Oral Microbiology Immunology 2006: 21: 84–92
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Porphyromonas gingivalis lipids and diseased dental tissues

F. C. Nichols[1], K. Rojanasomsith[1,2]
[1]Department of Oral Health and Diagnostic Sciences, University of Connecticut School of Dental Medicine, Farmington, CT, USA; [2]Chulalongkorn University, Faculty of Dentistry, Department of Periodontology, Bangkok, Thailand

Nichols FC, Rojanasomsith K. Porphyromonas gingivalis *lipids and diseased dental tissues.*
Oral Microbiol Immunol 2006: 21: 84–92. © Blackwell Munksgaard, 2006.

Background/aim: *Porphyromonas gingivalis* synthesizes several classes of dihydroceramides and at least one of these lipid classes promotes proinflammatory secretory reactions in gingival fibroblasts as well as alters fibroblast morphology in culture. The purpose of this investigation was to determine whether the dihydroceramide lipids of *P. gingivalis* are recovered in lipid extracts of subgingival plaque, diseased teeth, and diseased gingival tissue samples.
Methods: Lipids were extracted from *P. gingivalis*, subgingival plaque, subgingival calculus, teeth laden with gross accumulations of subgingival calculus, and gingival tissue samples obtained from chronic severe periodontitis sites. Lipid samples were analyzed by gas chromatography-mass spectrometry as trimethylsilyl derivatives or by electrospray-mass spectrometry as underivatized products. High-performance liquid chromatography fractions of *P. gingivalis* lipids and gingival tissue lipids were also analyzed by electrospray-mass spectrometry analysis.
Results: *P. gingivalis* phosphorylated dihydroceramides were recovered in lipid extracts of subgingival plaque, subgingival calculus, calculus contaminated teeth, and diseased gingival tissue samples. However, the distribution of phosphorylated dihydroceramides varied between these samples.
Conclusion: Subgingival plaque, subgingival calculus, diseased teeth, and gingival tissue are contaminated with phosphorylated dihydroceramides produced by *P. gingivalis*. The previously reported biological activity of these substances together with the recovery of these lipids at periodontal disease sites argues strongly for their classification as virulence factors in promoting chronic inflammatory periodontal disease.

Key words: dihydroceramides; gingival tissue; mass spectrometry; *Porphyromonas gingivalis*; teeth

Dr Frank C. Nichols, Department of Oral Health and Diagnostic Sciences, University of Connecticut School of Dental Medicine, 263 Farmington Avenue, Farmington, CT 06030, USA
Fax: +1 860 6791027;
e-mail: nichols@nso.uchc.edu
Accepted for publication September 26, 2005

Porphyromonas gingivalis synthesizes free, phosphoethanolamine and phosphoglycerol dihydroceramides that share a core lipid structure consisting of three long chain bases (17, 18 and 19 carbon aliphatic chains) in amide linkage to 3-OH isobranched (*iso*) $C_{17:0}$ (7, 10). The phosphoglycerol dihydroceramide class may be substituted with *iso*$C_{15:0}$ linked to the beta hydroxyl of 3-OH *iso*$C_{17:0}$ (10). Both phosphorylated dihydroceramide classes of *P. gingivalis* were shown to promote prostaglandin secretory responses in gingival fibroblasts in culture and the phosphoglycerol dihydroceramides caused substantial morphologic changes in gingival fibroblasts, suggesting either apoptosis, cell necrosis or both (10). These lipids therefore possess biological activity, supporting their classification as microbial virulence factors. Yet, little is known about the recovery of these lipids on diseased teeth and in diseased gingival tissues. A previous report demonstrated elevated levels of 3-OH *iso*$C_{17:0}$ in gingival tissue lipid extracts from periodontitis sites compared with healthy/mildly inflamed sites (6). A subsequent report indicated that the majority of 3-OH *iso*$C_{17:0}$ in lipid extracts of gingival tissue is amide-linked, suggesting contamination of diseased gingival tissues with bacterial ceramide lipids that contain 3-OH *iso*$C_{17:0}$ (9). As these lipids could be important in the pathogenesis of destructive periodontal diseases, the purpose of this investigation was to determine whether the dihydroceramide lipids of *P. gingivalis* could be detected in lipid extracts of periodontally diseased teeth and gingival tissues.

Material and methods

P. gingivalis (ATCC 33277, type strain) was grown in basal (peptone, trypticase and yeast extract (BBL)) medium supplemented with hemin and menadione (Sigma Co., St. Louis, MO) and brain heart

infusion as previously described (7, 10). Bacteria were harvested by centrifugation (3000 × g for 20 min) and lyophilized. Lipids were extracted from approximately 400 mg of *P. gingivalis* pellet using a modification of the phospholipid extraction procedure of Bligh & Dyer (1) and Garbus (2). Informed consent was obtained from patients in accordance with Institutional Review Board policy of the University of Connecticut Health Center before donation of dental tissues, teeth or subgingival plaque. Surgically removed teeth, pooled subgingival plaque or gingival tissue samples were processed using the same phospholipid extraction procedure. Diseased teeth and subgingival plaque samples were taken from sites demonstrating chronic severe periodontitis as described below. Subgingival plaque samples were obtained by placing coarse endodontic paper points into each diseased sulcus and collecting the subgingival plaque contents for approximately 10 s. The teeth or subgingival plaque samples from each donor were combined and the pooled samples were extracted. Gross adherent soft tissue was removed from calculus-contaminated teeth before lipid extraction but some adherent soft tissue remained attached to the teeth. Gingival tissue samples were surgically excised from chronic severe periodontitis sites and were immediately frozen. Chronic severe periodontitis sites demonstrated greater than 50% alveolar bone loss, periodontal pocket depth of at least 5 mm and bleeding on probing. The gingival tissue samples ($n = 8$) were thawed and homogenized in the first solvent phase of the Bligh & Dyer extraction procedure (1). The remaining phospholipid extraction procedure was completed once the gingival tissue samples were thoroughly homogenized.

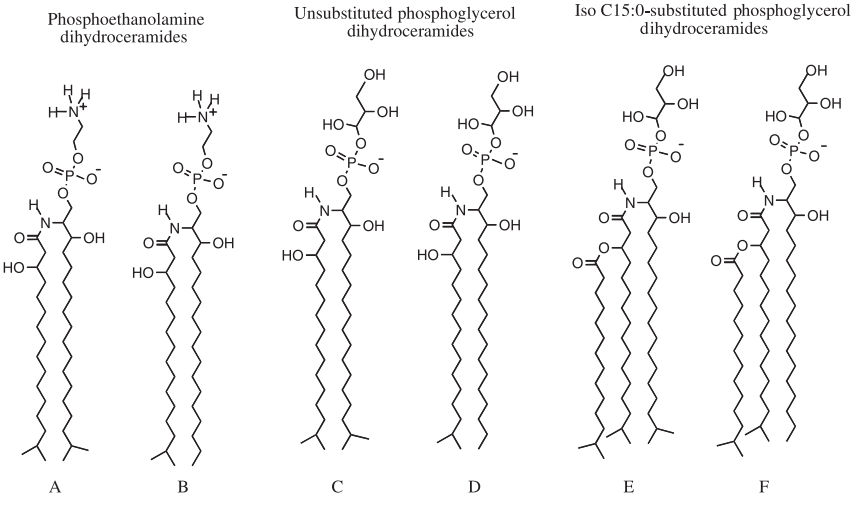
Fractionation of bacterial lipids and gingival tissue lipids by high-performance liquid chromatography (HPLC) was accomplished using a μPorosil HPLC column (0.4 × 25 cm silica gel, 5 μm, Waters Associates, Milford, MA) eluted isocratically with hexane-isopropanol-water (6 : 8 : 0.75, by vol.; Solvent A) (3). Lipid samples were dissolved in solvent A to achieve a concentration of approximately 3 mg/ml. For each chromatographic separation, 200 μg of lipid was applied and fractions were pooled for replicate fractionations. Lipid samples were eluted at 0.4 ml/min with 1 min fractions collected. The fractions were read at 206 nm and were dried under nitrogen and resuspended in $CHCl_3$.

Phosphoethanolamine and phosphoglycerol dihydroceramide recovery was examined in tooth lipids and gingival tissue lipids by treating approximately 1 mg of each lipid extract overnight with N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA, 100 μl) and analyzing for characteristic ions of the dihydroceramides of *P. gingivalis* using gas chromatography-mass spectrometry (GC-MS; see (10) for details of the structures and characteristic mass spectra of these dihydroceramides). Samples were applied to an SPB-1 column (15 m × 0.3 mm × 0.1 μm film thickness; Supelco, Bellefonte, PA). The inlet was maintained at 310°C using the splitless mode with a temperature program of 10°C/min from 200°C to 300°C followed by 5°C/min to 310°C and 4 min at 310°C. The mass spectrometer was used in the electron impact ionization mode with the ion source temperature of 200°C, an electron energy of 70 eV and emission current of 300 mA. For the GC-MS evaluation of teeth and gingival tissue lipid extracts, selected ion monitoring included acquisition of at least four characteristic ions for each major dihydroceramide lipids (7, 10).

Electrospray-MS analysis was accomplished using a Micromass Quattro II mass spectrometer system as previously described (10). Dihydroceramide lipid fractions were dissolved in hexane : isopropanol (6 : 8, by vol., elution solvent) and the samples injected at a maximum concentration of 100 μg/ml. Lipid samples (10 μl) were infused at a flow rate of 30 μl/min. For electrospray negative ion analyses, the desolvation and inlet block temperatures were 80°C and 100°C, respectively, and the transcapillary potential was 3000 V. The cone voltage was usually 30 V and the mass acquisition range was 0–2000 amu for all electrospray-MS analyses.

Additional samples of purified phosphorylated dihydroceramides, diseased teeth, and high-powered liquid chromatography (HPLC) fractions of gingival tissue lipids were treated with sodium methoxide (0.5 M in dry methanol, 40°C, 20 min). The reaction was terminated with the addition of 100 μl of glacial acetic acid

Table 1. Detection of *P. gingivalis* phosphorylated dihydroceramides by GC-MS in lipid extracts of dental samples. The structures of phosphoethanolamine dihydroceramides (A and B), unsubstituted phosphoglycerol dihydroceramides (C and D) and $isoC_{15:0}$-substituted phosphoglycerol dihydroceramides (E and F) are depicted below. Analysis of these lipids by GC-MS required the formation of volatile trimethylsilyl derivatives as described in the Material and methods. Lipid extracts from calculus-contaminated teeth, subgingival plaque, subgingival calculus, and diseased gingival tissue samples were shown to contain *P. gingivalis* lipid products when all characteristic ions were recovered at the expected retention time for each lipid derivative. See Fig. 2 and the Results section for a description of the mass spectra generated from phosphorylated dihydroceramides structures A and C or structures B and D



Source of lipid sample	*P. gingivalis* lipid type recovered
Tooth sample 1	A and/or C, B and/or D, E and F
Tooth sample 2	A and/or C, B and/or D, E and F
Subgingival calculus	A and/or C, B and/or D
Subgingival plaque	A and/or C, B and/or D
Diseased gingival tissue	A and/or C, B and/or D, and only E

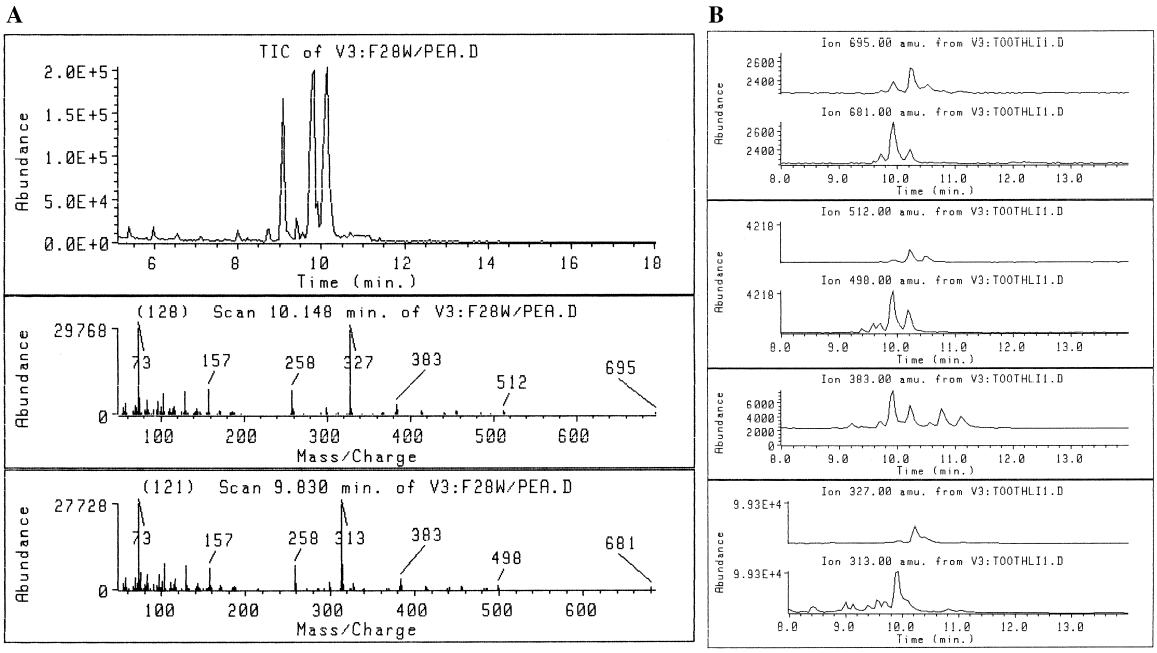
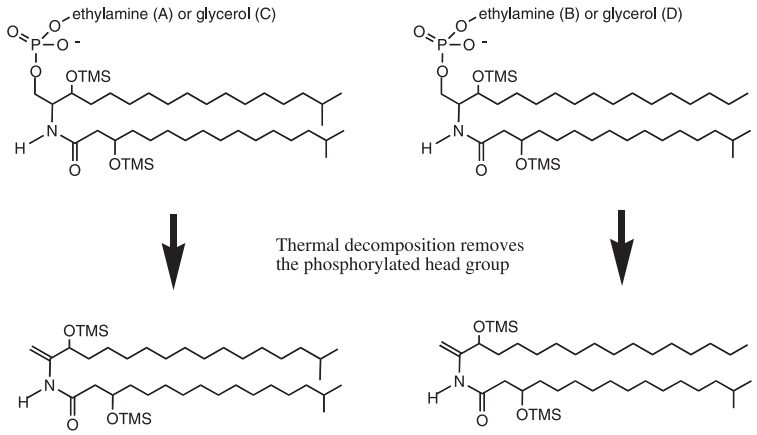


Fig. 1. Phosphorylated dihydroceramides of *P. gingivalis* recovered in lipid extracts of teeth covered with gross accumulations of subgingival calculus. Lipids were extracted from surgically removed teeth according the Bligh & Dyer procedure (1) as described under Material and methods. Phosphoethanolamine dihydroceramides of *P. gingivalis* were purified by HPLC, treated to form trimethylsilyl derivatives and subjected to GC-MS analysis. A) Characteristic mass spectra for the two major phosphoethanolamine dihydroceramides of *P. gingivalis*. See (9) for the interpretation of the mass spectra and Fig. 2 for a description of other products that can produce the same mass spectra. Selected ion monitoring of a lipid extract of calculus-contaminated teeth (B) revealed characteristic ions coeluting with retention times approximating those for the phosphoethanolamine dihydroceramides of *P. gingivalis*.



TMS derivatives of phosphorylated dihydroceramides A and C yield the same mass spectra. (See lipid peak emerging at 10.148 min, Figure 1A)

TMS derivatives of phosphorylated dihydroceramides B and D yield the same mass spectra. (See lipid peak emerging at 9.830 min, Figure 1A)

Fig. 2. Structural rearrangements of phosphorylated dihydroceramide derivatives with GC-MS analysis. The trimethysilyl derivatives of phosphoethanolamine dihydroceramides A and B and trimethysilyl derivatives of unsubstituted phosphoglycerol dihydroceramides C and D (shown in Table 1) undergo loss of phosphorylated head groups and desaturation by thermal decomposition with GC-MS analysis. Thermal decomposition of phosphoethanolamine dihydroceramide A or phosphoglycerol dihydroceramide C yields mass spectra identical to the lipid product emerging at 10.148 min (see Fig. 1A, middle frame). Thermal decomposition of phosphoethanolamine dihydroceramide B or phosphoglycerol dihydroceramide D yields mass spectra identical to the lipid product emerging at 9.830 min (see Fig. 1A, lowest frame).

and 2 ml of water. Lipid products were recovered by extracting twice with 2 ml of chloroform followed by drying the lipid extract. The resultant products were repurified by HPLC and analyzed directly by electrospray-MS.

Results

Trimethysilyl derivatives of HPLC-purified phosphoethanolamine dihydroceramides of *P. gingivalis* were analyzed by GC-MS (see Table 1 for these and other complex lipid structures). The total ion chromatogram in the uppermost frame of Fig. 1A shows that several major lipid products are detected in the phosphoethanolamine dihydroceramide fraction. The two dominant lipid products demonstrate retention times of 10.148 and 9.830 min and are designated as phosphoethanolamine dihydroceramide structures A and B, respectively (see bottom of Table 1). The mass spectra corresponding to these lipid products are shown in lower frames of Fig. 1A. The characteristic ions depicted for the lipid product emerging at 10.148 min include ions of 695, 512, 383, 327 and 258 m/z (10). This lipid product represents a dephosphorylated dihydroceramide with a 19-carbon iso-branched long chain base structure (Fig. 2) (10). The lipid product emerging at 9.830 min demonstrates characteristic ions of 681, 490, 383, 313 and 258 m/z and represents a dephosphorylated dihydroceramide with an 18-carbon unbranched long chain base (10). The two

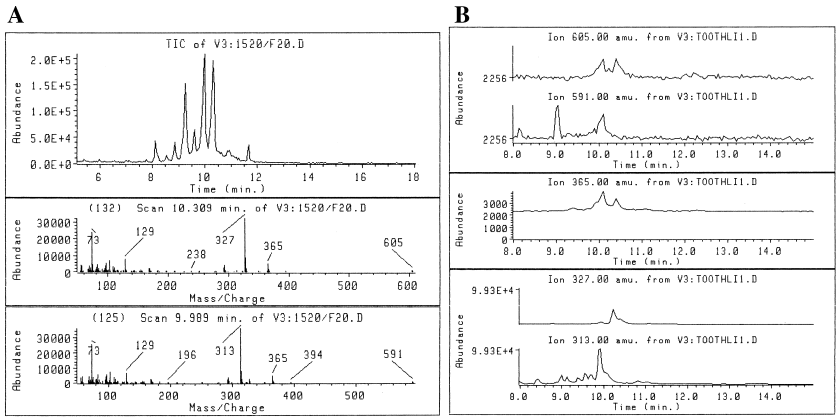


Fig. 3. Detection of *iso*$C_{15:0}$ -substituted phosphoglycerol dihydroceramides of *P. gingivalis* in lipid extract of calculus-contaminated teeth. Phosphoglycerol dihydroceramides substituted with *iso*$C_{15:0}$ were purified by HPLC fractionation of *P. gingivalis* lipids. A sample of the purified lipid was treated to form trimethysilyl derivatives and analyzed by GC-MS as described in the Material and methods. Fig. 2A shows the characteristic mass spectra produced from the two major phosphoglycerol dihydroceramides substituted with *iso*$C_{15:0}$ (see structures E and F at the bottom of Table 1). Selected ion monitoring of a lipid extract from calculus-contaminated teeth (Fig. 2B) revealed characteristic ions eluting with retention times approximating those for the major *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis*. Ions monitored for the high mass phosphoglycerol dihydroceramide of *P. gingivalis* include 605, 365 and 327 m/z ions. The lower mass phosphoglycerol dihydroceramide was monitored by the 591, 365 and 313 m/z ions. The 327 and 313 m/z ions demonstrated peaks with retention times corresponding to the phosphoethanolamine dihydroceramides shown in Fig. 1 but the shoulder regions to the right of the principal peaks correspond to the phosphoglycerol dihydroceramides.

major phosphoglycerol dihydroceramides of *P. gingivalis* that are not substituted with *iso*$C_{15:0}$ produce mass spectra identical to the phosphoethanolamine dihydroceramides shown in Fig. 1A (see Figs 2 and 5) (10). The reason that both lipid classes produce the same mass spectra is that the phosphorylated head group for both lipid classes is lost through thermal decomposition in the gas chromatograph. Loss of the phosphorylated head group is accompanied by desaturation of the dihydroceramide long chain base in a manner previously reported for various phospholipids analyzed by GC-MS (4, 7, 10). Although GC-MS analysis provides detailed structural confirmation of these lipid derivatives, this technique cannot distinguish phosphoethanolamine from unsubstituted phosphoglycerol dihydroceramides with identical long chain base and fatty acid constituents.

Calculus-contaminated teeth were removed from two patients due to generalized severe periodontitis and six teeth from each patient were pooled and extracted to yield two tooth lipid extracts. A sample (1 mg) of one lipid extract was treated to form trimethysilyl derivatives and was subjected to GC-MS analysis. Selected ion monitoring revealed that this lipid extract contained all six characteristic ions at the predicted retention times for the two major phosphoethanolamine and/or unsubstituted phosphoglycerol dihydroceramides of *P. gingivalis* (Fig. 1B). The second tooth lipid extract as well as lipid extracts from pooled subgingival plaque, subgingival calculus, and diseased gingival tissue samples were contaminated with phosphorylated dihydroceramides A and/or C as well as phosphorylated dihydroceramides B and/or D (Table 1).

Highly purified *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis* were prepared by HPLC previously and verified by electrospray-MS (10). Trimethylsilyl derivatives of *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis* fragment with GC-MS analysis to form a different group of characteristic ions. Figure 3A (lower frames) shows the characteristic mass spectra for the two major *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides (see structures E and F in Table 1) shown in the upper frame eluting at 10.309 and 9.989 min. The same tooth lipid sample shown in Fig. 1(B) was analyzed for the *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides (Fig. 3B). The selected ion chromatograms revealed that the characteristic ions of the two major phosphoglycerol dihydroceramides of *P. gingivalis* were observed in this tooth lipid extract.

The *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis* were detected in both lipid extracts from calculus-contaminated teeth but were not detected in lipid extracts of subgingival plaque and subgingival calculus as determined by GC-MS analysis (Table 1). Only the high mass *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramide (structure E, bottom of Table 1) was detected in the lipid extract from diseased gingival tissue samples. Recovery of *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis* dihydroceramides in dental samples was confirmed when all characteristic ions (lower frames, Fig. 3A) were demonstrated at the appropriate retention times for each *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramide product.

Because the trimethylsilyl derivatives of phosphoethanolamine or unsubstituted phosphoglycerol dihydroceramides of *P. gingivalis* can produce identical mass spectra using GC-MS analysis (Fig. 2), another analytical approach was needed to distinguish these lipid products. Electrospray-MS analysis was employed to confirm which phosphorylated dihydroceramides of *P. gingivalis* were present in these dental samples. Figure 4 compares the electrospray-MS spectra of the total lipid extracts from *P. gingivalis* (upper frame), two different tooth lipid samples, and an impacted third molar that was not exposed to the oral cavity before surgical removal (lowest frame). The 960 and 946 m/z ions represent the major *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramide structures E and F of *P. gingivalis* (Table 1) (10) and were observed in both lipid extracts of calculus-contaminated teeth but not in the lipid extract from the impacted tooth. The 736 and 722 m/z ions represent the unsubstituted phosphoglycerol dihydroceramide structures C and D of *P. gingivalis* (Table 1). This was confirmed by treating *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides (lower frame, Fig. 5) with sodium methoxide. This hydrolysis yielded three major products with negative ion masses of 736, 722 and 708 m/z as detected by electrospray-MS (upper frame, Fig. 5). The 736 and 722 m/z ions were detected in lipid extracts of *P. gingivalis* and diseased teeth but not in the impacted tooth lipid extract. The 705 and 691 m/z ions represent the two major phosphoethanolamine dihydroceramide structures A and B of *P. gingivalis* (10). These ions were detected in only one lipid extract of calculus-contaminated teeth and were not detected in the lipid extract from the impacted third molar.

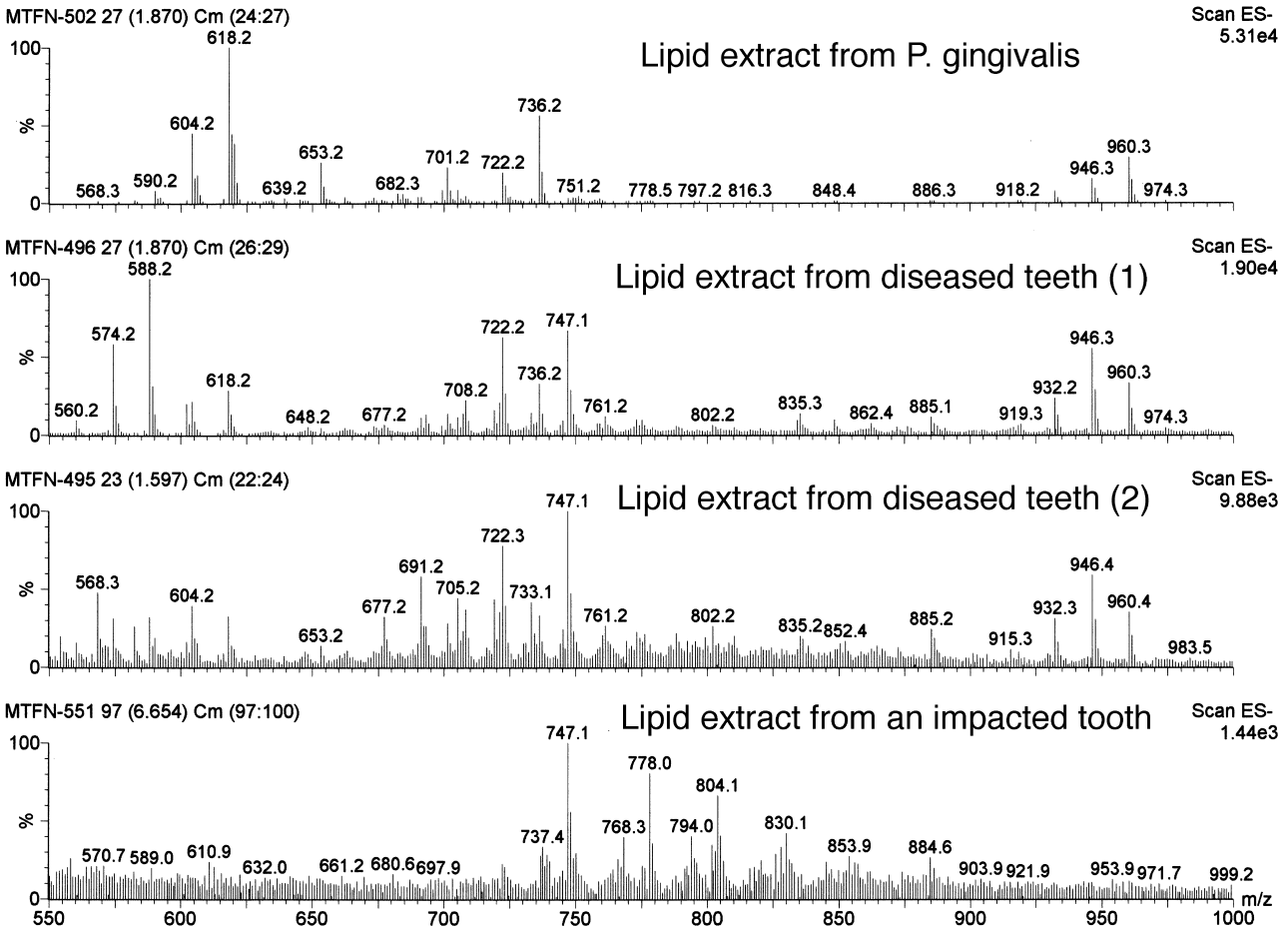


Fig. 4. Electrospray-MS analysis of lipid extracts recovered from *P. gingivalis*, calculus-contaminated teeth or an impacted third molar. Each lipid extract was dissolved in hexane : isopropanol (6 : 8, by vol.) at a concentration of approximately 100 μg/ml. Samples were introduced at a flow of 30 μl/min and analyzed as described in the Material and methods section. The partial mass spectra were 580–1000 m/z to better visualize the lipid ions of interest. The base peak ion abundance is shown in the upper right hand corner of each mass spectrum. Negative ions detected in lipid extracts of *P. gingivalis* and calculus-contaminated teeth include 960, 946, 932, 736, 722, 718, 618, and 604 m/z ions. The 960, 946, 932, 736, 722, and 708 ions represent phosphoglycerol dihydroceramides of *P. gingivalis*. The latter three ions represent phosphoglycerol dihydroceramides without *iso*$C_{15:0}$ substitution (see Table 2 for ion assignments). One of the lipid extracts from calculus-contaminated teeth demonstrated the 705, 691, and 677 ions indicating phosphoethanolamine dihydroceramides. The *P. gingivalis* lipid ions were not detected in the extract from the impacted third molar. The 747 and 885 m/z ions observed in the lipid extract from either the impacted third molar or the lipid extracts from the calculus-contaminated teeth were not observed in the lipid extract of *P. gingivalis*.

Table 2 summaries the recovery of *P. gingivalis* lipids in dental samples as determined by electrospray-MS analysis. Note that the unsubstituted phosphoglycerol dihydroceramides (structures C and D, Table 1) were not detected in lipid extracts of subgingival plaque samples and the phosphoethanolamine dihydroceramides (structures A and B, Table 1) were not detected in the pooled subgingival calculus deposits. Recovery of *P. gingivalis* lipids in diseased gingival tissue samples will be discussed below.

The lipid extracts depicted in Fig. 5 were evaluated for lipid A of *P. gingivalis* lipopolysaccharide by examining the mass scale from 1000 to 2000 amu. According to Kumada et al. (5) the major lipid A structures of *P. gingivalis* lipopolysaccharide are detected as either 1449 m/z or 1191 m/z negative ions in the nondeacylated forms. The 1449 m/z ion was detected in very low abundance in the lipid extract of *P. gingivalis* but was not detected in lipid extracts of calculus-contaminated teeth, subgingival plaque or subgingival calculus (data not shown). Furthermore, the 1191 m/z ion was not detected in any of the lipid extracts (data not shown).

Before examining gingival tissue lipids by electrospray-MS, the remainder of the lipid extract from pooled periodontitis gingival tissue samples was fractionated and a defined amount of each HPLC fraction was hydrolyzed and analyzed for recovery of 3-OH *iso*$C_{17:0}$. 3-OH *iso*$C_{17:0}$ is a constituent fatty acid of all dihydroceramides of *P. gingivalis* (see dihydroceramide lipid structures, Table 1). Peak recovery of 3-OH *iso*$C_{17:0}$ was observed in HPLC fractions 5, 8 and 12 of periodontitis gingival tissue lipids (data not shown). Lipids were extracted from gingival tissue samples excised from relatively healthy sites (crown lengthening procedures) and were fractionated by HPLC. After correction for lipid mass in all HPLC fractions, at least 10-fold greater levels of 3-OH *iso*$C_{17:0}$ were recovered in HPLC fractionated lipids of periodontitis tissue samples than from crown lengthening lipid extracts (data not shown). Furthermore, GC-MS analysis of lipids extracted from healthy gingival tissues (recovered during crown lengthening procedures) contained no detectable levels of *P. gingivalis* dihydroceramides (data not shown).

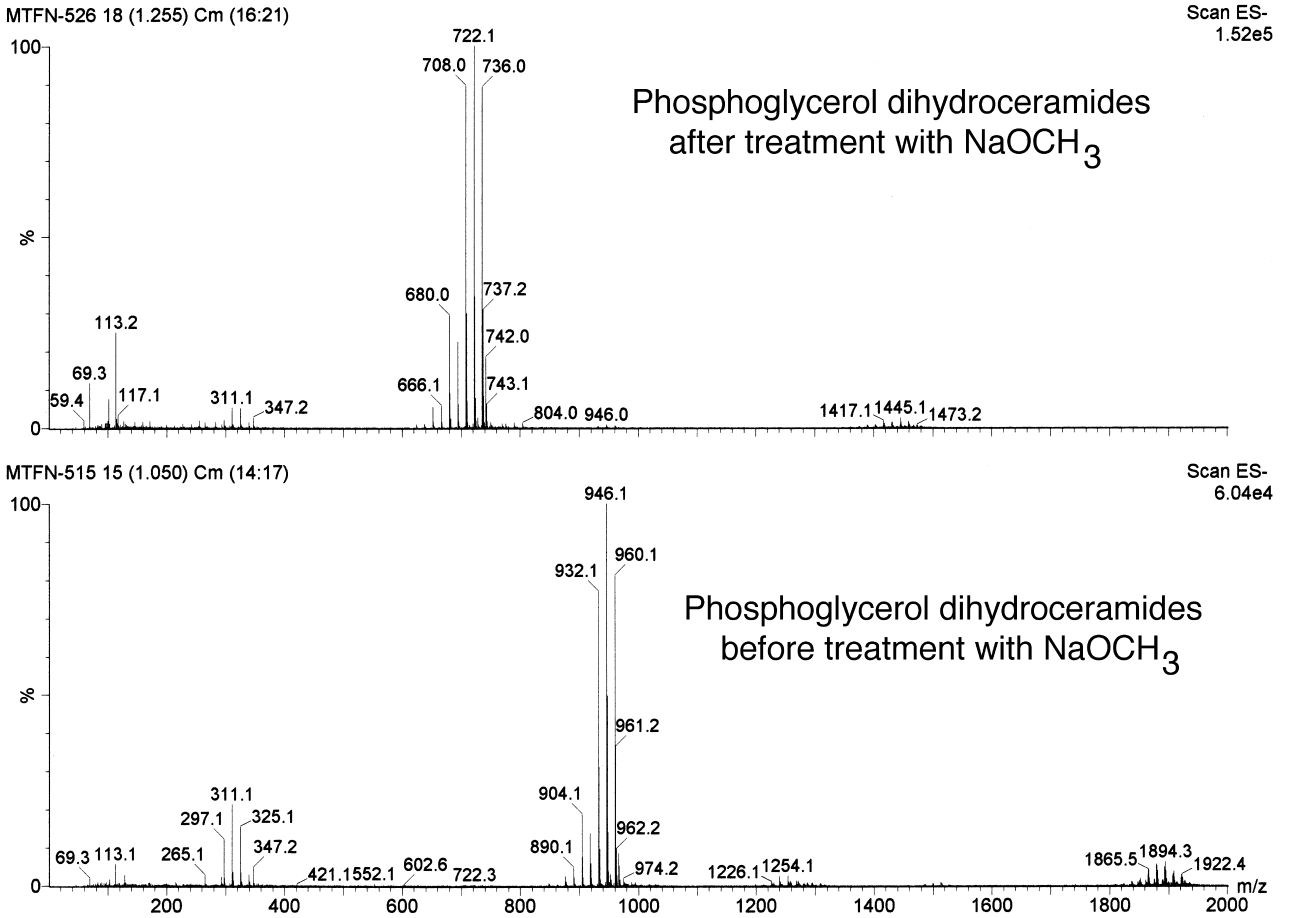


Fig. 5. Effect of sodium methoxide treatment on the *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis*. Purified *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis* were analyzed by electrospray-MS before (lower frame) and after (upper frame) treatment with sodium methoxide as described in the Material and methods. Loss of *iso*$C_{15:0}$ from the respective phosphoglycerol dihydroceramides decreased each ion mass by the expected 224 amu.

Table 2. Phosphorylated dihydroceramides or lipid A of *P. gingivalis* detected by electrospray-MS in lipid extracts from dental samples. Lipid A (1446 m/z) was identified in the lipid extract of *P. gingivalis* by extending the mass scale shown in Fig. 4 from 1000 to 2000 amu. The structures of phosphoethanolamine dihydroceramides (A and B), unsubstituted phosphoglycerol dihydroceramides (C and D) and iso$C_{15:0}$-substituted phosphoglycerol dihydroceramides (E and F) were identified by their characteristic molecular ions previously identified in HPLC purified lipid fractions of *P. gingivalis*. Lipid extracts from calculus-contaminated teeth, subgingival plaque, subgingival calculus, and diseased gingival tissue samples were examined for these characteristic ions as shown below

	Lipid class			
Characteristic molecular ions	Phosphoethanolamine dihydroceramides 705 m/z (A) 691 m/z (B)	Unsubstituted phosphoglycerol dihydroceramides 736 m/z (C) 722 m/z (D)	*iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides 960 m/z (E) 946 m/z (F)	Lipid A 1446 m/z
Source of lipid sample				
Tooth lipid 1	A and B	C and D	E and F	ND
Tooth lipid 2	A and B	C and D	E and F	ND
Plaque lipid 1	A and B	ND	E and F	ND
Plaque lipid 2	A and B	ND	E and F	ND
Subgingival calculus	ND	C and D	E and F	ND
Diseased gingival tissue	A	C and D	ND	ND
Healthy tooth/soft tissue	ND	ND	ND	ND

ND, lipid samples that did not yield the characteristic molecular ions for the indicated lipid products.

To calibrate which *P. gingivalis* lipids are present in HPLC fractionated lipids of diseased gingival tissues, a sample of *P. gingivalis* lipid was fractionated using the same HPLC system (10) and the lipids recovered in fractions 5, 8, and 12 were analyzed using electrospray-MS. Negative ion electrospray-MS spectra demonstrated that HPLC fraction 5 contained uncharac-

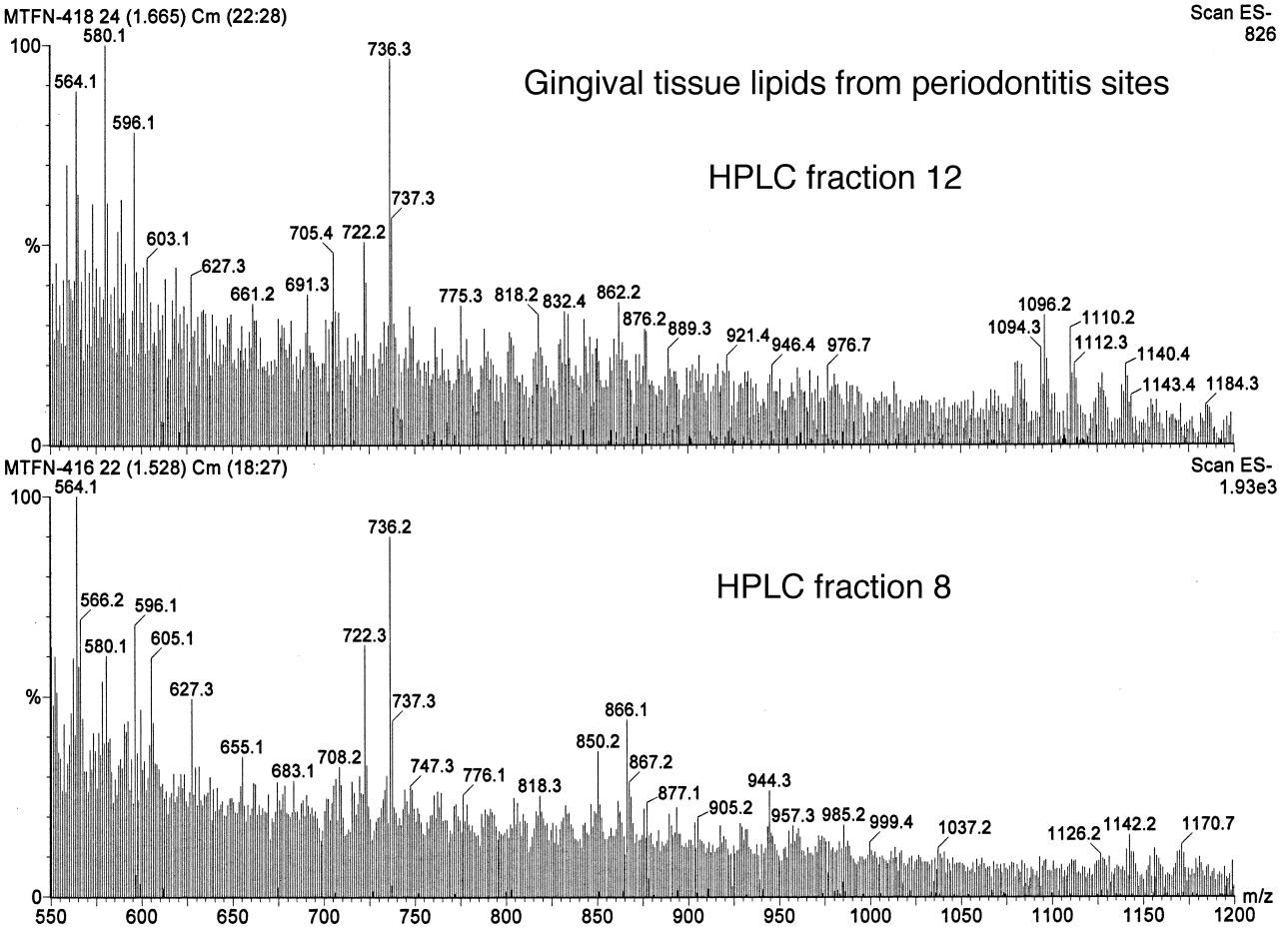


Fig. 6. Recovery of phosphorylated dihydroceramides in HPLC lipid fractions of diseased gingival tissues. Lipids of pooled gingival tissue samples were extracted as described in the Material and methods. The lipid sample was fractionated by HPLC, each fraction was treated with sodium methoxide and the resultant lipids were evaluated by negative ion electrospray-MS. HPLC fraction 8 and 12 lipids contained phosphoglycerol dihydroceramides (736 and 722 m/z ions) and fraction 12 contained slight amounts of the phosphoethanolamine dihydroceramides (705 and 691 m/z ions), although this fraction was also contaminated with the phosphoglycerol dihydroceramides recovered in fraction 8.

terized lipids of *P. gingivalis* that likely include free dihydroceramides (7). The major phosphoglycerol dihydroceramides of *P. gingivalis* substituted with *iso*$C_{15:0}$ (960 and 946 m/z ions, representing structures E and F, respectively) or the phosphoglycerol dihydroceramides without *iso*$C_{15:0}$ substitution (736 and 722 m/z ions, representing structures C and D, respectively) were recovered primarily in HPLC fraction 8. The major phosphoethanolamine dihydroceramides of *P. gingivalis* (705 and 691 m/z ions representing structures A and B, respectively) were recovered in HPLC fraction 12. The characteristic negative ions of the major phosphoethanolamine dihydroceramides (705 and 691 m/z) were the dominant ions recovered in fraction 12, although very low amounts of the 736 m/z ion and the 960, 946 and 922 m/z ions were also recovered.

Because gingival lipid extracts contain significant levels of mammalian phospholipids and other complex lipids, the lipid extract of diseased gingival tissue samples was first separated by HPLC followed by sodium methoxide treatment of all fractions before electrospray-MS analysis. Sodium methoxide released ester-linked fatty acids, thereby destroying contaminating phospholipids and triglycerides. Although sodium methoxide treatment of phosphoglycerol dihydroceramides released ester-linked *iso*$C_{15:0}$ from this lipid class (10), the remainder of the phosphoglycerol dihydroceramide structure was not affected, as demonstrated by the recovery of 736, 722, and 708 m/z ions (Fig. 5).

The recovery of phosphorylated dihydroceramides in lipid extracts from pooled diseased gingival tissue samples was examined next. HPLC fractionation of gingival lipids followed by sodium methoxide treatment and electrospray-MS analysis did not reveal neutral lipids of *P. gingivalis* in HPLC fraction 5 lipids (data not shown). HPLC fraction 8 of diseased gingival tissues revealed 736 and 722 m/z negative ions in significant abundance, indicating recovery of phosphoglycerol dihydroceramides of *P. gingivalis* (Fig. 6, lower frame). Peak recovery of 705 and 691 m/z negative ions was observed in HPLC fraction 12 (Fig. 6, upper frame). The 705 and 691 m/z ions were absent from HPLC fraction 10 and fractions 13–15, and only a slight amount was observed in HPLC fraction 11. HPLC fraction 12 also demonstrated quantitatively greater ion abundances of phosphoglycerol dihydroceramides indicated by recovery of the 736 and 722 m/z negative ions. Phosphoglycerol dihydroceramide detection in HPLC fraction 12 was

presumed to reflect the trailing edge of the lipid peak emerging in HPLC fraction 8. Of note, lipid extracts from pooled subgingival plaque samples demonstrated a greater abundance of phosphoethanolamine dihydroceramides than phosphoglycerol dihydroceramides (data not shown). Recovery of phosphorylated dihydroceramides in diseased gingival tissues does not appear to result from residual contamination with subgingival plaque organisms.

Discussion

This investigation demonstrated that specific phosphorylated dihydroceramides of *P. gingivalis* are detected in lipid extracts of calculus-contaminated teeth. The combined results from GC-MS and electrospray-MS analyses revealed that calculus-contaminated teeth harbor both phosphoethanolamine and phosphoglycerol dihydroceramides of *P. gingivalis*. GC-MS analysis of the total lipid extracts of teeth detected phosphoglycerol dihydroceramides substituted with *iso*$C_{15:0}$ as well as ions consistent with cleavage products of phosphoethanolamine dihydroceramides and/or phosphoglycerol dihydroceramides without *iso*$C_{15:0}$ substitution (10). Electrospray-MS confirmed that the lipid extracts of diseased teeth were contaminated with nonsubstituted and *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides, with smaller amounts of phosphoethanolamine dihydroceramides being detected. Both mass spectrometric detection techniques provide independent confirmation of *P. gingivalis* dihydroceramide lipid contamination of diseased teeth. In contrast to the lipid extract of *P. gingivalis*, lipid extracts from diseased teeth and subgingival plaque did not reveal ions indicating lipid A of *P. gingivalis* lipopolysaccharide. This demonstrates the relative abundance of phosphorylated dihydroceramides as potential virulence factors at periodontal disease sites.

GC-MS evaluation of total lipid extracts of diseased gingival tissue samples demonstrated phosphoglycerol dihydroceramides substituted with *iso*$C_{15:0}$ as well as cleavage products consistent with phosphoethanolamine dihydroceramides and/or unsubstituted phosphoglycerol dihydroceramides of *P. gingivalis*. However, the abundance of mammalian lipids in the total lipid extract of gingival tissues prevented the direct evaluation of *P. gingivalis* lipids by electrospray-MS analysis. To detect bacterial lipid contaminants, periodontitis tissue lipid samples were treated to destroy the background mammalian phospholipids before electrospray-MS detection of bacterial lipids. This was accomplished by hydrolyzing lipid fractions with sodium methoxide; however, this treatment also transesterified *iso*$C_{15:0}$ from phosphoglycerol dihydroceramides, resulting in loss of the high mass negative ions (960 and 946 m/z). Using this approach, diseased gingival tissues were shown to contain phosphoglycerol dihydroceramides and smaller amounts of phosphoethanolamine dihydroceramides of *P. gingivalis*. The primary disadvantage with this approach is that the sodium methoxide treatment removed *iso*$C_{15:0}$ from the phosphoglycerol dihydroceramides. However, GC-MS analysis (Fig. 2B and Table 1) demonstrated that the high mass phosphoglycerol dihydroceramide substituted with *iso*$C_{15:0}$ exists in lipid extracts of diseased gingival tissue samples. Therefore, unsubstituted and *iso*$C_{15:0}$-substituted phosphoglycerol dihydroceramides of *P. gingivalis* can be detected in diseased gingival tissues.

The primary dihydroceramide lipids of *P. gingivalis* recovered in diseased gingival tissue samples were the phosphoglycerol dihydroceramides; little phosphoethanolamine dihydroceramide was detected. Phosphoglycerol dihydroceramides were predominant over phosphoethanolamine dihydroceramides in total lipid extracts of calculus-contaminated teeth (Fig. 3) as determined by electrospray-MS analysis. In contrast, the phosphoethanolamine dihydroceramides were more abundant than the phosphoglycerol dihydroceramides in lipid extracts of subgingival plaque (data not shown). Therefore, the distribution of phosphorylated dihydroceramides in diseased gingival tissues is not similar to that observed with lipid extracts from subgingival plaque or calculus-contaminated teeth. This evidence suggests that diseased gingival tissues are not simply contaminated with subgingival plaque. Regardless of the processes resulting in bacterial lipid accumulation in periodontitis tissue samples or metabolic clearance of these bacterial lipids, the recovery of phosphorylated dihydroceramides in diseased gingival tissues indicates that the biological properties of the contaminating bacterial lipids must be understood within the context of disease promotion. A previous report indicated that lipids of subgingival calculus promote IL-1β-mediated prostaglandin secretion when fibroblasts were treated with a lipid concentration (μg/mm^2) approaching that observed on diseased teeth after scaling and root planing (8). More recently, *P. gingivalis* phosphoglycerol dihydroceramides substituted with *iso*$C_{15:0}$ were shown to markedly potentiate IL-1β-mediated prostaglandin secretion from gingival fibroblasts and to alter fibroblast morphology in culture (9). These bacterial lipids could alter the inflammatory processes associated with host–microbial interactions in periodontally diseased gingival tissues and could alter the capacity of host cells to promote regenerative processes on diseased root surfaces previously contaminated with subgingival calculus. Other preliminary work has demonstrated effects of phosphoglycerol dihydroceramides on epithelial cells, peripheral blood monocytes, endothelial cells and others. However, additional research is needed to thoroughly understand the biological responses in these cells and others.

In summary, novel phosphorylated dihydroceramides of *P. gingivalis* were detected in lipid extracts of calculus-contaminated teeth, subgingival calculus, subgingival plaque and in diseased gingival tissues. The phosphoglycerol dihydroceramides were the dominant *P. gingivalis* lipids recovered in lipid extracts of diseased gingival tissues. Given the reported capacity of phosphoglycerol dihydroceramides to promote cell activation and morphology changes in gingival fibroblasts, future studies should be directed toward understanding the potential of these lipids to participate in the pathogenesis of destructive periodontal diseases in adults.

Acknowledgment

The authors thank Mr. Marvin Thompson, Department of Chemistry, University of Connecticut, Storrs, CT, for his assistance in performing the electrospray-MS analyses.

References

1. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959: **37**: 911–917.
2. Garbus J, DeLuca HF, Loomas ME, Strong FM. Rapid incorporation of phosphate into mitochondrial lipids. J Biol Chem 1968: **238**: 59–63.
3. Hax WMA, Geurts Van Kessel WSM. High-performance liquid chromatographic separation and photometric detection of phospholipids. J Chromatogr 1977: **142**: 735–741.
4. Horning M, Casparrini G, Horning E. The use of gas phase analytical methods for the analysis of phospholipids. J Chromatogr Sci 1969: **7**: 267–275.
5. Kumada H, Haishima Y, Umemoto T, Tanamoto K. Structural study on the free lipid A isolated from lipopolysaccharide of *Porphyromonas gingivalis*. J Bacteriol 1995: **177**: 2098–2106.

6. Nichols FC. Distribution of 3-hydroxy iC17:0 in subgingival plaque and gingival tissue samples: relationship to adult periodontitis. Infect Immun 1994: **62**: 3753–3760.
7. Nichols FC. Novel ceramides recovered from *Porphyromonas gingivalis*: relationship to adult periodontitis. J Lipid Res 1998: **39**: 2360–2372.
8. Nichols FC, Levinbook H, Shnaydman M, Goldschmidt J. Prostaglandin E_2 secretion from gingival fibroblasts treated with interleukin-1beta: effects of lipid extracts from *Porphyromonas gingivalis* or calculus. J Periodontal Res 2001: **36**: 142–152.
9. Nichols FC, Maraj B. Relationship between hydroxy fatty acid and prostaglandin E_2 in gingival tissue. Infect Imun 1998: **66**: 5805–5811.
10. Nichols FC, Riep B, Mun J, Morton MD, Bojarski MT, Dewhirst FE, et al. Structures and biological activity of phosphorylated dihydroceramides of *Porphyromonas gingivalis*. J Lipid Res 2004: **45**: 2317–2330.