Collision-induced dissociation of F_2-isoprostane-containing phospholipids

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Abstract Free radical-induced lipid peroxidation results in the production of metabolites of arachidonic acid isomeric with prostaglandin $F_{2\alpha}$. The formation of these compounds, termed F_2-isoprostanes, occurs independent of the enzyme cyclooxygenase. The discovery that F_2-isoprostanes can exert potent biological activity has suggested that they may mediate, to some extent, the biological responses to oxidant injury. Collision-induced dissociation of the $[M\text{-}CH_3]^-$ ions from oxidized phospholipids isolated by extraction and normal phase high performance liquid chromatography from livers of rats treated with CCl_4 to induce lipid peroxidation revealed several molecular species of phospholipids that had the F_2-isoprostane esterified to the glycerophosphocholine backbone. Collision-induced dissociation of the $[M\text{-}CH_2CHN(CH_3)_3]^-$ ion revealed that the F_2-isoprostanes were primarily esterified at the *sn*-2 position of the glycerophospholipid as expected. Furthermore, tandem mass spectrometry of the carboxylate anion from the F_2-isoprostane (*m/z* 353) resulted in the unique loss of 44 u characteristic of a 1,2-cyclic diol moiety such as that found in the PGF_2-ring. These observations indicate that intact phospholipids containing fatty acyl groups of the isoprostane structure can be readily detected with tandem mass spectrometry even when present as minor components in a biological extract. Although no specific isomer identification can be made from the complex mixture, these techniques establish the existence of these novel metabolites of arachidonic acid esterified to glycerophospholipids. **—Kayganich-Harrison, K. A., D. M. Rose, R. C. Murphy, J. D. Morrow, and L. J. Roberts, II.** Collision-induced dissociation of F_2-isoprostane-containing phospholipids. *J. Lipid Res.* 1993. **34:** 1229–1235.

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Prostaglandins are potent biologically active metabolites of arachidonic acid formed by the cyclooxygenase enzyme found in most human cells. Free arachidonic acid, released from glycerophospholipid stores, is readily converted to PGH_2, the cyclic endoperoxide that is the progenitor of the various prostaglandins. Recently, it has been reported that similar prostaglandin-like compounds are produced in vivo in humans by a free radical nonenzymatic mechanism (1–3). These compounds have been termed isoprostanes because several positional isomers and stereoisomers can be produced by the free radical mechanism shown in **Scheme 1.** The common feature of all these isomers is the 1,3-dihydroxycyclopentane (F_2-ring). The demonstration that the formation of F_2-isoprostanes in animal models of lipid peroxidation is dramatically increased suggests that measurement of isoprostanes in biological fluids may provide a unique noninvasive means to assess oxidative stress in vivo (1–3).

Recent evidence has suggested that F_2-isoprostanes are initially formed in situ from arachidonate-containing glycerophospholipids and subsequently released in free form (4). Release of the isoprostanes from the glycerophospholipids by the action of phospholipases could represent a source of preformed lipid mediators if these isoprostanes have biological activity. Initial results indicate that at least one of the F_2-isoprostanes, 8-epi-$PGF_{2\alpha}$, exhibits potent biological activity (1, 5–7). Furthermore, recent results suggest that oxygenated arachidonic acids, such as the hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids, are released preferably to arachidonic acid (8, 9). Similarly, the phospholipid esterified F_2-isoprostanes may also be preferred substrates for phospholipase A_2.

Analysis of the F_2-isoprostanes has been accomplished by hydrolysis from phospholipids followed by derivatization and GC–MS for structural characterization (4, 10), although some of the derivatized isomers are not separable by capillary GC leading to mixed mass spectra after GC–MS analysis. We report here the use of FAB and

Abbreviations: HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; FAB, fast atom bombardment; CID, collision-induced dissociation; GPC, glycerophosphocholine.

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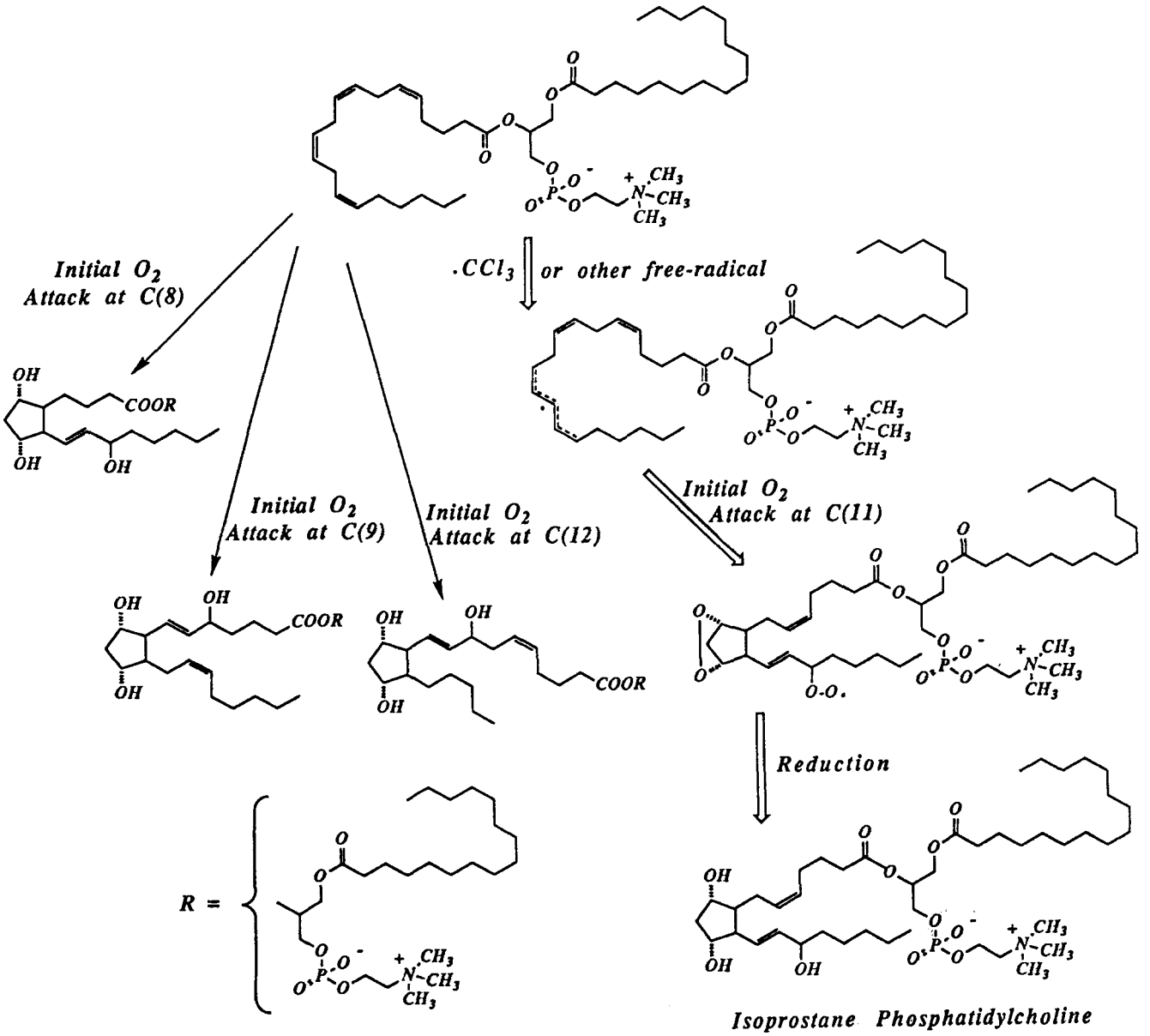

Scheme 1.

tandem mass spectrometry for the structural characterization of intact phospholipid esterified F_2-isoprostanes. The use of FAB-MS-MS for the direct analysis of these compounds circumvents the need for hydrolysis and derivatization, thus reducing artifacts and loss of information as to exact esterified phospholipid molecular species. The low energy CID mass spectra of nonesterified isoprostanes obtained from rat liver and authentic PGF_2 were also examined to determine whether specific fragment ions could be produced that will allow identification of these isomers, as was found previously for esterified epoxyeicosatrienoic acids (11).

EXPERIMENTAL

Materials

Authentic prostaglandin $F_{2\alpha}$ was purchased from Cayman Chemical (Ann Arbor, MI) and used without further purification. Diethanolamine was purchased from J. T. Baker Chemical.

Isolation of isoprostanes

Lipids were extracted from liver of rats (12) after administration of CCl_4 to induce endogenous lipid peroxidation. Putative F_2-isoprostane-containing species of

glycerophosphocholine were purified by normal phase HPLC as previously described (4). Briefly, normal phase HPLC separation of lipid extracts was carried out on an Econosil silica column 25 cm × 4.6 mm, Alltech Associates (Deerfield, IL) under isocratic conditions using hexane–isopropanol–water 4:6:1 (v/v/v) at a flow rate of 1 ml/min (4, 13, 14). Under the conditions used, F_2-isoprostane-containing species of phosphatidylcholine elute at a more polar retention volume (35–50 ml) than nonoxidized phosphatidylcholines which elute between approximately 18 and 25 ml. A representative HPLC separation has been previously published (4). Free F_2-isoprostanes were obtained for analysis by mass spectrometry by alkaline hydrolysis of lipids from liver of CCl_4-treated rats and subsequently purified as the free acid as described (4).

Mass spectrometry

All mass spectra were obtained with a Finnigan MAT TSQ70B triple quadrupole mass spectrometer (San Jose, CA) equipped with an Iontech, Ltd. (Toddington, UK) fast atom gun. The fast atom gun was operated at 1 mA to produce a 6 kV xenon atom beam. Diethanolamine was used for the fast atom bombardment ionization matrix. Collision-induced dissociation was carried out with 0.5 mTorr argon in the second quadrupole collision cell using a collision energy offset, E_{lab}, of 30 eV. The 20 kV conversion dynode was operated at 15 kV. Samples dissolved in chloroform–methanol were mixed on the probe tip with 1 μl diethanolamine. Ion currents lasted for 5–10 min.

RESULTS

The negative ion FAB mass spectrum of the normal phase HPLC fraction suspected of containing isoprostane phospholipids revealed intense high mass ions at *m/z* 687, 642, and 616 corresponding to $[M-CH_3]^-$, $[M-HN(CH_3)_3]^-$, and $[M-CH_2CHN(CH_3)_3]^-$ from sphingomyelin molecular species as well as ions expected for isoprostane glycerophosphocholine (GPC) species (**Fig. 1**). All phosphocholine-containing lipids yield this triplet set of negative ions resulting from losses from the choline moiety during FAB ionization (15). The abundance of the sphingomyelin-related ions compared to the glycerophosphocholine ions suggests that the glycerophosphocholine species were minor components of this HPLC fraction.

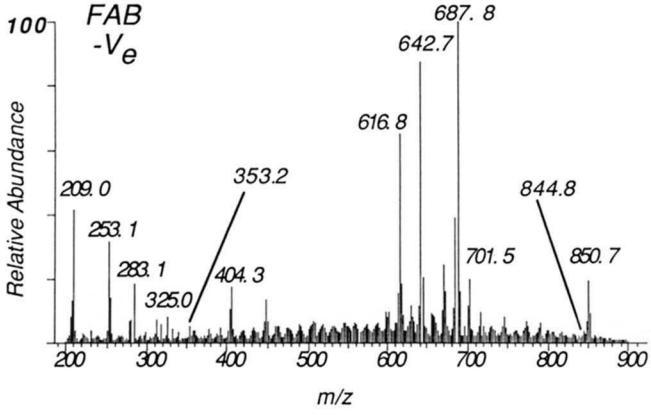

Fig. 1. Fast atom bombardment mass spectrum of a normal phase HPLC fraction of the lipids extracted from rat liver after CCl_4 treatment. GC–MS analysis of this fraction, following base hydrolysis, showed that it contained F_2-isoprostanes (1, 3).

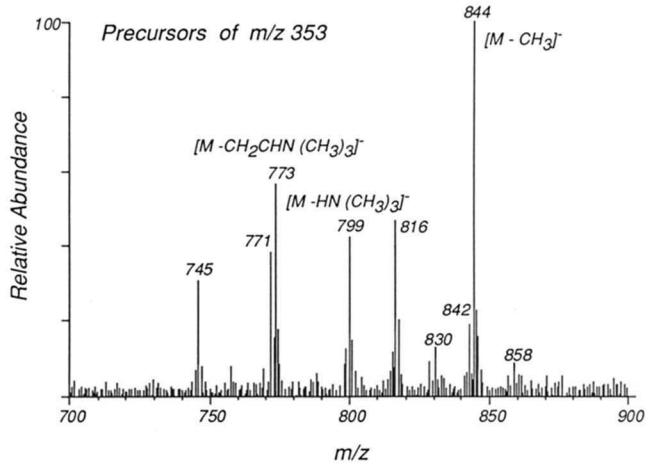

Fig. 2. Precursor ion scan for *m/z* 353 ($C_{20}H_{33}O_3^-$) from negative ions produced by FAB shown in Fig. 1. FAB of GPC produces high mass negative ions corresponding to losses of parts of the choline moiety. The ions at *m/z* 844, 799, and 773 correspond to the $[M-CH_3]^-$, $[M-HN(CH_3)_3]^-$, and $[M-CH_2CHN(CH_3)_3]^-$ ions from a major GPC molecular species that fragment to produce an ion with *m/z* 353. Ions at *m/z* 816, 771, and 745 correspond to these same ions for another GPC molecular species.

A precursor ion scan for the carboxylate anion ($C_{20}H_{33}O_5^-$) for F_2-isoprostane, *m/z* 353, was performed in order to identify the possible isoprostane-containing GPC molecular species in the sample. The rationale for this precursor scan was based on the fact that collision-induced dissociation of the negative ions from phospholipids produces abundant carboxylate anions from the esterified fatty acid moieties (15) and precursor ion scans for specific fatty acyl groups provide a sensitive means for detecting minor molecular species within complex mixtures (16). The isoprostane-containing molecular species suggested by the precursor ion scan are shown in **Fig. 2** with the most abundant ions at *m/z* 745, 771, 773, 799, 816, 842, and 844. Product ion spectra were recorded for these ions to further characterize the intact molecules.

The product ion spectrum of *m/z* 844, which would correspond to the $[M-CH_3]^-$ of a major isoprostane-containing molecular species, is shown in **Fig. 3A.** The isoprostane carboxylate anion is prominent as expected at *m/z* 353. The ion at *m/z* 283 [$C_{17}H_{35}COO^-$] indicates that the other fatty acyl moiety in this molecular species is the octadecanoyl group. The ion at *m/z* 168 [$(CH_3)_2NCH_2CH_2OPO_3H^-$]

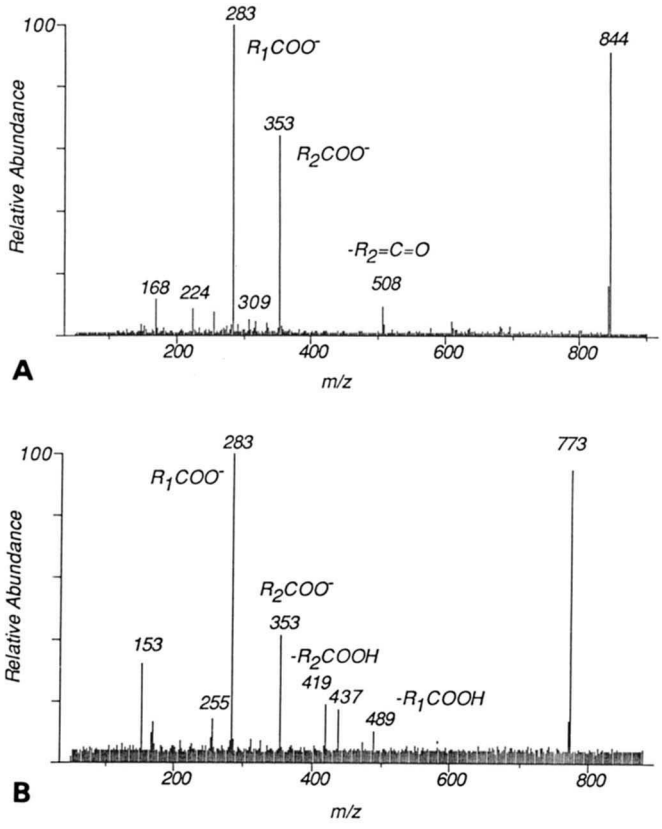

Fig. 3. CID product ion spectra of A) *m/z* 844, the $[M-CH_3]^-$ ion from a major precursor of *m/z* 353 and B) *m/z* 773, the corresponding $[M-CH_2CHN(CH_3)_3]^-$ from the same molecular species.

is derived from the phosphocholine head group (17). *M/z* 224 is likely derived from the glycerophosphocholine backbone after loss of both fatty acyl groups. Along with the fact that these phosphatidylcholine species exhibit more polar characteristics than unoxidized phosphatidylcholine on normal phase HPLC, these ions confirm that this molecular species is, in fact, an oxidized glycerophosphocholine. The ion at *m/z* 508 results from the neutral loss of 336 u corresponding to the loss of the isoprostane moiety as a ketene neutral species. Loss of the *sn*-2 fatty acyl substituent as a ketene has been observed for other GPC molecular species upon CID of the $[M-CH_3]^-$ anions (15, 17, 18) and the presence of this ion can be used to determine the positional identity of the *sn*-2 substituent (16, 19).

Further confirmation of the identity of the *sn*-2 substituent of this molecular species was obtained from the product ion spectrum the $[M-86]^-$ ion at *m/z* 773, corresponding to the $[M-CH_2CHN(CH_3)_3]^-$ ion, as is shown in Fig. 3*B*. The ions at *m/z* 419 and *m/z* 489 correspond to the neutral losses of fatty acyl moieties having 354 u and 284 u, respectively. The ion resulting from the neutral loss of 354 u is more abundant than the ion resulting from the loss of 284 u, indicating that the F_2-isoprostane (354 u) is esterified at the *sn*-2 position (19). Using these techniques, the other precursor ions to the F_2-isoprostane ion (*m/z* 353) shown in Fig. 2 were characterized as summarized in **Table 1.** The possibility does exist that an isomeric isoprostane constituent esterified to *sn*-1 (stearoyl at *sn*-2) may be present, but there is insufficient isomer present to substantially alter the expected ratio of *m/z* 419/489 from that observed with pure glycerophospholipid isomers (19).

Carboxylate anions from GPC fatty acyl substituents of the intact phospholipid molecular species are also formed by FAB ionization in the ion source and can be subjected to collisional activation in the tandem quadrupole instrument. The product ion spectrum from CID of the carboxylate ion at *m/z* 353 produced by FAB of the F_2-isoprostane-GPC HPLC fraction is shown in **Fig. 4.** The ion at *m/z* 309, loss of C_3H_4O, indicates that this could be an isoprostane with an F_2-type ring (20). Ions at *m/z* 335 and 317 indicate losses of one and two water molecules with a much less abundant ion at *m/z* 299 suggesting the presence of a third hydroxy moiety in the ion structure of *m/z* 353. The appearance of ions at *m/z* 335, 317, and 309 were also observed during decomposition of the $[M-15]^-$ from the isoprostane-GPC (Fig. 3A).

Low energy collision induced dissociation of the $[M-H]^-$ ion produced by FAB of authentic $PGF_{2\alpha}$ (*m/z* 353) is shown in **Fig. 5.** The loss of neutral molecules of H_2O (*m/z* 335) or C_2H_4O (*m/z* 309) from the isoprostane ring, has been previously described in high energy CID mass spectra of both positive and negative ions from $PGF_{2\alpha}$ (21, 22). The facile loss of 44 u (*m/z* 309) is somewhat specific for an F_2 prostane ring or thromboxane ring (21) and a mechanism for the loss of 44 u as C_2H_4O has been previously suggested for the 1,3 cyclic diol system (21). However, this fragment ion or the ions corresponding to the sequential loss of water do not provide information as to the alkyl side chains.

Fig. 6 is the product ion spectrum from $[M-H]^-$ of an F_2-isoprostane mixture isolated from different rat liver following hydrolysis of the phospholipid esters. Ions at *m/z* 335, 317, and 299 are present from the sequential losses

TABLE 1. Major molecular species of F_2 isoprostane-glycerophosphocholine found in rat liver after CCl_4 treatment

Ion m/z	*sn*-1[a] (Composition)[b]	*sn*-2[a] (Composition)[b]
816 (M-15)	*m/z* 255 ($C_{15}H_{31}COO^-$)	*m/z* 353 ($C_{19}H_{33}O_3COO^-$)
745 (M-86)	*m/z* 255	*m/z* 353
842	*m/z* 281 ($C_{17}H_{33}COO^-$)	*m/z* 353 ($C_{19}H_{33}O_3COO^-$)
844 (M-15)	*m/z* 283 ($C_{17}H_{35}COO^-$)	*m/z* 353 ($C_{19}H_{33}O_3COO^-$)
773 (M-86)	*m/z* 283	*m/z* 353

[a]Assignment of the esterification carbon obtained from the product ion spectrum of the $[M-86]^-$ ions with loss of the *sn*-2 group as ketene.
[b]Suggested elemental composition.

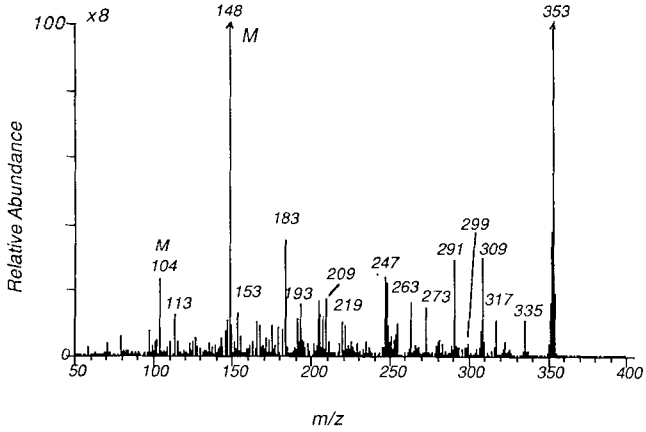

Fig. 4. CID product ion spectrum of *m/z* 353 produced by FAB of isoprostane-GPC fraction. Ions at *m/z* 335, 317, 309, 291, and 273 are indicative of the presence of an F_2-ring and three hydroxyl moieties. Several matrix fragment ions (marked M) are present due to the overlap of *m/z* 353 with matrix ions. Relative abundances are magnified 8-fold.

of the three hydroxyl moieties as H_2O molecules from the precursor carboxylate anion. Also present are the ions at *m/z* 309, 291, and 273 corresponding to the loss of 44 u, followed by losses of one and two molecules of water, respectively. These ions indicate the presence of the F_2-ring. An ion at *m/z* 255 is also present which suggests an additional loss of water and loss of 44 u as the loss of CO_2 from the carboxyl moiety. There are striking similarities in ions and ion abundances comparing Figs. 4, 5, and 6. Most notable among these are *m/z* 193, 209, 219, 247, 263, and 273 along with the above mentioned ions. From the free radical mechanism of isoprostane synthesis (Scheme 1), one expected product is $PGF_{2\alpha}$ and indeed all ions observed from the CID of authentic $PGF_{2\alpha}$ (Fig. 5) can be observed in the CID spectrum of the F_2-isoprostane mixture (Fig. 6) and even the CID of *m/z* 353 derived in the tandem mass spectrometry of the phospholipid extract (Fig. 4). Other ions present in Figs. 4 and 6 likely arise from specific mechanisms related to the different positional isomers present in these mixtures. Further understanding of the mechanisms of fragmentation involved in the generation of the lower mass fragments may give information about the alkyl chains extending from the prostane ring and the positional isomers of the F_2-isoprostanes when synthetic isomers become available.

DISCUSSION

Direct analysis of intact phospholipid molecular species by FAB–MS–MS adds a new dimension to the analysis of oxidized phospholipids. The analysis of oxidatively modified phospholipid molecular species can be a particularly difficult task because of the low levels of these compounds in biological mixtures of phospholipids which contain large quantities of the nonoxidized species. We have applied negative ion FAB and low energy collision-induced dissociation in order to simplify the analysis of F_2-isoprostane-esterified phospholipid molecular species, thought to be produced from arachidonate-containing phospholipids by a free radical mechanism. Several oxidized phospholipid molecular species were identified with this technique in a sample isolated by extraction and normal phase HPLC from the liver of a rat exposed to CCl_4. This sample contained predominantly sphingomyelin and the oxidized phospholipid molecular species represented minor components. The fact that these minor components could be identified is an indication of the sensitivity and specificity of this technique. This discovery provides additional evidence that F_2-isoprostane compounds are formed directly in situ from arachidonic acid esterified in phospholipids. The finding that the F_2-isoprostanes exist

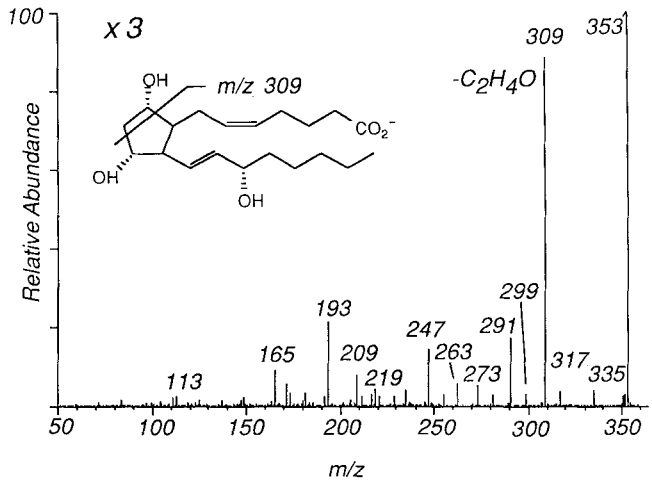

Fig. 5. CID product ion spectrum of *m/z* 353, $[M-H]^-$, from authentic $PGF_{2\alpha}$. Relative abundances are magnified threefold.

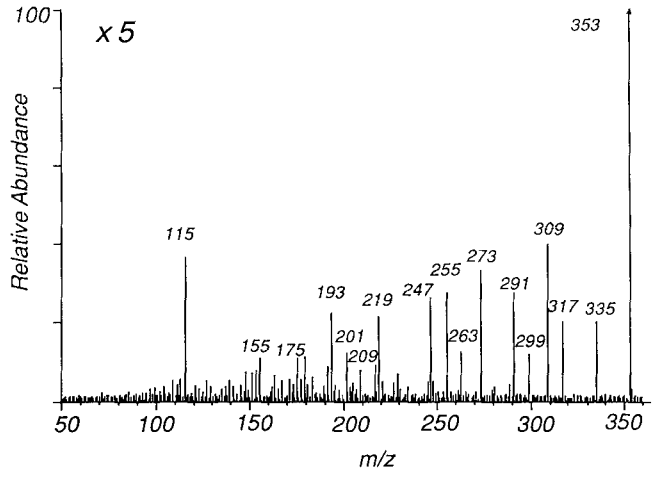

Fig. 6. CID product ion spectrum of *m/z* 353 from FAB of F_2-isoprostanes hydrolyzed from isoprostane-containing GPC. Relative abundances are magnified fivefold.

esterified to phospholipids is novel in that prostaglandins that are enzymatically produced by cyclooxygenase are not re-esterified to phospholipids following their synthesis (4, 22).

The precursor ion scan for *m/z* 353 permitted determination of the high mass ions that could result from F_2-isoprostane-containing phospholipid molecular species. Collision-induced dissociation of the $[M-CH_3]^-$ and $[M-86]^-$ ions for the major species established that the fatty acid group esterified in the *sn*-2 position had a mass of 353 u, as would an F_2-isoprostane or an odd-chain saturated fatty acid with 23 carbon atoms. As previously found for the more typical radyl groups found in phospholipids, the product ion produced upon neutral loss of the *sn*-2 substituent is more thermodynamically favored than the corresponding ion produced by loss of the *sn*-1 substituent (19). This appears to be the case for these more complex phospholipids as well. The *sn*-2 position for the F_2-isoprostanes is consistent with the known preference of biological systems to esterify arachidonic acid at the *sn*-2 position. This observation is also consistent with the release of F_2-isoprostanes from phospholipids using bee venom phospholipase A_2 (4). Determination of the *sn*-2 and *sn*-1 substituents of phospholipids by more traditional techniques can be a lengthy and tedious procedure, but the use of FAB-MS-MS provides a simpler and faster means for such determinations.

The additional ions produced at *m/z* 309, 335, and 317 from CID of $[M-CH_3]^-$ appear to arise after secondary fragmentation of the *m/z* 353 ion and are the same fragmentations seen upon CID of authentic $PGF_{2\alpha}$. Thromboxane B_2 also undergoes a loss of 44 u from its 6-membered dihydroxy acetal ring, but TxB_2 contains an additional oxygen atom and therefore would not produce a carboxylate anion isobaric with an isoprostane. Fatty acids not containing oxygen heteroatoms do not readily fragment under low energy CID conditions, therefore it is unlikely that *m/z* 353 is derived from tricosanoic acid (23:0, mol wt 354). In addition, the HPLC retention time for these GPC species indicated additional polarity in their structure such as that imparted by the oxygenated F_2-isoprostane moiety.

At the present time, this approach does not permit the direct determination of specific isomers of esterified F_2-isoprostanes. Diagnostic CID fragment ions for the different F_2-isoprostane isomers cannot be determined without the availability of pure standards. The presence of multiple double bonds and oxygen moieties make the low energy CID behavior of these negative ions difficult to predict without reference to standards because of the potential multiple sites for charge transfer reactions. However, the loss of 44 u and losses of H_2O molecules are rather specific indicators of the presence of the F_2-ring and the number of hydroxyl groups in the molecule. In addition, low energy CID of the F_2-isoprostanes, including $PGF_{2\alpha}$, yields a number of lower mass fragments that may be useful for structural elucidation as seen when comparing authentic $PGF_{2\alpha}$ with the isolated isoprostanes (Fig. 6). Further investigations of the mechanisms for these fragmentations are currently under investigation.

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