kinase inhibitors tyrphostin AG8 and AG213 as weak inhibitors of lysoPLD. Enzyme-coupled assays for the determination of choline (*2*, *3*) and LPA (*21*) are also applicable to monitoring of lysoPLD activity in animal biological fluids, but a possibility remains that the candidate compounds inhibit the activities of secondary enzymes such as choline oxidase, rather than the primary enzyme, lysoPLD. Thus, the first aim of this study was to devise a convenient and reliable assay to screen potential inhibitors of the lysoPLD activity of ATX.

LPA and its analogues, cyclic phosphatidic acid (PA) and its carba analogues, fatty alcohol phosphates as LPA analogues, acyl thiophosphates as LPA analogues, β-keto and β-hydroxy phosphonate analogues of LPA, α-substituted phosphonate analogues of LPA, α- and β-substitutes phosphonate analogues of LPA, *sn*-2-aminooxy analogue of LPA, Darmstoff analogues, and phosphonothioate and fluoromethylene phosphonate

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analogues of cyclic PA, were reported to be inhibitors of lysoPLD activity (*22*−*24*). However, little is known about the inhibitory effects of nonphospholipidic natural substances on the plasma lysoPLD activity of ATX. Thus, in the current study, we first attempted to explore the inhibitory potentials of natural phenolic antioxidants, including flavonols, on the plasma lysoPLD activity of ATX because flavonols have been shown to inhibit not only nucleotide phosphodiesterases (*25*) but also lipid-metabolizing enzymes such as lipoxygenases (*26*) and phospholipase A_2 (*27*). This novel effect of flavonols, if found, is expected to strengthen their beneficial effects including anticancer activity and therapeutic potential for human health. Therefore, we focused our attention on examining whether flavonols and related substances, which have a wide range of distribution in the plant kingdom, inhibit the lysoPLD activity of ATX, a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family (*28*).

MATERIALS AND METHODS

Materials. Lysophosphatidyl-*p*-nitrophenol [LPN, 2-linoleoyl (18:2)] was kindly given by Dr. T. Kishimoto (Alfresa Pharma, Osaka, Japan). 1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate sodium salt (16:0-LPA), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate sodium salt (18:1-LPA), and 1-*O*-alkyl-2-hydroxy-*sn*-glycero-3-phosphocholine (alkyl 16:0-LPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Phospholipase D from *Streptomyces chromofuscus*, daidzein, chrysin, *p*-coumaric acid, ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid), sinapic acid, chlorogenic acid, 3-(4-hydroxyphenyl)propionic acid, 4-hydroxyphenylacetic acid, *N*-(4-hydroxyphenyl)glycine, *N*-acetyl-L-phenylalanine, methyl (*R*)-(+)-2-(4-hydroxyphenoxy)propionate, 3-(4-hydroxyphenyl)-1-propanol, *N*-acetyl-L-tyrosine (2:0-NAT), and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Calcium chloride, 28% ammonia solution, 2,6-di-*tert*-butyl-*p*-cresol (BHT), gallic acid, L-tyrosine, L-(−)-phenylalanine, L-3-(3,4-dihydroxyphenyl)alanine, *n*-butyric acid (4:0-FA), *n*-hexanoic acid (6:0-FA), *n*-heptanoic acid (7:0-FA), *n*-decanoic acid (10:0-FA), lauric acid (12:0-FA), myristic acid (14:0-FA), propionyl chloride, *n*-butyryl chloride, and *n*-valeryl chloride were purchased from Nacalai Tesque (Kyoto, Japan). 4-Dimethylaminopyridine, rutin, caffeine monohydrate, apigenin, kaempferol, formic acid (1:0-FA), palmitic acid (16:0-FA), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Morin, quercetin dehydrate, isoquercitrin, 3-*tert*-butyl-4-hydroxyanisole (BHA), *n*-propyl gallate, 3-phenylpropionic acid, acetic acid (2:0-FA), *n*-octanoic acid (8:0-FA), *n*-decanoyl chloride, lauroyl chloride, myristoyl chloride, palmitoyl chloride, and methanol were purchased from Kanto Chemical Corp. (Tokyo, Japan). Filter-sterilized fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). *O*-Phosphorylethanolamine and stearic acid (18:0-FA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Palmitic anhydride was purchased from Bachem (Bubendolf, Switzerland). Quercetagetin, robinetin, isorhamnetin, isorhamnetin-3-*O*-glucoside, and tamarixetin were purchased from Extrasynthese (Genay Cedex, France). Myricetin was purchased from LKT Laboratories (St. Paul, MN). Fisetin, 4-(4-hydroxyphenyl)-2-butanone, and 8-hydroxyoctanoic acid (ωOH-8:0-FA) were purchased from Acros Organics (Geel, Belgium). (+)-Catechin hydrate was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). (−)-Epicatechin was purchased from Nagara Science (Gifu, Japan). *trans*-Resveratrol, luteolin, caffeic acid, eicosapentaenoic acid (20:5-FA), and docosahexaenoic acid (22:6-FA) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Genistein was purchased from Enzo Life Science International Inc. (Plymouth Meeting, PA). 3-(3,4-Dihydroxyphenyl)propionic acid was purchased from Alfa Aesar (Ward Hill, MA). Sodium oleate (18:1-FA), sodium α-linoleate (18:2-FA), sodium linolenate (18:3-FA), and sodium arachidonate (20:4-FA) were purchased from Nu-Chek Prep, Inc. (Elysian, MN).

Alkyl 16:0-LPA was prepared from alkyl 16:0-LPC using phospholipase D from *S. chromofuscus*, as previously reported (*29*). *N*-Palmitoylethanolaminephosphoric acid was prepared according to the modified method described by Sugiura et al. (*30*). Lipid−phosphorus was determined according to the method of Chalvardjian and Rudnicki (*31*). Other reagents were of analytical grade.

Assay of LysoPLD Activity. Test substances for assay of lysoPLD activity were dissolved in the following vehicles: saline for 16:0-LPA (∼3.4 mM), 18:1-LPA (∼3.4 mM), alkyl 16:0-LPA, and *N*-palmitoylethanolaminephosphoric acid (∼0.17 mM); double-distilled water for 16:0-LPA (∼1.2 mM), caffeine, L-(−)-phenylalanine, 4-(4-hydroxyphenyl)-2-butanone, 18:1-FA, 18:2-FA, 18:3-FA, and 20:4-FA (∼100 mM); diluted sodium hydroxide solution for L-tyrosine (∼100 mM); diluted hydrochloric acid for L-3-(3,4-dihydroxyphenyl)alanine (∼100 mM); and DMSO for 20:5-FA (∼10 mM), isorhamnetin-3-*O*-glucoside (∼30 mM), quercetagetin (∼60 mM), and all other substances tested (∼100 mM). The final concentration of DMSO was below 1% in all assay mixtures.

In a standard assay, 50 μL of diluted FBS (5% in saline; final, 0.83%) in each well of a 96-well microplate (Thermo Fisher Scientific, Waltham, MA) was mixed with 172 μL of saline. Then, the mixture was incubated with 3 μL of test solution in DMSO or other vehicle and 75 μL of the substrate solution (LPN; final, 75 μM) in 20 mM Tris-HCl buffer (pH 8.0) for up to 4 h at 37 °C. The absorbance at 405 nm was measured immediately on the microplate reader at 0 h and then every 20 min, 30 min, or 1 h for 4 h. The differences in absorbance with and without the substrate measured at different time intervals were calculated as the increase of absorbance due to the production of *p*-nitrophenol from LPN: Δ absorbance = absorbance (+ LPN) − absorbance (− LPN). On the basis of the graphs plotted with the increase in absorbance versus incubation time, the initial rate was calculated from the slope of the straight line extrapolated from the graph. A control solution containing no test substance was also tested similarly, and the inhibitory rate (percent) was calculated.

Synthesis of *N*-Acyltyrosine (NAT). Synthesis of NAT was based on the Schotten−Baumann reaction (*32*) as described below. Tyrosine (2.4−16.6 mmol) was dissolved in 4 N NaOH (3−4.5 equiv), and acyl chloride (1−4 equiv) was added in five portions with shaking to the solution (< 0 °C). After 1−2 h, the reaction mixture was treated with concentrated HCl and then extracted with ethyl acetate. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (Cosmosil 75C_{18}−OPN, H_2O/acetonitrile 3:1 for 3:0-NAT and D-3:0-NAT, 2:1 for 4:0-NAT and 5:0-NAT, and 1:1 for 5:0-NAT), preparative TLC (chloroform/methanol 9:1 for D-3:0-NAT, 4:0-NAT, and 5:0-NAT), recrystallization (methanol/ethyl acetate/*n*-hexane for 10:0-NAT, 12:0-NAT, and 14:0-NAT; methanol/diethyl ether for 14:0-NAT; methanol for 14:0-NAT and 16:0-NAT), or washing with *n*-hexane, diethyl ether, ethyl acetate, and methanol to afford NAT (in 0.1−30% yield).

In the cases of 10:0-NAT, 12:0-NAT, 14:0-NAT, 16:0-NAT, and D-16:0-NAT, *N*,*O*-diacylated derivatives were obtained as the major product in the reaction described above. Therefore, 4 N NaOH (4 equiv of diacyltyrosine) was added to the residue dissolved in tetrahydrofuran (15 mL for 10:0-NAT, 5 mL for 12:0-NAT) or dispersed in methanol (7 mL) for 14:0-NAT and/or tetrahydrofuran (42 mL for 14:0-NAT, 40 mL for 16:0-NAT, and 20 mL for D-16:0-NAT) at room temperature. After 0.5−0.8 h, the reaction mixture was treated with concentrated HCl and then extracted with ethyl acetate. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The subsequent purification afforded the desired NATs.

(*S*)-*3*-(*4-Hydroxyphenyl*)-*2-propionamidopropanoic acid* [*3:0-NAT*]: colorless oil, $[\alpha]_D^{29}$ +46.1 (*c* 0.41, CH_3OH); ^1H NMR (400 MHz, CD_3OD) δ 1.04 (t, *J* = 7.6 Hz, 3H), 2.17 (q, *J* = 7.6 Hz, 2H), 2.86 (dd, *J* = 13.9, 9.0 Hz, 2H), 3.10 (dd, *J* = 13.9, 5.1 Hz, 1H), 4.57−4.65 (m, 1H), 6.66−6.75 (m, 2H), 6.98−7.08 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 10.3, 29.8, 37.6, 55.1, 116.1, 129.0, 131.2, 157.1, 175.0, 176.9. ESIMS calcd for $C_{12}H_{15}NO_4Na$, MW 260.0899; found, *m*/*z* 260.0896 (M^+ + Na).

(*R*)-*3*-(*4-Hydroxyphenyl*)-*2-propionamidopropanoic acid* [*D-3:0-NAT*]: colorless oil, $[\alpha]_D^{29}$ −45.9 (*c* 1.04, CH_3OH). ESIMS calcd for $C_{12}H_{15}NO_4$-Na, MW 260.0899; found, *m*/*z* 260.0907 (M^+ + Na).

(*S*)-*2-Butyramido-3*-(*4-hydroxyphenyl*)*propanoic acid* [*4:0-NAT*]: brown oil, $[\alpha]_D^{29}$ +33.7 (*c* 1.30, CH_3OH); ^1H NMR (400 MHz, CD_3OD) δ 0.84 (t, *J* = 7.6 Hz, 3H), 1.54 (sext, *J* = 7.6 Hz, 2H), 2.13 (t, *J* = 7.6 Hz, 2H), 2.83 (dd, *J* = 14.2, 9.3 Hz, 1H), 3.10 (dd, *J* = 14.2, 4.9 Hz, 1H), 4.56−4.63 (m, 1H), 6.64−6.73 (m, 2H), 6.97−7.09 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 13.8, 20.1, 37.4, 38.5, 55.0, 116.1, 128.8, 131.1, 156.8,

175.0, 176.0. ESIMS calcd for $C_{13}H_{17}NO_4Na$, MW 274.1055; found, m/z 274.1059 ($M^+ + Na$).

(*S*)-*3*-(*4-Hydroxyphenyl*)-*2-pentanamidopropanoic acid* [*5:0-NAT*]: brown oil, $[\alpha]_D^{30}$ +27.8 (*c* 0.89, CH_3OH); ^1H NMR (400 MHz, CD_3OD) δ 0.86 (t, J = 7.3 Hz, 3H), 1.15−1.27 (m, 2H), 1.42−1.52 (m, 2H), 2.15 (t, J = 7.3 Hz, 2H), 2.82 (dd, J = 13.9, 9.5 Hz, 1H), 3.12 (dd, J = 13.9, 4.9 Hz, 1H), 4.55−4.64 (m, 1H), 6.64−6.72 (m, 2H), 6.97−7.07 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 14.1, 23.1, 28.9, 36.5, 37.5, 55.0, 116.1, 129.0, 131.1, 157.1, 175.0, 176.1. ESIMS calcd for $C_{14}H_{19}NO_4Na$, MW 288.1212; found, m/z 288.1205 ($M^+ + Na$).

(*S*)-*2-Decanamido-3-*(*4-hydroxyphenyl*)*propanoic acid* [*10:0-NAT*]: colorless powder, $[\alpha]_D^{23}$ +24.4 (*c* 1.00, CH_3OH); ^1H NMR (400 MHz, CD_3OD) δ 0.89 (t, J = 6.8 Hz, 3H), 1.14−1.37 (m, 12H), 1.44−1.55 (m, 2H), 2.15 (t, J = 7.6 Hz, 2H), 2.83 (dd, J = 13.9, 9.3 Hz, 1H), 3.10 (dd, J = 13.9, 4.9 Hz, 1H), 4.56−4.65 (m, 1H), 6.61−6.74 (m, 2H), 6.95−7.09 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.1, 30.41, 30.45, 30.5, 33.0, 36.8, 37.6, 55.1, 116.1, 129.1, 131.2, 157.3, 175.0, 176.1. ESIMS calcd for $C_{19}H_{29}NO_4Na$ MW 358.1994, found m/z 358.1997 ($M^+ + Na$).

(*S*)-*2-Dodecanamido-3-*(*4-hydroxyphenyl*)*propanoic acid* [*12:0-NAT*]: colorless needle, $[\alpha]_D^{22}$ +21.0 (*c* 1.00, CH_3OH); ^1H NMR (400 MHz, CD_3OD) δ 0.89 (t, J = 6.8 Hz, 3H), 1.10−1.39 (m, 16H), 1.42−1.58 (m, 2H), 2.15 (t, J = 7.6 Hz, 2H), 2.84 (dd, J = 13.9, 9.3 Hz, 1H), 3.10 (dd, J = 13.9, 4.9 Hz, 1H), 4.55−4.66 (m, 1H), 6.63−6.76 (m, 2H), 6.97−7.09 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.1, 30.5, 30.6, 30.74, 30.75, 33.1, 36.8, 37.7, 55.1, 116.2, 129.1, 131.2, 157.3, 175.0, 176.2. ESIMS calcd for $C_{21}H_{33}NO_4Na$ MW 386.2307, found m/z 386.2337 ($M^+ + Na$).

(*S*)-*3*-(*4-Hydroxyphenyl*)-*2-tetradecanamidopropanoic acid* [*14:0-NAT*]: white solid, $[\alpha]_D^{21}$ +24.3 (*c* 0.14, CH_3OH); ^1H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 7.1 Hz, 3H), 1.07−1.32 (m, 20H), 1.34−1.45 (m, 2H), 2.03 (t, J = 7.3 Hz, 2H), 2.71 (dd, J = 13.9, 9.8 Hz, 1H), 2.91 (dd, J = 13.7, 4.6 Hz, 1H), 4.27−4.38 (m, 1H), 6.58−6.70 (m, 2H), 6.94−7.06 (m, 2H), 8.01 (d, J = 8.3 Hz, 1H), 9.17 (s, 1H), 12.53 (br s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.0, 22.3, 25.3, 28.7, 28.9, 29.0, 29.1, 29.20, 29.23, 29.25, 31.5, 35.2, 36.2, 38.9, 53.7, 114.9, 127.7, 129.9, 155.8, 172.1, 173.2. ESIMS calcd for $C_{23}H_{37}NO_4Na$ MW 414.2620, found m/z 414.2601 ($M^+ + Na$).

(*S*)-*3*-(*4-Hydroxyphenyl*)-*2-palmitamidopropanoic acid* [*16:0-NAT*]: white solid, $[\alpha]_D^{21}$ +18.8 (*c* 0.13, CH_3OH); ^1H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 7.1 Hz, 3H), 1.05−1.43 (m, 26H), 2.03 (t, J = 7.3 Hz, 2H), 2.66−2.75 (m, 1H), 2.87−2.94 (m, 1H), 4.27−4.36 (m, 1H), 6.60−6.67 (m, 2H), 6.96−7.23 (m, 2H), 8.01 (d, J = 8.1 Hz, 1H), 9.17 (s, 1H), 12.32 (br s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 14.1, 22.4, 25.5, 28.9, 29.1, 29.2, 29.31, 29.38, 29.4, 31.6, 35.3, 36.3, 53.9, 115.2, 128.0, 130.2, 156.1, 172.7, 173.6. ESIMS calcd for $C_{25}H_{41}NO_4Na$, MW 442.2933; found, m/z 442.2900 ($M^+ + Na$).

(*R*)-*3*-(*4-Hydroxyphenyl*)-*2-palmitamidopropanoic acid* [*D-16:0-NAT*]: white solid, $[\alpha]_D^{19}$ −12.7 (*c* 0.11, CH_3OH). ESIMS calcd for $C_{25}H_{41}NO_4Na$, MW 442.2933; found, m/z 442.2971 ($M^+ + Na$).

Kinetic Analysis of Inhibition of Serum LysoPLD Activity. In our standard assay of lysoPLD activity in FBS, the final concentration of the substrate LPN was 0.075 mM. To analyze inhibition of the lysoPLD activity kinetically, we measured the initial rate for 4-h of incubation at 37 °C at different final concentrations of the substrate LPN (0.025, 0.0375, 0.075, 0.1, 0.15, and 0.3 mM). On the basis of the graphs plotted with the reciprocal of the initial rate versus that of the concentration of the substrate, a straight line was extrapolated from the graph. The inhibitors used for making Lineweaver−Burk plot were as follows: robinetin, 18:1-LPA, and 12:0-FA at 0.1 mM, and 2:0-FA, 6:0-FA, 2:0-NAT, 5:0-NAT, 12:0-NAT at 0.3 mM as representatives of flavonols, LPAs, fatty acids, and NATs, respectively.

Analysis of LPA by LC-MS/MS. Fifty microliters of 5% FBS in saline (final, 0.83%) as the enzyme source, 75 μL of 18:2-LPC and LPN in 20 mM Tris-HCl buffer, pH 8.0 (final, 75 μM), as the substrate, and 3 μL of the test solution in a vehicle such as DMSO or vehicle alone were mixed, and the mixture was incubated for 4−6 h at 37 °C. After incubation with LPA, lipids in the assay solution (100 μL) were extracted according to the modified method of Bligh and Dyer (*33*) after the addition of 100 pmol of 17:0-LPA as an internal standard. 18:2-LPA in the lipid extract from the acidified aqueous layer was quantified by obtaining the ratio of the peak areas of 18:2-LPA to 17:0-LPA in LC-MS/MS. Analytical conditions were described previously (*34*). For analysis of LPN, lipids in the assay solution (100 μL) were extracted according to the method of Bligh and Dyer (*33*). LPNs having an *sn*-1- or *sn*-2-linoleoyl group were analyzed by multiple reaction monitoring in a negative ion mode under the same conditions for LPA analysis by LC-MS-MS (*34*) except for setting Q_1 and Q_3 as $[M - H]^-$ at m/z 554 and $[linoleate]^-$ at m/z 279, respectively.

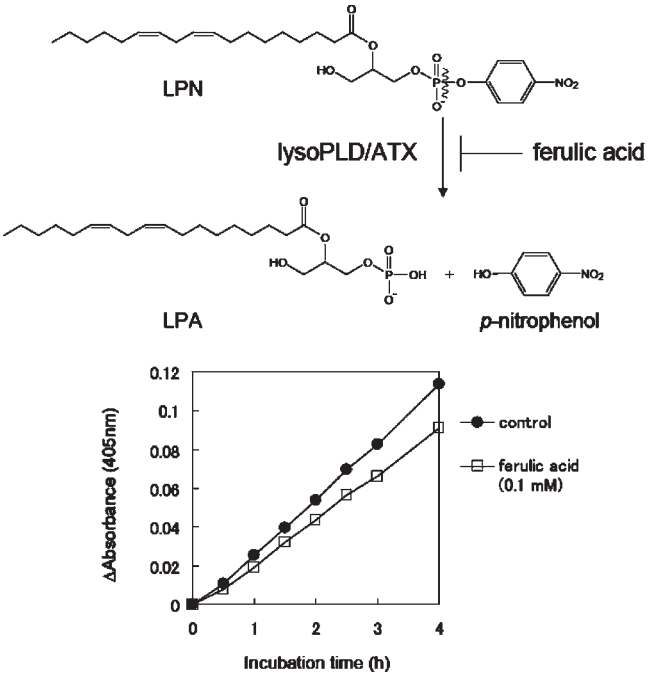


Figure 1. Time course of production of *p*-nitrophenol from LPN incubated in diluted FBS and its inhibition by ferulic acid. Diluted FBS (final, 0.83%) was incubated with LPN (final, 75 μM) at 37 °C for 4 h in the presence or absence of ferulic acid (final, 0.1 mM). The absorbance at 405 nm was measured every 30 min or 1 h for 4 h. Values are means of duplicate determinations.

RESULTS

Screening of Inhibitory Substances by a New Assay with a Chromogenic Substrate. We chose various antioxidants, including polyphenols, as the first screening target for development of lysoPLD inhibitors because polyphenols have attracted increasing attention as a dietary supplement that exerts beneficial effects on human health due to its antioxidant activity and enzyme−inhibitory activity [lipoxygenase (*26*), phospholipase A_2 (*27*), myeloperoxidase (*35*)]. Second, phenolic acids were examined as potential inhibitors of serum lysoPLD because their structures correspond to a partial structure of the flavonol skeleton. Because phenolic acids are known to be present at higher concentrations than flavonols in the blood after routine oral intake, their physiological inhibition of plasma lysoPLD is expected to be more significant. Third, we tested the lysoPLD-inhibiting activities of FAs with an aliphatic hydrocarbon chain as a series of homologues that structurally relate to a group of phenolic acids to assess the necessity of a benzene ring in the structures of a phenolic acid for its inhibitory action on lysoPLD activity. Last, we synthesized NATs with different acyl chains as structural analogues of phenolic acids and tested their inhibitory effects on lysoPLD activity. To test potential inhibitory effects of these compounds, we needed a convenient and reliable assay for lysoPLD activity. In the present assay system, we used LPN as an artificial chromogenic substrate. LysoPLD activity in FBS hydrolyzed LPN to LPA and *p*-nitrophenol (**Figure 1**), the

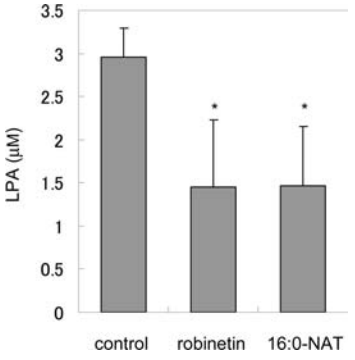


Figure 2. Inhibitory effects of robinetin and 16:0-NAT on lysoPLD activity toward exogenous 18:2-LPC, which is a physiologically more appropriate substrate than LPN. Five percent FBS was incubated with a solution of 18:2-LPC (final, 75 μM) in the presence and absence of robinetin or 16:0-NAT (final, 0.1 mM) at 37 °C for 6 h. After incubation, the incubation mixture was acidified, and lipids were extracted from the incubation mixture according to the method of Bligh and Dyer. 18:2-LPA was quantified by LC-MS/MS, as shown under Materials and Methods. Values are means ± SD of triplicate determinations. The results were compared by using an analysis of variance followed by Student's unpaired *t* test. The asterisks show statistical significance (∗, $P < 0.05$).

generation of which could be monitored by increased absorbance at around 405 nm. Thus, lysoPLD activity is continuously quantified as *p*-nitrophenol production for various periods. Using this assay, we tested the inhibitory activity of several compounds in a 96-well microplate, allowing the assay to be a time- and reagent-saving screening for lysoPLD inhibitors. We used FBS as an enzyme source, because we were able to purchase a large quantity in a single lot. In our standard assay, FBS was finally diluted to 0.83%. As shown in **Figure 1**, an almost linear increase in absorbance due to the production of *p*-nitrophenol was observed with time after incubation of diluted FBS with LPN in the presence and absence of ferulic acid (final, 0.1 mM). Thus, we set the final concentration of the substrate LPN as 75 μM, providing the absorbance change in a proper range. The maximal conversion of LPN to *p*-nitrophenol produced an absorbance increase of 0.1−0.2 at 405 nm for up to 4 h in diluted FBS, and an absorbance increase of 0.3−0.6 in undiluted FBS. The incubation time was up to 4 h. By LC-MS/MS, we found that percentages of peak area of LPN having an *sn*-2-lionoleoyl group, a faster migrating isomer than the corresponding *sn*-1 isomer on the LC column, were 49.0 ± 0.29, 21.9 ± 3.32 and 22.9 ± 6.82 (triplicate determinations) in the lipid extracts from the assay solution incubated with 75 μM LPN for 0, 2, and 4 h, respectively. This result showed the rapid equilibration between the *sn*-1 and *sn*-2 isomers (about 23:77) under our assay conditions, suggesting that serum lysoPLD acted on LPN with the constant isomer ratio during most of the assay time. Thus, we calculated the initial rate from the slope of a straight line on the graphs plotted with the increase of absorbance obtained versus the incubation time. By analyzing the results in a Lineweaver−Burk plot, we obtained a K_m value of 15.1 ± 1.3 μM (n = 21) for LPN as a substrate for the lysoPLD activity of ATX in FBS, which is about one-seventh of that for 16:0-LPC (*2*). However, it is about 5 times higher than the reported fluorogenic substrate for lysoPLD/ATX (*36*).

As noted above, the artificial substrate LPN was used in our assay. Thus, it was necessary to confirm whether the inhibitory effect of the hydrolysis of artificial substrate by lysoPLD activity corresponds to that of the hydrolysis of physiological substrate (LPC). Using LC-MS/MS, we directly measured the production

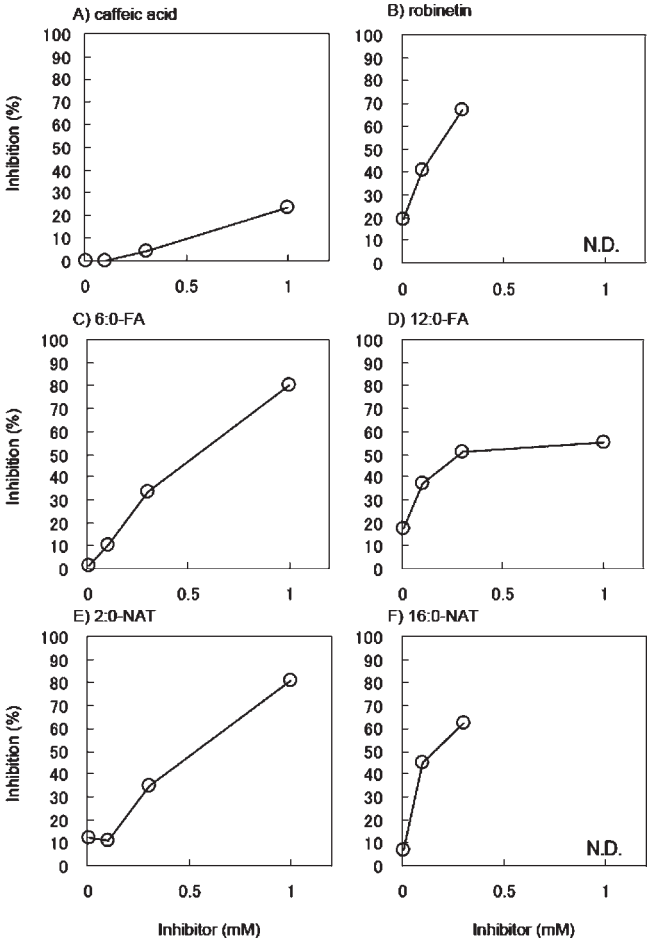


Figure 3. Comparisons of the concentration dependence of inhibition by polyphenols, FAs, and NATs on substances on lysoPLD. The final concentration of the substrate was 75 μM. Due to the low solubility of robinetin and 16:0-NAT, their results at 1 mM were not obtained. Values are means of duplicate determinations. N.D. = not determined.

of 18:2-LPA from exogenously added 18:2-LPC, a physiological substrate for plasma lysoPLD in the presence and absence of robinetin or 16:0-NAT as an inhibitor of lysoPLD, confirming that the test substances really inhibited the lysoPLD activity toward the physiological substrate LPC (**Figure 2**). Next, we tested effects of 16:0-NAT and robinetin on the equipotent lysoPLD activity of recombinant human ATX with that of diluted FBS in our standard assay with 75 μM LPN and found that 39.6 and 49.6% inhibitions were attained by 16:0-NAT and robinetin at 0.1 mM, respectively. This result suggests that the coexistence of components from diluted FBS in our assay system caused no serious interference over the lysoPLD activity ATX. Considering these results together, we concluded that the method using LPN is convenient and suitable for the screening of lysoPLD inhibitors.

The curves of concentration-dependent inhibition of lysoPLD activity by the eight test substances were different (**Figure 3**). The concentration dependences for robinetin, 12:0-FA, and 16:0-NAT were higher than those for caffeic acid, 6:0-FA, and 2:0-NAT. The former were found to function as competitive inhibitors, except for robinetin, whereas the latter were found to work mainly as noncompetitive inhibitors, except for the mixed type inhibition for 6:0-FA, which will be described later. LPA data are not shown because LPAs were hard to dissolve at final concentrations > 0.3 mM.

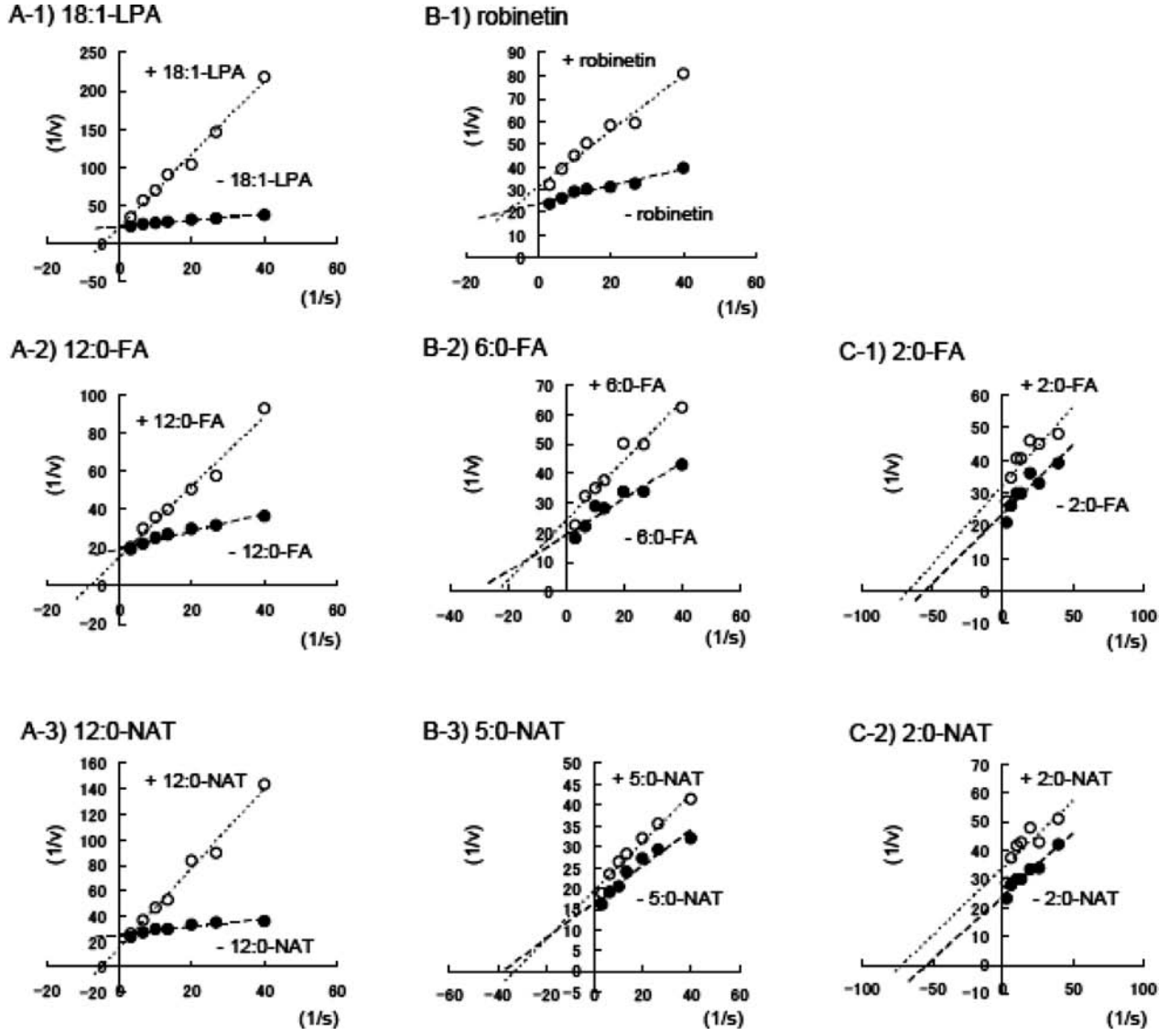


Figure 4. Lineweaver−Burk plot of inhibition by LPA, FAs, NATs, and robinetin on lysoPLD activity of diluted FBS. LysoPLD activity in diluted FBS (final, 0.83%) was calculated from the initial rate measured in 4 h of incubation at 37 °C. Concentrations of the substrate LPN were 0.025, 0.0375, 0.05, 0.075, 0.1, 0.15, and 0.3 mM. Concentrations of the inhibitor were 0.1 mM for robinetin, 18:1-LPA (**A-1**), and 12:0-FA (**A-2**) and 0.3 mM for 2:0-FA (**C-1**), 6:0-FA (**B-2**), 2:0-NAT (**C-2**), 5:0-NAT (**B-3**), and 12:0-NAT (**A-3**). Open and solid circles show values measured in the presence and absence of the inhibitor, respectively. Values are means of triplicate determinations. The mean and SD of K_m/V_{max} for the controls were 433 ± 86 (n = 8).

Kinetic Analysis of Inhibitions on LysoPLD Activity. We performed kinetic analysis of the inhibition of the lysoPLD activity, using a Lineweaver−Burk plot. We expected LPA to inhibit the activity of serum lysoPLD in a competitive manner, because LPA is the product generated by lysoPLD. As shown by the Lineweaver−Burk plot in **Figure 4A-1**, the intersection point of the graphs with and without LPA was just on the y-axis. This indicates that LPA behaves as a competitive inhibitor. On the other hand, the modes of action by flavonols such as robinetin were noncompetitive or mixed (**Figure 4B-1**). Interestingly, in the case of FAs and NATs, the kinetic type of enzyme inhibition was dependent on the length of the carbon chain of the fatty acid or fatty acyl moiety. Liposoluble substances with longer carbon chains (12:0-FA, 12:0-NAT) functioned mainly as competitive inhibitors as well as LPAs, whereas those with shorter carbon chains such as 6:0-FA and 5:0-NAT acted via a mixed inhibition. Both 2:0-FA and 2:0-NAT were noncompetitive inhibitors. Considering these results together, test substances could be classified by differences in their affinities to the catalytic and noncatalytic binding sites as a competitive, noncompetitive inhibitor and a mixed type inhibitor. These results suggest that lysoPLD has a catalytic binding site on which the substrate, competitive inhibitor, and mixed type inhibitor act and that it has at least one noncatalytic binding site on which the noncompetitive and mixed type inhibitors act. Even short fatty acids such as 2:0-FA were capable of inducing a conformational change of serum lysoPLD by binding to this noncatalytic binding site. Kinetic parameters of inhibition by test substances on hydrolysis of LPN by lysoPLD are shown in **Table 1**.

Dilution Dependence of Inhibition of LysoPLD Activity by Various Types of Substances. As shown in **Figure 5**, the increases in absorbance that correspond to the production of *p*-nitrophenol were observed with time after incubation of diluted and undiluted FBS with LPN in the presence and absence of a test substance. In the case of LPA used as an inhibitor, there was a large difference between its inhibitory effects when incubated with diluted and undiluted FBS. A significant inhibition of lysoPLD activity by LPAs at 0.1 mM was observed on lysoPLD activity when 0.5% FBS was used as the enzyme-containing solution (final, 0.83%). However, no significant inhibition by LPAs was observed at this

Table 1. Comparison of Kinetic Parameters Obtained by Lineweaver−Burk Plot Analysis of LPA-, FA-, NAT-, and Robinetin-Induced Inhibition of LysoPLD Activity against LPN[a]

	K_m (μM)			V_{max} (abs/h)		
test substance	(−)	(+)	ratio of K_m (±)	(−)	(+)	ratio of V_{max} (±)
18:1-LPA	12	163	13.1	0.040	0.038	1.0
12:0-FA	24	126	5.3	0.052	0.068	1.3
12:0-NAT	13	213	16.9	0.040	0.068	1.7
robinetin	14	35	2.6	0.043	0.033	0.8
6:0-FA	33	43	1.3	0.053	0.042	0.8
5:0-NAT	26	31	1.2	0.061	0.052	0.9
2:0-FA	18	15	0.8	0.043	0.031	0.7
2:0-NAT	19	14	0.7	0.042	0.030	0.7

[a] Kinetic parameters of inhibition by 18:1-LPA, 12:0-FA, 12:0-NAT, robinetin, 6:0-FA, 5:0-NAT, 2:0-FA, and 2:0-NAT were obtained by Lineweaver−Burk plot analysis (**Figure 4**). The upper, middle, and lower two or three substances in the table were competitive, mixed, and noncompetitive inhibitors, respectively. LysoPLD activity in 0.83% FBS was calculated from the initial rate measured for 4 h of incubation at 37 °C. Both K_m and V_{max} were measured in the presence (+) and absence (−) of the inhibitor, respectively. Values are means of duplicate determinations of one to four experiments.

concentration when undiluted FBS was used as the enzyme solution. Potent product inhibition by LPA in a mainly competitive manner was observed in a previous experiment in which conditioned medium from ATX-transfected insect sf9 cells was used as an enzyme source (*37*). The discrepancy between our result with undiluted FBS and the previous result may be explained by considering that higher concentrations of certain proteins in undiluted FBS than in conditioned medium of sf9 cells prevent the product inhibition by LPA in undiluted FBS. A similar diluted FBS-dependent inhibition was observed for FAs, whereas only a small inhibition of lysoPLD activity was observed in undiluted FBS. On the other hand, NATs inhibited lysoPLD in diluted and undiluted FBS to a similar extent. Among the test compounds, flavonols were the most refractory to reduction of their inhibitory actions when tested on undiluted FBS. These results indicate that the bioavailability of LPAs and FAs might be minimal in vivo. On the other hand, the flavonols and NATs tested were less sensitive to adsorption by serum proteins and, thus, may behave as more potent inhibitors in vivo than in the in vitro assay system. Flavonols and related substances including NATs may be more practical for development as a medical prototype candidate drug than LPAs and FAs.

Structure−Activity Relationship of Inhibition on LysoPLD Activity by Polyphenols. The structures of flavonols, phenolic acids, and related substances tested in this study are shown in **Table 2**. Some flavonols (kaempferol, tamarixetin), flavones (luteolin, apigenin, chrysin), flavanols [(+)-catechin, (−)-epicatechin], isoflavones (daidzein, genistein), other polyphenols (resveratrol), caffeine, L-amino acids [tyrosine, phenylalanine, and 3-(3,4-dihydroxyphenyl)alanine] and phenolic antioxidants (propyl gallate, BHT, BHA), methyl (*R*)-(+)-2-(4-hydroxyphenoxy)propionate, 3-(4-hydroxyphenyl)-1-propanol, and 4-(4-hydroxyphenyl)-2-butanone were inactive. The potencies of lysoPLD inhibition of active flavonols and phenolic acids relative to that of 16:0-LPA were obtained and are shown in **Figure 6A**,**B**, respectively. The open and shaded columns show results with the inhibitors at 0.1 and 0.3 mM, respectively. The pattern of relative inhibitory potency obtained at 0.3 mM was essentially similar to that at 0.1 mM. Among group A, flavonols such as myricetin, robinetin, and quercetagetin showed a stronger inhibitory effect on the lysoPLD activity than 16:0-LPA when assayed at 0.1 mM (**Figure 6A**). The inhibitory potencies of isoquercitrin and rutin on serum lysoPLD activity were roughly equal to its aglycone quercetin. Similarly,

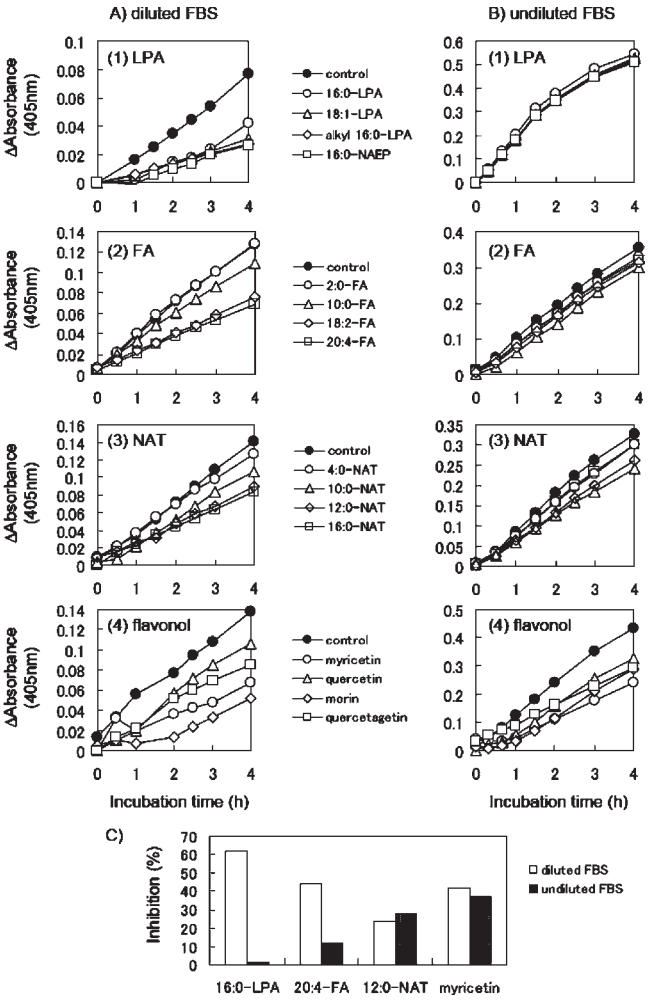


Figure 5. Different inhibitory potencies of LPAs (1), FAs (2), NATs (3), and flavonols (4) on lysoPLD activity in diluted and undiluted FBS. (**A**, **B**) Diluted (final, 0.83%) and undiluted (final, 16.7%) FBS was incubated with LPN (final, 75 μM) at 37 °C for 4 h in the presence or absence of the inhibitory substance (final, 0.1 mM). The absorbance at 405 nm was measured every 20 min, 30 min, or 1 h for 4 h. Values are means of duplicate determinations. (**C**) Inhibitory effects of 16:0-LPA, 20:4-FA, 12:0-NAT, and myricetin on lysoPLD activity are compared as representative of LPA, FA, NAT, and flavonol groups. White and black columns show percentages of inhibition by the test substances measured with diluted (final, 0.83%) and undiluted (final, 16.7%) FBS, respectively.

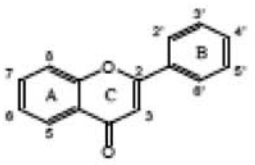
isorhamnetin-3-*O*-glucoside was almost equipotent to its aglycone isorhamnetin in inhibition of serum lysoPLD activity. These results indicate the free hydroxyl group at the 3-position on the C ring might be not essential to inhibit serum lysoPLD activity.

The inhibitory effects of various phenolic acids on lysoPLD activity were relatively lower than that of 16:0-LPA (**Figure 6B**). We confirmed that caffeic acid as a representative phenolic acid functioned as a noncompetitive inhibitor of the lysoPLD activity. In the FA and NAT classes, the substances with a longer methylene chains had stronger inhibitory effects on lysoPLD activity (**Figure 6C**,**D**). The inhibition of lysoPLD activity by substances functioning mainly as a noncompetitive inhibitors was rather moderate, except for some flavonols, whereas the substances that showed stronger inhibitory potency functioned as competitive inhibitors.

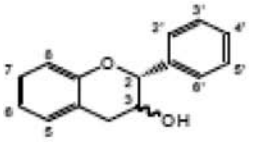
DISCUSSION

To identify nonlipid inhibitors of lysoPLD activity of ATX with satisfactory bioavailability, a virtual screening approach was

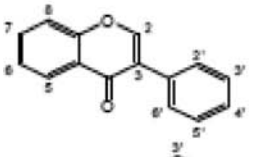
Table 2. Structural Features and Inhibitory Effects on LysoPLD Activity of Fiavonols, Phenolic Acids, and Their Related Substances Tested[a]



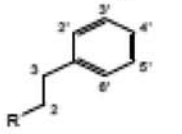
A) Flavonol and Flavone	Substituent								Inhibitory activity
	C3	C5	C6	C7	C2′	C3′	C4′	C5′	
morin	OH	OH	–	OH	OH	–	OH	–	+
quercetin	OH	OH	–	OH	–	OH	OH	–	+
quercetagetin	OH	OH	OH	OH	–	OH	OH	–	+
myricertin	OH	OH	–	OH	–	OH	OH	OH	+
robinetin	OH	–	–	OH	–	OH	OH	OH	+
fisetin	OH	–	–	OH	–	OH	OH	–	+
isorhamnetin	OH	OH	–	OH	–	OCH_3	OH	–	+
isorhamnetin-3-*O*-glucoside	O-glucose	OH	–	OH	–	OCH_3	OH	–	+
rutin	O-rutinose	OH	–	OH	–	OH	OH	–	+
isoquercitrin	O-glucose	OH	–	OH	–	OH	OH	–	+
luteolin	–	OH	–	OH	–	OH	OH	–	–
apigenin	–	OH	–	OH	–	–	OH	–	–
chrysin	–	OH	–	OH	–	–	–	–	–
keampferol	OH	OH	–	OH	–	–	OH	–	–
tamarixetin	OH	OH	–	OH	–	OH	OCH_3	–	–



B) Flavanol	Substituent								Inhibitory activity
	C3	C5	C6	C7	C2′	C3′	C4′	C5′	
(+)-catechin	OH	OH	–	OH	–	OH	OH	–	–
(−)-epicatechin	OH	OH	–	OH	–	OH	OH	–	–



C) Isoflavone	Substituent								Inhibitory activity
	C3	C5	C6	C7	C2′	C3′	C4′	C5′	
daidzein	–	–	–	OH	–	–	OH	–	–
genistein	–	OH	–	OH	–	–	OH	–	–



D) Phenolic acid analogs	Partial structure		Substituent				Inhibitory activity
	C2	C3	C3′	C4′	C5′	R	
caffeic acid	CH=CH		OH	OH	–	COOH	+
p-coumaric acid	CH=CH		–	OH	–	COOH	+
ferulic acid	CH=CH		OCH_3	OH	–	COOH	+
sinapic acid	CH=CH		OCH_3	OH	OCH_3	COOH	+
chlorogenic acid	CH=CH		OH	OH	–	CO-quinic acid	+
HPPA	CH_2	CH_2	–	OH	–	COOH	+
HPAA	CO	CH_2	–	OH	–	OH	+
DPPA	CH_2	CH_2	OH	OH	–	COOH	+
NHPG	CH_2	NH	–	OH	–	COOH	+
PPA	CH_2	CH_2	–	–	–	COOH	+
MHPP	CH_3	O	–	OH	–	$COOCH_3$	–
HPP	CH_2	CH_2	–	OH	–	CH_2OH	–
HPB	CH_2	CH_2	–	OH	–	$COCH_3$	–
L-DOPA	NH_2	CH_2	OH	OH	–	COOH	–
L-tyrosine	NH_2	CH_2	–	OH	–	COOH	–
L-phenylalanine	NH_2	CH_2	–	–	–	COOH	–

[a] The four compounds are abbreviated as follows: methyl (*R*)-(+)-2-(4-hydroxyphenoxy)propionate (MHPP); 3-(4-hydroxypheny)-1-propano1 (HPP); 4-(4-hydroxypheny)-2-butanone (HPB); and L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA). — shows substances providing <5% inhibition at the maximal concentration tested (0.3 or 1 mM).

developed based on the structural model of the ATX catalytic site that was simulated by computational strategies such as database screening guided by docked complexes of candidate structures and a binary QSAR model based on known substrates and inhibitors of ATX. Nine compounds were identified that inhibit lysoPLD activity toward the synthetic substrate 1 μM FS-3 at an IC_{50} value of <10 μM (*22*). A recent study with a high-throughput screening test assessment found a potent inhibitor of the lysoPLD activity of ATX purified from the culture medium of adipocytes, which were effective at nanomolar concentrations (*38*). In the current study, we found several substances inhibited lysoPLD activity under our assay conditions, although their potencies were not higher than the reported lysoPLD inhibitors with an IC_{50} value at submicromolar concentrations (*38*, *39*). Owing to a 75 times higher concentration of LPN than that of FS-3, however, the direct comparison of IC_{50} values of inhibitory substances obtained in this study with those reported previously (*22*, *39*) may not be useful. The IC_{50} values of several substances tested in the current study would be reduced considerably when assayed with LPN at the low concentration used for FS-3. Among flavonols tested in this study, myricetin, robinetin, and quercetagetin were found to exert the most potent inhibitory effect on lysoPLD activity in diluted FBS. As their common structural feature, they have three hydroxyl groups bound to the A or B ring, one double bond, and one carbonyl group in the C ring in the flavonol skeleton, allowing these three rings to exist on the same plane. Because flavanols such as catechin that have three hydroxyl groups on the A or B ring but no double bond and carbonyl group in the C ring failed to inhibit the lysoPLD activity (**Table 2**), the coplanarity of the A, B, and C rings is necessary to bind stereospecifically to the lysoPLD enzyme, inhibiting its activity in a noncompetitive fashion. Synthetic antioxidants such as BHA and BHT did not inhibit lysoPLD activity in this study, indicating that the observed inhibitory action of some flavonols was not due to their antioxidant activity. Judging from the reported structure−activity relationships of the metal-chelating activity of flavonols (*40*, *41*) that were distinct from that of the lysoPLD-inhibitory activity found in this study, the inhibition of lysoPLD activity of ATX by some flavonols is unlikely due to the flavonol-induced metal chelation from ATX, a metalloenzyme (*2*, *28*). Deletion of the C-3′ hydroxyl group of the B ring of luteolin to form apigenin was shown to abolish the inhibitory effects on phosphodiesterases 4 and 5 (*25*). By analogy with this paper, deletion of the C-3′

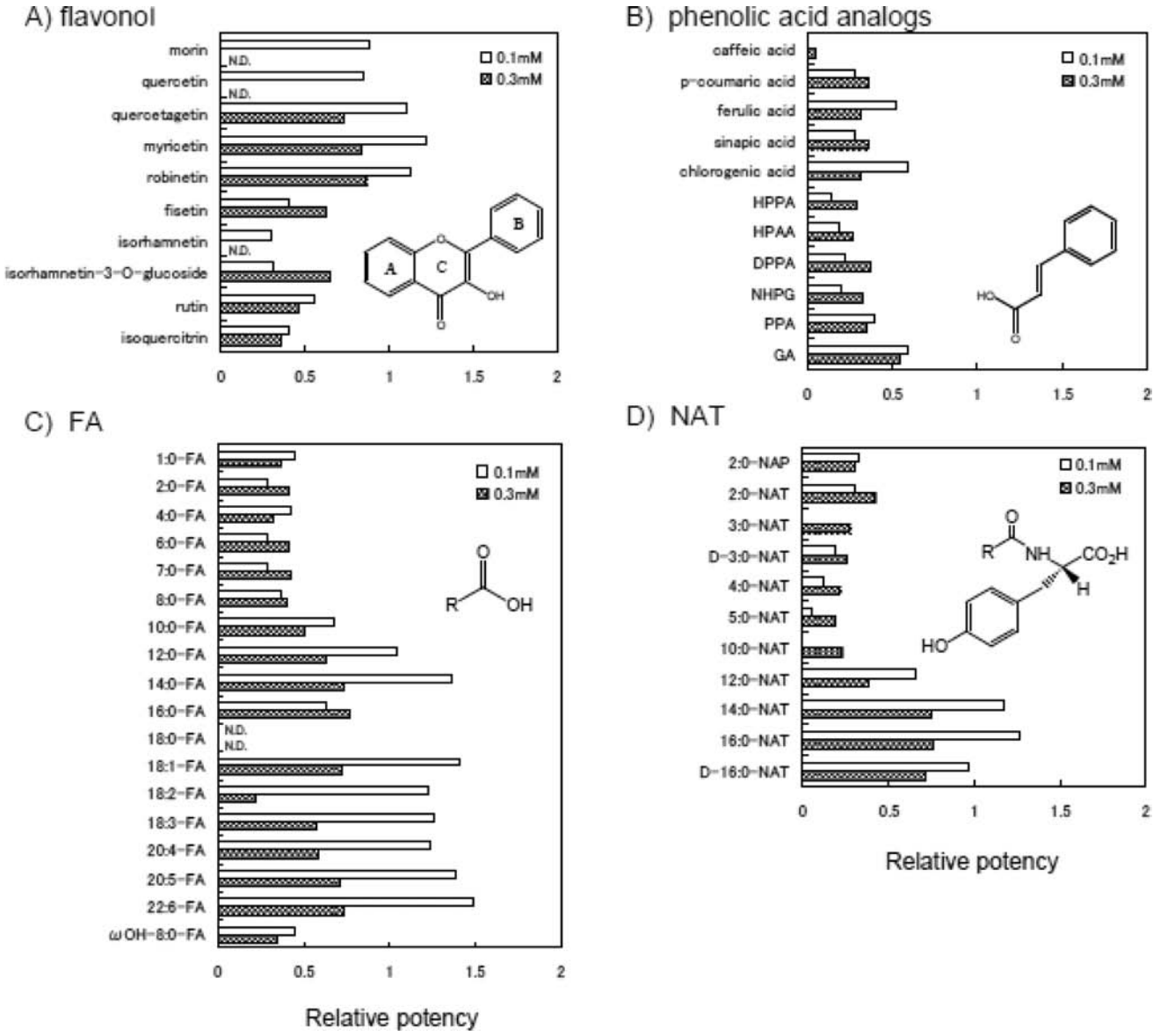


Figure 6. Relative potencies of inhibitory activity of different compounds on lysoPLD activity in 4 h of incubation of 0.83% FBS toward LPN to that of 16:0-LPA. The final concentrations of the substrate (LPN) and the inhibitors were 75 μM and 0.1 or 0.3 mM, respectively. Values are means of duplicate determinations. N.D. = not determined. The following abbreviations are used in this figure: HPPA, 3-(4-hydroxyphenyl)propionic acid; HPAA, 4-hydroxyphenylacetic acid; NHPG, *N*-(4-hydroxyphenyl)glycine; 2:0-NAP, *N*-acetyl-L-phenylalanine; GA, gallic acid; PPA, 3-phenylpropionic acid; DPPA, 3-(3,4-dihydroxyphenyl)propionic acid.

hydroxyl of the B ring of quercetin to form kaempferol was found to result in the loss of the inhibitory effect in this study. In addition, methylation of the C-4′ hydroxyl of the B ring of quercetin to form tamarixetin eliminated the inhibitory effect, indicating that an intact hydroxyl group is essential for the inhibitory action, whereas no significant reduction of the inhibitory effect due to methylation of the C-3′ hydroxyl group of quercetin to form isorhamnetin was observed. The lysoPLD-inhibiting activity of morin with C-2′- and C-4′- but not C-3′-hydroxyl groups on the B ring was equipotent to that of quercetin having C-3′- and C-4′-hydroxyl groups on the B ring.

Both LPA and FA, naturally occurring lipids, showed significant inhibitory effects on serum lysoPLD activity when FBS was diluted. However, we found that both acidic lipids failed to inhibit lysoPLD in undiluted serum. This result means that the inhibitory potency of LPA is masked by one or more components in serum ex vivo and plasma in the blood circulation in vivo, respectively. In fact, LPA accumulates at an almost constant rate for 1−2 days in serum and plasma preparations from various animal species during incubation at 25−37 °C, indicating no potent inhibition of lysoPLD activity by LPA produced by this enzyme during ex vivo incubations of plasma and serum (*8*,*18*,*42*). Some plasma proteins including albumin may sequester LPA away from its direct inhibitiory action toward lysoPLD activity of ATX. However, this might not be the case for some biological fluids, such as cerebrospinal fluid, that have a low protein concentration. Thus, it is likely that LPA regulates its own production by ATX/lysoPLD in certain local environments. On the other hand, inhibition by some flavonols was still observed in undiluted FBS, which reflects an in vivo pathological condition of blood circulation. Flavonols are taken in as food constituents and absorbed into the blood. In human blood circulation, flavonols from the diet may be physiologically more important in inhibiting plasma lysoPLD than endogenously circulating lipids such as LPA and FA, which are largely sequestered by plasma proteins including albumin. Interestingly, the inhibitory potencies of three

flavonol glycosides were almost equal to those of their aglycons in inhibiting plasma lysoPLD activity, suggesting that the sugar moiety of flavonol glycosides contributes to its inhibitory action to only a small extent. Because flavonoids such as quercetin are converted to glucuronidated or sulfated forms by intestinal and hepatic enzymes, flavonoids exist mainly as conjugated forms in the blood circulation. Our results showing essentially equipotent inhibitory actions by the glycosides of quercetin and isorhamnetin on serum lysoPLD activity to those of their aglycons suggest that glucuronized flavonols in the blood circulation would be effective in inhibiting plasma lysoPLD activity in vivo. In this sense, it should be mentioned that the inhibitory effect of chemically synthesized quercetin 3-D-*O*-glucuronide on myeloperoxidase activity was reported to be comparable to that of the quercetin aglycone (*35*). Although the blood concentration of flavonol conjugates may not reach an effective concentration to inhibit plasma lysoPLD significantly in vivo, an increase in its blood concentration is expected when a larger amount of flavonols is consumed as a dietary supplement. When our results are considered in combination with previous papers showing that most reported lysoPLD inhibitors have poor bioavailability (*20*,*22*,*38*), the water-soluble flavonol glycosides or conjugates with better bioavailability might be a prototype for developing a potential medical drug and food supplement, respectively, based on the inhibitory action on lysoPLD activity of ATX.

In this study, several phenolic acids were found to have a moderate inhibitory effect on lysoPLD activity in diluted FBS. In the case of phenolic acids, a free carboxylic group was necessary, but unlike the case of flavonols, multiple hydroxyl groups on a benzene ring were not a critical structural feature. The presence of a free amino group in the structure of phenolic acids was not effective in inhibiting plasma lysoPLD activity. Whereas the major structural unit of phenolic acids corresponds to the partial structural unit of flavonols, the mode of interaction of phenolic acids with lysoPLD enzyme would be different from that of flavonols.

The structure characterization and enzymatic properties of ATX/lysoPLD have been well characterized, but there are few reports on its three-dimensional structure. The information available now is all indirect and involves simulation of data obtained from crystallized bacterial NPPs, which have structural homology with ATX/lysoPLD (*43*). In addition, the molecular mechanism of its physiological regulation remains obscure. In this study, we obtained information about the sizes of the structural pockets of ATX/lysoPLD enzyme by systematically comparing the inhibitory potency and effect of different test substances on lysoPLD activity. Our results suggest the existence of pockets in which some small-molecule compounds can be sequestered, regulating the enzyme activity competitively and noncompetitively. LPA, a product of lysoPLD, was reported to behave as an inhibitor of lysoPLD itself in vitro, as well as sphingosine 1-phosphate (*37*). The mixed inhibition mode, which has a much larger contribution to competitive inhibition, was suggested for the inhibitory effect of these lipid phosphates on lysoPLD activity of recombinant ATX toward a fluorescence energy transfer-based phosphodiesterase sensor, because the intersection point of the graph with and without LPA deviated slightly from the *y*-axis of the Lineweaver−Burk plot (*37*). Liu et al. reported that 16:0-, 18:1-, 18:2-, and 20:4-LPA inhibited the activity of lysoPLD purified from FBS in a competitive fashion with K_i values of 1.4, 1.2, 0.21, and 0.55 μM, respectively (*44*). We confirmed that the main mode of LPA action on lysoPLD is competitive inhibition by the Lineweaver−Burk plot of data obtained under our assay conditions, indicating a much higher affinity of LPA to the catalytic binding site than to the noncatalytic binding site. The dual inhibition also seems to be the case of the lysoPLD substrate LPC. A recent study comparing the inhibitory effect of LPCs with a distinct acyl chain length showed that 14:0- and 16:0-LPCs more effectively reduced the hydrolysis of FS-3, a fluorogenetic synthetic substrate for ATX (*36*), than 12:0-LPC did, whereas the choline production from exogenous LPC by ATX was optimal for 12:0- and 14:0-LPC (*45*). They hypothesized that 16:0-LPC binds to ATX more efficiently than 12:0-LPC, but that the former is not well positioned for enzymatic hydrolysis. Thus, the substrate LPCs bound to both the catalytic and noncatalytic binding sites with distinct affinities, the ratios of which varied depending on its acyl chain length.

Several flavonols and phenolic acids were both found to inhibit serum lysoPLD activity, mainly in a noncompetitive or mixed inhibition manner. Interestingly, in the case of FAs and NATs, the main mode of lysoPLD inhibition was dependent on the length of the hydrocarbon chain of the free fatty acid or fatty acyl moiety. The major mode of inhibition by more liposoluble substances with longer carbon chains such as 12:0-FA and 12:0-NAT was competitive, whereas the mode of inhibition by FAs and NATs with shorter carbon chains (2:0-FA and 2:0-NAT) was noncompetitive. The mode of inhibition for the analogues having a medium chain length (6:0-FA and 5:0-NAT) was the mixed type. These results suggest that lysoPLD possesses at least one noncatalytic binding site on which several low molecular weight compounds bind to inhibit the lysoPLD enzyme noncompetitively. The noncatalytic binding site seems to accept smaller molecules, indicating a smaller entry cavity to the site, in comparison with the catalytic binding site. On the other hand, the test molecules with larger sizes correlate to the higher affinity to the catalytic binding site, suggesting that the size of this entry pocket is relatively large. Additionally, there were few differences in inhibitory potencies on lysoPLD activity between L- and D-forms of 3:0-NAT and 16:0-NAT. This poor stereospecificity for inhibition of lysoPLD activity might mean high flexibility of the catalytic and noncatalytic pockets, which can accept different types of molecules, leading to conformational changes around the catalytic site. In this sense, NATs and related substances seem to be useful synthetic compounds to evaluate the molecular mechanism of conformational changes of ATX/lysoPLD leading to inhibition and activation of the enzymatic activity. Consistent with this present result, ATX is not stereoselective in its recognition of glycerol-based phospholipids because none of the enantiomeric pairs of alkylglycerophosphonocholines (C16:0 and C18:0), analogues of natural alkyl-LPC, showed statistically different inhibitions of lysoPLD activity of ATX (*45*). Considering these findings together, the lysoPLD inhibitors found in this study were classified into three groups: inhibitors that exert their effect mainly in a competitive manner, a noncompetitive manner, or both (mixed-type inhibition).

LysoPLD/ATX is known to be up-regulated in different types of cancers and is considered to be an endogenous promoter of tumor metastasis. Thus, it is an attractive target for a therapy suppressing tumor aggressiveness. In the present study, we systematically investigated several groups of compounds as potential lysoPLD inhibitors and found that flavonols are the best candidates among those tested in this study. Unfortunately, however, the effective concentrations shown in this study are not easily reached under normal physiological conditions because the in vivo plasma concentration of total metabolites of flavonoids was estimated to vary from 0.1 to 10 μM even after the consumption of a flavonoid-rich meal (*46*, *47*). However, our results on the structure−activity relationships of flavonols and related substances will be useful for exploring more effective food components or developing compounds with higher

therapeutic potential. Further work is needed to extend the present results.

The lysoPLD activity of plasma ATX is constitutively active, but may be negatively regulated to slowly generate LPA in vivo, although the in vivo plasma level of LPC, the predominant physiological substrate for lysoPLD in human beings, is as high as its K_m value for highly purified lysoPLD/ATX from human plasma in vitro (*2*). In this study, we obtained useful information about the conformational structures of ATX/lysoPLD that are involved in the physiological regulation of the lysoPLD activity of ATX by using the inhibitory compounds as a useful tool for investigating physiological and pathophysiological significances of LPA production by ATX/lysoPLD in humans. Furthermore, our present findings will be useful for future identification of natural regulatory mechanisms and structural factors of ATX involved in lysoPLD activity in human biological fluids.

ABBREVIATIONS USED

ATX, autotaxin; BHA, 3-*tert*-butyl-4-hydroxyanisole; BHT, 2,6-di-*tert*-butyl-*p*-cresol; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FA, fatty acid; FBS, fetal bovine serum; LC-MS/MS, liquid chromatography−tandem mass spectrometry; LPA, lysophosphatidic acid; LPN, lysophosphatidyl-*p*-nitrophenol; lysoPLD, lysophospholipase D; NAT, *N*-acyltyrosine; NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; PA, phosphatidic acid; TLC, thin-layer chromatography. The fatty acyl moieties of lysophospholipids, free fatty acids, and *N*-acyl tyrosines are designated in terms of the numbers of carbon atoms and double bonds.

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