THE CDP-ETHANOLAMINE PATHWAY AND PHOSPHATIDYLSERINE DECARBOXYLATION GENERATE DIFFERENT PHOSPHATIDYLETHANOLAMINE MOLECULAR SPECIES

Onno B. Bleijerveld, Jos F.H.M. Brouwers, Arie B. Vaandrager, J. Bernd Helms and Martin Houweling

From the Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, and Institute of Biomembranes, University of Utrecht, Utrecht, The Netherlands

Running Title: Kennedy pathway versus phosphatidylserine decarboxylation

Address correspondence to: Martin Houweling, Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, P.O. Box 80176, 3508 TD Utrecht, The Netherlands; Tel. +31 30 2535379; Fax. +31 30 2535492; E-Mail: M.Houweling@vet.uu.nl

In mammalian cells, phosphatidylethanolamine (PtdEtn) is mainly synthesized via the CDP-ethanolamine (Kennedy) pathway and by decarboxylation of phosphatidylserine (PtdSer). However, the extent to which these two pathways contribute to overall PtdEtn synthesis, both quantitatively and qualitatively, is still not clear. To assess their contributions, PtdEtn species synthesized by the two routes were labeled with pathway-specific stable isotope precursors, D3-serine and D4-ethanolamine, and analyzed by high-performance liquid chromatography-mass spectrometry. The major conclusions from this study are that: i) in both McA-RH7777 and CHO-K1 cells, the CDP-ethanolamine pathway was favored over PtdSer decarboxylation, ii) both pathways for PtdEtn synthesis are able to produce all diacyl-PtdEtn species, but most of these species were preferentially made by one pathway. For example, the CDP-ethanolamine pathway preferentially synthesized phospholipids with mono- or di-unsaturated fatty acids on the sn-2 position (e.g. (16:0-18:2)PtdEtn and (18:1-18:2)PtdEtn) whereas PtdSer decarboxylation generated species with mainly poly-unsaturated fatty acids on the sn-2 position, (e.g. (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn in McArdle and (18:0-20:4)PtdEtn and (18:0-22:6)PtdEtn in CHO-K1), iii) the main PtdEtn species newly synthesized from the Kennedy pathway in the microsomal fraction appeared to equilibrate rapidly between the endoplasmic reticulum and mitochondria; iv) newly synthesized PtdEtn species preferably formed in the mitochondria, which is at least in part due to the substrate specificity of the phosphatidylserine decarboxylase, seemed to be retained in this organelle. Our data suggest a potentially essential role of the PtdSer decarboxylation pathway in mitochondrial functioning.

Phosphatidylethanolamine (PtdEtn) is the second most abundant phospholipid subclass in mammalian cells, comprising 15-25% of total phospholipids (1). Three pathways are present for PtdEtn biosynthesis. The majority of PtdEtn is synthesized via the CDP-ethanolamine pathway and PtdSer decarboxylation, whereas the third route, calcium stimulated base-exchange, is of little significance (2). In the CDP-ethanolamine (CDP-Etn) pathway, ethanolamine is converted to PtdEtn by the sequential actions of ethanolamine kinase, CTP:phosphoethanolamine cytidylyltransferase and finally choline-ethanolaminephosphotransferase (CEPT). CEPT has a dual specificity, as it can use both CDP-choline (CDP-Chol) and CDP-Etn as substrates for the biosynthesis of phosphatidylcholine (PtdCho) and PtdEtn, respectively (3). In addition to CEPT, a CDP-choline specific cholinephosphotransferase is available for PtdCho biosynthesis (4).

The PtdSer decarboxylation pathway for PtdEtn biosynthesis was first described by Borkenhagen et al. (5). In this pathway, PtdSer synthesized from PtdCho or PtdEtn by phosphatidylserine synthase-1 and -2, respectively (2;6;7), is decarboxylated by the enzyme phosphatidylserine decarboxylase (PSD)
to generate PtdEtn. To date, only one mammalian PSD has been cloned (8) and the enzyme was shown to be located on the external aspect of the inner mitochondrial membrane (9;10). Since PtdSer synthesis occurs in the ER and especially in ER-related membranes termed mitochondria-associated membranes (MAM) (2;11), PtdSer decarboxylation requires transport of PtdSer from its site of synthesis to the inner mitochondrial membrane, where PSD is located (12-14).

The relative importance of the CDP-Etn and PtdSer decarboxylation pathways to overall PtdEtn biosynthesis appears to vary, depending on cell type and the availability of the substrates ethanolamine and serine, respectively. From studies in Chinese hamster ovary (15;16) and baby hamster kidney (17) cells which were cultured in medium with FBS being the sole source of ethanolamine, it was concluded that PtdSer decarboxylation was the major pathway for PtdEtn synthesis. However, observations in hamster heart (18) and in rat liver, hepatocytes, heart and kidney (19;20) illustrated that the vast majority of PtdEtn is synthesized via the CDP-Etn pathway. A possible explanation for these opposite results is the availability of exogenous ethanolamine. All studies mentioned above employed incorporation of radioactive serine and ethanolamine into PtdEtn. Due to the presence of endogenous pools of unlabelled ethanolamine and serine, the quantitative contributions of both pathways to overall PtdEtn synthesis are still unclear.

An intriguing question is why two pathways for PtdEtn biosynthesis exist, whereas only one pathway is available for the de novo synthesis of PtdCho in most mammalian cells. First, a second biosynthetic pathway could serve as a backup pathway under conditions where one of the two pathways is not able to function properly. Second, the two pathways could serve mainly to ‘locally’ supply certain organelles with PtdEtn, for maintaining specific molecular species profiles within these organelles. Finally, it is possible that the two pathways yield different molecular species profiles as was shown for the synthesis of PtdCho (CDP-Cho pathway versus PtdEtn methylation) in rat hepatocytes (21). The large molecular diversity of PtdEtn and other phospholipid subclasses is dictated by the combination of different lengths, number of unsaturations and types of linkages of the hydrocarbon chains. An ester linkage at the sn-1 position defines a diacyl molecular subspecies, whereas an ether linkage at this position defines a plasmanyl subspecies and a plasmaloguen subspecies is defined by a vinyl ether bond at the sn-1 position, as in all three subspecies the hydrocarbon chain at the sn-2 position is known to be linked to the glycerol backbone via an ester bond (22).

The development and refinement of mass spectrometry in combination with the availability of deuterated pathway-specific precursors has opened the possibility of specifically displaying the PtdEtn species synthesized via the CDP-Etn or PtdSer decarboxylation pathways. We report here that McA-RH7777 cells, when cultured at equimolar concentrations of ethanolamine and serine, prefer the CDP-Etn pathway over PtdSer decarboxylation in a ratio of approximately 2:1, with the decarboxylation route having a preference for the synthesis of long chain, polyunsaturated species.

**EXPERIMENTAL PROCEDURES**

*Materials* - Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and horse serum (HS), were from Gibco BRL, Paisley, Scotland. D9-choline (HO(CH$_2$)$_3$N$^+$3H$_2$)), (2,3,3-D$_3$)-L-serine and D4-ethanolamine (HOCD$_2$CD$_2$NH$_2$) were from Cambridge Isotope Laboratories, Andover, MA, USA. Tissue culture flasks were from Corning Inc., Acton, MA, USA.

*Cell culture* - McArdle (McA-RH7777, ATCC no. CRL-1601) and Chinese hamster ovary cells (CHO-K1, ATCC no. CRL-9618) were cultured in DMEM supplemented with 6% FBS and 6% HS and Ham's F12 containing 10% FBS, respectively. The cells were maintained in 80 cm$^2$ culture flasks at 37°C, 5% CO$_2$ and 90% humidity.

*Incorporation of deuterium-labeled precursors into PtdEtn, PtdCho and PtdSer* - Cells were grown in ‘full’ DMEM to 60-80% confluency in 175 cm$^2$ culture flasks. For deuterium-label studies, cells were washed twice with phosphate-buffered saline (PBS) and incubated in serine- and choline-free DMEM (supplemented with serum as mentioned earlier) for 6, 24 or 72 hours in the presence of 400 µM D9-choline (D9-Cho) and either 400 µM D4-ethanolamine (D4-Etn) or D3-serine (D3-Ser). To maintain similar substrate concentrations during incubation, 400 µM unlabelled serine was supplemented to the culture medium of the D4-
ethanolamine incubations and vice versa. Incubations were stopped by washing the cells three times with ice-cold PBS and cells were scraped into methanol. Total lipids were extracted, phospholipid classes were isolated by normal-phase high-performance liquid chromatography (HPLC) and analyzed as described in one of the sections below.

**Subcellular fractionation of McA-RH7777 cells** - Mitochondrial and microsomal fractions were isolated from labeled McA-RH7777 cells, essentially as described for rat liver by Shiao et al. (12). Briefly, cells were scraped into PBS, pelleted and homogenized in ice-cold isolation medium (250 mM mannitol, 5 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% bovine serum albumin). After removal of nuclei and cell debris, the supernatant was centrifuged at 10,000 x g for 10 min. to pellet crude mitochondria. The resulting supernatant was centrifuged at 100,000 x g for 1 hr. to pellet microsomes. The mitochondrial pellet was further purified from MAM by hand-homogenization in isolation medium and layering of the homogenate on top of Percoll medium (225 mM mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA, and 0.1% bovine serum albumin and 30% (v/v) Percoll), followed by centrifugation for 30 min. at 95000 x g. Purity of mitochondria and characterization of microsomes was assessed by assaying for succinate dehydrogenase (mitochondrial marker enzyme) and aryl esterase (ER-marker) and by Western blotting for cytochrome c (mitochondrial marker) and calnexin (ER-marker).

**Analysis of phosphatidylethanolamine molecular species** - Total lipids were extracted from cells, mitochondrial- and microsomal fractions according to the method of Bligh and Dyer (23). The obtained total lipid extract was dissolved in hexane/isopropanol/acetone (82:17:1, v/v/v). Lipid classes were separated on a normal-phase HPLC column as described (24), the PtdEtn fraction was collected manually from the column effluent using a flow splitter, dried under nitrogen and stored at -20°C till analysis. The fraction was dissolved in chloroform/methanol (1:1, v/v) and PtdEtn species were separated on two Synergy 4μm MAX-RP 18A columns (250 x 3 mm) (Phenomenex, CA, USA) in series as described (25), with a slightly modified mobile phase of acetonitrile/methanol/triethylamine (25:24:1, v/v/v). Identification of molecular species was performed by on-line tandem mass spectrometry in the negative-ion mode on an API 4000 Q Trap mass spectrometer, fitted with an Atmospheric Pressure Chemical Ionization source (Sciex, Ontario, Canada). Analysis of PtdEtn molecular species compositions and deuterium labeling was performed by on-line single quadrupole mass spectrometry in the negative-ion mode on an API 3000 triple stage quadrupole mass spectrometer, fitted with an Atmospheric Pressure Chemical Ionization source (Sciex, Ontario, Canada). Nitrogen was used as nebulizer gas and curtain gas. PtdEtn molecular species compositions were determined by extracting the (labeled) molecular ions and isotope peaks of the various species from the negative Q1 chromatogram representing total PtdEtn, which was verified by detection with a Varlex MKIII evaporative light scattering detector (ELSD; Alltech, Deerfield, IL), operated at 100°C at a gas flow of 1.8 l/min. Determination of the position of the ester linkage of fatty acids to glycerophosphoethanolamine was performed according to Brouwers et al. (25). ELSD-data were analyzed using EZChrom software (Scientific Software, San Ramon, Canada) and mass spectrometric data were analyzed using Analyst1.4 software (Sciex, Ontario, Canada).

**Analysis of phosphatidylcholine molecular species** - The PtdCho fraction of cells, mitochondrial- and microsomal fractions was isolated from total lipid extracts by normal-phase HPLC as described in the previous section. The fraction was dissolved in chloroform/methanol (1:1, v/v) and PtdCho molecular species were separated on two LiChrospher 100 RP18-e columns (5 μm, 250 x 4.6 mm; Merck, Darmstadt, Germany) in series as previously (24), with a slightly modified mobile phase of acetonitrile/methanol/triethylamine (25:24:1, v/v/v). Identification of molecular species was performed by on-line tandem mass spectrometry in the positive-ion mode on an API 4000 Q Trap mass spectrometer, fitted with an electrospray ionization source (Sciex, Ontario, Canada). Deuterium labeling of the various molecular species was determined by on-line mass spectrometry in the positive-ion mode on an API 2000 Q Trap mass spectrometer operated in enhanced (trapping) mode, fitted with an electrospray ionization source (Sciex, Ontario, Canada). PtdCho molecular species compositions were determined by ELSD detection as described above. Determination of the position of the ester linkage of fatty acids to glycerophosphocholine was
performed according to Brouwers et al. (25) and manuscript in preparation). ELSD- and mass spectrometric data were analyzed using software as described above.

**Analysis of phosphatidylserine molecular species** - The PtdSer fraction of cells, mitochondrial- and microsomal fractions was isolated from total lipid extracts by normal-phase HPLC as described above. The fraction was dissolved in chloroform/methanol (1:1, v/v) and PtdSer molecular species were separated on a Synergi 4µm MAX-RP 18A column (250 x 3 mm) (Phenomenex, CA, USA) with a mobile phase of acetonitrile/methanol/H2O (15:22.5:12.5, v/v/v), containing 1 µM serine and 2.5 mM ammonium acetate. Analysis and identification of molecular species was performed by on-line (tandem) mass spectrometry in the negative Enhanced Mass Spectrometry ion mode on an API 4000 Q Trap mass spectrometer, fitted with an electrospray ionization source. PtdSer species compositions were determined in PtdSer fractions obtained from unlabeled cells, by extracting the molecular ions and isotope peaks of various species from the negative neutral loss 87-chromatogram representing total PtdSer.

**Interpretation of mass spectra** - The incorporation of D4-Etn into PtdEtn and D9-Cho into PtdCho was calculated for all molecular species, by determining the intensities of the unlabeled and labeled molecular ion peak in the negative or positive Q1 mass spectrum, and expressing the intensity of the labeled molecular ion as percentage of the sum of the unlabeled and labeled molecular ion. D3-Ser incorporation into PtdEtn and PtdSer was calculated similarly from (enhanced) negative Q1 spectra. Since D3-Ser labeling also, unexpectedly, yielded significant D2-Ser labeling (see ‘Discussion’ section), this D2-Ser incorporation had to be taken into account for determination of the total D3-Ser incorporation into the various PtdEtn and PtdSer species. All PtdCho, PtdEtn and PtdSer molecular ions display isotope peaks in Q1 mass spectra (Figure 1 C and D), originating from the natural presence of 13C in these large biomolecules. To determine total D3-Ser incorporation into PtdEtn and PtdSer molecular species, the natural contribution of 13C to the intensities of the isotope peaks of each molecular ion was subtracted from the actual, measured intensities of the isotope peaks in the Q1 mass spectra obtained, the remaining ‘isotope peak’ intensities (exemplified for PtdEtn in Figure 1 D) representing total D3-Ser incorporation into the various PtdEtn and PtdSer species.

**Measuring the intracellular deuterated to “cold” serine and ethanolamine ratio** - Cells were labelled with 400 µM D3-Ser or D4-Etn for various times up to 6 hours, washed twice with ice-cold PBS prior to extracting the water-soluble components (23). The water-methanol phase was collected, evaporated to dryness and the primary amines Ser and Etn were subsequently derivatized with fluorescamine exactly as described (26). The deuterated to “cold” ratio was determine using mass spectrometry.

**Determination of the substrate specificity of phosphatidylserine decarboxylase** - The substrate (D3-labeled egg-PtdSer and various PtdSer molecular species) required to determine the substrate specificity of phosphatidylserine decarboxylase (PSD) by using a mass spectrometry approach was synthesized from their respective PtdCho species. Briefly, 2.5 mg PtdCho was dissolved in 1 ml of chloroform, after which 25 mg of silica (kieselgel 60 for column chromatography) was added. The mixture was stirred for 30 min and carefully dried under a gentle stream of nitrogen. Subsequently, 250 µl of 100 mM acetate buffer (pH 5.6) containing 100 mM CaCl2, 50 mg/ml D3-serine and 10 units of phospholipase D (Streptomycins species) was added to the silica and the suspension was incubated for 36-48 hrs at 30 °C while shaking continuously. The reaction was stopped by adding 470 µl H2O and 80 µl 6M HCl and phospholipids were extracted (23;27). The amount of D3-PtdSer formed was quantified using the phosphorus assay (28).

Phosphatidylserine decarboxylase (PSD) activity was measured in mitochondria, prepared as described in the subcellular fractionation section, as the formation of D3-PtdEtn from D3-PtdSer (29). The reaction mixture (final volume 0.4 ml) consisted of 100 mM KH2PO4 (pH 6.8), 10 mM EDTA, 0.5 mg Triton X-100 per ml assay mixture, 50 µM PtdSer [D3-egg PtdSer or a mixture of equal amounts (10 µM) of D3-(16:0-16:0) PtdSer, D3-(16:0-20:4) PtdSer, D3-(18:0-18:1) PtdSer, D3-(18:0-18:2) PtdSer and D3-(18:0-20:4) PtdSer] and enzyme (approx. 250 µg of mitochondrial protein). Assays were carried out for 45 min at 37 °C and were terminated by adding 3.4 ml of chloroform-methanol/H2O (15:29:6, by vol.) followed by lipid extraction. After removing the triton X-100...
using small silica (kieselgel 60) columns, the amount of D3-PtdSer (substrate) and D3-PtdEtn (product) was quantified as described above.

RESULTS

Pathway-specific monitoring of phosphatidylethanolamine biosynthesis in McA-RH7777 cells

- To get insight into the qualitative and quantitative contributions of the CDP-Etn pathway and PtdSer decarboxylation to overall PtdEtn synthesis in mammalian cells, McA-RH7777 (McArdle) cells were incubated in the presence of deuterated, pathway-specific precursors. After various times, total lipids were extracted, subfractionated into phospholipid subclasses and the PtdEtn fraction was analyzed by high performance liquid chromatography-mass spectrometry (HPLC-MS). A typical chromatogram of the HPLC-separation of McArdle PtdEtn molecular species is shown in Figure 1A, with the peak identification given in Table 1. The major advantage of on-line HPLC-separation prior to MS is that isobaric molecular species, i.e. species having the same mass but different radiyl groups in their diacylglycerol backbones, are largely separated, thus allowing individual analysis.

Labeling of cells with D4-ethanolamine (D4-Etn) or D3-serine (D3-Ser) in combination with mass spectrometric phospholipid analysis allowed us to distinguish between PtdEtn species synthesized via the CDP-Etn pathway and species formed by PtdSer decarboxylation. D4-Etn labeled PtdEtn species, de novo synthesized via the CDP-Etn pathway, could be easily discriminated from unlabeled PtdEtn species because of a 4 Da mass difference (Fig. 1C) between the molecular ions in the (negative) Q1 spectrum (Fig. 1B). In D3-Ser, three carbon-bound protons of serine are replaced by deuterium atoms. Once D3-Ser label is incorporated into the head group of PtdSer species, the 3 Da-mass difference between labeled PtdSer species and their non-labeled counterparts is retained upon decarboxylation to PtdEtn (Fig. 1D; the unexpected appearance of D2-PtdSer will be explained in the ‘Discussion’ section and is corrected for, see ‘Experimental procedures’). In addition to PtdSer decarboxylation, (labeled) serine can be incorporated into PtdEtn via 2 other routes: i) it can enter the CDP-Etn pathway as (labeled) phosphoethanolamine, generated as an intermediate of sphingomyelin metabolism (30), and ii) it can be incorporated into the diacylglycerol moiety (31). However, experiments with β-chloro-L-alanine, a potent inhibitor of sphingomyelin synthesis (32), revealed that phosphoethanolamine liberated from sphingomyelin breakdown only marginally (< 2%) contributed to serine labeling of PtdEtn in our experimental system (data not shown). Furthermore, only a slight amount of deuterium label was detected in the diacylglycerol moiety of PtdEtn and PtdCho in McArdle cells labeled with D3-Ser for 24 h, which was clear from the fact that less than 4% of the PtdCho molecules were detected with a mass up to 3 mass units heavier than the parental molecular species. Therefore, it was concluded that in McArdle cells deuterated Ser-labeled PtdEtn species were derived from PtdSer decarboxylation (> 95%).

Qualitative and quantitative contributions of the CDP-ethanolamine pathway and PtdSer decarboxylation to overall phosphatidylethanolamine synthesis – We first determined a suitable labeling time to study the contribution of the various pathways to phospholipid biosynthesis, by labeling McArdle cells with 400 µM D9-Cho and 400 µM D4-Etn for 6, 24 and 72 hours. As McArdle cells have only one route for de novo PtdCho synthesis, namely the CDP-Cho pathway, it was expected that all PtdCho species would be labeled to the same extent when remodeling was completed. Figure 2A shows that already after 6 hours 24% of PtdCho mass in the cells is de novo synthesized, but not all species are equally labeled yet (28.1 ± 7.2%). After 24 h, approximately the doubling time of the McArdle cells under our growth conditions, 69% of the PtdCho mass is newly synthesized and all PtdCho species are equally labeled (70.4 ± 1.9) showing that remodeling was completed at this time. The observed increase, from 69% newly synthesized PtdCho at 24 h to 90% at 72 h is in agreement with the fact that after 3 cell divisions maximal 12.5% “old”-PtdCho (parental PtdCho) can still be present in the cells. In comparison with de novo PtdCho synthesis, the incorporation of D4-Etn into PtdEtn shows a totally different profile as not all PtdEtn species were equally labeled at 24 and 72 h. Furthermore, it is clear from Fig 2B that there is no or only a very modest increase in the percentage labeling of the diacyl-PtdEtn species between 24 and 72 h. As 72 h labeling shows exact the same distribution profile of newly synthesized PtdEtn as the 24 h time point, this
suggests that not all species are made via the CDP-Etn pathway in the same quantity and that this is not due to incomplete remodeling of some species. From here on, the 24 h time point was used to study PtdEtn synthesis via CDP-Etn pathway and PtdSer decarboxylation on a subcellular level.

To study the contribution of the two routes for de novo PtdEtn synthesis in detail, McArdle cells were incubated for 24 hours in the presence of 400 µM D4-Etn and 400 µM unlabeled Ser or D3-Ser and 400 µM unlabeled Etn, respectively. Under both conditions the medium was supplemented with 400 µM D9-Cho to profile PtdCho biosynthesis. The composition of the major PtdEtn and PtdCho molecular species in McArdle cells are displayed in Table 1 and 2 (column whole cell). The results clearly show that PtdEtn and PtdCho have distinct species profiles, PtdEtn containing significantly more polyunsaturated (ca. 30% of total PtdEtn versus 5% in PtdCho) and plasmalogen (ca. 10% of total PtdEtn) species than PtdCho (< 5%). In line with the data presented in Fig 2B, the various PtdEtn species were labeled with D3-Ser to a different level (Figs 3 and 4). PtdEtn species that were only modestly labeled with D3-Ser after 24 hours (e.g. (16:0-18:1)PtdEtn, D3-Ser labeled for 14%), displayed a high D4-Etn incorporation (66%) and vice versa (e.g. (18:0-20:5)PtdEtn was D4-Etn labeled for 21% and labeled with D3-Ser for 36%). To obtain the quantities (Figure 3) by which various phospholipid species are synthesized, label incorporation data were coupled to the molecular species compositions (Tables1-3).

Figure 4 displays the relative amounts by which the various PtdEtn species were de novo synthesized via the Kennedy pathway (grey bars) and PtdSer decarboxylation (black bars) in 24 hours. The contribution of PtdSer decarboxylation to PtdEtn synthesis is likely to be somewhat underestimated. This is due to the fact that, in contrast to Etn and Cho, the cells are able to synthesize this precursor de novo. The intracellular poolsize of Ser is for only 80 ± 5% deuterated (D3-Ser and D2-Ser), whereas in the case of Etn the pool is for 94 ± 2% deuterated. It can be concluded from Figure 4 in combination with Figure 2B that i) both routes seem capable of synthesizing all diacyl-PtdEtn species, whereas ethanolamine plasmalogens appeared to be predominantly synthesized via the Kennedy pathway and ii) overall, the Kennedy pathway was favored for PtdEtn biosynthesis over PtdSer decarboxylation in a ratio of approximately 2:1. Furthermore, Figure 4 clearly shows that the CDP-Etn pathway (grey bars) synthesized the largest amounts of PtdEtn in the form of (16:0-18:1)PtdEtn, (16:0-18:2)PtdEtn, (16:0-18:1)PtdEtn, (16:0-18:2/18:1-18:1)PtdEtn and (18:1-18:2)PtdEtn, whereas PtdSer decarboxylation (black bars) was the predominant route to synthesize (18:0-20:4)PtdEtn, (18:0-20:5)PtdEtn and (16:0-20:4)PtdEtn.

The D9-Cho data show (as already mentioned before, Fig. 2A) that as all PtdCho species were labeled to a similar extend, the picture illustrating the amount by which the various PtdCho species were synthesized de novo after 24 hours of incubation (Fig 3), reflected the PtdCho molecular species composition as presented in Table 2. Thus, the bulk of PtdCho synthesized by the CDP-Cho pathway is comprised of: (16:0-16:1)-PtdCho, (16:0-18:1) PtdCho, (16:1-18:1)PtdCho, (18:0-18:2)PtdCho and (18:1-18:1)PtdCho.

To determine whether the observed preference of the PtdSer decarboxylation route to synthesize PtdEtn species with polyunsaturated fatty acids at the sn-2 position is a more common phenomenon and not restricted to McArdle cells, a non rat-liver derived cell line was studied. CHO cells were labelled for 24 h with D4-Etn or D3-Ser and the amounts by which the various PtdEtn species were de novo synthesized via the CDP-Etn pathway and PtdSer decarboxylation are shown in Figure 5. In CHO cells the CDP-Etn pathway is favored over PtdSer decarboxylation in a ratio of 3:1. The higher contribution of the Kennedy pathway to PtdEtn synthesis in the CHO cells in comparison with McArdle cells is most likely due to the elevated amount of PtdEtn plasmalogens in CHO cells (CHO ca. 40% vs McArdle ca. 10%), as they are predominantly synthesized by the CDP-Etn pathway (Fig 5). The only PtdEtn species synthesized for more than 50% by the PtdSer decarboxylation route are (18:0-20:3)PtdEtn, (18:0-20:4)PtdEtn, (18:0-22:6)PtdEtn and (18:1-22:5)PtdEtn. Thus, in agreement with our observations in McArdle cells the PtdSer decarboxylation preferentially synthesizes PtdEtn species with a polyunsaturated fatty acid.

(Sub)cellular species composition and deuterium labeling of phosphatidylserine in McA-RH7777 cells - To gain more insight into the origin of
species selectivity in the PtdSer decarboxylation route (see Figures 3 and 4). PtdSer was isolated from i) McArdle cells, ii) the mitochondrial- and iii) the microsomal fraction after 24 hours of D3-Ser labeling, as described in ‘Experimental procedures’. The (sub)cellular composition of 6 selected PtdSer species are presented in Table 3. No significant difference in PtdSer species distribution between the whole cell, mitochondria and microsomes was observed. The PtdSer ‘parent-molecules’ of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn (the two PtdEtn species that are mostly synthesized by PtdSer decarboxylation) comprised only 2-3% of total PtdSer in the whole cell, mitochondria and microsomes. The PtdSer-precursors of the other two main “PSD-PtdEtn species”, (16:0-18:1)PtdSer and (18:0-18:2)PtdSer, were present in higher amounts. (18:0-18:1)PtdSer, precursor of a PtdEtn species predominantly synthesized via the CDP-Etn pathway, is, however, the most abundant PtdSer species in McArdle cells, comprising 24 ± 2% of total PtdSer in cells, mitochondria and microsomes. All PtdSer species were labeled with D3-Ser to a similar extent (50%) (Figure 3), which is lower than the amount of newly synthesized PtdCho (see Fig. 2A) due to the lower specific activity of deuterated Ser. The similar extend of labeling of all PtdSer species implicates that the relative amounts of the various PtdSer species, synthesized in 24 hours, reflected the PtdSer molecular species composition shown in Table 3.

**Contribution of CDP-Etn pathway and PtdSer decarboxylation to newly synthesized PtdEtn in McA-RH7777 mitochondria and microsomes** - PtdEtn synthesized via the CDP-Etn pathway originates from ER (1,10;33;34), whereas PtdSer decarboxylation occurs in the mitochondria (1,2,11). Shiao et al. (12) concluded from radioactive labeling studies that in Chinese hamster ovary cells, the majority of mitochondrial PtdEtn was derived from PtdSer decarboxylation and that essentially no PtdEtn was imported from the ER. To get a picture of ‘local’ PtdEtn and PtdCho synthesis in our cell system, a similar experiment as described in the previous section was performed using McArdle cells labeled with the three pathway-specific deuterated precursors (D4-Etn, D9-Cho and D3-Ser) for 24 hours.

No significant difference was detected in the PtdCho species profile between the whole cell, the mitochondrial- and microsomal fraction (Table 2), nor were any significant differences found in subcellular D9-Cho incorporation. As already described for the whole cell, PtdCho species in the mitochondria and microsomes were labeled to the same degree (70%) (data not shown). PtdCho is synthesized in the Golgi via CPT and in the ER via CEPT (33), therefore these data suggest a tendency of newly synthesized PtdCho to equilibrate relative quickly between its place of synthesis, mitochondria and presumably other cellular membranes.

In contrast to what was observed for PtdCho, the PtdEtn species distribution over the mitochondria and microsomes was very distinct (Table 1): whereas some species were more or less equally distributed over both fractions, (16:0-18:1)PtdEtn, (18:0-18:2/18:1-18:1)PtdEtn, (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn were 1.5-3 times more abundant in the ER compared to the mitochondria. On the contrary, (16:0-20:4)PtdEtn, (16:0-20:5)PtdEtn, (18:0-22:6)PtdEtn and (18:1-20:5)PtdEtn were enriched in the mitochondria. The most striking difference, however, was the distribution of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn: these species were 14- and 10-fold more abundant in the mitochondria compared to the microsomes, respectively.

The preferential synthesis of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn via PtdSer decarboxylation, as observed in whole cells (Figure 4, cf Figure 2B), was even more pronounced in mitochondria (Figure 4): approximately 80% of the newly synthesized molecules of these PtdEtn species were derived from PtdSer decarboxylation (black bars). In addition, newly synthesized PtdEtn of several other species was found to be preferentially derived from PtdSer decarboxylation in the mitochondria, including (16:0-20:4)PtdEtn, (18:0-20:3)PtdEtn, (18:0-22:6)PtdEtn, whereas almost half of newly synthesized (16:0-20:5)PtdEtn, (16:0-22:5)PtdEtn, (18:0-20:5)PtdEtn and (18:1-20:5)PtdEtn in the mitochondria was derived from this route. For all other newly synthesized PtdEtn species (except the ethanolamine plasmalogens) in the mitochondria, the balance was shifted towards PtdSer decarboxylation as well, but only to a modest extent: the vast majority of *de novo* synthesis for these species had taken place via the CDP-Etn pathway. In mitochondria the ratio CDP-Etn versus PtdSer decarboxylation derived PtdEtn was shifted towards the decarboxylation
route in comparison to the whole cell. The balance Kennedy pathway/PtdSer decarboxylation observed in the microsomal fraction (Figure 4) was the opposite of what was found in the mitochondrial fraction: all newly synthesized PtdEtn species in the microsomes, including (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn, were almost exclusively derived from the CDP-Etn pathway.

The PtdSer decarboxylation pathway generated preferentially PtdEtn species with a poly-unsaturated (e.g., 20:4 or 20:5) fatty acid at the sn-2 position. The preference of phosphatidylserine decarboxylase (PSD) for various PtdSer species was determined to get insight whether the substrate specificity of the enzyme contributes to this observation. Using egg-D3-PtdSer containing different concentrations of various PtdSer molecular species (Figure 6A) and equal amounts of five D3-labeled PtdSer species (Figure 6C) as substrates it was shown that the PtdSer species with a 20:4 fatty acid on the sn-2 position were preferentially decarboxylated by mitochondrial PSD in vitro (Figures 6B and 6D).

DISCUSSION

The CDP-Etn pathway and PtdSer decarboxylation are present in all mammalian cells. Here, we described the detailed analysis of the qualitative and quantitative contributions of the various pathways responsible for PtdEtn, PtdSer and PtdCho biosynthesis in McA-RH7777 cells by pathway-specific deuterium labeling coupled to HPLC-MS analysis. At the 24 h labeling time-point, the amount of newly synthesized PtdSer is somewhat lower than that of PtdCho and PtdEtn, via their respective CDP-pathways (Figs 2A and B). This can be explained as, in contrast to Cho and Etn, McArdle cells synthesize Ser de novo. As a result, the intracellular poolsize of Ser, which is comprised of D3-Ser, D2-Ser and Ser is only for 80 ± 5% deuterated (ratio D3-Ser to D2-Ser is 1:1), whereas in the case of Etn the pool is for 94 ± 2 deuterated. Therefore, it can be concluded that in the Figs 3 and 4 the contribution of the PtdSer decarboxylation pathway to PtdEtn synthesis is somewhat underestimated. However, this lower specific activity of the deuterated intracellular Ser pool does not influence the major conclusions drawn from Fig. 3 and 4. Furthermore, Figure 2B shows the quantitative contribution of the CDP-Etn route to the synthesis of the various PtdEtn species independent of the D3-Ser labeling experiments. We expected the observation that not all PtdEtn species were labeled to the same extent at 24 and 72 h, to be due to the fact that not all PtdEtn species were made via the CDP-Etn pathway in the same quantity and not due to incomplete remodeling of some species. This was supported by the observations that: i) the two plasmalogen PtdEtn species show a similar profile as PtdCho (see Figs. 2A and 2B), which is in agreement with the fact that plasmalogens are exclusively synthesized via the CDP-Etn pathway and ii) the PtdEtn species with a relative low labeling percentage from the CDP-Etn pathway were rapidly synthesized via the decarboxylation route, as was confirmed by the D3-Ser incorporation studies (Fig 3 and 4).

Preferential synthesis of polyunsaturated PtdEtn species via the PtdSer decarboxylation route - Although the PtdSer decarboxylation pathway is capable of synthesizing all diacyl species analyzed in CHO-K1 and McArdle cells, it generated preferentially PtdEtn species with a poly-unsaturated fatty acids at the sn-2 position (CHO-K1: 20:3, 20:4 and 22:6 and McArdle; 20:4 and 20:5). Where does the distinct species profile synthesized by PtdSer decarboxylation in McArdle cells originate from? Three possibilities were considered: i) certain PtdSer species are preferentially translocated from the site of PtdSer biosynthesis (ER/MAM) to the organelle where PtdSer decarboxylation takes place (mitochondria), or equally imported into mitochondria and subjected to remodeling prior to decarboxylation; ii) all PtdSer species are transported from ER/MAM to mitochondria with equal efficiency and selectivity is introduced by substrate specificity of the PtdSer decarboxylase (PSD); iii) PSD is indiscriminative towards imported PtdSer species and selectivity is introduced by remodeling of PtdEtn synthesized by PSD in the mitochondria.

To assess the possibility that PtdSer species were preferentially imported into the mitochondria (i), the PtdSer species composition and D3-Ser incorporation into this phospholipid class was determined in cells, mitochondria and microsomes. There was no difference in PtdSer species composition between the whole cell, mitochondria and microsomes and, more importantly, all PtdSer species were labeled with D3-Ser with equal efficiency in all three fractions. Based on these results, it seems that
PtdSer, once synthesized in the ER/MAM, readily equilibrates over the cell. Therefore, we consider it unlikely that the species selectivity observed in the PSD pathway originates from selectivity in PtdSer-import into mitochondria or specific remodeling of PtdSer in the mitochondria.

The second possibility (ii), i.e. that the species selectivity originates from substrate specificity of the PSD seems a likely explanation as an in vitro PSD assay, using isolated mitochondria as source for the decarboxylase, showed a clear substrate preference for PtdSer species with a 20:4 fatty acyl chain at the sn-2 position (see Figure 6). So the substrate specificity of the decarboxylase explains, at least in part, the preferential synthesis of PtdEtn species with long-chain polyunsaturated fatty acids at the sn-2 position. This observation is in contrast to earlier reports, where no clear preference of PSD for polyunsaturated fatty acids was detected in in vitro studies (35;36). We have no ready explanation for this discrepancy except that different tissue, cell lines and methodologies were used.

Alternatively, selectivity is introduced by rapid remodeling of PtdEtn generated by decarboxylation of PtdSer (iii). Although it has been shown that the de novo synthesized phospholipids are extensively remodeled by deacylation-reacylation (37-39), studies on phospholipid remodeling have yielded conflicting results. As the enzymes involved in remodeling are reported to reside in several subcellular compartments (40-43), including microsomes and mitochondria, remodeling of PtdEtn, ‘freshly’ synthesized by PtdSer decarboxylation in the mitochondria, is a possible explanation for the preferential synthesis of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn via this route which cannot be excluded by our data.

The molecular species profiles synthesized by the Kennedy pathways are similar - The final reaction of the Kennedy pathways for PtdEtn and PtdCho synthesis is catalyzed by a phosphotransferase. Mammalian cells express a phosphotransferase with a dual specificity for CDP-Etn and CDP-Cho. As the phosphotransferase reaction supplies the diacylglycerol part of the PtdEtn and PtdCho molecules, one would expect, when remodeling is of minor contribution, the species profiles of PtdCho and PtdEtn made by the respective Kennedy pathways to be similar. As shown in Fig 3, this seems to be largely the case, as the CDP-Etn/-Cho pathways synthesize predominantly saturated and mono- and di-unsaturated phospholipid species. Thus, the large variety of PtdEtn species (versus PtdCho) (Tables 1 and 2) is mainly caused by the presence of an additional pathway, the PtdSer decarboxylation route.

Involvement of a serine racemase explains the synthesis of D2-PtdSer from D3-serine - In mammalian cells, PtdSer is synthesized by a calcium-dependent base-exchange reaction in which the head-group of pre-existing PtdCho and PtdEtn is replaced with L-serine, catalyzed by PtdSer synthase 1 and -2, respectively (44). The molecular species profile of PtdSer agrees with that of its substrate, microsomal PtdCho and PtdEtn, except for the enrichment in (18:0-18:1)PtdSer (Table 1-3). Whether this is caused by a substrate preference of the PtdSer synthases or by the existence of specific PtdSer pools between endoplasmic reticulum and MAM, cannot be concluded from our data. The analysis of D3-Ser incorporation into PtdSer and PtdEtn showed an unexpected finding: in addition to endogenous, unlabeled phospholipid species and D3-Ser labeled species, significant D2-Ser incorporation in all PtdEtn and PtdSer species was detected (Figure 1D). This phenomenon could not be correlated to impurity of the D3-Ser (L-Serine 2,3,3-D3) label used, nor to a-specific, non-enzymatic exchange of deuterium atoms from D3-Ser with protons from water molecules during incubation (not shown). One possible explanation is that during the exchange of the head group from PtdCho or PtdEtn with serine a deuterium atom is lost. This seems unlikely, as the hydroxyl group participating in the PtdSer synthase-catalyzed reaction is not deuterated in the precursor (L-Serine 2,3,3-D3). More likely may be the involvement of a serine racemase activity. This enzyme, which is present in various tissues like brain, heart, kidney and liver (45), catalyzes the conversion of L-serine to D-serine and vice versa. The reaction mechanism of amino acid racemases, such as alanine- and serine racemase (46;47) shows that the hydrogen attached to the C2 (α-atom) of the amino acid gets detached from the molecule, resulting in the conversion (2,3,3-D3-labeled) L-serine to (3,3-D2-labeled) D-serine. The presence of a substantial amount of D2-Ser labeled PtdSer and PtdEtn suggests that one or both PtdSer
synthases may possibly use D-serine as substrate, provided that a racemase is indeed the cause of the D2-Ser formation. However, Kuksis and Itabashi recently showed that in rat brain, a tissue with a relatively high serine racemase activity, the serine in PtdSer was exclusively in the L-configuration (48). This observation suggests that D2-serine is most likely converted back to L2-serine—despite a 10 times higher \( k_m \) for the D- to L-serine than for the L- to D-serine conversion—before it is used by the PtdSer synthase.

Local phosphatidylethanolamine synthesis suggests an essential role of the PtdSer decarboxylation route in mitochondria - The main PtdEtn species newly synthesized from the Kennedy pathway in the ER/microsomes appeared to rapidly equilibrate between ER and mitochondria and seemed to be actively imported into mitochondria. The rapid equilibration of PtdEtn \( de \) novo synthesized by the CDP-Etn pathway, but also of the newly formed PtdSer and PtdCho observed in our study, is in agreement with previous reports (13). As the Kennedy pathway was found to be responsible for the bulk of the PtdEtn synthesis, rapid equilibration over the whole cell is probably required to keep up with the cellular demand for this phospholipid. In contrast, (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn, mainly formed by PtdSer decarboxylation in the mitochondria, appeared to be actively retained within this organelle, as these two species are notably enriched in the mitochondria but hardly present in the microsomal fraction. Although the biochemical significance of this observation is not known yet, Steenbergen et al. (49) recently showed that embryo’s of mice lacking PSD activity die in the uterus and have fragmented and misshapen mitochondria, an abnormality that likely contributes to the embryonic lethality of the PSD knock-out.

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FOOTNOTES

1 Abbreviations used are: CDP-Cho, CDP-choline; CDP-Etn, CDP-ethanolamine; Cho, choline; CEPT, choline-/ethanolaminephosphotransferase; ELSD, evaporative light scattering detector; ER, endoplasmic reticulum; Etn, ethanolamine; MAM, mitochondria-associated membrane PSD, phosphatidylserine decarboxylase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; Ser, serine.

FIGURE LEGENDS

Figure 1. HPLC-MS analysis of McA-RH7777 phosphatidylethanolamine molecular species.
McA-RH7777 cells were labeled with D4-Etn or D3-Ser as described in ‘Experimental procedures’. The PtdEtn fraction was isolated from total lipid extracts and analyzed for its molecular species composition and deuterium labeling by HPLC-APCI-MS. Representative figures are shown in panel A, B, C and D. A. Negative-ion Q1 (-Q1) chromatogram of PtdEtn molecular species (Peak identification is given in Table 1); B. Typical –Q1 mass spectrum, showing fatty acids -originating from the eluted PtdEtn molecular species- in the region m/z 200-300 and D4-Etn labeled PtdEtn molecular species -eluting in peak 7 of the chromatogram- in the region m/z 650-850; C. Close-up view of D4-Etn labeled (16:0-18:1)PtdEtn, showing the molecular ions of the labeled (‘new’) and
unlabeled (‘old’) species and isotope peaks originating from the natural presence of $^{13}$C in (large) biomolecules. D. Example: close-up view of D3-Ser labeled (18:0-20:4)PtdEtn, showing the total D3-Ser labeling (both D2-Ser and D3-Ser labeling) of this molecular species.

**Figure 2.** Phosphatidylcholine and phosphatidylethanolamine synthesis in McArdle cells via their respective CDP-pathway. McArdle cells were grown at 37 °C in 175 cm$^2$ flasks. When the cells were about 75% confluent, cells were incubated with D9-Cho (A) or D4-Etn (B) and after 6, 24 and 72h of labelling with the deuterated precursors, cells were harvested for lipid extraction. Subsequently lipids were separated into the major phospholipid classes by HPLC followed by molecular species profiling of the various phospholipids classes by HPLC-MS as described in ‘Experimental procedures’. PtdCho synthesis is shown in (A) and PtdEtn in (B) at 6h (open bars), 24 h (grey bars) and 72 h (black bars). Results (mean ± S.D. of triplicate) of a representative experiment are shown, which was repeated once with similar results.

**Figure 3.** Amount of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine in McArdle cells and the contribution of various synthetic routes to de novo phospholipid synthesis. McArdle cells were grown at 37 °C in 175 cm$^2$ flasks. When the cells were about 75% confluent, cells were labeled with deuterated pathway specific precursors for 24 h to monitor phospholipids composition (open bars) and de novo synthesis (D9-Chol, grey insert PtdCho (PC)-bar; D3-Ser, black insert of PtdSer (PS)- and PtdEtn (PE) bar; D4-Etn, grey insert PE bar. After labeling the cells were collected, lipids extracted and separated into the major phospholipid classes by HPLC prior to quantification of the molecular species composition of the various phospholipids classes by HPLC-MS as described in ‘Experimental procedures’. Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

**Figure 4.** Amount of phosphatidylethanolamine synthesized via the CDP-ethanolamine pathway and phosphatidylserine decarboxylation. McArdle cells were grown at 37 °C in 175 cm$^2$ flasks. When the cells were about 75% confluent, cells were labeled with D3-Ser or D4-Etn for 24 h. After labeling the cells (C) were collected for lipid extraction or sub-cellular fractions (mitochondria, M and microsomes E) were prepared prior to lipid extraction. Subsequently lipids were separated into the major phospholipid classes by HPLC followed by molecular species profiling of the various phospholipids classes by HPLC-MS as described in ‘Experimental procedures’. The black bars represent the contribution of PtdSer decarboxylation and the grey bars the contribution of the CDP-Etn pathway to newly synthesized PtdEtn. Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

**Figure 5.** Amount of phosphatidylethanolamine in CHO-K1 cells and contribution of the CDP-ethanolamine pathway and phosphatidylserine decarboxylation to its de novo synthesis. CHO-K1 cells were grown at 37 °C in 100 mm dishes. When the cells were approximately 75% confluent cells were labelled with deuterated pathway specific precursors for 24 h to monitor the PtdEtn composition (open bars) and de novo PtdEtn synthesis from D3-Ser (black insert) and D4-Etn (grey insert). After labelling the cells were collected, lipids extracted and the major PtdEtn species were separated and quantified using HPLC-MS as described in ‘Experimental Procedures’. Data are expressed as the mean ± S.D. of three experiments performed in triplicate.

**Figure 6.** Substrate specificity of phosphatidylserine decarboxylase. McArdle cells were grown at 37 °C in 175 cm$^2$ flasks. When the cells were about 75% confluent, cells were incubated in the presence of 400 µM ethanolamine and serine for 24 h. Cells were harvested, mitochondria prepared and used in the PSD-assay. Lipids were extracted and quantified as described in Experimental Procedures. (B) D3-PtdEtn formed in 45 min from its substrate D3-egg PtdSer (A). (D) D3-PtdEtn formed from its 5 synthesized D3-PtdSer species (C). The amount of PtdEtn formed is expressed as the percentage of its corresponding PtdSer species. Data are expressed as the mean ± S.D. of one representative experiment, which was repeated twice with similar results.
Table 1. Phosphatidylethanolamine molecular species composition in McA-RH7777 cells, mitochondria and microsomes.
The PtdEtn fraction was collected from total lipid extracts of McA-RH7777 cells, mitochondria and microsomes and the molecular species compositions were determined as described in ‘Experimental procedures’. Shown in this table is the PtdEtn molecular species composition (first row) of the total cell homogenate (third row), the mitochondrial fraction (fourth row) and microsomal fraction (fifth row). The second row indicates the chromatogram peak in which the various molecular species elute in the HPLC-MS chromatogram displayed in Figure 1A. Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

| PtdEtn species   | Peak | Whole cell (% of total PtdEtn) | Mitochondria (% of total PtdEtn) | Microsomes (% of total PtdEtn) |
|-----------------|------|-------------------------------|----------------------------------|-------------------------------|
| 16:0-18:1       | 7    | 18.2 ± 0.1                    | 14.4 ± 1.0                       | 19.5 ± 3.7                    |
| 16:0-18:2       | 5    | 6.1 ± 0.4                     | 5.7 ± 0.6                        | 6.2 ± 1.6                     |
| 16:0-20:4       | 3    | 1.7 ± 0.8                     | 2.6 ± 1.0                        | 0.6 ± 0.4                     |
| 16:0-20:5       | 1    | 0.4 ± 1                       | 1.4 ± 0.7                        | 0.3 ± 0.3                     |
| 16:0-22:6       | 2    | 0.8 ± 0.2                     | 1.2 ± 0.2                        | 1.3 ± 0.5                     |
| 18:0-18:1       | 9    | 6.1 ± 2.0                     | 5.5 ± 2.6                        | 7.8 ± 1.4                     |
| 18:0-18:2/18:1  | 7    | 20.9 ± 1.4                    | 15.4 ± 0.9                        | 27.5 ± 3.6                    |
| 18:1-18:1       | 8    | 0.5 ± 0.1                     | 0.5 ± 0.2                        | 0.2 ± 0.1                     |
| 18:0-20:3       | 6    | 5.1 ± 0.5                     | 12.3 ± 3.3                       | 0.9 ± 0.8                     |
| 18:0-20:5       | 4    | 8.7 ± 1.3                     | 15.3 ± 3.5                       | 1.6 ± 1.3                     |
| 18:0-22:5/20:1  | 6    | 0.8 ± 0.2                     | 1.3 ± 0.1                        | 0.4 ± 0.3                     |
| 20:1-20:4       | 5    | 2.1 ± 0.1                     | 2.3 ± 0.1                        | 1.3 ± 0.4                     |
| 18:0-22:6       | 5    | 5.8 ± 0.3                     | 4.6 ± 0.2                        | 5.7 ± 1.6                     |
| 18:1-18:2       | 2    | 0.2 ± 0.1                     | 0.3 ± 0.2                        | 0.2 ± 0.1                     |
| 18:1-20:1/18:1  | 9    | 1.0 ± 0.3                     | 0.5 ± 0.2                        | 1.5 ± 0.2                     |
| 18:0-20:2       | 3    | 1.7 ± 0.6                     | 1.4 ± 0.5                        | 1.0 ± 0.6                     |
| 18:1-20:5       | 1    | 0.3 ± 0.1                     | 1.1 ± 0.5                        | 0.3 ± 0.2                     |
| 18:1-22:6       | 2    | 0.4 ± 0.1                     | 0.6 ± 0.1                        | 0.5 ± 0.1                     |
| Plas(16:0-18:1) | 8    | 0.5 ± 0.2                     | 0.2 ± 0.1                        | 0.8 ± 0.3                     |
| Plas(16:0-22:6) | 3    | 1.8 ± 0.51                    | 1.3 ± 0.1                        | 1.9 ± 0.9                     |
Table 2. Phosphatidylcholine molecular species composition in McA-RH7777 cells, mitochondria and microsomes.
The PtdCho fraction was collected from total lipid extracts of McA-RH7777 cells, mitochondria and microsomes and the molecular species compositions were determined as described in ‘Experimental procedures’. Shown in this table is the PtdCho molecular species composition (first row) of the total cell homogenate (second row), the mitochondrial fraction (third row) and microsomal fraction (fourth row). Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

| PtdCho species | Whole cell (average ± SD) | Mitochondria (average ± SD) | Microsomes (average ± SD) |
|----------------|--------------------------|-----------------------------|--------------------------|
| 14:0-16:0      | 1.1 ± 0.1                | 1.4 ± 0.2                   | 1.4 ± 0.2                |
| 16:0-16:0      | 1.0 ± 0.2                | 0.6 ± 0.2                   | 1.3 ± 0.4                |
| 16:0-16:1/18:1 | 7.6 ± 1.3                | 8.1 ± 0.6                   | 8.0 ± 0.4                |
| 14:0-18:1      | 41.7 ± 1.9               | 39.6 ± 0.9                  | 39.0 ± 1.9               |
| 16:0-18:2      | 5.4 ± 0.9                | 5.8 ± 0.4                   | 5.7 ± 0.2                |
| 16:0-20:1      | 0.7 ± 0.1                | 0.6 ± 0.1                   | 0.7 ± 0.1                |
| 16:0-20:2      | 3.2 ± 0.1                | 2.9 ± 0.1                   | 3.0 ± 0.1                |
| 16:0-20:4      | 0.2 ± 0.1                | 0.1 ± 0.0                   | 0.1 ± 0.0                |
| 16:0-22:6      | 0.3 ± 0.1                | 0.3 ± 0.0                   | 0.3 ± 0.1                |
| 16:1-18:1      | 5.1 ± 0.5                | 6.5 ± 0.4                   | 5.6 ± 1.6                |
| 18:0-18:1      | 1.0 ± 0.2                | 1.1 ± 0.1                   | 1.1 ± 0.0                |
| 18:0-18:2      | 7.8 ± 0.2                | 8.9 ± 0.3                   | 7.8 ± 0.6                |
| 18:1-18:1      | 5.8 ± 0.7                | 6.7 ± 0.5                   | 6.3 ± 1.2                |
| 18:1-18:2      | 1.1 ± 0.1                | 1.4 ± 0.1                   | 1.2 ± 0.4                |
| 18:1-20:1      | 1.2 ± 0.3                | 1.4 ± 0.1                   | 1.3 ± 0.1                |
| 18:1-20:5      | 0.2 ± 0.1                | 0.2 ± 0.0                   | 0.1 ± 0.0                |
Table 3. Composition of some phosphatidylserine molecular species in McA-RH7777 cells, mitochondria and microsomes.
The PtdSer fraction was collected from total lipid extracts of McA-RH7777 cells, mitochondria and microsomes and the molecular species compositions were determined as described in ‘Experimental Procedures’. Shown in this table is the composition of 6 selected PtdSer molecular species (first row), which were abundant and/or interesting with respect to the research question, in the total cell homogenate (second row), the mitochondrial fraction (third row) and microsomal fraction (fourth row). Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

| PtdSer species | Whole cell | Mitochondria | Microsomes |
|----------------|------------|--------------|------------|
| % of Total PtdSer (average ± SD) |
| 16:0-18:1 | 9.4 ± 0.4  | 9.4 ± 0.7  | 9.1 ± 0.5  |
| 18:0-18:1 | 24.3 ± 2.1 | 23.8 ± 2.5 | 23.5 ± 3.7 |
| 18:0-18:2 | 10.4 ± 0.6 | 10.0 ± 0.6 | 10.1 ± 0.8 |
| 18:1-18:1 | 4.6 ± 0.1  | 4.7 ± 0.4  | 5.1 ± 0.4  |
| 18:0-20:3 | 2.5 ± 0.3  | 2.5 ± 0.2  | 2.7 ± 0.5  |
| 18:0-20:4 | 2.1 ± 0.5  | 2.0 ± 0.3  | 2.1 ± 0.4  |
| 18:0-20:5 | 2.1 ± 0.5  | 2.0 ± 0.3  | 2.1 ± 0.4  |
Bleijerveld et al. Figure 1
A

Newly synthesized PtdCho (% of total species)

B

Newly synthesized PtdEtn (% of total species)

Bleijerveld et al. Figure 2
Species composition (% of the total phospholipid class)

Bleijerveld et al. Figure 3

16:0-18:1  18:0-18:1  18:0-18:2  18:0-20:3  18:0-20:4  18:0-20:5
18:1-18:1  18:1-18:1
Bleijerveld et al. Figure 4
Bleijerveld et al. Figure 5

Species composition (% of total phosphatidylethanolamine)

A

16:0-18:1
18:0-18:1
18:0-18:2
18:1-18:1
Plas(16:0-18:1)
Plas(18:0-20:2)
Plas(16:0-20:4)
Plas(16:0-22:6)

B

16:0-18:2
16:0-20:4
18:0-20:3
18:0-20:4
18:1-18:2
18:1-20:3
18:1-22:5
Plas(16:0-18:2)
Figure 6

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