Metabolomic analysis and identification of a role for the orphan human cytochrome P450 2W1 in selective oxidation of lysophospholipids[S]

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Abstract **Human cytochrome P450 (P450) 2W1 is still considered an "orphan" because its physiological function is not characterized. To identify its substrate specificity, the purified recombinant enzyme was incubated with colorectal cancer extracts for untargeted substrate searches using an LC/MS-based metabolomic and isotopic labeling approach. In addition to previously reported fatty acids, oleyl (18:1) lysophosphatidylcholine (LPC, lysolecithin) was identified as a substrate for P450 2W1. Other human P450 enzymes tested showed little activity with 18:1 LPC. In addition to the LPCs, P450 2W1 acted on a series of other lysophospholipids, including lysophosphatidylinositol, lysophosphatidylserine, lysophosphatidylglycerol, lysophosphatidylethanolamine, and lysophosphatidic acid but not diacylphospholipids. P450 2W1 utilized *sn*-1 18:1 LPC as a substrate much more efficiently than the *sn*-2 isomer; we conclude that the *sn*-1 isomers of lysophospholipids are preferred substrates. Chiral analysis was performed on the 18:1 epoxidation products and showed enantio-selectivity for formation of (9*R*,10*S*) over (9*S*,10*R*). The kinetics and position specificities of P450 2W1-catalyzed oxygenation of lysophospholipids (16:0 LPC and 18:1 LPC) and fatty acids (C16:0 and C18:1) were also determined. Epoxidation and hydroxylation of 18:1 LPC are considerably more efficient than for the C18:1 free fatty acid.**—Xiao, Y., and F. P. Guengerich. **Metabolomic analysis and identification of a role for the orphan human cytochrome P450 2W1 in selective oxidation of lysophospholipids. *J. Lipid Res.* 2012.** 53: **1610–1617.**

Supplementary key words gas chromatography-mass spectrometry • liquid chromatography-mass spectrometry • metabolomics • oleyl lysophosphatidylcholine • chirality • enzyme kinetics

Although the genomic sequences of human and numerous other organisms have been established, the functions of less than one-half of the proteins have been annotated, even in *Escherichia coli*. Thus, an important and challenging task in modern biochemistry is the elucidation of protein functions, including the establishment of the catalytic activities of novel enzymes with unknown substrates (1, 2). P450 enzymes play important roles in the metabolism of a large number of compounds, including sterols, fatty acids, eicosanoids, vitamins, and xenobiotics (3). It has been estimated that P450 reactions are involved in ~75% of the enzymatic transformations of small molecule drugs (4, 5). There are 57 human P450 genes identified in the human genome, and about one fourth of them can be termed "orphans" because of their unknown physiological or other functions (5, 6).

Human P450 2W1 is considered one of the orphan P450 enzymes. It is preferentially expressed in colorectal cancer tissue (7, 8), and it is regulated by gene methylation and reverse membrane orientation (8, 9). Moreover, expression of the P450 2W1 variant allele G541A (Ala181Thr) in tumors has been reported to be associated with lower survival rates (10). Interestingly, P450 2W1 expression is seen in colon, ileum, and testes in mice (11), but more sensitive searches in corresponding human tissues have not been reported. Some P450 2W1-catalyzed reactions have been identified, including *N*-demethylation of benzphetamine (12), reduction of the drug candidate 1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione (AQ4N) (13), oxidation of indole and its derivatives (8, 12, 14), oxidation of the FFA C20:4[2] (at very low rates) (7, 12), and activation of a variety of chemical carcinogens to genotoxic forms (12).

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Abbreviations: APCI, atmospheric pressure chemical ionization; BSTFA, *N,O-bis*-(trimethylsilyl)-trifluoroacetamide; HRMS, high resolution mass spectrometry, LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PC, phosphatidylcholine; pFBB, pentafluorobenzyl bromide; P450, cytochrome P450; TMCS, trimethylchlorosilane; TMSI, trimethylsilylimidazole; UPLC, ultra-performance liquid chromatography.

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[2] Individual FFAs are designated by the chain length:number of double bonds.

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of text, two tables, and two figures.

LC/MS is one of the most widely used analytical methods for metabolomic analysis and has proved to be a powerful approach in substrate searches (15–18). Recently we developed a general strategy for the identification of endogenous substrates of human P450s in tissue extracts using LC/MS assays and the program DoGEX (19, 20). The approach is based on the fact that the majority of P450-mediated reactions involve the incorporation of an oxygen atom into the substrate, i.e., the product is 16 amu heavier than the substrate. Incubation of a 1:1 mixture of ^{18}O- and ^{16}O-labeled oxygen gas with tissue extracts generates products as M/M+2 doublets in the MS spectra, which can be identified by the program DoGEX (19, 20). This strategy has been validated (19) and used to identify endogenous substrates for P450 4F11 (i.e., FFAs) (21).

The aim of the present work was to identify endogenous substrates for human P450 2W1. The purified enzyme was used to conduct untargeted substrate searches in human colorectal cancer samples (i.e., site of P450 2W1 expression) using the LC/MS metabolomic and isotopic labeling approach (19, 21). A series of lysophospholipids and FFAs were identified as novel substrates for P450 2W1, and the isomer- and enentiomer-selectivity of P450 2W1-catalyzed lysophospholipid oxidations have been characterized. The identities of the oxidation products were defined, and steady-state kinetics of the P450 reactions were determined.

EXPERIMENTAL PROCEDURES

Materials and reagents

P450s 1A2 (22), 2A6 (23), 2C8 (24, 25), 2D6 (26), 2E1 (27), 2S1 (12), 2W1 (12), 3A4 (28), 7A1 (29), and rat NADPH-P450 reductase (30) were expressed in *E. coli* and purified as previously described. P450 2C19 was expressed (31) and purified using the same protocol as that for P450 2C9 (32). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). The BSTFA:TMCS:TMSI:pyridine mixture (3:2:3:10, v/v/v/v) was purchased from Regis Technologies (Morton Grove, IL). Preparative TLC was conducted on precoated 2,000 μm silica gel GF-254 plates (Analtech Inc., Newark, DE). All other reagents and solvents were obtained from general commercial suppliers.

Colorectal cancer extracts

Malignant human colorectal cancer samples were obtained from the Translational Pathology Shared Resource, Vanderbilt University School of Medicine. Human liver samples (from organ donors) were obtained from Tennessee Donor Services. Extracts were prepared from pooled samples from five individuals using Folch reagent ($CHCl_3$:CH_3OH, 2:1, v/v) as previously described (19).

LC/MS metabolomics and data analysis

In vitro P450 incubations were performed in 1.0 ml of 100 mM potassium phosphate buffer (pH 7.4) each containing purified human P450 enzyme (1.0 μM), NADPH-P450 reductase (2.0 μM), L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine (150 μM), and an aliquot of an ethanolic solution of tissue extracts (1%, v/v). For $^{16}O_2$/$^{18}O_2$ isotopic labeling experiments, reactions were performed using the method described previously (19), except that 100% $^{16}O_2$ and 97% $^{18}O_2$ gas were used in two individual Thunberg tubes. The enzyme reactions were initiated by the addition of an NADPH-generating system including 100 μl of 100 mM glucose 6-phosphate, 50 μl of 10 mM $NADP^+$, and 2 μl of a 1 mg ml^{-1} solution of yeast glucose 6-phosphate dehydrogenase (33). After incubation at 37°C for 60 min, the contents of the two Thunberg tubes were combined (equal volumes) and quenched with CH_2Cl_2. After centrifugation at 2×10^3 *g* for 10 min, the organic phase (lower) was carefully separated, taken to dryness under a N_2 stream, and redissolved in CH_3CN for LC/MS analysis. The oxidation products were identified as M and M+2 doublets (19) with a newly developed approach, which was based on the software MZmine2 (34) and an in-house made Matlab program, as described in the supplementary data.

LC separation was performed with a Waters Acquity UPLC system (Waters, Milford, MA) with an Acquity BEH octadecylsilane (C_{18}) UPLC column (1.7 μm, 1.0 mm × 100 mm) at 50°C. Samples (10 μl) were injected onto the UPLC column, and components were eluted with a linear gradient increasing from 95% (v/v) mobile phase A (10 mM $NH_4CH_3CO_2$ in a 5:95 (v/v) CH_3CN/H_2O mixture) to 100% mobile phase B (10 mM $NH_4CH_3CO_2$ in a 95:5 (v/v) CH_3CN/H_2O mixture) over 20 min, and held at 100% mobile phase B for 5 min at a flow rate of 0.15 ml min^{-1}. Data was collected with a ThermoFinnigan LTQ ion trap mass spectrometer (ThermoFisher, Watham, MA) equipped with an ESI source or APCI source scanning from *m/z* 80 to 800 in the profile mode, using the same instrument parameters as previously described (19). A ThermoFinnigan Orbitrap mass spectrometer was used for the collection of HRMS data.

Characterization of oxidation products

Characterization of oxidation products was performed by GC/MS after preparing the corresponding TMS ethers. Oxidation products of FFAs were obtained by incubating each FFA (100 μM) in 1.0 ml of reaction mixture containing phosphate buffer, P450 2W1, NADPH-P450 reductase, L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and an NADPH-generating system (see above). The products were extracted with 2.0 ml of CH_2Cl_2 and dried under a N_2 stream. Epoxides were converted to dihydrodiols after incubation with H_2O (adjusted to pH 2) at 23°C for 10 min and extracted again with CH_2Cl_2. Oxidation products of LPCs were obtained by incubating each LPC (100 μM) with 1.0 ml of the reaction mixture (see above). The reactions were quenched with 2.0 ml of CH_3OH containing butylated hydroxytoluene (0.005%, w/v) and 1.0 ml of aqueous KOH (15%, w/v). The mixtures were then mixed with a vortex device, purged with Ar, and incubated at 37°C for 30 min to hydrolyze the oxidized LPCs and release the oxidized fatty acids (35). The mixtures were acidified to pH 2 with HCl, and the oxidized fatty acids were extracted into CH_2Cl_2. TMS derivatization was performed with 20 μl of silylation reagent (BSTFA/TMCS/TMSI/pyridine, 3:2:3:10, v/v/v/v) at 60°C for 30 min. The resulting TMS derivatives were analyzed by GC/MS in the electron impact mode as previously described (19).

Kinetic analysis of P450 reactions

Substrate concentrations ranging from 0 to 200 μM were used for steady-state kinetic studies. Reactions were run in duplicate at 37°C for 15 min. Oxidation products of FFAs and LPCs were extracted as described above. The products were derivatized with 20 μl of 10% (v/v) *N,N*-diisopropylethylamine in CH_3CN and 40 μl of 10% (v/v) pFBB in CH_3CN at 37°C for 20 min (35). pFBB-derivatized samples were dried under a N_2 stream and then derivatized with 20 μl of BSTFA and 7 μl of dry dimethylformamide at 37°C for 20 min (35) and analyzed by GC/MS in the chemical ionization mode. FFA C17:0 and C19:0 standards were used to

prepare calibration curves for kinetic analysis of FFA C16:0 and C18:1 oxidation; 17:0 LPC and C19:0 LPC were used to prepare calibration curve for kinetic analysis of 16:0 LPC oxidation. Epoxy-18:1 LPC and epoxy-16:1 PC, purified and quantified by a phosphorus assay (36), were used to prepare calibration curves for kinetic analysis of 18:1 LPC oxidation. Epoxy-18:1 LPC was chemically synthesized by incubating 18:1 LPC with an excess amount of *m*-chloroperoxybenzoic acid. The reaction mixture was streaked on a preparative fluorescent TLC plate, developed with CH_3OH:$CHCl_3$ (1:1 v/v), and visualized by UV light. The lower band was eluted by the same solvent, taken to dryness using a rotary evaporator, and dissolved in C_2H_5OH containing 1% diisopropylethylamine (v/v). LC/MS analysis confirmed that all 18:1 LPC was converted into epoxy-18:1 LPC. Epoxy-16:1 PC was synthesized and quantified with the same method (see above). For rate comparisons of different lysophospholipids, P450 2W1 was incubated with 100 μM 18:1 LPC, 18:1 LPI, 18:1 LPS, 18:1 LPG, 18:1 LPE, or 18:1 LPA in triplicate, and the rates were determined as described above.

Purification of *sn*-1 and *sn*-2 LPC

HPLC was used to separate the two isomers. *sn*-1 and *sn*-2 LPCs were monitored at 196 nm and baseline separation was achieved with a Phenomenex prodigy ODS (3) HPLC column (5 μm, 2.0 mm × 150 mm). An isocratic solution of 1:1 (v/v) CH_3CN/H_2O (pH adjusted to 5 with NH_4CO_2H) was used to resolve the two isomers at 40°C, at a flow rate of 0.5 ml min^{-1}. The collected *sn*-1 and *sn*-2 LPC fractions were frozen and concentrated by lyophilization prior to enzymatic reaction.

Chiral analysis

Optically pure (9*S*,10*R*)- and (9*R*,10*S*)-epoxystearic acids were produced by hydrogenating pure (9*S*,10*R*)-epoxy-12*Z*-octadecenoic acid and (9*R*,10*S*)-epoxy-12Z-octadecenoic acid (37) with Pd powder under a H_2 stream for 3 min (19). The enantiomers of 9,10-epoxystearic acid were separated by normal phase HPLC with a Waters Alliance 2695 HPLC pump (Waters, Milford, MA) and a Chiralpak AD column (5 μm, 4.6 mm × 25 cm). An isocratic solvent of a 100:2:0.05 (v/v/v) hexanes/CH_3OH/CH_3CO_2H mixture was used to resolve the enantiomers at a flow rate of 1 ml min^{-1} at room temperature. The retention times of (9*S*,10*R*)- and (9*R*,10*S*)-epoxystearic acids were determined to be 16.9 min and 18.7 min, respectively. Epoxide generated from FFA C18:1 was extracted with CH_2Cl_2 after enzymatic reaction. Epoxide generated from 18:1 LPC was subjected to hydrolysis as described above, and five volumes of 1 M potassium phosphate buffer (pH 7.4) was added to neutralize the pH before extraction with CH_2Cl_2. After dried under a N_2 stream, the epoxide was analyzed as described above and detected with the APCI negative ion mode.

Other methods

UV-visible spectra were recorded using an Aminco DW-2a/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA). P450 concentrations were estimated spectrally as previously described (38).

RESULTS

Searches for P450 2W1 substrates in malignant human colorectal cancer extracts

Purified P450 2W1 was incubated with malignant human colorectal cancer extracts, NADPH, and $^{16}O_2$/$^{18}O_2$ gas mixtures. In principle, all doublets (*m/z* M/M+2) in LC/MS data result from the addition of an oxygen atom to endogenous substrates, with the general concept described previously (20). To profile as many metabolites as possible, samples were analyzed with both ESI and APCI sources in both the positive and negative ionization modes. A new approach, based on the software MZmine2 and an in-house made Matlab program, was used for doublet searches due to its improved performance regarding both precision and recall (supplementary Table I).

The doublets *m/z* 538/540 (**Fig. 1A**) in the ESI positive ion mode and *m/z* 269/271, 271/273, 295/297, 297/299, and 319/321 in the ESI negative ion mode were identified (supplementary Table II). Product candidates were found only in the samples incubated with P450 2W1, NADPH-P450 reductase, and NADPH but not in the samples absent any of these. All doublets were further confirmed to be oxidation products by comparison with the incubations done only with $^{16}O_2$ gas, in which the *m/z* M+2 peaks were absent.

The *m/z* values of the respective substrates can be deduced from the *m/z* values of the products by subtracting 16 amu (oxygen). Therefore, the molecular masses of the putative substrates were calculated to be 521, 254, 256, 280, 282, and 304. MS fragmentation of the *m/z* 538 ion in the ESI positive ion mode produced a daughter ion of *m/z* 184 (Fig. 1B), indicative of a phosphocholine group (39). The LIPIDMAPS database (http://www.lipidmaps.org) suggested that 18:1 LPC (*m/z* 521) was a likely substrate. MS fragmentation analysis of the products detected in the ESI negative ion mode and the search of LIPIDMAPS database suggested that FFAs C16:0, C16:1, C18:1, C18:2, and C20:4 were likely substrates. To confirm the identities of the substrates, 18:1 LPC and five FFAs were incubated with P450 2W1 and NADPH, and the extracted products were analyzed by LC/MS/MS. All of the product peaks formed in the incubations with the authentic compounds yielded exactly the same peaks identified by the new software.

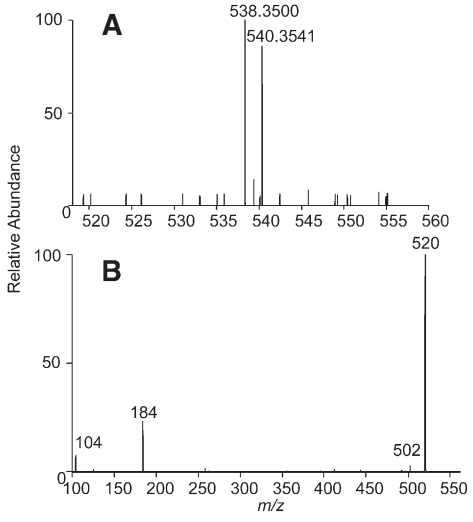

Fig. 1. LC/MS/MS analysis of the *m/z* 538/540 doublet. (A) HRMS of *m/z* 538/540 produced from the incubation of P450 2W1, NADPH, colon cancer extracts, and $^{18}O_2$/$^{16}O_2$ gas (1:1, v/v). (B) MS/MS fragmentation of the 18:1 LPC oxidation product *m/z* 538 in the ESI positive ion mode.

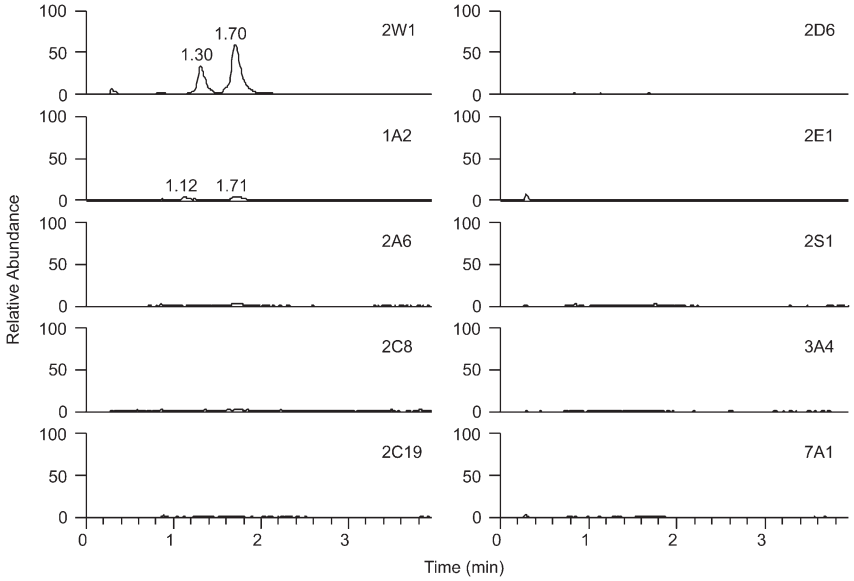

Fig. 2. Specificity of 18:1 LPC as a substrate for human P450 enzymes. Selected ion chromatograms of 18:1 LPC oxidation products (*m/z* 538) after incubating 18:1 LPC with purified P450 enzymes. Retention times (t_R) are indicated on the chromotograms.

These results identified 18:1 LPC and the C16:0, C16:1, C18:1, C18:2, and C20:4 FFAs as substrates for P450 2W1 in malignant human colorectal cancer tissue.

Specificity of 18:1 LPC as a substrate for human P450 enzymes

The specificity of 18:1 LPC as a substrate for different human P450 enzymes was investigated. 18:1 LPC was incubated with purified human P450s 1A2, 2A6, 2C8, 2C19, 2D6, 2E1, 2S1, 2W1, 3A4, and 7A1, and the extracted products were analyzed by LC/MS (**Fig. 2**). Although many other human P450 enzymes share similar catalytic efficiencies toward fatty acids (19, 21), they showed little activity with 18:1 LPC. We conclude that 18:1 LPC is a substrate relatively specific for P450 2W1.

Characterization of oxidation products

GC/MS assays of TMS derivatives were used to characterize the oxidation products of P450 2W1 reactions. For FFAs, oxidation products were extracted with CH_2Cl_2 and their identities were established after silylation. For LPCs, base hydrolysis was performed to release the oxidized fatty acids prior to silylation. Multiple doublets in the LC/MS data shared the same *m/z*, suggesting that each substrate may have multiple products (supplementary Table II). As summarized in **Table 1**, P450 2W1 catalyzed both hydroxylation and epoxidation at the middle of fatty acid chains.

Steady-state kinetic analysis of P450 2W1 reactions

Kinetic studies were performed for 16:0 LPC and 18:1 LPC, as well as for the FFAs C16:0 and C18:1. The kinetic parameters k_{cat} and K_m were estimated based on Michaelis-Menten plots and nonlinear regression analysis (**Table 2**). The catalytic efficiencies (k_{cat}/K_m) of P450 2W1-catalyzed fatty acid oxidations were comparable with those catalyzed by other human P450 enzymes (19, 21). The catalytic efficiency of 18:1 LPC oxidation was ~6-fold greater than that of the FFA C18:1.

Isomer specificity of lysophospholipid oxidation

Commercially available LPCs are mixtures of both *sn*-1 and *sn*-2 isomers due to acyl migration, which occurs even at neutral pH (40). The interconversion can be attenuated at pH 4–5 (40), and baseline separation could be achieved

TABLE 1. EI GC/MS analysis of TMS derivatives of the oxidation products of fatty acids and LPCs produced by human P450 2W1

Substrate		[M^+]	Major Fragment Ions (*m/z*)	Product
16:0[a, b]	Hydroxylation	416	145, 159, 173, 345, 359, 373	11-OH, 12-OH, 13-OH
18:1[a, b]	Hydroxylation	442	201, 241, 303, 343	8-OH, 11-OH
18:1[a, b]	Epoxidation	532	215, 317	9, 10-epoxide
16:0 LPC[a]	Hydroxylation	416	215, 229, 243, 275, 289, 303	6-OH, 7-OH, 8-OH
18:0 LPC[a]	Hydroxylation	444	229, 243, 257, 289, 303, 317	7-OH, 8-OH, 9-OH
18:1 LPC[a]	Hydroxylation	442	201, 241, 303, 343	8-OH, 11-OH
18:1 LPC[a]	Epoxidation	532	215, 317	9, 10-epoxide
20:0 LPC[a]	Hydroxylation	472	243, 257, 271, 303, 317, 331	8-OH, 9-OH, 10-OH

[a] See supplementary Fig. II for fragmentation patterns.
[b] FFA.

TABLE 2. Steady-state kinetics of P450 2W1-catalyzed oxidations

Substrate		k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} mM^{-1})
16:0[a]	Hydroxylation	0.77 ± 0.06	83 ± 17	9.3 ± 0.7
18:1[a]	Hydroxylation	0.63 ± 0.09	101 ± 31	6.2 ± 0.9
18:1[a]	Epoxidation	0.044 ± 0.003	95 ± 13	0.46 ± 0.03
16:0 LPC	Hydroxylation	0.012 ± 0.002	14 ± 5	0.9 ± 0.1
18:1 LPC	Hydroxylation	1.36 ± 0.16	38 ± 12	36 ± 4
18:1 LPC	Epoxidation	0.21 ± 0.02	34 ± 11	6.2 ± 0.6

[a] FFA.

using a pH 5 solvent in HPLC (supplementary Fig. I). The identities of recovered isomers were confirmed by MS/MS fragmentation patterns, due to the different ratios of fragmentation ions at *m/z* 184 and 104 (39): the *sn*-1 isomer produces more *m/z* 104 daughter ion (**Fig. 3D, E**) than the *sn*-2 isomer (Fig. 1B). A mock incubation of pure *sn*-1 and *sn*-2 isomers in a P450 reaction mixture was performed for 10 min, and little interconversion between the two isomers was observed. To determine the isomer specificity of lysophospholipid oxidation, equivalent amounts of *sn*-1 and *sn*-2 isomers were incubated with P450 2W1 and NADPH for 10 min. The reactions were quenched with three volumes of CH_3CN, and the pH was decreased to 5 with one-half volume of 1 M NH_4CO_2H buffer (pH 5). Oxidation of 18:1 LPC was observed only in the incubation with *sn*-1 isomer (Fig. 3A). The *sn*-1 isomeric nature of the oxidation product, confirmed by MS/MS fragmentation (Fig. 3D, E), also indicated that *sn*-1 18:1 LPC is the preferred substrate. We conclude that the *sn*-1 isomers of lysophospholipids are preferred substrates.

Substrate specificity for other phospholipids

Diacylphospholipids, including 16:0 PC, 16:0-18:1 PC, and 16:0-20:4 PC, were incubated with the P450 2W1 reaction mixture and analyzed using the same conditions as for LPCs; no oxidation products were detected. Other classes of lysophospholipids, including 18:1 LPI, 18:1 LPS, 18:1 LPG, 18:1 LPE, and 18:1 LPA, were also confirmed to be substrates for P450 2W1, with similar rates of oxidation (**Fig. 4**).

Chiral analysis

P450 2W1-catalyzed epoxidation was investigated with chiral HPLC. The epoxide generated from FFA C18:1 was a mixture of (9*S*,10*R*) and (9*R*,10*S*) in the ratio of 1:10 (**Fig. 5A**). The epoxide generated from 18:1 LPC was extracted after hydrolysis, and chiral analysis showed that epoxy-18:1 LPC was also a mixture of (9*S*,10*R*) and (9*R*,10*S*) but in the ratio of 1:3 (Fig. 5B).

DISCUSSION

To elucidate the substrate specificity and possible physiological function of P450 2W1, untargeted substrate searches were performed using an LC/MS-based metabolomic and isotopic-labeling approach (19). In addition to FFAs, 18:1 LPC was identified as a substrate for P450 2W1

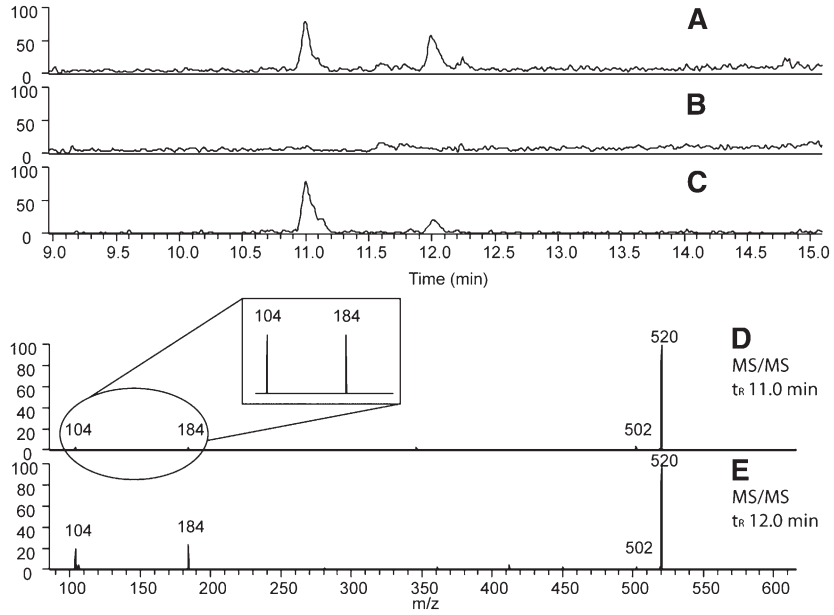

Fig. 3. Isomer specificity of lysophospholipid oxidation. Selected ion chromatograms of 18:1 LPC oxidation products (*m/z* 538) after incubating P450 2W1 with (A) *sn*-1 18:1 LPC, (B) *sn*-2 18:1 LPC, or (C) an equilibrated mixture of *sn*-1 and *sn*-2 18:1 LPC (containing 90% *sn*-1 18:1 LPC and 10% *sn*-2 18:1 LPC). Fragmentation of (D) the peak at t_R 11 min and (E) the peak at t_R 12 min.

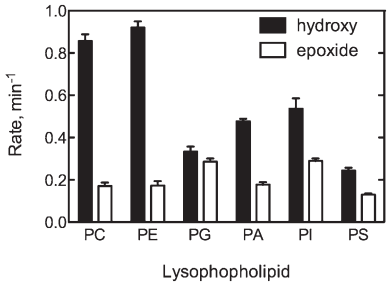

Fig. 4. Rates of P450 2W1-catalyzed oxidation with different 18:1 lysophospholipids.

(supplementary Table II). Other human P450 enzymes tested showed little activity toward 18:1 LPC (Fig. 2). In addition to LPCs, a series of other lysophospholipids, including 18:1 LPI, 18:1 LPS, 18:1 LPG, 18:1 LPE, and 18:1 LPA (but not diacylphospholipids), were identified as substrates for P450 2W1. *sn*-1 18:1 LPC was the preferred substrate over the *sn*-2 isomer (Fig. 3), and we conclude that the *sn*-1 isomers of lysophospholipids are the preferred substrates. For the 18:1 epoxidation product, chiral analysis showed enantio-selectivity for formation of (9*R*,10*S*) over (9*S*,10*R*). The position specificities (Table 1) and the kinetics (Table 2) of P450 2W1-catalyzed oxygenation of lysophospholipids (16:0 LPC and 18:1 LPC) and FFAs (C16:0 and C18:1) were also determined. The epoxidation and hydroxylation for 18:1 LPC were considerably more efficient than for the FFA C18:1. The reaction with an unsaturated acyl LPC (18:1 LPC) was also considerably more efficient than with a saturated acyl LPC (16:0 LPC).

FFAs are common substrates for a number of human and other P450 enzymes (12, 21, 41–43). Among the five fatty acids identified in our study, only C20:4 has been reported as a substrate for P450 2W1 previously (12). This is

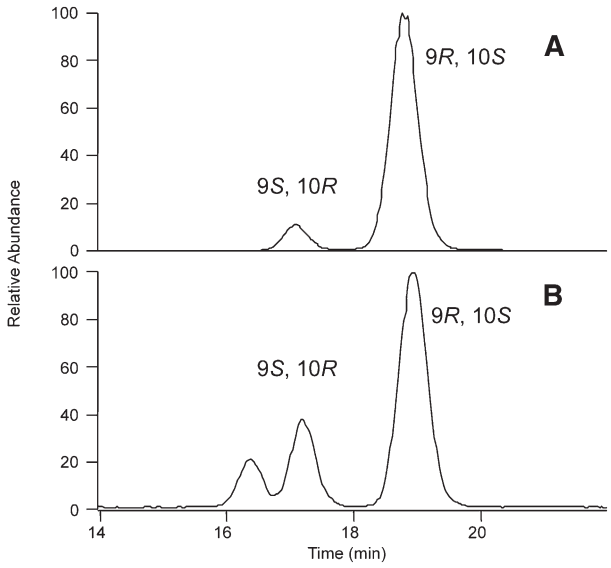

Fig. 5. Chiral analysis of P450 2W1-catalyzed epoxidation of FFA C18:1 and 18:1 LPC. Selected ion chromatograms of P450 2W1-catalyzed epoxidations (*m/z* 297 > 171) of (A) FFA C18:1 and (B) 18:1 LPC. The identity of the peak at t_R 16.5 min is unknown.

the first report that the FFAs C16:0, C16:1, C18:1, and C18:2 are substrates for P450 2W1. P450 2W1 catalyzes both hydroxylation and epoxidation at the middle of fatty acid chains (Table 1). Although some of oxidation products of FFAs have been shown to have interesting physiological functions in vivo (44), the fact that many P450 enzymes catalyze FFA oxidations at very slow rates raises doubts about the physiological importance of many of the products (e.g., ω-1, ω-2).

To our knowledge, this is the first report that lysophospholipids are substrates for any P450 enzyme. The most abundant LPCs in plasma are 16:0 LPC, 18:0 LPC, 18:1 LPC, 18:2 LPC, and 20:4 LPC (45). All of these, except 18:2 LPC and 20:4 LPC, were confirmed to be substrates for P450 2W1. 18:2 LPC and 20:4 LPC are also likely to be substrates for P450 2W1, but these were not commercially available and, therefore, were not tested. The results of our kinetic studies suggest that unsaturated acyl LPCs are more efficiently oxidized by P450 2W1 than are saturated acyl LPCs.

Although commercial LPCs are composed of ~90% *sn*-1 and ~10% *sn*-2 isomers, the percentage of each isomer in plasma has been reported to be ~50% (46). In plasma, 90% of the unsaturated acyl LPCs were *sn*-1 isomers (46), which can be more efficiently oxidized by P450 2W1 compared with saturated acyl LPC.

Lysophospholipids are lipid mediators involved in a vast variety of biological functions (47). In particular, LPCs are endogenous proinflammatory lipids that stimulate chemotaxis of T lymphocytes (48) and macrophages (49). Decreased concentrations of LPCs have been identified in the plasma of colorectal cancer and lung cancer patients (39, 50). Thus, decreased LPC levels may be an important contributing factor for tumor development. LPAs are also potent lipid mediators that lead to a plethora of biological actions, including cell proliferation, survival, motility, and invasion, which are critically required for tumor initiation and progression (51, 52). 18:1 LPA, one of the substrates of P450 2W1, has been reported to enhance the metastatic potential of human colon cancer cells and to protect them from apoptosis (53–55). One aspect of LPA action is its role as a ligand for several cell surface G-protein coupled receptors [e.g., LPA1, LPA2, LPA3, LPA4/GPG23, and LPA5/GPR92 (56)]; it is not known whether these receptors are isomer-selective or enantio-selective. LPA enhances cell proliferation by activation of the transcription factor Krüppel-like fractor 5 (KLF5) (54, 57). Considering the presence of hydroxyl- and epoxy-lysophospholipids/phospholipids in vivo (58, 59), it is possible that P450 2W1-catalyzed lysophospholipid oxidations are involved in inflammation and tumor development by producing ligands to these receptors and modulate downstream signaling pathways, although further information is not available.

The balance of concentrations of oxidized lysophospholipids is very delicate. They can be synthesized from at least three pathways: P450 2W1-catalyzed lysophospholipid oxidation, deacylation from oxidized phospholipids (59), and esterification of oxidized fatty acids to the glycerol derivatives. At the meantime, they can be degraded by several

lysophospholipase-based pathways. It is possible that the upregulation of P450 2W1 expression in colorectal cancer tissues disturbs the balance of oxidized lysophospholipids and leads to pathological consequences, but further conjecture about the role of P450 2W1 in cancer is speculative.

In conclusion, we have identified P450 oxidation reactions that selectively occur on lysophospholipid fatty acid chains (but not on diacylphospholipids). They were found to be selectively catalyzed by an orphan human P450, P450 2W1, that had not been clearly shown to have definitive catalytic activities with physiological substrates previously. The physiological functions of these oxidized lysophospholipids, if any, remain to be established. We have also introduced new software for the analysis of isotopic compositions of compounds in MS, which can be used in other metabolomic studies.

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REFERENCES

1. Hughes, T. R., M. D. Robinson, N. Mitsakakis, and M. Johnston. 2004. The promise of functional genomics: completing the encyclopedia of a cell. *Curr. Opin. Microbiol.* **7:** 546–554.
2. Carpenter, A. E., and D. M. Sabatini. 2004. Systematic genome-wide screens of gene function. *Nat. Rev. Genet.* **5:** 11–22.
3. Ortiz de Montellano, P. R., editor. 2005. Cytochrome P450: Structure, Mechanism, and Biochemistry. 3rd edition. Kluwer Academic/Plenum Publishers, New York.
4. Williams, J. A., R. Hyland, B. C. Jones, D. A. Smith, S. Hurst, T. C. Goosen, V. Peterkin, J. R. Koup, and S. E. Ball. 2004. Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC_i/AUC) ratios. *Drug Metab. Dispos.* **32:** 1201–1208.
5. Guengerich, F. P. 2005. Human cytochrome P450 enzymes. *In* Cytochrome P450: Structure, Mechanism, and Biochemistry. 3rd edition. P. R. Ortiz de Montellano, editor. Kluwer Academic/Plenum Publishers, New York. 377–530.
6. Guengerich, F. P., and Q. Cheng. 2011. Orphans in the human cytochrome P450 superfamily: approaches to discovering functions and relevance in pharmacology. *Pharmacol. Rev.* **63:** 684–699.
7. Karlgren, M., A. Gomez, K. Stark, J. Svard, C. Rodriguez-Antona, E. Oliw, M. L. Bernal, S. Ramon y Cajal, I. Johansson, and M. Ingelman-Sundberg. 2006. Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1. *Biochem. Biophys. Res. Commun.* **341:** 451–458.
8. Gomez, A., J. Nekvindova, S. Travica, M. Y. Lee, I. Johansson, D. Edler, S. Mkrtchian, and M. Ingelman-Sundberg. 2010. Colorectal cancer-specific cytochrome P450 2W1: intracellular localization, glycosylation, and catalytic activity. *Mol. Pharmacol.* **78:** 1004–1011.
9. Gomez, A., M. Karlgren, D. Edler, M. L. Bernal, S. Mkrtchian, and M. Ingelman-Sundberg. 2007. Expression of CYP2W1 in colon tumors: regulation by gene methylation. *Pharmacogenomics.* **8:** 1315–1325.
10. Gervasini, G., S. G. de Murillo, J. M. Ladero, and J. A. Agundez. 2010. CYP2W1 variant alleles in Caucasians and association of the CYP2W1 G541A (Ala181Thr) polymorphism with increased colorectal cancer risk. *Pharmacogenomics.* **11:** 919–925.
11. Renaud, H. J., J. Y. Cui, M. Khan, and C. D. Klaassen. 2011. Tissue distribution and gender-divergent expression of 78 cytochrome P450 mRNAs in mice. *Toxicol. Sci.* **124:** 261–277.
12. Wu, Z. L., C. D. Sohl, T. Shimada, and F. P. Guengerich. 2006. Recombinant enzymes overexpressed in bacteria show broad catalytic specificity of human cytochrome P450 2W1 and limited activity of human cytochrome P450 2S1. *Mol. Pharmacol.* **69:** 2007–2014.
13. Nishida, C. R., M. Lee, and P. R. Ortiz de Montellano. 2010. Efficient hypoxic activation of the anticancer agent AQ4N by CYP2S1 and CYP2W1. *Mol. Pharmacol.* **78:** 497–502.
14. Yoshioka, H., N. Kasai, S. Ikushiro, R. Shinkyo, M. Kamakura, M. Ohta, K. Inouye, and T. Sakaki. 2006. Enzymatic properties of human CYP2W1 expressed in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **345:** 169–174.
15. Long, J. Z., J. S. Cisar, D. Milliken, S. Niessen, C. Wang, S. A. Trauger, G. Siuzdak, and B. F. Cravatt. 2011. Metabolomics annotates ABHD3 as a physiologic regulator of medium-chain phospholipids. *Nat. Chem. Biol.* **7:** 763–765.
16. Saghatelian, A., S. A. Trauger, E. J. Want, E. G. Hawkins, G. Siuzdak, and B. F. Cravatt. 2004. Assignment of endogenous substrates to enzymes by global metabolite profiling. *Biochemistry.* **43:** 14332–14339.
17. Smith, C. A., E. J. Want, G. O'Maille, R. Abagyan, and G. Siuzdak. 2006. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **78:** 779–787.
18. Cheng, Q., D. C. Lamb, S. L. Kelly, L. Lei, and F. P. Guengerich. 2010. Cyclization of a cellular dipentaenone by *Streptomyces coelicolor* cytochrome P450 154A1 without oxidation/reduction. *J. Am. Chem. Soc.* **132:** 15173–15175.
19. Tang, Z., M. V. Martin, and F. P. Guengerich. 2009. Elucidation of functions of human cytochrome P450 enzymes: identification of endogenous substrates in tissue extracts using metabolomic and isotopic labeling approaches. *Anal. Chem.* **81:** 3071–3078.
20. Sánchez-Ponce, R., and F. P. Guengerich. 2007. Untargeted analysis of mass spectrometry data for elucidation of metabolites and function of enzymes. *Anal. Chem.* **79:** 3355–3362.
21. Tang, Z., S. G. Salamanca-Pinzón, Z. L. Wu, Y. Xiao, and F. P. Guengerich. 2010. Human cytochrome P450 4F11: heterologous expression in bacteria, purification, and characterization of catalytic function. *Arch. Biochem. Biophys.* **494:** 86–93.
22. Sandhu, P., Z. Guo, T. Baba, M. V. Martin, R. H. Tukey, and F. P. Guengerich. 1994. Expression of modified human cytochrome P450 1A2 in *Escherichia coli*: stabilization, purification, spectral characterization, and catalytic activities of the enzyme. *Arch. Biochem. Biophys.* **309:** 168–177.
23. Yun, C. H., K. H. Kim, M. W. Calcutt, and F. P. Guengerich. 2005. Kinetic analysis of oxidation of coumarins by human cytochrome P450 2A6. *J. Biol. Chem.* **280:** 12279–12291.
24. Schoch, G. A., J. K. Yano, S. Sansen, P. M. Dansette, C. D. Stout, and E. F. Johnson. 2008. Determinants of cytochrome P450 2C8 substrate binding. *J. Biol. Chem.* **283:** 17227–17237.
25. Schoch, G. A., J. K. Yano, M. R. Wester, K. J. Griffin, C. D. Stout, and E. F. Johnson. 2004. Structure of human microsomal cytochrome P450 2C8. *J. Biol. Chem.* **279:** 9497–9503.
26. Gillam, E. M., Z. Guo, M. V. Martin, C. M. Jenkins, and F. P. Guengerich. 1995. Expression of cytochrome P450 2D6 in *Escherichia coli*, purification, and spectral and catalytic characterization. *Arch. Biochem. Biophys.* **319:** 540–550.
27. Gillam, E. M., Z. Guo, and F. P. Guengerich. 1994. Expression of modified human cytochrome P450 2E1 in *Escherichia coli*, purification, and spectral and catalytic properties. *Arch. Biochem. Biophys.* **312:** 59–66.
28. Gillam, E. M., T. Baba, B. R. Kim, S. Ohmori, and F. P. Guengerich. 1993. Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Arch. Biochem. Biophys.* **305:** 123–131.
29. Shinkyo, R., and F. P. Guengerich. 2011. Cytochrome P450 7A1 cholesterol 7α-hydroxylation: individual reaction steps in the catalytic cycle and rate-limiting ferric iron reduction. *J. Biol. Chem.* **286:** 4632–4643.
30. Hanna, I. H., J. F. Teiber, K. L. Kokones, and P. F. Hollenberg. 1998. Role of the alanine at position 363 of cytochrome P450 2B2 in influencing the NADPH- and hydroperoxide-supported activities. *Arch. Biochem. Biophys.* **350:** 324–332.
31. Cuttle, L., A. J. Munns, N. A. Hogg, J. R. Scott, W. D. Hooper, R. G. Dickinson, and E. M. J. Gillam. 2000. Phenytoin metabolism by human cytochrome P450: Involvement of P450 3A and 2C forms in secondary metabolism and drug-protein adduct formation. *Drug Metab. Dispos.* **28:** 945–950.
32. Sandhu, P., T. Baba, and F. P. Guengerich. 1993. Expression of modified cytochrome P450 2C10 (2C9) in *Escherichia coli*, purification, and reconstitution of catalytic activity. *Arch. Biochem. Biophys.* **306:** 443–450.

33. Guengerich, F. P., and C. J. Bartleson. 2007. Analysis and characterization of enzymes and nucleic acids. *In* Principles and Methods of Toxicology. 5th edition. A. W. Hayes, editor. CRC Press, Boca Raton, FL. 1981–2048.
34. Pluskal, T., S. Castillo, A. Villar-Briones, and M. Oresic. 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics.* **11:** 395.
35. Liu, W., J. D. Morrow, and H. Yin. 2009. Quantification of F2-isoprostanes as a reliable index of oxidative stress in vivo using gas chromatography-mass spectrometry (GC-MS) method. *Free Radic. Biol. Med.* **47:** 1101–1107.
36. Rouser, G., S. Fkeischer, and A. Yamamoto. 1970. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
37. Gao, B., W. E. Boeglin, Y. Zheng, C. Schneider, and A. R. Brash. 2009. Evidence for an ionic intermediate in the transformation of fatty acid hydroperoxide by a catalase-related allene oxide synthase from the cyanobacterium *Acaryochloris marina*. *J. Biol. Chem.* **284:** 22087–22098.
38. Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239:** 2370–2378.
39. Dong, J., X. Cai, L. Zhao, X. Xue, L. Zou, X. Zhang, and X. Liang. 2010. Lysophosphatidylcholine profiling of plasma: discrimination of isomers and discovery of lung cancer biomarkers. *Metabolomics.* **6:** 478–488.
40. Plückthun, A., and E. A. Dennis. 1982. Acyl and phosphoryl migration in lysophospholipids: importance in phospholipid synthesis and phospholipase specificity. *Biochemistry.* **21:** 1743–1750.
41. Nakano, M., E. J. Kelly, and A. E. Rettie. 2009. Expression and characterization of CYP4V2 as a fatty acid ω-hydroxylase. *Drug Metab. Dispos.* **37:** 2119–2122.
42. Chuang, S. S., C. Helvig, M. Taimi, H. A. Ramshaw, A. H. Collop, M. Amad, J. A. White, M. Petkovich, G. Jones, and B. Korczak. 2004. CYP2U1, a novel human thymus- and brain-specific cytochrome P450, catalyzes ω- and (ω-1)-hydroxylation of fatty acids. *J. Biol. Chem.* **279:** 6305–6314.
43. Höfer, R., I. Briesen, M. Beck, F. Pinot, L. Schreiber, and R. Franke. 2008. The *Arabidopsis* cytochrome P450 CYP86A1 encodes a fatty acid ω-hydroxylase involved in suberin monomer biosynthesis. *J. Exp. Bot.* **59:** 2347–2360.
44. Capdevila, J., V. R. Holla, and J. R. Falck. 2005. Cytochrome P450 and the metabolism and bioactivation of arachidonic acid and eicosanoids. *In* Cytochrome P450: Structure, Mechanism, and Biochemistry. 3rd edition. P. R. Ortiz de Montellano, editor. Kluwer Academic/Plenum Publishers, New York. 531–551.
45. Rabagny, Y., W. Herrmann, J. Geisel, S. H. Kirsch, and R. Obeid. 2011. Quantification of plasma phospholipids by ultra performance liquid chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.* **401:** 891–899.
46. Croset, M., N. Brossard, A. Polette, and M. Lagarde. 2000. Characterization of plasma unsaturated lysophosphatidylcholines in human and rat. *Biochem. J.* **345:** 61–67.
47. Makide, K., H. Kitamura, Y. Sato, M. Okutani, and J. Aoki. 2009. Emerging lysophospholipid mediators, lysophosphatidylserine, lysophosphatidylthreonine, lysophosphatidylethanolamine and lysophosphatidylglycerol. *Prostaglandins Other Lipid Mediat.* **89:** 135–139.
48. Ryborg, A. K., B. Deleuran, K. Thestrup-Pedersen, and K. Kragballe. 1994. Lysophosphatidylcholine: a chemoattractant to human T lymphocytes. *Arch. Dermatol. Res.* **286:** 462–465.
49. Yang, L. V., C. G. Radu, L. Wang, M. Riedinger, and O. N. Witte. 2005. G_i-independent macrophage chemotaxis to lysophosphatidylcholine via the immunoregulatory GPCR G2A. *Blood.* **105:** 1127–1134.
50. Zhao, Z., Y. Xiao, P. Elson, H. Tan, S. J. Plummer, M. Berk, P. P. Aung, I. C. Lavery, J. P. Achkar, L. Li, et al. 2007. Plasma lysophosphatidylcholine levels: potential biomarkers for colorectal cancer. *J. Clin. Oncol.* **25:** 2696–2701.
51. van Corven, E. J., A. Groenink, K. Jalink, T. Eichholtz, and W. H. Moolenaar. 1989. Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell.* **59:** 45–54.
52. Moolenaar, W. H., L. A. van Meeteren, and B. N. Giepmans. 2004. The ins and outs of lysophosphatidic acid signaling. *Bioessays.* **26:** 870–881.
53. Sun, H., J. Ren, Q. Zhu, F. Z. Kong, L. Wu, and B. R. Pan. 2009. Effects of lysophosphatidic acid on human colon cancer cells and its mechanisms of action. *World J. Gastroenterol.* **15:** 4547–4555.
54. Zhang, H., A. Bialkowska, R. Rusovici, S. Chanchevalap, H. Shim, J. P. Katz, V. W. Yang, and C. C. Yun. 2007. Lysophosphatidic acid facilitates proliferation of colon cancer cells via induction of Kruppel-like factor 5. *J. Biol. Chem.* **282:** 15541–15549.
55. Shida, D., J. Kitayama, H. Yamaguchi, Y. Okaji, N. H. Tsuno, T. Watanabe, Y. Takuwa, and H. Nagawa. 2003. Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. *Cancer Res.* **63:** 1706–1711.
56. Choi, J. W., S. Lim, Y-S. Oh, E-K. Kim, S-H. Kim, Y-H. Kim, K. Heo, J. Kim, J. K. Kim, Y. R. Yang, et al. 2010. Subtype-specific role of phospholipase C-β in bradykinin and LPA signaling through differential binding of different PDZ scaffold proteins. *Cell. Signal.* **22:** 1153–1161.
57. Lee, S-J., and C. C. Yun. 2010. Colorectal cancer cells–proliferation, survival and invasion by lysophosphatidic acid. *Int. J. Biochem. Cell Biol.* **42:** 1907–1910.
58. Adachi, J., M. Asano, N. Yoshioka, H. Nushida, and Y. Ueno. 2006. Analysis of phosphatidylcholine oxidation products in human plasma using quadrupole time-of-flight mass spectrometry. *Kobe J. Med. Sci.* **52:** 127–140.
59. Karara, A., E. Dishman, J. R. Falck, and J. H. Capdevila. 1991. Endogenous epoxyeicosatrienoyl-phospholipids. A novel class of cellular glycerolipids containing epoxidized arachidonate moieties. *J. Biol. Chem.* **266:** 7561–7569.