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Novel carbonyl and nitrile products from reactive chlorinating species attack of lysosphingolipid

Viral V. Brahmbhatt [a], Fong-Fu Hsu [c], Jeff L.-F. Kao [b], Erin C. Frank [a], David A. Ford [a,*]

[a] *Department of Biochemistry and Molecular Biology, St. Louis University Medical Center, 1402 South Grand Blvd., St. Louis, MO 63104, United States*
[b] *Department of Internal Medicine, Washington University, St. Louis, MO 63110, United States*
[c] *Department of Chemistry, Washington University, St. Louis, MO 63110, United States*

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Abstract

Lysosphingolipids are important lipid signaling molecules that are associated predominantly with high density lipoproteins (HDL) in human plasma. Further, HDL has been shown to be a target for the reactive chlorinating species (RCS) produced by myeloperoxidase (MPO). Accordingly, RCS attack of lysosphingolipids was characterized in these studies. It was shown that RCS attack of sphingosylphosphorylcholine results in the formation of 2-hexadecenal and 1-cyano methano phosphocholine. The structures were identified and confirmed predominantly using mass spectrometric analyses. Further, it was demonstrated that RCS attack of another bioactive lysosphingolipid sphingosine 1-phosphate also results in the formation of 2-hexadecenal from its sphingosine base. Using a synthetically prepared, deuterated 2-hexadecenal internal standard, it was determined that 2-hexadecenal quickly accumulated in HDL treated with MPO/RCS generating system. Thus, the present studies characterize the formation of a novel group of lipid products generated following RCS attack of lysosphingolipids.

Keywords: Myeloperoxidase; Reactive chlorinating species; Lysosphingolipids; Mass spectrometry; 2-Hexadecenal; 1-Cyano methano phosphocholine

1. Introduction

Myeloperoxidase (MPO), a heme-containing enzyme, is present in the α granules of neutrophils and is released into phagolysosomes (Hampton et al., 1998; Jiang et al., 1997) as well as the extracellular space by activated phagocytes (Edwards et al., 1987). MPO catalyzes formation of the reactive chlorinating species (RCS), hypochlorous acid (HOCl) and its conjugate base (OCl)-, from hydrogen peroxide and chloride ion (Hampton et al., 1998; Harrison and Schultz, 1976). Hypochlorous acid, is a strong oxidizing agent and can react with primary amines to generate chloramines (R-NHCl), which retain their oxidizing potential (Lampert and Weiss, 1983; Weiss et al., 1983). *In vivo*, the primary role of MPO is bactericidal. In fact, individuals with MPO deficiency show decreased capacity of microbial killing by their neutrophils (Cech et al., 1979; Kitahara et al., 1981; Lehrer and Cline, 1969). However, RCS also attack the host tissue (Weiss, 1989) and several studies have established a role of MPO in heart disease. For example, MPO polymorphisms have been shown

* Corresponding author. Tel.: +1 314 977 9264; fax: +1 314 977 9205.
E-mail address: fordda@slu.edu (D.A. Ford).

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to affect coronary artery disease (Asselbergs et al., 2004; Nikpoor et al., 2001) and expression of human MPO has been shown to promote atherosclerosis in mice (McMillen et al., 2005). Still, the role of MPO in cardiovascular disease remains to be fully elucidated.

MPO-derived RCS attack a wide variety of molecules including nucleic acids, proteins and lipids. Previous studies have established plasmalogens as important targets of MPO-derived RCS. MPO-derived RCS attack on plasmalogens generates 2-chlorohexadecanal (Albert et al., 2001), which is increased in atherosclerotic tissue (Thukkani et al., 2003) and can inhibit endothelial nitric oxide synthase activity (Marsche et al., 2004). MPO-derived RCS also attack the unsaturated –C=C– bonds within aliphatic chains of phospholipids and lipoprotein-associated cholesterol which leads to the production of chlorohydrins (Winterbourn et al., 1992) and dichlorinated products (Hazen et al., 1996). Furthermore, RCS attack the primary amine group of phosphatidylethanolamine to produce chloramines (Carr et al., 1998).

Lysosphingolipids such as D-erythro sphingosylphosphorylcholine (SPC) and sphingosine 1-phosphate (S1P) as well as sphingosine, have emerged as a new class of signaling molecules (Alewijnse et al., 2004). These lipids associate predominantly with high-density lipoproteins (HDL) in the plasma (Nofer et al., 2001). HDL-associated lysosphingolipids have been suggested to mediate, in part, the atheroprotective effects of HDL such as nitric oxide dependent vasorelaxation (Nofer et al., 2004), decrease of E-selectin expression on human endothelial cells (Nofer et al., 2003) and decrease of endothelial cell apoptosis (Kimura et al., 2001; Nofer et al., 2001). Other atheroprotective effects of lysosphingolipids include inhibition of thrombin generation (Deguchi et al., 2004) and inhibition of platelet function (Altmann et al., 2003). Additionally, it is known that MPO derived RCS targets HDL and affects cholesterol efflux (Bergt et al., 2004). Accordingly, the present study tested the hypothesis that MPO-derived RCS can attack HDL-associated lysosphingolipids. The results here demonstrate that MPO-derived RCS attack SPC to generate 2-hexadecenal and 1-cyano methano phosphocholine. Moreover, we demonstrate that 2-hexadecenal is increased when MPO-derived RCS react with HDL.

2. Experimental procedures

2.1. Materials

Human myeloperoxidase (MPO), catalase and HDL were purchased from Calbiochem. Sphinganylphosphorylcholine, D-erythro sphingosylphosphorylcholine (SPC), sphingosine and sphingosine 1-phosphate (S1P) were purchased from Matreya, Inc. Sodium(meta)periodate was purchased from Fluka. [13,13,14,14,14-d_5]-tetradecanoic acid was purchased from C/D/N isotopes. All other reagents were purchased from Aldrich, Sigma or Fisher.

2.2. Analysis of reaction products from RCS treatment of sphingolipids

Incubations were performed in chlorine-demand-free and chloride-free reaction vessels (Weil and Morris, 1949). One hundred micrograms of lipid or 200 μg of protein (for HDL) was incubated in 1 ml of 20 mM phosphate-buffer comprised of 0.1 mM diethylenetriamine-pentaacetic acid (pH 4.0–7.0) in the presence or absence of indicated amounts of MPO (1–2 U/ml), hydrogen peroxide (H_2O_2) (1 mM) or NaCl (100 mM) for 15 min at 37 °C. Synthetic lipids were sonicated in buffer, prior to initiating reactions. Reactions were terminated by the addition of methanol. The reaction products were extracted into chloroform by the method of Bligh and Dyer (Bligh and Dyer, 1959). Thin-layer chromatography (TLC) analysis was done on silica gel 60 Å plates (Whatman) utilizing a mobile phase comprised of petroleum ether/ethyl ether/acetic acid (90/10/1, v/v/v) for neutral lipid separation. To separate polar lipids, chloroform/acetone/methanol/acetic acid/water (6/8/2/2/1, v/v/v/v/v) was used as mobile phase and the TLC plate was run twice in the same direction after drying in between runs. In both cases, the reaction products were visualized by charring concentrated sulfuric acid-treated plates.

2.3. Preparation of 2-hexadecenal

Sphingosine was reacted with sodium(meta)periodate to generate 2-hexadecenal (Baumann et al., 1969). 2-Hexadecenal was purified by high-pressure liquid chromatography (HPLC) using a solid-phase silica column (250 mm × 4.6 mm, 5 μm; Beckman). An isocratic gradient of hexane/isopropanol (100/0.5, v/v) was utilized at a flow rate of 2 ml/min. UV monitoring was set at 206 nm for detection of 2-hexadecenal.

2.4. Synthesis of 2-[d_5]-hexadecenal

Synthesis of 2-[15,15,16,16,16-d_5]-hexadecenal (2-[d_5]-hexadecenal) was achieved by the following scheme: (1) [13,13,14,14,14-d_5]-tetradecanoic acid was converted to [13,13,14,14,14-d_5]-tetradecanol

using sodium bis(2-methoxyethoxy)aluminum hydride, which was further converted to [13,13,14,14,14-d_5]-tetradecanal using oxalyl chloride-activated dimethyl sulfoxide as described earlier (Mancuso et al., 1978); (2) [13,13,14,14,14-d_5]-tetradecanal was converted to 2-[15,15,16,16,16-d_5]-hexadecenoic acid by addition of propanedioic acid in the presence of pyridine and piperidine as described by Hino et al (Tohru Hino et al., 1986); (3) cis and trans forms of 2-[15,15,16,16,16-d_5]-hexadecenoic acid were separated by HPLC using reverse-phase solid-phase (Econosil C18 10 μm, 250 mm × 10 mm) with gradient elution using water (pH 2 using phosphoric acid) and acetonitrile as mobile phase A and B, respectively. Following injection of the reaction mixture, the column was eluted with 50% A and 50% B for 15 min, followed by linear gradient from 50% A to 100% B over 15 min. Following the gradient the column was further eluted with 100% B for 90 min. UV was set at 192 nm. Trans 2-[15,15,16,16,16-d_5]-hexadecenoic acid elutes at ∼34 min; (4) Trans 2-[15,15,16,16,16-d_5]-hexadecenoic acid was converted to 2-[15,15,16,16,16-d_5]-hexadecenol using sodium bis(2-methoxyethoxy)aluminum hydride (Thukkani et al., 2002); (5) 2-[15,15,16,16,16-d_5]-hexadecenol was oxidized to 2-[d_5]-hexadecenal using pyridinium chlorochromate and extracted from silica after TLC purification in neutral lipid solvent system as described above; (6) 2-[d_5]-hexadecenal purity was confirmed by GC–MS of its pentafluorobenzyl (PFB) oxime derivative (see below) and quantified by gas chromatography with flame ionization detection of its acid methanolysis derivatives using arachidic acid as an internal standard.

2.5. Gas chromatography-mass spectrometry (GC–MS) analysis of reaction products

TLC-purified reaction products were extracted from silica into chloroform by a modified Bligh and Dyer technique. TLC-purified reaction products were analyzed either directly, or following derivatization using methanolic HCl or PFB hydroxylamine. For GC–MS electron impact (EI) ionization analysis, reaction products in chloroform were dried under nitrogen and resuspended in 50–100 μl ethyl acetate. One microliter was injected using a Hewlett Packard 7683 series injector. The products were separated on a DB-1 column (12.0 m, 0.2 mm inner diameter, 0.33 μm methyl silicone film coating; Cobert, St. Louis, MO) utilizing a Hewlett Packard 6890 gas chromatograph. The injector temperature was set to 250 °C and transfer lines were maintained at 280 °C. The GC oven was maintained at 125 °C for 2 min and temperature was increased at a rate of 25 °C/min to 300 °C. The oven temperature was held at 300 °C for an additional 4 min. All spectra were acquired on a Hewlett Packard 5973 mass spectrometer. The source temperature was set at 230 °C. The electron energy was 69.9 eV and the emission current was 34.6 μA.

For PFB hydroxylamine-derivatized products, either TLC-purified reaction products or crude reaction product extracts were derivatized using PFB hydroxylamine and analyzed as previously described (Thukkani et al., 2002). Briefly, extracts were dried under nitrogen and resuspended in 300 μl of ethanol. Three hundred microliters of PFB hydroxylamine solution (6 mg/ml) was added, vortexed for 5 min and allowed to incubate for 25 min at room temperature. Following the addition of 1.2 ml of water, the reaction products were sequentially extracted into 2 ml of cyclohexane: diethyl ether (4:1, v/v), dried under a stream of nitrogen and resuspended in 50–100 μl of petroleum ether for GC–MS analysis. GC–MS analysis was performed in the negative ion chemical ionization mode (NICI) with methane as the reagent gas. The instruments and the separating column used were the same as described above. The source temperature was set at 150 °C. The electron energy was 193.3 eV and the emission current was 49.4 μA. The injector and transfer lines were maintained at 250 °C and 280 °C, respectively. The GC oven was maintained at 150 °C for 3.5 min, increased at the rate of 25 °C/min to 310 °C and held at 310 °C for another 5 min. Quantification of 2-hexadecenal was performed utilizing selected ion monitoring (SIM) by comparing the integrated area corresponding to $m/z = 413$ to that produced from the deuterated internal standard 2-[d_5]-hexadecenal at $m/z = 418$.

For methanolic HCl-derivatized products, crude reaction product extracts or TLC-purified reaction products were resuspended in 1 ml of 1N methanolic HCl and incubated at 90 °C for 90 min under nitrogen. The reaction was allowed to cool for 10 min before addition of sodium carbonate (∼200 mg). For GC–MS analysis, 1 ml of water and 2 ml of petroleum ether was added to extract the derivatized reaction products into organic phase. The organic phase was evaporated under nitrogen and the products were resuspended in 50 μl of petroleum ether. The derivatized products were separated using DB-1 column and analyzed with GC–MS EI ionization as described earlier. Alternatively, for electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis, 5 ml of methanol was added after derivatization and sodium carbonate addition. This mixture was centrifuged at 1000 rpm for 2 min. The supernatant was then analyzed by ESI-MS/MS.

2.6. ESI-MS/MS

The crude reaction product extracts were dried under nitrogen and resuspended either in methanol or methanol/water/acetic acid (50/50/1, v/v/v). These were then injected into the electrospray ionization interface in the direct infusion mode (TSQ quantum ultra, Thermo Electron Corporation) at a flow rate of 3 μl/min. Tandem mass spectrometry (MS/MS) was performed with collision energies ∼22 eV. Typically, spectra were averaged 3–5 min and processed utilizing Xcalibur software (Finnigan). Ions were monitored in positive ion mode. In some cases, ESI-MS/MS was performed on TSQ 7000 (Finnigan). Skimmer collision induced dissociation (CID)–MS/MS was performed at 25 eV.

2.7. HPLC-ESI-MS/MS of 1-cyano methano phosphocholine and SPC

Crude reaction product extracts collected as described earlier were dried under nitrogen and resuspended in 10 ml methanol. Ten microliters was injected on a solid-phase reverse-phase Discovery HS C18 column (5 μm, 150 mm × 2.1 mm). The products were eluted using solvent A: water/methanol/acetic acid (69/30/1, v/v/v) with 5 mM ammonium acetate and solvent B: methanol/acetic acid (99/1, v/v) with 5 mM ammonium acetate as described by Sullards et al for SPC analysis (Sullards and Merrill, 2001). Following injection of the reaction mixture, the column was eluted with 50% solvent A and 50% solvent B for 2 min, the gradient was increased to 100% B over the next 3.6 min and held at 100% B for the next 2.4 min, the gradient was again brought to 50% solvent A and 50% solvent B over the next 0.1 min and held for the next 6.9 min. The 223 → 136.9 transition reaction (for 1-cyano methano phosphocholine) and the 465 → 184.1 transition reaction (for SPC) were monitored in positive ion mode at 28 eV collision energy. Argon gas pressure was set at 1.5 mTorr.

2.8. H-D exchange

Crude reaction products generated by MPO treatment were extracted by Bligh and Dyer technique as mentioned earlier. Chloroform was evaporated and the products resuspended in deuterated methanol with 0.1 mM ammonium deuteroxide and analyzed by ESI-MS/MS.

2.9. Proton NMR spectrometry

Crude reaction products from multiple reactions were extracted in hexane, pooled together and purified by HPLC to separate 2-hexadecenal, as above. Proton NMR spectrometry and analysis were performed at ambient temperature in $CDCl_3$ using Varian Unity 300 spectrometer (7.05 T). ^1H shifts were referenced to solvent peak at 7.24 ppm. The magnetic field strength used was 14.1 T.

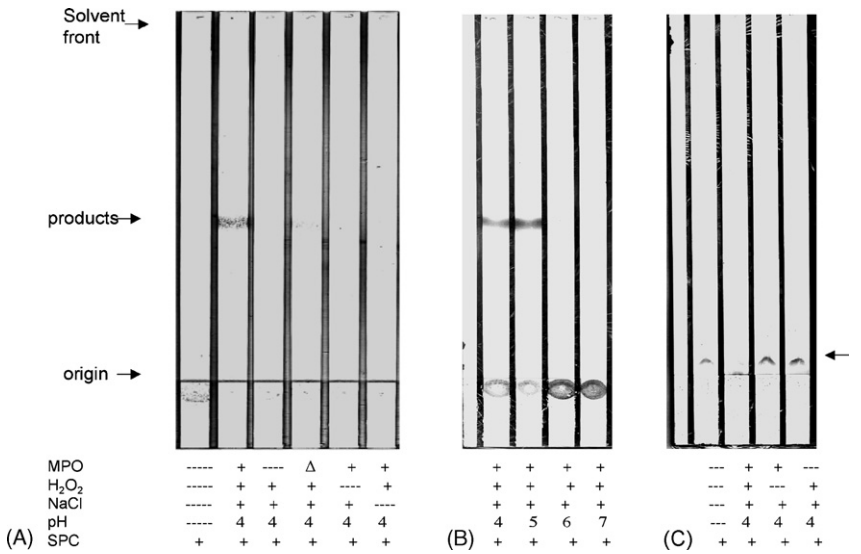


Fig. 1. TLC analysis of MPO-treated SPC. SPC (100 μg) was incubated for 15 min at 37 °C in the presence or absence of each of the MPO-derived RCS generating reagents including, MPO (1U), heat-inactivated (Δ) MPO, H_2O_2 (1 mM), NaCl (100 mM) and 20 mM phosphate buffer at pH 4–7 as indicated. Reactions were terminated by the addition of methanol and products were sequentially extracted into chloroform and separated by TLC using either petroleum ether/ethyl ether/acetic acid (90/10/1, v/v/v) (A and B) or chloroform/acetone/methanol/acetic acid/water (6/8/2/2/1, v/v/v/v/v) as mobile phase (C). The reaction products were visualized by sulfuric acid treatment and charring.

3. Results

MPO is thought to have important roles in the pathophysiology of atherosclerosis. The biological effects of MPO are mediated by RCS. In vascular disease it is likely that MPO-derived RCS attack lipoprotein lipids and proteins (Bergt et al., 2004; Marsche et al., 2004). Since lysosphingolipids are biologically active lipids present in HDL (Nofer et al., 2001), initial experiments were performed to determine the stability of SPC in presence of RCS. TLC analysis revealed that in presence of the complete MPO/RCS generating system (MPO, H_2O_2 and Cl^-) SPC is degraded (Fig. 1C, lane 2) resulting in the production of a neutral lipid (Fig. 1A, lane 2). This neutral lipid was not observed in the absence of MPO, H_2O_2 or sodium chloride from the complete MPO/RCS generating system. Additionally, this neutral product was not produced when heat inactivated MPO was used (Fig. 1A, lane 4). Since acidic conditions are optimal for MPO-derived RCS generation at the concentration of H_2O_2 and sodium chloride used (Hollenberg et al., 1974; Zgliczynski et al., 1977), we characterized the effect of pH on the generation of the neutral lipid product. It is observed that the neutral lipid product was present at pH 4 and 5 but absent at pH 6 and 7 (Fig. 1B). This is most likely due to the decrease in generation of MPO-derived RCS at neutral pH (Hollenberg et al., 1974; Zgliczynski et al., 1977). SPC incubation with sodium hypochlorite also results in the production of this neutral lipid product (data not shown).

The TLC-purified neutral lipid product generated by RCS-attack of SPC was analyzed by GC–MS using electron impact ionization. Fig. 2 shows the mass spectra of the TLC-purified product having a retention time of 5.6 min. The molecular ion at *m/z* 238 and its fragmentation pattern is depicted in Scheme 1, showing the McLafferty rearrangement ion as the base peak at *m/z* 70. The molecular ion and fragmentation pattern is consistent with a 16 carbon unsaturated aldehyde, 2-hexadecenal, generated from the degradation of the sphingosine base of SPC.

To further confirm the structure and presence of the carbonyl group, TLC-purified products were derivatized using PFB hydroxylamine (Hsu et al., 1999). The PFB derivatives were analyzed by GC–MS in NICI mode. Fig. 3 shows the mass spectrum and fragmentation scheme of PFB-oxime of 2-hexadecenal having a retention time of 8.24 min. The spectrum shows the structurally informative ions at *m/z* 433 ($[M]\bar{\bullet}$), *m/z* 413 ($[M - HF]\bar{\bullet}$), *m/z* 383 ($[M - HF - NO]\ \bar{\bullet}$) and *m/z* 252 ($[M - 181]\bar{\bullet}$), along with the ions at *m/z* 178 and *m/z* 196 that arise from the dissociation of the PFB residue (Hsu et al., 1999). The presence of the carbonyl group in the molecule was further confirmed by acid methanolysis that results in the formation of a dimethyl acetal from TLC-purified material. The dimethyl acetal derivative was analyzed by GC–MS with electron impact ionization. A peak at 6.5 min that gives a molecular ion and fragmentation pattern corresponding to a dimethyl acetal of 2-hexadecenal was observed (Fig. 4).

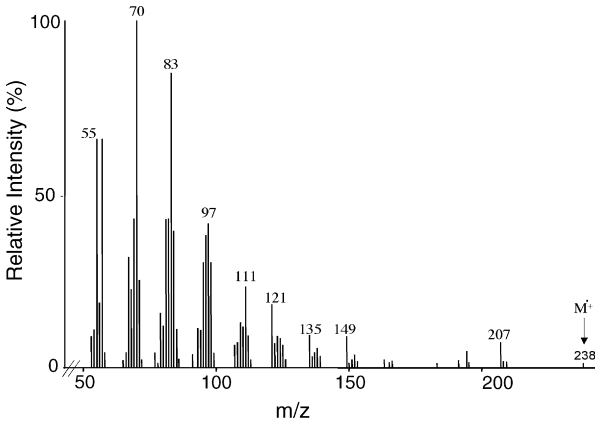
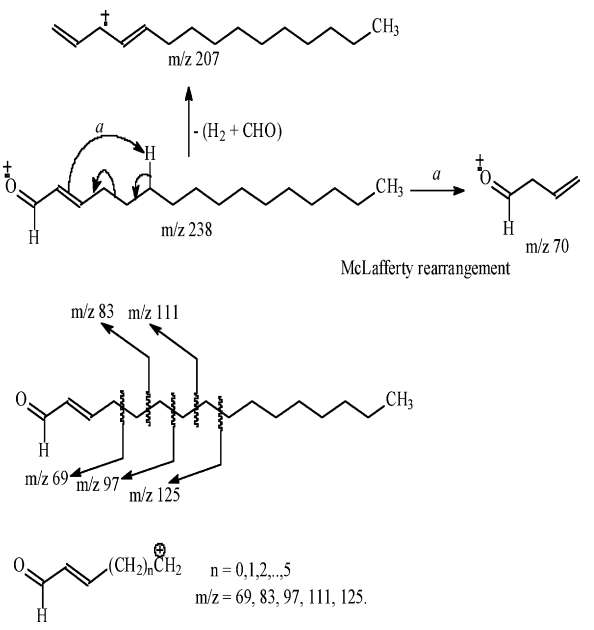


Fig. 2. Electron impact GC–MS analysis of MPO-treated SPC. SPC (100 μg) was incubated with MPO-derived RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4. The reaction products were extracted into chloroform. The neutral lipid product was purified by TLC and subsequently analyzed by GC–MS using electron impact (EI) ionization as described under Section 2. The EI spectra of the neutral lipid product is depicted.



Scheme 1. Fragmentation scheme for 2-hexadecenal.

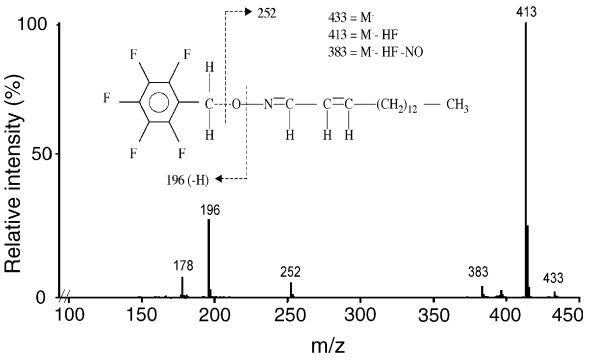


Fig. 3. PFB hydroxylamine derivatization and GC–MS analysis of MPO-treated SPC. SPC (100 μg) was incubated with MPO-derived RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4. The reactions were terminated with methanol. Reaction products were sequentially extracted into chloroform and derivatized with pentafluorobenzyl hydroxylamine and analyzed by GC–MS in negative ion chemical ionization (NICI) mode. The mass spectra for the PFB oxime of 2-hexadecenal is shown along with the fragmentation scheme (inset).

To further confirm the position of the double bond of 2-hexadecenal, proton NMR was performed. The 2-hexadecenal generated by MPO-derived RCS treatment of SPC was HPLC purified for NMR analysis. Semi-synthetic 2-trans-hexadecenal was used as reference (Baumann et al., 1969). The NMR spectrum of HPLC purified 2-hexadecenal derived from SPC and MPO-derived RCS reaction was identical to that of the

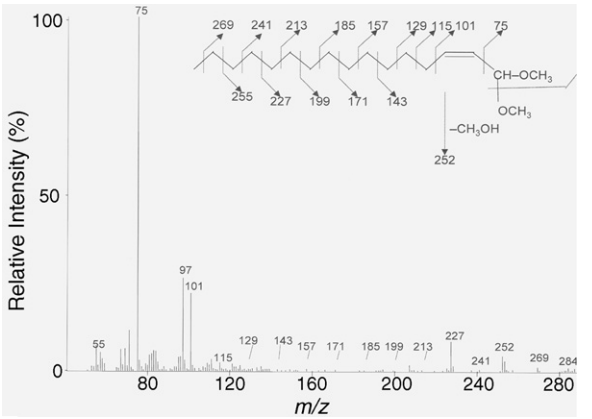


Fig. 4. Methanolic HCl derivatization and GC–MS analysis of MPO-treated SPC. SPC (100 μg) was incubated with MPO-derived RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4. The reaction products were extracted into chloroform and purified by TLC. The TLC-purified neutral lipid product was subjected to acid methanolysis and the derivatization product was analyzed by GC–MS using electron impact ionization mode. The fragmentation spectra and fragmentation scheme (inset) are depicted.

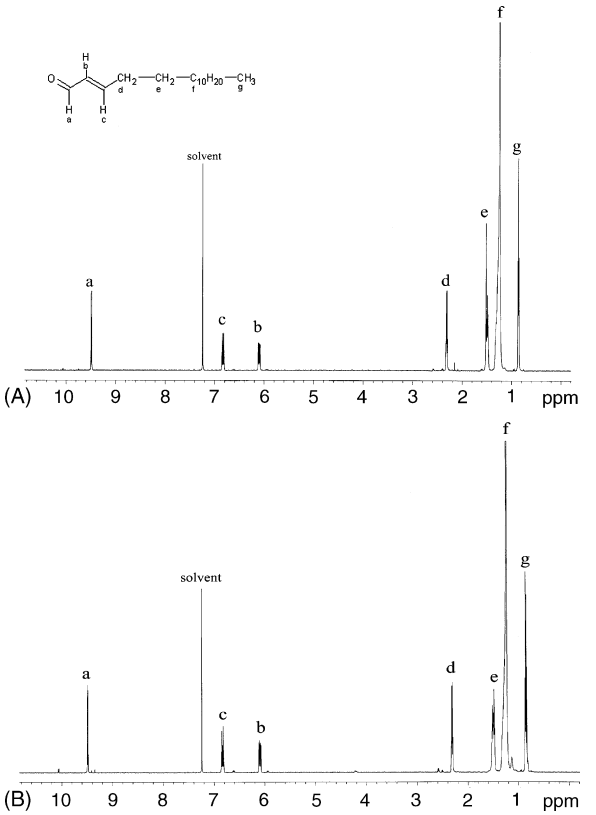


Fig. 5. NMR analysis of 2-hexadecenal. Semi-synthetic 2-hexadecenal (A) and the putative 2-hexadecenal product from MPO-derived RCS targeting of SPC (B) were prepared and subjected to proton NMR as described in Section 2. Peak assignments are shown in inset in Fig. 4A.

reference (Fig. 5). This confirmed that MPO-derived RCS generate 2-hexadecenal from SPC.

Having identified the neutral lipid product as 2-hexadecenal, we confirmed the involvement of MPO-derived RCS in reactions including inhibitors of MPO production of RCS. SPC was incubated with MPO-derived RCS generating system including MPO, H_2O_2 and sodium chloride in phosphate buffer (pH 4.0). Before starting the reactions, MPO was pre-incubated either with aminotriazole or sodium azide, while catalase was pre-incubated with hydrogen peroxide. The reactions were initiated by addition of MPO and allowed to incubate for 15 min at 37 °C. At the end of the incubation period the reaction products were extracted and analyzed by TLC. As observed in Fig. 6 the presence of aminotriazole, sodium azide and catalase inhibited the formation of 2-hexadecenal by MPO-derived RCS.

In order to test whether other bioactive lysosphingolipids, sharing the common sphingosine base, can be attacked by MPO-derived RCS to generate 2-hexadecenal, we incubated S1P with MPO-derived RCS

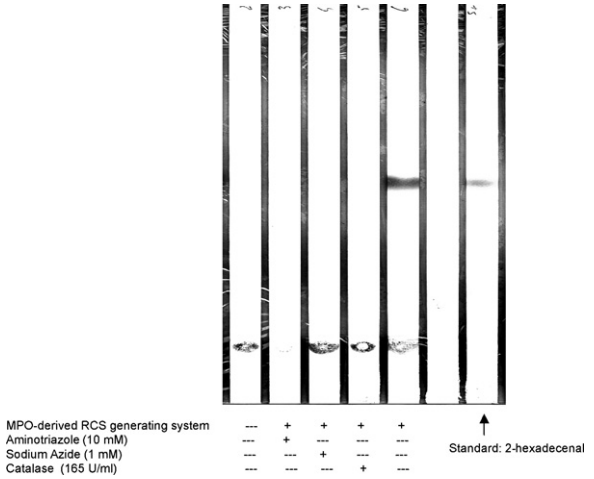


Fig. 6. MPO-dependence for 2-hexadecenal generation. SPC (100 μg) was incubated with MPO/RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4. 3-Amino triazole and sodium azide were preincubated with MPO for 5 min and catalase was preincubated with H_2O_2 prior to addition of MPO to the reaction mixture as described in Section 2. Reaction products were sequentially extracted into chloroform followed by TLC separation using a mobile phase of petroleum ether/ethyl ether/acetic acid (90/10/1, v/v/v). The products were visualized by sulfuric acid treatment and charring.

generating system. At the end of the incubation period the reaction products were analyzed after derivatization with PFB hydroxylamine utilizing GC–MS. Fig. 7 shows two peaks generated from the *syn*- and the *anti*-isomers

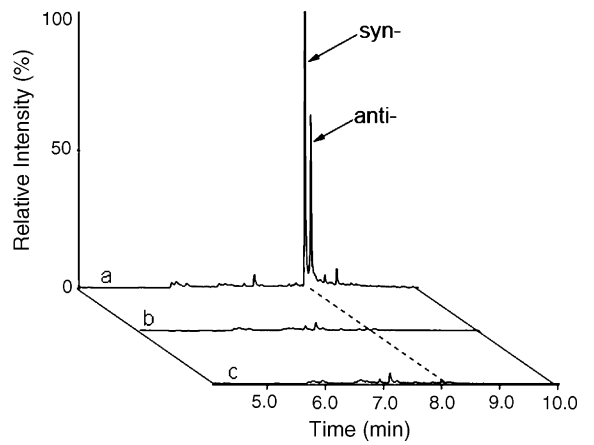


Fig. 7. MPO-derived RCS targets S1P: S1P (100 μg) was incubated with MPO-derived RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer (pH 4) for 15 min. At the end of the incubation period the reaction products were derivatized by PFB hydroxylamine and analyzed by GC–MS as detailed under Section 2. Figure shows the total ion chromatogram generated by the derivatized reaction products of S1P incubated with MPO-derived RCS (trace a), in the absence of H_2O_2 (trace b) or in the absence of MPO (trace c). The *anti*- and *syn*-isomers of PFB oxime of 2-hexadecenal are labeled (trace a).

of PFB oxime of 2-hexadecenal in presence of the MPO-derived RCS generating system (Fig. 7, trace a). In the absence of either H_2O_2 or MPO from the reaction mixture these peaks are also absent (Fig. 7, trace b and c). Further, the identities of these peaks as PFB oximes of 2-hexadecenal have been confirmed by mass spectrometry. Thus, it is believed that 2-hexadecenal can be generated from MPO-derived RCS attack on lysosphingolipids containing a sphingosine base.

We have thus far confirmed the identity of 2-hexadecenal utilizing TLC, GC–MS analysis, GC–MS analysis of PFB oxime derivative and dimethyl acetal derivative of 2-hexadecenal and by NMR studies. We have also confirmed the involvement of MPO using heat-treated MPO and MPO inhibitors. Furthermore, it has been shown that MPO-derived RCS attack both SPC and S1P to generate 2-hexadecenal.

To identify the by-product of SPC and MPO-derived RCS reaction generating 2-hexadecenal, we performed ESI-MS/MS analysis by direct infusion of the crude reaction product extracts derived from SPC and MPO-derived RCS reaction. In the positive ion mode, two ions at *m/z* 223 and 245 were indicated as probable candidates. The fragmentation spectra of these ions are depicted in Fig. 8A and B. The presence of a peak at *m/z* 23 in the product-ion spectra of *m/z* 245 identified the ion at *m/z* 245 as a sodiated adduct ion of *m/z* 223 (Fig. 8B). The product-ion spectra of $[M+H]^+$ ion at *m/z* 223 and of the $[M+Na]^+$ ion at *m/z* 245 show the base peak fragment ion at *m/z* 86, corresponding to an ethylene-trimethyl ammonium ion (Scheme 2). Additionally, fragment ions at *m/z* 164 and 186 arising from loss of the trimethylamine residue (59 Da), from $[M+H]^+$ and $[M+Na]^+$, respectively, are observed. These fragment ions are characteristic of the phosphocholine head group (Ayanoglu et al., 1984). To further reveal the structure, we also obtained the product-ion spectra of the ion at *m/z* 164 generated by skimmer CID (Fig. 8C). The spectra contains ions at *m/z* 137 probably arising from a loss of HCN residue and at *m/z* 107 arising from the cleavage of the P-O bond (Scheme 2). The combined information suggested that the compound might represent 1-cyano methano phosphocholine. The structure assignment is also consistent with the results obtained from similar ESI-MS/MS analysis of the corresponding ions after H-D exchange. The results indicate the presence of one exchangeable hydrogen and an analogous loss of DCN is also observed (data not shown).

To confirm the presence of the cyano group in the compound we also performed an acid methanolysis reaction using methanolic HCl on the crude reaction product extracts followed by structural analysis

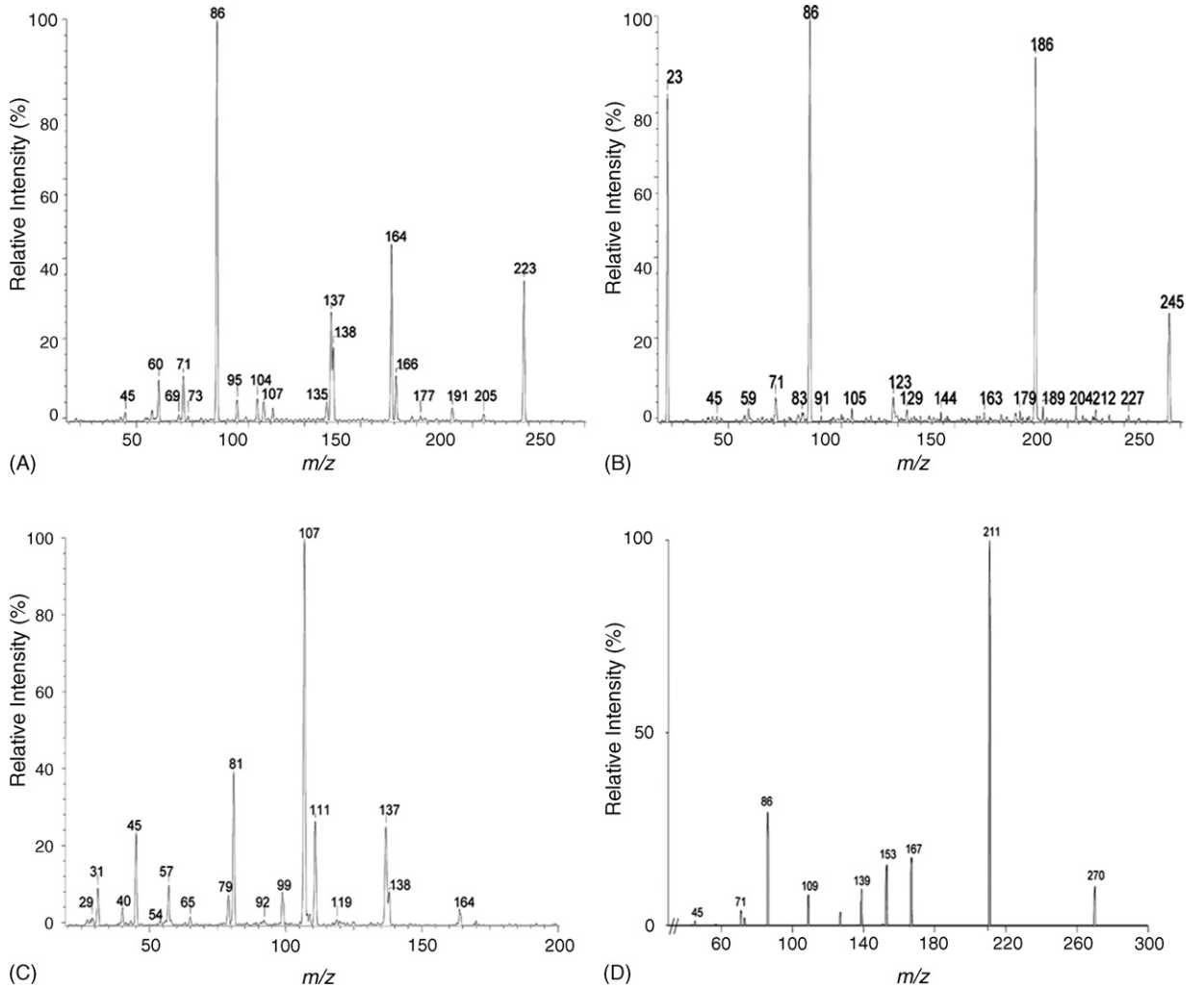
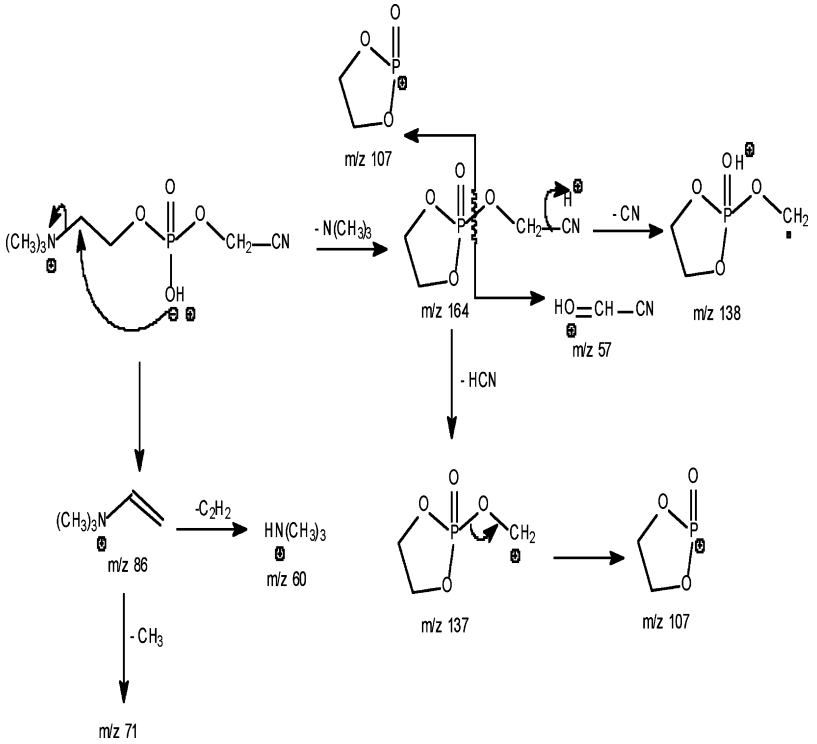


Fig. 8. Identification of 1-cyano methano phosphocholine. SPC (100 μg) was incubated with MPO-derived RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4. The products were extracted and analyzed by ESI-MS/MS; (A) Product-ion spectra of *m/z* 223 $[M+H]^+$; (B) Product-ion spectra of *m/z* 245 $[M+Na]^+$; (C) Product-ion spectra of *m/z* 164 generated by skimmer CID. The extracts were also derivatized by acid methanolysis and analyzed by ESI-MS/MS. (D) Shows the product-ion spectra for methyl ester of 1-cyano methano phosphocholine at *m/z* 270 $[M]^+$ species.

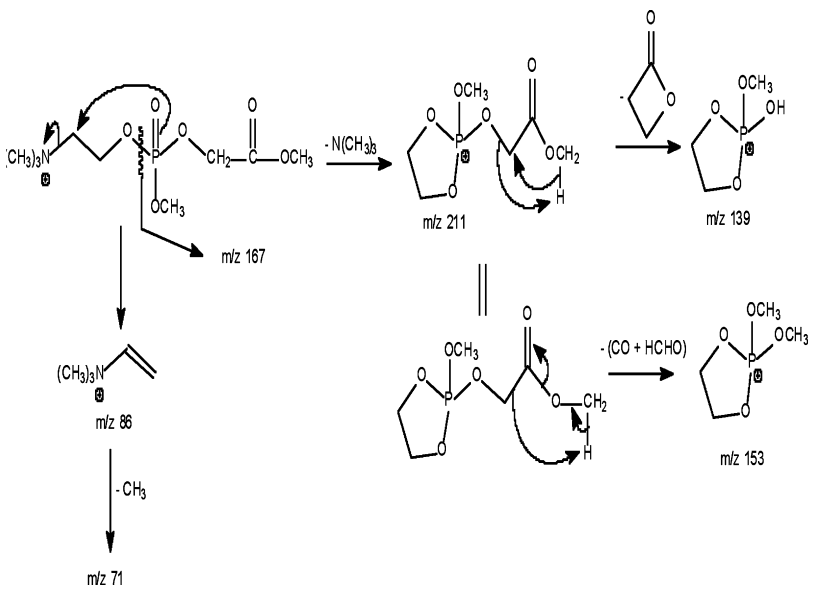
with ESI-MS/MS. Indeed, a molecular species and its product-ion spectra that are consistent with the structure of a methyl ester of 1-cyano methano phosphocholine were observed (Fig. 8D, Scheme 3). Having identified the fragment ions, we utilized HPLC-MS/MS selected reaction monitoring technique (Fig. 9) to confirm that 1-cyano methano phosphocholine is generated with a corresponding reduction in SPC when MPO-derived RCS reacts with SPC. In contrast, 1-cyano methano phosphocholine is not formed in the absence of either MPO or H_2O_2.

Previously hypochlorous acid has been shown to attack –C=C– bonds in phosphatidylcholine to form monochlorinated and chlorohydrin products (Winterbourn et al., 1992). Since the sphingosine base in SPC is also unsaturated we addressed the possibility of monochlorinated and chlorohydrin product formation from MPO-derived RCS attack on SPC. SPC was incubated with MPO-derived RCS generating reagents as described earlier and the reaction products were analyzed with ESI-MS/MS. Fig. 10 shows the product-ion spectra of the monochlorinated SPC (*m/z* = 499) (Fig. 10A) and SPC chlorohydrin (*m/z* = 517) (Fig. 10B). The product-ion spectra of the $[M+H]^+$ at *m/z* 499 shows the base peak at *m/z* 184 along with ions at *m/z* 440 and 316 which arise from neutral losses of 59 and 183, respectively. Additionally, an ion at *m/z* 463, arising from the neutral loss of 36 (loss of HCl) was also observed. The



Scheme 2. Fragmentation scheme for 1-cyano methano phosphocholine.

spectrum suggested that the compound is a monochlorinated SPC. The product-ion spectra of the $[M+H]^+$ ion at *m/z* 517 contains ions at *m/z* 499 (517—H_2O) and 463 (499—$H^{35}Cl$) consistent with the suggested structure as a chlorohydrin. It should be mentioned however, we did not confirm the positions assigned for chlorine and hydroxyl group in these structures. Moreover, the possibility that this monochlorinated SPC is a chloramine cannot be excluded. Although, on incubating sphinganylphosphorylcholine, which has a free amino



Scheme 3. Fragmentation scheme for methyl ester of 1-cyano methano phosphocholine.

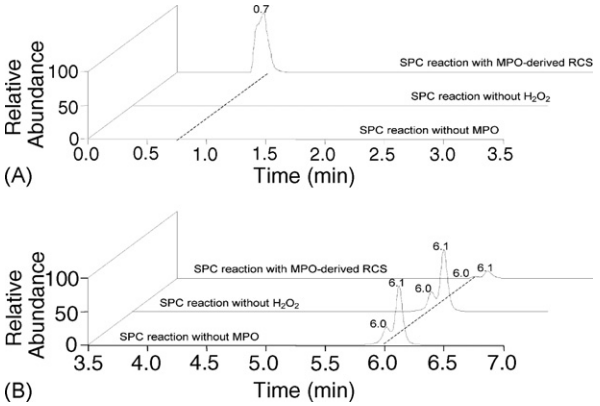


Fig. 9. LC/ESI-MS/MS of 1-cyano methano phosphocholine and SPC. SPC (100 μg) was incubated with or without MPO/RCS generating reagents, which includes MPO (2U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4 as described in Section 2. The reaction products were subjected to HPLC/ESI-MS/MS analysis as described in Section 2 with SRM of 223 → 136.9 (A) for 1-cyano methanophosphocholine and the 465 → 184.1 (B) for SPC as described in Section 2. The chromatograms shown are representative of three independent experiments.

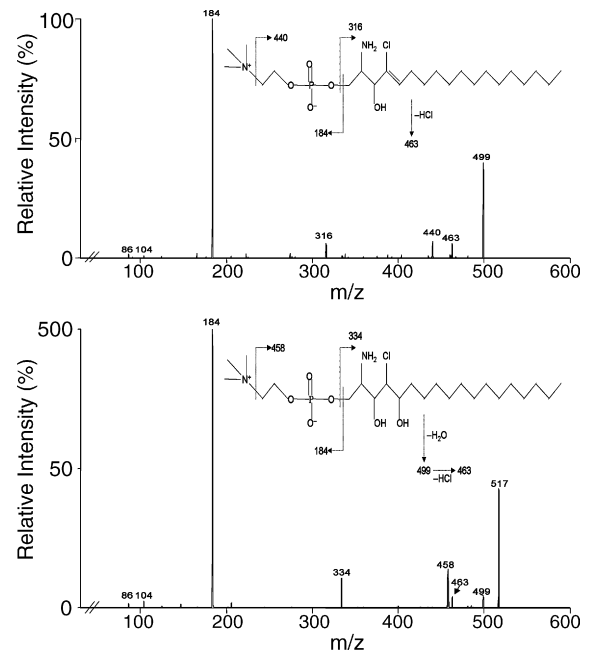


Fig. 10. Identification of monochlorinated SPC and SPC chlorohydrin. SPC (100 μg) was incubated with MPO-derived RCS generating reagents, which includes MPO (1U), H_2O_2 (1 mM) and NaCl (100 mM) in 20 mM phosphate buffer at pH 4. The products were extracted and analyzed by ESI-MS/MS in positive ion mode; (A) Depicts the fragmentation spectrum of monochlorinated SPC (*m/z* 499 $[M+H]^+$); (B) Depicts the fragmentation spectrum of SPC chlorohydrin (*m/z* 517 $[M+H]^+$). The insets show putative fragmentation schemes for respective compounds.

group similar to SPC but does not possess the –C=C– in the sphingosine base, with MPO-derived RCS generating system does not yield the corresponding ion at *m/z* = 501 (data not shown).

Since *in vitro* experiments revealed the production of 2-hexadecenal from SPC and S1P, further studies were performed to determine the susceptibility of HDL-associated lysosphingolipids to RCS attack. In these studies, we used GC–MS selected ion monitoring for the quantification of PFB-oxime derivatives of 2-hexadecenal with synthetic 2-[d_5]-hexadecenal as an internal standard. Base peaks observed at *m/z* 413 and 418 in the NICI spectra for the PFB oxime derivative of 2-hexadecenal and 2-[d_5]-hexadecenal, respectively were used for quantification of 2-hexadecenal. A linear relationship is observed between the ratio of the integrated peak area of *m/z* 413 and *m/z* 418 using SIM GC–MS (data not shown). We have previously used a similar technique for quantification of 2-chlorohexadecanal in biological tissue (Thukkani et al., 2002). The generation of 2-hexadecenal was then quantified after HDL was treated with MPO and H_2O_2 in phosphate-buffered saline at pH 4.0, using SIM GC–MS for *m/z* 413 (from PFB oxime of 2-hexadecenal) and *m/z* 418 ions (from PFB oxime of 2-[d_5]-hexadecenal). As shown in Fig. 11, 2-hexadecenal was increased when HDL was treated with complete reaction mixture capable of generating RCS. In contrast a similar increase in 2-hexadecenal was not detected when MPO or H_2O_2 is not present in the reaction mixture. Taken together with studies using SPC, this leads us to conclude that MPO-derived RCS can attack HDL-associated lysosphingolipids.

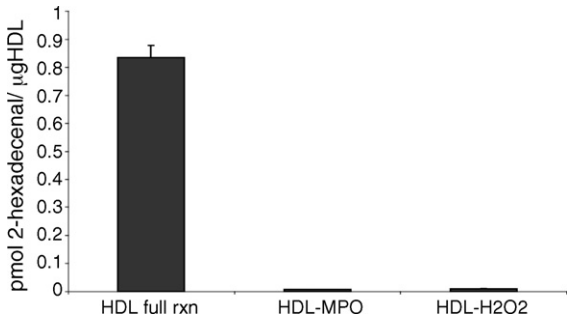


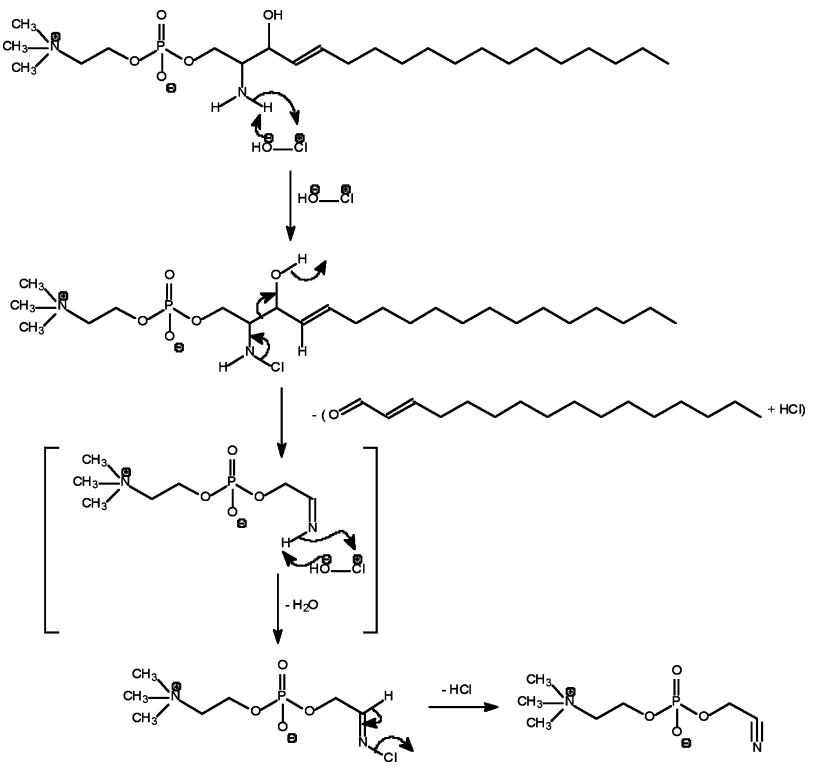
Fig. 11. HDL (200 μg) protein was incubated in the presence or absence MPO/RCS generating reagents including MPO (2U), H_2O_2 (1 mM), NaCl (100 mM) in 20 mM phosphate buffer at pH 4 for 25 mins at 37 °C. For each condition, 2-hexadecenal was quantified following derivatization with pentafluorobenzyl hydroxylamine and analysis by NICI GC–MS using 2-[d_5]-hexadecenal as internal standard. Each value represents the mean ± S.E.M of three independent experiments.

4. Discussion

MPO-derived RCS are generated as a part of host defense mechanism (Albrich et al., 1981), however they are also known to attack host tissue (Weiss, 1989) and may be involved in pathogenesis of atherosclerosis (Podrez et al., 2000). In this study we describe a possible mechanism through which MPO-derived RCS can react with HDL associated lysosphingolipids to generate 2-hexadecenal, an α,β-unsaturated aldehyde. First, we identified the products that can be generated from MPO-derived RCS attack on SPC as 2-hexadecenal and 1-cyano methano phosphocholine. Structural confirmation for 2-hexadecenal was obtained by NMR and GC–MS studies, while 1-cyano methano phosphocholine was confirmed with ESI-MS/MS studies of the parent compound, after H-D exchange and after acid methanolysis derivatization. We propose the formation of 2-hexadecenal and 1-cyano methano phosphocholine from MPO-derived RCS attack of SPC as shown in Scheme 4. The initial attack of hypochlorous acid would form a chloramine leading to formation of 2-hexadecenal as a stable intermediate with loss of HCl. Secondary attack by hypochlorous acid then leads to formation of 1-cyano methano phosphocholine through an imine intermediate.

HDL-associated lysosphingolipids have been shown to partly account for the atheroprotective effects of HDL (Kimura et al., 2001; Nofer et al., 2003; Nofer et al., 2001; Nofer et al., 2004). Lysosphingolipids also display other atheroprotective actions such as inhibition of thrombin generation (Deguchi et al., 2004) and inhibition of platelet activation (Altmann et al., 2003). It is already known that MPO-derived RCS can attack HDL-apolipoprotein A-I in the arterial wall and affect cholesterol transport (Bergt et al., 2004; Zheng et al., 2004). Herein, we show that MPO-derived RCS can attack HDL-associated lysosphingolipids. HDL has been reported to contain 1.2 and 4.7 μg/mg protein of SPC and lysosulfatide, respectively (Nofer et al., 2001). It is likely that these are the primary HDL sphingolipids attacked by RCS. It is also possible that HDL S1P and sphingosine are attacked by RCS as well. Taken together these data suggest that during vascular wall injury, MPO-derived RCS can attack HDL-associated lysosphingolipids and alter the atheroprotective potential of HDL.

Lysosphingolipids may be attacked by MPO-derived RCS *in vivo* and MPO-derived RCS oxidation might provide an alternative pathway of lysosphingolipid degradation under these conditions. It is likely that 2-hexadecenal is metabolized by cellular enzymes such as 2-alkenal reductase and aldehyde dehydrogenase (van



Scheme 4. Reaction mechanism.

Veldhoven and Mannaerts, 1993). It is also possible that, 2-hexadecenal, being an α,β-unsaturated aldehyde forms Schiff base adducts and Michael adducts with primary amino and thiol groups similar to 4-hydroxynonenal and might affect protein function (Crabb et al., 2002; Okada et al., 1999).

Recently, it has been shown that S1P/S1P lyase products have similar proliferative actions as S1P (Kariya et al., 2005). Moreover, a role for a ceramide metabolite in cell toxicity has also been suggested (Tserng and Griffin, 2004). Thus, it is likely that 2-hexadecenal and 1-cyano methano phosphocholine possess biological activity and it would be of interest to learn these in an effort to understand the pathogenesis of atherosclerosis.

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