

# Glycerophospholipid metabolism

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Petition for Inter Partes Review  
Of U.S. Patent 8,278,351  
Exhibit

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## I. Introduction

Glycerophospholipids are the major class of complex lipids characterized by a glycerol backbone with one of the primary hydroxyl groups (*sn*-3) esterified to phosphoric acid (Fig. 1). The secondary hydroxyl group in glycerophospholipids (*sn*-2) is always esterified to a long-chain fatty acid, which in the majority of instances is monounsaturated or polyunsaturated. The *sn*-1 hydroxyl group is most commonly esterified to another fatty acid, generally saturated or monounsaturated, forming the diacyl glycerophospholipids (or phosphoglycerides). However, the *sn*-1 position can also contain a long aliphatic chain in *cis*  $\alpha,\beta$ -unsaturated ether linkage in the case of the plasmalogens (alk-1-enyl acyl derivatives), or a saturated aliphatic chain in simple ether linkage (alkyl acyl derivatives) (Fig. 1).

Phosphatidic acid (PtdA), a quantitatively minor glycerophospholipid, is nonetheless an important intermediate in the biosynthesis of glycerophospholipids and can be regarded as the simplest. In PtdA the glycerol backbone is esterified to two fatty acids and phosphoric acid (Fig. 1). The quantitatively important glycerophospholipids contain nitrogenous bases, esterified to the phosphoric acid, such as choline (phosphatidylcholine, PtdCho), ethanolamine (phosphatidylethanolamine, PtdEtn) and serine (phosphatidylserine, PtdSer) or polyalcohols, such as inositol (phosphatidylinositol, PtdIns) or glycerol (phosphatidylglycerol) (Fig. 1). Cardiolipin

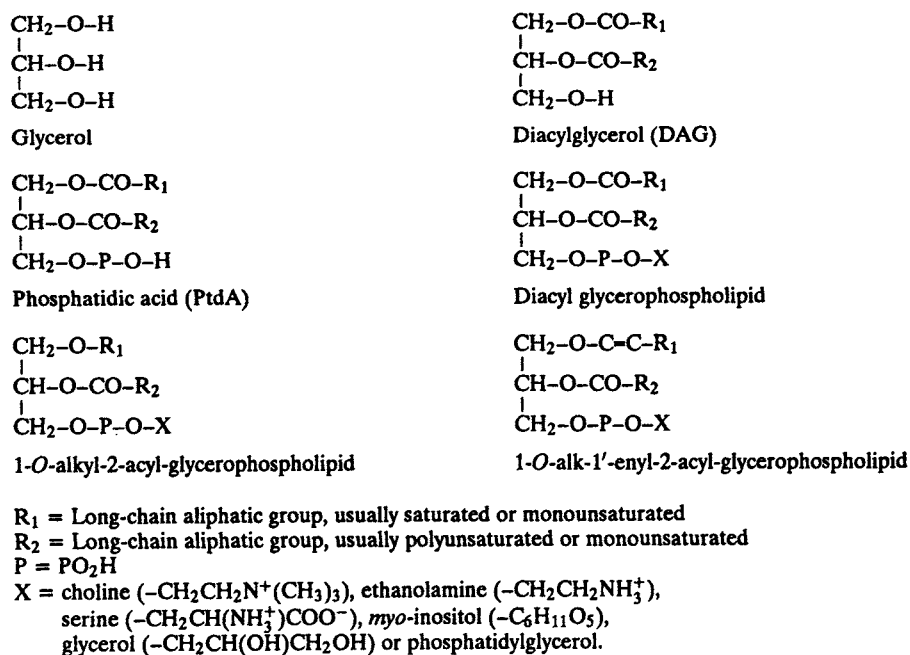


Fig. 6.1. Basic structures of the glycerophospholipids, their precursors and head groups.

(diphosphatidylglycerol) is formed when the phosphate groups of two phosphatidic acid molecules are bridged by a third glycerol moiety esterified at the 1 and 3 positions. All the glycerophospholipids are important components of biological membranes and are not found in high concentrations elsewhere in the cell.

Glycerophospholipids, phosphoglycerides and phospholipids are not synonymous terms although the terminologies are still used in a confused manner today. Not all glycerophospholipids are phosphoglycerides, as the term phosphoglyceride should, strictly speaking, be reserved for the diacyl derivatives alone and should not include the ether-linked derivatives. Similarly, not all phospholipids are glycerophospholipids. For instance, sphingomyelin contains phosphorus and so is a phospholipid but the phosphoric acid is esterified to a sphingosine backbone and not glycerol and so is correctly termed a sphingolipid. This chapter deals exclusively with the metabolism of the major classes of glycerophospholipids (PtdCho, PtdEtn, PtdSer and PtdIns), including the ether-linked derivatives, although, due to the dearth of information on the metabolism of the ether-linked derivatives in fish, the focus will be on the diacyl derivatives. I have endeavored to maintain this nomenclature throughout this chapter, but there are many instances where the use of phospholipid rather than glycerophospholipid has been more appropriate in the discussion of previous work and therefore for simplicity the term 'phospholipid' is often used.

## II. Biosynthesis, turnover and catabolism

The pathways of glycerophospholipid biosynthesis have not been extensively studied or elucidated in fish<sup>83</sup>. However, the existing evidence strongly suggests that the same pathways operate in fish as in mammals. Holub *et al.*<sup>100</sup> demonstrated the existence of glycerol-3-phosphate acyltransferase in the liver of rainbow trout (*Oncorhynchus mykiss*). When liver microsomes were incubated with *sn*-[U-<sup>14</sup>C]glycerol-3-phosphate in the presence of activated fatty acid, palmitoyl-CoA, 77% of the radioactivity was recovered in total glycerophospholipids with the remainder recovered in neutral lipids. PtdA and lysoPtdA were also labeled, supporting the conclusion that glycerophospholipid and lipid biosynthesis in general proceeded *via* a PtdA intermediate in fish.

The presence of cytidine diphosphate (CDP)-choline-1,2-diacylglycerol choline phosphotransferase has been demonstrated in the microsomes of trout liver<sup>101</sup> and brain and liver from goldfish (*Carassius auratus*)<sup>129</sup>. The synthesis of PtdCho from <sup>14</sup>C-CDP-choline and 1,2-diacylglycerol (diolein) in the presence of Mg<sup>2+</sup> established that the CDP-choline pathway for the biosynthesis of PtdCho, as studied in detail in mammals, also operated in fish<sup>101,129</sup>. There have been few studies in fish to fully characterize the biosynthetic pathways for PtdCho, PtdEtn, PtdSer, PtdIns and cardiolipin or the pathways, known in mammals, for interconversion between the glycerophospholipids. However, in a recent study, the *de novo* pathways of glycerophospholipid biosynthesis were investigated in trout hepatocytes

PtdEtn-methyltransferase (PtdEtn  $\rightarrow$  PtdCho) and PtdSer-decarboxylase (PtdSer  $\rightarrow$  PtdEtn) were demonstrated<sup>90</sup>.

In mammals, ether-linked glycerophospholipids are formed solely *via* the dihydroxyacetone phosphate pathway (see ref. 194). Briefly, fatty alcohol is formed by the NADPH reduction of fatty acyl-CoA. Fatty alcohol then reacts with fatty acyldihydroxyacetone phosphate to form alkyldihydroxyacetone phosphate which is then reduced, specifically with NADPH, to form alkylglycerophosphate. The enzymes responsible for the synthesis and reduction of alkyldihydroxyacetone phosphate are located in the peroxisomes. Reaction with fatty acyl-CoA, removal of phosphate and reaction with CDP-base results in the formation of alkyl glycerophospholipid. Alk-1-enyl acylglycerophospholipids (plasmalogens) are formed by oxidation of the corresponding alkyacylglycerophospholipid by a microsomal enzyme requiring NADPH and molecular oxygen. Little of the above pathway has been characterized in fish, but the available evidence from studies with spiny dogfish (*Squalus acanthias*) appears to suggest that the biosynthesis of ether-linked glycerophospholipids in fish is *via* a pathway similar to that outlined above (see ref. 194).

A review of muscle lipase activities in various fish species indicated that the catalytic hydrolysis of phospholipids was primarily under the control of phospholipases A<sub>1</sub> and A<sub>2</sub><sup>205</sup>. Intracellular phospholipase A activities have been demonstrated in muscle tissue from rainbow trout<sup>112</sup>, pollock (*Gadus pollachius*)<sup>11</sup>, winter flounder (*Pseudopleuronectes americanus*)<sup>206</sup> and Atlantic cod (*Gadus morhua*)<sup>48</sup>. Neas and Hazel<sup>154-156</sup> studied the activity of phospholipase A<sub>2</sub> towards PtdCho in the microsomes of trout liver. Activity of phospholipase C has been demonstrated directly in isolated olfactory cilia from the channel catfish (*Ictalurus punctatus*)<sup>39</sup> and has been implicated indirectly in other tissues by the demonstration of a phosphoinositide cycle (see section V.2.2)<sup>10,207</sup>, but it appears that phospholipase D activity has not been investigated in fish. Holub *et al.*<sup>102</sup> showed that trout liver microsomes also contained acylCoA: 1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase activity. Therefore, enzymes required for partial catabolism of glycerophospholipids and for the reacylation of lyso-glycerophospholipids, and thus for the turnover of glycerophospholipids, have been demonstrated in fish.

Catabolism of ether-linked glycerophospholipids hinges on the cleavage of the ether bond. Enzymic cleavage of the *O*-alkyl bond has been demonstrated in fish<sup>194</sup>. The cleavage is considered to occur in two steps, whereby the alkyl bond is first oxidized to alk-1-enyl *via* a reaction involving NADPH, molecular oxygen and a pteridine cofactor, before cleavage to generate fatty aldehyde<sup>194</sup>. The above enzyme system has yet to be directly studied in fish, and so it is not known if it will also cleave plasmalogens. Plasmalogenases, as described in mammalian brain, do not appear to have been studied in fish<sup>194</sup>.

The specificities of the enzymes involved in both *de novo* synthesis of the glycerophospholipids and in the turnover processes of deacylation/reacylation with respect to both head group and fatty acyl chains have important consequences in maintaining the normal glycerophospholipid class composition, the fatty acyl dis-

changes. Some of the more direct enzyme studies, discussed above, addressed this problem in relation to environmental temperature<sup>101–103,129,154</sup>. However, there has been a considerable amount of data obtained from more indirect studies of the effects of environment on glycerophospholipid metabolism and this is summarized later (see section IV.6).

### III. Digestion, absorption and transport

#### 1. Digestion and absorption

Depending upon the precise nature of the diet, a significant and potentially large and consistent portion of the lipid component in the natural food of fishes will be biomembrane lipids, primarily glycerophospholipids. Unfortunately, the lack of a discrete pancreas in most teleost species has hampered studies on intestinal lipolysis in fish. In consequence, even less is known about the digestion and absorption of dietary glycerophospholipids than is known about the biosynthetic pathways. There are virtually no studies on the intestinal digestion of glycerophospholipids in fish, but it could be presumed that the mechanisms are similar to those in mammals. Therefore dietary glycerophospholipids are presumably digested by pancreatic or intestinal phospholipases resulting in the formation of 1-acyl lyso-glycerophospholipids and free fatty acids that are absorbed by the intestinal mucosal cells<sup>96,198</sup>. Mankura *et al.* studied the hydrolysis of L-1-palmitoyl-2-[1-<sup>14</sup>C]arachidonyl-3-*sn*-glycerophosphatidylcholine by carp hepatopancreas preparations<sup>141</sup>. They found phospholipase A<sub>2</sub> activity distributed in all the subcellular fractions, although the highest activity was located in the 10,000 g supernatant. The activity was dependent upon Ca<sup>2+</sup> and bile salt, consistent with a pancreatic enzyme, but had a conflicting acidic pH optimum of 5.0<sup>141</sup>. Whether this phospholipase activity reflects an intestinal activity or an intracellular phospholipase is, therefore, unclear. Recent work has suggested that cod pyloric caeca/pancreas contains a single, bile salt-activated, lipase activity with a wide substrate specificity including triacylglycerols, steryl esters, fatty acid methyl esters and carboxyl esters<sup>79,80</sup>. Whether this enzyme is also active towards phospholipid and whether cod intestine actually lacks phospholipase A<sub>2</sub> activity is unclear.

The concentration of lyso-glycerophospholipids is very low in fish plasma and so it has also been assumed that the majority of lysophospholipid is re-esterified within the intestinal mucosa before export into the circulatory system. However, studies on the incorporation of [1-<sup>14</sup>C]palmitate and [U-<sup>14</sup>C]L-glycerol-3-phosphate into lipids in carp (*Cyprinus carpio*) intestinal homogenates in the presence of CTP, CDP-choline and CDP-ethanolamine showed that glycerophospholipid biosynthesis proceeded *via* PtdA and diacylglycerol (DAG) intermediates<sup>110</sup>. Therefore, mechanisms may exist in fish intestinal mucosa for the synthesis of glycerophospholipids from moieties more degraded than lyso-glycerophospholipids. Iijima and coworkers<sup>108</sup> have also studied the absorption of radioactivity from [1-<sup>14</sup>C]dioleoyl PtdCho force-fed to carp. At 20–28 h after dosing radioactivity in the lipids of

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