Structures and biological activities of novel phosphatidylethanolamine lipids of *Porphyromonas gingivalis*

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Abstract The Gram-negative periodontal pathogen *Porphyromonas gingivalis* synthesizes several classes of novel phosphorylated complex lipids, including the recently characterized phosphorylated dihydroceramides. These sphingolipids promote the interleukin-1 (IL-1)-mediated secretion of inflammatory mediators from fibroblasts, including prostaglandin E_2 and 6-keto prostaglandin $F_{2\alpha}$, and alter gingival fibroblast morphology in culture. This report demonstrates that one additional class of phosphorylated complex lipids of *P. gingivalis* promotes IL-1-mediated secretory responses and morphological changes in cultured fibroblasts. Structural characterization identified the new phospholipid class as 1,2-diacyl phosphatidylethanolamine, which substituted predominantly with isobranched $C_{15:0}$ and $C_{13:0}$ fatty acids. The isobranched fatty acids, rather than unbranched fatty acids, and the phosphoethanolamine head group were identified as the essential structural elements required for the promotion of IL-1-mediated secretory responses. These structural components are also observed in specific phosphorylated sphingolipids of *P. gingivalis* and likely contribute to the biological activity of these substances, in addition to the phosphatidylethanolamine lipids described in this report.—Nichols, F. C., B. Riep, J. Mun, M. D. Morton, T. Kawai, F. E. Dewhirst, and M. B. Smith. **Structures and biological activities of novel phosphatidylethanolamine lipids of *Porphyromonas gingivalis*. *J. Lipid Res.* 2006.** 47: **844–853.**

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Porphyromonas gingivalis is a Gram-negative anaerobic organism that resides in the gingival sulcus surrounding the teeth, particularly at sites of chronic inflammatory or destructive periodontal diseases. A recent report demonstrated that *P. gingivalis* synthesizes two major phosphorylated dihydroceramides, one of which potentiates interleukin-1 (IL-1)-mediated prostaglandin E_2 (PGE_2) secretory responses in gingival fibroblasts and markedly alters gingival fibroblast morphology in culture (1). These lipids are also recovered from diseased tooth roots (2), and indirect evidence indicates that the phosphoglycerol dihydroceramide lipids are the primary ceramides of *P. gingivalis* recovered in gingival tissues at disease sites (3). Therefore, *P. gingivalis* produces biologically active complex lipids that are recovered at periodontal disease sites.

Recent investigations have demonstrated that *P. gingivalis* produces one additional class of phospholipids that also demonstrate substantial capacity to promote IL-1β-mediated prostaglandin secretion from gingival fibroblasts. However, this lipid class is not a sphingolipid, because it lacks amide-linked fatty acid. This report describes the structural characterization of this new phospholipid class and demonstrates the capacity of this lipid to promote prostaglandin secretory responses and morphological changes in cultured gingival fibroblasts. Lastly, using a structural analog as a control for the bacterial lipid fraction as well as the hydrolysis products of this bacterial lipid class, this report defines the critical structural components of the phosphorylated lipids of *P. gingivalis* that appear to account for their biological activity.

MATERIALS AND METHODS

P. gingivalis (ATCC 33277, type strain) was grown in batch suspension culture after verification of anaerobic growth of Gram-negative rods in thioglycollate medium and demonstration of black-pigmented bacterial colonies on blood agar plates only with anaerobic culture conditions. *P. gingivalis* was grown under anaerobic conditions in basal (peptone, trypticase, and yeast ex-

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tract) medium supplemented with hemin and menadione (Sigma-Aldrich, St. Louis, MO) and brain heart infusion as described previously (1). The suspension cultures were incubated in an anaerobic chamber flushed with N_2 (80%), CO_2 (10%), and H_2 (10%) at 37°C for 5 days, and the bacteria were harvested by centrifugation (3,000 *g* for 20 min). A sample of *P. gingivalis* pellet was extracted using a modification of the phospholipid extraction procedure of Bligh and Dyer (4) and Garbus et al. (5). *P. gingivalis* lipid extract was fractionated by HPLC using a semipreparative HPLC column (1 × 25 cm silica gel, 5 μm; Supelco, Inc., Bellefonte, PA) and eluted isocratically with hexane-isopropanol-water (6:8:0.75, v/v/v; solvent A) (6). Replicate fractionations were pooled by fraction number, and lipid recovery was determined for each fraction as described previously (1). GC-MS and electrospray-MS analysis (see below) demonstrated that the phosphatidylethanolamine lipids were recovered in greatest abundance and highest purity in HPLC fractions 23 and 24.

Lipid film preparation

Phosphatidylethanolamine lipids were dissolved in ethanol to achieve a final concentration of 0.5 μg/μl, and 20 μl of each lipid fraction was deposited in 35 mm plastic culture wells, as described previously (1). The ethanol was evaporated overnight, leaving a lipid residue covering most of the culture well surface. Vehicle control wells received only ethanol solvent. Primary cultures of gingival fibroblasts were obtained by explant culture using gingival tissue samples recovered from healthy sites during periodontal surgical procedures, as described previously (7). Gingival tissue samples were obtained according to a protocol approved by the Institutional Review Board, and participants provided written informed consent. Gingival fibroblasts were inoculated into culture wells to achieve a cell number/surface area equivalent to confluent cultures. After 2 h of exposure to bacterial lipids, the culture medium was supplemented with either control medium or medium containing recombinant human IL-1β or IL-1α (final concentration, 10 ng/ml; Immunex, Seattle, WA). The cultures were then incubated for an additional 24 h, after which the culture medium was recovered and frozen.

Quantification of prostaglandins in culture medium samples

$PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, and PGE_2 were quantified in medium samples using a modification of the method of Luderer, Riley, and Demers (8), as described previously (1, 9). Each medium sample was supplemented with 100 ng of D_4-$PGF_{2\alpha}$, D_4-6-keto $PGF_{1\alpha}$, and D_4-PGE_2 and the pH adjusted to pH 3.5 with concentrated formic acid. The acidified medium samples were then applied to reverse-phase preparative columns (Supelclean, LC-18 SPE tubes, 6 ml; Supelco, Inc.) mounted on a vacuum manifold, the columns were washed, and enriched prostaglandins were eluted with 100% methanol (1). The eluted prostaglandins were then supplemented with 2 ml of 1% formic acid in water, and the prostaglandins were extracted twice with 2 ml of chloroform. The chloroform extracts were dried under nitrogen.

All derivatizing agents were obtained from Pierce Chemical Corp. (Rockford, IL). Prostaglandin samples were derivatized using the method of Waddell, Blair, and Wellby (10). Prostaglandin samples were first treated with 2% methoxylamine hydrochloride in pyridine (30 μl). After standing overnight at room temperature, the samples were dried under nitrogen, dissolved in acetonitrile (30 μl), and treated with pentafluorobenzyl bromide (35%, v/v, in acetonitrile; 10 μl) and diisopropylethylamine (10 μl). The samples were vortexed, incubated for 20 min at 40°C, and evaporated under nitrogen. The residue was then treated with bistrimethylsilyl-trifluoroacetamide (50 μl) and allowed to stand at room temperature for 4–5 days.

Synthesis of isobranched $C_{15:0}$ (13-methyl tetradecanoic acid)

A suspension of Mg turnings (1.21 g, 49.8 mmol) in tetrahydrofuran (10 ml) was treated with 1-bromo-3-methylbutane (5 ml, 41.5 mmol) with vigorous stirring at room temperature. After initiation of the reaction, tetrahydrofuran (25 ml) was added and stirred until the reaction mixture reached ambient temperature. A solution of 10-bromo-1-decanol (3 g, 12.6 mmol) in tetrahydrofuran (27 ml) was cooled to −78°C and treated with the Grignard reagent described above together with Li_2CuCl_4 (0.1 M, 4 ml) at −78°C. The reaction mixture was stirred overnight, during which time the temperature increased to room temperature, giving a dark solution. After stirring for 5 min with saturated aqueous NH_4Cl (15 ml), water (150 ml) and ethyl acetate (150 ml) were added. The organic layer was separated and washed with saturated aqueous $NaHCO_3$ and brine, dried with $MgSO_4$, and concentrated in vacuo. Purification with silica column chromatography gave 13-methyl-1-tetradecanol as a colorless oil (2.74 g, 12.0 mmol, 95%). The 13-methyl-1-tetradecanol was added to a 50 ml flask containing chromium trioxide in aqueous sulfuric acid (Jones' reagent) and allowed to stir at ambient temperature for 2 h. The solution was extracted with 3 × 50 ml of dichloromethane, the organic fractions were dried ($MgSO_4$) and filtered, and the solvent was removed in vacuo to give 2.79 g (11.5 mmol, 96%) of 13-methyltetradecanoic acid. The free fatty acid was treated with thionyl chloride to form the acid chloride, followed by treatment with methanol to form the methyl ester. The fatty acid methyl ester was evaluated by GC-MS as described below.

Hydrolysis of *P. gingivalis* phosphatidylethanolamine lipids

Analysis of esterified fatty acids was accomplished by treating a sample of the phosphatidylethanolamine lipid fraction with 0.5 N $NaOCH_3$ in anhydrous methanol (0.5 ml, 40°C for 20 min). The reaction was stopped with the addition of 100 μl of glacial acetic acid and 1 ml of water. The sample was then extracted twice with hexane, and the contents were dried under nitrogen. The sample was reconstituted in hexane for GC-MS analysis.

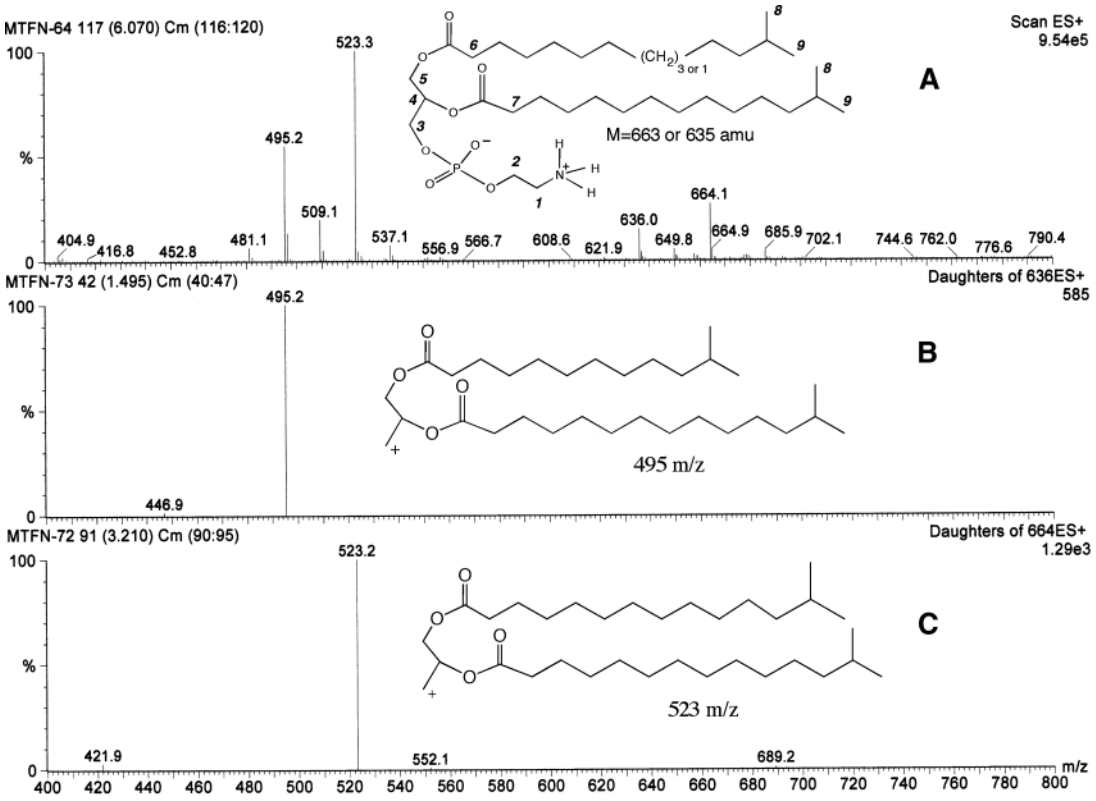
Approximately 1 mg of *P. gingivalis* phosphatidylethanolamine lipid was dissolved in anhydrous ether (1 ml) and combined with 1% calcium chloride (1.3 ml) and 2 units of phospholipase C (*Clostridium perfringens*; 4.6 U/mg; Sigma-Aldrich) in Tris buffer (17.5 mM, 4 ml, pH 7.3) (11). The mixture was incubated for 2 h at 30°C with frequent vortexing, and the reaction was stopped by adding 0.1 M HCl (0.2 ml). The hydrolysis products were extracted and treated with bistrimethylsilyl-trifluoroacetamide (Pierce Chemical Corp.; 40 μl, overnight).

GC-MS analysis

GC-MS was carried out on a Hewlett-Packard 5890 gas chromatograph interfaced with a 5988A mass spectrometer. Prostaglandin samples were applied to an SPB-1 column (12 m × 0.2 mm, 0.33 μm film thickness; Supelco, Inc.) held at 100°C. Prostanoid samples were analyzed using a temperature program of 2°C/min from 100°C to 240°C. The injector block was held at 260°C, and the transfer tube was maintained at 280°C. Prostaglandin derivatives were detected using electron capture-negative chemical ionization, as described previously (1). Prostaglandin levels were quantified using selected ion monitoring of the characteristic base peak ions of the deuterated and authentic prostaglandins.

Fatty acid methyl esters recovered from *P. gingivalis* phosphatidylethanolamine lipids were analyzed using electron-impact GC-MS. The sample was applied to an SPB-1 column (12 m × 0.2 mm, 0.33 μm film thickness; Supelco, Inc.) held at 100°C, and the column was heated at 10°C/min to 270°C. The injector block was held at 260°C, and the transfer tube was maintained at 280°C. The ion source temperature was 150°C, the electron energy was 70 eV, and the emission current was ~300 mA. The mass spectrometer was used in the electron-impact mode with ion acquisition of 50 to 700 amu. The retention time and mass spectrum of synthetic isobranched (*iso*)$C_{15:0}$ methyl ester were used to verify the identity of the dominant $C_{15:0}$ methyl ester fatty acid recovered from *P. gingivalis* phosphatidylethanolamine lipids. Straight-chain fatty acid standards were also used to verify the identity of the remaining fatty acid methyl esters recovered from phosphatidylethanolamine lipids.

For diglyceride analyses, the trimethylsilyl lipid derivatives were analyzed by GC-MS using a SPB-1 column (15 m × 0.25 mm × 0.1 mm film; Supelco, Inc.) and a temperature program of 200°C to 290°C at 10°C/min using the mass spectrometer in the electron-impact mode.



	^{1}H ($CDCl_3$)	^{13}C (HMQC data in $CDCl_3$:MeOH)
H-4	5.22 (m, 1H)	69.8
H-5	4.37 (dd, 1H, J=12.0 and 2.8 Hz)	60.9
H-5	4.15 (dd, 1H, J=12.2 and 5.3 Hz)	60.9
H-2	4.09 (bs, 1H)	61.9
H-3	3.95 (bs, 2H)	63.1
H-1	3.17 (bs, 2H)	39.9
H-6/7	2.30 (q, 4H, J=8Hz)	33.6, 33.4
Lipid	1.54 (bs, 4H)	29.0
	1.26 (bs, 32H)	29.3-26.5
	1.14 (bs, 4H – CH_2 adjacent to distal branch)	38.4
H-8	1.49 (sept., 2H, J=6.4 Hz)	24.2
H-9	0.86 (d, 6H, J=6.4 Hz)	21.7

Fig. 1. Positive ion electrospray-MS and electrospray-tandem mass spectrometry (MS/MS) analysis of phosphatidylethanolamine lipids of *P. gingivalis*. Lipids were separated by semipreparative HPLC, as described previously (1). GC-MS and electrospray-MS analyses demonstrated that the phosphatidylethanolamine lipids of interest were recovered in greatest abundance and highest purity in HPLC fractions 23 and 24. A sample of phosphatidylethanolamine lipids was subjected to positive ion electrospray analysis (A) and electrospray-MS/MS analysis (B, C). A: Positive ion mass spectrum of phosphatidylethanolamine lipids and the proposed structures of the major components. B, C: Daughter positive ions produced from the major high-mass ions, *m/z* 636 and 664, respectively. Proton/carbon NMR assignments are listed in the table at bottom, and the corresponding assignments are listed on the chemical structure shown in A. MeOH, methanol.

Electrospray-tandem mass spectrometry analysis of dihydroceramide lipids

Electrospray-MS analysis was accomplished using a Micromass Quattro II mass spectrometer system. The *P. gingivalis* phosphatidylethanolamine lipid fraction as well as all HPLC lipid fractions were dissolved in hexane-isopropanol (6:8, v/v; elution solvent) and injected at a maximum concentration of 100 μg/ml. Lipid samples (10 μl) were infused at a flow rate of 20 μl/min. For electrospray positive ion analyses, the desolvation and inlet block temperatures were 100°C and 150°C, respectively, and the transcapillary potential was 3,500 V. For electrospray negative ion analyses, the desolvation and inlet block temperatures were 80°C and 100°C, respectively, and the transcapillary potential was 3,000 V. The cone voltage was usually 30 V, and the mass acquisition range was 0–2,000 amu for initial electrospray MS analyses. Tandem mass spectrometry (MS/MS) analysis used a collision energy of between 28 and 30 V, and argon was introduced at a pressure of 10^{-2} to 10^{-4} torr. The gas and collision energies were adjusted to minimize parent ion recoveries and maximize daughter ion recoveries. These conditions were used for both positive and negative ion electrospray-MS/MS analyses.

NMR analyses

NMR data were collected on a Bruker DRX-400, a Bruker Avance 500, and a Varian INOVA 600 (operating frequencies of 400.144, 500.13, and 599.75 megahertz, respectively). One-dimensional ^{1}H, ^{13}C, and ^{31}P, as well as two-dimensional ^{1}H-^{1}H Correlated Spectroscopy (COSY), ^{1}H-^{13}C Heteronuclear Multiple Quantum Correlation (g-HMQC), ^{1}H-C-C-^{13}C Heteronuclear Multiple Bond Correlation Spectroscopy (g-HMBC), Total Correlated Spectroscopy (TOCSY), Nuclear Overhauser Spectoscopy (NOESY), and ^{1}H-^{31}P (gHMBC), were acquired for *P. gingivalis* phosphatidylethanolamine lipids dissolved in deuterated solvents of specified composition ($CDCl_3$ or CD_3OD). These data allowed the assignment of proton and carbon resonances of the dihydroceramide base and the phosphate side chain of the molecule, as well as specific components of the aliphatic chains.

Data analysis

Statistical tests included one-factor ANOVA comparing prostaglandin secretion between culture treatment groups and the Fisher PLSD or Scheffe *F*-test for significant differences between treatment categories.

RESULTS

The phosphatidylethanolamine class was isolated as a highly enriched sample using semipreparative HPLC. The phosphatidylethanolamine lipids of *P. gingivalis* were recovered in highest concentration in HPLC fractions 23 and 24 (~15 mg recovered), as determined by GC-MS and electrospray-MS analysis of HPLC fractions. Positive ion electrospray-mass spectra and tandem mass spectra of phosphatidylethanolamine lipids are shown in **Fig. 1**. Phosphatidylethanolamine lipids isolated by HPLC were analyzed without derivatization, and the resulting ion products shown in Fig. 1A suggest that this lipid class consists of either two or four major products. However, positive ion electrospray-MS/MS demonstrated that the *m/z* 664 and 636 parent ions produce the *m/z* 523 and 495 daughter ions, respectively (Fig. 1B, C). Therefore, the number of major lipid products shown in Fig. 1A is limited to two major lipid products of 663 and 635 amu (positive ions are generated through the formation of proton adducts, resulting in ion masses of *m/z* 664 and 636). Additional evidence provided below confirms the proposed lipid structures shown in Fig. 1.

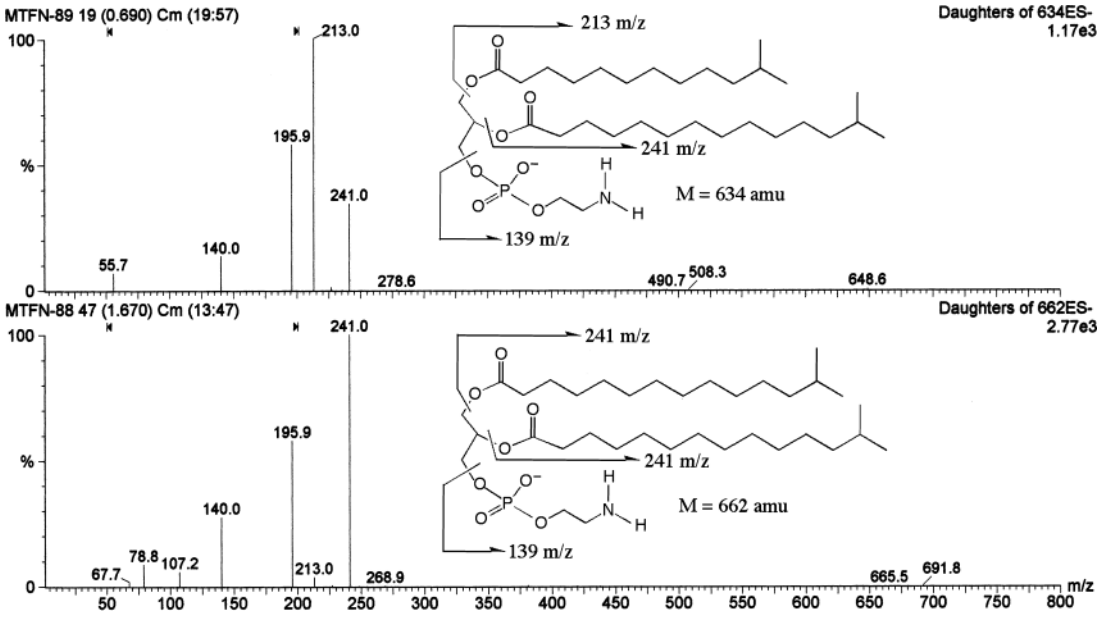


Fig. 2. Negative ion electrospray-MS/MS analysis of phosphatidylethanolamine lipids of *P. gingivalis*. The lipid sample described for Fig. 1 was shown to contain two dominant negative ions (*m/z* 662 and 634). The *m/z* 662 parent negative ion produced daughter ions of *m/z* 241 and 140. The *m/z* 634 parent ion produced daughter ions of *m/z* 241, 213, 196, and 140. The *m/z* 213 and 241 daughter ions represent $C_{13:0}$ and $C_{15:0}$ fatty acids, respectively. The *m/z* 140 ion represents the phosphoethanolamine head group. See Results for the proposed structure of the *m/z* 196 ion.

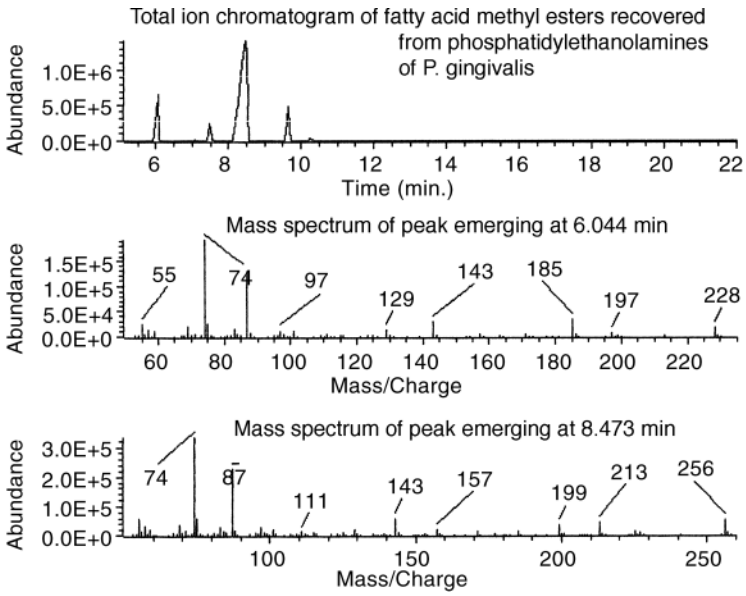


Fig. 3. GC-MS analysis of fatty acid methyl esters recovered from phosphatidylethanolamine lipids after hydrolysis with sodium methoxide. Phosphatidylethanolamine lipids were treated with fresh 0.5 N sodium methoxide in anhydrous methanol, and the fatty acid methyl esters were recovered in hexane. The methyl ester fatty acids were then analyzed by GC-MS, as described in Results. The mass spectrum of isobranched (*iso*)$C_{13:0}$ is depicted in the middle, and *iso*$C_{15:0}$ is depicted at bottom. The abundances of the dominant fatty acid methyl esters are described in Results.

By NMR (see table at the bottom of Fig. 1), the proton resonance at H-4 (5.21 ppm) showed TOCSY and COSY correlations to protons at 3.94 ppm (H-3, 2H), 4.37, and 4.18 ppm (H-5 protons) (proton and carbon assignments are listed on the chemical structure shown in Fig. 1A). Proton resonances on H-3, H-2, and H-1 were broadened by ^{31}P coupling at 3.94, 4.09, and 3.17 ppm, respectively. The 4.09 ppm protons represent the -CH_2-OP protons of the phosphoethanolamine head group. The 3.17 ppm proton signal represents -CH_2-N of the ethanolamine moiety. Because proton signals of the glycerol chain are asymmetric and the most upfield signal is coupled to ^{31}P, this evidence confirms the position of the phosphoethanolamine head group at the terminal end of the glycerol. The COSY also indicated that the fatty acid aliphatic chains are not hydroxy substituted, as was observed with other dominant

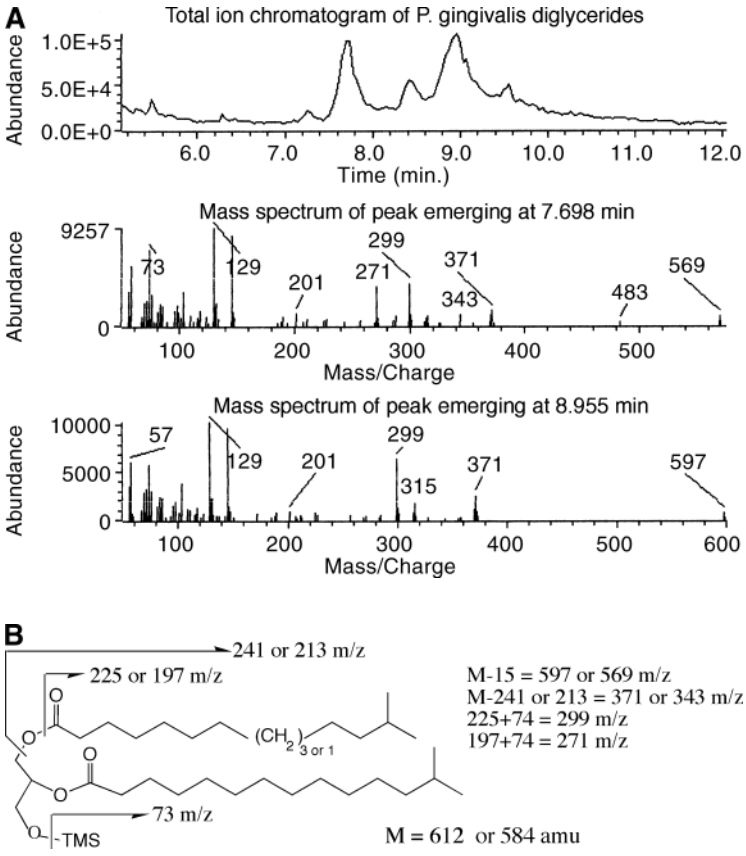


Fig. 4. GC-MS analysis of diacylglycerols after treatment of phosphatidylethanolamine lipids with phospholipase C. Approximately 1 mg of phosphatidylethanolamine lipids was treated with phospholipase C, as described in Materials and Methods. Dimyristoyl phosphatidylethanolamine lipid standard (Avanti Polar Lipids, Inc., Alabaster, AL) treated with phospholipase C verified that the diacylglycerols of *P. gingivalis* phosphatidylethanolamine lipids represent 1,2-di*iso*$C_{15:0}$ glycerol (lipid emerging at 8.955 min) or 1-*iso*$C_{13:0}$,2-*iso*$C_{15:0}$ glycerol (lipid emerging at 7.698 min) (13, 14). The mass spectrum of the major lipid product emerging at 8.95 min (bottom spectrum; 45.12% of diglyceride ion abundance) is consistent with a 1,2-diacylglycerol rather than a 1,3-diacylglycerol-substituted structure, as reported by others (13, 14). See Results for a description of the fatty acid substitutions within the observed diglyceride products.

complex lipid classes of *P. gingivalis* (1), but are branched at the distal end. The ^{1}H-^{13}C HMBC analysis revealed that the four protons at 2.30 ppm correlate with the inequivalent carbonyl carbons at 173.4 and 173.0 ppm. Therefore, the phosphatidylethanolamine lipids of *P. gingivalis* exist as 1,2-diacyl glycerol 3-phosphoethanolamine lipids substituted predominantly with isobranched fatty acids. The chirality of carbon 2 of the glycerol chain remains to be characterized, although hydrolysis with cobra venom factor completely destroyed phosphatidylethanolamine lipids of *P. gingivalis*. If the carbon 2 chirality were of the unnatural type (D-glycerophosphatides), cobra venom factor would not destroy this lipid class (12).

Negative ion electrospray-MS of *P. gingivalis* phosphatidylethanolamine lipids demonstrated two dominant lipid ions of *m/z* 662 and 634 (data not shown). Electrospray-MS/MS analysis revealed daughter negative ions of *m/z* 241, 196, and 140 produced from the *m/z* 662 parent ion (**Fig. 2**, bottom). The *m/z* 241 ion is consistent with the negative ion of $C_{15:0}$, and the *m/z* 140 ion is consistent with a protonated phosphoethanolamine moiety (1). The *m/z* 195 ion likely results from the loss of both fatty acids, with oxygen retention on the phosphoethanolamine-glycerol moiety. In turn, the *m/z* 634 parent ion (Fig. 2, top) produces daughter ions of *m/z* 140 and 196 as well. However, the *m/z* 634 parent ion produces daughter negative ions of *m/z* 213 and 241, suggesting the presence of both $C_{13:0}$ and $C_{15:0}$, respectively. Therefore, both parent ions contain similar phosphorylated head groups but differ with regard to fatty acid substitutions.

P. gingivalis phosphatidylethanolamine lipids were treated with sodium methoxide, and the fatty acid methyl esters were analyzed by GC-MS (**Fig. 3**). Four fatty acid methyl esters were identified. A synthetic standard of *iso*$C_{15:0}$ methyl ester demonstrated the same retention time and identical mass spectrum as the dominant fatty acid methyl ester recovered from *P. gingivalis* phosphatidylethanolamines. Therefore, *iso*$C_{15:0}$ (retention time = 8.47 min; 79.02% of the total major fatty acid ion abundance) is the dominant fatty acid methyl ester recovered after transesterification of *P. gingivalis* phosphatidylethanolamines. Based on the retention times and mass spectra of straight-chain and isobranched synthetic standards, *P. gingivalis* phosphatidylethanolamines were also shown to contain ester-linked *iso*$C_{13:0}$ (retention time = 6.04 min; 12.01%) and lower amounts of $C_{14:0}$ (retention time = 7.45 min; 0.52%) and $C_{16:0}$ (retention time = 9.60 min; 8.44%).

P. gingivalis phosphatidylethanolamine lipids were hydrolyzed with phospholipase C according to the method of Kuksis et al. (11). **Figure 4** shows the trimethylsilyl derivatives of the major lipid products recovered after phospholipase C hydrolysis. Dimyristoyl phosphatidylethanolamine lipid standard was treated with phospholipase C, and the mass spectrum of the hydrolysis products verified the diacylglycerol structure of *P. gingivalis* phosphatidylethanolamine lipids. The mass spectrum of the lipid product emerging at 8.95 min (Fig. 4, bottom frame; 45.12% of diglyceride ion abundance) is consistent with a 1,2-diacylglycerol rather than a 1,3-diacylglycerol-substituted structure, as reported by others (13, 14). The lipid product emerging at 7.698 min (39.09% abundance) produced a mass spectrum consistent with diacylglycerol that is substituted with both *iso*$C_{13:0}$ and *iso*$C_{15:0}$ rather than substituted with only *iso*$C_{15:0}$ (lipid product emerging at 8.955 min; 45.12% ion abundance). Odd-chain fatty acids were shown to be isobranched in the phosphatidylethanolamine fraction of *P. gingivalis* lipids (see above). Based on the partial mass spectra of the other three minor diglycerides recovered, the lipid product emerging at 9.56 min (2.95% ion abundance) contains *iso*$C_{15:0}$ and $C_{16:0}$, the lipid product emerging at 8.43 min (9.71% ion abundance) contains $C_{14:0}$ and $C_{16:0}$, and the lipid product emerging at 7.27 min

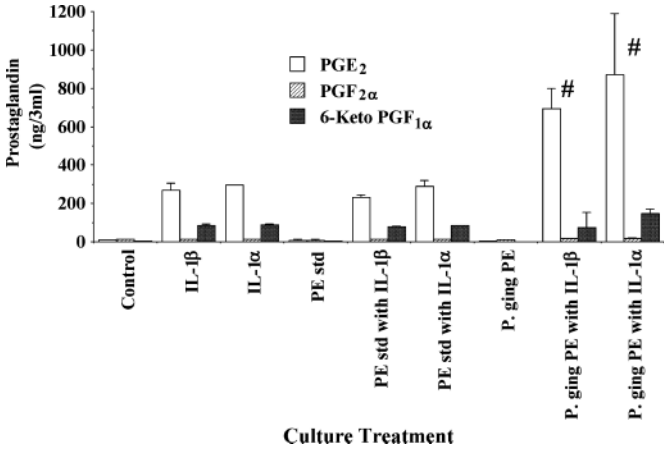


Fig. 5. Effects of *P. gingivalis* phosphatidylethanolamine lipids on interleukin-1 (IL-1)-mediated prostaglandin secretion from gingival fibroblasts. *P. gingivalis* phosphatidylethanolamine lipids (10 μg in 20 μl of 100% ethanol) or dimyristoyl phosphatidylethanolamine lipid standard (Avanti Polar Lipids, Inc.) were dispersed into 35 mm plastic culture dishes and air-dried, as described previously (1, 9). Gingival fibroblasts were grown, harvested, and treated with lipids as described (1, 9). Approximately 2×10^6 cells were deposited into each culture well suspended in Minimum Essential Medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After a 2 h exposure to bacterial lipids, the culture medium was supplemented with either control medium (30 μl) or medium containing recombinant human IL-1β or IL-1α (1 mg/ml, 30 μl; Immunex). The cultures were then incubated for an additional 24 h at 37°C in a humidified 5% CO_2 incubator, after which the medium samples were harvested and frozen. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto $PGF_{1\alpha}$, and PGE_2 were quantified in medium samples using stable isotope dilution GC-MS analysis, as described previously (1, 9). Each histogram bar depicts the mean ± SD for two trials. *P. gingivalis* phosphatidylethanolamine (P. ging PE) lipids or phosphatidylethanolamine lipid standard alone (PE std) did not significantly stimulate prostaglandin secretion from gingival fibroblasts compared with the control cells treated with ethanol vehicle alone. Significantly greater PGE_2 release was observed from fibroblasts treated with *P. gingivalis* phosphatidylethanolamine lipids (10 μg) supplemented with either IL-1β or IL-1α compared with fibroblasts treated with IL-1β or IL-1α alone or treated with dimyristoyl phosphatidylethanolamine plus IL-1β or IL-1α ($^{\#}P < 0.001$ by one-factor ANOVA between treatment categories; significantly different at 99% confidence interval by Fisher PLSD). These results were replicated in two additional experiments. Secretion of the other major prostaglandins was not significantly different when comparing the treatment categories.

(3.13% ion abundance) contains $C_{14:0}$. Positive ion chemical ionization GC-MS using methane as the reagent gas produced M+1 *m/z* proton adduct ions, confirming the masses of the major diacylglycerols as 612 and 584 amu (data not shown). Although the proposed lipid structures place the *iso*$C_{13:0}$ on the terminal glycerol carbon, the possibility that the central glycerol carbon is substituted with *iso*$C_{13:0}$ cannot be excluded.

The GC-MS results together with the electrospray-MS results support the structural reconciliation proposed in Fig. 1A. Therefore, the major phosphatidylethanolamine lipids of *P. gingivalis* are substituted only with *iso*$C_{15:0}$ or with *iso*$C_{15:0}$ and *iso*$C_{13:0}$. Low amounts of phosphatidylethanolamine lipids containing $C_{16:0}$ and $C_{14:0}$ are also present.

P. gingivalis phosphatidylethanolamine lipids were tested for activity in promoting IL-1β- or IL-1α-mediated prostaglandin secretion from gingival fibroblasts, as shown in **Fig. 5**. Included in this trial was a dimyristoyl phosphatidylethanolamine synthetic standard used at the same concentration (10 μg/35 mm culture well) as the phosphatidylethanolamine HPLC fraction. The *P. gingivalis* phosphatidylethanolamine lipids significantly potentiated IL-1β- and IL-1α-mediated PGE_2 secretion from gingival fibroblasts, whereas the dimyristoyl phosphatidylethanolamine standard did not stimulate PGE_2 release either alone or together with IL-1β or IL-1α. The levels of PGE_2 shown in Fig. 5 are substantially higher than those shown in **Fig. 6**. Fibroblasts from a healthy gingival tissue specimen were used to generate Fig. 5, whereas cells from a diseased gingival tissue specimen were used to generate Fig. 6. Additional work has shown that PGE_2 secretion from gingival fibroblasts varies depending on the donor of the gingival tissue, the disease status of the tissue specimen, and the number of cell passages in culture, but the significant trends shown in Figs. 5 and 6 were observed in at least three replicate experiments.

Note that the primary effect of *P. gingivalis* phosphatidylethanolamines on prostaglandin secretion is limited to PGE_2, as described previously for phosphoglycerol dihydroceramides (1). Treatment of *P. gingivalis* phosphatidylethanolamine lipids with phospholipase C produced the expected diacylglycerols, as demonstrated in Fig. 4. Gingival fibroblasts treated with the diacylglycerols of *P. gingivalis* followed by IL-1β or IL-1α did not secrete increased levels of

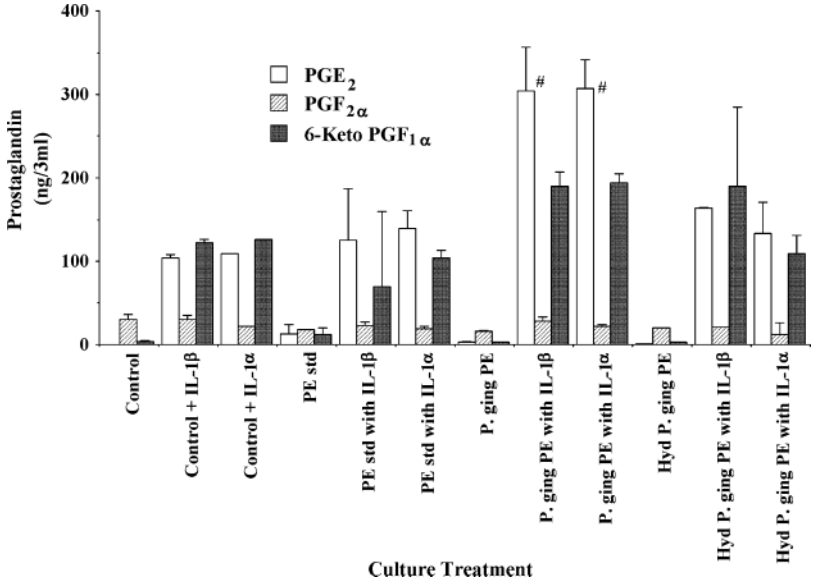


Fig. 6. Biofunctional relevance of the phosphoethanolamine moiety of *P. gingivalis* phosphatidylethanolamines on the production of prostaglandins by gingival fibroblasts. A sample of *P. gingivalis* phosphatidylethanolamines was treated with phospholipase C, as described for Figure 4. *P. gingivalis* phosphatidylethanolamine lipids or their diacylglycerol products (10 μg in 20 μl of 100% ethanol) were dispersed into 35 mm plastic culture wells. Dimyristoyl phosphatidylethanolamine lipid standard (Avanti Polar Lipids, Inc.) was used as the control lipid preparation. Fibroblasts were grown from a fresh gingival tissue explant using the method described for Figure 5. After a 2 h exposure to bacterial lipids, the culture medium was supplemented with either control medium (30 μl) or medium containing recombinant human IL-1β or IL-1α (1 mg/ml, 30 μl; Immunex). The cultures were then incubated for an additional 24 h at 37°C in a humidified 5% CO_2 incubator, after which the medium samples were harvested and frozen. $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, and PGE_2 were quantified as described for Figure 5. Results are depicted as means ± SD for two trials. *P. gingivalis* phosphatidylethanolamine lipids (P. ging PE) or PE lipid standard alone (PE std) did not significantly stimulate prostaglandin secretion from gingival fibroblasts compared with vehicle control cells. Fibroblasts treated with IL-1β or IL-1α alone or dimyristoyl phosphatidylethanolamine standard plus IL-1β or IL-1α released significantly less PGE_2 than cells treated with *P. gingivalis* phosphatidylethanolamine lipids plus IL-1β or IL-1α ($^{\#}P < 0.001$ by one-factor ANOVA between treatment categories; significantly different at 99.9% confidence interval by Fisher PLSD). The diacylglycerol lipid preparation of *P. gingivalis* phosphatidylethanolamine lipids (10 μg) together with IL-1β or IL-1α did not potentiate PGE_2 secretion over that observed with IL-1β or IL-1α alone.

PGE_2 compared with IL-1 alone (Fig. 6), indicating the involvement of the phosphoethanolamine moiety in the biofunctional activity of the phosphatidylethanolamine lipids of *P. gingivalis*. Phosphatidylethanolamine lipids of *P. gingivalis* alone did not significantly stimulate prostaglandin secretion from gingival fibroblasts but did alter the morphology of gingival fibroblasts in culture, as shown in **Fig. 7**. *P. gingivalis* phosphatidylethanolamine lipids caused significant morphological changes in fibroblasts and cell detachment regardless of treatment with IL-1β or IL-1α (data not shown). Therefore, the phosphatidylethanolamine lipids of *P. gingivalis* markedly potentiate IL-1β- and IL-1α-mediated PGE_2 secretion from gingival fibroblasts but also induce substantial changes in fibroblast shape and adherence in culture.

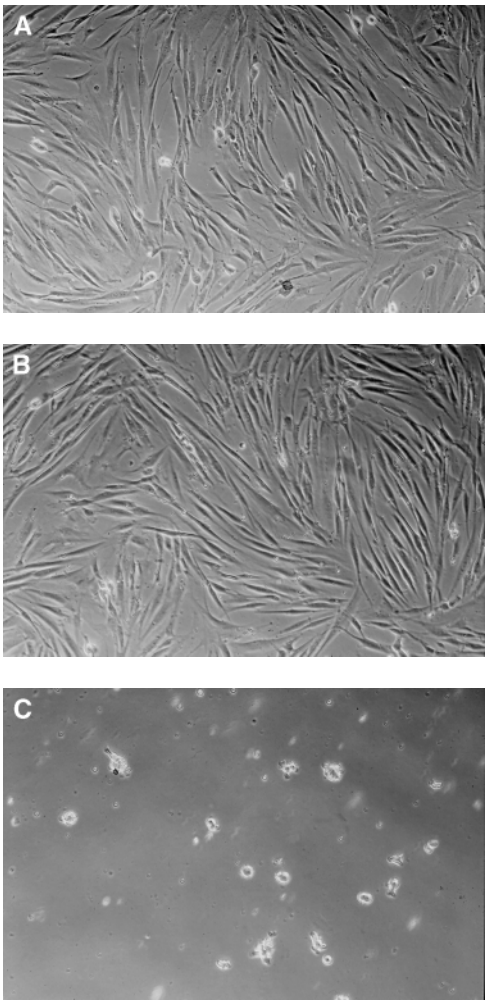


Fig. 7. Morphological appearance of gingival fibroblasts after treatment with lipid preparations. The cell cultures described for Figure 5 were photographed at the end of the culture period (26 h). A: Control cells exposed to culture wells previously coated with ethanol vehicle alone. B: Cells exposed to culture wells coated with dimyristoyl phosphatidylethanolamine lipid standard (10 μg; Avanti Polar Lipids, Inc.). C: Cells exposed to phosphatidylethanolamine lipids from *P. gingivalis* (10 μg). Supplementing cultures with IL-1β or IL-1α treatment did not affect the appearance of fibroblasts compared with cells exposed to dimyristoyl phosphatidylethanolamine lipid standard or *P. gingivalis* phosphatidylethanolamine lipids alone (data not shown).

DISCUSSION

Although previous reports demonstrated phosphatidylethanolamines in lipid extracts of *P. gingivalis* (15) and a related organism, *Bacteroides melaninogenicus* (16), this study identifies the novel structural characteristics of *P. gingivalis* phosphatidylethanolamines as well as the role of these novel structural constituents in promoting biological activity. Based on the evidence provided here, the isobranched fatty acids together with the phospholipid head group dictate the biological activity of the phosphatidylethanolamine lipids of *P. gingivalis*. Substitution of straight-chain fatty acids for isobranched fatty acids or removal of the phosphoethanolamine head group virtually eliminated the biological activity observed with phosphatidylethanolamine lipids of *P. gingivalis*. The isobranched fatty acids, therefore, confer biological activity through the branched aliphatic chains. Although the phosphorylated head group is essential for the biological activity, the phosphoethanolamine group is not structurally unique.

Complex lipids of *P. gingivalis*, including phosphoethanolamines, are believed to be constituents of cell walls and membrane vesicles that are shed from this organism (17–19). *P. gingivalis* is unusual among periodontal organisms in its capacity to shed membrane vesicles or blebs. Complex lipids of *P. gingivalis* are also recovered at periodontal disease sites, as demonstrated in lipid extracts of teeth covered with subgingival calculus as well as gingival tissues afflicted with chronic inflammatory periodontal disease (3). Several plausible mechanisms could account for periodontal tissue contamination with lipids of *P. gingivalis*, including direct adhesion between bacteria and host epithelial cells in the gingival sulcus surrounding each tooth (20–23), direct contact between host tissues and lipid-contaminated diseased teeth, or invasion of periodontal tissues by *P. gingivalis* (24–31). Because complex lipids of *P. gingivalis* are most prevalent on periodontally diseased tooth roots (3, 9), this study exposed gingival fibroblasts to lipid films that recreate the approximate lipid levels observed on calculus-contaminated tooth roots. A previous report indirectly estimated *P. gingivalis* lipid levels on calculus-contaminated tooth roots by quantifying 3-OH *iso*$C_{17:0}$ on root sections of known surface area (9). Replicate HPLC fractionations of total lipid extracts also demonstrated that at least 15% of *P. gingivalis* lipids represent phosphatidylethanolamine lipids. Based on these findings, we estimated that calculus-contaminated tooth root surfaces retain *P. gingivalis* phosphatidylethanolamine lipids at levels that exceed a dose of 10 μg of phosphatidylethanolamine lipid per 35 mm culture dish. This was the dose of *P. gingivalis* phosphatidylethanolamine lipids selected for the experiments reported here. However, another experiment demonstrated that exposure of fibroblasts to 5 μg of *P. gingivalis* phosphatidylethanolamine lipids promoted IL-1-mediated PGE_2 secretion (increased

PGE_2 release by >40% over IL-1 controls) to a lesser extent than the 10 μg dose.

Other work has shown that exposure of cells to lower levels of *P. gingivalis* phosphatidylethanolamine lipids will promote biological responses. However, it is not established what levels of *P. gingivalis* phosphatidylethanolamines will mimic target cell lipid exposures typical of periodontal disease tissue levels or systemic bacteremias. Future studies will quantify periodontal tissue and systemic exposures to these bacterial lipids, and will use these levels to examine the biological effects of these lipid preparations in promoting autoimmune, atherosclerosis, and degenerative systemic diseases at the relevant exposure levels.

The capacity of *P. gingivalis* phosphatidylethanolamines to promote PGE_2 secretion and alter fibroblast morphology is very similar to that reported for phosphoglycerol dihydroceramides of *P. gingivalis* (1). *P. gingivalis* produces free, phosphoethanolamine, and phosphoglycerol dihydroceramide classes all with core structures consisting of 3-OH *iso*$C_{17:0}$ in amide linkage to *iso*$C_{17:0}$, $C_{18:0}$, and *iso*$C_{19:0}$ long-chain bases. Of these dihydroceramide classes, only the phosphoglycerol dihydroceramide class markedly stimulates PGE_2 secretion and alters fibroblast morphology. Only the phosphoglycerol dihydroceramides contain *iso*$C_{15:0}$ linked to the β-hydroxyl of 3-OH *iso*$C_{17:0}$, and the proximity of branched aliphatic chains within this lipid class likely contributes to the biological activity of these phosphoglycerol dihydroceramides. Further support for the role of the branched aliphatic chains in promoting biological activity comes from recent work demonstrating that removal of *iso*$C_{15:0}$ from the phosphoglycerol dihydroceramides substantially reduces the capacity of this lipid class to promote IL-1-mediated prostaglandin secretion (data not shown). Additional preliminary work indicates that both the phosphatidylethanolamine and phosphoglycerol dihydroceramide lipid classes activate the phosphorylation of mitogen-activated protein kinase family proteins in RAW264.7 cells, including p38 mitogen-activated protein kinase, extracellular signal regulated protein kinases (ERK1/2), and C-jun kinase (JNK). Therefore, the isobranched aliphatic chains of phosphoglycerol dihydroceramides and phosphatidylethanolamines of *P. gingivalis* appear to be critical to the biofunctional activity of these lipids. However, the mechanisms by which host cells recognize isobranched fatty acids of *P. gingivalis* phosphatidylethanolamines and promote PGE_2 and morphological changes in fibroblast cells remain to be characterized.

In summary, the results of this study indicate that the close proximity of isobranched fatty acid aliphatic chains with the phosphorylated head group accounts for the biological responses observed in cultured gingival fibroblasts after exposure to phosphatidylethanolamines of *P. gingivalis*. Activation of host cells likely involves a microbial pattern receptor that specifically recognizes the combination of isobranched aliphatic chains together with the phosphorylated head group in lipids from *Porphyromonas* species. Therefore, these findings, together with previously reported evidence demonstrating biological effects of phosphorylated dihydroceramides, suggest a new paradigm for microbial lipids to act as virulence factors in promoting inflammatory diseases, including but not limited to chronic inflammatory periodontal diseases.

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