Minireview

Metabolic Insights into Phospholipid Function Using Gene-targeted Mice*

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Few human diseases have been described that are due to a defect in phospholipid biosynthesis. Phospholipids are required for providing the essential milieu of biological membranes and are important precursors of signaling molecules. One would anticipate that a null mutation in an enzyme required for the biosynthesis of a phospholipid would not be compatible with life, particularly if that mutation resulted in complete elimination of that phospholipid. However, for several of the major mammalian phospholipids (e.g., phosphatidylcholine (PC),1 phosphatidylethanolamine (PE), and phosphatidylserine (PS)) more than one biosynthetic pathway operates (1). Consequently, the possibility exists that humans might have deficiencies in phospholipid biosynthesis that would not be detectable if alternative enzymes that catalyzed the same reaction were active or if alternative pathways were available for making that phospholipid. In the last two decades, advances in gene targeting in mice have made it possible to determine whether or not a particular enzyme or protein is essential for mammalian life. This article will summarize the current state of knowledge about induced mutations in murine genes involved in phospholipid biosynthesis and intracellular transport. To date, gene-targeting experiments have revealed that mice can survive without certain phospholipid biosynthetic enzymes; in each case, an alternative pathway or enzyme exists for making that phospholipid.

The major biosynthetic routes for the biosynthesis of PC, PE, and PS were elucidated in the 1950s largely by Eugene Kennedy and co-workers (2). In subsequent decades, attempts were made to purify the enzymes involved in these pathways. However, because many of the enzymes are integral membrane proteins, their purification to homogeneity represented an enormous task that has been aptly described as “maasochistic enzymology” (3). Nevertheless, despite these travails, several of these enzymes were purified from mammalian sources during the 1980s. Sequencing of the proteins led to the cloning and expression of the corresponding cDNAs and subsequently to the cloning and characterization of the corresponding genes. With the advent of techniques for generating genetically modified mice, it became possible to construct targeted mutations of selected genes of phospholipid metabolism. We shall discuss the results obtained from experiments in which specific genes involved in phospholipid biosynthesis/transport have been “knocked-out” in mice.

Transbilayer Movement of Phospholipids

The first mammalian gene of phospholipid metabolism that was disrupted was murine Mdr2 (AbcB4). Prior to these experiments, Borst and colleagues (4) suggested that Mdr2 played any role in phospholipid function. It was known, however, that Mdr2 was a hepatic P-glycoprotein belonging to the class of vectorial transport proteins known as ATP-binding cassette (ABC) transporters and that this protein was found on canalicular membranes. Thus, these investigators speculated that Mdr2 might play a role in biliary excretion. To test their hypothesis, Mdr2−/− mice were generated. Only the livers of Mdr2−/− mice showed histological abnormalities, and during the second week after birth these mice developed liver disease that appeared to be caused by a complete inability to export PC from hepatocytes into bile (4). Strikingly, phospholipid and cholesterol were virtually absent from the bile of Mdr2−/− mice. Heterozygous Mdr2+/− mice did not display hepatic abnormalities, but phospholipid output into bile was ~50% less than in Mdr2+/+ mice. Excretion of bile salts was unaffected by disruption of the Mdr2 gene. These observations strongly suggested that Mdr2 was involved in transferring phospholipid from hepatocytes into bile. The experiments demonstrated that Mdr2 was required for PC excretion into bile but did not define the mechanism of action of the protein. The function of Mdr2 was addressed by Ruetz and Gros (5, 6) who expressed murine Mdr2 in secretory vesicles in yeast. They demonstrated that a fluorescent derivative of PC was translocated in an ATP-dependent manner from the outer to the inner leaflet of the membrane of these vesicles. In contrast, a related P-glycoprotein, Mrdr3, did not catalyze PC translocation. The conclusion was that Mdr2 translocates PC to the luminal side of the canalicular membrane where bile salts extract the PC in the form of micelles into bile.

ABC transporters comprise a large family of transmembrane proteins, several of which have been implicated in the ATP-dependent transbilayer movement of lipids. Mutations in ABC transporters in humans are responsible for diseases such as Tangier disease (7–9), Stargardt’s macular dystrophy (10), sitosterolemia (11), and X-linked adrenal hyperplasia (12). One of these transporters, ABCA1, is widely expressed in plasma membranes of mammalian cells and is defective in Tangier disease (7–9). This disorder is characterized by extremely low levels of plasma high density lipoproteins. The formation of high density lipoproteins requires the efflux of PC and cholesterol from cell surfaces to an acceptor protein, apolipoprotein (apo) A1. Mice with targeted disruption of the Abca1 gene have been generated (13–15) and, as in Tangier disease patients, have an almost complete lack of high density lipoproteins as well as a major reduction in the efflux of PC and cholesterol to apoA1. The precise mechanism by which ABCA1 stimulates PC efflux from the cell surface is not yet understood although the presumed function is to directly translocate PC across the plasma membrane. The possibility that ABCA1 is an auxiliary protein that is required for PC efflux, however, cannot be discounted. ABCA1 has also been proposed to promote the outward movement of PS across the plasma membrane, thereby increasing the exposure of PS at the cell surface (16). Such a perturbation of the normal distribution of plasma membrane phospholipids might favor the release of PC and cholesterol for high density lipoprotein formation (16) although this concept has been challenged (17).

A group of proteins that have also been implicated in the transbilayer movement of phospholipids in the plasma membrane is the scramblases of which four murine members have been identified (18). The generally accepted view has been that scramblases act as bi-directional phospholipid flippases in the plasma membrane, but recent data indicate that this might not be their primary function.

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1 The abbreviations used are: PC, phosphatidylcholine; ABC, ATP-binding cassette; apo, apolipoprotein; CT, CTP; phosphocholine cytidylyltransferase; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; FTPP, phosphatidylinositol transfer protein; PS, phosphatidylserine; PSS, phosphatidylserine synthase; TG, triacylglycerol.
In addition to its presence in the plasma membrane, scramblase-1 is also present in the nucleus and participates in signaling pathways related to cell proliferation (19). Consistent with this finding, mice with targeted deletion of scramblase-1 exhibit perinatal granulocytopenia but have no defect in phospholipid scramblase activity in the plasma membrane (20). A flipase function for scramblase-1 cannot, however, be ruled out because other scramblase family members might compensate for a loss of phospholipid scramblase activity contributed by scramblase-1.

Scramblase-3-deficient mice have also recently been generated (21). In contrast to scramblase-1, which is expressed primarily in blood cells, scramblase-3 is also highly expressed in muscle and fat cells (18). In scramblase-3 knock-out mice, phospholipid scramblase activity is normal, but the animals accumulate abdominal fat and develop insulin resistance and dyslipidemia (21). In addition, primary adipocytes and bone marrow-derived macrophages from these mice are engorged with neutral lipids, suggesting a role for scramblase-3 in normal development and/or function of these cells. Thus, although the mechanism of action of scramblases has not yet been defined, studies with scramblase knock-out mice do not provide evidence that these proteins function physiologically in transbilayer movement of phospholipids.

Mice That Lack Phosphatidylethanolamine N-Methyltransferase Are Viable but Metabolism of Lipoproteins and Homocysteine Is Abnormal

Phosphatidylethanolamine N-methyltransferase (PEMT) catalyzes the methylation of PE to PC (22, 23). Although PEMT activity is detectable in non-hepatic tissues, the activity is usually less than 1% of that in the liver. Thus, PEMT is essentially a liver-specific enzyme. The PEMT gene was selected as a target for gene ablation in mice because the liver can use an alternative route for making PC (the CDP-choline pathway) that might compensate for loss of PEMT. Disruption of murine Pemt was the first example in which an enzyme of phospholipid biosynthesis was eliminated in an intact animal (24). Pemt−/− mice are viable and do not exhibit any obviously abnormal phenotype. In the livers of Pemt−/− mice, the amount of PC is the same as in Pemt+/+ mice. The CDP-choline pathway can apparently compensate for the lack of PEMT because the membrane-associated activity of CTP-phosphocholine cytidylyltransferase (CT), which under most metabolic states catalyzes the regulated and rate-limiting step in the CDP-choline pathway for PC biosynthesis (25), is increased by 60%.

PC synthesis via the CDP-choline pathway requires the input of an exogenous source of choline. Best and Huntsman (26) first described choline as an important dietary component in 1932. Therefore, an obvious question was: how would Pemt−/− mice fare if they were fed a choline-deficient diet? Choline deficiency in rodents has frequently been used as an experimental model for studies on PC function. Rats fed a choline-deficient diet can survive for at least a year but eventually develop hepatic steatosis and, often, hepatic cancer (27, 28). When Pemt−/− mice were fed a choline-deficient diet for 3 days, the PC content of the liver decreased by 50%, the level of hepatic triacylglycerol (TG) increased by 3–6-fold, and liver failure ensued (Fig. 1) (29). In contrast, when Pemt+/+ mice were fed the same diet for 3 days there was no obvious liver pathology or TG accumulation. The damage to the liver and the decreased PC levels, caused by simultaneously eliminating dietary choline and PEMT activity, were reversed when the mice were subsequently fed a choline-supplemented diet (30). The specificity of the requirement for choline in Pemt−/− mice is remarkable. Dimethylaminoethanol, a choline analog that contains two, rather than three, methyl groups can be readily incorporated in vivo into the phospholipid phosphatidyl(dimethyl)aminoethanol (31). When fibroblasts are cultured in the presence of dimethylaminoethanol instead of choline, the cells grow normally implying that in these cells phosphatidyl(dimethyl)aminoethanol can substitute for PC (32, 33). Thus, the expectation was that dimethylaminoethanol would be able to substitute for choline in Pemt−/− mice. However, when the Pemt−/− mice were fed dimethylaminoethanol instead of choline, liver failure still occurred but 1 day later than in Pemt−/− mice fed the choline-deficient diet without supplementation (34).

Whereas choline-deficient Pemt−/− mice rapidly develop liver failure, the PC content of other organs is not reduced nor is obvious damage noted to any other tissues (29). Interestingly, the choline-deficient diet did not reduce the amount of PC secreted into bile of Pemt−/− mice (24). A possible reason for the rapid onset of liver failure in choline-deficient Pemt−/− mice is that a 29-g mouse normally excretes 23 mg of biliary PC/day (35), whereas the total amount of PC in the liver is ~20 mg. Therefore, a mouse secretes the equivalent of its entire hepatic pool of PC into bile each day; some of this PC is reabsorbed by the intestine (35). Therefore, the possibility that the large drain of PC from the liver into bile was responsible for the liver failure was investigated. Pemt−/− mice were bred with Mdr2−/− mice to generate a strain of mice that lacked both PEMT and MDR2. As noted above, Mdr2−/− mice have a defect in the secretion of PC into bile. Remarkably, the double knock-out mice usually survive for longer than 3 months when fed a choline-deficient diet (36). These studies demonstrate that elimination of biliary PC secretion protects Pemt−/− mice from liver failure induced by lack of dietary choline and confirm that excretion of PC into bile greatly aggravates the potential for liver damage in these mice.

Because Pemt−/− mice do not show an obvious phenotype when fed laboratory chow, the mice were challenged with a high fat/high cholesterol “Western-style” diet for 3 weeks (36). Livers from male, but not female, Pemt−/− mice showed a striking 4–6-fold accumulation of TG and cholesteryl esters compared with Pemt+/+ mice but no decrease in the amount of PC (36). Correspondingly, the plasma concentration of TG was 60% lower in male Pemt−/− mice than in Pemt+/+ mice. The plasma level of apoB100 was also reduced by 50% by PEMT deficiency (36). Based on experiments with intact mice and cultured hepatocytes (36–38), the reduction in plasma TG and apoB100 was attributed to a defect in hepatic secretion of very low density lipoproteins. The mechanism by which a lack of PEMT inhibits lipoprotein secretion and the basis for the sexual dimorphism remain to be elucidated. A lack of PEMT also abolished the secretion of lipoproteins in low density lipoprotein receptor-deficient mice. Furthermore, when these double knock-out mice were fed a high fat/high cholesterol diet for 12 weeks, the development of atherosclerosis was greatly attenuated compared with that of low density lipoprotein receptor-deficient mice.

In mouse plasma, PC and cholesterol are largely present in high density lipoproteins. In Pemt−/− mice fed either chow or a high fat/high cholesterol diet, plasma levels of PC and cholesterol were 25–45% lower than in Pemt+/+ mice (36). The mechanism responsible for this reduction is not known.

For each PC molecule generated, the PEMT reaction produces 3 molecules of S-adenosylhomocysteine that are subsequently catabolized to homocysteine in the liver. Homocysteine is then converted to either methionine or cysteine or is secreted from the liver into plasma. A high level of plasma homocysteine is an independent risk factor for development of cardiovascular disease (40). Unexpectedly, plasma homocysteine was 50% lower in Pemt−/− mice than in Pemt+/+ mice (39). Moreover, cultured hepatocytes from Pemt−/− mice secreted ~50% less homocysteine than did Pemt+/+ hepatocytes. These experiments demonstrate that PEMT is a more important source of plasma homocysteine than was previously recognized.

From these studies with Pemt−/− mice unexpected roles for PEMT have been discovered in liver viability, regulation of plasma homocysteine levels, and lipoprotein metabolism.

**Disruption of Murine CTα and CTβ Genes**

CT is present in all nucleated mammalian cells and catalyzes the rate-limiting reaction of the CDP-choline pathway for PC biosyn-
thesis. Eagle in 1955 demonstrated that choline was necessary for growth of cells in culture (41). Chinese hamster ovary cells with a temperature-sensitive mutation in CT undergo apoptosis at 40 °C (42, 43). Thus, it was anticipated that global disruption of the CT gene in mice would be embryonic lethal. However, since a second murine CT gene, Pcyt2, is present in the murine genome, the possibility was raised that embryonic lethality would not necessarily occur in CTβ-deficient mice (44). Alternative splicing of the CTβ gene in mice yields two mRNAs that encode CTβ2 and CTβ3 (45, 46). The CTβ gene, Pcyt1a, has been classically studied and resides on murine chromosome 16. Recent studies from Jackowski’s laboratory show that disruption of Pcyt1a in mice is embryonic lethal.4

Tabas and colleagues (47) used the Cre-lox system specifically to disrupt the Pcyt1a gene in macrophages in mice. Despite the absence of CTα, cultured peritoneal macrophages derived from these mice appeared normal. CT activity was decreased by 70–90%, with residual activity ascribed to enhanced expression of CTβ2. Macrophages that are incubated with acetylated low density lipoproteins take up cholesterol, and CT activity is increased (48). The increased CT activity was postulated to protect the macrophages from toxic effects of cholesterol accumulation. This hypothesis was tested by comparing the extent of cell death in wild-type and CTα-deficient macrophages. After incubation with acetylated low density lipoproteins, 29% of CTα-deficient macrophages died compared with only 2% of wild-type macrophages (47). Thus, increased CT activity provided protection against cholesterol-induced death of cultured murine macrophages.

The Cre-lox system was similarly used to inactivate CTα selectively in murine livers in which Cre expression was governed by the promoter of the large (65–95%) reduction of PS degradation is an important homeostatic response. These observations confirm that maintenance of a normal cellular phospholipid composition is an important feature. Studies with primary hepatocytes show that PS degradation is slowed by PSS2 deficiency, presumably as a mechanism for maintaining normal levels of PS.5 Future studies are expected to establish whether or not PSS1 is required for embryonic development and if Pss1−/− mice are viable.

Phospholipid Transfer Protein Genes

In vivo, there is no persuasive evidence that these proteins perform such a function.

The PC transfer protein is an abundant cytosolic protein that is highly specific for PC in transfer assays in vitro. One function suggested for this protein was to catalyze the net transfer of PC from the endoplasmic reticulum, a site of PC synthesis, to other intracellular membranes including the bile canalicular membranes. Another conclusion was drawn in PC secretion for lung surfactant (57). Consequently, Borst and colleagues (57) investigated if ablation of the PC transfer protein gene in mice resulted in viable mice and if bile excretion and/or lung surfactant production were compromised. Remarkably, no defect was observed in PC secretion into bile or lung surfactant. Apparently the PC transfer protein is not essential for intermembrane trafficking of PC in vivo, and the physiological function of this protein remains unknown.

Phosphatidylinositol transfer proteins (PITPs) transfer phosphatidylinositol from one membrane to another. Three mammalian PITPs, designated PITPα, PITPβ, and rdgBβ have been identified. PITPα and PITPβ share 77% sequence identity (58). Both proteins catalyze PC and phosphatidylinositol transfer between membranes in vitro. Although PITPβ also catalyzes PS transfer (59), it cannot gain insight into the function of these proteins. Bankaitis and colleagues (58) generated mice that lacked PITPα. Pitpα−/− mice survived the prenatal period but developed severe neurodegenerative disease as well as intestinal and hepatic steatosis. Moreover, Pitpα−/− mice were severely hypoglycemic. Nevertheless, elimination of PITPα in murine cells produced no obvious defects in bulk phospholipid metabolism (58). However, PITPα does appear to function in lipoprotein assembly and/or secretion from the intestine and liver, as well as in maintaining plasma glucose levels. How, or if, these functions relate to a role of PITPs in mediating the intermembrane transfer of PC and/or phosphatidylinositol is not clear. Attempts to generate Pitpβ−/− mice and to obtain murine embryonic stem cells that lack both copies of the PITBβ gene have been unsuccessful, suggesting that PITBβ is an essential gene for early embryonic murine development (59).

Another putative phospholipid transfer protein is the plasma phospholipid transfer protein that also catalyzes PC transfer between membranes in vitro (60). Plasma PC transfer protein plays an important role in plasma by mediating phospholipid transfer among lipoproteins (60), and ablation of the gene in mice markedly reduced plasma high density lipoprotein levels (61). An additional role for this protein was uncovered because secretion of apoB-

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4 L. Wang, S. Magdaleno, I. Tabas, and S. Jackowski, unpublished results.
5 R. Steenbergen and J. E. Vance, unpublished results.
containing lipoproteins was decreased in mice lacking plasma phospholipid transfer protein (62) and significant plasma phospholipid transfer activity was detected within the Golgi. These findings suggest that in addition to its role in mediating lipoprotein homeostasis in plasma, the plasma phospholipid transfer protein is involved in adding phospholipids to nascent apolipoprotein-containing lipoproteins in the Golgi (62).

Future Directions

Targeting of genes involved in phospholipid biosynthesis and transport has provided novel insights into the functions of these proteins. Surprisingly, however, gene targeting of the so-called phospholipid transport proteins has not provided insights into the functions of these proteins. Many more genes of phospholipid metabolism remain to be ablated so that functions of the corresponding proteins and their phospholipid products can be defined. For example, PE is made by two completely independent pathways located in distinct intracellular organelles (endoplasmic reticulum and the mitochondrion). Are these pathways both required? Do mice need both genes that encode choline kinase activity? Is each of these pathways disrupted in specific tissues, but not in others, without compromising viability? Is the biosynthesis of cardiolipin involved in adding phospholipids to nascent apoB-containing lipoproteins in the Golgi (62)?

Minireview: Knock-out Mice in Phospholipid Metabolism

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