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Review

Biosynthesis and possible biological functions of plasmalogens

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Contents

1. Introduction

Both choline and ethanolamine glycerophospholipids in mammalian tissues consist of three subclasses, namely, 1,2-diacyl-*sn*-glycero-3-phosphocholine (-ethanolamine), 1-alkyl-2-acyl-*sn*-glycero-3-phospho-choline (-ethanolamine), and 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phospho-choline (-ethanolamine). In order to provide a simple term for the 'diradylglycerophospho-' radicals that would be comparable to 'phosphatidyl,' which designates the '1,2-diacyl-*sn*-glycero-3-phospho' radical, the Working Group on Lipid Nomenclature for the IUPAC-IUB Commission on Biochemical Nomenclature in 1976 [1] recommended the terms plasmanyl(alkyl) and plasmenyl (alk-1-enyl) for the 1-alkyl-2-acyl and 1-alk-1′-enyl-2-

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acyl types of glycerophospholipids, respectively. Thus, plasmanic acid represents the alkyl analog, and the plasmenic acid represents the alk-1-enyl analog of phosphatidic acid. Plasmanylcholine and plasmenylcholine can be interchanged with 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) and 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine (alkenylacyl-GPC), respectively, whereas plasmenylethanolamine is 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (alkenylacyl-GPE). Those glycerophospholipids with alk-1-enyl groups at the *sn*-1 position of the glycerol are also referred to as plasmalogens (i.e. choline plasmalogens or ethanolamine plasmalogens) (Fig. 1).

In general, the alkyl moieties are found in both choline and ethanolamine glycerophospholipids, whereas the alk-1-enyl linkage occurs mainly in ethanolamine glycerophospholipids [2–4]. However, notable exceptions do exist. For instance, the content of the alkylacyl-subclass is relatively low in most tissues examined [2–6], but cells that can be stimulated to produce the potent biological mediator, platelet-activating factor (PAF), contain elevated levels of alkylacyl-GPC [4–6]. Among these are human polymorphonuclear leukocytes and rat alveolar macrophages where alkylacyl-GPC represents 50.2 and 35.2% of the total choline glycerophospholipids, respectively [4–6]. Similarly, the plasmenylcholine content of hearts from several species (except rat) and bovine epididymal spermatozoa can be as high as 33–63% of the total diradyl-GPC fraction (also see Table 1, and [4]).

Table 1
Plasmenylcholine (alk-1-enylacyl-GPC) levels in the choline glycerophospholipids of selected mammalian tissues and cells

Tissue	Percentage of total diradyl-GPC
Mouse brain	4.4
Human heart	36–41
Bovine/ox heart	33–52
Rabbit heart	39–41
Guinea pig heart	36
Canine myocardial sarcolemma	57
Rat heart	2–4
Rat small intestinal smooth muscle	7.2
Human polymorphonuclear leukocytes	9.4
Bovine epididymal spermatozoa	63
Porcine spermatozoa	23

Adapted from the review by Sugiura and Waku [4].

Extensive research studies in the late sixties and early seventies identified the enzyme activities that catalyze the biosynthesis of the ether linkage and generate the alk-1-enyl bond in plasmenylethanolamine [7,8]. However, the origin of the alk-1-enyl linkage of plasmenylcholine has only recently begun to unravel. The intent of the present review is to briefly summarize the current available knowledge regarding the biosynthesis of alkyl glycerophospholipids and ethanolamine plasmalogens (plasmenylethanolamine), to highlight the evidence indicating that choline plasmalogens (plasmenylcholine) are not made directly from plasmanylcholine via microsomal Δ1 alkyl desaturase as shown for that of ethanolamine plasmalogens, and to provide an overview showing that ethanolamine plasmalogens are converted through a series of metabolic reactions to choline plasmalogens. Finally, the possible biological functions of ethanolamine and choline plasmalogens will be discussed.

2. Biosynthesis of alkyl glycerophospholipids

2.1. Enzymes participating in the committed steps of introducing the ether-linkage in lipids

Two substrates, namely long chain fatty alcohols (ROH) and acyldihydroxyacetone phosphate (acyl-DHAP), are required in order to introduce the ether-linkage into the glycerophospholipids. Fig. 2 illustrates a brief schematic representation for the biosynthesis of alkylglycerophosphate (alkyl-GP, an ether analog of the lysophosphatidic acid) from acyl-CoA and DHAP (a glycolytic intermediate).

Acyl-CoA reductase catalyzes the two consecutive reductions of acyl-CoA to alcohols with NADPH serving as the specific coenzyme (acyl-CoA → aldehyde → alcohol) (Fig. 2, reaction I) [8]. DHAP acyltransferase catalyzes esterification of the free hydroxyl group of DHAP by utilizing long chain ($>C_{10}$) acyl CoAs to form acyl-DHAP (Fig. 2, reaction II) [9]. Alkyl-DHAP synthase then replaces the acyl chain in acyl-DHAP with a long-chain fatty alcohol to form alkyl-DHAP, the first detectable ether-linked product in the ether lipid biosynthetic pathway (Fig.

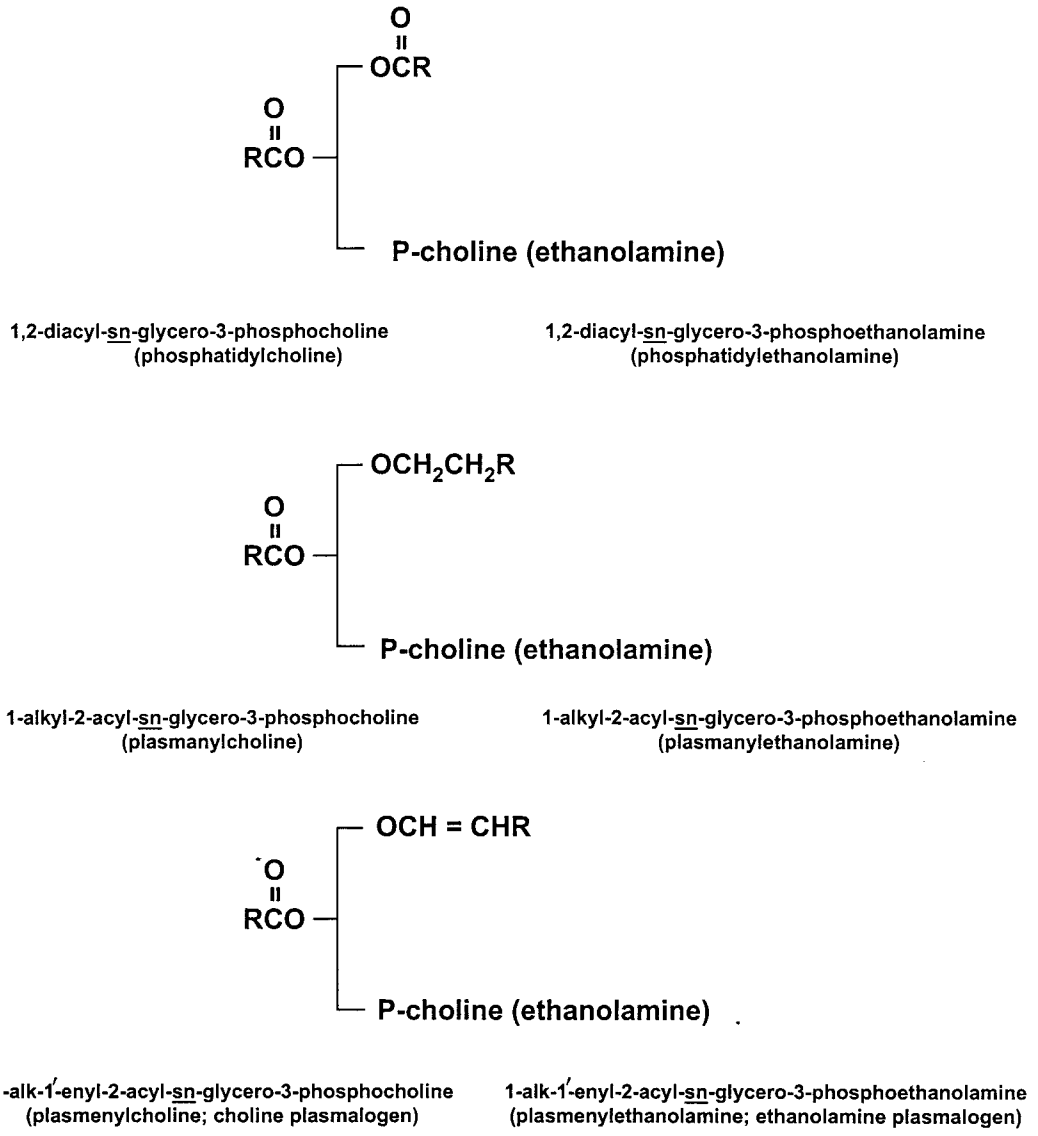

Fig. 1. Subclasses of choline and ethanolamine glycerophospholipids.

2, reaction III) [8]. Finally, alkyl-DHAP reductase reduces the ketone group of alkyl-DHAP using NADPH to generate alkyl-GP (Fig. 2, reaction IV) [8].

It has been postulated that the low activity of the acyl-CoA reductase accounts for this enzyme as the rate-limiting reaction for the overall biosynthesis of ether lipids in animal tissues [10]. In addition, this reductase is shown to be fairly specific for palmitate (16:0), stearate (18:0), and oleate (18:1). Similarly, the alkyl and alk-1-enyl moieties of both choline and ethanolamine phospholipids in a variety of tissues and tumors are also mainly 16:0, 18:0, and 18:1 [10]. On the other hand, alkyl-DHAP synthase incorporates both polyunsaturated and shorter chain alcohols [8]. Therefore, acyl-CoA reductase is believed to determine the distribution of ether chains found in tissues.

A recent report by Hayashi and Sato [11] indicates that rat liver peroxisomes are able to catalyze the chain elongation of dodecanoyl-CoA by acetyl-CoA, which is supplied by β-oxidation, and then subsequently form hexadecanol in the presence of a reducing factor (NADH/NADPH). It has been fur-

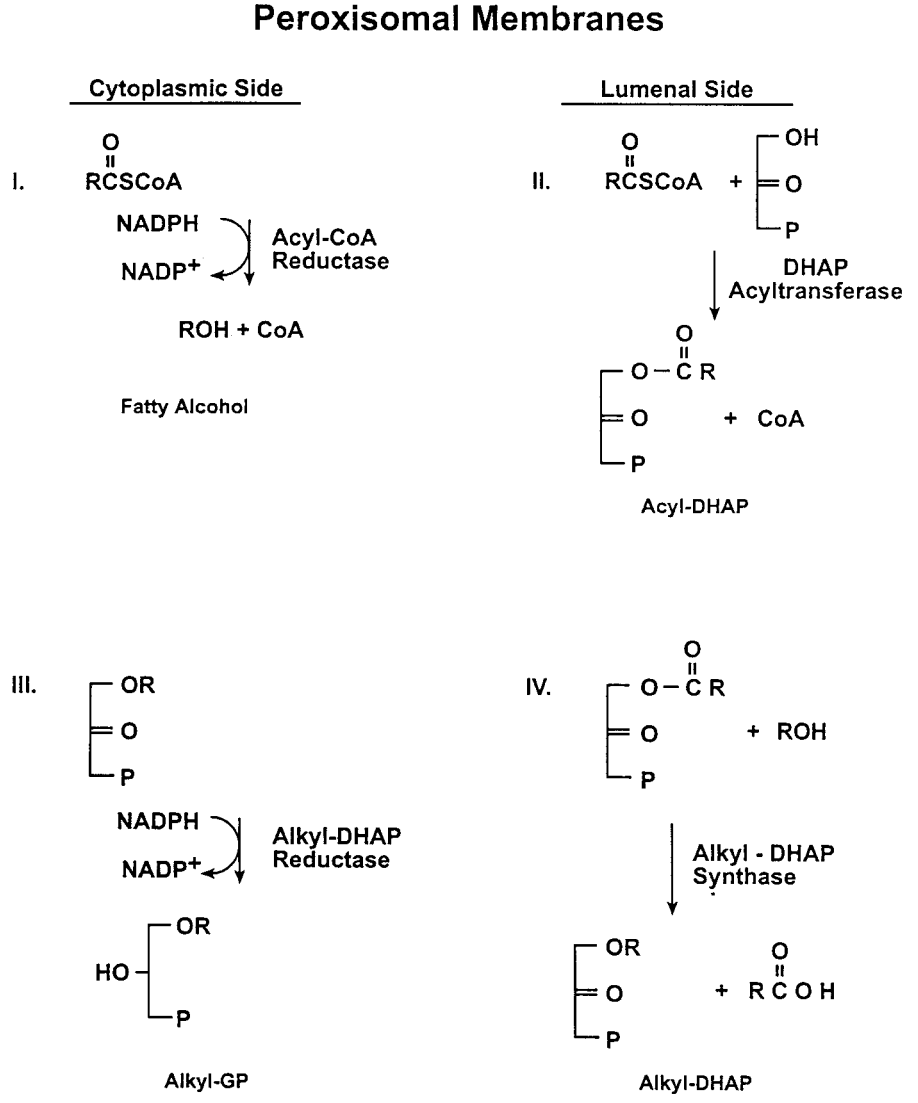

Fig. 2. Peroxisomal enzymes participating in the committed reaction steps responsible for introducing the ether-linkage into lipids.

ther shown that acetyl-CoAs derived from peroxisomal β-oxidation are readily incorporated into the alk-1-enyl group of ethanolamine plasmalogens [12]. However, the quantitative contribution of this pathway to the synthesis of ether lipids is currently unclear.

DHAP acyltransferase has been purified to apparent homogeneity from guinea pig liver [13] and human placental [14] peroxisomes with a single 69- and 65-kDa band on SDS-PAGE, respectively. Using antibodies raised against the purified DHAP acyltransferase in rabbits, Ofman and Wanders [14] showed, by immunological titration with the prepared antiserum, that fibroblasts from Zellweger cerebrohepatorenal syndrome (ZS) patients (whose liver and kidney tissues lack peroxisomes) contain only ~10% of the DHAP acyltransferase found in normal fibroblasts.

Recently, Thai et al. [15] have been able to extract and partially purify the DHAP acyltransferase from rabbit Harderian gland peroxisomes. From peptide sequences, matching EST-clones were obtained, which allowed cloning and sequencing of the cDNA from a human cDNA library. The nucleotide-derived amino acid sequence revealed a protein consisting of 689 amino acid residues of molecular mass 77 187 containing a C-terminal type 1 peroxisomal targeting signal. Screening of protein data-

bases revealed homologies of human DHAP acyltransferase with glycerol-3-phosphate acyltransferase established for a wide variety of species. The homologies are restricted to distinct domains of about 35–80 amino acid residues in lengths with 78% similarities and up to 45% identities.

Alkyl-DHAP synthase prepared from a peroxisomal membrane fraction of guinea pig liver has also been purified to a single 65-kDa band on SDS-PAGE [16]. Both cDNAs of alkyl-DHAP synthase from guinea-pig liver [17] and human fibroblasts [18] have been cloned and revealed the presence of a peroxisomal targeting signal 2.

There has been a steady debate over the years concerning the subcellular localizations of DHAP acyltransferase and alkyl-DHAP synthase [8,13–17] and whether mitochondrial and microsomal *sn*-glycerol-3-phosphate (GP) acyltransferase also catalyze the acylation of DHAP [13,18]. The question also still remains as to the extent, in addition to its main function in the formation of the obligate precursor of ether lipid (acyl-DHAP), that DHAP acyltransferase is involved in the biosynthesis of non-ether lipids in animals.

It is generally accepted now that all animal peroxisomes contain anabolic acyl-DHAP pathway enzymes. Acyl-CoA reductase is localized on the outside (cytosolic side) of the peroxisomal membrane, and DHAP acyltransferase and alkyl-DHAP synthase are localized on the inside face (lumenal side) of the peroxisomal membrane [7]. Alkyl-DHAP reductase, the same enzyme that also catalyzes the reduction of acyl-DHAP [19], is found in both peroxisomes (cytosolic side) and the endoplasmic reticulum (ER) [7]. These results are consistent with the findings that both DHAP acyltransferase [15] and alkyl-DHAP synthase [17,18] have type 1 and type 2 peroxisomal targeting signals, respectively.

Both mitochondria and microsomes were reported to have DHAP acyltransferase activities [7]. However, it has not been resolved whether these activities are derived from the presence of GP acyltransferases present in those fractions using DHAP as a non-specific substrate. Mammalian mitochondrial GP acyltransferase has recently been purified and its gene has been cloned and expressed in baculovirus-infected cells [20]. However, it has not been reported whether this enzyme will acylate DHAP or not.

In a number of tissues including tumors, alkyl-DHAP synthase was shown to be enriched in the microsomal fractions [8]. Considering the microperoxisomes (smaller than peroxisomes in liver and kidney, <0.1 to 1.0 μm) [21] in these tissues whose sedimentation properties are similar to that of ER vesicles (i.e. brain and intestine mucosa), it turns out that the presence of alkyl-DHAP synthase in microsomes is due to the contaminating microperoxisomes in the ER [7]. However, tumor cells have no demonstrable peroxisomes [7], but nevertheless contain high concentrations of ether lipids and high levels of alkyl-DHAP synthase in the microsomes [8]. It will be interesting to determine where alkyl-DHAP synthase is located in cancer cells enriched in ether lipids.

It is not clear whether non-ether glycerolipids are synthesized via a combination of peroxisomal DHAP acyltransferase and acyl-DHAP reductase to generate acyl-GP (lysophosphatidic acid) intermediate; however, 50–60% of non-ether glycerolipids was estimated to be synthesized by this particular pathway using D-[U-^{14}C,3-^{3}H]glucose as a labeled precursor [7,22]. This substrate generates intracellular labeled glycerol, and also [4-^{3}H]NADPH. The latter selectively transfers hydrogen to C-2 glycerol in glycerolipid via the acyl-DHAP pathway and thus the ^{3}H/^{14}C ratio can serve as an indicator for the participation of the acyl-DHAP pathway in non-ether glycerolipid synthesis. However, it should be pointed out that this method may be subject to isotope effects, and the acyl-DHAP reductase may not be absolutely specific for NADPH [23]. Recently, a mutant isolated from the fibroblast-like cell line, CHO-K1 (Chinese hamster ovary cells), with a deficiency in acyl/alkyl-DHAP reductase activity showed a decrease of 45–50% in overall phospholipid biosynthesis [24]. Both diacyl- and ether-linked species were affected. Thus, these results support the notion and also provide direct evidence that the 'DHAP pathway' contributes significantly to diacyl phospholipid biosynthesis.

2.2. Enzymes involved in the biosynthesis of alkyl glycerophospholipids

There are three/four enzymes, namely, alkyl-GP acyltransferase, alkylacyl-GP phosphohydrolase, and alkylacyl-G:CDP-choline (CDP-ethanolamine)

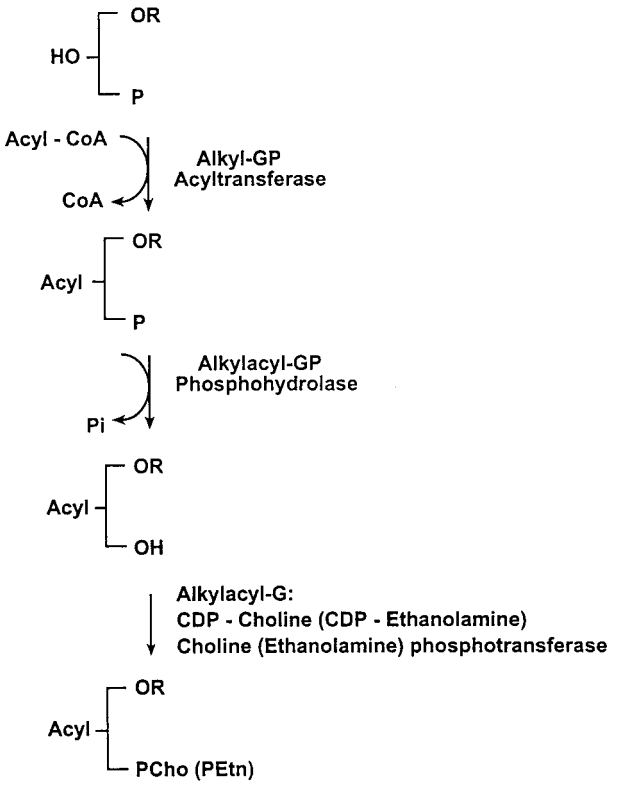

Fig. 3. Microsomal enzymes involved in the biosynthesis of alkyl glycerophospholipids containing choline or ethanolamine.

choline (ethanolamine) phosphotransferase, involved in the conversion of alkyl-GP to two major alkyl glycerophospholipids (alkylacyl-GPC/alkylacyl-GPE) (Fig. 3). It appears that all of these enzymes are located in the microsomes [8]. Based on the fact that alkyl-DHAP synthase is located at the luminal side of the peroxisomal membrane, and alkyl-DHAP/acyl-DHAP reductase is positioned at the cytoplasmic side of peroxisomal membrane and ER, it is reasonable to assume that alkyl-DHAP has to be translocated across the peroxisomal membrane for further conversions to other phospholipids in the ER.

Alkyl-GP acyltransferase is not identical to acyl-GP (lysophosphatidic acid) acyltransferase based on indirect data showing that these two enzyme activities have different relative specific activities in different tissues and different kinetic parameters towards a series of acyl-CoA donors [25]. Human lysophosphatidic acid acyltransferase has recently been cloned and expressed; however, whether this enzyme could use alkyl-GP as a substrate requires further testing [26].

Phosphohydrolase activities that can remove the phosphate moiety from alkylacyl-GP have been reported to be present in microsomes and lysosomes [8]. However, properties of these alkylacyl-GP phosphohydrolase activities have not been characterized in detail. Also, no studies are currently available to show the relationships between alkylacyl-GP phosphohydrolase and the well-characterized diacyl-GP (phosphatidate) phosphohydrolases [27,28].

The requirement for CDP-choline or CDP-ethanolamine and magnesium, and the involvement of choline- or ethanolamine-phosphotransferase in the synthesis of alkylacyl-GPC or alkylacyl-GPE, is analogous to the requirement established much earlier for the diacyl phospholipid counterparts [8]. Substantial evidence, including genetic criteria, support the notion that the cholinephosphotransferase and ethanolaminephosphotransferase are separate enzymes [8,29–31].

Similar pH optima, thermolabilities, inhibitions by Mn^{2+}, and dithiothreitol exhibited by diacyl-G and alkylacyl-G cholinephosphotransferases indicate that a closely related or identical enzyme catalyzes the formation of diacyl-GPC and alkylacyl-GPC [32]. Besides, diacyl-G, alkylacyl-G, and alkenylacyl-G are labeled in a common fashion from metabolic precursors (i.e. [^{3}H]choline) in the heart and liver that are high (32% of choline glycerophospholipids) and low (3% of choline glycerophospholipids) in plasmenylcholine content, respectively, and are competitive inhibitors in vitro. This also implies that one enzyme may catalyze all three cholinephosphotransferase reactions [30]. In addition, results from metabolic labeling of the ethanolaminephosphotransferase mutants is consistent with the notion that ethanolaminephosphotransferase utilizes both diacyl-G and alkenylacyl-G as substrates in vivo [29]. The availability of diradylglycerols and the turnover rate of ether-linked lipids are important factors in controlling the diradyl-GPC levels in tissues [32,33].

It has been a difficult task to purify membrane-bound cholinephosphotransferase or ethanolaminephosphotransferase to homogeneity from any source [30,31]. However, a cholinephosphotransferase-encoding gene and a dual specificity choline-/ethanolamine-phosphotransferase gene have been isolated

from *Saccharomyces cerevisiae*, and the structure and properties of these two genes have been characterized extensively [30,31]. Furthermore, a CDP-ethanolamine:diglyceride ethanolaminephosphotransferase from a bovine liver has recently been purified with a purification factor of about 13 000-fold and analyzed to be a single protein band of 38 kDa on SDS-PAGE [34]. This isolated protein exhibits both ethanolaminephosphotransferase and cholinephosphotransferase activity [34].

3. Biosynthesis of alk-1-enyl (plasmalogen) glycerophospholipids

3.1. Ethanolamine plasmalogens

Conversion of alkylacyl-GPE to alk-1-enylacyl-GPE (ethanolamine plasmalogen) is carried out by a cytochrome b_5-dependent microsomal electron transport system (Fig. 4). This electron transport system is analogous to that of stearoyl-CoA desaturase containing three components, namely cytochrome b_5, NADH:cytochrome b_5 reductase, and a cyanide-sensitive Δ1 alkyl desaturase [8]. These two desaturase systems respond differently to fat-free diets [35]. The stearoyl-CoA desaturase activity was increased by the fat-free diet, while no increase in the desaturation of alkylacyl-GPE was observed. This study suggests that the two desaturase systems are not identical and probably depend on different cyanide-sensitive terminal desaturases.

3.2. Choline plasmalogens

It has been known since the early seventies that in various compounds tested in enzyme systems that converted up to 50% of the corresponding ethanolamine precursors to ethanolamine plasmalogens, no synthesis of choline plasmalogens was observed [8]. Indeed, the biosynthesis of choline plasmalogens was poorly understood for many years. This is partly due to the small amounts of choline plasmalogens present in most mammalian tissues, in addition to the slow conversion of radioactive precursors into alk-1-enylacyl-GPC. As evident from the descriptions below, the biosynthesis of choline plasmalogens seems to involve several complex metabolic pathways. The first conclusive evidence to establish that choline plasmalogens are not directly derived from alkylacyl-GPC or alkyllyso-GPC in intact cells is based on results obtained in 1991 from experiments where [1′2′-^{3}H]hexadecyllyso-[*N*-methyl-^{14}C]-GPC (^{3}H/^{14}C = 2.7) was incubated with neonatal rat myocytes for various times up to 24 h [36]. Under these conditions, the ^{3}H/^{14}C ratios remained relatively constant in alkyllyso-GPC and alkylacyl-GPC throughout the incubation times, but decreased in the alk-1-enylacyl-GPC to less than the expected ratio of 1.35, assuming that half of the radiolabel is lost from the [1′2′-^{3}H]alkylacyl-GPC due to desaturation and that there is no isotope effect of the substrate on the desaturase. These data further imply the incorporation of the [*N*-methyl-^{14}C]choline polar-head group of alkyllyso-GPC into alk-1-enylacyl-GPC is considerably faster than that of the [^{3}H]alkyl portion of alkyllyso-GPC. Consistent with these results is the metabolic conversion of [^{3}H]alkylacyl-GPC, or [^{3}H]alkylacetyl-GPC, via [^{3}H]alkylacyl-GPE to [^{3}H]alk-1-enylacyl-GPE instead of [^{3}H]alk-1-enyacyl-GPC reported in Madin Darby canine kidney (MDCK) cells [37] and an amnion-derived cell line [38], respectively,

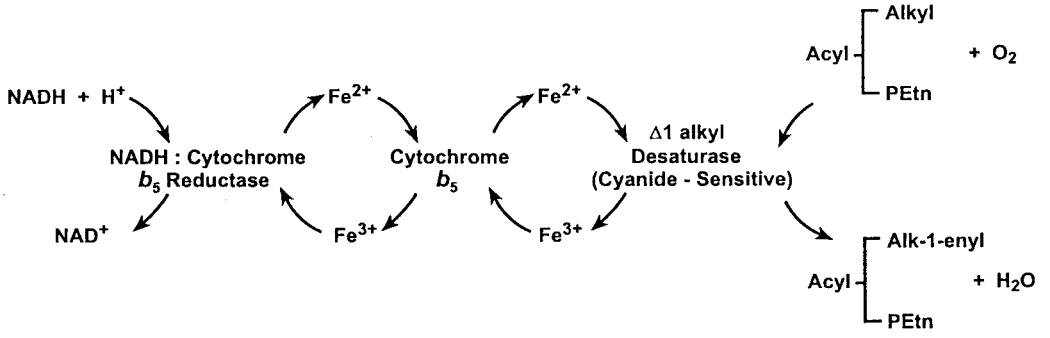

Fig. 4. Microsomal electron transport system that contains Δ1 alkyl desaturase complex.

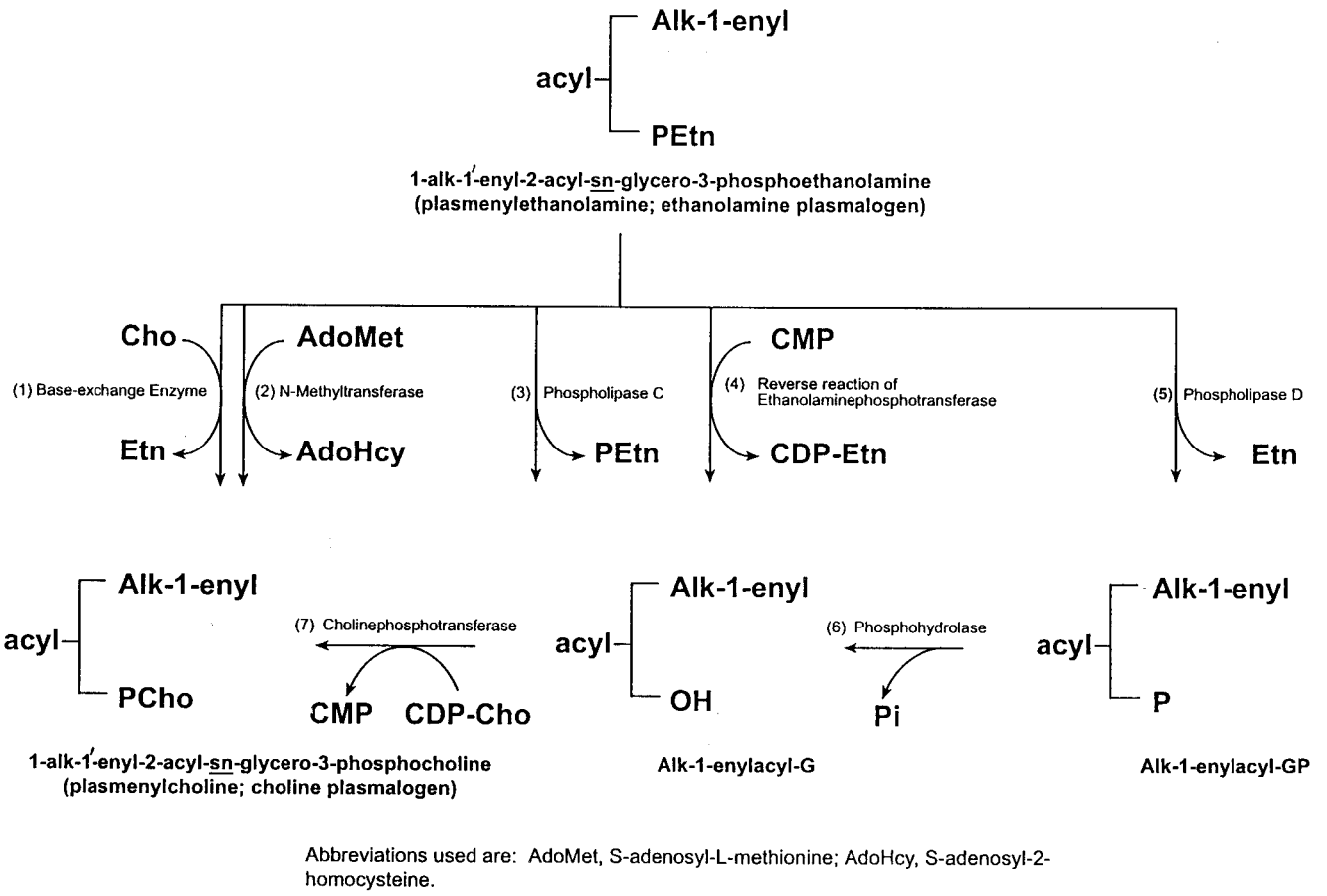

Fig. 5. Metabolic pathways for the biosynthesis of choline plasmalogens from ethanolamine plasmalogens involving direct polar-head group modification. Adapted from the previous review by us [83].

throughout a 24-h incubation. In addition, Strum et al. [37] established that it is not the phospholipase C or phospholipase D, but the reverse reaction of cholinephosphotransferase that is responsible for the conversion of [^{3}H]alkylacyl-GPC to [^{3}H]alkylacyl-GPE.

The first indication suggesting that the alk-1-enyl linkage in choline plasmalogens originates from ethanolamine plasmalogens came from the observations made by Lee et al. [36]. These results showed that when equal concentrations of [^{3}H]alkyllyso-GPC or [^{3}H]alkyllyso-GPE were incubated with neonatal rat myocytes for 4, 12, and 24 h, the conversion sequence to [^{3}H]alk-1-enylacyl-GPC appeared to be alkyllyso-GPC → alkylacyl-GPC → alkylacyl- GPE → alk-1-enylacyl- GPE → alk-1-enylacyl-GPC or via alkyllyso-GPE → alkylacyl-GPE → alk-1-enylacyl- GPE → alk-1-enylacyl-GPC [36]. These data clearly show that alk-1-enylacyl-GPE (ethanolamine plasmalogens) serves as a precursor of alk-1-enylacyl-GPC (choline plasmalogens). A similar conclusion on the precursor–product relationship between plasmenylethanolamine and plasmenylcholine was also reached with pulse-chase experiments in rabbit hearts using [1-^{3}H]hexadecanol as the precursor [39].

There are several metabolic routes that could lead to the biosynthesis of plasmenylcholine (alk-1-enyacyl-GPC) from plasmenylethanolamine (alk-1-enylacyl-GPE. These metabolic pathways can be divided into two major groups. Fig. 5 illustrates the enzymatic reactions that involve only the polar-head group modifications such as: (1) base-exchange enzyme; (2) *N*-methyltransferase; (3) phospholipase C/cholinephosphotransferase; (4) the reverse reactions of ethanolaminephosphotransferase/cholinephosphotransferase; and (5) phospholipase D/phosphohydro-

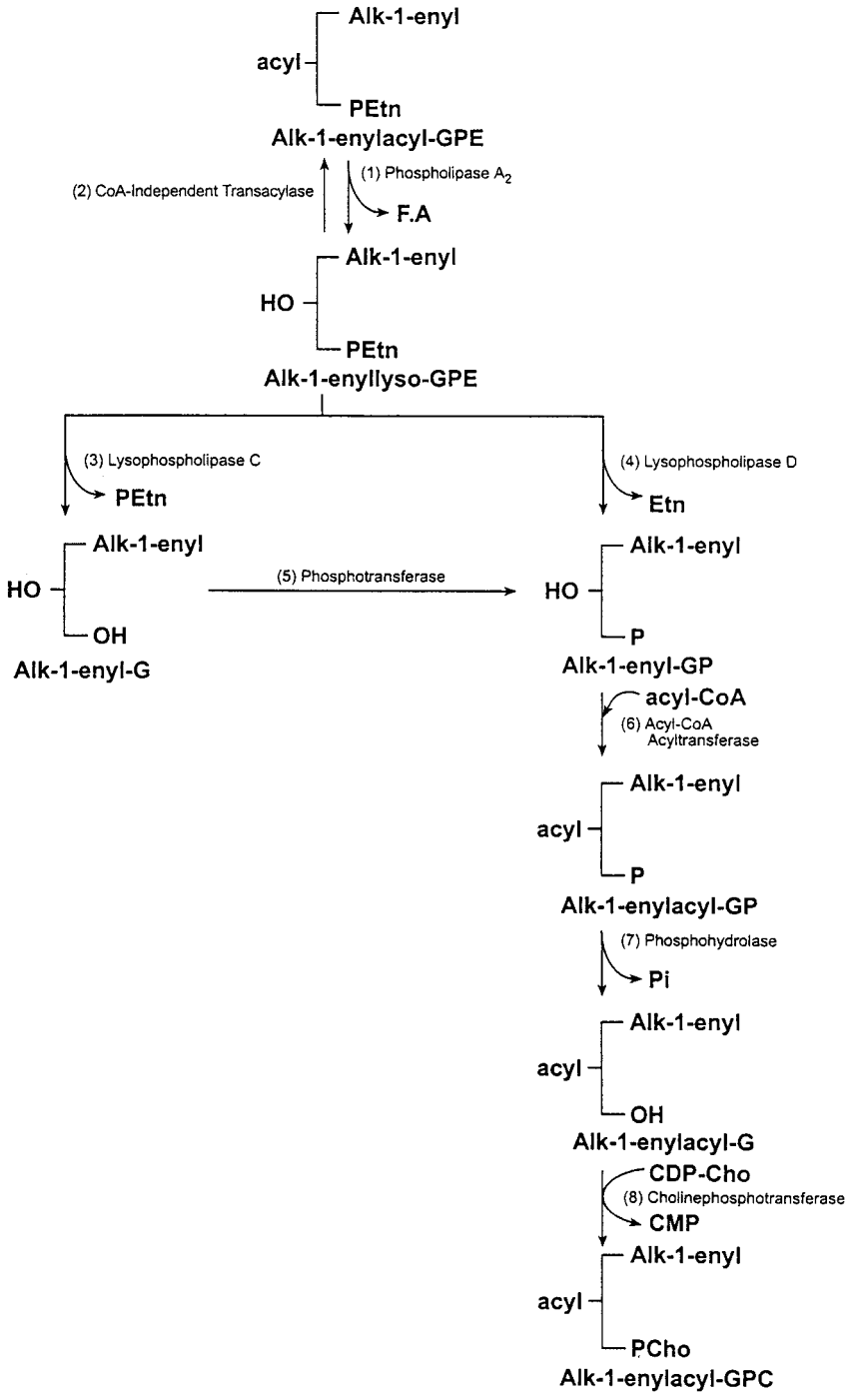

Fig. 6. Metabolic pathways for the biosynthesis of choline plasmalogens from ethanolamine plasmalogens involving both the *sn*-2 and polar-head group modifications. Adapted from the previous review by us [83].

lase/cholinephosphotransferase. In contrast, Fig. 6 shows the enzymatic reactions that require a series of both *sn*-2 position and polar-head group modifications. In this scheme, alk-1-enylacyl-GPE is first converted to alk-1-enyl-GP via a sequence of reactions catalyzed by phospholipase A_2/lysophospholipase D or phospholipase A_2/lysophospholipase C/phosphotransferase. The product, alk-1-enyl-GP,

could then be converted to alk-1-enylacyl-GPC through the classic de novo 'Kennedy's' pathway.

Reactions catalyzed by *N*-methyltransferase and the base-exchange enzyme do not seem to contribute significantly to the synthesis of alk-1-enylacyl-GPC from alk-1-enylacyl-GPE. Although enzymatic methylation of alk-1-enylacyl-GPE has been reported in rabbit myocardial membranes [40] and human platelet lysates [41], the activity was relatively low (3.9 pmol/mg/h) in rabbit myocardial membranes, and diacyl-GPE was the preferred substrate for the methylation rather than alk-1-enylacyl-GPE. In addition, alk-1-enylacyl-GPC only contained 0.3% of the radioactivity incorporated into alk-1-enylacyl-GPE when guinea pig hearts were perfused with radiolabeled ethanolamine for up to 120 min [42]. Furthermore, when the alk-1-enylacyl-GPE pool in myocytes was first prelabeled with [^{3}H]alkyllyso-GPE, and then the prelabeled cells were incubated further in the presence of either [^{14}C]choline or [^{14}C]methionine, only an estimated 7–17% of the synthesized alk-1-enylacyl-GPC was derived from methylation through methionine [36]. Finally, when MDCK cells were incubated with [^{3}H]alk-1-enyllyso-[^{32}P]GPE for up to 24 h, only 0.4% of the total cellular [^{32}P]radioactivity was recovered in alk-1-enylacyl-GPC [43]. Collectively, these results not only imply that *N*-methyltransferase (Fig. 5, reaction (2)) and the base-exchange enzyme (Fig. 5, reaction (1)) play a minor role in the biosynthesis of alk-1-enylacyl-GPC from alk-1-enylacyl-GPE, but they also indicate that a sequence of reactions results in the replacement of both the phosphate and ethanolamine groups with different phosphate and choline groups.

Wolf and Gross [44] have partially purified a neutral phospholipase C from canine myocardium. This enzyme can not only use phosphatidylcholine and plasmenylcholine as substrates, but it also appears that the same enzyme can hydrolyze plasmenylethanolamine to alk-1-enylacyl-G and phosphoethanolamine (Fig. 5, reaction (3)). Noteworthy, as that the reverse reaction of alk-1-enylacyl-G:CDP-ethanolamine ethanolaminephosphotransferase (Fig. 5, reaction (4)) has been observed in the presence of CMP and [^{3}H]alk-1-enylacyl-GPE in MDCK cell microsomes [43]. Also, the further addition of CDP-choline to the incubation mixture results in the generation of a small, but significant, amount of [^{3}H]diradyl-GPC. These data indicate that alk-1-enylacyl-GPE can be converted to alk-1-enylacyl-GPC by a combination of the reverse reaction of ethanolaminephosphotransferase and the forward reaction of the cholinephosphotransferase (Fig. 5, reactions (4) and (7)) in vitro.

Substantial advances have been made in the enzymology and regulation of phospholipase D, and several phospholipase D isoforms have been purified and cloned from many sources [45]. The ability of these various types of phospholipase D to cleave plasmenylethanolamine has not been described [45]. However, the hydrolysis of alk-1-enylacyl-GPE with the liberation of ethanolamine (an indication of phospholipase D activity) was shown to be stimulated by 12-*O*-tetradecanoylphorbol-13-acetate in NIH 3T3 cells [46] and MDCK cells [47]. Additionally, when a phosphono analog of alkyllyso-GPE ([^{3}H]alkyllyso-*sn*-glycero-3-phosphonoethanolamine) was used to investigate the contribution of phospholipase D/phosphohydrolase (Fig. 5, reactions (5) and (6)) to the synthesis of choline plasmalogen, significant amounts of this phosphono analog were converted to [^{3}H]alk-1-enylacyl-GPC by the MDCK cells following 24 h of incubation [43]. The fact that the phosphono analog is not a substrate for phospholipase D indicates that alk-1-enylacyl-GPE can be converted in the absence of phospholipase D/phosphohydrolase activities to alk-1-enylacyl-GPC via alternate routes.

Quantitative assessments on the contributions of various metabolic pathways depicted in Figs. 5 and 6 to the biosynthesis of choline plasmalogens from ethanolamine plasmalogens were derived from the work by Blank et al. reported in 1993 [48]. When either [^{3}H]alk-1-enylglycerol or [^{3}H]alk-1-enyllyso-GPE was incubated with intact HL-60 cells, both [^{3}H]plasmenylethanolamine and [^{3}H]plasmenylcholine were produced [48]. However, molecular species analysis showed the dissimilarities in distribution between [^{3}H]alk-1-enylacyl-GPC and [^{3}H]alk-1-enylacyl-GPE that originated from both substrates. Importantly, a closer agreement of tritium distribution in the molecular species of [^{3}H]alk-1-enylacyl-GPC synthesized from the [^{3}H]alk-1-enylacyl-GPE would be obtained if one assumes that 70% of the [^{3}H]alk-1-enylacyl-GPC was produced from a

pool originated from alkylglycerols and 30% was from a pool of alk-1-enylacyl-GPE [48].

Direct conversion of alk-1-enylacyl-GPE to alk-1-enylacyl-GPC can be achieved through base exchange (Fig. 5, reaction (1)) or methylation (Fig. 5, reaction (2)). On the other hand, conversion of alk-1-enylglycerol to alk-1-enylacyl-GPC requires a series of reactions catalyzed by alk-1-enylglycerol:ATP phosphotransferase (Fig. 6, reaction (5)), alk-1-enyl-GP:acyl-CoA acyltransferase (Fig. 6, reaction (6)), alk-1-enylacyl-GP phosphohydrolase (Fig. 6, reaction (7)), and alk-1-enylacyl-G:CDP-choline cholinephosphotransferase (Fig. 6, reaction (8)). All the enzymes mentioned above have been proven to be present in mammalian tissues [14]. In addition, Xu et al. [49] concluded that cholinephosphotransferase (Fig. 5, reaction (7) and Fig. 6, reaction (8)) catalyzes the formation of both phosphatidylcholine and plasmenylcholine and that the rate of plasmenylcholine biosynthesis is dependent on the availability of alk-1-enylacyl-G. Furthermore, Strum and Daniel [43] have detected a Mg^{2+}-dependent lysophospholipase C (Fig. 6, reaction 3) in the microsomes of MDCK cells that catalyzes the conversion of alk-1-enyllyso-GPE to alk-1-enylglycerol. The participation of this enzyme in the synthesis of choline plasmalogens from ethanolamine plasmalogens was suggested by the fact that the conversion of alk-1-enylacyl-GPE to alk-1-enylacyl-GPC in MDCK cells prelabeled with [^{3}H]alk-1-enyllyso-GPE was stimulated with a concomitant increase in alk-1-enylglycerol following 12-*O*-tetradecanoylphorbol-13-acetate treatment. Additionally, Ford and Gross [39] observed that there were discordant rates between *sn*-1 aliphatic chain incorporation and polar head group/*sn*-2 remodeling into plasmalogens; polar head group/*sn*-2 remodeling occurred at a rate of 100- to 300-fold greater than that of the *sn*-1 aliphatic chain incorporation (de novo plasmalogen biosynthesis) into plasmalogens. These results also emphasize the fundamental importance of polar-head group/*sn*-2 remodeling metabolic pathways in both choline and ethanolamine plasmalogen biosynthesis.

The properties of CoA-independent transacylase (Fig. 6, reaction (2)) that are involved in the conversion of alk-1-enyllyso-GPE to alk-1-enylacyl-GPE have been reviewed extensively [50–52]. A diversified group of phospholipase A_2 has also been recently identified [53,54]; however, the phospholipase A_2 responsible for the hydrolysis of alk-1-enylacyl-GPE to alk-1-enyllyso-GPE (Fig. 6, reaction (1)) has not been firmly established. It is not known whether or not lysophospholipase D (Fig. 6, reaction (4)) is involved in the biosynthesis of choline plasmalogens from ethanolamine plasmalogens.

In light of the above summaries on the possible metabolic pathways involved in the biosynthesis of choline plasmalogens, it seems most likely that choline plasmalogens are formed via both the *sn*-2 and polar-head group modifications when ethanolamine plasmalogens of the cells undergo high turnover through activation by various agonists. In part, the balances among activities of PLA_2 (Fig. 6, reaction (1)), lysophospholipase C (Fig. 6, reaction (3)), and CoA-independent transacylase (Fig. 6, reaction (2)) will determine the amounts of ethanolamine plasmalogens that convert to choline plasmalogens. On the other hand, when phospholipase C, phospholipase D/phosphohydrolase, or CDP-choline formation are activated under certain cellular conditions, one can envision the conversion of ethanolamine plasmalogens through polar-head group modification as described in Fig. 5.

4. Possible biological functions of plasmalogens

4.1. Generation of potent lipid mediators

The possible role of ether lipids as membrane constituents has been determined from studies on the biophysical properties of model membranes (see [55] for a review). However, other biological functions of plasmalogens, especially choline plasmalogens, are not firmly established or well understood.

Plasmenylethanolamines are the major storage depot for arachidonic acid [56,57]. Lysoplasmalogens generated from ethanolamine plasmalogens via phospholipase A_2 hydrolysis can induce the biosynthesis of the potent lipid mediator, platelet-activating factor, through CoA-independent transacylases [58–60]. Furthermore, several plasmalogen-selective and calcium-independent phospholipase A_2s have been found and identified in mammalian cells, namely, a 40-kDa myocardial cytosolic phospholipase A_2 [61] and a 39-kDa bovine brain cytosolic phospholipase

A_2 [62]. It is hypothesized that stimulation of receptors on the cell surface by an agonist results in the stimulation of a calcium-independent plasmalogen-selective phospholipase A_2 that hydrolyzes plasmalogens into lysoplasmalogens and arachidonic acid [62]. Arachidonic acid is metabolized to eicosanoids and the lysoplasmalogen is either reacylated or degraded by lysoplasmalogenase [62]. However, Williams and Ford [63] recently reported lysoplasmenylcholine can activate myocardial cAMP-dependent protein kinase. This suggests that a potential role for lysoplasmalogen is to serve as a second messenger in signal transduction [63].

4.2. Peroxisomal disorders and defect in plasmalogen biosynthesis

The genetic diseases in humans involving peroxisomes can be classified into three groups depending on the degree to which peroxisomal functions are deficient: (1) generalized impairment of peroxisomal functions (absence of peroxisomes) consisting of disorders such as the Zellweger syndrome, the neonatal type of adrenoleukodystrophy, and the infantile type of Refsum disease; (2) a multiple loss of peroxisomal functions (peroxisomes are present) comprising the rhizomelic type of chondrodysplasia punctata and Zellweger-like syndrome; and (3) a single loss of peroxisomal functions [21]. In addition, six complementation groups of disorders of peroxisome biogenesis have been established by cell fusion experiments using the restoration of plasmalogen biosynthesis and the ability to form peroxisomes, as evidenced by the formation of particle-bound catalase as criteria for successful genetic complementation [21].

An early indication suggesting that plasmalogens may have important functions is apparent from the studies of Datta et al. [64] and Schrakamp et al. [65]. These investigators demonstrated that in tissues of patients with the cerebrohepatorenal (Zellweger) syndrome, the plasmalogen content is very low and that peroxisomes are absent. Furthermore, fibroblasts from patients with Zellweger syndrome had deficient activities in DHAP acyltransferase and alkyl-DHAP synthase. In parallel, incorporation of [^{14}C]hexadecanol into the alk-1-enyl moiety of plasmalogens was strongly reduced in Zellweger patients as compared to controls. On the other hand, [^{3}H]alkylglycerol was incorporated into plasmalogens with the same efficiency in Zellweger patients as in controls [65]. These results imply that only the reaction(s) involved in the introduction of the ether bond (i.e. DHAP acyltransferase, and alkyl-DHAP synthase) in the process of plasmalogen synthesis are deficient in Zellweger patients. Similar incorporation patterns of [^{14}C]hexadecanol and [^{3}H]alkylglycerol were also found in the fibroblasts of patients with other peroxisomal disorders (one or more dysfunctions of peroxisomal metabolism, [21]), such as rhizomelic chondrodysplasia punctata, neonatal adrenoleukodystrophy, and infantile Refsum disease; these results also indicated a deficient formation of the glycero–ether bond [66]. Using an identical approach, normal de novo plasmalogen biosynthesis was found in X-linked chondrodysplasia punctata, adult Refsum disease, as well as in heterozygotes of Zellweger syndrome and infantile Refsum disease [63]. Similar conclusions were reached when a plasmalogen ratio (defined as area ratio of lysophosphatidylethanolemine produced from plasmenylethanolamine to the diacyl form of phosphatidylethanolamine) was used as an index [67].

It should be pointed out that the demonstration of plasmalogen deficiency in the tissues from patients suffering from Zellweger syndrome emphasizes the indispensable role of peroxisomes for ether phospholipid biosynthesis. On the other hand, the pathological consequences associated with peroxisomal disorders may not necessarily reflect the effect of plasmalogen insufficiency, but may be the result of the loss of other peroxisomal functions, such as a defect in the degradation of very long-chain fatty acids. Recently, de Vet et al. [68] identified a point mutation in a patient with an isolated deficiency in alkyl-DHAP synthase activity, but not in the level of this protein. Also, a fibroblast cell line, which is only defective in alkyl-DHAP synthase but contains intact, functional peroxisomes, has been isolated and characterized [69]. These cell systems will allow us to examine the role of ether lipids in cellular function without complications associated with peroxisome deficiency.

4.3. Potential antioxidant property of plasmalogens

Zoeller and Raetz [70] have isolated several CHO

cell mutants that are deficient in both plasmalogen biosynthesis and peroxisome assembly. CHO cells could incorporate 12-(1′-pyrene)dodecanoic acid (P12) into membrane lipids [71]. Exposure of P12-labeled cells to long wavelength ultraviolet (UV) light causes cell killing, presumably because excitation of the pyrene moiety (a photosensitizer) leads to the generation of oxygen species (i.e. singlet oxygen and radicals). In contrast, when mutant CHO cells lacking plasmalogens are labeled with the photosensitizer, P12, they are rendered much more susceptible to killing by long wavelength UV light than are P12-labeled, plasmalogen-containing cells. Supplementation of the mutants with 1-*O*-hexadecyl-*sn*-glycerol restores plasmalogen levels and nearly normal resistance to P12/UV treatment, whereas the biogenesis of peroxisomes is not restored. Therefore, it is reasonable to conclude that plasmalogen deficiency is not the cause of the failure of peroxisome biogenesis in the mutant. Moreover, plasmalogens are selectively decomposed in P12/UV-induced wild-type cells. These data suggest that plasmalogens might protect animal cell membranes from singlet oxygen and/or radical-initiated oxidation by functioning as scavengers and decomposing to products that can be reutilized [71]. Subsequently, Morand et al. [72] confirmed that plasmenylethanolamine is rapidly and preferentially destroyed to 2-monoacyl-GPE, formic acid, and pentadecanal during P12/UV treatment of CHO-K1 cells.

The influence of pyrene-fatty acids on the resistance of cells to UV radiation was investigated in cultured fibroblasts from patients with five types of peroxisomal disorders [73]. Reduced survival was observed in cells deficient in plasmalogens (rhizomelic chondrodysplasia punctata) and in cells deficient in peroxisomal fatty acid oxidation (bifunctional enzyme deficiency), which accumulated pyrene-fatty acids. X-linked adrenoleukodystrophy fibroblasts accumulated pyrene-fatty acids and showed increased UV sensitivity only when exposed to longer-chain pyrene fatty acids. UV radiation resistance was lowest in cells with combined impairment of plasmalogen synthesis and fatty acid oxidation (Zellweger syndrome, neonatal adrenoleukodystrophy), suggesting that UV sensitivity correlates inversely with the ratio of plasmalogens to radical-producing substances.

Vance [74] was the first investigator to report that ethanolamine plasmalogens comprise 20–30% of total ethanolamine glycerophospholipids in nascent lipoproteins secreted by cultures of rat hepatocytes. Likewise, about 60% of radylacyl-GPE is plasmenylethanolamine, and 4% of radylacyl-GPC is plasmenylcholine in LDL [75]. The sum of plasmenylcholine and plasmenylethanolamine constitutes approximately 4.5% of total phospholipids in this particle. The content of total plasmalogen phospholipids is in the same concentration range as the amount of vitamin E in LDL. In vitro oxidation of LDL by 2,2′-azobis-(2-amidinopropane hydrochloride) (AAPH) or copper induced a selective reduction of plasmalogen level. The sensitivities of plasmalogen towards an oxidative attack by AAPH and copper are in a comparable range with that of α-tocopherol (vitamin E). In vitro enrichment of LDL with lysoplasmenylcholine or lysoplasmenylethanolamine increases the lag phase of formation of conjugated double bonds (a measure of the resistance of LDL lipids against oxidation) induced by oxidation of the particles with copper. Thus, choline plasmalogens and ethanolamine plasmalogens may play a significant role in the defense of LDL particles against oxidative stress. Compared to that of LDL, HDL has a lower susceptibility to autoxidation initiated with Fe^{2+}/ascorbate [76]. Mechanistically, when liposomal suspension of egg yolk phosphatidylcholine was used as a system to study the effect of ethanolamine plasmalogen on lipid peroxidation, it was found that ethanolamine plasmalogens inhibited iron- and copper-dependent peroxidation in the presence of preformed hydroperoxides through inhibition of preformed peroxide decomposition by trapping transition metal ions [77]. However, ethanolamine plasmalogens were not effective in preventing radical initiator mediated lipid peroxidation [77].

4.4. Preferential plasmalogen–protein interactions

Several additional studies suggest that preferential plasmalogens and protein interactions may explain the correlation between the cellular plasmalogen contents and their cellular functional roles. For instance, two host human hepatoma cell lines associated with hepatitis B virus (HBV) infection contained extremely high concentrations (more than 60% of cho-

line- and ethanolamine-containing glycerophospholipids) of ether-linked phospholipids and an elevated level of DHAP acyltransferase activities [78]. These investigators [78] suggest the existence of a possible relationship between HBV-induced hepatocellular carcinogenesis and massive changes in ether lipid metabolism. Also, the increase in the percentage of hexadec-1-enylarachidonoyl-GPE (plasmenylethanolamine) in red blood cells was shown to be significantly related to the increase in maximal activity of the Na^+/K^+ pump [79]. It was proposed that the putative preferential lipid–protein interaction of this plasmalogen species with membrane-embedded portions of the pump molecule could induce a conformational change of the protein, thereby hindering the access of intracellular Na+ to its binding site. Similarly, plasmenylcholine has the ability to facilitate the gramicidin ion channel function [80].

Reconstituted sodium–calcium exchanger (enriched in the sarcolemma of excitory cells) activity increased as the ratio of plasmalogen to diacyl molecular subclasses of phospholipids increased in the vesicles [81]. The addition of phosphatidylserine to plasmalogen vesicles enhanced the observed sodium–calcium exchange activity further. These results suggest that the biophysical conformation of plasmalogen vesicles provided an enhancement of sodium–calcium exchange activity either directly or indirectly through alterations in ionic phospholipid membrane dynamics in plasmalogen vesicles. Since plasmalogens are selectively hydrolyzed during myocardial ischemia [82], it is possible that alterations in sarcolemmal plasmalogen content during ischemia may contribute to ischemia-induced alterations in calcium metabolism through effects on sarcolemmal sodium–calcium exchange.

5. Concluding remarks

Even though the metabolic pathways involved in the biosynthesis of choline plasmalogen have begun to unravel in recent years, the exact route(s), quantitative contributions, or regulatory control of individual pathways are far from firmly established. Besides, most of the enzymes that participate in these pathways are associated with membranes, and, therefore, are not well characterized or purified. Additionally, the physiological functions of choline plasmalogens are poorly defined. Using the techniques of isolating and characterizing mutants defective in plasmalogen biosynthesis should help to elucidate the role of ether lipids in cellular functions. Furthermore, using molecular biological approaches, such as cloning the cDNAs and functional expressing the enzymes, generating mice deficient in specific enzyme(s), or infusion of animals with recombinant enzyme(s) that are involved in the production of plasmalogens etc., would be expected to provide a more in-depth understanding of plasmalogen functions and biosynthesis.

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