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Identification and characterization of plasmalogen fatty acids in swine heart*

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Abstract: Reperfusion of ischemic swine myocardium is associated with the loss of sarcolemmal phospholipids resulting in the accumulation of amphiphilic metabolites, lysophosphoglycerides and free fatty acids, especially arachidonic acid, causing electro-physiological dysfunction and cell death. Recently, phospholipids containing a vinyl ether bond at the Sn-1 position, commonly known as plasmalogens, have been identified as major constituents of heart, which contain a large amount of arachidonic acid in the Sn-2 position. Because of the potential importance of plasmalogens, the fatty acid composition of the choline and ethanolamine phosphoglycerides in swine heart was determined. Lipids were extracted from the left ventricular biopsies from swine heart, phosphoglycerides were separated from the neutral lipids by thin layer chromatography, converted into methyl derivatives and analysed by GC. The peaks for fatty acid methyl ester (FAME) and dimethylacetal (DMA) derivatives of choline and ethanolamine phosphoglycerides were confirmed using GC–MS. The results showed high amounts of 18:1 (17 mol %), 18:2 (24 mol %) FAME in choline phosphoglycerides in contrast to the occurrence of a high amount of 20:4 (28 mol %) FAME in ethanolamine phosphoglycerides, suggesting that plasmenylethanolamine, and not plasmenylcholine, may serve as the depot for arachidonic acid in swine heart.

Keywords: *Plasmalogens; glycerophospholipids; swine; heart; fatty acids.*

Introduction

Myocardial sarcolemmal phospholipids are predominantly comprised of plasmalogen molecular species, and a growing body of evidence indicates that plasmalogen phospholipids may play a rôle in the pathophysiology of many heart diseases. Thus, plasmalogens are believed to be involved in the sarcolemmal dysfunction associated with myocardial ischemia and reperfusion [1, 2]. It has been speculated that the distribution of plasmalogens in the heart might affect the bilayer stability, which may be instrumental for the sarcolemmal destruction during ischemia and reperfusion.

The amount of plasmalogen glycerophospholipids varies from organ to organ as well as from species to species. For example, the plasmalogen content of liver is only 0.7–0.8% of the total phospholipids [3], while in heart over 25% of the phospholipids are plasmalogens [4]. Besides heart, retina, brain and kidney also contain a significant amount of their phosphoglycerides in the plasmalogen form [5].

Reperfusion of ischemic myocardium is associated with the loss of membrane phospholipids resulting in the accumulation of free fatty acids, especially arachidonic acid [6]. The molecular composition of the plasmalogen phospholipid species is characterized by the presence of a relatively higher amount of arachidonic acid esterified to the Sn-2 position [7]. However, the exact source of the arachidonic acid among the phosphoglyceride classes are not known.

Experimental

Materials

The authentic standards of the fatty acids, and the internal standards, diarachidyl PC, diheptadecanoic PE, and acetyl chloride were purchased from the Sigma Chemical Co. (St Louis, MO). Fatty acid methyl esters were purchased from Nucheck Lipids. All solvents used were from Burdick and Jackson, and

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obtained from Baxter Diagnostics (McGaw Park, IL).

Methods

Animal preparation. Yorkshire pigs weighing 20–25 kg were anaesthetized with an i.v. injection of Nembutal (30 mg kg^{-1}), incubated, and ventilated with room air by a Harvard respirator. The chest was opened with a median sternotomy incision. After heparinization with sodium heparin (500 units kg^{-1}), the animals were placed on cardiopulmonary bypass with a bubble oxygenator and a roller pump. The heart was isolated *in situ* from the systemic circulation by cross-clamping the ascending aorta as described previously [8]. After 10–15 min of stabilization, transmural myocardial biopsies were obtained, instantly frozen in liquid N_2, and stored at −70°C for subsequent assay of plasmalogen glycerophospholipids.

Processing of biopsies and extraction and separation of lipid. About 0.2 g of a biopsy was homogenized in 20 vol of ice-cold $CHCl_3$–CH_3OH (2:1, v/v) solution containing 0.005% BHT using a Polytron homogenizer as described previously [9]. Diheptadecanoyl phosphatidylethanolamine (25 nmol) and diarachidyl phosphatidylcholine (50 nmol) were used as internal standards. Phospholipid classes were separated on silica gel K6 plates (Whatman, Clifton, NJ) using a mixture of chloroform–methanol–petroleum ether–acetic acid–boric acid (40:20:30:7:1.8, v/v/v/v/wt) as a solvent system. The lipids on the silica gel plates were identified by cochromatography with authentic lipid standards after brief exposure with iodine vapour.

Derivatization of lipids. The phosphoglycerides were scraped off from the TLC plates into a screwcap test tube containing 0.5 ml of anhydrous CH_3OH with 0.01% BHT; 0.5 ml of 20% acetylchloride in anhydrous CH_3OH was added to the tubes, and placed in a water bath (90–100°C) for 90 min. The tubes were then cooled to the room temperature. The methanolyzate was neutralized with an excess of anhydrous Na_2CO_3. Fatty acid methyl esters (FAME) and dimethyl acetal (DMA) derivatives of phosphoglyceride acyl and alk-1-enyl groups, respectively, were extracted from the methanolyzate by the addition of 2 ml hexane, and the tubes were vortexed. One millilitre of NaCl saturated water was then added and vortexed again. The hexane phase was removed following centrifugation to aid phase separation. The derivatized product was extracted in hexane, evaporated to dryness under a stream of N_2, and the methyl derivatives reconstituted in 0.5 ml of hexane.

Gas chromatography–mass spectroscopy (GC–MS). One microlitre from the above hexane extract was injected onto the injection port and run in a splitless mode at 225°C using a Hewlett Packard gas chromatograph, model 5890A equipped with a 7693 automatic injector and a SP 2330 polar phase capillary column (Beleonte, PA). The temperature program was as follows: beginning temperature 120°C for 3 min; then increased at 5°C min^{-1} until it reached 160°C; then increased at 10°C min^{-1} until it reached 250°C; the oven temperature was maintained at 250°C for a few minutes and then cooled down to the initial temperature of 120°C at 20°C min^{-1}.

In order to confirm the DMA and FAME peaks, the remaining hexane extract after the GC was run spotted on an activated silica gel G plate, and developed with benzene. FAME (R_f = 0.45) and DMA (R_f = 0.25) were confirmed from R_f values. The spots were scraped off from the TLC plate, extracted according to Otani *et al.* [11], the derivatives were eluted from the silica gel with $CHCl_3$–CH_3OH (2:1, v/v), and analysed by GC under the same conditions as the original chromatography. The chromatograms were compared, and peaks identified as FAME or DMA.

The hexane extract was also analysed by MS to confirm the identity of the derivatized peaks. A Supelco SP-2330 capillary column having a film thickness of 0.2 μm was connected from the GC to a Finnigan MAT quadrupole mass spectrometer (Palo Alto, CA) via a heated transfer line. For data capture, the system was coupled to a Data General computer model DG 10 and a Printronix model MVP printer. The GC–MS transfer line temperature was maintained at 250°C while the head pressure on the inlet was maintained at 62 kPa (8 psi) using helium as the carrier gas. A 4-μl sample was injected in each case. The injector temperature was 230°C and the injector was operated in a splitless mode. The temperature program consisted of a starting temperature of 140°C which was held for 4 min, and then increased to 180°C at an

increment of 10°C min^{-1}. The temperature was finally raised from 180 to 260°C at a rate of 8°C min^{-1}. The total run time was 14 min. The Finnigan MAT quadrupole mass spectrometer was used in combination with an INCOS 50B data system. The instrument was set on electron ionization mode: the ion source temperature was 180°C, and the ionization energy was 70 eV.

Results

Fatty acid profiles of phosphoglycerides

Representative gas chromatograms corresponding to the DMA and FAME of glycerophospholipids derived from pig heart are shown in Figs 1 and 2, respectively. DMA derivatives of ethanolamine glycerophosphates contained relatively higher amounts 18:0 and 18:1 fatty acids compared to those of choline glycerophosphates, while FAME derivatives of choline contained higher amounts of 16:0, 18:1 and 18:2; but lower amounts of 18:0, 20:4 and 22:6 fatty acids compared with those of ethanolamine phosphoglycerides. Ethanolamine glycerophosphates contained a higher amount of 16:0 DMA compared with 16:0 FAME, but a lower amount of 18:0 DMA compared with 18:0 FAME. Choline glycerophosphates contained higher amounts of 16:0, 18:0 and 18:1 FAMEs as compared with 16:0, 18:0 and 18:1 DMAs. The results derived from Fig. 1, and normalized corresponding to the internal standards are shown in Tables 1 and 2.

Identification of fatty acids by MS

Mass spectra of the FAMEs and DMAs are shown in Figs 2 and 3, respectively. The

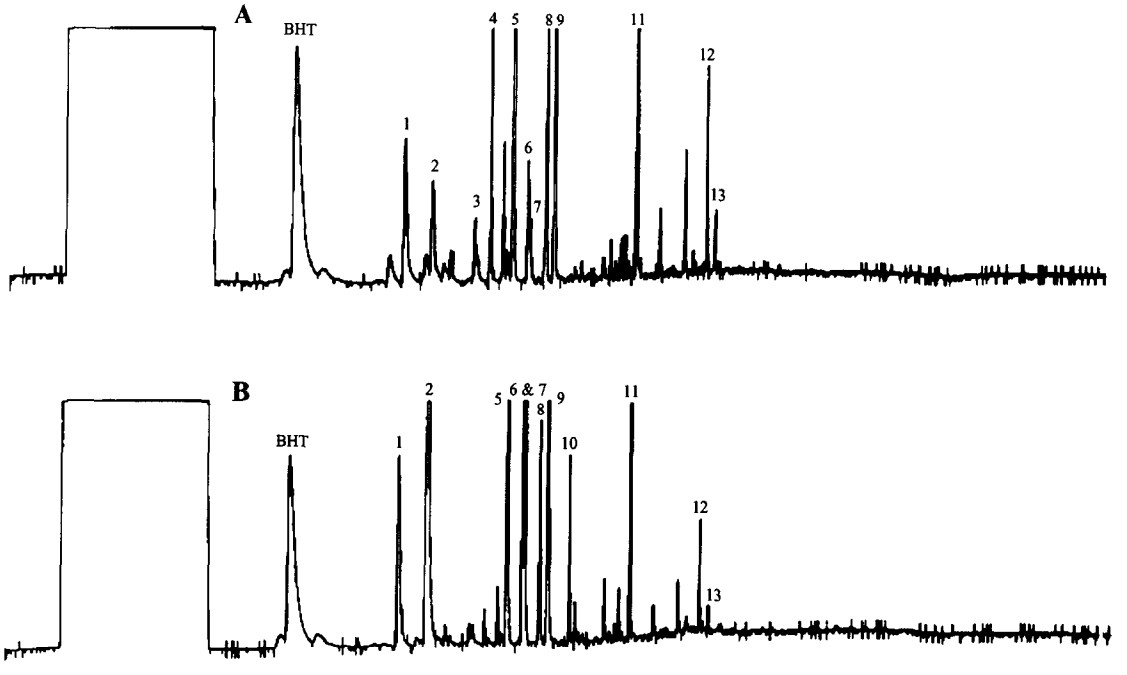

Figure 1
A typical gas chromatograph of DMA and FAME derived from pig myocardium. (A) Ethanolamine glycerophosphates and (B) choline glycerophosphates separated using a polar phase capillary column and using a flame ionization detector. (1) 16:0 DMA, (2) 16:0 methyl ester, (3) 18:0 DMA, (4) 18:1 DMA, (5) 18:0 methyl ester, (6) 18:1 d7 methyl ester, (7) 18:1 d9 methyl ester, (8) 19:0 methyl ester (internal standard used to check recoveries), (9) 18:2 methyl ester, (10) 20:0 methyl ester (internal standard), (11) 20:4 methyl ester, (12) unidentified fatty acid methyl ester, (13) 22:6 methyl ester.

Table 1
Dimethylacetyl (DMA) derivatives of choline and ethanolamine glycerophosphates

Glycerophosphates	16:0	18:0	18:1
Choline (mol %)	12.0 ± 1.3	1.1 ± 0.3	1.5 ± 0.1
Ethanolamine (mol %)	12.0 ± 0.4	10.9 ± 1.7	5.3 ± 0.5

Results are expressed as mean ± SE of at least six determinations.

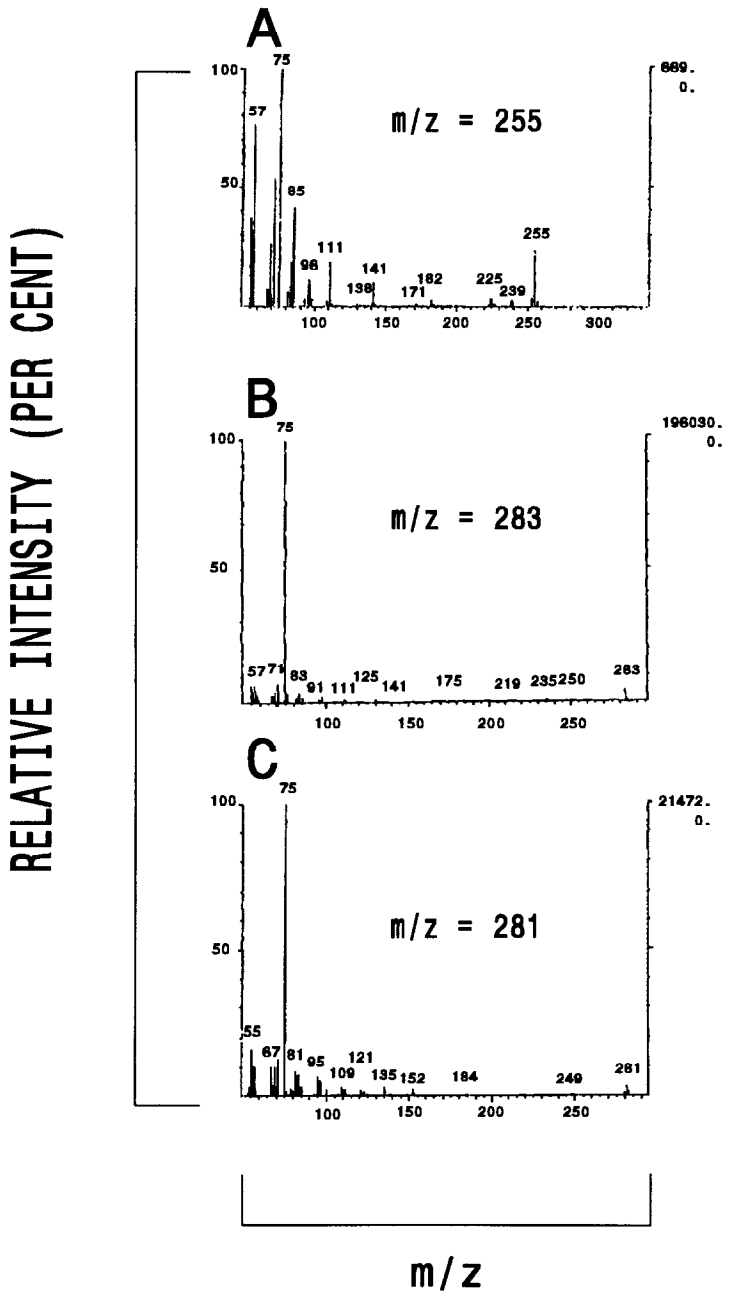

Figure 2
Representative mass spectrum for the DMAs corresponding to the designated peaks from Fig. 1. (A) 16:0 Fatty acid (peak 1); (B) 18:0 fatty acid (peak 3); (C) 18:1 fatty acid (peak 4).

Table 2
Fatty acid methyl ester (FAME) derivatives of choline and ethanolamine glycerophosphates

Glycerophosphates	16:0	18:0	18:1	18:2	20:4	22:6
Choline (mol %)	24.1 ± 1.0	10.0 ± 1.3	16.9 ± 1.3	23.7 ± 2.0	9.2 ± 0.7	0.5 ± 0.3
Ethanolamine (mol %)	3.3 ± 0.04	19.3 ± 1.5	4.8 ± 0.4	12.2 ± 1.2	27.9 ± 0.8	1.5 ± 0.8

Results are expressed as mean ± SE of at least six determinations.

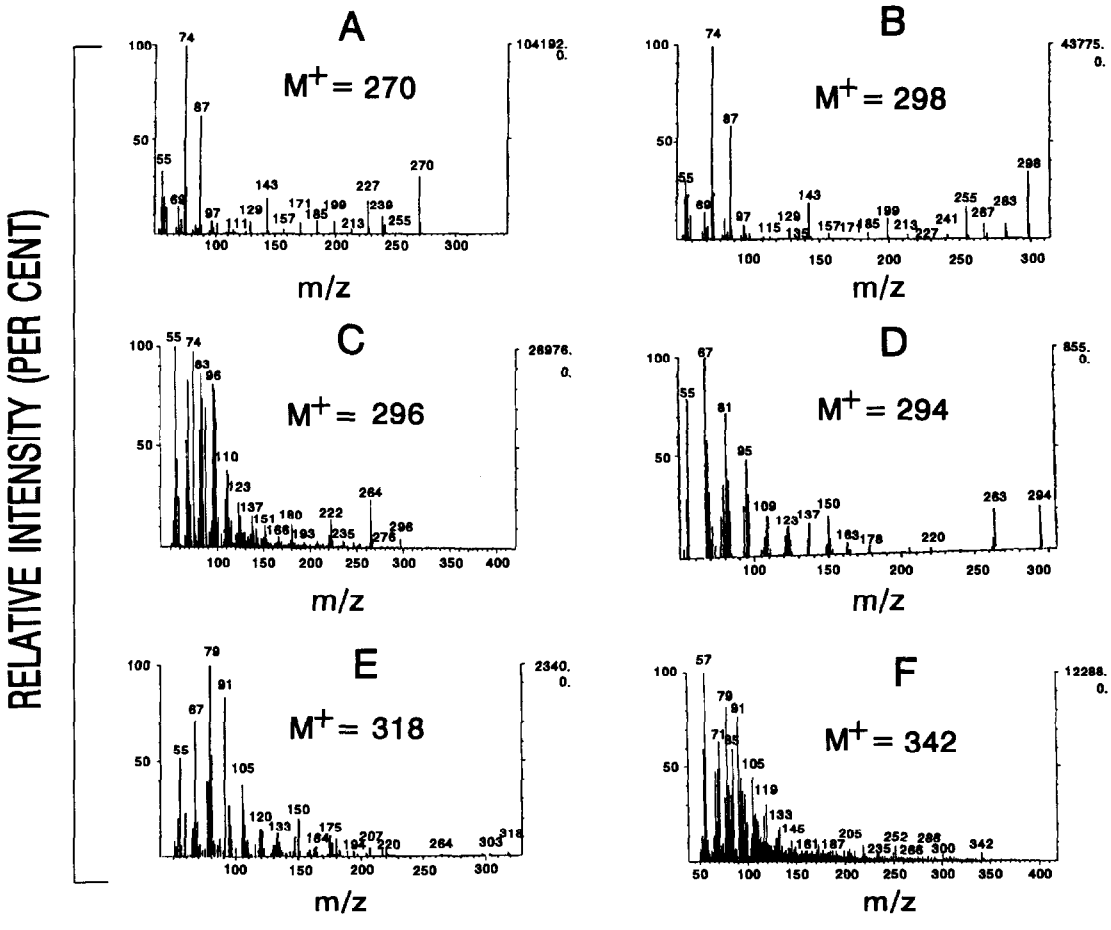

Figure 3
Representative mass spectrum for the FAMEs of the designated peaks from Fig. 1. (A) 16:0 Fatty acid (peak 2); (B) 18:0 fatty acid (peak 5); (C) 18:1 fatty acid (peak 6); (D) 18:2 fatty acid (peak 9); (E) 20:4 fatty acid (peak 11); (F) 22:6 fatty acid (peak 13).

molecular ion (M^+) peak of the FAME is distinct and characterized by the well known McLafferty rearrangement and cleavage one bond removed from the carbonyl group. The methyl ester of the aliphatic acids unbranched at the α-carbon giving a strong peak at *m/z* 74, usually base peaks, as can be seen in 16:0 and 18:0 methyl esters. These methyl esters also produced an excellent diagnostic peak at M-31. Furthermore, the cleavage at each C—C bond gave an alkyl ion (*m/z* 29, 43, 57, 71, . . . , etc.), and an oxygen containing ion, $C_nH_{2n-1}O_2$. The peak *m/z* 87 representing the ion $[CH_2CH_2COOCH_3]^+$ was always more intense than its homologues, especially for saturated FAMEs. The mass spectra of DMAs were characterized by $(M\text{-}OCH_3)^+$ peaks and this cleavage was mediated by an oxygen atom, and thus facile. The DMAs unbranched at the α-carbon producing a strong base peak at *m/z* 75, which is evident in the 16:0, 18:0 and 18:1 DMAs. Mass spectral analyses and comparison with authentic GLC standards confirmed the identity of the compounds analysed by GC.

Discussion

Since the identification of plasmalogens as the predominant phospholipid constituent of several mammalian myocardial sarcolemma and sarcoplasmic reticulum [10], interest has grown significantly to examine any potential rôle of these plasmalogens in the pathogenesis of myocardial diseases. The fact that arachidonic acid is accumulated in ischemia reperfused tissue at the expense of sarcolemmal phospholipids [11], and that plasmalogens contain large amounts of arachidonic acid in the Sn-2 position [12], led us to speculate this arachidonic acid to be the possible depot for the production of prostaglandin hydroperoxides, which are known to regulate the

vascular tone of the coronary circulation during the reperfusion of ischemic myocardium [13]. This study was specifically designed to analyse the fatty acid composition of two major ether-linked phosphoglycerides, plasmenyl choline and plasmenyl ethanolamine phosphoglycerides in swine heart.

The results of this study demonstrated that ethanolamine glycerophospholipids are rich in 18:2 and 20:4, but low in 16:0 fatty acids, while choline glycero-phosphates contain a higher amount of 16:0, and lower amounts of 18:2 and 20:4 fatty acids. A low amount of 22:6 fatty acid is present in both choline and ethanolamine phosphoglycerides, a significantly lower amount being present in the former.

The molecular ion peaks (M^+) of fatty acid methyl esters are usually distinct. The most characteristic peak is due to the familiar McLafferty rearrangement and cleavage one bond removed from the carbonyl group. Thus, a methyl ester of an aliphatic acid unbranched at the α-carbon gives a strong base peak at *m/z* 74; which is the case in both 16:0 and 18:0 methyl esters. The fatty acid methyl esters also give an excellent diagnostic peak at M-31. Furthermore, cleavage at each C—C band gives an alkyl ion (*m/z* 29, 43, 57, . . .), and an oxygen containing ion, $C_nH_{2n-1}O_2$ + (*m/z* 59, 73, 87, . . .). Thus, there are hydrocarbon clusters at intervals of 14 mass units in the case of saturated FAMEs; in each cluster there is a prominent peak at $C_nH_{2n-1}O_2$. The peak *m/z* 87, representing the $[CH_2CH_2COOCH_3]^+$ is always more intense than its homologues.

The mass spectra of DMAs are characterized by $(M\text{-}OCH3)^+$ peaks, and in DMA samples the cleavage is mediated by an oxygen atom, and therefore facile. The DMAs are unbranched at the α-carbon producing a strong base peak at *m/z* 75, which is evident in the 16:0, 18:0 and 18:1 DMAs. Mass spectral analyses and comparison with authentic GLC standards confirmed the identity of the compounds analysed by GC.

The results of this study reflect a gross analysis of the phosphoglyceride acyl and alk-1-enyl moities of heart, rather than accurate estimation of plasmanyl glycerophosphates, because this study does not include the isolation of different membrane fractions and characterization of 1,2-diester phosphoglycerides and 1-alk-1-enyl, 2-ester phosphoglycerides. Further studies using preparative TLC isolating the choline phosphoglycerides and ethanolamine phosphoglycerides fractions followed by hydrolysing the polar head groups using phospholipase C thereby liberating the DGs (which can be readily acetylated) are required to obtain the exact fatty acid composition of the plasmalogens.

In summary, the present data suggest that unlike the diacyl glycerolipids where arachidonic is likely to be derived from the breakdown of choline phosphoglyceride during ischemia and reperfusion [14], plasmenyl ethanolamine may serve as the source of arachidonic acid in ether-linked phosphoglycerides. Indeed a plasmalogen-selective phospholipase A_2 has recently been found in the myocardial membrane, which becomes activated during ischemia [15]. It is tempting to speculate that plasmenylethanolamine becomes deacylated by this phospholipase A_2 during ischemia reperfusion leading to the generation of arachidonic acid. However, further study is necessary to confirm this hypothesis by examining the selectivity of this plasmalogen-specific phospholipase A_2 towards choline and ethanolamine phosphoglycerides.

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References

[1] D.A. Ford, S.L. Hazen, J.E. Saffitz and R.W. Gross, *J. Clin. Invest.* **88**, 331–335 (1991).
[2] K.J. Kako, *Can. J. Cardiol.* **2**, 184–194 (1986).
[3] R.M.C. Dawson, N. Hemington and J.B.F. Davenport, *Biochem J.* **84**, 497–501 (1962).
[4] R.W. Gross, *Biochemistry* **23**, 158–165 (1984).
[5] Y. Masuzawa, T. Sugiura, Y. Ishima and K. Waku, *J. Neurochem.* **42**, 961–968 (1984).
[6] D.K. Das and R.M. Engelman, in *Pathophysiology of Reperfusion Injury* (D.K. Das, Ed.), pp. 149–180. CRC Press, Florida (1992).
[7] M.L. Brown, J.A. Jakubowski, L.L. Leventis and D. Deykin, *Biochim. Biophys. Acta* **921**, 159–166 (1987).
[8] Y. Kimura, J. Iyengar, R.M. Engelman and D.K. Das, *J. Cardiovasc. Pharmacol.* **16**, 992–999 (1990).
[9] R. Prasad, R.M. Jones, H.S. Young, L.B. Kaplinsky and D.K. Das, *J. Chromatogr.* **428**, 221–228 (1988).
[10] R.W. Gross, *Biochemistry* **24**, 1662–1668 (1985).
[11] H. Otani, R. Prasad, R.M. Jones and D.K. Das, *Am. J. Physiol.* **257**, 252–258 (1989).
[12] D.A. Ford and R.W. Gross, *Proc. Natn Acad. Sci. USA* **36**, 3479–3483 (1989).
[13] H. Otani, R.M. Engelman, J.A. Rousou, R.H. Breyer and D.K. Das, *J. Molec. Cell. Cardiol.* **18**, 953–961 (1986).
[14] D.K. Das, R.M. Engelman, J.A. Rousou, R.H. Breyer, H. Otani and S. Lemeshow, *Am. J. Physiol.* **251**, 71–79 (1986).
[15] R.A. Wolf and R.W. Gross, *J. Biol. Chem.* **260**, 7295–7303 (1985).

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