Synthesis of plasmalogens in eye lens epithelial cells

Thanh-Phuong Thai[a], Claus Rodemer[a], Jutta Worsch[a], Andreas Hunziker[b], Karin Gorgas[c], Wilhelm W. Just[a,*]

[a] *Biochemiezentrum Heidelberg (BZH), Universität Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany*
[b] *Deutsches Krebsforschungszentrum Heidelberg, Abteilung Zellbiologie, Heidelberg, Germany*
[c] *Institut für Anatomie und Zellbiologie, Universität Heidelberg, Im Neuenheimer Feld 307, D-69120 Heidelberg, Germany*

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Abstract The present paper describes cloning and sequencing of the mouse cDNA encoding dihydroxyacetonephosphate acyltransferase (DAPAT), the peroxisomal key enzyme of plasmalogen (PM) biosynthesis. Using monospecific antibodies, we localized DAPAT and alkyl dihydroxyacetonephosphate synthase to peroxisomes of mouse lens epithelial cells (LECs) and determined their enzymatic activity. By electrospray ionization mass spectrometry of mouse lens lipid extracts, we identified phosphatidyl ethanolamine including plasmenyl ethanolamine species as major constituents. Our data demonstrate the capacity of LECs to synthesize PMs and the high coincidence between deficiency of PM and early manifestation of cataract in patients with peroxisomal disorders suggests that ether-bonded lipids may play an important role in maintaining lens transparency.

Key words: Plasmalogen; Peroxisome; Dihydroxyacetonephosphate acyltransferase; Electrospray ionization mass spectrometry; Eye lens; Lens epithelial cell

1. Introduction

Metabolic pathways located in peroxisomes of higher eukaryotes are not only catabolic in nature. There are several anabolic reactions which serve the synthesis of a number of essential cellular constituents, such as ether lipids including ether phospholipids and plasmalogens (PMs) [1–4], platelet activating factor [5–7], distinct glycosyl phosphatidyl inositol-anchored proteins [8–10], farnesyl pyrophosphate, cholesterol [11,12] and unconjugated and conjugated bile acids [13–15]. In humans, a number of peroxisomal disorders are known. They are subdivided into two major categories according to multiple or single defects in peroxisomal functions and are distinguished by the fact that distinct peroxisomal activities and their related products are strongly reduced or even absent [16,17]. PMs, for example, are strongly deficient in patients with generalized peroxisomal defects, such as the cerebro-hepato-renal (Zellweger) syndrome (ZS) and related disorders, and in patients with isolated defects in the peroxisomal enzymes dihydroxyacetonephosphate acyltransferase (DAPAT) or alkyl dihydroxyacetonephosphate synthase (ADAPS), both involved in the formation of ether-bonded lipids. These isolated defects transmit the clinical manifestation of rhizomelic chondrodysplasia punctata (RCDP) [16–18] and, like the ZS, belong to the class of particularly severe peroxisomal disorders usually fatal within the first 2 years of life [19]. Among the major clinical features of peroxisomal disorders, hypotonia, psychomotor retardation, liver disease, cataract and retinopathy were noted. Cataract, for example, is a common clinical feature of RCDP and is well-documented in about 80% of the patients with ZS [16–18,20].

Since one of the physiological functions of PMs may be protecting animal cell membranes against oxidative stress [21,22], the frequent appearance of cataract in patients with peroxisomal disorders prompted us to study the peroxisome content and PM biosynthesis in cells derived from mouse lens epithelium. In the present paper, we describe the sequence of mmDAPAT cDNA and the cDNA-derived amino acid sequence. C-terminal DAPAT and ADAPS tail peptides were used to raise monospecific antisera [23] which allowed us to localize these enzymes to peroxisomes of cultured lens epithelial cells (LECs). Measurements of DAPAT activity demonstrated a 5–6 times higher activity in LECs than in human fibroblasts. Ultrastructural analysis of the mouse lens revealed that peroxisomes are preferably of tubular shape, randomly distributed within the cytoplasm and frequently associated with cytoskeletal elements. By electrospray ionization mass spectrometry (ESI-MS) of lipid extracts of mouse lens epithelial tissue, we identified plasmenyl ethanolamine (plasmenyl-PE) as a major component. The data suggest that mouse LECs contain peroxisomes capable of synthesizing PMs.

2. Material and methods

2.1. Molecular biology

Based on the previously established hsDAPAT cDNA sequence (GenBank number AJ002190) [23], we used the BLAST algorithm to probe the database of expressed sequence tags (dBEST). Initially, we identified five murine cDNAs (GenBank number AA015262, AA690646, AA061254, AA444471 and AI286730) which covered about 80% of the entire cDNA. Whereas the last three clones showed overlapping sequences representing the C-terminal third of DAPAT, clone AA015262 contained the N-terminal end. The full-length cDNA was obtained by screening a mouse brain cDNA library (Stratagene, Heidelberg, Germany) using oligonucleotides prepared according to the N- and C-terminal sequences (N-terminal sense: 5′-ATGGACGTTCCTAGCTCCTCCAGCTCCCGATTCTCG-3′; C-terminal sense: 5′-AAGAAGCCAATAGGAAAACCAGCCACTGCAAAACTA-3′). Five clones expected to contain the full-length open reading frame were identified of which one comprising 2.48 kb was entirely sequenced from both ends. Sequencing revealed a 164 bp 5′-

*Corresponding author. Fax: (49) (6221) 54 43 66.
E-mail: cl4@popix.urz.uni-heidelberg.de

Abbreviations: ADAPS, alkyl dihydroxyacetonephosphate synthase; DAPAT, dihydroxyacetonephosphate acyltransferase; LEC, lens epithelial cell; ESI-MS, electrospray ionization mass spectrometry; FITC, fluorescein isothiocyanate; PM, plasmalogen; RCDP, rhizomelic chondrodysplasia punctata; TRITC, tetramethylrhodamine isothiocyanate; ZS, Zellweger syndrome

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and a 288 bp 3′-untranslated region and a 2034 bp open reading frame encoding a polypeptide of 678 amino acid residues (GenBank number AJ132012).

2.2. Preparation and culture of mouse LECs

Using a low-resolution microscope, 4–6 mouse lenses (NIH strain, 3–7 days post-natal) were prepared under sterile conditions and incubated in 1–1.5 ml of 0.125% collagenase type CLS III (Biochrom KG, Berlin, Germany) in α-MEM containing 25 mM HEPES/NaOH, pH 7.4, at 37°C for 30 min. After 15–20 min of incubation, the tissue was gently dissociated using a Pasteur pipette. This treatment was repeated at the end of the incubation. The cells released were pelleted by centrifugation at $300 \times g$ for 5 min and were resuspended after several washes in α-MEM containing 20% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin and plated onto 5 cm Petri dishes.

2.3. Enzymatic determinations

Mouse lenses or LECs were homogenized in a buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM leupeptin, 1 mM dithiothreitol, 1 mM EDTA, 1% CHAPS and subjected to sonication (Branson sonifier B 30, Branson, Danbury, CT, USA) at 30% duty and output 2, 3 and 4, subsequently, each for 20 s on ice. The total homogenates were used for enzyme determinations. Activities of DAPAT and ADAPS were assayed as described previously [23–25]. Protein was determined by the Lowry method [26].

2.4. Immunological techniques

The C-terminal peptides of mmDAPAT (omitting the peroxisomal targeting signal 1) and mmADAPS, to which N-terminally a cysteine residue was added, were coupled to keyhole limpet hemocyanin (Sigma, Deisenhofen, Germany) at a 20-fold molar excess. The coupled peptides (0.2 mg) were mixed with adjuvant and used for the immunization of four rabbits [23]. Double immunofluorescence was performed as previously reported [27,28], decorating the primary rabbit anti-DAPAT and anti-ADAPS IgG with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Sigma, Deisenhofen, Germany) and the primary sheep anti-catalase IgG (Biotrend, Cologne, Ger-

Polypeptide Sequence Alignment of Human and Murine DAP-AT

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          *   *** * ****  *** * *** * ********* ****** ** *********  *
hsDAPAT   MESSSSSNSYFSVGPTSPSAVVLLYSKELKKWDEFEDILEERRHVSDLKFAMKCYTPLVY  60
mmDAPAT   MDVPSSSSSRFSVGSASPSSV-LLYAKDLKKWDEFEDLLEERRHISDFKFAMKCYTPPLY  59

           ******* ***  ** **** ******* ***  ******* **** ******* ****
hsDAPAT   KGITPCKPIDIKCSVLNSEEIHYVIKQLSKESLQSVDVLREEVSEILDEMSHKLRLGAIR  120
mmDAPAT   RGITPCKPGDIKSIVLSSEEINYVIKQLSRESLTGVDVLREEASEILEEMSHKLRIGAIR  119

          * ** *** ********************* ********************** ** ***
hsDAPAT   FCAFTLSKVFKQIFSKVCVNEEGIQKLQRAIQEHPVVLLPSHRSYIDFLMLSFLLYNYDL  180
mmDAPAT   FFAFVLSKIFKQIFSKVCVNEEGIQKLQRAVQEHPVVLLPSHRSYIDFLMLSFILYSYDL  179

          ***************  * ********************************** *******
hsDAPAT   PVPVIAAGMDFLGMKMVGELLRMSGAFFMRRTFGGNKLYWAVFSEYVKTMLRNGYAPVEF  240
mmDAPAT   PVPVIAAGMDFLGMRVVSELLRMSGAFFMRRTFGGNKLYWAVFSEYVKTMLRCGYAPVEF  239

          ******** *************************** ************* ** ** ***
hsDAPAT   FLEGTRSRSAKTLTPKFGLLNIVMEPFFKREVFDTYLVPISISYDKILEETLYVYELLGV  300
mmDAPAT   FLEGTRSRAAKTLTPKFGLLNIVMEPFFKREVFDTYFVPISISYDKILEESLYAYEILGV  299

          ************** ***************************  *  ****** ***** *
hsDAPAT   PKPKESTTGLLKARKILSENFGSIHVYFGDPVSLRSLAAGRMSRSSYNLVPRYIPQKQSE  360
mmDAPAT   PKPKESTTGLLKARRILSENFGSIHVYFGDPVSLRSLAAGRLNRNTYNLVPRCIPQKQPE  359

          *  ** ******* ******  **** * *  ****  ***************** ** **
hsDAPAT   DMHAFFTEVAYKMELLQIENMVLSPWTLIVAVLLQNRPSMDFDALVEKTLWLKGLTQAFG  420
mmDAPAT   DVQAFVTEVAYKMQLLQIENLALSPWLLVVTILLQNQLSMDFDALVEKTLWLKGVTQVFG  419

          *** *****  ****  ******* ******** **  ** * ** **   ** ******
hsDAPAT   GFLIWPDNKPAEEVVPASILLHSNIASLVKDQVILKVDSGDSEVVDGLMLQHITLLMCSA  480
mmDAPAT   GFLLWPDNKLPEEVVQSSILLHSNLASLVKDQVVLKMNSGSSQVVNGLVPEHIALLMCSA  479

          ********* ****** ** **** ***** *** *** **************** ******
hsDAPAT   YRNQLLNIFVRPSLVAVALQMTPGFRKEDVYSCFRFLRDVFADEFIFLPGNTLKDFEEGC  540
mmDAPAT   YRNQLLNIFARPSLVALALHMTPGLRKEDVFSCFSFLRNVFSDEFIFLPGNTLRDFEEGC  539

          ***** ** *   ***  * **  ** **  **********  * ***  *** * *  *
hsDAPAT   YLLCKSEAIQVTTKDILVTEKGNTVLEFLVGLFKPFVESYQIICKYLLSEEEDHFSEEQY  600
mmDAPAT   YLLCKAEAMQMAGKDIILTDKGTAVLQFLTSLFKPFVESYQLLCRYLL-HEEDYFGEKEY  598

          * * **** ****** ***** ***  ******* **********        ** ***
hsDAPAT   LAAVRKFTSQLLDQGTSQCYDVLSSDVQKNALAACVRLGVVEKKKINNNCIFNVNEPATT  660
mmDAPAT   LVAARKFTRQLLDQGSSQCYDALSSELQKNALAAFVRLGVVEKKKVDSKYVYYVNGPATS  658

          ********* **********
hsDAPAT   KLEEMLGCKTPIGKPATAKL                                          680
mmDAPAT   KLEEMLGCKKPIGKPATAKL                                          678
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Fig. 1. cDNA-derived amino acid sequences of human and mouse DAPAT exhibiting 81% identity. Identical amino acid residues are marked with an asterisk. The mouse sequence is shortened by two residues lacking V-22 and S-587 of the human sequence. Both polypeptides carry a peroxisomal targeting signal 1 at the C-terminal end (printed in bold). The C-terminal tail peptide used for raising anti-DAPAT antibodies is underlined.

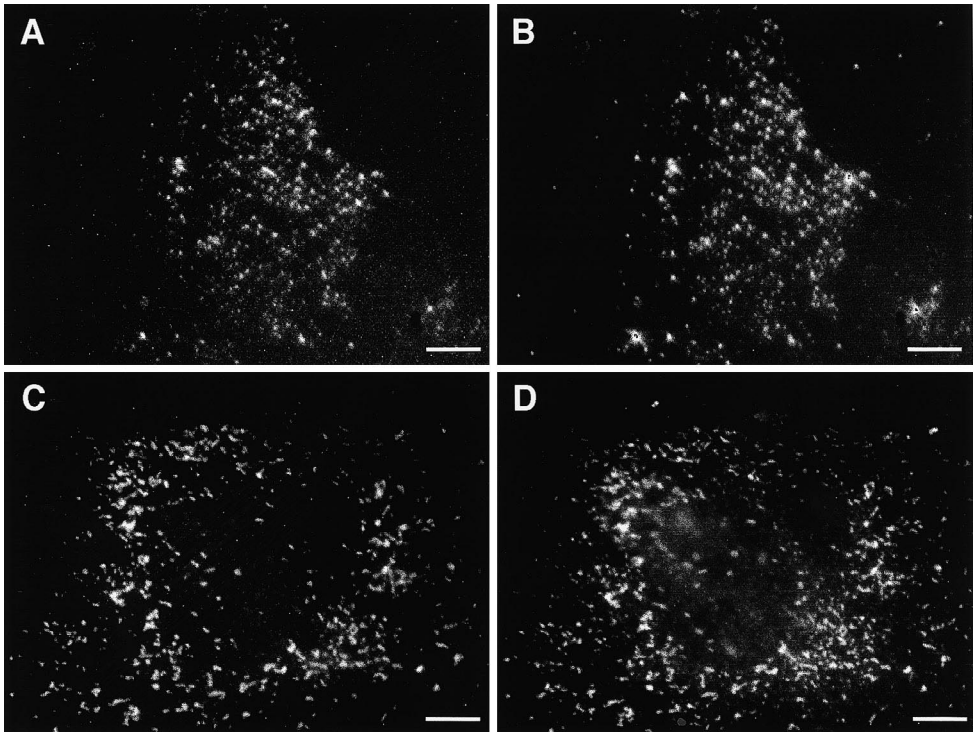

Fig. 2. Co-localization of DAPAT (A) and ADAPS (C) with catalase (B and D) in cultured mouse LECs. The primary anti-DAPAT and anti-ADAPS antibodies were decorated with FITC-labelled second antibodies and primary anti-catalase antibodies with TRITC-labelled second antibodies. Both DAPAT and ADAPS co-localized with catalase, demonstrating their peroxisomal localization. The bars represent 2 μm.

many) with tetramethylrhodamine isothiocyanate (TRITC)-labelled goat anti-sheep IgG (Sigma, Deisenhofen, Germany).

2.5. Nano-ESI-MS

Lipids of 4–6 mouse lenses were extracted as described [29] and after perchloric acid hydrolysis, the phosphate content was determined [30]. Extracted lipids were dried under a stream of nitrogen and redissolved in $CHCl_3$/MeOH 1:2 at a concentration of about 500 nmol/ml. For positive ion mode scans, 10 mM ammonium acetate was added to the extracted lipids. Following centrifugation of the samples at $13\,000 \times g$ for 5 min, measurements were done in a Quattro II nano-ESI mass spectrometer (Micromass, Manchester, UK).

2.6. Electron microscopy

Mouse lenses were fixed in a 1:1 mixture of 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M PIPES/NaOH, pH 7.6, containing 2% sucrose and saturated picric acid (200 μl/10 ml fixative) for 15 min. The fixed tissue was cut into 50–100 μm thick slices using a microslicer and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 2% polyvinyl pyrrolidone and 0.05% $CaCl_2$ for an additional 15 min. After cytochemical catalase staining with 3,3′-diaminobenzidine [31], the samples were embedded in Epon as described [32,33]. Ultrathin sections were prepared and viewed in a Zeiss EM 10 electron microscope.

3. Results

3.1. Cloning of mmDAPAT cDNA

In a previous study, we described isolation of DAPAT from peroxisomes of the rabbit Harderian gland and cloning of the hsDAPAT cDNA by screening the dBEST using rabbit sequences [23]. For the present experiments, we used the human sequence to probe dBEST and found several clones containing partial sequences of mmDAPAT cDNA. By screening a mouse brain cDNA library with synthetic oligonucleotide probes, five clones expected to contain the entire sequence were identified. One clone of the size of 2.48 kb was sequenced. It was composed of a 2034 bp open reading frame encoding a polypeptide of 678 amino acid residues (Fig. 1). The first methionine was included in a consensus Kozak sequence (5′-GCGCCATGG-3′). Compared with the human ortholog, the mmDAPAT sequence which is shortened by two amino acid residues is 81% identical. Like the human protein, mmDAPAT is targeted to peroxisomes via a type 1 peroxisomal targeting signal [34] comprised of the last three C-terminal residues AKL.

3.2. Peroxisomal localization and enzymatic activity of DAPAT and ADAPS in LECs

By using antibodies raised against synthetic C-terminal tail peptides, DAPAT and ADAPS containing structures were identified in cultured mouse LECs by immunofluorescence (Fig. 2A and C). The identified structures also contain catalase (Fig. 2B and D), demonstrating that they are peroxisomes.

Enzymatic activity of DAPAT and ADAPS was determined in both mouse lens homogenates and LECs (Table 1). Where-

Table 1
Specific activities (nmol/h/mg) of DAPAT and ADAPS in mouse lens homogenate, cultured mouse LECs and human fibroblasts

	DAPAT	ADAPS
Lens homogenate	1.35 ± 0.06	2.01 ± 0.21
Cultured LECs	10.10 ± 0.51	13.10 ± 0.72
Cultured human fibroblasts	6.20 ± 0.28	2.63 ± 0.04

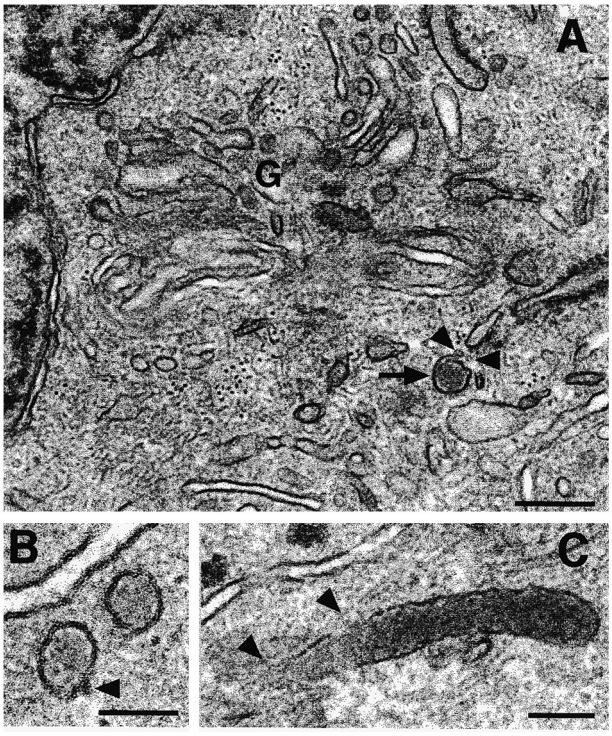

Fig. 3. Ultrastructural cytochemistry of mouse lens epithelial tissue. Peroxisomes are spherical (arrow in A) and tubular (C) in shape and randomly distributed within the cytoplasm with a higher frequency close to the nucleus. They are often found attached to microtubules (arrowheads in A and C) and to filamentous structures of about 10 nm diameter, resembling intermediate filaments (arrowhead in B). (G) Golgi apparatus. The bars represent 250 nm (A), 100 nm (B) and 125 nm (C).

as specific activities measured in lens homogenates were comparable to those present in human fibroblasts, the activities in LECs were about 7-fold higher.

3.3. Ultrastructure of mouse lens epithelial peroxisomes

Both anterior LECs and peripheral cells in the early stages of fiber cell differentiation contain DAB-stained peroxisomes. They are spherical as well as tubular in shape and their size ranges between 50–200 nm in diameter (Fig. 3A and C). Frequently, peroxisomes are associated with microtubules (Fig. 3A) [35,36] and/or closely attached to yet unidentified filamentous structures exhibiting diameters of about 10 nm (Fig. 3B and C).

3.4. Identification of plasmenyl-PE in mouse lens tissue

PMs of mammalian tissues predominantly belong to the class of ethanolamine phospholipids [1,2,37], which are readily detected by negative ion mode scanning [38]. Fig. 4 shows the single stage negative ion spectrum of a total unprocessed lipid extract from mouse lenses. The following major plasmenyl-PE species were identified: plasmenyl-PE 34:1, plasmenyl-PE 36:2, plasmenyl-PE 44:4. The major PE species were PE 34:1, PE 36:2, PE 40:6 and the major phosphatidyl choline (PC) species measured by positive ion mode scanning (not shown) were PC 32:0, PC 34:1, PC 36:4 and PC 40:6. These data demonstrate that membranes of mouse lens tissue contain plasmenyl-PE of various chain lengths and degree of desaturation.

4. Discussion

In reviewing the literature, we noticed a striking coincidence between deficiency of PMs, as they occur in distinct peroxisomal disorders, and the early onset of cataract and/or retinopathy in afflicted patients [16–18,20]. The peroxisomal disorders involved are caused by genetic defects in both the assembly of the peroxisomal compartment, e.g. ZS, neonatal adrenoleukodystrophy or RCDP, and single peroxisomal enzymes, such as DAPAT and ADAPS. All these disorders have in common that tissue levels of PMs are more or less severely decreased and activities of DAPAT and ADAPS are heavily reduced or absent.

Synthesis of PMs requires the concerted action of the peroxisomal compartment and the endoplasmic reticulum. Synthesis starts within peroxisomes in which DAPAT and ADAPS mediate the formation of the C1 long chain alkyl ether of DAP [1,2]. The subsequent steps to complete the phospholipid molecule all occur in the endoplasmic reticulum. Since these peroxisomal activities are the only ones known so far leading to formation of ether lipids in mammalian tissues, the unimpaired activities of DAPAT and ADAPS are essential for PM biosynthesis.

Using the cDNA sequence of hsDAPAT [23,39], we screened the EST database and a mouse brain cDNA library and identified the mouse ortholog. The cDNA-derived amino acid sequences of the human and mouse enzyme are about 80% identical and hence, the protein seems to be highly conserved within mammals. We used the sequence information of mouse DAPAT and ADAPS to raise antibodies which reliably recognized the antigens in Western blots [23] and in immunofluorescence studies with cultured mouse LECs. DAPAT and ADAPS staining in these cells resulted in punctate patterns characteristic for peroxisomal localization [27,28,40]. Specific peroxisomal location was verified by co-localizing the DAPAT and ADAPS staining with that of catalase, a specific peroxisomal marker.

Peroxisomes of mouse LECs are spherical to tubular structures. Tubules exhibit an average diameter of 85 nm, exceeding a length of 1 μm. The organelles are randomly distributed

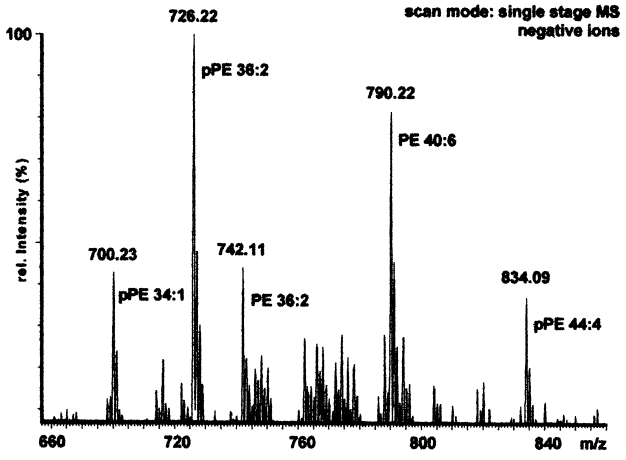

Fig. 4. Single stage ESI-MS of a lipid extract of mouse lens tissue, analyzed in the negative ion mode for the detection of plasmenyl-PE (pPE) and PE species. The phospholipid molecules detected revealed a variable chain length and degree of desaturation within their fatty acid moieties.

within the cytosol and often localize adjacent to the nucleus (Fig. 3B and C) [41]. Frequently, they were found closely attached to microtubules, suggesting their active movement within LECs [35,36,42]. However, microtubules were not the only cytoskeletal elements found in close proximity to LEC peroxisomes. A number of organelles were associated with filamentous structures exhibiting diameters of about 10 nm and resembling intermediate filaments. Their exact characterization remains to be done.

To compare the PM synthetic capacity of mouse LECs with that of other tissues, we measured DAPAT and ADAPS activities in total homogenates of mouse lenses, LECs and human fibroblasts. Due to the low amount of metabolically active cells, DAPAT and ADAPS activities in lens homogenates were rather low, however, they could be measured reliably and were in the range comparable to that in human fibroblasts, demonstrating the potency of these cells to synthesize the PM precursor 1-*O*-alkyl DAP.

Final proof that mouse lens epithelial tissue contains PMs was obtained by ESI-MS analysis of mouse lens lipid extracts. These studies revealed the presence of various species of plasmenyl-PE including plasmenyl-PE C 34:1, plasmenyl-PE C 36:2 and plasmenyl-PE C 44:4. The occurrence of plasmenyl-PE in bovine and rat lenses was previously reported [43] and recently confirmed by a [^{31}P]NMR analysis [44]. Besides plasmenyl-PE, we found PE, PC, phosphatidyl serine and sphingomyelin as major constituents.

The physiological role of PMs is still not clear, although various functions have been attributed to them. Their role in protecting animal cell membranes against oxidative stress is highly attractive and seems to be well documented [21,22,45,46]. Due to the reactivity of the enolether moiety, PMs seem to be particularly suited for defense against oxidative damage compared to their fatty acid ester analogs, thereby effectively inhibiting iron- and copper-dependent lipid peroxidation rather than radical initiator-mediated reactions [47] and cf. [17,20]. Thus, PMs may be indispensable for the lens. However, besides the anti-oxidative capacity of PMs, ether lipids may exert essential physiological functions, as for example in the form of PAF [5–7], 1-*O*-alkyl 2-acyl glycerol [48] or distinct GPI-anchored proteins [8–10].

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