Physiological consequences of disruption of mammalian phospholipid biosynthetic genes

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Abstract By 1959, Eugene Kennedy and coworkers had outlined most pathways of phospholipid biosynthesis. In the next four decades, the emphasis was on enzymology and regulation of these pathways. In the last 12 years, several lines of mice with disrupted genes of phospholipid biosynthesis were generated. From this research, we have learned that embryonic lethality occurs in mice that lack choline kinase (CK) α, CTP:phosphocholine cytidylyltransferase α, CTP:phosphoethanolamine cytidylyltransferase, or phosphatidylserine decarboxylase. Whereas mice that lack CK β are viable but develop hindlimb muscular dystrophy and neonatal bone deformity. Mice that lack CTP:phosphocholine cytidylytransferase β have gonadal dysfunction and defective axon branching. Mice that lack phosphatidylethanolamine *N*-methyltransferase exhibit no phenotype until fed a choline-deficient diet, which leads to rapid liver failure. Future research should extend our knowledge about the function of these and other enzymes of phospholipid biosynthesis.—Vance, D. E., and J. E. Vance. **Physiological consequences of disruption of mammalian phospholipid biosynthetic genes. *J. Lipid Res.* 2009.** 50: **S132–S137.**

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In 1959, the general outline of biosynthesis of major phospholipids had been established in studies pioneered by Eugene Kennedy (1–3). A key finding was that CTP, not ATP, was the ribonucleoside triphosphate required for phospholipid biosynthesis (1). The next major focus was to purify enzymes involved in phospholipid biosynthesis, many of which were integral membrane proteins. Thus, success was limited. Choline kinase (CK) was purified in 1984 (4), CTP:phosphocholine cytidylyltransferase (CT) was purified in 1987 (5), and the integral membrane protein, phosphatidylethanolamine (PE) *N*-methyltransferase (PEMT) that converts PE to PC was purified in 1987 (6). Several phospholipid biosynthetic enzymes have never been purified.

In the 1970s, investigations into the regulation of phospholipid biosynthesis were initiated. In 1975, primary rat hepatocytes were used to demonstrate that the second enzyme in the Kennedy pathway for PC biosynthesis, CT, catalyzed the rate-limiting step (7). These findings were confirmed under most metabolic conditions (8). In addition, the rate-limiting enzyme of PE synthesis via CDP-ethanolamine was identified as CTP:phosphoethanolamine cytidylyltransferase (ET) (7). A striking regulatory feature of PC biosynthesis is that CT is inactive in the cytosolic fraction of cells but is activated by translocation to membranes (9). Insights into the mechanism of translocation of CT to membranes have been largely provided by Cornell and Northwood (10) and Taneva et al. (11). Studies on regulation of expression of mRNAs encoding CTα have concluded that CTα expression is linked to the cell cycle, cell growth, and differentiation rather than energy metabolism (12).

Clearly, significant progress has been made during the last 50 years in understanding the enzymology and regulation of PC biosynthesis. Moreover, some progress was made in understanding the enzymology and regulation of the biosynthesis of other phospholipids. The intent of this article is to focus on physiological consequences of deletion of genes of phospholipid metabolism in mice that have been generated in the last 12 years.

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Abbreviations: apo, apolipoprotein; CD, choline-deficient; CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; ET, CTP:phosphoethanolamine cytidylyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase: PS, phosphatidylserine; PSD, PS decarboxylase; PSS, PS synthase; TG, triacylglycerol.

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DISRUPTION OF GENES ENCODING ENZYMES OF PC BIOSYNTHESIS

PEMT and liver failure

PEMT converts PE to PC in three sequential methylation reactions that use S-adenosylmethionine as methyl group donor (**Fig. 1**). PEMT is expressed mainly in liver (13) and produces ~30% of hepatic PC, whereas the CDP-choline pathway produces 70% of PC (7). The mouse gene encoding PEMT (*Pemt*) was disrupted (14). *Pemt*$^{-/-}$ mice that were fed chow exhibited no obvious phenotype. When *Pemt*$^{-/-}$ mice were fed a choline-deficient (CD) diet, the concentration of hepatic PC in *Pemt*$^{-/-}$ mice was ~50% lower than in *Pemt*$^{+/+}$ mice and liver failure occurred after 3 days (15). Thus, PEMT appears to have survived during evolution to provide PC/choline when dietary choline is inadequate.

The mechanism responsible for the dramatic loss of hepatic PC, and how this led to liver failure, has been elucidated. The liver secretes lipoproteins and bile, both of which contain significant amounts of PC. Thus, there is a tremendous drain of PC from the liver into bile. Hence, it was postulated that when PC biosynthesis was attenuated by simultaneous lack of PEMT and dietary choline, the amount of PC secreted into bile might not be replaced by new synthesis. To test this hypothesis, *Pemt*$^{-/-}$ mice were bred with a mouse in which the PC flippase (Mdr2) on the bile canalicular membrane was eliminated and PC secretion into bile was prevented (16). The *Mdr2*$^{-/-}$ mice and the double knockout mice were fed the CD diet. The rapid decrease in PC that occurred in livers of *Pemt*$^{-/-}$ mice was attenuated in *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice (17). These mice did not did not experience liver failure after 3 days of choline deficiency but instead lived for >90 days.

Unexpectedly, the PC in livers of *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice that had been fed the CD diet for 21 days was also 50% lower than in choline-supplemented mice. Clearly, the liver failure was not simply due to decreased hepatic PC. Moreover, liver failure could not be attributed to steatosis, because *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice accumulated more hepatic triacylglycerol (TG) after 21 days of the CD diet than did *Pemt*$^{-/-}$ mice after 3 days. Thus, the mechanism that caused liver failure in *Pemt*$^{-/-}$ mice fed the CD diet was investigated (18). The conclusion was that a decreased molar ratio of PC/PE was responsible for development of steatohepatitis and liver failure. Thus, the PC/PE ratio in livers of *Pemt*$^{-/-}$ mice fed the CD diet was ~0.8 compared with ~1.8 for mice fed the choline-supplemented diet. These and other findings led to the hypothesis that some cell surface PC (a cylindrical molecule) in *Pemt*$^{-/-}$ hepatocytes was replaced by PE (an inverted cone-shaped molecule), resulting in imperfect packing of the plasma membrane bilayer so that molecules leaked through the plasma membrane and promoted inflammation associated with steatohepatitis. Subsequent studies substantiated the hypothesis (18). Thus, the PC/PE ratio appears to play a fundamental role in maintaining hepatocyte integrity.

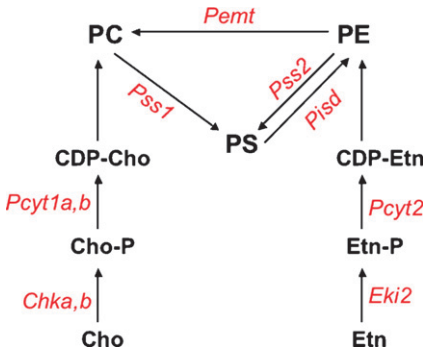

Fig. 1. Genes of phospholipid synthesis that have been disrupted in mice. Cho, choline; Etn, ethanolamine. Names in italics are genes encoding the following enzymes: *Chka,b*, choline kinase; *Eki2*, ethanolamine kinase; *Pcyt1a,b*, CTP:phosphocholine cytidylyltransferase; *Pcyt2*, CTP:phosphoethanolamine cytidylyltransferase; *Pemt*, phosphatidylethanolamine *N*-methytransferase; *Pisd*, phosphatidylserine decarboxylase; *Pss1,2*, phosphatidylserine synthase.

PEMT, lipoprotein metabolism, and homocysteine homeostasis

Whereas *Pemt*$^{-/-}$ mice do not exhibit an obvious phenotype when fed a chow diet, when challenged with a high-fat/high-cholesterol diet, the amount of TG and apolipoprotein (apo) B100 in plasma lipoproteins of male mice was less than in *Pemt*$^{+/+}$ mice fed the same diet (19, 20). Subsequently, the lower levels of plasma TG and apo B100 were attributed to a reduced rate of hepatic VLDL secretion (19, 20). PC and cholesteryl ester in plasma of mice are mainly carried in HDLs. In *Pemt*$^{-/-}$ mice fed either chow or a high-fat/high-cholesterol diet, PC and cholesteryl in plasma were 25%–45% lower than in *Pemt*$^{+/+}$ mice fed the same diet (20). The reduction in plasma lipids was due to increased expression of the scavenger receptor-B1 (21).

The products of the PEMT reaction are PC and *S*-adenosylhomocysteine. For each molecule of PC generated, three *S*-adenosylhomocysteine molecules are formed, from which homocysteine is derived by hydrolysis. The level of homocysteine in plasma of *Pemt*$^{-/-}$ mice is 50% lower than in *Pemt*$^{+/+}$ mice (22). Furthermore, hepatic PEMT activity is 2-fold higher in liver-specific CTα knockout mice than in wild-type mice, and the plasma homocysteine level is 20–40% higher (23). These and other studies suggest that PEMT generates ~50% of homocysteine in plasma and have clinical relevance, because elevated plasma homocysteine is a risk factor for cardiovascular disease (24).

CT

CT is encoded by two genes, *Pcyt1a* and *Pcyt1b*. CTα (product of the *Pcyt1a* gene) is largely localized to the nucleus of many different cell types (25). Disruption of the *Pcyt1a* gene in mice resulted in early embryonic death (26). CTβ (encoded by *Pcyt1b*) is localized to the cytoplasm (27). Mice express two isoforms of CTβ, CTβ2 and CTβ3, that are formed by alternative splicing of the CTβ transcript (28).

The *Pcyt1b* gene was disrupted in mice. Mice that lack CTβ2 survive but exhibit gonadal dysfunction (29). The major site of expression of CTβ is the brain. Although *Pcyt1b*$^{-/-}$ mice do not have obvious neurological problems,

axon branching of neurons cultured from CTβ2-deficient mice is severely impaired (Demizieux, L. and Vance, J.E., unpublished results). This finding is consistent with experiments in which reduction of CTβ2 expression in PC12 cells by RNA interference decreased neurite branching by ~60% (30).

Because *Pcyt1a*$^{-/-}$ mice do not survive during development, the Cre-lox system was used to disrupt CTα expression specifically in several cell types in vivo. *Pcyt1a*$^{-/-}$ macrophages appeared normal, although CT activity was decreased by 70–90% (28). When macrophages take up acetylated LDLs, CT activity is increased (31). It was postulated that increased CT activity would enhance PC synthesis and protect the macrophages from cell death induced by increased cellular cholesterol delivered from the lipoproteins. In support of this hypothesis, only 71% of CTα-deficient macrophages survived after incubation with acetylated LDL, whereas 98% of wild-type macrophages survived (28). Lipopolysaccharide stimulation of macrophages induces the secretion of tumor necrosis factor-α and interleukin-6. Lack of CTα expression in mouse macrophages impaired secretion of these cytokines, whereas secretion of interleukin-1β and apo E was unaffected (32). It appears that PC generated via CTα is required for normal secretion of these two cytokines.

The Cre-lox system was also used to inactivate CTα in mouse livers. Hepatic CTα-deficient mice are viable and fertile (33, 34) even though CT activity is reduced by 85%. The amounts of plasma PC, cholesterol, and TG were ~50% lower in knockout mice than in controls, as were HDLs (cholesterol and apo A1) and VLDLs (TG and apo B100). Moreover, secretion of both apo B100 and apo B48 was decreased in hepatic-specific CTα knockout mice (33) and efflux of cholesterol and PC was lower in knockout, than in control, hepatocytes. Thus, the CTα-PC biosynthetic pathway appears to be a key player in maintaining homeostasis of plasma lipoproteins.

The *Pcyt1a* gene was selectively inactivated in mouse lung epithelial cells (35). Lack of CTα did not impair lung development, but at birth, the mice suffered respiratory failure. Dipalmitoyl-PC in lungs of the knockout mice, as well as in the alveolar lavage, was significantly reduced. Thus, CTα is not required for proliferation or differentiation of lung epithelia but is essential for normal PC secretion by the lung (35).

CK

CK exists in three distinct isoforms (α1, α2, and β) encoded by two genes, *Chka* and *Chkb* (36). CKα is highly expressed in testis and liver, whereas CKβ is abundant in heart and liver. Gene disruption of *Chka* in mice caused embryonic lethality, highlighting the importance of CKα for development (37). A spontaneous recessive mutation in the *Chkb* gene was identified in mice that unexpectedly led to progressive muscular dystrophy and neonatal bone deformity (38). CK activity was almost completely eliminated from forelimb and hindlimb muscles (38). The hindlimb muscular dystrophy in *Chkb*$^{-/-}$ mice appears to be caused by attenuated PC biosynthesis and enhanced PC catabolism (Wu, G., Sher, R.B., Cox, G.A. and Vance, D.E., unpublished results).

GENES ENCODING ENZYMES OF PS AND PE BIOSYNTHESIS

Mammalian cells have two major PE biosynthetic pathways: the CDP-ethanolamine pathway (1) on the endoplasmic reticulum (ER) and the PS decarboxylase (PSD) pathway in mitochondria (39) (Fig. 1). This spatial separation of the two pathways suggests that pools of PE might be compartmentalized according to biosynthetic origin. The quantitative contribution of the PE biosynthetic pathways in mammalian cells has not been rigorously investigated, although in several cell types, >80% of PE is made from PSD (40).

Genes of the CDP-ethanolamine pathway

Two genes encode ethanolamine kinase (EK). One is a dual-specificity ethanolamine/CK, whereas the other (EK-2) is specific for ethanolamine (41). Two lines of mice have been generated with targeted disruption of the EK-2 gene. In one, litter size was reduced and ~20% of pups died perinatally (42). In the other, litter size and pup mortality and fertility were normal, as was morphology of embryonic and adult testis (43). The reason for differences between the two lines of EK knockout mice remains to be determined.

In contrast to CT, ET is encoded by a single gene, *Pcyt2* (44). ET-deficient mice were generated (45). *Pcyt2*$^{+/-}$ mice are outwardly normal and tissue levels of PE are normal. However, ET is essential for mouse development, because *Pcyt2*$^{-/-}$ embryos died prior to embryonic day 8.5. Thus, PE synthesized from PSD cannot substitute for PE made via CDP-ethanolamine.

Phosphatidylserine decarboxylase

PSD activity in mammalian cells (Fig. 1) is encoded by a single gene. PE synthesis from PSD occurs in mitochondrial inner membranes, and the conversion of PS to PE is regulated by transport of newly-made PS from the ER to mitochondria (46). The transport likely involves transient contact between mitochondria and an ER domain called mitochondria-associated membranes (MAM) (47) Almost all mitochondrial PE is made in situ in mitochondria by PSD (48). Elimination of PSD in mice causes embryonic lethality, whereas *Psd*$^{+/-}$ mice appear normal. PSD-null embryos die around embryonic day 9.5 and have morphologically aberrant, fragmented mitochondria, indicative of a defect in mitochondrial fusion (49). It is likely, therefore, that PSD is required to provide sufficient mitochondrial PE for normal mitochondrial function and fusion. Thus, cellular pools of PE are compartmentalized according to biosynthetic origin, and both pathways of PE synthesis are essential for mouse development.

Genes of phosphatidylserine synthesis

Studies in Chinese hamster ovary mutant cells demonstrated that PS is synthesized by two distinct PS synthases

(PSSs) (Fig. 1) (50). PSS1 exchanges serine for choline of PC, whereas PSS2 exchanges serine with PE. PSS1 and PSS2 are localized to MAM and are largely absent from the bulk of ER (51). PSS1 is ubiquitously expressed in mouse tissues, but PSS2 is most highly expressed in testis (52). PSS2-null mice are outwardly normal. However, testes of male *Pss2*$^{-/-}$ mice are smaller than in wild-type littermates and ~10% of males are infertile (52). Despite up to 90% reduction in serine-exchange activity (contributed by both PSS1 and PSS2), amounts of PS and PE are normal in *Pss2*$^{+/-}$ and *Pss2*$^{-/-}$ tissues (53). Thus, PSS2 is not required for mouse viability, and PSS1 can, for the most part, substitute for PSS2.

Pss1$^{-/-}$ mice were generated and were also viable. *Pss1*$^{-/-}$ mice have a normal lifespan, and males and females are fertile (54). Thus, PSS1 is not essential for mouse development or viability. Although total serine-exchange activity in PSS1-deficient tissues was markedly reduced, the PS/PE content of tissues was not significantly altered. *Pss1*$^{-/-}$ mice were bred with *Pss2*$^{-/-}$ mice, and, as expected, no *Pss1*$^{-/-}$/*Pss2*$^{-/-}$ double knockout mice were born (54). Nevertheless, mice with three inactivated *Pss* alleles are viable despite some tissues having only ~10% of normal PSS activity and a reduction in PS/PE content of up to 40%. Thus, mice can tolerate as little as 10% of normal PSS activity and significantly reduced amounts of PS and PE, but a minimum threshold of PS/PSS appears to be essential. These studies reveal significant redundancy in the two PS biosynthetic pathways. The evolutionary pressures that preserved expression of two PSSs remain unclear.

CONCLUSION AND THE FUTURE

Disruption of phospholipid biosynthetic genes in mice has greatly expanded our understanding of the physiological requirements for phospholipids (**Table 1**). Many gene disruptions were, not unexpectedly, embryonic lethal, because phospholipids maintain cell integrity. Several enzyme activities involved in the biosynthesis of PC, PE, and PS are encoded by multiple genes. Whereas PEMT deficiency is, for the most part, compensated by the CDP-choline pathway, ET and PSD do not substitute for one another, suggesting compartmentalization of PE pools made by these pathways. Similarly, lack of CKα is not compensated by CKβ, whereas in most tissues, CKβ can be replaced by CKα. Moreover, PSS1 can apparently substitute for PSS2 and vice versa. Thus, only PE appears to have two functionally distinct biosynthetic routes.

If, 50 years ago, Eugene Kennedy were asked to speculate about where the phospholipid metabolism field would be in 2009, it is unlikely he would have predicted the striking development of knockout mice as a tool for understanding phospholipid function. In 1989, Kennedy wrote: "the dramatic development of eukaryotic genetics in recent years, however, will surely prove a great stimulus to studies of phospholipid biosynthesis in animal cells, as well as in yeast." (3). Twenty years later, the impact of knockout studies on understanding phospholipid metabolism and function has exceeded expectations. If, in 1959, the question were asked: "what would be the biological impact of deleting a mouse gene of PC biosynthesis?" the answer would probably have been: embryonic death. Even in 1989, the idea was not on the radar screen that disruption of PC biosynthesis would lead to hepatic steatohepatitis, lipoprotein abnormalities, bone growth defects or muscular dystrophy, and improved protection against diet-induced obesity and type 2 diabetes (Jacobs, R.L., Zhao, Y., Koonen, D.P.Y., Kennedy, B., Dyck, J.R.B. and Vance, D.E., unpublished results).

In the next decade, the remaining genes of phospholipid biosynthesis will likely be disrupted in mice. Perhaps therapies for human diseases will be developed based on findings in mice with disrupted genes of phospholipid

TABLE 1. Physiological consequences of gene disruptions in phospholipid biosynthesis

Mouse gene	Enzyme deleted	Diet	Organ/cell	Physiological consequence
Chka	CKα	Chow	All	Embryonic lethality
Chkb	CKb	Chow	All	Hindlimb muscular dystrophy, neonatal bone deformity
Eki2	EK2	Chow	All	No apparent phenotype? Neonatal lethality?
Pcyt1a	CTα	Chow	All	Embryonic lethality
Pcyt1a	CTα	Chow	Hepatocyte	Reduced plasma lipoproteins
Pcyt1a	CTα	HF	Hepatocyte	Steatosis/steatohepatitis
Pcyt1a	CTα	Chow	Macrophage	Resistant to cholesterol-induced cell death; impaired secretion of cytokines
Pcyt1a	CTα	Chow	Lung epithelial cells	Neonatal respiratory failure
Pcyt1b	CTβ2	Chow	All	Gonadal dysfunction, defective axon branching
Pcyt2	ET	Cho	All	Embryonic lethality
Pemt	PEMT	Chow	All	No apparent phenotype
Pemt	PEMT	Choline deficiency	All	Liver failure (steatohepatitis) after 3 days
Pemt	PEMT	HF/HC	All	Reduced plasma lipoproteins, decreased atherosclerosis
Pemt	PEMT	HF	All	Decreased obesity; increased insulin sensitivity
Pisd	PSD	Chow	All	Embryonic lethality
Pss1	PSS1	Chow	All	No apparent phenotype
Pss2	PSS2	Chow	All	Male subfertility
Pss1/Pss2	PSS1/PSS2	Chow	All	Embryonic lethality

References for these data are cited in the text. Abbreviations: HF, high fat; HF/HC; high fat/high cholesterol. Other abbreviations are as used in text.

biosynthesis. There is no doubt that scientists in 2059 will experience the same thrill of discovery as in the past 50 years.

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