CONSTRUCTION OF BACULOVIRUS TRANSFER VECTORS FOR INSECT CELL EXPRESSION OF HUMAN CHOLINE AND ETHANOLAMINE KINASE ISOFORMS

By

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CERTIFICATE

This is to certify that the dissertation entitled "Construction Of Baculovirus Transfer Vectors for Insect Cell Expression of Human Choline and Ethanolamine Kinase Isoforms" is the bonafide record of research work done by Mr. Chang Chiat Han during the period from July 2008 to October 2008 under my supervision.

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LIST OF SYMBOLS AND ABBREVIATIONS

AcNPV	=	*Autographa californica* Nuclear Polyhedrosis Viruses
ATP	=	Adenosine triphosphate
CCT	=	CTP:phosphocholine cytidylyltransferase
CDP-Choline	=	Cytidine diphosphate-Choline
CK	=	Choline kinase
CMP	=	Cytidine monophosphate
CPT	=	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CTP	=	Cytidine triphosphate
DAG	=	Diacylglycerol
dATP	=	Deoxyadenosine triphosphate
dCTP	=	Deoxycytidine triphosphate
dGTP	=	Deoxyguanosine triphosphate
dNTP	=	Deoxyribonucleotide triphosphate
dTTP	=	Deoxythymidine triphosphate
ECT	=	CTP:phosphoethanolamine cytidylyltransferase
EK	=	Ethanolamine kinase
EPT	=	CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
ER	=	Endoplasmic reticulum
HC-3	=	Hemicholinium-3
his-tag	=	histidine-tag
LB	=	Luria-Bertani

LB+Amp	=	Luria-Bertani agar with ampicillin
LCAT	=	Lecithin:cholesterol acyltransferase
LVLDL	=	Very low density lipoprotein
Mg^{2+}	=	Magnesium ion
Na^{+}	=	Sodium ion
OD	=	Optical density
PChol	=	Phosphocholine
PEtn	=	Phosphoethanolamine
PS	=	Phosphatidylserine
PtdCho	=	Phosphatidylcholine
PtdEtn	=	Phosphatidylethanolamine
RE	=	Restriction enzyme
SAM	=	S-adenosylmethionine
VLDL	=	Very low density lipoprotein

PEMBINAAN VEKTOR PEMINDAHAN BACULOVIRUS UNTUK PENGEKSPRESAN ISOFORM KOLINA DAN ETANOLAMINA KINASE MANUSIA DALAM SEL SERANGGA

ABSTRAK

Fosfatidikolina dan fosfatidietanolamina merupakan fosfolipid yang paling banyak terdapat di dalam sel eukariot. Kolina kinase dan etanolamina kinase manusia memainkan peranan yang amat penting dalam biosintesis fosfatidikolina dan fosfatidietanolamina dengan merangsangkan fosforilasi pertama dalam rangkaian tersebut. Dalam kajian ini, fragmen gen isoform kolina kinase-β dan etanolamina kinase-2β manusia dimasukkan ke dalam plasmid yang baru dibina. Plasmid ini dibina daripada plasmid pemindahan pBAC4x-1 yang dibeli. Rangka bacaan terbuka kolina kinase-β dan etanolamina kinase-2β manusia dimasukkan selepas promoter polihedrin untuk dihasilkan sebagai protein yang berlabel histidin. Pembinaan tersebut sedia untuk rekombinasi homologus dengan genom Virus *Autographa californica* Nuclear Polyhedrosis. Sel serangga yang dijangkiti oleh Virus *Autographa californica* Nuclear Polyhedrosis rekombinan dijangka akan menghasilkan protein kolin kinase-β dan etanolamin kinase-2β manusia.

CONSTRUCTION OF BACULOVIRUS TRANSFER VECTORS FOR INSECT CELL EXPRESSION OF HUMAN CHOLINE AND ETHANOLAMINE KINASE ISOFORMS

ABSTRACT

Phosphatidylcholine and phosphatidylethanolamine are the most abundant phospholipid in eukaryotic cell membrane. Human choline kinase and ethanolamine kinase play a very important role in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine by catalyzing in the first phosphorylation step of the pathway. In this study, genes encoding human choline kinase-β (hCK-β) and ethanolamine kinase-2β (hEK-2β) isoform gene fragments were inserted into newly constructed plasmid. The plasmid was constructed from commercially purchased transfer plasmid pBAC4x-1. Open reading frames (ORF) of hCK-β and hEK-2β were introduced into the downstream of polyhedrin promoter for expression as histidine-tagged (his-tag) proteins. The constructs were ready for homologous recombination with *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) genome. Insect cells infected with the recombinant AcNPV were expected to overexpress the hCK-β and hEK-2β proteins.

1.0 Introduction

1.1 Phosphatidylcholine

Formerly known as lecithin, phosphatidylcholine (PtdCho) was firstly discovered by Gobley in 1847 which presented as a component in egg yolk (Vance and Vance, 2008). It is the major component of phospholipid in eukaryote, constituting for about 50% of phospholipid in the cells and more than half of serum phospholipids (Kent, 1990). PtdCho plays a major structural role in cell membrane structure by forming the bilayer matrix (Kent, 1990), particularly at the outer leaflet of plasma membrane.

In mammalian respiratory system, PtdCho (in dipalmitoyl-PtdCho form) interacts with surfactant-associated protein B to form pulmonary surfactant. Pulmonary surfactant is important in lowering alveoli surface tension at the pulmonary air and water interface, thus preventing alveolar collapse (van Golde1988). The importance of PtdCho in forming pulmonary surfactant is no doubt because study showed that the secretion of PtdCho was inhibited when surfactant was added in alveolar type II cells, suggesting the surfactant synthesis and secretion was under feedback inhibitory control (Dobbs *et al.*, 1987).

PtdCho acts as the substrate for lecithin:cholesterol acyltransferase (LCAT). It is important as a regulator for the activity of LCAT (Parks *et al.*, 2000). LCAT transfers the fatty acyl group of PC to cholesterol, forming cholesterol ester (Aron *et al.*, 1978) which can then be transported from peripheral tissues to liver for excretion through reverse cholesterol transport. LCAT is also predominant in HDL maturation and distribution in the body.

Moreover, PtdCho is the major component of human plasma lipoprotein. Together with other phospholipids, they form outer coat of mature lipoprotein particles surrounding the core of non-polar lipids for lipid transport.

Exton (1990) reported that the breakdown product of PtdCho caused cell signaling, rendering it to be secondary cell signal messenger (Figure 1.1). PtdCho can be hydrolyzed by Phospholipase A_2, C and D. Arachidonic acid, diacylglycerol (DAG) LysoPtdCho and phosphatidic acid are among the signaling molecules generated from PtdCho (Cui and Houweling, 2002), which serve some physiological functions.

1.2 Phosphatidylethanolamine

Phosphatidylethanolamine (PtdEtn), formerly known as cephatin, appears to be the most abundant phospholipid in mammalian cell membrane after PtdCho, where it consists of about 20 to 50% of total phospholipids. However, the distribution of PtdEtn in mammalian is distinct. As much as 45% phospholipids of the brain are consisted of PtdEtn. The level of PtdEtn is relatively low in hepatocytes where it constitutes only about 20% of total phospholipid. (Vance, 2008)

It should be noted that the distribution and composition of PtdEtn is not only happened in different mammalian cells. For each cell, the distribution of PtdEtn in between inner leaftlet and outer leaftet of cell membrane is asymmetrical. It was reported that more than 80% of PtdEtn were located within the inner leaflet of cell membrane. This is in contrast with PtdCho where the majority of PtdCho is located at the outer leaftlet of cell membrane. This asymmetric distribution is maintained partly by aminophospholipid translocase that catalyzes PtdEtn to be transferred from outer leaflet into inner leaflet of

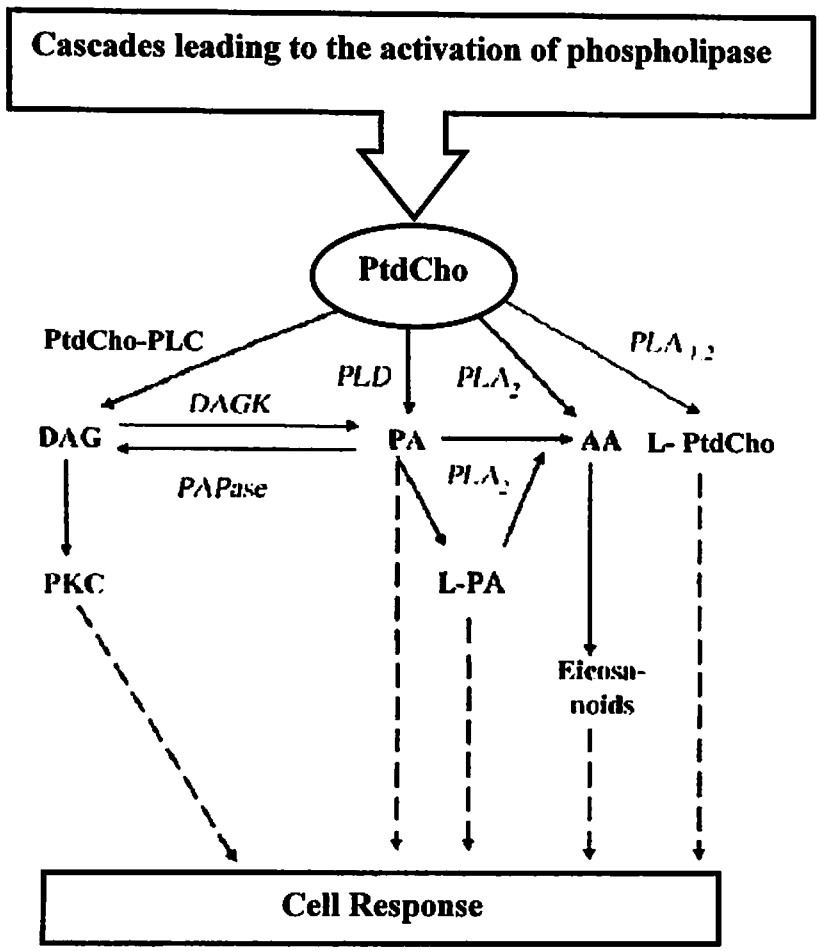

Figure 1.1: Signaling mediators generated from PtdCho upon cell stimulation. Abbreviations are as follows: AA, arachidonic acid; DAG, diacylglycerol; DAGK, diacylglycerol kinase; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAPase, phosphatidic acid phosphohydrolase; PtdCho, phosphatidylcholine; PtdCho-PLC, phosphatidylcholinespecific phospholipase C; PKC, protein kinase C; PLA_2, phospholipase A_2; PLD, phospholipase D (Cui and Houweling, 2002).

cell membrane. In intracellular, the PtdEtn content of mitochondrion, especially in its inner membrane, is particularly higher than the other cell organelles (Vance, 2008).

PtdEtn may involve in lipoprotein secretion in the liver. It was reported that nascent hepatic very low density lipoprotein (VLDL) was enriched with PtdEtn in the Golgi fraction of mice hepatocytes upon treated with lipase inhibitor. However, this VLDL with PtdEtn enrichment was not being demonstrated to be transferred into plasma, indicated by lower total phospholipid content of VLDL secreted from hepatocytes. This nascent VLDL might undergo secretory pathways before being secreted into plasma. (Agren *et al.*, 2005)

Besides that, it has been observed that sarcolemmal disruption and irreversible cell damage occurred during ischemic heart disease because the distribution of PtdEtn in sarcolemmal membranes was altered, indicating the important role of PtdEtn metabolism in heart. (Post *et al.*, 1995)

Emoto *et al.* (1997) reported that during early cell apoptosis, PtdEtn as well as phosphatidylserine (PS) were exposed on the cell surface. This led to the loss of asymmetric bilayer distribution of aminophospholidpid of cell membrane bilayer, which caused the apoptotic cells to be recognized and eventually engulfed by phagocytes.

PtdEtn also involves in cell surface signaling. The ethanolamine moiety is donated by PtdEtn for glycosylphosphatidylinositol to anchor cell surface signaling proteins to the lipid bilayer of plasma membrane (Menon and Stevens, 1992).

1.3 Biosynthesis of Phosphatidylcholine

PtdCho biosynthesis is achieved through three distinct pathways. CDP-choline pathway, which is also known as Kennedy pathway in the honor of Kennedy and co-worker to describe the pathway (Kennedy and Weiss, 1956) is the main pathway of PtdCho biosynthesis. Choline served as the precursor for the pathway. In animal cells, most of the choline is not made *de novo*. As the first precursor in CDP-choline pathway, animal cell choline is supplied mainly through diet, even though choline deficiency is unlikely to occur. For CDP-choline pathway to be initiated, choline is needed to be transported, mainly by facilitated diffusion from extracellular matrix into the cells. This is achieved by two mechanisms, which are high affinity Na^+-dependent transporter and low affinity Na^+-independent transporter (Haeffner, 1975). Upon being transported from extracellular into cytosol, choline will be phosphorylated by choline kinase (CK) in the presence of adenosine triphosphate (ATP) to produce phosphocholine (PChol). Then, cytidine monophosphate (CMP) is transferred from cytidine triphosphate (CTP) to PChol to form cytidine diphosphate-choline (CDP-choline). This step is catalyzed by CTP:phosphocholine cytidylyltransferase (CCT), which is also the key step described by Kennedy and Weiss (1956) leading to the completion of CDP-choline pathway. This activated headgroup is condensed with diacylglycerol by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) in endoplasmic reticulum (ER) to produce the final product of the pathway – PtdCho.

CDP-choline pathway has been studied well by researchers. Each and every step in the pathway has been well studied and reported as a regulatory key enzyme for the pathway. However, in most of metabolic pathways, the regulation is determined by many enzymes in a variety of conditions. Haeffner (1975) reported that extracellular choline

uptake by the cells was found to be a rate-limiting step. This was due to the fact that the rate of choline phosphorylation was determined by the extracellular choline concentration. However, estrogen stimulation on young rooster caused an increase in PChol which was due to increased activity of choline kinase and resulted a doubling rate of PtdCho biosynthesis (Vigo and Vance, 1981). In murine 3T3 cells, choline kinase activity in the cells and cell-free extract were shown to increase by 2-fold after introducing mitogenic growth factors (Warden and Friedkin, 1984). When C3H10T1/2 cells were transfected with Harvey-*ras* oncogene, CK activity was observed to increase 2-fold but CCT activity was reduced to 50%, where the authors proposed this down regulation may be related to fine tuning of PtdCho biosynthesis (Teegarden *et al.*, 1990). CCT was firstly shown to be a rate limiting enzyme of CDP-choline pathway by Vance *et al.* (1980). They reported an increase of CPT levels in HeLa cells transfected by poliovirus. The turnover rate of CCT was also being enhanced more than 2-fold by poliovirus, which led to a conclusion that poliovirus stimulated the rate of CCT reaction, causing the rate of PtdCho synthesis to increase. Another research group performed study of PtdCho biosynthesis regulation on embryonic chick muscle cells treated with phospholipase C. Activities of CK, CPT and some other enzymes were observed to be similar with control cells, except for CCT where its activity increased 3-fold. Supported by the level of PChol decreased and the level of CDP-choline increased, they identified that CCT was the regulatory enzyme for choline flux in the cells (Sleight and Kent, 1980). In conjunction with previous study, Wright *et al.* (1985) proposed the CCT translocation hypothesis after the group showed that CCT activity increased when it reversibly associated with cellular membrane upon phospholipase C treatment. The active form of CCT was membrane-bound while free form of CCT was located in the cytosol. The reversible association of CCT with cellular membranes was involved in

regulating PtdCho biosynthesis. The sole study by Bjornstad and Bremer (1966) showed that CCT was at equilibrium *in vivo*, so it was unlikely to be involved in the regulatory.

The second PtdCho biosynthesis pathway involves the conversion of PtdEtn to PtdCho by PtdEtn methylation (Bremer and Greenberg, 1959). This pathway is mainly involved in mammalian liver and relatively quantitative insignificance in other cells. It is achieved in three successive methylation by a single enzyme phosphatidylethanolamine N-methyltransferase (PEMT), with S-adenosylmethionine (SAM) as the source of methyl group to produce PtdCho.

Another pathway which is currently only discovered to be working in bacteria involves the reaction of choline with CDP–DAG to form PtdCho and CMP (Sohlenkamp *et al.*, 2003).

The pathways of PtdCho biosynthesis are shown in Figure 1.2.

1.4 Biosynthesis of Phosphatidylethanolamine

In eukaryotic cells, there are four major pathways which led to PtdEtn biosynthesis (Figure 1.3). The first and foremost pathway is named as CDP-ethanolamine pathway. The CDP-ethanolamine pathway, also known as Kennedy pathway, was elucidated by Kennedy and Weiss in 1956. CDP-ethanolamine pathway uses ethanolamine as the precursor for PtdEtn synthesis. Ethanolamine is mostly obtained from diet, while PtdEtn degradation contributes to the minor.

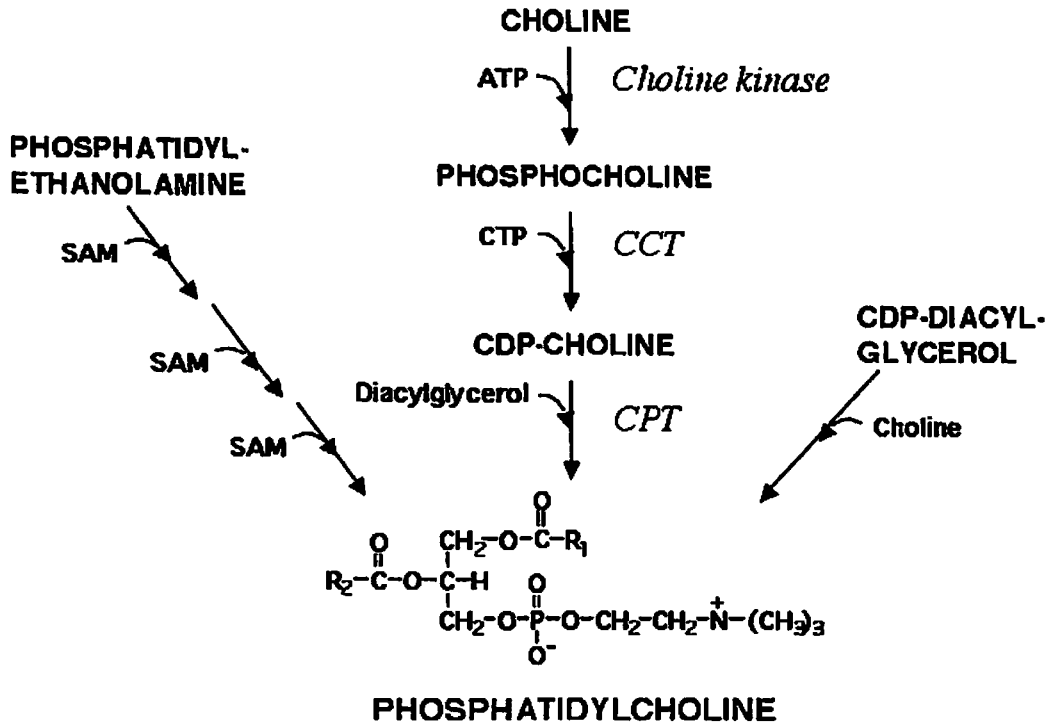

Figure 1.2: Pathways of PtdCho biosynthesis (Kent, 1990). ATP, adenosine triphosphate; CCT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; CTP, cytidine triphosphate; SAM, S-adenosylmethionine.

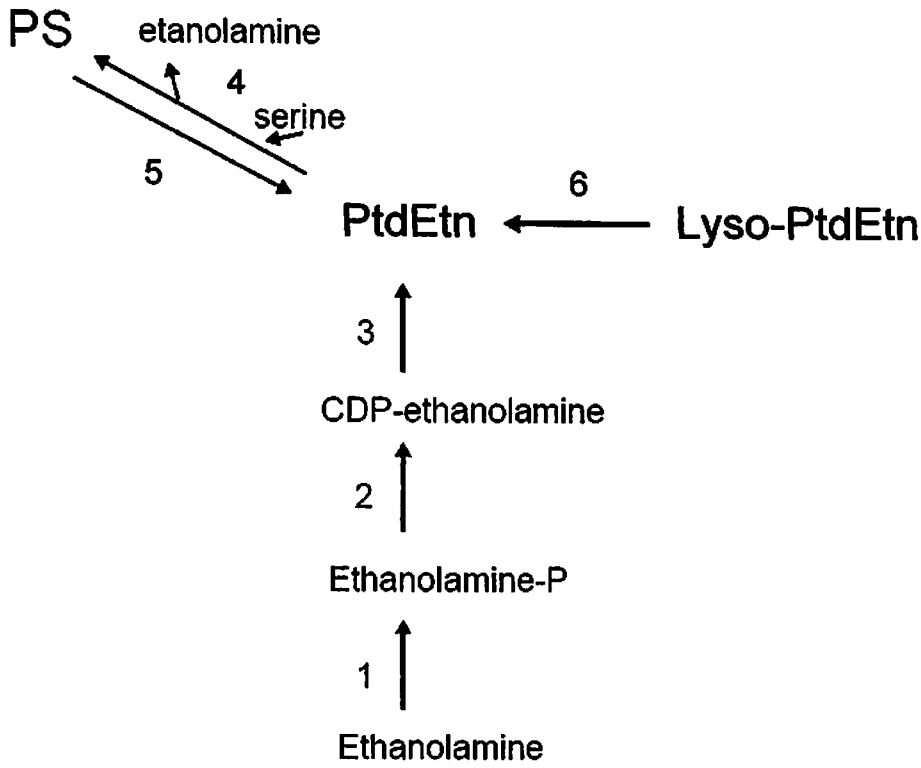

Figure 1.3: Biosynthesis of PtdEtn. The numbers indicate the enzymes. 1, ethanolamine kinase; 2, CTP:phosphoethanolamine cytidylyltransferase; 3, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; 4, phosphotidylserine synthase-2; 5, phosphotidylserine decarboxylase; 6, acyl-CoA:lyso-phosphatidylethanolamine acyltransferase (Vance and Vance, 2008).

In the pathway, the first reaction involves the phosphorylation of ethanolamine by ethanolamine kinase (EK), producing phosphoethanolamine (PEtn). After that, PEtn is converted by cytosolic CTP:phosphoethanolamine cytidylyltransferase (ECT) to form CDP-ethanolamine. Lastly, with the catalysis of CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) which is located mainly in ER and nuclear envelope membrane, PEtn moiety of CDP-ethanolamine is transferred to DAG to synthesize the final product of the pathway, PtdEtn.

The regulation of CDP-ethanolamine pathway is controversial. It has been reported that ECT was a rate limiting enzyme in the pathway (Bladergroen and van Golde, 1997). However, McMaster and Choy (1992) reported the accumulation of radiolabelled ethanolamine occurred in the Hamster heart, indicating that the phosphorylation of ethanolamine by EK was the rate limiting step. Besides that, DAG was showed to be a regulator CDP-ethanolamine pathway in the condition of high glucagon level which caused DAG supply to decrease (Tijburg *et al.*, 1989b).

The other three PtdEtn biosynthesis pathways involve the modification of pre-existing phospholipid. PS decarboxylation pathway is the second major PtdEtn biosynthesis pathway after CDP-ethanolamine pathway (Kanfer and Kennedy, 1964). The enzyme that involves in this pathway is PS decarboxylase which is located at the outer leaflet of mitochondrion inner membrane (Zborowski *et al.*, 1983). The origin of PS synthesis is in ER. So in order to initiate this pathway, PS is needed to be transported from ER into mitochondrial inner membrane, where PS is decarboxylated into PE upon reacted with PS decarboxylase.

Other PtdEtn synthesis pathways are acylation of lyso-PtdEtn and base-change reaction between ethanolamine and serine of PS, where the quantity is minor and insignificant.

In comparing with CDP-ethanolamine pathway where PtdEtn synthesis occurs at ER, PS decarboxylation pathway is limited in mitochondrion, suggesting two different PtdEtn biosynthesis pathways in the cells (Vance and Vance, 2008). Most of PtdEtn synthesized from PS decarboxylation pathway retain in the mitocondria, so it is possible that PS decarboxylation pathway is potentially important for mitocondria (Bleijerveld *et al.*, 2007).

PtdEtn biosynthesis pathway depends on the cell type. Zalinski and Choy (1982) studied the PtdEtn biosynthesis pathway of isolated hamster heart using radiolabeled ethanolamine perfusion. They found out that CDP-ethanolamine pathway was the main pathway of PtdEtn biosynthesis in hamster heart while PS decarboxylation and base-change reaction lead to minor contribution of PtdEtn synthesis. Study on rat isolated hepatocytes also revealed that CDP-ethanolamine pathway was the main PtdEtn biosynthesis pathway for rat hepatocytes compared with PS decarboxylation pathway (Tijburg *et al.*, 1989a). However, PS decarboxylation pathway was important for PtdEtn biosynthesis in many types of cells even though ethanolamine was abundant (Voelker, 1984).

The species of PtdEtn synthesized by CDP-ethanolamine pathway and PS decarboxylation pathway are different. Although both pathways produce diacyl-PtdEtn species, most of the species are prone to be synthesized by one pathway. It was reported that PtdEtn with mono or diunsaturated fatty acids at *sn*-2 position was preferentially