Analysis by Fast-Atom Bombardment Tandem Mass Spectrometry of Phosphatidylcholine Isolated from Heart Mitochondrial Fractions: Evidence of Incorporation of Monohydroxylated Fatty Acyl Moieties

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Phosphatidylcholine (PC) is one of the main phospholipids present in mitochondrial membranes. According to current knowledge, the predominant fatty acyl moieties in this phospholipid are 16, 18, 20, or 22 carbon atoms long with chains that contain only carbon and hydrogen atoms. We have conducted a detailed analysis of the fatty acid substituents of the phospholipids present in mitochondrial fractions by using fast-atom bombardment tandem mass spectrometry. Six monohydroxylated C-16 and C-18 fatty acyl moieties were found in PC extracted from mitochondrial fractions of rat heart. The structure of one of these monohydroxylated fatty acids has been elucidated and corresponded to 12-hydroxy 9-octadecenoic acid. Indications that concern the structure of the five other monohydroxylated fatty acids are presented. These monohydroxylated fatty acyl groups are preferentially associated in the PC molecule with C-18 and C-20 fatty acyl moieties. We present arguments to suggest that the formation of these compounds is probably not due to a free-radical initiated mechanism. The potential implication of these monohydroxylated fatty acids in several physiological functions is suggested by the fact that free hydroxylated fatty acids that are identical or closely related to those found in the mitochondrial fractions possess various biological activities. *(J Am Soc Mass Spectrom 1996, 7, 50–58)*

Glycerophospholipids are the major components of cell membranes in living organisms. There are five main forms: phosphatidylcholine (PC), phosphatidylethanolamine, cardiolipin, phosphatidylinositol, and phosphatidylserine. In mammalian cell membranes, PC is the most abundant phospholipid [1]; it accounts for up to 41% of the total phospholipid content of rat heart mitochondrial membranes [2].

The chemical and physical properties of phospholipids make these compounds difficult to analyze. Classical methods of analysis are generally time-consuming and multistep processes usually require degradation of the complex lipid into simpler constituents and analysis of the component parts via chromatographic or spectroscopic techniques.

In 1986, Jensen et al. were one of the first groups to recognize the usefulness of fast-atom bombardment (FAB) ionization and tandem mass spectrometry for the analysis of phospholipids and for the chemical characterization of the carboxylate components of these complex lipids. They used a three-sector mass spectrometer with an electric sector to analyze the product ions after collision-induced dissociation (CID) of negative ions from PC. The product ion resolution of this instrument was approximately 100, which limited to

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some extent the possible analysis of fatty acyl components of phospholipids that differ by a single double bond. Later, several groups that used a tandem instrument with better product ion resolution confirmed the observations of Jensen et al. and extended the analysis to other PC molecular species [4–9]. The Murphy group [5], for example, successfully used negative-ion fast-atom bombardment tandem mass spectrometry (FAB/MS-MS) to characterize arachidonate-containing phosphatidylcholines. With the same technique, they studied choline phospholipid molecular species related to platelet activating factor [6] and oxidatively modified phospholipids [7, 8]. The use of FAB/MS-MS for the study of phospholipids has the advantage that it provides structural information about the fatty acid substituents esterified in phospholipid molecules. Indeed, the determination of the presence of structural modifications on the hydrocarbon chain can be performed by collisional activation of FAB-desorbed carboxylate anions [10–14].

Tandem mass spectrometry is also a useful technique for the analysis of complex mixtures of PC molecular species extracted from biological samples. FAB/MS-MS and FAB/MS^3, used in both positive- and negative-ion modes, allowed the characterization of PC molecules extracted from a purified preparation of human immunodeficiency virus type I (HIV-1), the etiologic agent of acquired immune deficiency syndrome [15]. Exploitation of this technique of tandem mass spectrometry enabled direct determination of phospholipid structures in microorganisms [16] and structural characterization of F2-isoprostane-containing phospholipids [17].

We report the use of FAB/MS-MS for the analysis of PC isolated from rat heart mitochondrial fractions. This technique allowed us to detect and characterize six novel fatty acid substituents in PC molecules. These substituents, which are C-16 and C-18 monohydroxylated fatty acyl groups, are present under physiological conditions. Several biological activities have been described for free hydroxylated fatty acids that are identical [18–23] or closely related [24–28] to those we observed; thus their potential biological importance is emphasized.

Materials and Methods

Chemicals

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, cardiolipin [*R*]-12-hydroxy-*cis*-9-octadecenoic acid, and Nagarse were purchased from Sigma Chemical Co. (St. Louis, MO). High-performance thin-layer chromatography (HPTLC) silica gel 60 aluminum sheets were purchased from Merck (Darmstadt, Germany). Chloroform, methanol, and other chemicals of analytical grade were obtained from either Merck or Boehringer (Mannheim, Germany), 2,6-Di-*tert*-butyl-*p*-cresol (BHT) was obtained from Fluka (Bornem, Belgium) and *m*-nitrobenzyl alcohol was obtained from Janssen Pharmaceutica (Beerse, Belgium).

Cell Fractionation

Fed adult male Wistar rats, maintained on a standard laboratory chow diet (AO4, U.A.R., Epinay-sur-Orge, France), were used in all experiments. To prepare heart mitochondrial fractions, freshly isolated hearts were minced with scissors and subjected to proteolytic digestion by Nagarse for 5 min at 0 °C. They were then homogenized in a glass–Teflon Potter–Elvehjem device and the mitochondrial fractions were obtained by centrifugation [29].

Heart Perfusion

Rat hearts were perfused in a Langendorff system at 37 °C with a standard Krebs–Henseleit bicarbonate buffer that contained 11-mM glucose and 1.25-mM $CaCl_2$ as previously described [29]. The perfusion protocol included 15 min of equilibration followed by 45 min under normoxic conditions (controls) or by 45 min of global ischemia (ischemics) followed in some cases by reperfusion for 10 min under normoxic conditions (reperfused). At the end of these perfusions, the hearts were used immediately to prepare the mitochondrial fraction [29].

Extraction and Separation of Phospholipids

The subcellular fractions were extracted immediately after their preparation [30] with chloroform–methanol (2:1, vol/vol) that contained butylated hydroxytoluene (BHT; 0.007%). The phospholipids were then separated by HPTLC [31] and their phosphorus content was measured [32]. Whenever possible, the preparative steps were performed under N_2 to avoid lipid peroxidation.

Analysis of Fatty Acids by Gas Chromatography–Mass Spectrometry

The fatty acid composition of each phospholipid was determined by gas chromatography (GC) analysis of either the methyl ester or trimethylsilyl derivatives in a Hewlett-Packard (Brussels, Belgium) model 5890 gas chromatograph coupled to a Hewlett-Packard model 5971 mass spectrometer. PC fractions were transmethylated with methanol–HCL (3 M) at 90 °C for 15 min [33] and the fatty acid methyl esters were subjected to GC on a Hewlett-Packard (Brussels, Belgium) Ultra II capillary column (25 m × 0.11 μm), and the N_2 flow at 1 mL/min. The temperature gradient was as follows: 2 min at 50 °C, then stepwise increases to 150 °C (20 °C/min), to 240 °C (5 °C/min), and finally to 310 °C

(25 °C/min). The trimethylsilylated derivatives were obtained, after alkaline hydrolysis of the phospholipid (NaOH 4N for 4 h at 95–100 °C), by derivatization in the presence of bis-(trimethylsilyl)trifluoroacetamide at 70 °C for 40 min. These fatty acid derivatives were subjected to gas chromatography-mass spectrometry (GC/MS) as described in preceding text. Derivatized fatty acids were identified by comparison of both their electron impact mass spectra and their relative retention times with those of standards under the same conditions.

Analysis of the Phospholipids by Fast-Atom Bombardment Tandem Mass Spectrometry

All mass spectra were obtained with a Finnigan TSQ 70 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA). The FAB gun (Ion Tech, Teddington, UK) was operated at 50 mA with xenon accelerated to 8 keV. Xenon was used as the collision gas at a pressure of approximately 0.8 mtorr in the second quadrupole region of the mass spectrometer. The collision offset voltage was between 3 and 10 V. The purified preparation of PC was dissolved in methanol and mixed in *m*-nitrobenzyl alcohol, which was used as a liquid matrix on the probe tip. Unit resolution was utilized for both precursor and product ions. The scan time was 3 s per full scan. Five to ten spectra were averaged.

Results

Relative Proportions of Phospholipids in Heart Mitochondrial Fractions

The total content of phospholipids from heart mitochondrial fractions measured by phosphorus assay [32] amounted to 352 ± 11-nmol/mg protein [mean ± standard error of the mean (SEM), $n = 6$], which is similar to the values of 327–340-nmol/mg protein reported by Palmer et al. [2]. When the phospholipids were separated into their different subclasses by HPTLC, phosphatidylcholine represented 42.4 ± 2.4%, phosphatidylethanolamine represented 42.0 ± 1.8%, and cardiolipin represented 10.7 ± 0.8% of the total phospholipids (mean ± SEM, $n = 6$). Phosphatidylinositol and phosphatidylserine, which co-migrated and were not separated in our system, together represented 4.9 ± 0.6% (mean ± SEM, $n = 6$) of the total phospholipids. This is consistent with published values [2, 34].

Determination of the Fatty Acid Composition of Phosphatidylcholine by Gas Chromatography–Mass Spectrometry

For the subsequent studies of the fatty acid components via GC/MS, we focused on PC because of its relative abundance. The trimethylsilylated derivatives obtained after hydrolysis and derivatization of PC extracted from rat heart mitochondrial fractions were separated by GC and identified by comparison of their relative retention times and mass fragmentation patterns with those of standards. In agreement with published data [2, 34], the most abundant fatty acids were 16:0, 18:2, 18:1, 18:0, and 20:4. However, detailed analysis of the chromatogram revealed the presence of traces of compounds that did not correspond to any fatty acyl groups previously described in phospholipids. One of these compounds had the same retention time and mass fragmentation pattern as the trimethylsilylated derivative of authentic 12-hydroxy 9-octadecenoic acid (12-HOME). Traces of a compound that had the same retention time and mass fragmentation pattern as authentic 12-HOME methyl ester also were detected in the chromatogram of the fatty acid methyl esters obtained after transmethylation of PC. The unexpected presence of this fatty acid in mammalian tissues prompted us to analyze intact PC by FAB/MS-MS.

Analysis of Heart Mitochondrial Phosphatidylcholine by Fast-Atom Bombardment Tandem Mass Spectrometry

The characteristics of PC analyzed by negative-ion FAB/MS have been described in detail [3]. PC is fragmented in the source to generate low mass ions that correspond to free fatty acid carboxylates and high mass ions that represent losses of 15, 60, and 86 μ from the molecular ion. All phosphorylcholine-containing lipids yielded this triplet set of anions, which corresponds to $[M - CH_3]^-$, $[M - CH_3 - NH(CH_3)_2]^-$, $[M - CH_3 - CH_2{=}CHN(CH_3)_2]^-$, that represents losses from the choline moiety during FAB ionization.

In the low mass region, the negative-ion FAB mass spectrum of PC isolated from rat heart mitochondrial fractions displayed peaks at *m/z* 255, 279, 281, 283, 303, and 305 (Figure 1), which corresponds to 16:0, 18:2, 18:1, 18:0, 20:4, and 20:3 carboxylate anions. These results are in good agreement with our data obtained by GC/MS and with data reported in the literature [2]. Groups of high mass ions also appeared in this spectrum. These ions corresponded to losses of 15, 60 and 86 u from different PC molecular species (Figure 1 and Table 1).

Detailed examination of the negative-ion FAB mass spectrum, combined with analysis of product ion spectra obtained by CID of the high mass ions that correspond to M-15, M-60, and M-86 ions of the PC molecular species, allowed us to determine how the fatty acyl pairs are associated in the PC molecule. Palmitate may be accompanied, in the PC molecule, by linoleate, oleate, or stearate, as evidenced by the presence in the FAB mass spectra of triplet sets of high mass ions at

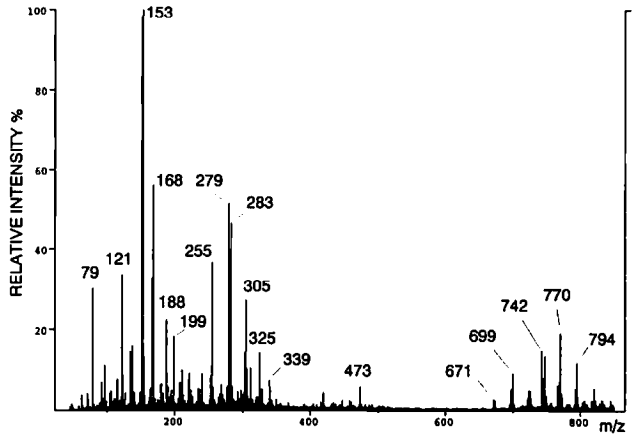

Figure 1. Negative-ion FAB mass spectrum of PC isolated from a rat heart mitochondrial fraction. The spectrum displays low mass ions that correspond to free fatty acid carboxylates and high mass ions that correspond to M-15, M-60, and M-86 PC molecular species. The fragment at *m/z* 153 corresponds to the liquid matrix and peaks that appear at *m/z* 121, 151, 168, 188, 199, 311, 325, and 339 were also present in the blank and correspond to substances present in silica gel plates. The fatty acid compositions of the most intense M-15, M-60, and M-86 ions present in this spectrum are reported in Table 1.

m/z 742, 744, 746, *m/z* 697, 699, 701, and *m/z* 671, 673, and 675, which correspond to M-15, M-60, and M-86 ions of these molecular species. Palmitate also may be associated with arachidonate in the PC molecule as indicated by the presence of three ions at *m/z* 766, 721, and 695. At the *sn*-1 and *sn*-2 positions, phosphatidylcholine molecules also may contain two C-18 fatty acyl groups, either of linoleate, oleate, or stearate, as indicated by the presence of triplet sets of ions at *m/z* 766, 768, 770, and 772, *m/z* 721, 723, 725, and 727, *m/z* 695, 697, 699, and 701. Finally, peaks at *m/z* 792, 794, *m/z* 747, 749, and *m/z* 721, 723 confirmed with presence of PC molecular species with C-18 and C-20 fatty acids as substituents. The definitive confirmation of the fatty acid composition of the M-15, M-60, and M-86 ions present in the negative-ion FAB mass spectrum was obtained by submitting these ions to collision-induced dissociation. The fatty acid composition of some of these molecular species ions is reported in Table 2.

Detailed analysis of the negative-ion mass spectrum of PC isolated from rat heart mitochondrial fractions also revealed the presence of six carboxylate ions at *m/z* 267, 269, 271, 295, 297, and 299, which did not correspond to known substituents of mammalian phospholipids. Collision-induced dissociation of these carboxylate anions was performed to identify them. In agreement with the preliminary results obtained by GC/MS analysis, the ion at *m/z* 297 was identified as 12-HOME on the basis of the comparison of its mass fragmentation pattern with that of authentic 12-HOME. As indicated in Figure 2 both spectra displayed a characteristic peak at *m/z* 183 that resulted from the fragmentation of the C-11—C-12 bond, an allylic position with a proton transfer to the observed carboxylate fragment. The other peak at *m/z* 279 resulted from loss of H_2O. The mechanism for the fragmentation of this compound already has been described [35].

Analysis of the fragmentation pattern obtained by CID of the anions at *m/z* 295 and 299 indicated that these ions only differ from 12-HOME in the number of double bonds; thus, they correspond to hydroxy octadecadienoate and hydroxy octadecanoate, respectively. The ions at *m/z* 267, 269, and 271 have masses that correspond to hydroxy hexadecadienoate, hydroxy hexadecenoate, and hydroxy hexadecanoate, respectively. The presence of the typical fragment at *m/z* 183 (see Figure 2) in the spectrum of the four unsaturated fatty acids indicates that the hydroxyl group is on C-12 and that differences in chain length or saturation occur outside this fragment. For hydroxy hexadecanoic and hydroxy octadecanoic acids, the two saturated fatty acids, the hydroxyl position could not be specified. The main fragments that appear in the CID product ion spectra of the carboxylate ions *m/z* 295, 299, 267, 269, and 271 are reported in Table 3.

Confirmation that these monohydroxylated fatty acyl groups were incorporated into PC molecules was obtained by FAB/MS-MS. The precursor ions scans of these six fatty acids revealed the corresponding typical PC molecular species (Figure 3).

The precursor spectrum of the ion at *m/z* 297, for example, displayed high mass ions at *m/z* 690, 715, 737, 763, 784, and 808. Analysis of the spectra obtained

Table 1. Mass-to-charge ratios of the most abundant M-15, M-60, and M-86 ions present in the negative-ion FAB mass spectrum of PC isolated from rat heart mitochondrial fractions

PC molecular species	Mass-to-charge ratios		
	M-15	M-60	M-86
16:0/18:2	742	697	671
16:0/18:1	744	699	673
18:0/18:2	770	725	699
18:1/18:1	770	725	699
18:0/20:4	794	749	723
18:1/20:3	794	749	723

Table 2. Fatty acid composition of some PC molecular species isolated from rat heart mitochondrial fractions; when the ions with the indicated *m/z* are selected as precursors (left column), the *m/z* of the given fatty acid anions are observed (right column)

Ion: m/z (assignment)	Molecular species composition
671 (M-60)	16:0 & 16:1
(M-86)	16:1 & 18:1
(M-86)	16:0 & 18:2
673 (M-60)	16:0 & 16:0
(M-86)	16:1 & 18:0
(M-86)	16:0 & 18:1
699 (M-60)	16:0 & 18:1
(M-86)	18:2 & 18:0
(M-86)	18:1 & 18:1

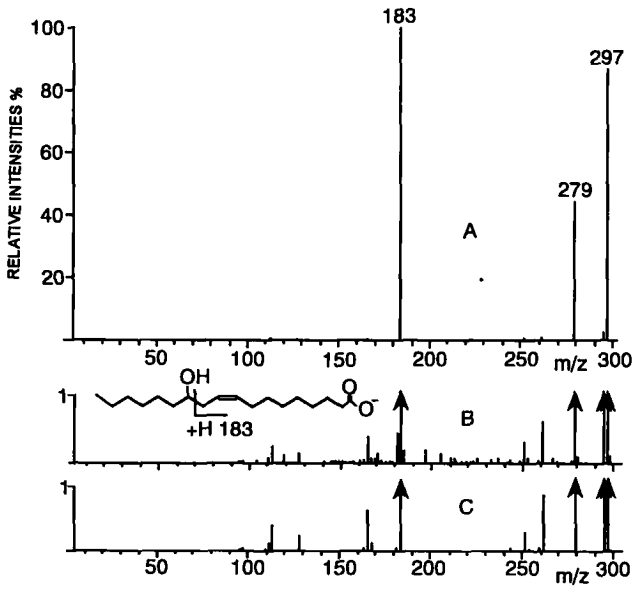

Figure 2. Comparison of the product ion spectrum of putative 12-HOME in PC with the spectrum of authentic [*R*]-12-hydroxy-*cis*-9-octadecenoic acid. The product ion spectrum of putative 12-HOME, which is the ion at *m/z* 297 in the negative-ion mass spectrum of PC, displays a prominent peak at *m/z* 183 that results from the fragmentation of the C-11—C-12 bond and another peak at *m/z* 279 that results from loss of H_2O (**A**). Amplification of the signal to reveal those peaks detectable at the 1% level of relative intensity in (**A**) gives spectrum (**C**). This spectrum is almost identical to spectrum (**B**), which results from the same amplification of the product ion spectrum of authentic 12-HOME. The arrows indicate that the signal is out of scale. The inset shows the structure of 12-HOME and its fragmentation product.

by CID of these high mass ions allowed us to determine their fatty acid composition. This analysis revealed that 12-HOME may be accompanied in the phospholipid molecule by arachidonate or eicosatrienoate, as shown by the presence of ions at *m/z* 808, 810, *m/z* 763, 765, and finally *m/z* 737, 739, which represent the M-15, M-60, and M-86 ions of PC species with 12-HOME and arachidonate or eicosatrienoate, respectively. Linoleate, oleate, and stearate also may be esterified together with 12-HOME at the glycerol backbone of PC from rat heart mitochondria, as evidenced by the presence of high mass negative ions at *m/z* 784, 786, and 788, *m/z* 739, 741, and 743, and finally *m/z* 713, 715, and 717. Finally, 12-HOME also may be associated in the PC molecule with palmitate, as attested by the presence of M-60 and M-86 ions at *m/z* 715 and 689.

Similar analysis of the precursor ion spectra of the other five monohydroxylated fatty acids indicated that they were incorporated in PC molecules and were accompanied by the same fatty acyl groups (Figure 3 and Table 4).

The precursor ion spectrum of *m/z* 297 is represented in detail in Figure 4. The ions at *m/z* 355, 434, and 522 correspond to fragments of PC that contain 12-HOME, which have lost the second fatty acyl moiety. Indeed, the ion at *m/z* 355 corresponds to a PC molecular species that contains 12-HOME, which has lost the second fatty acyl moiety and the phosphocholine group $(PO_3CH_2CH_2N(CH_3)_3)^+$. The ion at *m/z* 434 also represents a fragment of a PC molecule that contains 12-HOME, which has lost both the second fatty acyl moiety and the choline group $(CH_2CH_2N(CH_3)_3)^{\cdot+}$. Finally, the ion at *m/z* 522 is the result of the fragmentation of a PC molecular species that contains 12-HOME, which has lost both the second fatty acid substituent in the form of a ketene neutral species and one methyl group of the choline moiety. Indeed, loss of a fatty acid substituent as a ketene has been observed already for other PC molecular species upon CID of the $[M - CH3]^-$ anions, and the presence of this ion has been used to determine the positional identity of the *sn*-2 substituent [3, 9]. The ion at *m/z* 450 represents the adduct of 12-HOME and one matrix molecule.

The definitive proof of the incorporation of the monohydroxylated fatty acids in PC molecules was

Table 3. Mass-to-charge ratios of the main fragments present in the product ion spectra of the carboxylate ions *m/z* 295, 299, 267, 269, and 271; relative intensities (expressed in percent of precursor ion abundance) are in parentheses[a]

Carboxylate ion (m/z)	Fragments (m/z)									
295	277 (4.8)	265 (4.0)	238.8 (6.4)	223 (4)	221 (3.1)	209 (3.5)	195 (6.7)	183 (17.6)	153 (4)	
299	281 (6.5)	269 (8.7)	253 (14.7)	241 (71.2)	225 (5)	196.7 (6.5)	185 (5.5)	165.8 (5.5)	137 (8.8)	123 (5)
267	249 (3.5)	237 (17.4)	221.3 (4.8)	209.1 (11.0)	195 (9.9)	183.1 (12.0)	153.1 (3.5)			
269	251 (4.6)	239 (25.1)	223 (7.4)	221 (12.7)	194.9 (4.2)	183 (6.7)	153 (2.3)			
271	253 (7.2)	241 (20.8)	225 (9.8)	213 (7.1)	195 (3.5)	151 (2.9)				

[a]The product ion spectrum of the m/z 297 ion is illustrated in Figure 2.

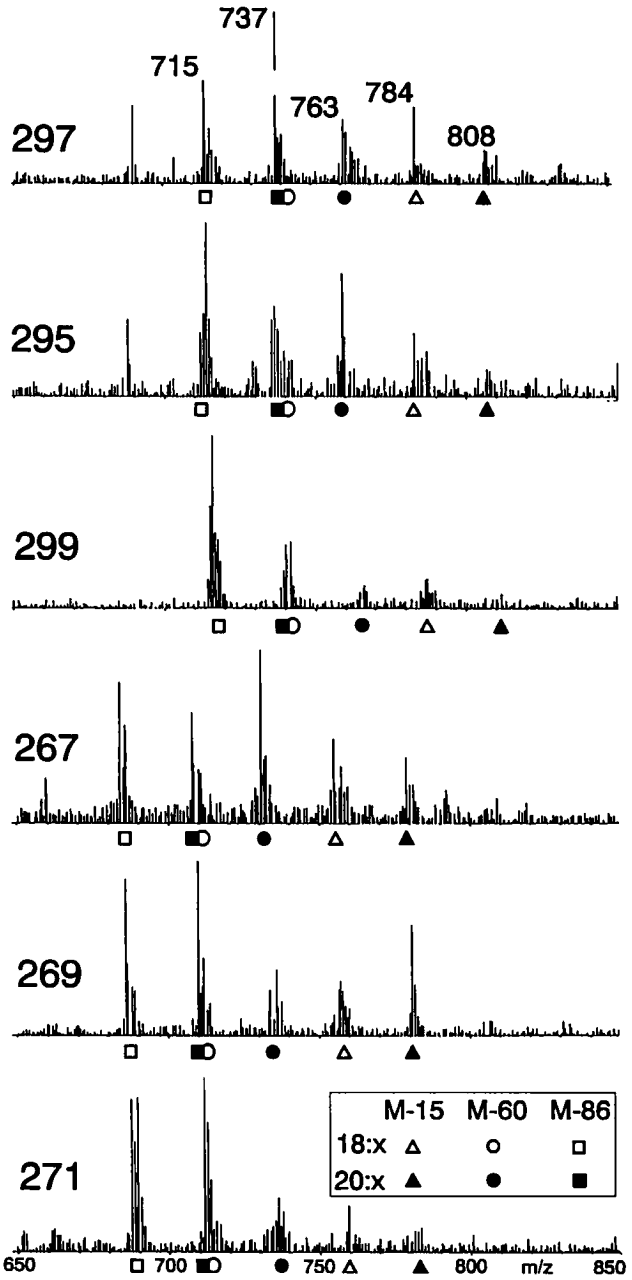

Figure 3. Precursor ion scans of the six monohydroxylated fatty acids. Parent ion scans of the ions at m/z 297, 295, 299, 267, 269, and 271 revealed the presence of groups of high mass anions that correspond to PC molecular species that contain one of the six monohydroxylated fatty acyl moieties and another fatty acyl group. For each PC molecular species, three fragments are obtained that correspond to $[M - CH_3]^-$, $[M - CH_3 - NH(CH_3)_3]^-$, and $[M - CH_3 - CH_2{=}CHN(CH_3)_2]^-$. The precursor spectrum of each monohydroxylated fatty acid is illustrated and shows peaks that correspond to the M-15, M-60, and M-86 fragments of PC that contain this particular fatty acid and another acid, which was either 18:x (open symbols) or 20:x (filled symbols). For the first spectrum, the mass-to-charge ratio of the major peaks is indicated. The mass-to-charge ratios for all the various PC fragment ions observed in the precursor ion scans are given in Table 4. These spectra are typical of those obtained from at least 10 different preparations. For this experiment, the resolution has been decreased to increase the sensitivity.

obtained by submitting the M-15, M-60, and M-86 ions that appear in their precursor spectra to CID. The product ion spectrum of m/z 713, for example, is illustrated in Figure 5. The carboxylate ions incorporated in this M-60 or M-86 PC molecular species appeared at m/z 253, 255, 279, 281, 283, 269, 295, and 297.

Effect of Ischemia and Reperfusion on the Monohydroxylated Fatty Acid Content of Phosphatidylcholine from Heart Mitochondrial Fractions

It is generally accepted that oxidized fatty acyl groups within phospholipids result from attack by free radicals, usually during pathological conditions, such as ischemia, hypoxia, or reoxygenation [36, 37]. If the hydroxylated fatty acids we have detected were formed in this way, higher concentrations of these acids would be expected in conditions that favor the formation of oxygen reactive species [38, 39]. Therefore, we studied the effect of ischemia and reperfusion on the content of the monohydroxylated fatty acids in heart mitochondrial phospholipids. In agreement with a previous observation [29], a 45-min period of ischemia caused oxidative damage as shown by a decrease in oxygen consumption in isolated heart mitochondrial fractions when glutamate–malate was used as substrates (not shown). We attempted to obtain a semiquantitative estimation of the proportion of the monohydroxylated fatty acyl groups in PC from rat heart mitochondrial fractions. This was based on the estimation of their proportion in the FAB mass spectrum and on the phosphorus content. These monohydroxylated fatty acids amounted to 0.7–1.8% of the total fatty acid content of PC in control normoxic hearts. No significant difference could be detected in the PC fraction of ischemic hearts (0.7–1.9%) or even after 10 min of reperfusion (0.4–1.7%), which exacerbated the damage caused to the mitochondrial electron transport chain.

Evidence that the Monohydroxylated Fatty Acids are not Artefacts of the Preparative Procedures

To exclude the possibility that these fatty acyl moieties were formed artefactually during the preparation of the mitochondrial fraction, a portion of the heart of an anesthetized rat was freeze-clamped in vivo between aluminum blocks pre-cooled in liquid N_2. The phospholipids were immediately extracted from whole tissue and separated by HPTLC, exactly as for the subcellular fractions. The six hydroxylated fatty acyl groups were clearly identified in the PC fraction, which demonstrated that their presence did not result from cell fractionation procedures.

Similarly, their presence did not result from an artefact that occurred during the separation of the

Table 4. Mass-to-charge ratios of the PC fragment ions that contain the monohydroxylated fatty acyl groups[a]

	Fatty acyl moieties			
Monohydroxylated (m/z)	Other	M-15 (▲, △)	M-60 (●, ○)	M-86 (■, □)
297	(18:2)	784	739	713
12-Hydroxy 9-octadecenoate	(18:1)	786	741	715
	(18:0)	788	743	717
	(20:4)	808	763	737
	(20:3)	810	765	739
295	(18:2)	782	737	711
Hydroxy octadecadienoate	(18:1)	784	739	713
	(18:0)	786	741	715
	(20:4)	806	761	735
	(20:3)	808	763	737
299	(18:2)	786	741	715
Hydroxy octadecanoate	(18:1)	788	743	717
	(18:0)	790	745	719
	(20:4)	810	765	739
	(20:3)	812	767	741
267	(18:2)	754	709	683
Hydroxy hexadecadienoate	(18:1)	756	711	685
	(18:0)	758	713	687
	(20:4)	778	733	707
	(20:3)	780	735	709
269	(18:2)	756	711	685
Hydroxy hexadecenoate	(18:1)	758	713	687
	(18:0)	760	715	689
	(20:4)	780	735	709
	(20:3)	782	737	711
271	(18:2)	758	713	687
Hydroxy hexadecanoate	(18:1)	760	715	689
	(18:0)	762	717	691
	(20:4)	782	737	711
	(20:3)	784	739	713

[a]The symbols ▲, △, ●, ○, ■, □ refer to those used in Figure 3.

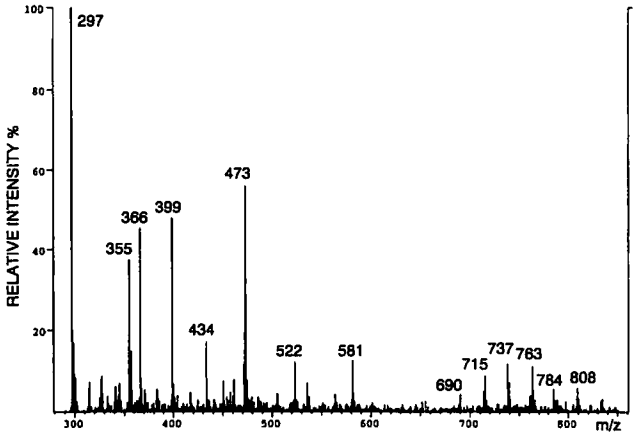

Figure 4. Precursor ion scan of m/z 297. Ions at m/z 366, 399, and 473 probably result from adducts with the matrix. Indeed, they were not present in the precursor ion scan of m/z 297 when glycerol was used as a matrix. The spectrum is a record of the relative intensities of ions versus mass-to-charge ratio; the most intense ion is set at 100.

phospholipids by HPTLC on silica gel plates, because 12-HOME could be detected in the total phospholipid fraction extracted from the heart.

It also is worth mention here that C-20 monohydroxylated fatty acyl groups could not be detected in rat heart mitochondrial fractions. Moreover, we were unable to detect any of the C-16 and C-18 monohydroxylated fatty acids in the animal diet.

Discussion

The use of FAB/MS-MS to analyze membrane phospholipids opens up new perspectives: the sensitivity and specificity of this technique allow the detection and structural châracterization of new molecular species present in very low concentrations in biological samples. The procedure is quite simple when compared with tedious traditional procedures classically used to study membrane phospholipids; indeed, it only requires extraction of phospholipids from the membrane by the classical Folch method, separation of these phospholipids into different subclasses by HPTLC, and direct analysis by tandem mass spectrom-

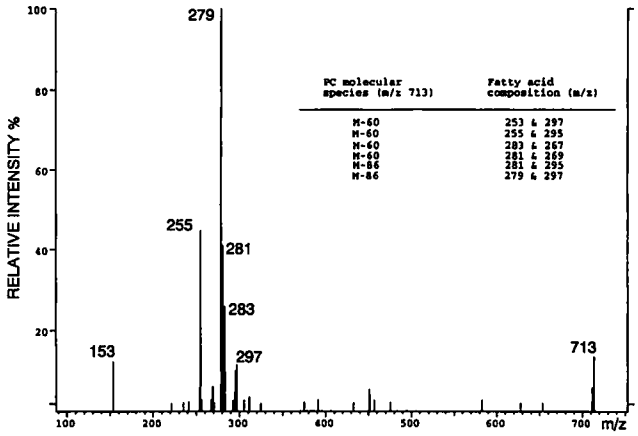

Figure 5. CID product ion spectrum of m/z 713. The CID spectrum of the ion m/z 713, which would correspond to an $[M - CH_3 - NH(CH_3)_2]^-$ or $[M - CH_3 - CH_2 = CHN(CH_3)_2]^-$ ion, displays carboxylate ions at m/z 253, 255, 279, 281, 283, 295, and 297, which confirms the incorporation of these carboxylates into the ion m/z 713. The spectrum also displays small peaks at 267, 269, and 271. These three C-16 monohydroxylated fatty acid indeed could be incorporated together with 18:0, 18:1, and 18:2, respectively, into the ion m/z 713. The spectrum is a record of the relative intensities of ions versus mass-to-charge ratio; the most intense ion is set at 100.

etry. Furthermore, this procedure does not require any derivatization, which thus reduces the risk of artefacts.

Analysis of the negative-ion mass spectrum (Figure 1) reveals that PC isolated from rat heart mitochondrial membranes is an extremely complex mixture of several molecular species: the most abundant are 16:0/18:2, 16:0/18:1, 18:0/18:2, and 18:0/20:4. Exploitation of the sensitivity of tandem mass spectrometry in this complex mixture of highly concentrated nonoxidized phosphatidylcholines enabled us to detect very low concentrations of oxidized phospholipids without any prior separation of oxidized and nonoxidized species. We have demonstrated, for the first time, the existence of C-16 and C-18 monohydroxylated fatty acyl groups in mitochondrial membranes. These monohydroxylated derivatives are preferentially associated in the PC molecule with C-18 and C-20 fatty acyl groups. Surprisingly, associations with C-16 fatty acyl moieties, although possible, are less frequent.

The originality of this work lies in the fact that we have described, for the first time, the existence of monohydroxylated fatty acids under physiological conditions, thereby questioning the belief that oxidized phospholipids are always the result of oxidative damage. The formation, in membranes, of oxidized fatty acid derivatives that include hydroxides and hydroperoxides already has been described to occur under pathological conditions such as oxidative stress. Polyunsaturated fatty acids are indeed recognized targets of oxidant damage; they readily undergo peroxidation upon exposure to free radicals. We postulate that the formation of the C-16 and C-18 monohydroxylated fatty acyl groups we discovered in membranes is not the result of lipid peroxidation initiated by free radicals or from an artefact that occurs during the preparation of the membranes. Indeed, the positional specificity of the hydroxyl group on C-12, observed for the unsaturated fatty acids, argues against its formation by a poorly specific free radical-initiated mechanism. Moreover, the content of the monohydroxylated fatty acids was not increased by ischemia and reperfusion—conditions that are known to increase the production of reactive oxygen species. Finally, we could not detect C-20 monohydroxylated fatty acyl moieties in our mitochondrial fractions.

Our discovery has another important biological implication: It implies that mitochondrial membranes could serve as a source of hydroxylated C-16 and C-18 fatty acids that, like hydroxylated derivatives of arachidonic acid, could have potent biological activities. Several biological roles indeed already have been ascribed to fatty acids that are identical [18–23] or closely related [24–28] to those we found in the mitochondrial fractions.

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References

1. Yorek, M. A. In *Phospholipids Handbook*; Cevc, G., Ed.; Dekker: New York, 1993; pp 745–775.
2. Palmer, J. W.; Schmid, P. C.; Pfeiffer, D. R.; Schmid, H. H. O. *Arch. Biochem. Biophys.* **1981**, *211*, 674–682.
3. Jensen, N. J.; Tomer, K. B.; Gross, M. L. *Lipids* **1986**, *21*, 580–588.
4. Murphy, R. C. *Handbook of Lipid Research*, Vol. 7; Snyder, F., Ed.; Plenum: New York, 1993; pp 214–231.
5. Kayganich, K.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1991**, *2*, 45–54.
6. Zirrolli, J. A.; Clay, K. L.; Murphy, R. C. *Lipids* **1991**, *26*, 1112–1116.
7. Kayganich-Harrison, K. A.; Murphy, R. C. *Anal. Biochem.* **1994**, *221*, 16–24.
8. Bernstrom, K.; Kayganich, K.; Murphy, R. C. *Anal. Biochem.* **1991**, *198*, 203–211.
9. Huang, Z. H.; Gage, D. A.; Sweeley, C. C. *J. Am. Soc. Mass Spectrom.* **192**, *3*, 71–78.
10. Tomer, K. B.; Crow, F. W.; Gross, M. L. *J. Am. Chem. Soc.* **1983**, *105*, 5487–5488.
11. Jensen, N. J.; Tomer, K. B.; Gross, M. L. *Anal. Chem.* **1985**, *57*, 2018–2021.
12. Tomer, K. B.; Jensen, N. J.; Gross, M. L. *Anal. Chem.* **1986**, *58*, 2429–2433.
13. Jensen, N. J.; Gross, M. L. *Lipids* **1986**, *21*, 362–365.
14. Adams, J.; Gross, M. L. *Anal. Chem.* **1987**, *59*, 1576–1582.

15. Bryant, D. K.; Orlando, R. C.; Fenselau, C.; Sowder, R. C.; Henderson, L. E. *Anal. Chem.* **1991**, *63*, 1110–1114.
16. Cole, M. J.; Enke, G. G. *Anal. Chem.* **1991**, *63*, 1032–1038.
17. Kayganich-Harrison, K. A.; Rose, D. M.; Murphy, R. C.; Morrow, J. D.; Roberts, L. J., II. *J. Lipid Res.* **1993**, *34*, 1229–1235.
18. Capasso, F.; Tavares, I. A.; Tsang, R.; Bennett, A. *Prostaglandins* **1985**, *30*, 119–124.
19. Tiruppathi, C.; Miyamoto, Y.; Ganapathy, V.; Leibach, F. H. *Biochem. Pharmacol.* **1988**,, *37*, 1399–1405.
20. Hwang, T. C.; Guggino, S. E.; Guggino, W. B. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5706–5709.
21. Capasso, F.; Tavares, I. A.; Bennett, A. *Drugs Exptl. Clin. Res.* **1991**, *XVII*, 351–353.
22. Witt, M. R.; Nielsen, M. *Biochem. Pharmacol.* **1994**, *47*, 742–744.
23. Izzo, A. A.; Mascolo, N.; Viola, P.; Capasso, F. *Eur. J. Pharmacol.* **1993**, *243*, 87–90.
24. Buchanan, M. R.; Haas, T. A.; Lagarde, M.; Guichardant, M. *J. Biol. Chem.* **1985**, *260*, 16056–16059.
25. Setty, B. N. Y.; Berger, M.; Stuart, M. J. *Biochem. Biophys. Res. Commun.* **1987**, *146*, 502–509.
26. Henricks, P. A. J.; Engels, F.; van der Vliet, H.; Nijkamp, F. P. *Prostaglandins* **1991**, *41*, 21–27.
27. De Meyer, G. R. Y.; Bult, H.; Verbeuren, T. J.; Herman, A. G. *Br. J. Pharmacol.* **1992**, *107*, 597–603.
28. Buchanan, M. R.; Bertomeu, M. C.; Haas, T. A.; Orr, W.; Eltringham-Smith, L. L. *Blood* **1993**, *81*, 3303–3312.
29. Veitch, K.; Hombroeckx, A.; Caucheteux, D.; Pouleur, H.; Hue, L. *Biochem. J.* **1992**, *281*, 709–715.
30. Folch, J.; Lees, M.; Stanley, G. H. S. *J. Biol. Chem.* **1957**, *226*, 497–509.
31. Schlame, M.; Horvath, L.; Vigh. L. *Biochem. J.* **1990**, *265*, 79–85.
32. Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 466–468.
33. Morrison, W. R.; Smith, L. M. *J. Lipid Res.* **1964**, *5*, 600–608.
34. Daum, G. *Biochim. Biophys. Acta* **1985**, *822*, 1–42.
35. Wheelan, P.; Zirrolli, J. A.; Murphy, R. C. *Biol. Mass Spectrom.* **1993**, *22*, 465–473.
36. Freeman, B. A.; Crapo, J. D. *Lab. Invest.* **1982**, *47*, 412–426.
37. Halliwell, B.; Gutteridge, J. M. C. *Lancet* **1984**, *i*, 1396–1397.
38. McCord, J. M. *N. Engl. J. Med.* **1985**, *312*, 159–163.
39. Turrens, J. F.; Beconi, M.; Barilla, J.; Chavez, U. B.; McCord, J. M. *Free Rad. Res. Commun.* **1991**, *12–13*, 681–689.