Regulation of apoptosis, induced by phosphatidylcholine synthesis inhibition

Regulatie van celdood, veroorzaakt door de
remming van de fosfatidylcholine synthese

(met een samenvatting in het Nederlands)

Proefschrift

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Michiel Henrik Marie van der Sanden
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Promotor:

Prof. Dr. J.B. Helms
Hoofdafdeling Biochemie & Celbiologie
Faculteit der Diergeneeskunde, Universiteit Utrecht

Co-promotor:

Dr. A.B. Vaandrager
Hoofdafdeling Biochemie & Celbiologie
Faculteit der Diergeneeskunde, Universiteit Utrecht

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Contents

Abbreviations

AARE	amino acid response element
ADP	adenosine 5’-diphosphate
ALP	alkyl-lysophospholipid analogue
ASK	apoptosis signal-regulating kinase
ATP	adenosine 5’-triphosphate
ATF	activating transcription factor
CDP	cytidine 5’diphosphate
C/EBP	CCAAT/enhancer-binding protein
CHO	Chinese hamster ovary
CHO-K1	wild-type Chinese hamster ovarian K1 cells
CHOP	C/EBP-homologous protein
CK	choline kinase
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine 5’triphosphate
DAG	diacylglycerol
EDTA	ethylenediamine tetraacetate
EM	electron microscopy
ER	endoplasmic reticulum
FCS	fetal calf serum
GADD	growth arrest and DNA damage
GPAT	glycerol-3-phosphate acyltransferase
GPC	glycerophosphocholine
HePC	hexadecylphosphocholine
HPLC	high-performance liquid chromatography
HSP	heat-shock protein
JNK	C-Jun N-terminal kinase
LUC	luciferase
LysoPC	lysophosphatidylcholine
MT58	mutant 58 cells
PBS	phosphate buffered saline
PA	phosphatidic acid
PC	phosphatidylcholine
PCD	programmed cell death
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine-N-methyltransferase
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
p38 MAPK	p38 mitogen-activated protein kinase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SM	sphingomyelin
TAG	triacylglycerol
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UPR	unfolded protein response

CHAPTER 1

General introduction

into

Phosphatidylcholine

and

Apoptosis

Introduction

Almost all organisms on earth consist of cells. Some organisms are so small that they just exist of one cell, for example bacteria and protozoa. Mammalians, including humans, are composed of trillions of cells, differentiated in many specific cell-types. One universal feature of all cells is an outer limiting membrane called the plasma membrane. The plasma membrane protects the cell from harmful influences of its environment. In addition, eukaryotic cells contain elaborate systems of internal membranes, which form various membrane-enclosed compartments within the cell. Therefore, eukaryotic cells are highly organized with many functional units or organelles. Membranes are specialized in that it contains specific proteins and lipid components that enable it to perform its unique roles for that cell or organelle. In essence membranes are essential for the integrity and function of the cell.

The basic structural unit of biomembranes is the lipid bilayer, of which the lipid composition varies among different membranes. Membrane lipids are amphipathic, having both a hydrophilic head group and hydrophobic hydrocarbon tails (see Fig. 1).

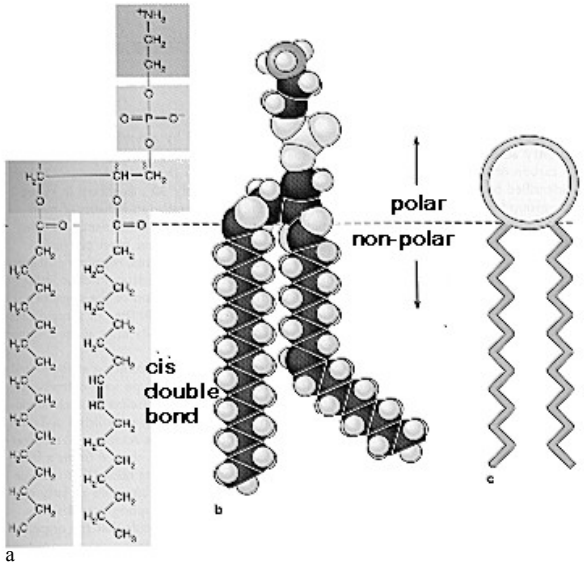

Figure 1. The structure of phosphatidylcholine Phosphatidylcholine (PC) consists of a hydrophilic choline-headgroup and two hydrophobic acyl-chains, connected to each other by a glycerol backbone. PC is represented; in formula (A), as a space filling model (B) and as a symbol (C). The kink in the space-filling model represents the *cis*-double bond.

The bilayer is a sheet like structure composed of two layers of lipid molecules whose polar head groups face the surrounding water and whose fatty acyl chains form a continuous hydrophobic interior. Each lipid layer in this lamellar structure is called a leaflet. The major driving force for the formation of lipid bilayers is hydrophobic interaction between the fatty acyl chains of glycolipid- and lipid molecules. Van der Waals interactions among the hydrocarbon chains favor close packing of these hydrophobic tails. Hydrogen bonding and

electrostatic interactions between the polar head groups and water molecules also stabilize the bilayer. Mammalian bilayers contain a substantial proportion of phospholipids, predominantly phosphoglycerides, which have a glycerol backbone.

Phospholipids

Phospholipids make up an essential part of cellular membranes that act as a barrier for entry of compounds into cells. Another function of phospholipids in the bilayer is that they serve as precursor of second messenger molecules like diacylglycerol (DAG) and inositol 1,4,5,-trisphosphate (IP_3). A third role of phospholipids is storage of energy in the form of fatty acyl components. The majority of phospholipids in cellular membranes consists of glycerophospholipids. A key building block of all mammalian glycerophospholipids is phosphatidic acid (PA) (Fig. 2).

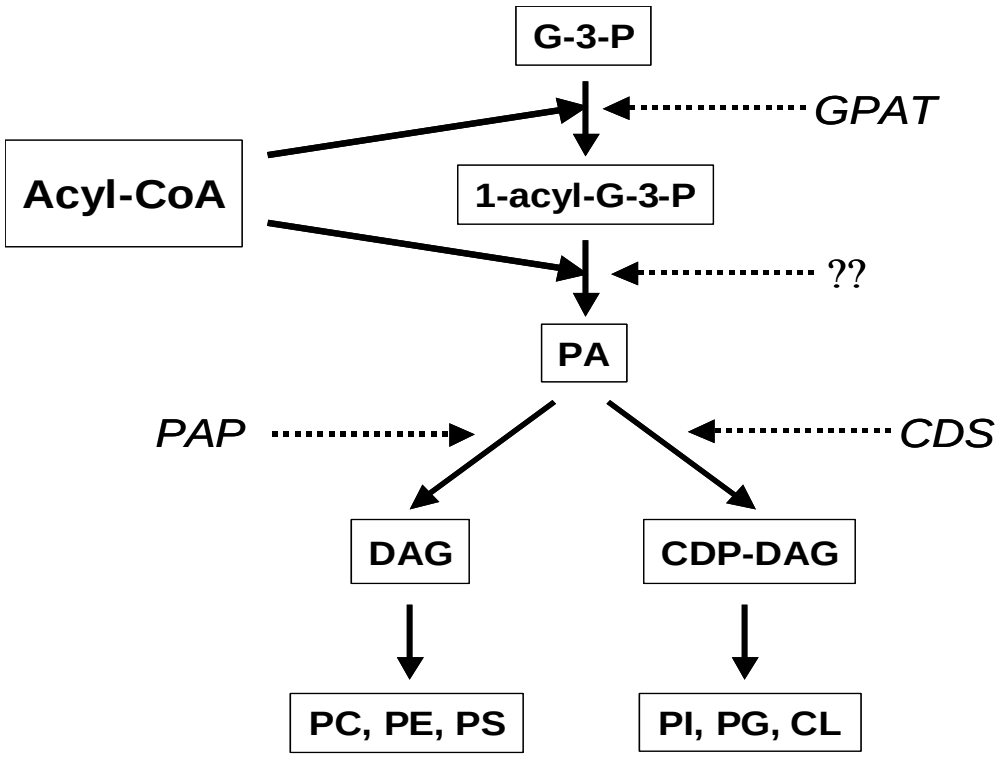

Figure 2. Biosynthesis of phosphatidic acid. Phosphatidic acid is synthesized by two acylation reactions of glycerol-3-phosphate. See text for details. The abbreviations are: G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; DAG, diacylglycerol; CDP-DAG, cytidine diphosphodiacylglycerol; CDS, cytidine diphosphodiacylglycerol synthase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin.

For PA synthesis, glycerol-3 phosphate is first converted to 1-acylglycerol-3-phosphate (lysophosphatidic acid), using acyl-CoA as donor of the fatty acyl chain. The key enzyme in this reaction is glycerol-3-phosphate acyltransferase (GPAT). In mammals, two isoforms of GPAT have been identified that are encoded by different genes and have distinct subcellular localizations. (1, 2). One GPAT isoform is associated with mitochondria and the other on the endoplasmic reticulum (ER). The mitochondrial acyltransferase prefers palmitoyl-CoA (saturated) as an acyl donor as compared to oleoyl-CoA (unsaturated), whereas the ER enzyme does not show a preference of saturated versus unsaturated acyl-CoAs (1, 2). Most glycerophospholipids have a saturated fatty acid chain at the *sn*-1 position and the mitochondrial GPAT is proposed to be responsible for this fatty acid distribution (1, 2). The second acylation step in PA synthesis mainly takes place in the ER. It is presumed that the majority of lysoPA formed in the mitochondria is transferred to the ER for the second acylation, although there is a low acylation activity in mitochondria (3). The acyltransferase of the second acylation primarily utilizes unsaturated fatty acyl-CoAs, resulting in glycerophospholipids with mainly unsaturated fatty acid chain at the *sn*-2 position. The variation of acyl chains at the *sn*-1 and *sn*-2 positions results in a diverse mixture of PA species.

This newly synthesized PA is an intermediate that occurs at a branchpoint in the glycerophospholipid synthesis. *De novo* synthesis of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) requires DAG as donor for acyl chains. DAG is generated through the hydrolysis of PA (see Fig. 2). Essential in this reaction is the enzyme phosphatidic acid phosphohydrolase. The activity of this enzyme can be regulated reversible translocation between cytosol and ER (4). In the cytosol it is in a less active form and translocation to the membranes of the ER results in its activation. PA can also be converted to CDP-diacylglycerol (see Fig. 2). CDP-diacylglycerol is the main precursor in the synthesis of diphosphatidylglycerol (cardiolipin), phosphatidylglycerol (PG) and phosphatidylinositol (PI). The conversion of PA into CDP-diacylglycerol depends on the activity of CDP-diacylglycerol synthase. Mammalian CDP-diacylglycerol synthase is present in both mitochondrial and microsomal fractions, although 90-95% of the activity resides in microsomes (5). Two genes encoding human CDP-diacylglycerol synthase have been found (5). It is hypothesized that the two isoforms are compartmentalized according to their function (6). The CDP-diacylglycerol synthase located in the microsomal fraction is primarily responsible for the synthesis of PI in the ER, whereas the mitochondrial isoform is used for synthesis of the mitochondrial glycerophospholipids phosphatidylglycerol and cardiolipin (6).

Thus, one feature that distinguishes glycerophospholipid classes from one-another is the use of different acyl chain donors, namely CDP-diacylglycerol or DAG.

Classification of various glycerophospholipids is based on the structure of their polar head groups. The head group determines the charge of the glycerophospholipid at neutral pH. Some phosphoglycerides (phospatidylcholine, phosphatidylethanolamine) have no net electric charge; others (phosphatidylglycerol, cardiolipin, phosphatidylserine) have a net negative charge. A few rare phospholipids carry a net positive charge at neutral pH. Other lipids like sphingomyelin (SM) and glycolipids are similar in shape to phosphoglycerides and can also form mixed bilayers with them. Cellular membranes are a diverse mixture of phospholipid species because of the variation in acyl chains and polar head groups. PC is the major phospholipid class in most eukaryotic cell membranes generally comprising ~50% of the total phospholipid mass, followed by PE, PS, PI and SM. An exception is *Drosophila* in which PE comprises around 55% of total membrane phospholipids (7). Therefore, PC has a major structural role in maintaining cell and organelle integrity. Synthesis and degradation of PC must be tightly regulated to ensure appropriate PC levels are present to allow its coordination with cell homeostasis and cell growth.

PC and the CDP-choline pathway

PC was first decribed by Gobley in 1847 as a constituent of egg yolk and named it ‘lecithin’ after the Greek equivalent for egg yolk (*lekithos*). In the 1860s Diakonow and Strecker demonstrated that lecithin contained two fatty acids linked to glycerol by an ester-linkage and that choline was attached to the third hydroxyl by a phosphodiester linkage. This composition of PC was confirmed ninety years later by Baer and Kates by the chemical synthesis of PC. Besides being the most abundant building block of cellular membranes, PC also functions as a precursor of the membrane phospholipid PS (8) and as a choline donor for the synthesis of SM (9). PC has also been identified as a major source of intracellular second messenger molecules in signal transduction pathways (10, 11, 12, 13). In conclusion, PC has a central role in cell structure as well in cell signalling and hence pathways of PC synthesis and breakdown contribute to cellular homeostasis.

De novo synthesis of PC predominately occurs via the CDP-choline pathway, also known as the Kennedy pathway (see Fig. 3) (14).

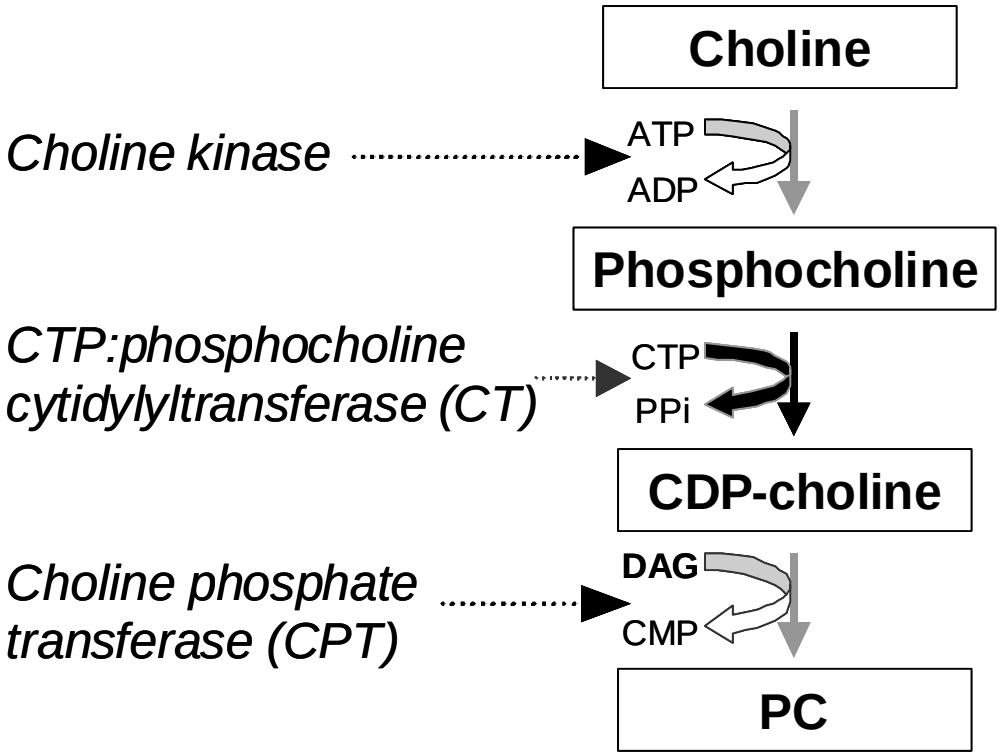

Figure 3. De novo synthesis of PC via the CDP-choline pathway. See text for details. The abbreviations are; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; PPi, diphosphate; CMP, cytidine monophosphate; DAG, diacylglycerol; PC, phosphatidylcholine.

The CDP-choline pathway was first described in the 1950s by Eugene Kennedy, who discovered that CTP, rather than ATP, was the activating nucleotide for this biosynthetic pathway (15). Alternatively, a small percentage of PC is formed by base-exchange, a process causing head-group exchange between phospholipids. The CDP-choline pathway is present in all mammalian cells and consists of three steps:

I. Choline kinase

In the first reaction choline is converted to phosphocholine by choline kinase (CK) (16-19). Choline, used in PC synthesis, mainly originates from exogenous sources as a supplement in cell culture or in the diet of an organism. There appear to be two distinct mechanisms for transport of choline into the cell; a high affinity (K_m < 5 μM), Na-dependent transporter and a lower affinity (K_m > 30 μM) Na-independent transporter (17). Once choline is inside the cell, it is rapidly converted to phosphocholine by CK which is also shown to have phosphorylation activity towards ethanolamine (16, 18, 19). There are two distinct genes identified for CK in

eukaryotic cells, namely Chok/Etnk-α and Chok/Etnk-β (21). Alternative splicing of Chok/Etnk-α results in two isoforms, CK-α1 (50 kDa) and CK-α2 (52 kDa), which are both primarily present in testis and liver (20). Chok/Etnk-β only codes for one isoform, CK-β, which is expressed in most tissues (21). CK only functions as a dimer and α/α and β/β homodimers as well as α/β heterodimers have been found in murine tissues (21).

II. CTP:phosphocholine cytidylyltransferase

The second step is synthesis of CDP-choline from phosphocholine by the enzyme CTP:phosphocholine cytidylyltransferase (CT). CT was first purified from rat liver in 1986 (22) and its cDNA was cloned from rat liver in 1990 (23). Sequence analysis revealed the presence of several distinct structural domains were present in CT: a *N*-terminal nuclear targeting sequence, followed by a catalytic domain (residues 75-235), a membrane/lipid-binding domain, and a *C*-terminal phosphorylation site. Two CT genes, *Pcyt1a* and *Pcyt1b* encoding CTα and CTβ respectively have been identified (24, 25). The murine CTβ gene produces two different CTβ proteins, CTβ2 and CTβ3. CTβ2 is highly expressed in the brain and CTβ3 is expressed in most tissues, but especially in testes (25). CTβ1 is only found in humans and is different from the other CTβ isoforms in the fact that it lacks the *C*-terminal phosphorylation domain (26). Beside from their different subcellular and tissue distribution, CTα and CTβ have a similar catalytic domain and lipid-binding domain (26). Furthermore the activity of the isoforms is similarly regulated by lipids (26).

CT is considered to be the rate-limiting step of the CDP-choline pathway (27), hence, events that regulate CT activity also influence the cellular pool of PC. CT activity in cells is regulated primarily by association with membrane lipids, by translocation between endoplasmic reticulum, cytoplasm and nucleus (27-31), but also by phosphorylation, and by gene expression (26, 31, 32, 34). Especially the regulation of CT activity via its lipid-binding domain has been well characterized. Activation of CT takes places by translocation from an inactive soluble state to a membrane bound form (34). Binding of CT to membranes depends on an α-helical lipid-binding domain and is initiated by an electrostatic absorption, followed by hydrophobic interactions with the core of the bilayer. The insertion of CT is favoured in membranes that mainly consist of lipids with small head groups, resulting in packing defects of the membrane (35). Enrichment of the membrane with hexagonal phase II-preferring lipids like PE and DAG results in loose packaging of lipids and therefore cause a low surface pressure of the membrane (36). This event, along with a disordering of acyl chains of

phospholipids caused by oxidation, creates a favourable curvature stress for the insertion of CT (37, 38). As stated above, DAG is an important factor determining PC synthesis as it can promote translocation to membranes (35), as well as by serving as substrate for the cholinephosphotransferase reaction in the last step of the CDP-choline pathway (see Fig. 3). Other lipids that stimulate translocation of CT to membranes are free fatty acids, PE and anionic phospholipids (35). On the other hand, increasing levels of PC in a membrane results in a dissociation of CT from the membrane. Therefore, PC itself is responsible for a negative feed-back system on CT activity (39). CT localizes mainly to the endoplasmic reticulum, cytosol and nucleus (28). CTα is predominately located in the nucleus with membrane-associated CT being at the nuclear envelope in cells in culture (28, 30). The *N*-terminal nuclear targeting sequence is responsible for the subcellular distribution of CTα to the nucleus (28). Activity of CT is mostly needed during early stages of cell cycle (G_0 to G_1 transition). At this stage of the cell cycle, rapid PC synthesis is occurring by shuttling CT out of the nucleus into the cytosol with subsequent association with membranes of the ER (28).

Besides translocation and membrane association, activity of CT is regulated by its phosphorylation status. Modulation of CT activity by phosphorylation depends on 16 serine residues located in the carboxyl terminal of the protein (residues 315-367). The kinases that phosphorylate CT *in vivo* have not been identified. Likewise, the phosphatases that dephosphorylate CT are still unknown. It has been described that the membrane-bound CT enzyme is less phosphorylated than the soluble CT, although dephosphorylation is not required for membrane association (40). Despite extensive mutagenesis studies on the phosphorylation domain of CT (27), a clear picture of the regulatory mechanism of phosphorylation status of CT is still lacking. It is generally thought that phosphorylation of CT has an inhibitory effect.

Changes in gene expression is the third way by which CT activity is regulated. In the promoter of CTα several activating and repressing regions are present. With the help of promoter-reporter constructs three motifs have been identified that bind the transcription factor Sp-1, resulting in an increase in expression of the CTα gene (41). Two related transcription factors, Sp-2 and Sp-3, are responsible for inhibition and activation of expression of the CTα gene, respectively (42). Transcription factors TEF-4 and Ets-1 were also found to regulate CTα gene expression (43, 44). TEF-4 has a dual regulatory function: when it binds to the promoter region, it results in suppression of CTα gene expression, but it functions as an activator when it binds to the basal transcriptional machinery (43).

Furthermore, TEF-4 enhances the activity of the transcription factor Ets-1 which also can activate transcription of CTα by itself (44). There is also a sterol response element present in the promoter of CTα, but the expression of the CTα gene is only modestly regulated by this element *in vivo* (45, 46).

III. CDP-choline:1,2-diacylglycerol cholinephospho-transferase

In the last step of the CDP-cholinepathway, phosphocholine is transferred from CDP-choline to DAG to form PC. The enzyme CDP-choline:1,2-diacylglycerol cholinephospho-transferase (CPT) mediates this process (47). CPT is an integral membrane protein that is mainly localized to the ER. Two isoforms of CPT have been identified and one isoform is specific for CDP-choline, whilst the other isoform can also convert CDP-ethanolamine and DAG to PE (48, 49). The dual specificity CPT is expressed in all tissues whilst the CDP-choline specific CPT has a more restricted tissue distribution and is highly expressed in testis and small intestine.

Most nucleated cells synthesize PC only via the CDP-choline pathway. However, in hepatic cells an alternative pathway for PC synthesis exists, consisting of three sequential methylation events of PE (50). Substantial amounts of PC from PE can be synthesized by these methylations that are catalysed by phosphatidylethanolamine-N-methyltransferase (PEMT). Two isoforms of PEMT, both originating from a single gene, have been identified and named PEMT-1 and PEMT-2 (51, 52). PEMT-1 is mainly found in the ER, whilst PEMT-2 is bound to mitochondria-associated membranes (MAM) (52). PE that functions as a substrate for these methylation reactions is generated by the CDP-ethanolamine pathway (53) or by the decarboxylation of PS (54). An explanation for the existence of this alternative pathway for PC synthesis in the liver is that this organ must synthesize a relatively large amount of PC for bile production even when choline supply is low (55). This can be deduced from studies in mice in which the PEMT gene was deleted (56). $PEMT^{-/-}$ mice, fed a choline-deficient diet, had extreme liver failure within three days compared to wild-type mice (56). During this choline starvation, The PC content in livers of $PEMT^{-/-}$ mice was depleted by 50% (56). This observation suggests that PC export into bile has the potential to deplete the liver of PC and that PEMT must provide sufficient PC for normal bile production during choline deprivation in the diet.

Catabolism of PC

The majority of newly synthesized PC from the CDP-Choline pathway is required for the assembly of biological membranes, especially during cell division. During this process a cell must double its phospholipid mass to form two daughter cells. The cell cycle is organized in four phases: G_1 phase, S phase, G_2 phase and M phase. During these phases phospholipid metabolism has a key role in the transition of one phase to the other. The G_1 phase is characterized by a high rate of membrane phospholipid turnover (32, 57, 58). There is a high increase of choline incorporation into PC, simultaneously observed with a high degradation of PC via hydrolysis by phospholipase C and D (59). This turnover of PC is thought to be an important aspect of phospholipid metabolism during G_1 phase that is necessary for entry into the S phase. During the S phase there is still an accelerated PC synthesis, but the rate of PC degradation is decreased by an order of magnitude (59). The cessation of PC degradation is an important contributor to the net accumulation of phospholipids, resulting in a doubling of total phospholipid mass specifically during the S phase (59, 60). This duplication of phospholipid content is not coupled to the duplication of DNA that also occurs during the S phase. Treatment of cells with dibutyryl-cAMP or aphidicolin prevented S phase DNA synthesis under these conditions, however, net phospholipid accumulation continued (59), indicating that this process is independent of DNA replication. During the G_2 and M phase there is little overall phospholipid synthesis or degradation occurring. One explanation could be that during these phases an attenuation of membrane trafficking occurs (61). Furthermore a maximum of CT phosphorylation, that results in an inhibition of the CDP-choline pathway, is observed during the G_2/M phase, correlating with the cessation of phospholipid synthesis (59). It is apparent that the bulk of PC for cell division is synthesized via the CDP-choline pathway during the G_1 and S phase of the cell cycle and that PC accumulates only during the S phase.

The majority of PC is used for membrane assembly, either as a PC molecule or as a precursor of other membrane phospholipid species like PS, PE and SM. PS is synthesized in mammalian cells by a calcium stimulated base-exchange of L-serine with pre-existing phospholipids. Both PC and PE can act as substrates for this reaction (62). Base-exchange is the main synthesis pathway of PS and performed by the enzyme PS synthase. There are two distinct PS synthases with different substrate specificity identified in mammalians (62). PS synthase-1 exchanges serine for the choline head-group of PC, whereas PS synthase-2 is responsible for the exchange with PE (62). PE can also be formed via calcium stimulated base-exchange of free ethanolamine with PC (63). The amount of PE made by base- exchange

under normal circumstances in eukaryotic is rather limited to a small percentage and most of the PE is synthesized *de novo* via the CDP-ethanolamine pathway or PS decarboxylation. SM, a phospholipid that lacks a glycerol backbone, also is commonly found in plasma membranes, especially in regions known as rafts. Instead of a glycerol backbone it contains sphingosine, an amino alcohol with a long unsaturated hydrocarbon chain. A fatty acyl side chain is linked to the amino group of sphingosine by an amide bond to form a ceramide (64, 65). The terminal hydroxyl group of sphingosine is esterified to phosphocholine to form SM. PC functions as the donor of the phosphocholine head-group in this reaction that is mediated the enzyme SM synthase (64, 65). Approximately 90% of SM synthesis takes place in the *cis* and *medial* Golgi apparatus (66, 67). Synthesis of SM from PC and ceramide links glycerolipid and sphingolipid signaling pathways, although it is not know if cells capitalize on this relationship for signaling purposes.

PC is described to be an important source for signaling molecules, like DAG, PA, LysoPC and arachidonic acid (10-13). Especially one group of enzymes, the phospholipases, is responsible for the generation of these signaling molecules from PC degradation. Most research has been performed on DAG. The generation of DAG in response to cellular changes is often biphasic. Hydrolysis of phosphatidylinositol(4,5) bisphosphate (PIP_2) to DAG and inositol 1,4,5,-trisphosphate (IP_3) results in a fast generation of DAG in the cell. This increase in DAG pool-size, along with a Ca^{2+} release from the ER, induced by IP_3, leads to the activation of protein kinase C (PKC) and downstream signal cascades (68, 69). Prolonged increases of DAG after stimulation are generated primarily from PC via PC-specific phospholipase C (PLC) and proceeds without an elevation of intracellular Ca^{2+} (3, 4, 70). This event might be related to the activation of Ca^{2+}-independent PKC isoforms (70). Beside PLC, activation of phospholipase D (PLD) and PA phosphohydrolase (PAP) result in the generation of DAG. The majority of the DAG species originating from the PLC pathway are polyunsaturated, whilst DAG species generated via PLD and PAP are saturated or monounsaturated and these species are not capable of PKC activation (71).

PA that is generated directly from PC by PLD has been proposed to have direct signaling roles, or it can be metabolized to lysophosphatidic acid by a PA specific lipase A_2. Lysophosphatidic acid can function as a signal molecule or mitogen. PA and lysophosphatidic acid can act extracellulary through a G protein-coupled receptor, eventually resulting in growth regulation (72). Both molecules can also act as intracellular regulatory molecules that stimulate a phosphatidic acid-activated protein kinase. PA is suggested to be a key regulatory molecule in phagocyte respiratory burst (73).

LysoPC is a key regulatory molecule in a variety of biological processes including cell proliferation, tumour invasiveness and inflammation (74, 75). LysoPC is produced by the action of phospholipase A_2 (PLA_2) on PC (76). LysoPC has been identified as a regulatory ligand for G2A, a lymphocyte-expressed G protein-coupled receptor (77). The lysoPC-G2A interaction is thought to be involved in inflammatory autoimmune disease and atherosclerosis.

PC and cell death

Signaling events involving activation of lipases for PC are implicated in a broad array of cellular processes, including cell proliferation and cell death. Activation of PC-specific PLC by 1,4-benzothiazine analogues is described to induce apoptosis (78) and in mature B-lymphocytes, PC-specific PLD may transduce negative immunomodulatory signals via PA (79). Furthermore, PC metabolism plays a crucial role in the cell division and stringent control mechanisms must be in place to keep the PC content in tune with the cell cycle. Disruption of PC synthesis would have a general effect on membrane structure and function, cell division and signaling pathways in the cell. Eventually, perturbations in PC homeostasis will disturb cell growth and leads to the induction of a mechanism, called programmed cell death (PCD).

PCD is the ability of cells to self-destruct by activation of an intrinsic cell suicide program when they are no longer needed or have become seriously damaged. The execution of this death program is often associated with characteristic morphological and biochemical changes within the dying cell. PCD can be divided into apoptosis, an event in which cellular membrane integrity is maintained and necrosis, whereby cell death is observed with rupture of membranes and release of cell content into its environment (Fig. 4). A Chinese hamster ovary (CHO) cell line MT58, which contains a thermo-sensitive inhibitory mutation in CT, has been established and was shown to have severely reduced levels of PC at the non-permissive temperature (80-82). This inactivation of CT and subsequent decrease in PC activates a PCD, which bears all characteristics of apoptosis (83). Furthermore, apoptotic processes were observed in cells deprived of choline (84) or cells treated with drugs that inhibited enzymatic steps of the CDP-choline pathway (85-88). From these studies it seems that apoptosis is the main cell death process in cells with disturbed PC homeostasis.

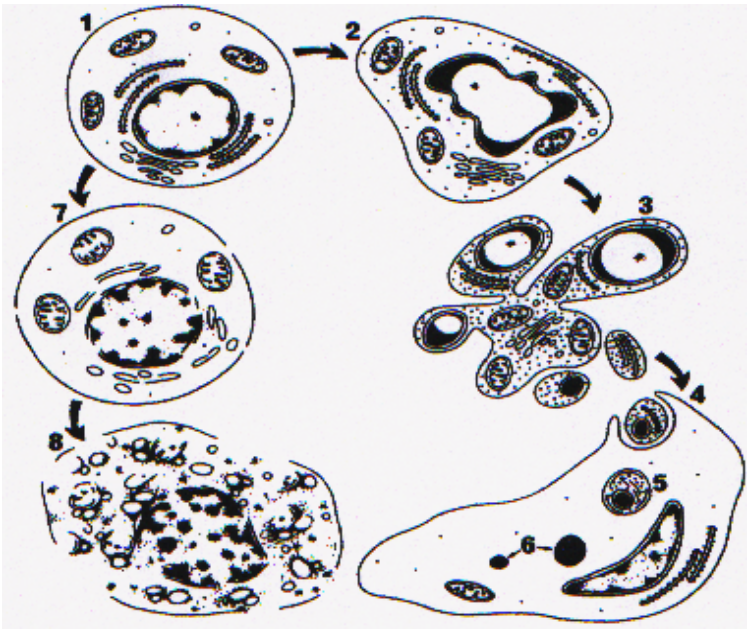

Figure 4. Sequence of ultra-structural changes in apoptosis (2-6) and necrosis (7 and 8).
(1) Normal cell, committed to die. (2) Early apoptosis is characterized by compaction and margination of nuclear chromatin, condensation of cytoplasm and convolution of nuclear and cellular outlines. (3) Nuclear fragments and protuberances that form on the cell surface separate to produce apoptotic bodies. (4) Apoptotic bodies are phagocytosed by nearby cells. (5 and 6) The apoptotic bodies are degraded within the lysosomes of the cell.
(7) The development of necrosis is associated with irregular clumping of chromatin, marked swelling of organelles and focal disruption of membranes. (8) Membranes subsequently disintegrate, but the cell usually retains its overall shape until removed by mononuclear phagocytes.

Apoptosis

Apoptosis is a fundamental elimination process that plays an important role in development, tissue homeostasis and immune regulation. The basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells at all times. The activation of this suicide program is regulated by many different signals that originate from the intra- and extracellular milieu. Apoptosis can be divided into four distinct stages. These include; (I) the decision of whether a given cell will die or adopt another fate; (II) the death of the cell; (III) engulfment of the cell by phagocytosis; and (IV) degradation of the engulfed body. Dysregulation of apoptosis may lead to deviations from normal development and may cause or contribute to a variety of diseases, including cancer, acquired immunodefiency syndrome (AIDS), neurodegenerative diseases and ischemic stroke (89, 90).

Apoptosis induces a sequence of ultra-structural changes in the cell in time (see Fig. 4; 1-6). The first morphologic changes that occur in the apoptotic cell are condensation of the cytoplasm and formation of granular mass that is localized to the nuclear membrane. This granular mass is caused by DNA fragmentation, compaction and segregation of nuclear chromatin. Progression of cellular condensation is accompanied by convolution of nuclear and cellular outlines, followed by breaking up of the nucleus into discrete fragments that are surrounded by a double-layered envelope. Membrane-bound apoptotic bodies are formed by budding of the dying cell. The size and composition of these bodies vary considerably: many contain several nuclear fragments, whereas others lack a nuclear component. The cytoplasmic organelles remain well preserved during these morphological changes and can be found unchanged in the apoptotic bodies. The apoptotic bodies arising in tissues are quickly ingested by nearby cells and degraded within their lysosomes or they are removed by engulfment of macrophages. This phagocytotic process occurs without inflammation, in contrast to necrosis.

Biochemical mechanisms involved in apoptosis

The ultra-structural changes that occur during apoptosis are regulated by well-defined biochemical mechanisms. These mechanisms are activated by executioners of apoptotic signal pathways, namely members of the caspase family (91). Caspases belong to a highly conserved family of cysteine proteases with specificity for aspartic acid residues in their substrates. Caspases are responsible for the cleavage of certain key substrates that orchestrate the death and packaging of the cell for clearance. Genomic DNA fragmentation starts with cleavage of nuclear DNA into 300- or 50- kilo base pair fragments, followed by double strand cleavage of the nuclear DNA in linker regions between nucleosomes. Eventually, all DNA fragments formed are multiples of units comprising 180-200 base pairs, which give a characteristic ladder on ethidium bromide-stained gels. This nucleosomal ladder is almost always observed in apoptosis and is widely accepted as a biochemical hallmark of apoptotic death (92). Enzymes responsible for inter-nucleosomal cleavage are still a subject of considerable debate. DNA cleavage at an early stage seems to serve a protective function in preventing the transfer of potentially active and possible dangerous genetic material to nearby cells when apoptotic bodies are phagocytosed (93).

Together with nuclear DNA degradation, cytoplasmic condensation takes place, which is accompanied by an increase in cellular density (94). Just before this event a raise in β-tubulin messenger RNA has been noticed and at a later stage increased amounts of β-tubulin

appear in the cytoplasm (95). β-tubulin is a cytoskeletal protein and a major participation of cytoskeletal elements in cytoplasmic condensation has been suggested, although the mechanisms of this event are still unclear. Separation of discrete apoptotic bodies from the condensing cell is accompanied by an increase in transglutaminase, which is preceded by an increase in the level of its messenger RNA (96). This enzyme is involved in cross-linking of intracellular proteins and highest concentrations of this enzyme are consistently present in discrete apoptotic bodies (96). It is proposed that transglutaminase activity leads to a formation of a highly cross-linked rigid framework within apoptotic bodies, which maintains the integrity and prevents leakage of the content of the apoptotic body into the extra-cellular space.

The rapid phagocytosis of apoptotic bodies without inflammation is necessary to prevent injury to healthy cells in the surrounding tissue. The swiftness of phagocytosis by nearby cells suggests a highly specific recognition mechanism. Lectin-like receptors of surrounding cells recognize changes in carbohydrates exposed on the surface of the apoptotic body (97). Exposure of PS on the apoptotic body surface is also thought to be involved in recognition by macrophages (98). It seems that multiple and perhaps overlapping mechanisms are operating together to ensure the immediate recognition by adjacent cells.

The observation of raised levels of mRNA for proteins, like transglutaminase and β-tubulin, may suggest that newly synthesized proteins are required for execution of apoptotic process. However, it was shown that inhibitors of RNA or protein synthesis fail to block apoptosis or may even induce it in many situations. Furthermore, apoptosis can be induced in cells whose nuclei have been removed (99). These observations suggest that the apoptotic effector molecules are always present and are constitutively expressed in most mammalian cells (89). If these apoptotic effector proteins are present in living cells, their potentially lethal activities must be suppressed in cells that normally survive and close regulation of this system is needed. RNA and protein synthesis is more necessary for assembly of control molecules that activate or depress the existing cell death machinery, rather than the making any components for the basic cell death program itself. The apoptotic program is controlled by many different, distinct signal pathways that ultimately converge to activate the death of a cell.

Functions of apoptosis

Besides the clearance of damaged and potentially dangerous cells, apoptosis plays an essential role in the normal development of vertebrates and control of cell numbers. Apoptosis serves as a prominent force in sculpting the developing organism. In the frog it is responsible for the regression of the tadpole tail that takes place during metamorphosis (100). In mammalian embryos apoptosis is responsible for the removal of inter-digital webs during limb development and regression of the Müllerian duct by males (101). Furthermore, it is involved in the closure of the neural tube (102) and is important in other processes involving cell movements. In neural tube closure, cells programmed to die, may help in rolling up and coming together of the neural folds to form the neural tube. In adult mammals apoptosis is linked with cell proliferation and therefore regulates cell numbers. There is growing evidence that apoptosis is regulated in a reciprocal fashion to mitosis by growth factors and trophic hormones and most cells in higher animals may require continuous trophic stimulation to survive. Furthermore, potent inducers of cell proliferation, like the proto-oncogene c-Myc and the adenovirus oncoprotein E1A, possess pro-apoptotic activity. It seems that proliferative pathways are coupled with apoptotic pathways. The balance between the two processes is regulated by competition of trophic factors that stimulate mitosis and inhibit apoptosis, leading to an increase in cell numbers or a stabilisation of the cell population to its former level. A number of involutional processes occurring in normal adult mammals have been shown to be associated with marked enhancement of apoptosis. Examples are ovarian follicular atresia, reversion of lactating breast to its resting stage after weaning, and catagen involution of hair follicles.

The main task of apoptosis is homeostasis of cells of metazoan animals, acting as a defense mechanism against unwanted, damaged or potentially dangerous cells, such as self-reactive lymphocytes or cells infected by viruses and tumour cells. Survival of these cells might be harmful to the organism and could disturb homeostasis of the organism. The homeostasis of tissues is maintained by a balance between survival signals that inhibit cell death and signals that activate PCD. A special emphasis in homeostasis by apoptosis is made for malignant tumours. Apoptosis can be found in virtually all untreated malignant tumours, with in some cases apoptotic activity approaching levels that have been observed for rapidly involuting tissues. The factors responsible for the spontaneous occurrence of apoptosis in tumours are diverse. Apoptosis often is present near foci of confluent necrosis where mild ischemia is likely to be involved in its initiation. Apoptosis in tumours can be induced by

extracellular factors like tumour necrosis factor α (TNFα) (89) or in other instances by a result of an attack on the tumour by cytotoxic T-lymphocytes. It is thought that apoptosis in malignant tumours is already initiated during an early stage of the process of carcinogenesis. Increased apoptosis in this early stage temporarily balances increased cell proliferation of the tumour. In surviving invasive tumours the rate of cell proliferation overtakes apoptosis.

PC and apoptosis

As mentioned earlier proliferation of cells depends on *de novo* synthesis of PC. In a number of tumour cells higher levels of phosphocholine were detected (103). Furthermore, transformation of cells with the *ras* oncogene results in a constitutive expression of CK (104). Several drugs that are able to inhibit the CDP-choline pathway are shown to possess a potent anti-neoplastic activity. For instance, alkyl-lyso-phospholipid analogs (ALPs) can inhibit CT and were tested with encouraging results in clinical settings as a purging agent in the treatment of skin metastases of breast cancer (105). Therefore, drugs that inhibit the CDP-choline pathway could provide a possible therapy against cancer. At this moment several inhibitors of the CDP-choline pathway have been found (85-88). All of these inhibitors eventually induce apoptosis. It is, however, a concern whether these inhibitors are specific for the enzymes of the intended pathways. In the context of PC, this concern could be addressed by determining whether cells can be rescued from apoptosis by exogenous PC or lysoPC. For each of the three steps of the CDP-choline pathway pharmacological inhibitors are described.

The most potent inhibitors of CK, the first enzyme in the CDP-choline pathway, are hemicholium-3 (HC-3) derivates (106). Inhibition of CK by HC-3 results in growth arrest and apoptosis. However, no depletion of PC has been observed in cells treated with this agent (106). CK is not the rate-limiting enzyme in PC synthesis and therefore a partial inhibition of CK may still provide sufficient activity for rest of the pathway. Another explanation could be the presence of mutiple isoforms of CK with different sensitivities to CK inhibitors (107). However, HC-3 has anti-neoplastic activity *in vivo* against human tumours transplanted in mice (106) and treatment of cancer cells in culture with CK inhibitors results in growth arrest and apoptosis (108).

A number of alkyl-lysophospholipid analogs (ALPs) are shown to inhibit CT and also induce apoptosis (85, 86, 103). These anticancer lipids include 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3) and hexadecylphosphocholine (HePC), which are both used as anti-cancer agents (103). HePC inhibits CT activity via reduction of CT

translocation to membranes (85). The choline head group of HePC is important in interference with translocation since hexadecylphospoethanolamine (HePE) or hexadecylphosphoserine (HePS) were not active as inhibitors (109). The cytotoxic effect of HePC and ET-18-OCH_3 can be attenuated by lysoPC. LysoPC even restores normal proliferation in HL60 cells that were treated with these inhibitors (85). Furthermore, overexpression of CT prevents apoptosis, but fails to restore proliferation of ET-18-OCH_3 treated Hela cells (110). In addition to their inhibition of CT, these alkyl-lysophospholipids affect other processes in cells, such as activation of the SAPK/JNK pathway (111), stimulation of FAS clustering (112), inhibition of MAPK/ERK (113), inhibition of PLC (114), PKC activation (115) and ceramide formation (116).

Another class of lipid inhibitors of CT are short chain ceramides, like C2-ceramide. They have potent inhibitory effects on CT in baby hamster kidney (BHK) cells (117). This inhibition of CT is suggested to lead eventually to apoptosis in BHK cells (117). But in other cell lines, like rat-2 fibroblast, short chain ceramides inhibit CPT instead of CT (118). This observation is consistent with the finding that natural occurring ceramides do not inhibit CT *in vitro* (118). Furthermore, C2-ceramide also inhibits the synthesis of DNA and proteins (119), so the specificity of these synthetically made short-chain ceramides is a concern. Further research on the role of short chain ceramides is necessary to elucidate their role in PC synthesis inhibition.

The last step of the PC synthesis can be inhibited with the drugs farnesol, geranylgeranol and chelerythrine, also resulting in apoptosis (87, 88, 120). Farnesol and geranylgeranol are known to compete with DAG for binding to CPT (120). Although the specificity of these inhibitors is a concern, addition of exogenous PC or lysoPC does rescue the cells after treatment with these chemicals. However, expression of recombinant human CPT fails to rescue the cells from farnesol-induced apoptosis and farnesol also fails to inhibit recombinant CPT in a mixed micelle assay (121). It seems that the inhibition of CPT by farnesol in intact cells or isolated microsomes may be indirect. As mentioned earlier, synthetic short chain ceramides are capable of inhibition of CPT in rat-2 fibroblast (118). But, like the inhibition of CT by short chain ceramides, the inhibitory mechanisms on CPT are not well understood.

In conclusion, inhibitors of all three steps of the CDP-choline pathway can also induce apoptosis. More important, in MT58, a mutant Chinese hamster ovarian cell line with a thermo-sensitive mutation in CT, inhibition of PC synthesis at the non-permissive temperature results in apoptosis. So far, little is known about which specific pathway is involved in

induction of apoptosis by PC pertubation. Caspases seems to be involved, because caspase inhibitors were able to block apoptosis induced by choline deficiency (84). The pathway up-stream of caspases triggered by PC depletion, however, is not known. A likely candidate could be the ER stress response, since most of enzymes of PC synthesis, CT and CPT, are located to ER membranes. Furthermore, the ER has been identified to mediate several perturbations in the cell, like glucose starvation, CA^{2+} overload and accumulation of unfolded or misfolded proteins. When faced with one of these stresses, the ER induces growth arrest to restore homeostasis or apoptosis by prolonged and severe stress.

The ER stress response and apoptosis

The ER serves several important functions, including folding and assembly of newly-synthesized transmembrane and secretory proteins, and their post-translational modification. The ER also functions as a cellular calcium store. Various conditions can disturb ER functions and are collectively termed “ER stress”. ER stress events include accumulation of misfolded or unfolded proteins, caused by inhibition of protein glycosylation or a reduction in disulfide bond formation, impairment of protein transport from the ER to the Golgi, calcium depletion or overload of the ER lumen and glucose starvation.

To survive and adapt under ER stress conditions, cells have a self-protective mechanism against ER stress (122, 123). The classical ER stress response was first described for accumulation of unfolded or misfolded proteins in the organelle. Therefore the ER stress response is also known as the unfolded protein response. The ER stress response consists of four functionally different phases (Fig. 5). The first phase involves the up-regulation genes encoding ER chaperone proteins to increase protein folding activity and to prevent protein aggregation. In the second phase the general protein translation machinery is attenuated to reduce the load newly synthesized proteins and thereby preventing further accumulation of unfolded proteins. The third phase is characterized by degradation of misfolded proteins in the ER and is known as ER-associated degradation (ERAD). The first three steps are orientated on repair and restoration of cellular homeostasis. In the fourth and final step apoptotic pathways are induced. This phase occurs when the functions of the ER are extensively impaired.

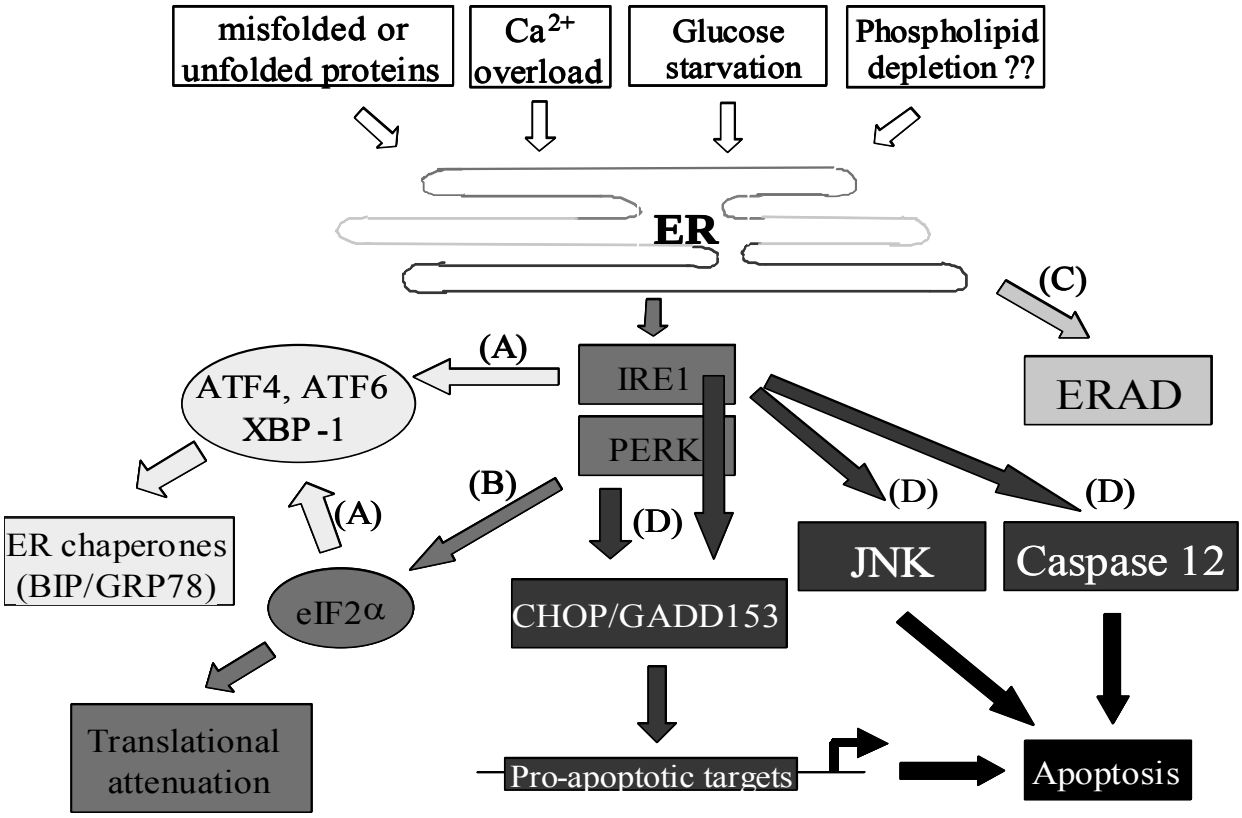

Figure 5. Mechanism of the ER stress response pathway. Accumulation of stress in the ER, like unfolded proteins, Ca^{2+} overload or glucose deprivation, activates ER stress transducer proteins such as IRE1, ATF6 and PERK, resulting in the induction of the ER stress response. IRE1 and PERK are triggered by dissociation of Bip from their ER lumenal domains. ATF6 is activated by a two-step cleavage by Site-1 protease (S1P) and Site-2 protease (S2P). The ER stress response can be divided in four independent steps.

(A) During the early stage of ER stress, transcriptional induction of ER chaperones like Bip/GRP78 occurs to prevent protein aggregation. Transcription factors that bind and activate induction of target chaperones are XBP-1, ATF6 and ATF4. XBP-1 is generated by IRE1-dependent XBP-1 mRNA splicing, p50ATF6 is generated by proteolysis of p90ATF6, and ATF4 is up-regulated by eIF2α phosphorylation by PERK. (B) Translational attenuation in ER stress prevents further accumulation of unfolded proteins. This process is mediated through phosphorylation of eIF2α and thereby blocking translation initiation. The phosphoryaltion of eIF2α is mediated by the ER-resident kinase PERK (C) The ER-associated degradation (ERAD) pathway eliminates misfolded proteins by the ubiquitin-proteasome system. (D) If the functions of the ER are severely impaired during prolonged stress, apoptosis is induced by transcriptional activation of CHOP, activation of the JNK pathway and activation of caspase-12. IRE1, ATF6 and ATF4 are involved in the induction of CHOP. IRE1 is responsible for the activation of the JNK kinase and caspase-12.

Transcriptional induction of ER chaperones in ER stress

Proper folding of proteins in the ER depends on ER chaperone proteins BIP/GRP78, GRP94 and on proteins such as protein disulfide isomerase (PDI) and peptidyl-prolyl isomerase. During ER stress transcriptional activation of these proteins occurs. ER stress results in the activation of an intracellular signaling pathway from the ER to the nucleus. Proteins induced by the ER stress response share a consensus sequence in their promoters, the *cis*-acting ER stress response element (ERSE).

The key players in this intracellular signaling pathway are the transcription factors ATF6 (124) and XBP-1 (125-127) (see Fig. 5). ATF6 is synthesized as a type II transmembrane protein (p90ATF6) and is localized in the ER and activated by ER stress-induced proteolysis (128). Proteolysis of p90ATF6 takes place in the Golgi after translocation of the enzyme to this organelle, a process that is induced by ER stress (129, 130). ATF6 is processed by two enzymes, Site-1-protease (S1P) and Site-2-protease (S2P). The N-terminal cytosolic domain of p90ATF6 is cleaved off and this peptide is a functional transcription factor. It contains a ZIP domain and after translocation to the nucleus it can heterodimerize with transcription factor NF-Y. The ATF6/NF-Y dimer binds to the ERSE of target proteins, resulting in a upregulation of these ER stress proteins.

XBP-1 is a transcription factor of the bZIP protein family. Activation of XBP-1 depends on its splicing by IRE1α. IRE1α on his turn is activated by its dissociation from Bip resulting in oligomerization of IRE1α and subsequent autophosphorylation (131). This activated IRE1α complex is capable of splicing XBP-1 mRNA. Splicing of XBP-1 mRNA results in a frame-shift causing the formation of an active form with the original N-terminal DNA binding domain and a new C-terminal transactivation domain. The spliced form of XBP-1 has a higher transcriptional activity resulting in the upregulation of ER stress proteins. XBP-1 itself is induced during ER stress, by binding of ATF6 to the XBP-1 promoter. ATF6 mRNA, in contrast, is not induced during ER stress. Therefore, it is suggested that the early phase of ER stress only results in the activation of ATF6, and that during prolonged ER stress both ATF6 and XBP-1 are activated (125).

Translational attenuation during ER stress

The presence of misfolded or unfolded proteins result in activation of a specific ER stress pathway causing attenuation of mRNA translation. Purpose of this translation attenuation is the prevention of further accumulation of unfolded proteins in the ER. Essential in this process is the phoshorylation of eIF2α.

The factor eIF2 plays a vital role in translational initiation, because it mediates the binding of the initiator Met-tRNA to the ribosome. Activity of eIF2 is regulated by phosphorylation at residue Ser51 of its α subunit, which results in an inhibition of the factor. PERK has been identified as the kinase responsible of the phosphorylation of eIF2α during ER stress (132, 133). PERK is a type I transmembrane serine/threonine kinase localized in the ER. Like IRE1α, activation of PERK is triggered by the dissociation of Bip from its ER lumenal domain, also resulting in autophosphorylation of the intracellular regions (131). Termination of PERK signaling depends on reformation of the PERK-Bip complex and on dephosphorylation of eIF2α by GADD34 (132). GADD 34 transcription is induced during ER stress and forms a complex with protein phosphatase 1 (PP1c), which specially is responsible for the dephosphorylation of eIF2α. Therefore, GADD34 is suggested to provide a negative feedback loop that promotes recovery from translational inhibition.

ER-associated degradation (ERAD) during ER stress

Misfolded proteins which can not be restored to their proper conformation have to be removed from the ER to the cytosol and subsequently degradated. This process is known as ER associated degradation (ERAD).

In the first phase of ERAD misfolded proteins have to be recognized. Some ideas exists about the recognition of misfolded glycoproteins, but virtually nothing is known about the ERAD signal of non-glycoproteins. The second phase is the capture of the misfolded proteins in the ER. Calnexin (CNX) and calreticulin (CRT), ER resident lectin-like chaperones are observed to bind to misfolded proteins and mediate their ER retention in order to prevent premature degradation (134). Furthermore, Bip and J-domain containing heat-shock proteins 40 (HSP40), like Jem1p and Scj1p are also responsible to maintain ERAD substrates in a retrotranslocation-competent state (135). Translocation of the ERAD substrates to the cytosol is the last phase. Transport of the misfolded proteins out of the ER is mediated by a protein complex consisting of central components of the Sec61p channel. After transport,

ERAD substrates are targeted for degradation by the 26S proteasome by ubiquitin-conjugating enzymes. This group of proteins include Ubc6p (136), Ubc7p (137, 138) and ER membrane-bound ubiquitin ligases such as Der1p (139), Der3p/Hrd1p (140, 141) and Hrd3p (140). The genes for this degradation phase have been identified in yeast (142), but their some of their human homologues are still unknown.

Induction of apoptosis during ER stress

In the first phases of ER stress the cell tries to restore homeostasis by activating repair mechanisms, chaperones, and prevention of accumulating stress. This is often observed with the simultaneously induction of growth arrest. However, when the ER is faced with prolonged and severe ER stress, it will result in cell death via apoptosis.

The first step in apoptosis induction is transcriptional induction of the gene for C/EBP homologues protein (CHOP), also known as GADD153, which encodes a bZip transcription factor (143-145). CHOP is either not expressed or at low levels under normal physiological circumstances, but it is strongly induced in response to ER stress (146). Overexpression of CHOP has been shown to induce growth arrest and apoptosis (143) and CHOP knock-out mice are not capable of inducing apoptosis upon ER stress (147). CHOP/GADD153 expression is induced by both the IRE1 pathway (148) and the ATF6 pathway (149). Furthermore, CHOP can also be induced via the PERK pathway through translational induction of a transcription factor ATF4 (150). CHOP expression can be followed by posttranslational events, such as phosphorylation at Ser 78 and Ser 81 by p38 MAPK (151-153). CHOP is a nuclear protein that can dimerize with C/EBP transcription factors (151, 153, 154). These stable heterodimers are capable of recognizing novel DNA targets (154-156) and CHOP may mediate the induction of ER stress inducible apoptotic genes. One group of target genes of CHOP are referred to as DOC (for downstream of CHOP) (157). One of the DOCs identified is a stress-induced form of carbonic anhydrase VI, which is suggested to promote apoptosis by increasing proton concentrations and decreasing intracellular pH (158). This event can facilitate the pore forming activity of proapoptotic regulator Bax, which has a relatively lower pH optimum (159). CHOP has been also reported to downregulate Bcl-2 protein, a survival factor, and to increase production of reactive oxygen species (ROS) (160).

The second apoptotic pathway involved in ER stress is the cJun NH_2-terminal kinase (JNK) pathway. JNK belongs to the family of mitogen-activated protein kinases (MAPKs). JNK phosphorylates specific serines and threonines of target protein substrates and thereby

regulate cellular activities, including gene expression and apoptotic processes. ER stress activates JNK through activation of IRE1α or IRE1β (161). The cytosolic kinase domains of IRE1s bind TRAF2, an adaptor protein that couples plasma membrane receptors IRE1α or IRE1β to JNK, resulting in its phosphorylation and thus activation (161) Furthermore, sustained activation of the JNK or p38 MAPK pathway during ER stress requires activation of Apoptosis signal-regulating kinase (ASK1) (162). The physiological significance of JNK activity during ER stress is unknown, but studies on the role of ASK1 in ER stress may shed light on the role of JNK in ER stress.

The final step of execution of cell death in ER stress is via increased expression and activation of caspase-12, an ER-situated caspase (163). Caspase-12 is localized on the cytosolic site of the ER membrane and prolonged ER stress results in the cleavage of the prodomain of caspase-12 from the ER membrane by caspase-7 (164). Prolonged ER stress results in a movement of active caspase-12 to the cytosol where it interacts with caspase-9 (165). Caspase-9 cleaves the prodomain of capase-12, thereby forming an active caspase12 of 35 kD, possessing protease activity.

Oxidative stress and JNK

Although the ER stress response is the most likely candidate for mediating stress induced by the inhibition of PC synthesis, other stress-induced pathways exist in the cell that function independently of the ER. For instance, mitochondria are described to play an essential role in the activation of apoptosis of cells exposed to ultraviolet light or deprived of essential growth factors. These insults evoke a change in mitochondrial permeability and consequently results in leakage or rupture of the mitochondria, thereby releasing cytochrome C from this organelle. Cytochrome C can bind to Apaf-1 and pro-caspase 9, forming a complex known as the apoptosome (166). The apoptosome activates downstream effector caspases and is thought to account for most caspase-depended apoptosis. Furthermore, mitochondria also mediate stress responses that are a result of the accumulation of reactive oxygen species (ROS) in the cell. High concentrations of ROS in the cell are cytotoxic and can induce apoptosis. This event is known as oxidative stress.

Oxidative stress plays a causative role in a variety of human diseases, and is believed to contribute to the degenerative changes that occur with aging (167, 168). Normally the cell is capable of detoxification of minor concentrations of ROS, such as molecular oxygen, (O_2),

superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-), often produced in the course of aerobic metabolism in the mitochondria. Only when the generation of ROS and other oxidizing species exceeds the cellular capacity for detoxification, like during mitochondrial permeability transition, it results in oxidative stress and it ensues damage to DNA, proteins and lipids. The mechanisms controlling the cellular response to oxidative stress have been extensively investigated at the molecular level in bacterial systems, resulting in the identification of regulatory proteins known as the OxyR and Sox/S regulons (169). Based on this bacterial work identical pathways have been identified in mammals and the signaling pathway of oxidative stress is becoming unraveled.

The sensing mechanism of oxidative stress seems to depend on ASK1. ASK1 is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family and is an upstream activator of p38 MAPK or JNK through MAP kinase kinases 3/6 and MAP kinase kinases 4/7, respectively (170) (see Fig. 6).

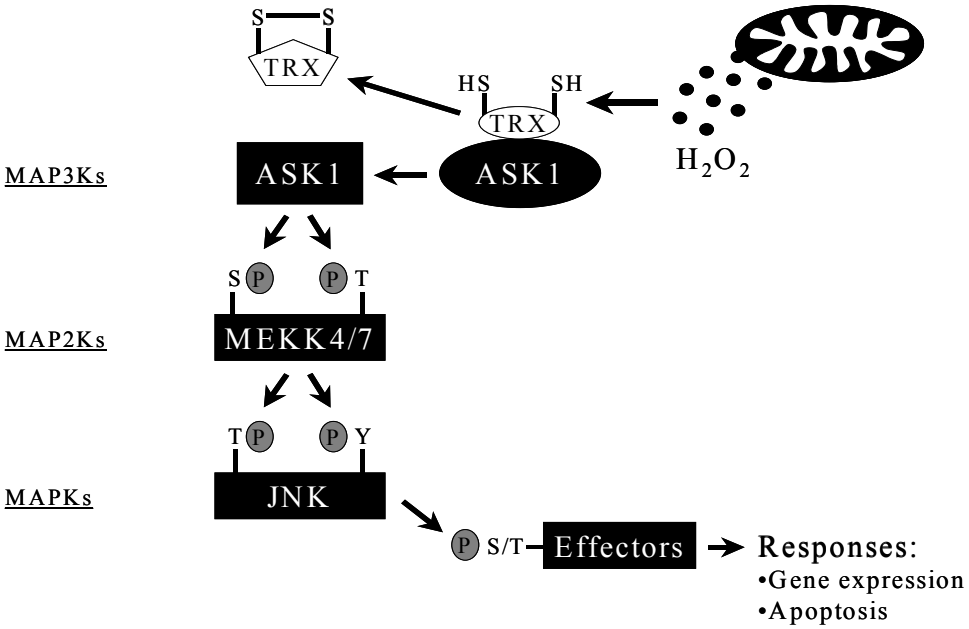

Figure 6. Activation of a JNK MAP kinase module during oxidative stress. Under normal circumstances ASK1 is associated with TRX (or GRX) via two mixed disulfide bonds that alter the conformation of both proteins. Upon oxidative stress (H_2O_2), reactive oxygen species cause the oxidation-mediated dissociated of TRX from ASK1, resulting in an conformational change of ASK1 to an active kinase. Consequently JNK is activated by sequential protein phosphorylation through the MAP kinase module, in which MAP3Ks like ASK1 phosphorylates and activates dual specificity MAP2Ks such as MKK4 and MKK7 by phosphorylation of serine (S) and threonine (T) residues. MKK4 and MKK7 phosphorylate JNK on both threonine and tyrosine (Y), thereby stimulating JNK activity. Activated JNK phosphorylates downstream effectors on serine or threonine residues, resulting in changes in patterns of gene expression and other cellular responses like apoptosis.

Beside oxidative stress, ASK1 can be activated by cytokine exposure or binding of tumour necrosis factor-α (TNF-α) to its receptor (171). Furthermore ASK1 plays a role in the activation of JNK during ER stress as mentioned earlier. Under normal conditions ASK1 is bound to small redox-sensitive proteins, thioredoxin (TRX) (172) or glutaredoxin (GRX) (173). Binding to these proteins is possible when they are in their reduced state and this inhibits ASK1. TRX, for example, binds to the amino terminus of ASK1 by forming an inter-molecular disulfide bridge between Cys32 and Cys35 in the catalytic site of TRX with putative reactive groups in ASK1 (172). The binding between TRX and ASK1 alters the conformation of both proteins, making the catalytic domain of ASK1 inaccessible for substrates. Oxidation of TRX by ROS results in the formation of an intra-molecular disulfide bridge between Cys32 and Cys35 of TRX, forcing its dissociation from ASK1. Besides making ASK1 unavailable for substrates, binding of TRX targets ASK1 for ubiquitination, thereby reducing ASK1 levels in the cell. A similar model has been proposed for the interaction of GRX with ASK, which is also depending on disulfide bridge formation. Oxidative stress induces dissociation of the redox proteins from ASK1, allowing ASK1 to phosphorylate JNK/stress-activated protein kinase-associated protein 1 (JSAP1) (174). This protein is a scaffold protein and phosphorylation triggers the conformational change of JSAP1 to an active configuration (174). JSAP1 induces the formation of a signaling complex composed of JSAP1, ASK1, MKK4/MKK7 and JNK. This complex is responsible of the subsequent activation of JNK. It is thought that p38 MAPK is activated by ASK1 in a similar way, although the interacting scaffold proteins are not yet identified. Once activated, JNK and p38 MAPK enhance expression and/or induce DNA binding of many transcription factors, such as Nrf2, c-myc, c-fos, c-jun, ATF2, CHOP/GADD153 and NFκB, leading to changes in gene expression profiles and a molecular response to cellular stress (175, 176). Several different genes are up-regulated as a result of ASK1 activation: Phase II detoxifying enzymes, such as glutathione S-transferase (GST), heme oxygenase 1 (HO-1) and quinone reductase (QR) (177-179) and pro-apoptotic proteins like CHOP/ GADD153 (180) and Fas ligand (Fas-L) (181).

Central in the activation of the oxidative stress pathway are the stress activated protein kinases JNK and p38 MAPK. As earlier mentioned JNK is also identified to mediate stress events during the ER stress response. The JNK signaling pathway is involved in the regulation of many cellular events, including gene expression, mitosis, metabolism, cellular stress and cell death. Beside ER stress and oxidative stress, JNK is also stimulated in response to inflammatory cytokines of the tumour necrosis factor (TNF) family (TNFα/β, interleukin-1,

CD-40, Fas ligand), by vasoactive peptides (endothelin and angiotensin II) and by a variety of stresses like heat shock, DNA damage, reperfusion injury and protein synthesis inhibition (182-184). Malfunction of JNK signaling is implicated to be responsible for deformation during early embryonic development (185), oncogenic transformation (186) and immune deficiency (187). Three genes (jnk1, jnk2 and jnk3) have been identified, encoding three JNK protein kinases. JNK1 and JNK2 protein kinases are expressed ubiquitously, while JNK3 is expressed primarily in the brain and with extremely low expression in the kidney and testis (188). Alternative splicing of the genes yields four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms (189). To date, no functional differences have been ascribed to these isoforms, but JNK2 is described to have a lower substrate binding compared to JNK1 and JNK3 (189). There is a strong homology between the isoforms and the primary difference between the jnk3 gene and jnk1 and jnk2 genes is that the protein product for JNK3 has an extra 39 amino acids in its N-terminus, but a functional role for this N-terminus difference has not yet been ascribed.

All three JNK kinases are capable of binding and phosphorylation of many substrates like transcription factors, c-jun (190-192), ATF2 (193), Elk-1, (194), NFAT (195), as well as tumour suppressor protein p53 (196, 197) and a cell death domain protein, MADD (197). In addition, the neurofilament heavy subunit (NFH) is identified as a target substrate of JNK, implicating that JNK is involved in neurite outgrowth or regeneration (198, 199). Activation of transcription factors, such as ATF2 and c-jun regulates gene expression in response to cytokines, growth factors and cellular stress stimuli. ATF-2 and c-jun, along with other transcription factors comprise the AP-1 transcriptional activator complex. It is thought that induction of apoptotic genes is primarily mediated via the AP-1 activator complex because inhibition of JNK3 phosphorylation of c-jun and subsequently AP-1 transcriptional activity results in an inhibition of neuronal apoptosis (200).

Regulation of the JNK pathway is extremely complex and is influenced by many MAP3Ks. So far, 13 different MAP3Ks have been identified that regulate JNK. It is this diversity of MAP3Ks that allows a wide range of stimuli to activate the JNK pathway. The most import MAP3ks in JNK signaling are members of the MEKK family, ASK1, MLK, TAK1 and TPL-2 (170, 201-203). JNK is activated by sequential protein phosphorylation through a MAP kinase module, i.e., MAP3K → MAP2K → MAPK (see Fig. 6). Two MAP2Ks (JNKK1/MKK4/SEK1 and JNKK2/MKK7) for JNK have been identified (170) These kinases are believed to be dual specificity kinases in that they are capable of phosphorylating JNK on both Threonine and Tyrosine (204, 205). Activation of the MAP

kinase module seems to depend on scaffold proteins such as JIP, β-arrestin and JSAP1, as mentioned earlier (174). Termination of JNK activation can be achieved by protein phosphatases like mitogen-activated protein phosphatases (175).

So how can we place the stress of PC depletion in context with oxidative stress and JNK-mediated apoptosis? Inhibition of PC synthesis might result in a disturbed composition of mitochondrial membranes, evoking a change in mitochondrial permeability. This event could induce oxidative stress and consequent activation of JNK. Alternatively, PC synthesis inhibition might (also) lead to an accumulation of ceramides, which are normally used for synthesis of SM using PC as donor of the phosphocholine head-group. MLKs, a family member of MAP3Ks, are activeated by an increase of ceramides in the cell and consequently activate JNK.

Outline of this thesis

PC is the most abundant phospholipid in cellular membranes of mammalian tissues. In addition to its structural role in membranes and lipoproteins, PC functions as a major source of intracellular signaling molecules. All eukaryotic cell types and tissues display unique and stable profiles of PC. Perturbation of PC homeostasis by pharmacological agents or by genetic engineering, leads to cell death via a process called apoptosis. Although several studies describe a relation between inhibition of PC synthesis and apoptosis, the underlying signaling pathways that mediate this cell death have not been identified. Therefore, the aim of this study was to identify signaling pathways that mediate apoptosis during the inhibition of PC. With the help of a genetic model, a temperature sensitive mutant, MT58 we investigated the effect of PC depletion on several potential stress pathways. Furthermore, we tried to elucidate which cellular event might be the trigger for the induction of apoptotic processes during prolonged inhibition of PC synthesis.

First we analysed the effect of prolonged inhibition of PC synthesis (24 h) on PC breakdown, the composition of PC species in cellular membranes and the consequence of these changes on cellular structures that require PC as structural component of their membrane (**chapter 2**). Despite the fact that PC synthesis is severely decreased after 24h at 40 °C, PC breakdown is even increased in MT58 cells. This results in a decrease of PC and especially in PC species, which are mono-saturated at the sn-1 position. Overall, cellular membranes in MT58 cells have the tendency to maintain a more saturated composition during incubation at the non-permissive temperature. The morphology of the cell also changes drastically as a result of these metabolic changes. PC depletion results in a dramatic disturbance of ER and Golgi structures and an accumulation of lipid droplets in the cell. Because of the dramatic impact of PC depletion on ER and Golgi structures, the role of the ER stress response in the apoptotic process of PC depleted cells was investigated (**chapter 3).** Inhibition of PC synthesis leads to the induction of the ER stress-related, pro-apoptotic transcription factor CHOP/GADD153, but does not influence canonical ER stress events, like induction of the ER chaperone Bip, inhibition of protein translation or caspase 12 activation. The putative regulatory element in the CHOP promoter that contributes to PC depletion induced expression was identified (**chapter 4)**. CHOP expression is mediated via activation of a conserved region, the C/EBP-ATF site. Activation of this site might depend on the binding of transcription factor ATF2. Phosphorylation of ATF2 is essential for binding to the

C/EBP site, but during PC depletion ATF2 phosphorylation is not mediated by JNK kinase. Chemical agents that inhibit PC synthesis are often used to investigate the role of PC depletion in apoptosis. Furthermore they are used as neoplastic agents during anti-cancer therapy. We compared the effects of a familiar used pharmacological drug of PC synthesis inhibition, HePC with the results of our genetic model (**chapter 5)**. Although both systems lead to an inhibition of PC synthesis and the induction of apoptosis, fundamental differences between them were observed. Treatment of HePC did not result in depletion of PC, apoptotic processes were not preceded by an induction of CHOP and HePC treated cells can be rescued by both LysoPC and LysoPE. Therefore HePC treated cells induce apoptosis probably via other pathways than the inhibition of PC. This thesis concludes with a summarizing discussion (**chapter 6)** in which an overview of the major findings is presented and placed in a broader perspective.

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CHAPTER 2

Inhibition of phosphatidylcholine synthesis results in specific changes in cellular morphology and lipid composition and in the generation of lipid droplets

Michiel H.M. van der Sanden, Onno B. Bleijerveld, Jos F. Brouwers, Martin Houweling, J. Bernd Helms and Arie B. Vaandrager

Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, and Institute of Biomembranes, University of Utrecht, The Netherlands

Manuscript in preparation

Synopsis

Inhibition of phosphatidylcholine (PC) synthesis in a mutant Chinese hamster ovary cell line MT58, containing a thermo-sensitive mutation in CTP: phosphocholine cytidylyltransferase, the regulatory enzyme in the CDP-choline pathway, causes PC depletion and eventually leads to apoptosis. Whereas the synthesis of PC is inhibited, the degradation of PC continues at the normal rate as compared to wild type cells, inducing a strong decrease in cellular PC within 24 h. The inhibition in PC synthesis did not cause an equal decrease in all PC species. The relative amounts of dipalmitoyl PC and arachidonic acid containing species were higher in MT58 cells grown at 40 °C, whereas 18:1/18:1 PC was preferentially degraded. Analysis of PC depleted MT58 cells by EM and fluorescence microscopy revealed a disruption of the structures of ER and Golgi, but not of mitochondria or the plasma membrane. Also the presence of lipid droplets was noticed. These results suggest that lipid droplets are organelles active in phospholipid metabolism and that PC depletion results in the preferential degradation of intracellular membranes of the ER and Golgi.

Introduction

Phosphatidylcholine (PC) is the major phospholipid species in eukaryotic cell membranes, generally comprising ±50% of the total phospholipid mass of most cells and their organelles. PC exists of two hydrocarbon chains attached to glycerophosphocholine via acyl, alkyl or alkenyl linkages. The molecular diversity of PC species is dictated by the combination of different length and number of double bonds of the two hydrocarbon chains. In most mammalian cells PC is mainly synthesized *de novo* via the CDP-choline pathway, which is regulated by the enzyme CTP:phosphocholine cytidylyltransferase (CT) (1). CT localizes primarily to the endoplasmic reticulum (ER), cytoplasm and nucleus (2), but it is also found associated with the Golgi and transport vesicles (1, 3). CT activity in cells is controlled by association with membrane lipids and by gene expression (4-6). The majority of PC produced by the CDP-choline pathway is used for membrane assembly, either as a PC molecule or as precursor of other membrane phospholipid species like PS, PE and SM. In addition to its structural role, PC also functions as a major source of intracellular signaling molecules (7-9). Agonist-stimulated hydrolysis of PC by phospholipase C (PLC) or by the combined action of phospholipase D (PLD) and phosphatidate phosphohydrolase results in an elevation of diacylglycerol (DAG) levels in the cell (7). This hydrolysis is essential for activating protein kinase C (PKC), an enzyme involved in biological processes such as proliferation and differentiation (10). Therefore it has been suggested that PC synthesis is tightly regulated in accordance with requirements for membrane turnover and cell proliferation (11). Stringent control mechanisms must be in place to keep the phospholipid content in tune with the cell cycle. Inhibition of PC synthesis with pharmacological drugs like hexadecylphosphocholine (HePC), geranylgeraniol or farnesol leads to inhibition of cell growth and increased apoptosis (12,13). Furthermore, a Chinese hamster ovary (CHO) cell line MT58, which contains a thermo-sensitive inhibitory mutation in CT, has been established and was shown to have severely reduced levels of PC at the non-permissive temperature of 40 °C (14-16). This inactivation of CT and subsequent decrease in PC activates a programmed cell death (PCD) mechanism, which bears all characteristics of apoptosis (17). The death of MT58 reveals a direct link between PC biosynthesis and apoptosis. In a previous study we have shown that shifting MT58 cells to 40 ˚C leads to an inhibition of PC synthesis within 5 h, which results in a subsequent rapid depletion in the amount of PC within 24 h at which time more than 50% of PC is lost (18). However, the point where MT58 cells become committed to die lies between

30 and 48 h and addition of LysoPC before this point of no-return can still reverse the apoptotic effect of PC depletion by precluding the onset or interrupting the progression of the apoptotic cascade (18).

The present study was designed to investigate the effects of a prolonged inhibition of PC synthesis on PC breakdown, the composition of PC species in cellular membranes and morphological changes in viable MT58 cells. Identification of abnormalities in MT58 cells that are depleted of PC might help elucidate the central events triggering apoptosis.

Here we report that inhibition of PC synthesis resulted in a changed composition of PC species in MT58 cells. Especially, dipalmitoyl-PC or PC species containing arachidonic acid were significantly enriched, whilst 18:1/18:1 PC was preferentially declined during PC depletion. Furthermore, inhibition of PC synthesis in MT58 resulted in the disturbance of the structure of the endoplasmic reticulum (ER) and the Golgi apparatus and in an accumulation of lipid droplets.

Material and methods

Materials

Ham's F12 medium, fetal bovine serum (FBS), calcium-free phosphate-buffered saline were purchased from Gibco BRL (Grand Island, NY), and culture dishes and flasks were from Nunc Inc (Rochester, NY). [*methyl*-^{3}H] Choline chloride (83.0 Ci/mmol) was obtained from Amersham Pharmacia Biotech. (Little Chalfont, UK), penicillin, streptomycin, trypsin/EDTA solution, Nile red colour dye and all other chemicals were from Sigma (Poole, UK). Mouse monoclonal anti GM130 (Golgi marker) was purchased by BD transduction Lab. Mouse monoclonal anti calnexin (ER marker) was purchased from Santa Cruz. Prefab Silica Gel G thin-layer chromatography plates were purchased from Merck (Darmstad, Germany). Coomassie® Plus Protein assay reagent kit was supplied by Pierce (Cheshire, UK). All other not specified chemicals were of analytical grade.

Cell Culture

Wild-type CHO-K1 and CHO-MT58 (15, 16) cell lines were cultured in Ham's F-12 medium supplemented with 7.5% FBS, 100-units/ml penicillin and 100 μg/ml streptomycin. All cells were maintained in 80 cm^2 flasks at 33 ˚C, 5% CO_2 and 90% relative humidity, sub-cultured twice a week and media changed every 2-3 days.

Determination of PC degradation

The PC pool was labeled by incubation of the cells for 48 hr at 33 ˚C in 60 mm dishes containing 3 ml of DMEM containing 7.5 % FCS and 2 μCi [^{3}H]choline. After removal of the label by three washes with DMEM, cells were incubated in 3 ml Ham's F-12 medium containing 7.5 % FCS and 100 μM unlabeled choline for 2 or 22 hr at either 33 ˚C or 40 ˚C. Subsequently, the cells were washed with ice-cold PBS and 1 ml of methanol and 0.8 ml of water were added. Lipids and water-soluble products were extracted from the cells by the method of Bligh and Dyer (19). The extracted lipids and water-soluble products were separated by TLC on prefab silica G plates in a solvent system of chloroform/methanol/water 65:35:4 (v/v/v) or methanol/1.2 % NaCl in water/25% ammonia in water 50:50:5 (v/v/v), respectively. [^{3}H] choline containing spots were scraped off and radioactivity in the spots was measured by liquid scintillation counting.

Analysis of molecular PC species

PC species of cells incubated for a period of 24 h at 33 ˚C or 40 ˚C were analyzed essentially as described previously (20). In brief, total lipid extracts were dissolved in chloroform: methanol (1:2, v/v) and separated on two Lichrospher RP-18e columns (Merck, Darmstad, Germany) in series (500 x 4.6 mm total dimensions). Acetonitrile: methanol: triethylamine (50:47:3 v/v/v) was used as a mobile phase, and the column effluent was introduced into a Sciex API 365 mass spectrometer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) via a TurboIon spray operating at 350°C and +5.5 kV. Mass spectra were recorded at a scan speed of 150 amu/s. The declustering potential (cone voltage) was set at 37V and the focussing potential at 185V. For quantitative purposes, extracted ion chromatograms (1 Da mass width around maximum) were constructed and integrated with Analyst software.

Electron microscopy

After an incubation for 24 h at 33 ˚C or 40 ˚C, cells were fixed with karnovsky for 24 h, postfixed in 2% OsO4 buffered in 0,1M caodylaat buffer, block stained in 2% UAC, dehydrated in a graded series of acetone and embedded in Durcupan AMC. Ultrathin cryosections were prepared according to Liou *et al.* (21). Briefly, the specimens were infused with 2.3 M sucrose overnight, placed on an aluminum specimen holder and frozen in liquid nitrogen. Ulthrathin sections (50 nm) were cut with a diamond knife (Drukker International,

Cuijck, The Netherlands) on a Leica Ultracut T equipped with a cryo-attachment (Leica Microsystems, Vienna, Austria). After etching with sodium periodate, sections were rinced with buffer and stained with 25 aqueous uranyl acetate and with 0.5% lead citrate. The sections were examined and photographed using a EM Philips CM 10 EX electron microscope (Philips, Eindhoven) at 75 kV.

Immunofluorescence microscopy

CHO-K1 and MT58 cells were grown on glass coverslips. Incubations were started by shifting the cells to 40 ^{o}C or leaving them at 33 ^{o}C (control). After 24 h of incubation, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. After pre-incubation with PBS containing 0.1 bovine serum albumin (BSA), cells were incubated with the primary antibodies for 2 h at room temperature (mouse monoclonal anti-GM130 for Golgi structures and mouse monoclonal anti-calnexin for ER structures). Cells were washed three times 5 min each with PBS containing 0.1% BSA, then incubated with fluorophore-conjugated secondary antibodies for 1 h. After PBS washes, Slow-Fade reagent was used as an anti-fading reagent. Fluorescently labeled proteins were visualized by placing the specimen under a LEICA DMR fluorescence microscope.

For Nile red staining of the lipid droplets cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS, followed by Nile red staining (final concentration 1 μg/ml) for 10 min at room temperature. Imaging of Nile red was visualized by excitation at wavelength 563 nm on a LEICA DMR fluorescence microscope.

Results

MT58 cells with an inhibited PC synthesis have a normal degradation of PC molecules

The CHO-mutant cell line MT58 has a temperature sensitive mutation in the CT-α gene (22). In a previous study we have shown that this mutation results in a decrease of PC synthesis activity by 80 % in MT58 cells grown at the non-permissive temperature of 40 °C (18). Cellular membranes have a high turnover in PC, caused by a high rate of both synthesis and breakdown (11). Since these two processes are supposed to be closely connected, we investigated which effect inhibition of *de novo* synthesis of PC would have on the degradation of cellular PC in MT58 cells. Therefore MT58 cells and the parental CHO-K1 cells were labelled with [^{3}H] choline for 48 h and breakdown of PC was measured after a chase with cold choline for 20 h. As a second indicator of PC turnover cellular levels of glycerol-

phosphocholine (GPC), one of the intermediates formed by PC breakdown, were also determined. As shown in Fig. 1, MT-58 cells incubated at 33 °C for 20 h contained still 74 ± 7% of labelled PC compared to 58 ± 1% of label present in CHO-K1 cells at 33 °C, indicating that less PC was broken down in MT58 than in CHO-K1. In contrast, MT58 cells incubated at 40 °C had only 42 ± 4% of the [^{3}H] labelled PC remaining after 20 h and similar results were found for the wild-type CHO-K1 cells incubated at 40 °C. MT58 and CHO-K1 cells incubated at 33 °C have low levels of labelled GPC, 8 ± 1% and 11 ± 0,5%, respectively, whereas MT58 and CHO-K1 cells incubated at 40 °C had a 4-fold and 5-fold higher GPC level after 20 h at 40 °C in comparison to 33 °C. These results indicate that the breakdown of PC is relatively high in CHO-K1 and MT58 cells at the non-permissive temperature and therefore contributes to the rapid decline of the cellular PC content in the cell, as observed in previous studies (14,18).

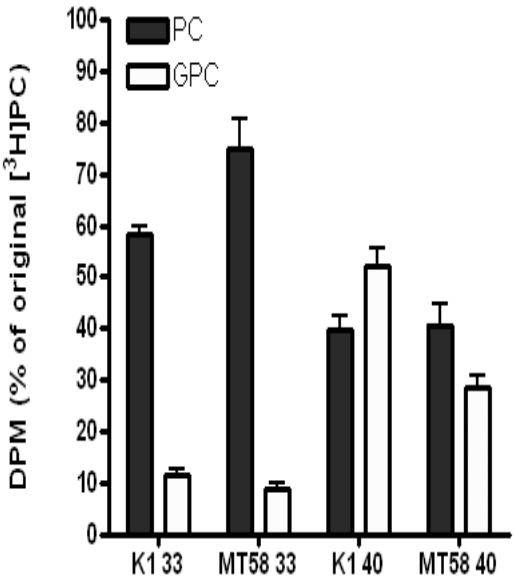

Figure 1. Effect of the non-permissive temperature on PC breakdown in MT58 cells. CHO-K1 and MT58 cells were grown at 33 ˚C for 24 h. When the cells were about 50% confluent, cells were labeled with 2 μCi [^{3}H] choline for 48 h as described in the method section. After labelling the cells were incubated at 40°C for a period of 2 or 22 h in choline-containing medium. The label present in PC or GPC after 22 h incubation was expressed relative to the amount of label present in PC after 2 h. The results represent the means ± S.E. of a representative experiment, performed in triplicate.

Molecular species composition in MT58 differs from CHO-K1 at 40 ℃.

To evaluate whether all PC species were decreased to a similar extent in MT58 cells cultured at 40 °C, we determined the PC species composition in these cells and compared it to the PC composition of CHO-K1 cells. As shown in Tabel I, the relative abundance of many PC species differed between MT58 and CHO-K1 cells grown for 24 h at the non-permissive temperature. In both cell lines 16:0/18:1 was the most prominent species, although it was approximately 20 % less abundant in MT58 cells compared to K1. An even larger decrease was observed for the second most abundant species in K1 cells, 18:1/18:1 PC, which had an intermediate abundance in MT58 cells. In contrast the second most abundant species in MT58

cells, 16:0/16:0 PC, did only account for approximately 5 % in CHO-K1 cells. The amounts of arachidonic acid containing PC species were also anomalous in MT58 cells, as they were 2-5 fold enriched in comparison to CHO-K1 cells. Most of the above mentioned differences in PC composition between MT58 and K1 cells were already visible at 33 °C, although to a lesser extent, suggesting that they were enhanced by the PC depletion occurring in the MT58 cells at the non-permissive temperature. An effect of the temperature shift on the lipid composition independent of PC depletion was also observed. For instance, in both cell lines 16:1/18:1 PC and 16:0/16:1 PC were less abundant at 33 °C in comparison to 40 °C.

Molecular PC species	CHO-K1 33 °C	CHO-K1 40 °C	MT58 33 °C	MT58 40 °C
16:0 / 22:6	**1,8 ± 0,05**	**2,3 ± 0,05**	**2,9 ± 0,1**	**2,9 ± 0,6**
16:0 / 20:4	**2,7 ± 0,1**	**2,8 ± 0,1**	**6,7 ± 0,1**	**9,6 ± 0,6 ***
16:0 / 18:2	**4,1 ± 0,2**	**4,7 ± 0,1**	**4,0 ± 0,1**	**2,9 ± 0,3**
16:0 / 18:1	**29,6 ± 0,3**	**35,1 ± 0,2**	**27,8 ± 0,2**	**28,1 ± 0,7**
16:0 / 16:1	**12,8 ± 0,05**	**8,8 ± 0,2**	**10,1 ± 0,1**	**5,8 ± 0,4**
16:0 / 16:0	**4,6 ± 0,1**	**5,3 ± 0,4**	**6,5 ± 0,2**	**13,6 ± 0,9 ***
18:0 / 22:6	**0,8 ± 0,05**	**1,0 ± 0,05**	**1,4 ± 0,2**	**2,3 ± 0,4**
18:0 / 20:4	**0,9 ± 0,05**	**1,3 ± 0,2**	**2,7 ± 0,7**	**7,3 ± 1,2 ***
18:0 / 18:2	**1,7 ± 0,2**	**2,6 ± 0,4**	**2,3 ± 0,1**	**2,4 ± 0,4**
18:0 / 18:1	**5,6 ± 0.2**	**7,4 ± 0,6**	**3,8 ± 0,3**	**5,4 ± 0.4**
16:1 / 18:1	**10,2 ± 0,7**	**5,1 ± 0,05**	**6,0 ± 0,1**	**2,3 ± 0,6**
18:1 / 22:6	**1,1 ± 0,05**	**0,9 ± 0,1**	**1,8 ± 0,1**	**0,9 ± 0,1**
18:1 / 20:4	**3,1 ± 0,3**	**3,3 ± 0,05**	**7,9 ± 0,3**	**7,6 ± 0.6**
18:1 / 18:2	**3,1 ± 0,2**	**2,7 ± 0,2**	**4,0 ± 0,3**	**1,4 ± 0,3 ***
18:1 / 18:1	**17,8 ± 0,8**	**16,5 ± 0,2**	**12,2 ± 0,8**	**7,1 ± 0,5 ***
Molecular SM species	CHO-K1 33 °C	CHO-K1 40 °C	MT58 33 °C	MT58 40 °C
SM 16:0	**9,9 ± 0,2**	**12,1 ± 0,4**	**13,4 ± 3,1**	**27,4 ± 1,0**
SM 18:0	**0,5 ± 0,05**	**0,6 ± 0,1**	**0,5 ± 0,1**	**0,9 ± 0,1**

Table 1 Effect of inhibition of CT on specific PC species compositions. CHO-K1 and MT58 cells were incubated for 24 h at 33 °C and 40 °C and lipid extracts were prepared as described under "Material and methods". PC species are expressed as % of the total of all the PC species determined, which accounted to more than 80 % of the total mass of PC in the cell. The total mass of PC in MT58 cells grown at 40 °C was more than 50 % lower than in the other conditions as indicated by the higher relative abundance of sphingomyelin (SM), whose levels are is known to hardly affected by the temperature shift (14). * indicate PC species that are affected the most by PC depletion as judged by their different abundance compared to wild-type K1 cells and to MT58 cells at 33 °C.

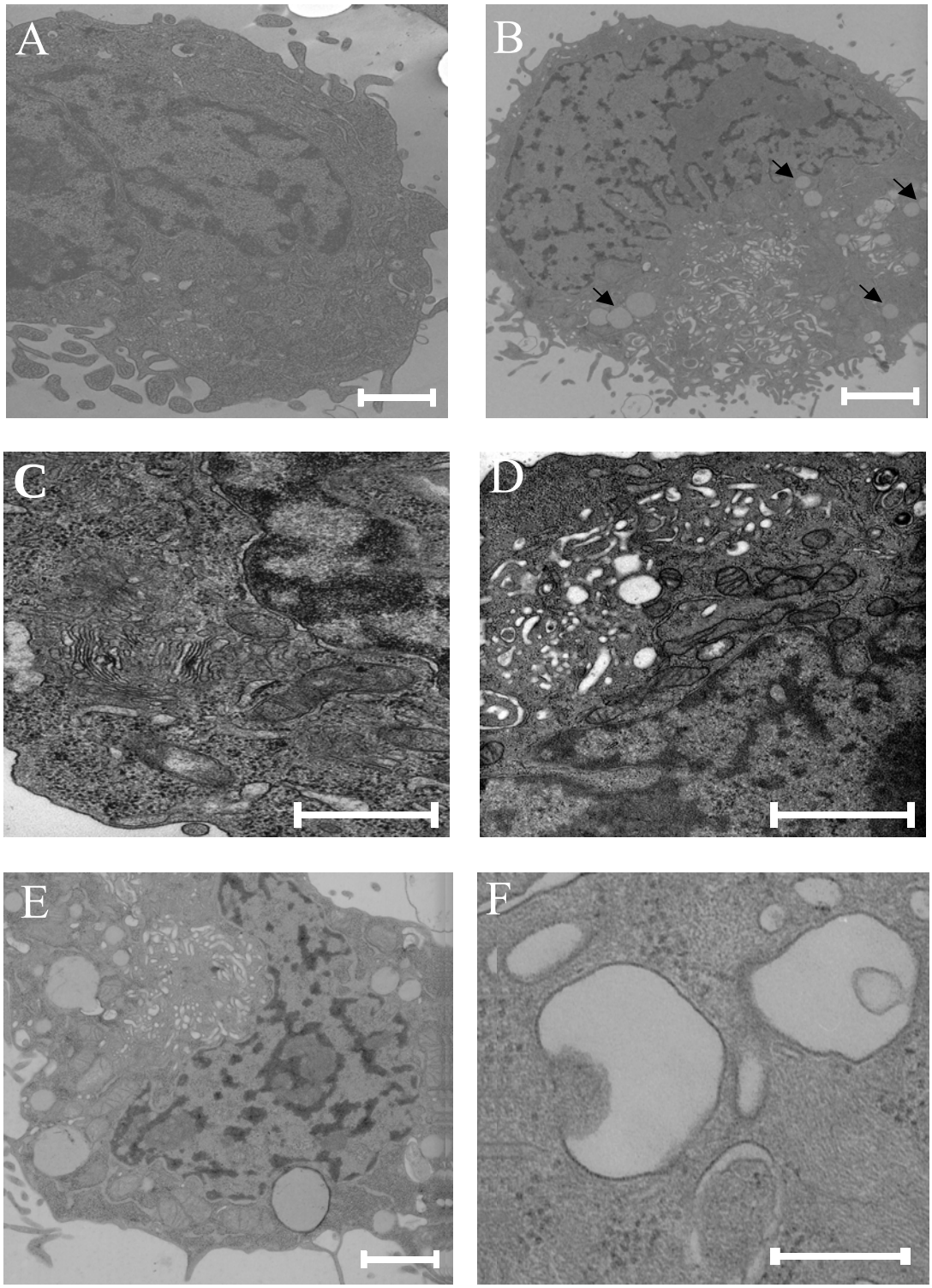

Figure 2. Morphological analysis of PC depleted MT58 cells.

A + B: CHO-K1 (A) and MT58 (B) cells were grown for 24 h by 40 °**C,** fixed and processed for EM as described in the Material and methods. The arrows indicate vacuole-like structures. Scale bars, 1 μm.

C + D: After 24 h at the non-permissive temperature, CHO-K1 (C) and MT58 (D) cells were fixed and processed for EM as described in the Material and methods**.** Scale bars, 0.5 μm.

*E + F:*After 24 h at the non-permissive temperature, MT58 cells (E) were fixed and processed for EM as described in the Material and methods**.** The arrows indicate vacuole-like lipid droplet structures, scale bar, 1 μm. Vacuole-like lipid droplets structures (F) were studied more intensively using a higher magnification, scale bar, 0.5 μm.

PC depletion results in perturbations of ER and Golgi and accumulation of lipid droplets

The incapability of MT58 to generate PC for membrane structures is expected to influence the (sub)cellular organization. To investigate the effect of PC depletion on cell morphology we performed EM studies on MT58 and CHO-K1 cells incubated at 40 °C for 24 h. The EM pictures clearly depict several changes in MT58 cells at 40 °C, compared to wild-type CHO-K1 cells (see Fig. 2) or to MT58 cells incubated for 24 h at 33 °C (data not shown). In both cell-lines the mitochondria, the nuclear and the plasma membrane appeared intact and were apparently not affected by the decrease in PC. In contrast, internal structures like ER and especially the Golgi appeared to be disrupted in MT58 cells at 40 °C. After 24 h of PC depletion at 40 °C, Golgi structures could hardly be observed in MT58 cells. Instead, a mixture of uncommon membrane structures and vesicles were visible, which could represent remnants of Golgi and/or ER structures (see Fig. 2B and 2D). To evaluate the structural changes of ER and Golgi observed in the EM studies, fluorescence microscopy using organelle specific antibodies was performed (see Fig. 3).

In contrast to CHO-K1 cells incubated for 24 h at 40 °C or to control MT58 cells grown at 33 °C, PC-depleted MT58 cells labelled with a Golgi-specific antibody showed almost no punctuated perinuclear staining typical for Golgi. Incubation of MT58 at 40 °C resulted in a less intensive staining of the ER compared to MT58 grown at 33 °C, but were still present. These results may indicate that MT58 cells first reduce selective internal membranes during PC depletion along the secretory pathway, involved in synthesis of PC.

Beside the observed abnormal membrane structures, several vacuole-like structures of variable size were present in PC-depleted MT58 cells (see Fig. 2B, 2E and 2F; indicated by the arrows). It has been described that inhibition of PC synthesis results in an increase of TAG due the diversion of DAG to the TAG pool (23,24). The rise of TAG in these cells might result in the formation of lipid storage droplets. To determine if the vacuole-like structures observed by EM, are indeed lipid storage droplets, MT58 cells and CHO-K1 cells

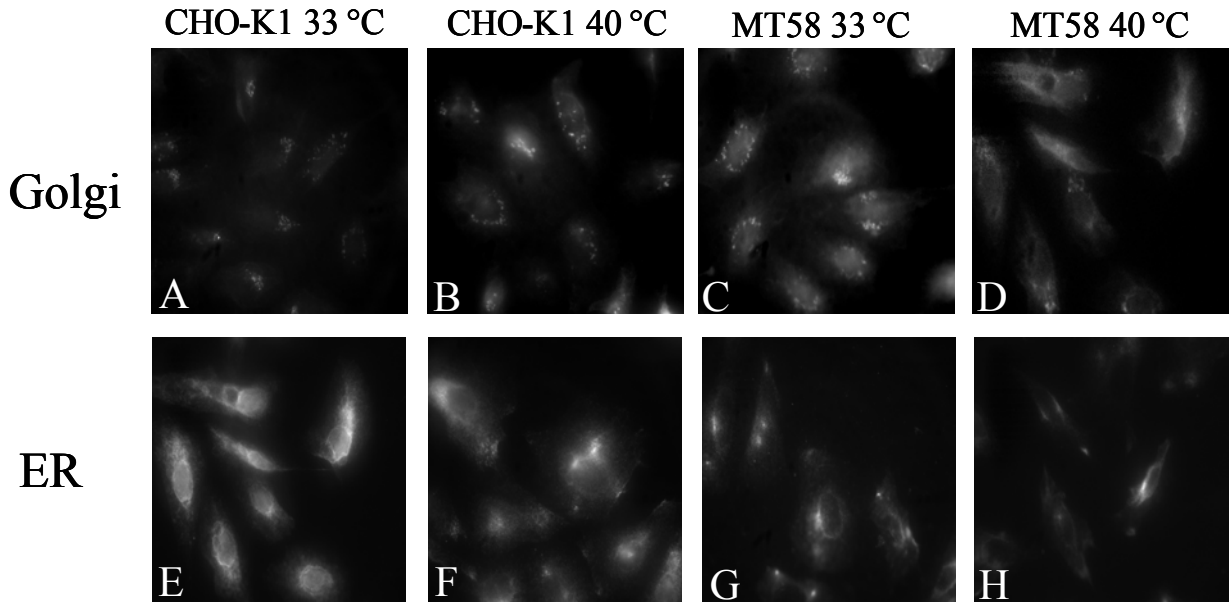

Figure 3. PC synthesis inhibition results in a disorded ER and Golgi structure. CHO-K1 and MT58 were incubated for 24 h at 33 °C or 40 °C. Cells were fixed and Golgi (A-D) and ER (E-H) structures were visualized with anti-GM130 (Golgi-marker) and anti-calnexin (ER marker), respectively, in combination with Alexa Fluor 488 rabbit anti-mouse IgG.

were incubated for 24 h at 40 °C and afterwards stained for lipid droplets with Nile red dye. As shown in Fig. 4, MT58 cells, incubated at 40 °C, contained several Nile red stained lipid storage droplets (see Fig. 4D). In contrast, lipid storage droplets were only incidentally observed in CHO-K1 cells at 33 °C or 40 °, or in MT58 cells at 33 °C (see Fig. 4A, B and C).

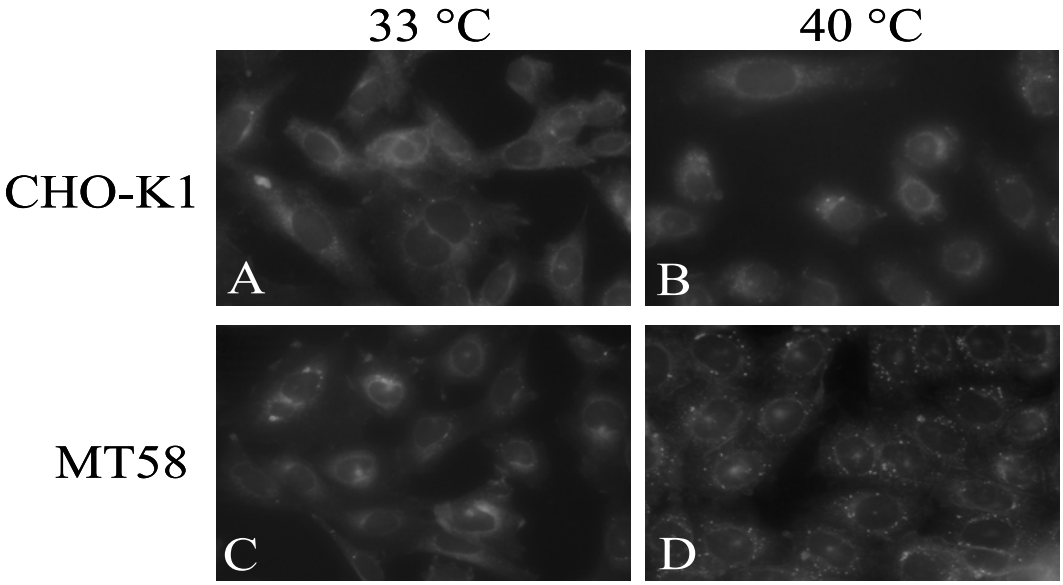

Figure 4. PC synthesis inhibition results in lipid droplets formation. CHO-K1 (A and B) and MT58 (C and D) were incubated for 24 h at 33 °C (A and C) or 40 °C (B and D). Lipid droplets were visualized using Nile red staining.

Discussion

MT58 cells incubated for 24 h at non-permissive temperature have a drastically decreased cellular PC content but are still viable and can even proliferate if PC homeostasis is restored by LysoPC addition (14,18). Therefore we used this model to investigate the effects of inhibition of PC synthesis on PC breakdown, PC species distribution and on morphological changes.

MT58 cells are shown to have an impaired CT activity at 33 ˚C, but these cells can maintain their PC levels and grow at a rate almost similar to the parent CHO-K1 cell line (18). In this study we show that the breakdown at 33 ˚C is lower than that in CHO-K1 at 33 ˚C, thereby compensating the reduced synthesis. However, culturing MT58 cells at the non-permissive temperature leads to a further inhibition of CT activity and this is not compensated by a further reduction of the PC breakdown. The inhibited synthesis combined with a normal rate of breakdown at 40 ˚C as compared to wild-type, will result in a subsequent rapid decrease in PC levels. The accumulation of GPC in MT58 and CHO-K1 cells at 40 ˚C is in line with the suggested role for a calcium-independent phospholipase A_2 during enhanced PC turnover (24-26). It has been described that inhibitors of this enzyme, methyl arachidonyl fluorophosphonate (MAFP) and manoalide can attenuate the decrease in PC levels, caused by the inhibition of CT in MT58 cells grown at 40°C (24).

The decrease in PC synthesis does not result in an equal reduction of all PC species in MT58 cells. Mass spectrometry analysis of the PC species revealed that especially 18:1/18:1 PC is reduced preferentially upon inhibition of PC synthesis. In contrast, the cellular PC content of MT58 cells incubated at 40 °C is enriched with dipalmitoyl (16:0/16:0) PC, but also with arachidonic acid containing PC species. The relative increase in both classes of PC species might be a result of increased PC synthesis via an alternative pathway not involving CT. For instance PC synthesis by methylation of PE resulted in a different PC species profile compared to PC synthesized by the CDP-choline pathway (27). Since MT58 cells have no PE methylation pathway, other PC forming pathways, like base-exchange, might be relatively more active in MT58 cells compared to CHO-K1 cells. Other possibilities to explain the different PC species profiles between MT58 cells and CHO-K1 cells are differences in the breakdown of specific PC species or differences in PC remodeling. The physiological relevance of the different species profile in PC-depleted MT58 cells, if any, is not clear. We may speculate that the relative increase in arachidonic acid containing PC species is related to the role of this lipid in cellular signaling. In order to conserve this bioactive lipid, MT58 cells

may degrade PC species containing arachidonic acid more slowly. This also would prevent this potent signaling precursor to accumulate in the cell. To compensate for the higher membrane fluidity caused by the increase in PC species with poly-unsaturated arachidonic acid, other PC species should contain relatively more saturated fatty acids like palmitate.

Incubation of MT58 cells at 40 ^{o}C leads to the formation of lipid droplets as observed by EM and fluorescence microscopy. The surplus of DAG, which cannot enter the PC synthesis pathway in MT58 cells, is likely converted into TAG in MT58 cells incubated at 40 ^{o}C (23, 24). Here we suggest that the surplus of TAG is stored in MT58 cells in lipid droplets. Lipid droplets are in general regarded as storage depots for neutral lipids in mammalian cells. Recently, there are indications that metazoan lipid droplets might also be metabolically active. Cholesterol ester-rich lipid droplets in macrophages (28) as well as cytosolic lipid droplets in mammary epithelial cells (29) contain cholesterol esterase. Furthermore the caveolae marker protein caveolin has been shown to localize to lipid droplets (30,31) suggesting a direct role for lipid droplets in supplying cholesterol to membranes. Recently, CGI-58, a protein thought to be involved in phospholipid recycling, has been isolated from lipid droplets in CHO-K2 cells that were grown in the presence of oleate (32). These observations suggest that lipid droplets appear to be complex, metabolically active organelles that are directly involved in membrane traffic and possible phospholipid recycling. The MT58 cells might offer a good model to study the formation of this organelle.

The inhibition of PC synthesis is expected to influence the synthesis of new membranes, but also existing membranes might be affected. After 24 h of PC depletion the structure of the plasma membrane, the membrane of the nucleus and mitochondrial structures seem to be intact. In contrast, the structure of the Golgi seemed disrupted and also ER structures seemed to be affected, although to a lesser extent. This suggests that the dynamic processes at the early secretory pathway are affected the most. Disruption of the structure of both organelles could be a possible trigger for the induction of stress pathways that eventually result in apoptosis in PC depleted MT58 cells. The ER has been identified as the origin of the response to several perturbations in the cell, like glucose starvation, Ca^{2+} overload and accumulation of unfolded or misfolded proteins. When cells are faced with one of these stresses, the ER induces growth arrest or initiates apoptosis by a process known as the ER stress response (33-36). The ER stress response was also shown to be involved in the apoptotic response to cholesterol loading in macrophages (37). Cholesterol overload is suggested to cause a misbalance between cholesterol and phospholipids in biological membranes (37) and since PC depletion is also likely to cause a change in the phospholipid-

to-cholesterol ratio, the ER stress pathway could be a likely candidate to play a role in the stress response to PC depletion. Furthermore, most of enzymes of PC synthesis, CT and CPT, are located in the ER. The ER stress response is characterized by specific events like the up-regulation of ER chaperone Bip/GRP78 and CHOP/GADD153, protein translation attenuation and activation of caspase 12. However, in a previous study we did not observe any of these ER stress responses except for CHOP induction (18). Therefore it is not likely that the canonical ER stress response is involved in the activation of apoptosis during PC depletion. The ER and the Golgi complexes are also the compartments in the cell responsible for the storage of cellular Ca^{2+}. Efflux of Ca^{2+} from these stores induced by arachidonic acid, oxidative stress and ceramides is also described in several stress-induced apoptotic pathways (38, 39). Besides the ER and Golgi, the newly formed lipid droplets may also play a role in the mechanism by which PC depletion finally results in apoptosis.

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CHAPTER 3

Inhibition of phosphatidylcholine synthesis induces expression of the endoplasmic reticulum stress and apoptosis related protein C/EBP-Homologous Protein (CHOP/GADD153)

Michiel H.M. van der Sanden, Martin Houweling, Lambert M.G. van Golde and Arie B. Vaandrager

Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, and Institute of Biomembranes, University of Utrecht, The Netherlands

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Synopsis

Inhibition of *de novo* synthesis of phosphatidylcholine (PC) by some anti-cancer drugs like hexadecylphosphocholine (HePC) leads to apoptosis in various cell lines. Likewise in MT58, a mutant Chinese hamster ovary (CHO) cell line, containing a thermo-sensitive mutation in CTP: phosphocholine cytidylyltransferase (CT), an important regulatory enzyme in the CDP-choline pathway, inhibition of PC synthesis causes PC depletion. Cellular perturbations like metabolic insults and unfolded proteins can be registered by the endoplasmic reticulum and result in ER stress responses, which eventually can lead to apoptosis. In this study we investigated the effect of PC depletion on the ER stress response and ER related proteins. Shifting MT58 cells to the non-permissive temperature of 40 ˚C, resulted in PC depletion via an inhibition of CT within 24 h. Early apoptotic features appeared in several cells around 30 h, and most cells were apoptotic within 48 h. The temperature shift in MT58 led to an increase of pro-apoptotic C/EBP-homologous protein (CHOP/GADD153) after 16 hours, to a maximum at 24 h. Incubation of wild type CHO-K1 or CT-expressing MT58 at 40 ˚C did not induce differences in CHOP protein levels in time. In contrast, expression of the ER chaperone BiP/GRP 78, induced by an increase in misfolded/unfolded proteins (UPR), and caspase 12, a protease specifically involved in apoptosis that results from stress in the ER, did not differ between MT58 and CHO-K1 in time when cultured at 40 ˚C. Furthermore HSP 70 a protein that is stimulated by accumulation of abnormal proteins and heat stress, displayed similar expression patterns in MT58 and K1. These results suggest that PC depletion in MT58 induces the ER stress-related protein CHOP, without raising a general ER stress response.

Introduction

Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotic cells and the major structural component of cellular membranes. Furthermore PC serves as reservoir of the lipid second messenger diacylglycerol (DAG) and of phosphatidic acid in several signal transduction pathways (1). PC synthesis may also influence the levels of the signal molecule ceramide since sphingomyelin (SM) biosynthesis depends on the transfer of the phosphocholine head-group from PC to ceramide (2, 3).

De novo synthesis of PC by the CDP-choline pathway is tightly regulated (4). The rate-limiting step of this pathway is the conversion of phosphocholine to CDP-choline, which is catalysed by the enzyme CTP: phosphocholine cytidylyltransferase (CT) (5). CT localizes mainly to the endoplasmic reticulum and nucleus (6). CT activity in cells is regulated primarily by association with membrane lipids, translocation between endoplasmic reticulum, cytoplasm and nucleus (7-11) and by gene expression (9, 10, 12, 13). An important role of PC biosynthesis has been implicated both in the control of cell proliferation and cell-death (14-17). Inhibition of CT by hexadecylphosphocholine (HePC), an alkylphosphocholine or inhibition of CDP-choline: 1,2 diacylglycerol cholinephosphotransferase (CPT) by geranylgeraniol and farnesol result in inhibition of PC biosynthesis and was paralleled by inhibition of cell growth and increased apoptosis (18-21). Moreover PC depletion in a Chinese hamster ovary (CHO) cell line MT58, which contains a thermo-sensitive mutation in CT (22-24) resulted in apoptosis (16). The death of MT58 reveals a direct link between PC biosynthesis and apoptosis. However the molecular mechanism by which the depletion of PC is sensed and transduced to the death pathway is unknown.

Cellular perturbations like metabolic and toxic insults, increased production of free radicals and unfolded proteins can be registered by the endoplasmic reticulum (ER), and might lead to an induction of ER stress, the so-called ER stress response (ERSR) (25-28). The ER can respond to different insults in several manners. Accumulation of proteins with wrong conformations or unfolded proteins in the ER results in the induction of several proteins, including the ER chaperone BiP, also known as GRP 78 (29). BiP facilitates proper protein folding, by interacting with exposed hydrophobic patches on protein-folding intermediates and is thought to prevent their aggregation while maintaining the protein in a folding-competent state (30). BiP interaction ensures that only proteins in their right conformation exit the ER compartment. This stress response, involving BiP/GRP78, is known as unfolded

protein response (UPR) (28, 29, 31). BiP has a great homology to the cytosolic HSP 70 stress protein. (29, 31) HSP 70, which protects proteins against heat damage by its chaperone activity with the folding, assembly and degradation of proteins (32, 33). This cytoprotective mechanism of HSP 70 is able to rescue cells from apoptosis at a late stage of the process (34). Another factor that is induced by cellular stress and is involved in mediating apoptosis is C/EBP homologues protein (CHOP), also known as GADD153, which encodes a bZip transcription factor (35-37). The CHOP gene is most responsive to perturbations that culminate in the induction of stress in the ER, although the mechanism by which ER stress leads to CHOP gene expression is not known (38-40). CHOP/GADD153 expression can be followed by posttranslational events, such as phosphorylation (36, 41, 42). CHOP is a nuclear protein that can dimerize with C/EBP transcription factors (36, 42, 43). These stable heterodimers are capable of recognizing novel DNA targets (43-45) and suggest that CHOP may have a role in transducing signals from the stressed ER to changes in gene expression, which eventually may lead to apoptosis. Alternatively the ER can also induce apoptosis through activation of caspases via increased expression and activation of caspase-12, an ER-situated caspase (46-48). ER stress results in a translocation of caspase-7 to the ER surface where it cleaves caspase-12 (46). Prolonged ER stress results in a movement of active caspase-12 to the cytosol where it interacts with caspase-9 (46). Finally, ER stress is known to inhibit protein synthesis by phosphorylation of eIF2α by the ER resident protein kinase PERK (49).

As depletion of the main phospholipid PC may lead initially to a disturbance in the functioning of the ER, where PC is synthesized, we explored the effect of inhibition of PC synthesis on the ER-stress-related proteins, using the conditionally CT deficient cell line MT58 (22-24). Here we report for the first time that inhibition of PC synthesis leads specifically to induction of the ER stress-related protein CHOP, but not to the canonical ER stress proteins BiP or caspase 12 or to the inhibition of protein synthesis.

Material and methods

Materials

Ham's F12 medium, fetal bovine serum (FBS), calcium-free phosphate-buffered saline were purchased from Gibco BRL (Grand Island, NY), and culture dishes and flasks were from Nunc Inc (Rochester, NY). [*methyl*-^{3}H] Choline chloride (83.0 Ci/mmol) was obtained from

Amersham Pharmacia Biotech. (Little Chalfont, UK), Boc-D-fluoromethyl ketone (FMK) was purchased from Alexis (Läuflingen, Switserland), penicillin, streptomycin, trypsin/EDTA solution, and all other chemicals were from Sigma (Poole, UK). Prefab Silica Gel G thin-layer chromatography plates were purchased from Merck (Darmstad, Germany). Polyclonal anti-actin, polyclonal anti-GRP78 (BiP), polyclonal anti-caspase-12 and polyclonal anti-GADD153 (CHOP) were provided by Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). A monoclonal anti-HSP 70 was purchased from Sigma. The Supersignal chemiluminescent substrate kit (ECL) for detection of proteins on immunoblots and Coomassie® Plus Protein assay reagent kit were supplied by Pierce (Cheshire, UK).

Cell Culture

Wild type CHO-K1, CHO-MT58 (16, 22, 24) and CT-expressing CHO-MT58 (50) cell lines were cultured in Ham's F-12 medium supplemented with 7.5% FBS, 100-units/ml penicillin and 100 μg/ml streptomycin. All cells were maintained in 80 cm^2 flasks at 33 ˚C, 5% CO_2 and 90% relative humidity, sub-cultured twice a week and media changed every 2-3 days.

Growth curves and quantification of apoptosis

For growth curves, cells were plated at a density of 5 x 10^4 cells in 60 mm dishes containing 5 ml of medium and incubated at 33 ˚C or at the non-permissive temperature for the CT-mutant MT58 cells of 40 ˚C. At the indicated time points, culture medium was removed, cells were washed twice with PBS and fixed in methanol, prior to staining with propidium-iodide at a final concentration of 2.4 μM. Stained cells were observed using LEICA DMR fluorescence microscope. Cell numbers were established by counting total population of nuclei and cells that contained condensed or fragmented nuclei were scored as apoptotic.

Cell rescue experiments

Cells were plated at 5 x 10^4 cells in 60 mm dishes containing 5 ml of medium and incubated at 33 ˚C or the non-permissive temperature of 40 ˚C. At indicated time-points 25 μM lysophosphatidylcholine (LysoPC) was added to the cell cultures. Cells were fixed in methanol, prior to staining with propidium-iodide at a final concentration of 2.4 μM, 72 h after switching cells to 40 ˚C or 33 ˚C. Cell numbers and apoptosis were established as described above.

[^{3}H]Choline chloride incorporation into PC

100 mm dishes were incubated at 33 ˚C or 40 ˚C for the indicated time periods, prior to labelling with 1μCi/ml [methyl-^{3}H] choline in complete medium for 1 h. Incorporation of label was quenched by removing the medium, washing the cells two times with ice-cold PBS and addition of methanol. Lipids and water-soluble precursors were extracted from the cells by the method of Bligh and Dyer (51). The extracted lipids were separated by TLC on prefab silica G plates in a solvent system of chloroform/methanol/water 65:35:4 (v/v/v). PC spots were scraped off and radioactivity in the spots was measured by liquid scintillation counting.

Determination of PC pool size

5 x 10^4 cells were plated in 60 mm dishes and incubated for various time periods at 40 ˚C or 33 ˚C. Cells were collected in 1 ml Tris-buffer (150 mM; PH 7.5). The protein content was determined using the Coomassie® Plus protein assay reagent kit (Pierce) according to the manufacturer's instructions. Lipids were extracted according to Bligh and Dyer (51). Lipid separation was accomplished by straight-phase HPLC on a LIChrosphere 100 Diol column (5 μm), (Merck, Darmstad, Germany), using two solvent delivery pumps, (Kontron Instruments), and an external solvent mixer (Kontron Instruments). The flow-rate was 1ml/min and column temperature was kept at 40 ˚C during all runs. Elution was performed with a gradient of hexane: isopropanol: aceton (82:17:1 v/v/v) to isopropanol: chloroform: aceton (85:12:3 v/v/v). A Varex MKIII light scattering detector was used for the detection of (phospho)lipids. The detector signal was recorded and integrated by the software program EZ Chrom™ chromatography DATA system (Scientific Software Inc.). The results were quantified using a mass-response curve according to the method of Rouser *et al* (52).

Western Blot

Cells were washed with cold PBS and scraped off the plate in 100 μl PBS. Aliquots were used to measure protein content and the remaining 50 μl of cell suspension in PBS was lysed with 50 μl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentration; 62.5 mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.003% bromophenol blue, pH 6.8). After boiling samples for 10 min, 4 μg protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 1% western blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 1 h and exposed to rabbit polyclonal anti-GADD153 (CHOP), dilution 1:750, or goat

polyclonal anti-GRP 78 (BiP), dilution 1:1000, or mouse monoclonal anti-HSP 70, dilution 1:2500. Following four washing steps with TBS-Tween (50 mM Tris; 150 mM NaCl; 1 % Tween20, pH 7.5), blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were washed four times with TBS-Tween and CHOP, BiP or HSP 70 proteins were displayed by a reaction on Supersignal chemiluminescent substrate (Pierce, Cheshire, UK) and exposure to x-ray films.

Measurement of protein synthesis.

Cells were washed twice in PBS and incubated in methionine-free RPMI 1640 medium/10% dialyzed FCS (1 h, 40 °C) prior to labelling (1 h, 40°C, 100 µCi ml-1 [^{35}S]-methionine). Cells were washed with cold PBS and scraped off the plate in 200 µl PBS. 100 µl suspension was used for TCA precipitation of the proteins and incorporation of radioactivity into TCA-precipitated proteins was measured with a scintillation-counter. The remaining 100 µl of cell suspension was lysed with 100 µl SDS-PAGE sample buffer (as described above) and labelled proteins were separated by SDS-PAGE under reducing conditions. The proteins were visualized by autoradiography.

Results

MT58 cells have an impaired PC biosynthesis at 40 ˚C, leading to reduction of PC pool-size.

The CHO-MT58 cell line has been suggested to contain a single point mutation in the CT-α gene (53), causing the gene product to be less stable, especially at an elevated temperature of 40 ˚C. To confirm the effect of the unstable CT-α on the biosynthesis of PC via the CDP-choline pathway, we measured the incorporation of [^{3}H] choline into PC in CHO-MT58 cells, the parental CHO-K1 cells, and in MT-58 cells, stably transfected with wild-type CT-α gene, at 33 ˚C and 40 ˚C As shown in Fig. 1, already at the permissive temperature of 33 ˚C the amount of [^{3}H]-choline incorporated into PC was found to be 2.3 fold lower in MT58 cells, than in CHO-K1 control cells or in the CT-expressing MT58 cells. After incubation for 5 h at 40 ˚C, PC synthesis in MT-58 cells was decreased even further to only approximately 15 % of that in the parental CHO-K1 cells or the CT-expressing MT-58 cells. In contrast, the temperature shift to 40 ˚C only marginally affected the [^{3}H]choline incorporation into PC in wild-type CHO or in the CT-expressing MT58 cells (Fig. 1).

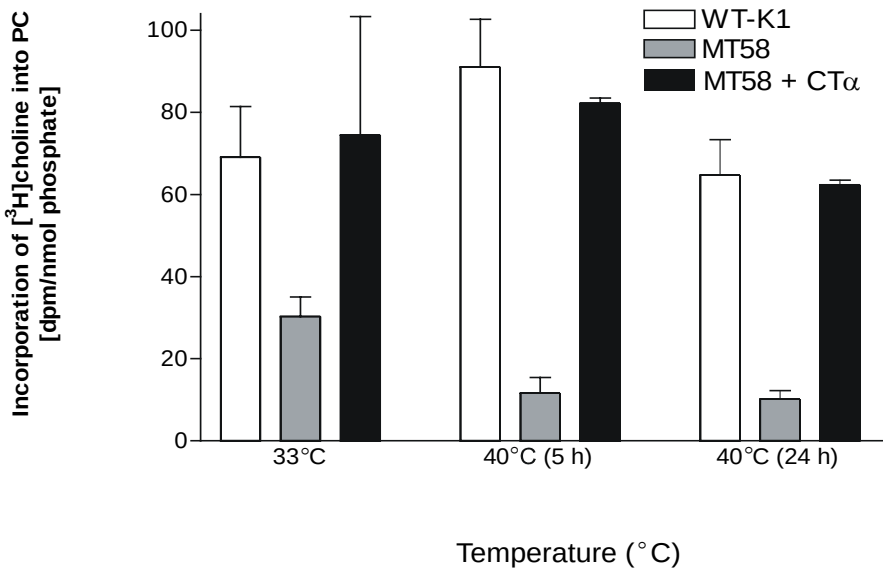

Figure 1. Effect of the non-permissive temperature on PC biosynthesis in CHO cells. CHO-K1, MT58 and CT expressing MT58 were grown at 33 ˚C on 100-mm dishes for 24 h. When the cells were about 50% confluent, 2/3 of the dishes was shifted to 40°C for a period of 5 or 24 h, and the remaining cultures were left at 33 ˚C as control. Afterwards PC biosynthesis was measured by labelling with 2.5 μCi [^{3}H] choline. After 1 h, lipids were extracted using a Bligh-Dyer method and an aliquot of the lipids was taken to measure total phosphate concentration. The remainder of the lipid extraction was used to determine the amount of radiolabel incorporated into PC. The results represent the means ± S.E. of three independent experiments, each performed in triplicate

Cellular membranes have a high turnover in PC, caused by a high rate of synthesis and breakdown (14). Therefore we investigated the result of inhibition of *de novo* synthesis of PC on the cellular PC pool-size. Despite its reduced capacity to incorporate choline into PC, MT58 cells were found to have a similar PC content as CHO-K1 cells when grown at 33 ˚C. However, incubation at 40 ˚C led to a dramatic reduction of the cellular PC pool-size (Fig. 2). Already after 8 hours, a 40 % decrease in PC content was observed, and after 24 hours at 40 ˚C only 20 % of the PC was left in the MT-58-cells. The decrease in PC mass was apparently not compensated by other phospholipids as the total amount of phosphorous in the lipid fraction also decreased from 135 nmol/mg protein to 75 nmol/mg protein after the 24 h incubation at 40 ˚C (data not shown). As shown in Fig. 2, incubation of the control CHO-K1 cells at 40 ˚C did not lead to changes in PC content.

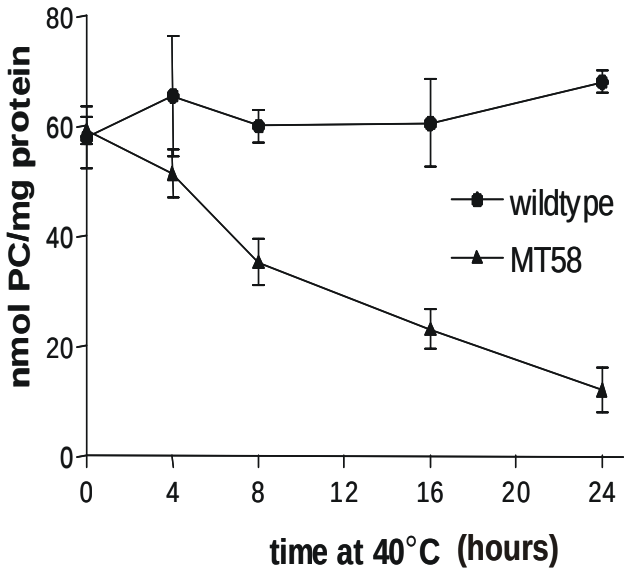

Figure 2. PC biosynthesis inhibition leads to reduction in PC pools. CHO-K1 and MT58 cells were grown in 60 mm dishes at 33 ˚C or 40 ˚C. At the indicated time points, cells were harvested and aliquots were taken to determine protein concentrations (μg/ml) of samples. After lipid extraction PC mass was determined by HPLC as described in methods. The PC concentration per μg protein is plotted for K1 (circles) and MT58 (triangles) cells in time. The results represent the means ± S.E. of three independent experiments.

Inhibition of PC synthesis leads to apoptosis in MT58 cells.

As shown above, shifting MT58 cells to 40 ˚C leads to an inhibition in PC synthesis via the CDP-choline pathway within 5 h, which results in a subsequent rapid depletion in the amount of PC. Next we assessed the time required for the PC depletion to affect cell growth and apoptosis in the MT-58 cells. As shown in Fig. 3A, MT58 cells grow at 33 ˚C at a slightly reduced rate compared to wild type CHO-K1 cells. However, when shifted to 40 ˚C, MT58

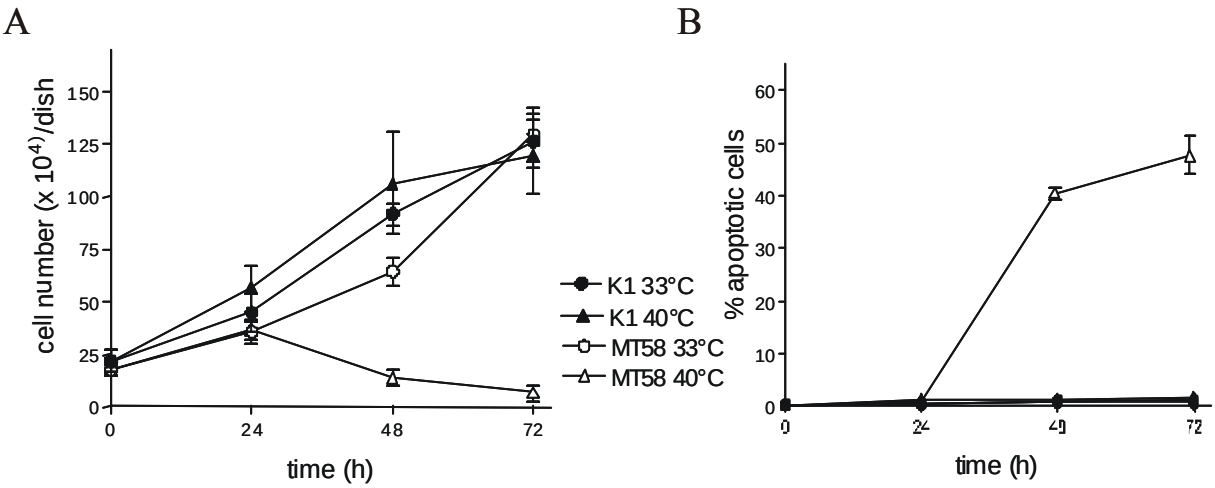

Figure 3. Inhibition of cell proliferation and induction of apoptosis in MT58 at 40 ˚C. CHO-K1 and MT58 cells were plated on 60-mm dishes with 5 ml of Ham's F12 medium at a density of 5 X 10^4 cells/dish. After 24h at 33 ˚C, cells were then incubated at either 40 ˚C (open *triangles*) or 33 ˚C (open *circles*). At the indicated times some dishes were fixated and stained with PI. Total cell populations (*panel A*) and apoptotic cells percentage (*panel B*) were quantified as described in experimental procedures. A minimum of 100 cells from 5 different areas of each plate was evaluated for quantification of apoptosis. Shown are the means ± S.E. of three independent experiments.

cells grew normally up to 24 h, but started dying by apoptosis between 24 and 48 h (Fig. 3A + B). This apoptotic process was found to depend on caspases for the final execution, because MT58 cells pretreated with the broad-spectrum caspase inhibitor Boc-D-fluoromethylketone (Boc-D-FMK) showed an increased survival after incubation at 40 ˚C. In the presence of FMK only 13,2 % ± 1,5 of the MT-58 cells became apoptotic after 48 h at the non-permissive temperature (data not shown). In contrast, 45-50% of the MT-58 cells died in the absence of the caspase inhibitor (Fig. 3B).

To investigate at which time point the PC depletion has irreversibly triggered apoptosis, we determined the latest time-point at which MT58 cells could be rescued by adding LysoPC, a compound known to compensate for the decrease in PC synthesis by the CDP-choline pathway [18,22]. MT58 cells grown at 40 ˚C could be rescued almost completely if LysoPC was added within 30 hours (Fig. 4A + B). However addition of LysoPC at 48 h could not reverse the apoptotic process. These results suggest that the point where cells become committed to die lies between 30 and 48 h and that adding LysoPC before this point of no-return can still reverse the apoptotic effect of PC depletion by precluding the onset or interrupting the progression of the apoptotic cascade.

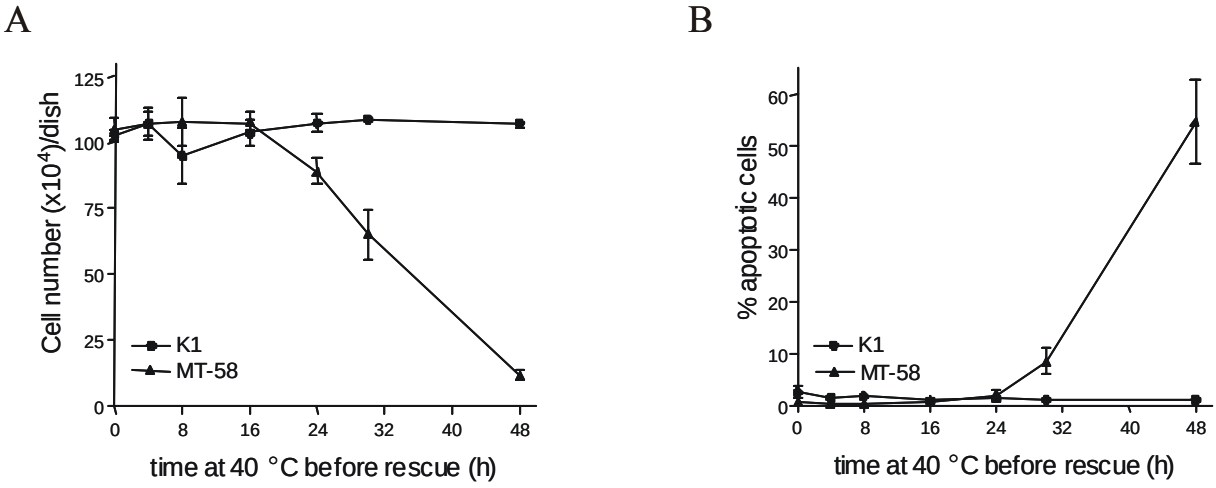

Figure 4. Rescue from apoptosis, caused by PC depletion, with LysoPC. To investigate if LysoPC can rescue MT58 cells from apoptosis, 50-55% confluent cells were shifted to 40 ˚C. LysoPC was added to a final concentration of 25 µM at the indicated time-points to the cells (points of rescue) and refreshed every 24 h afterwards. Cells were harvested 72 h after shifting cells to 40 ˚C, and viability and apoptosis were assessed. Closed circles = WT-K1 and closed triangles = MT58 cells. Total cell populations (*panel A*) and percentages of apoptotic cells (*panel B*) were quantified as described in experimental procedures. Shown are the means ± S.E. of three independent experiments.

CHOP is induced in MT58 cells grown at the non-permissive temperature.

To determine the possible mechanisms of the induction of apoptosis by PC depletion in MT58 cells we assessed the effect of inhibition on PC synthesis on various ER stress response proteins implicated in apoptosis, at time points before the onset of apoptosis. First, we investigated the influence of PC depletion on ER-chaperone protein BiP/GRP 78. BiP is a protein, responsible for the proper folding of proteins and over-expression of BiP can lead to cell survival (28), and high levels of BiP protein expression is indicative of ER stress (28, 40). However, no differences in expression levels of BiP were observed between CHO-K1 cells and MT58 cultured at 40 ˚C (Fig. 5A).

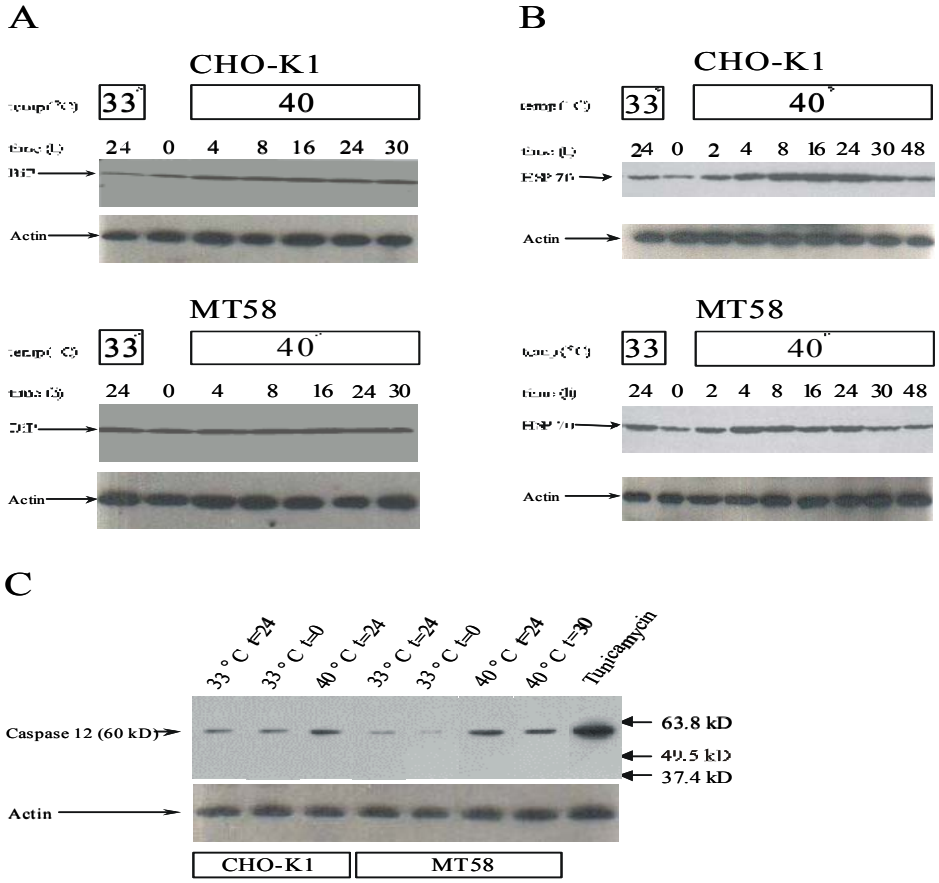

Figure 5. The effect of PC depletion on BiP, HSP 70 and caspase-12 response. The role of ER stress response in PC depletion was investigated by analysis of two ER-related proteins, BiP (Panel A) and caspase-12 (Panel B). Furthermore the expression of HSP 70, also induced by stress, like malfolded proteins and heat is investigated. CHO-K1 and MT58 cells were cultured in 60 mm dishes at 33 ˚C or switched to 40 ˚C for indicated time periods. Cells were harvested and aliquots were taken to determine the protein concentration (data not shown). Total cell homogenates (4 μg of protein) were separated on SDS-PAGE and the amount of BiP, caspase-12, HSP 70 and actin (internal control) were detected by western blotting using goat polyclonal α-GRP78/BiP antibody, rabbit polyclonal α-caspase-12 antibody, mouse monoclonal α-HSP 70 antibody and a goat polyclonal α-actin antibody antibody, respectively. Results shown are representative for three independent experiments.

Like BiP, HSP 70 is induced by accumulation of abnormal proteins, but also by heat stress. Furthermore HSP 70 is suggested to rescue cells from apoptosis at a late stage in the process. As shown in Fig. 5B, HSP 70 levels are increased in both MT-58 and CHO-K1 cells as early as 2 hours after shifting the cells to 40 ˚C, presumably as reaction to the heat shock. As there was no correlation between the response of both BiP and HSP 70 and the depletion of PC only in MT-58 cells, these stress proteins are unlikely to have a causative role in the apoptosis induced by the inhibition of PC synthesis. Another protein reported to couple ER stress to the activation of the apoptotic machinery is caspase-12 (44, 46, 47). In our cell lines, a relatively small induction of the ER bound caspase-12 is noticed for both K1 and MT58 after 24 h, when compared to the robust induction of caspase-12 by tunicamycin, a typical ER stress-inducing agent (Fig. 5C).

In contrast, culturing CHO-K1 and MT58 cells at the non-permissive temperature of 40 ˚C leads to an induction of CHOP expression only in MT58 cells within 16 h, to a maximum at 24 h (Fig. 6A). In several experiments CHOP expression was increased as soon as 8 h after shifting to 40 ˚C (results not shown). The induction of CHOP at 40 ˚C observed in MT-58 cells was clearly correlated with the defect in PC synthesis, as MT-58 cells stably expressing recombinant CT-α showed only a slight increase in CHOP expression (Fig. 6B). Furthermore, the increase in CHOP expression in MT58 at 40 °C is not the result of a small difference in confluency between MT-58 and K1 cells (data not shown) or to an enhanced sensitivity of the MT-58 cells to ER stress. In both cell lines incubation with tunicamycin (10 µg/ml) or DTT (1 mM) increased the expression of CHOP to a similar level, starting at 6 h with a maximum after 24 h (data not shown). Also no difference in the apoptotic response to tunicamycin between CHO-K1 and MT58 cells was observed (data not shown).

To unveil more about the role of the ER stress response in the induction of CHOP by PC depletion, we investigated the effect of PC depletion on protein synthesis. It is known that ER stress results in inhibition of protein synthesis by phosphorylation of eIF2α by the ER resident protein kinase PERK (49). We found that protein synthesis was not inhibited in MT58 cells at 40 °C. The incorporation of [^{35}S]methionine was 130 ± 18% and 95 ± 15% after 16 and 24 h at 40 °C, respectively, of that of MT58 cells incubated for 24 h at 33 °C (n=2). Similar results were found for the wild-type cells (106 ± 2% after 16 h at 40 °C, and 118 ± 10% after 24 h at 40 °C). In contrast, induction of ER stress in wild-type and MT-58 cells by incubation with tunicamycin (10µg/ml) for 16 h, provoked a strong inhibition of protein synthesis. The incorporation of [^{35}S]methionine in the presence of tunicamycin was

only 27 ± 4% and 30 ± 2%, respectively, of that of wild-type and MT-58 cells incubated for 24 h at 33 °C in the absence of this ER stress-inducing agent. Treatment with cycloheximide (50 μg/ml; 4 h) almost completely abolished protein synthesis in both wild-type and MT58 cells. Also no changes in the pattern of [^{35}S]methionine labelled proteins were observed on SDS-PAGE after incubation of MT58 and K1 cells at 40 °C (results not shown).

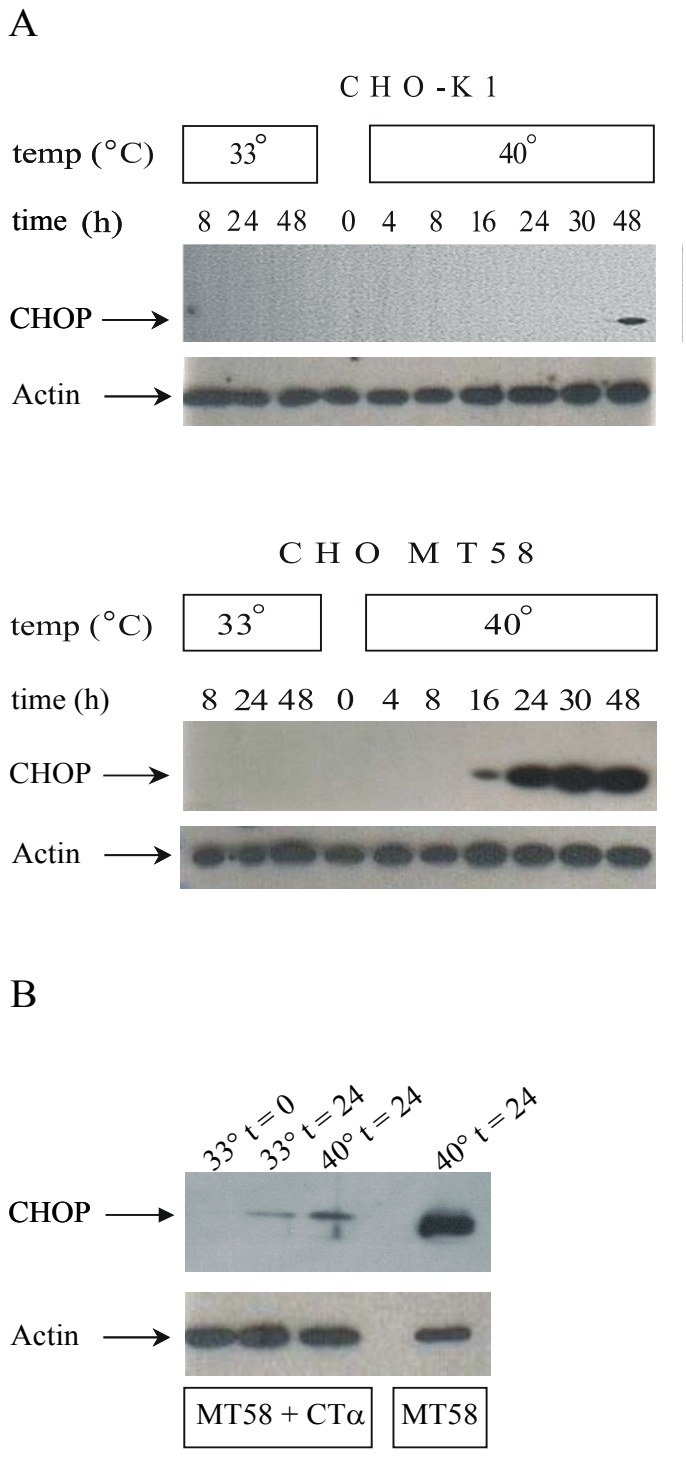

Figure 6. CHOP induction in PC depleted MT58 cells.

(A) CHO-K1 and MT58 cells were cultured in 60 mm dishes at 33 ˚C or switched to 40 ˚C for the indicated time periods. Induction of CHOP expression was estimated by western blotting on equal amounts of proteins (4 μg) from whole cell extracts, using specific rabbit polyclonal α-GADD153/CHOP as first antibody, and goat-anti-rabbit horseradish peroxidase-conjugated antibody as secondary antibody. Identical blots were probed for actin as internal control. These finding were reproduced in five independent experiments.

(B) Western Blot analysis on cell homogenates of MT58 or CT-expressing MT58 cells were performed as described under A. MT58 + CT cultures (MT58 + CT) were incubated for 24 h at 33 ˚C or 40 ˚C and compared with MT58 at 40 ˚C for 24 h. These finding were reproduced in two independent experiments.

Discussion

Inhibition of PC biosynthesis at the level of the rate-limiting enzyme, CTP: phosphocholine cytidylyltransferase was shown to correlate with the induction of apoptosis in a number of cell lines (15, 16, 18). Likewise in MT58, a mutant Chinese hamster ovary cell line, containing a thermo-sensitive mutation in CT, we were able to confirm that PC depletion induces apoptosis (16). Although MT58 already display impaired CT activity at 33 ˚C, the cells can maintain their PC levels and can grow at a rate almost similar to the parent CHO-K1 cell line. However culturing MT58 cells at the non-permissive temperature leads to a further inhibition of CT activity and a subsequent rapid decrease of PC levels, which eventually is somehow sensed by the cell and triggers an apoptotic pathway. In this study we describe for the first time that CHOP/GADD153 might be involved in the apoptotic route, induced by PC depletion. The induction of CHOP expression is a relatively early event, beginning 8 - 16 h after shifting the temperature to the non-permissive temperature, and precedes the onset of terminal execution phase of the apoptotic process, as LysoPC can rescue the cells from apoptosis up to 30 h. The relatively short latency period between the temperature-induced PC depletion and the appearance of CHOP protein suggests that CHOP is upstream in the signaling cascade, which eventually leads to apoptosis. CHOP is suggested to be pro-apoptotic, as mouse embryonic fibroblasts (MEFs) derived from CHOP-/- animals exhibited significantly less programmed cell death when challenged with ER disrupting agents, compared to the wild-type animals (35). Furthermore over-expression of CHOP in growth-factor dependent 32D myeloid precursor cells leads to the induction of apoptosis (54). As we did not perform an extensive search for apoptotic factors, it is possible that other pro-apoptotic factors besides CHOP are induced in the MT58 cells in response to PC depletion.

CHOP is a transcription factor that is induced by cellular stress especially by agents that adversely affect the function of the ER (35, 38-40). For example, blocking N-linked glycosylation, a post-translational event specific for proteins entering the ER, by tunicamycin results in a strong induction of CHOP (38, 40) along with an up-regulation of BiP and GRP94, specific proteins of the UPR (40). The fact, that N-glycosylation is ER specific, suggests that a stress signal emanating from that site induces CHOP. Other chemicals, that interfere with ER related processes, like thapsigargin (inhibition of the ER Ca^{2+}-ATPase), brefeldin A (an inhibitor of vesicle transport between ER and Golgi) and AIF4- (inhibition of trimeric G-proteins) also induce CHOP (40). Therefore, CHOP is suggested to have a role in

signal transduction from the stressed ER to apoptosis. Upon stress an as yet unidentified protein is released from the ER that binds to the ER stress element (ERSE) of the CHOP promoter, inducing CHOP transcription (45). The increased expression of CHOP leads to the formation of heterodimers of CHOP with C/EBP transcription factors (36). Although a set of genes, called DOC's (for downstream of CHOP) has been identified as targets of the CHOP hetero-dimer (37), it has been suggested that there are more unidentified targets. The tight linkage between ER stress response and CHOP expression in combination with the induction of CHOP by inhibition of PC synthesis suggests that PC depletion may induce an ER stress response.

In many forms of cellular stress, CHOP expression is shown to be coordinately regulated with the ER chaperone BiP (42, 55). Both proteins have a shared upstream signaling component, Ire1, which participates in the co-induction of CHOP and BiP genes in response to ER stress-inducing agents like the protein glycosylation inhibitor tunicamycin (40). In MT58 cells cultured at 40 ^{o}C we observed a modest increase in BiP expression. However, the increase in BiP preceded the induction of CHOP and was also observed in the control CHO-K1 cells. Therefore the expression of BiP is unlikely to be regulated by PC depletion in MT58 cells. This is enforced by the fact that we did not observed strong caspase-12 induction in MT58. Caspase-12 induction has been observed to be co-regulated with BiP expression and mediates the activation of the caspase cascade by prolonged ER stress, eventually resulting in apoptosis. ER stress has also been shown to lead to the induction of CHOP expression by a pathway involving translational control and the ER protein PERK (49, 56). Activated PERK phosphorylates the ribosomal initiation factor eIF-2α, resulting in a general inhibition of protein synthesis and a preferential translation of the transcription factor ATF 4, which subsequently increases the expression of CHOP. However we found no evidence for a general inhibition of protein synthesis upon PC depletion in the MT58 cells. Taken together, these results strongly suggest the absence of a canonical ER stress response by PC depletion. Furthermore the expression of HSP 70 protein, another protein often associated with protein-misfolding and stress, is not significantly different between K1 and MT58 when grown at the non-permissive temperature.

Although CHOP is often implicated with ER stress response, its induction has also been observed in apoptotic pathways, independent of the ER. The group of Fafournoux reported that amino acid limitation, especially leucine and arginine, up-regulates CHOP expression, but not BiP expression (57). Glutamine deprivation also results in a rapid elevation of CHOP mRNA in cells that are dependent on glutamine for growth and viability,

and is accompanied by a modest increase of BiP mRNA levels (58). A signal cascade from the Fas receptor via the G-proteins Ras and Rac to JNK/p38-K leads to an increase and activation of CHOP, resulting eventually in Fas-regulated apoptosis (41). Cell death mediated by C6-ceramides also involves increased expression of CHOP (41). So there are several mechanisms leading to the induction of CHOP by PC depletion in the cell; one that might involve a specific ER stress response, independent of BiP induction or an ER-independent apoptotic pathway. Analysis of the CHOP promoter suggests that both pathways can induce CHOP expression, but each pathway requires a different *cis* acting DNA elements (44, 45), to execute its action. Therefore, determining the effect of inhibition of PC synthesis on the various cis-acting elements of the CHOP promoter might be helpful to elucidate the mechanism of how PC depletion is sensed by a cell.

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CHAPTER 4

Induction of CHOP/GADD153 expression during inhibition of phosphatidylcholine synthesis is mediated via activation of a C/EBP-ATF responsive element

Michiel H.M. van der Sanden, Henriët Meems, Martin Houweling, J. Bernd Helms and Arie B. Vaandrager

Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, and Institute of Biomembranes, University of Utrecht, The Netherlands

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Synopsis

The gene for the pro-apoptotic transcription factor CHOP/GADD153 is induced by various cellular stresses. Previously, we described that inhibition of phosphatidylcholine (PC) synthesis in MT58 cells, that contain a temperature-sensitive mutation in CTP:phosphocholine cytidylyltransferase (CT), results in apoptosis preceded by the induction of CHOP. Here we report that prevention of CHOP induction, by expression of anti-sense CHOP, delays the PC depletion-induced apoptotic process. By mutational analysis of the conserved region in the promoter of the CHOP gene, we provide evidence that the C/EBP-ATF composite site, but not the ER-stress responsive element or the AP-1 site, is required for the increased expression of CHOP during PC-depletion. Inhibition of PC synthesis in MT58 cells also led to an increase in phosphorylation of the stress-related transcription factor ATF2 and the stress kinase JNK after 8 and 16 hours, respectively. In contrast, no phosphorylation of p38 MAPK was observed in MT58 cultured at the non-permissive temperature. Treatment of MT58 cells with the JNK inhibitor SP600125 could rescue the cells from apoptosis, but did not inhibit the phosphorylation of ATF2 or the induction of CHOP. Taken together, our results suggest that increased expression of CHOP during PC depletion depends on a C/EBP-ATF element in its promoter and might be mediated by binding of ATF2 to this element.

Introduction

Phosphatidylcholine (PC) constitutes the major portion of cellular phospholipids in eukaryotic cells. PC serves as a major structural building block of biological membranes and PC has also been recognized as an important source for signaling molecules (1-3). In eukaryotic cells, PC is made primarily through the CDP-choline pathway in which CTP:phosphocholine cytidylyltransferase (CT) is the key enzyme (4). CT localizes mainly to the endoplasmic reticulum (ER) and nucleus (5). CT activity in cells is regulated primarily by association with membrane lipids, translocation between ER, cytoplasm and nucleus (6-10) and by gene expression (8, 9, 11, 12). Inhibition of PC biosynthesis leads to pertubations in PC homeostasis, which eventually influences cell proliferation and cell-death (13-15). Treatment of cells with inhibitors of the enzymes of the CDP-choline pathway results in an inhibition of cell growth and increased apoptosis (16-18). Likewise in MT58, a mutant Chinese hamster ovary (CHO) cell line, containing a thermo-sensitive mutation in CT (19-21), inhibition of PC synthesis causes apoptosis (14). The death of MT58 cells reveals a link between PC homeostasis and apoptosis. In a previous study we have shown that MT58 cells, incubated at the non-permissive temperature, express a high level of C/EBP homologues protein (CHOP), also known as growth arrest and DNA damage-inducible protein 153 (GADD153), preceding apoptosis (22).

CHOP encodes a nuclear, pro-apoptotic, bZip transcription factor of the CCAAT/enhancer-binding protein (C/EBP) transcription factor family which is often induced by cellular stress (23-25). CHOP activity is regulated by both changes in expression level and by post-translational events, such as phosphorylation (24, 26, 27). Upon phosphorylation, CHOP dimerizes with other C/EBP transcription factors and these stable heterodimers are capable of recognizing novel DNA targets (24, 26-30). In general, CHOP induction in cells is often associated with perturbations that culminate in the induction of stress in the ER, the so-called unfolded protein response (UPR) (31-34). The classical ER stress response results in transcriptional activation of genes coding for the ER chaperone BiP (also known as GRP78), CHOP and caspase 12 (34-38), and inhibition of protein synthesis by phosphorylation of eIF2α by the ER resident protein kinase PERK (39). However, CHOP induction in PC depleted MT58 cells was not accompanied by any of these classical ER stress response events (22). Therefore, the route to induction of CHOP by PC depletion seems distinct from the ER stress signaling cascade, but the exact pathway is still not identified.

The present study was designed to identify possible transcription activating pathways that induce CHOP expression during inhibition of PC synthesis. The CHOP promoter region contains several elements, responsive to various stresses. Transcriptional induction of the CHOP gene in response to ER stress is mediated by a defined DNA element, the ER stress responsive element (ERSE) (30). This ERSE containing region in the human CHOP promoter, comprising nucleotides –104 to –75, is required for both constitutive and ER stress induced expression. Other important stress responsive elements in the promoter of the CHOP gene are an AP-1 site (40) and a C/EBP-ATF composite site (41-43). The AP-1 site, located between nucleotides –250 and –225 of the human CHOP promoter, has been described to have a significant role in transcriptional activation of CHOP during oxidative stress and DNA damage (40). UV, arsenite and H_2O_2 treatment of cells stimulate c-Fos and c-Jun phosphorylation and the formation of heterodimeric c-Jun/c-Fos complexes. Binding of the c-Jun/c-Fos heterodimer to the AP-1 site results in transcriptional activation of CHOP (40). C-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (p38 MAPK), both belonging to the family of stress-activated MAP protein kinases, were identified to be responsible for the phosphorylation of c-Jun during CHOP induction (27, 44). Furthermore both kinases were described to play a role in the post-translational phosphorylation of CHOP upon activation during stress (26, 27). The C/EBP-ATF composite site has been described to regulate the expression of CHOP during arsenite treatment as well (43). Furthermore, a C/EBP-ATF composite site was found to be responsible for the induction of CHOP by amino acid deprivation (29, 42). The C/EBP-ATF within the amino acid response element (AARE) is located upstream of the transcription initiation site, between nucleotides –313 and –295, and has some homology with specific binding sites for the C/EBP and ATF/CREB transcription families (42). The C/EBP-ATF composite site was shown to bind ATF2 and ATF4 during leucine starvation, resulting in CHOP expression (29, 45). However, the transactivity capacity of ATF4 depends on its expression level and that of ATF2 on its phosphorylation state (45). Therefore two different pathways, one leading to ATF4 induction and the other to ATF2 phosphorylation, are present for CHOP induction during amino acid starvation. Upstream regulatory proteins of ATF2 phosphorylation and ATF4 induction during amino acid deprivation are not known, although JNK is suggested to be involved (45).

In order to understand the individual steps in the cellular response to inhibition of PC synthesis, we investigated the role of CHOP and its induction by analyzing several potential binding sites for transcription factors in the CHOP promoter for the induction of this protein. We have identified for the first time that the C/EBP-ATF composite site plays a role in the

induction of CHOP expression during PC depletion. Furthermore we found that ATF2, which binds to this C/EBP-ATF site is activated during PC depletion and might therefore be involved in the transcriptional activation of the CHOP gene. We also observed that JNK is active in MT58 cells at the non-permissive temperature, but this stress kinase seems not to be involved in the induction of CHOP.

Material and methods

Materials

Ham's F12 medium, fetal bovine serum (FBS), calcium-free phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY), and culture dishes and flasks were from Nunc Inc (Rochester, NY). SP600125 JNK/SAPK kinase inhibitor and SB203580 p38 MAPK inhibitor were obtained from Tocris (Bristol, UK). Penicillin, streptomycin, G418 neomycin, trypsin/EDTA solution, and all other chemicals were from Sigma (Poole, UK). Polyclonal anti-actin, polyclonal anti-phospho-ATF2, polyclonal anti-GADD153 (CHOP) and monoclonal anti-phospho-c-Jun were provided by Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Polyclonal antibodies against JNK/SAPK kinases, phospho-JNK/SAPK kinases and phospho-p38 MAPK were purchased from New England Biolabs (Hertfordshire, UK). The Supersignal chemiluminescent substrate kit (ECL) for detection of proteins on immunoblots and Coomassie® Plus Protein assay reagent kit were supplied by Pierce (Cheshire, UK).

Generation of anti-sense CHOP expressing MT58 cells

For the construction of an anti-sense CHOP expression vector, a 847 bp DNA fragment encoding the CHOP gene was excised with *Bam*HI from pcD-X vector containing the cDNA insert for hamster CHOP (kindly donated by Dr. N. Holbrook) and subcloned into *Bam*HI digested pcDNA3.1 expression vector. Clones were checked for the proper anti-sense orientation by digestion with *Hind*III and *Pvu*II. Positive clones were sequenced to confirm the construction of an anti-sense CHOP containing expression vector using the ABI PRISM Bigdye terminator cycle sequencing reaction kit and the ABI PRISM 310 genetic analyser (Applied Biosystems) according to the manufacturer's instructions. MT58 cells were transfected with 4 μg of anti-sense CHOP vector or empty pcDNA3.1 vector using Lipofectamine Reagent, Gibco BRL (Grand Island, NY). Individual neomycin-resistant

colonies were selected with 600 μg/ml G418 in Ham's F12 medium. Single colonies were picked and grown as individual cell lines in the presence of 300 μg/ml G418. Positive clones were identified by western blotting for CHOP protein after induction by shifting cells to the non-permissive temperature.

Cell Culture

Wild-type CHO-K1, MT58 (15, 20, 22) and CT-expressing MT58 (46) cell lines were cultured in Ham's F-12 medium supplemented with 7.5% FBS, 100-units/ml penicillin and 100 μg/ml streptomycin. Stable cell lines expressing anti-sense CHOP vector or containing empty pcDNA3.1 were grown in Ham's F-12 medium supplemented with 7.5% FBS, 100-units/ml penicillin, 100 μg/ml streptomycin and 300 μg/ml G418. All cells were maintained in 80 cm^2 flasks at 33 ˚C, 5% CO_2 and 90% relative humidity, sub-cultured twice a week and media changed every 2-3 days.

Plasmids used for analysis of the CHOP promoter

pGL3 basic plasmids with the –649 to +91, -442 to +91 and –221 to +91 human CHOP promoter region fused to the luciferase gene were a gift of Dr. P. Fafournoux (29, 42). The 2X C/EBP-ATF-TATA*TK*-LUC, 2XE4ATF-TATA*TK*-LUC and 2Xjun2TRE-TATA*TK*-LUC plasmids fused to the luciferase gene were also donated by Dr. P. Fafournoux (29). 5' Mutations in the C/EBP-ATF, AP-1, and the ERSE sites were made using a site-directed mutagenesis kit of Stratagene (La Jolla, CA). The mutant C/EBP-ATF CHOP promoter construct was generated by substitution of the nucleotides TGCA of the core sequence in the C/EBP-ATF site into GAAT using a phosphorylated mutagenesis primer, nucleotide sequence: 5'-AGACACCGGTTGCCAAACAT**GAAT**TCATCCCCGCCCCCCTTTCC-3'. The mutant AP-1 CHOP promoter construct was generated by substitution of the nucleotides ACTC of the core sequence in the AP-1 site into CAGA with mutagenesis primer, nucleotide sequence: 5'-GCGCGCGCGCATG**CAGA**ACCCACCTCCTCC-3'. The mutant ERSE CHOP promoter was constructed by changing the nucleotides ATAC of the ERSE core sequence into GAAT with the help of a mutagenesis primer, coded by the following nucleotide sequence: 5'-GCCGGCGTGCCACTTTCT**GAAT**TCTAGGTTTTGGGGTCCCG-3'. Al steps of the mutagenesis reaction were performed according to the manufacturer's instructions. All the constructs were sequenced before utilization.

DNA transfection and luciferase assay

Cells were plated at 5 x 10^4 cells per well of a six-well plate with 4 ml of medium and incubated at 33 ˚C and transfected by using Lipofectamine plus reagent according to the manufacturer's instructions (Gibco). One microgram of luciferase plasmid was transfected into the cells along with 0.5 μg of pCMV-βGal (Promega), a plasmid carrying the bacterial β-galactosidase gene fused to the human cytomegalovirus immediate-early enhancer promoter region, as an internal control. Cells were exposed to the precipitate for 3 h, washed once with PBS, and then incubated with Ham's F12 medium, supplemented with 7,5% FBS. 48 hours after transfection, cells were shifted to 40 ˚C. After 24 h of incubation at the non-permissive temperature, CHO-K1 and MT58 cells were harvested in 300 μl of lysis buffer (Promega). From the lysate of the samples, all in quadruplicate, 20 μl of the supernatant was used to measure luciferase activity using the Luciferase assay system of Promega. β-galactosidase activity was measured according to the protocol supplied by Promega. Relative luciferase activity was given as a ratio of relative light units (DPM) to relative β-galactosidase units (ONPG). All values are the means of at least three independent experiments.

Western blot

Cells were washed with cold PBS and scraped off the plate in 100 μl PBS. Aliquots were used to measure protein content and the remaining 50 μl of cell suspension in PBS was lysed with 50 μl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentration; 62.5 mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.003% bromophenol blue, pH 6.8). Cell protein was measured using the Coomassie^R Plus protein assay reagent kit (Pierce) according to the manufacturer's instructions, using BSA as a standard. After boiling samples for 10 min, 8-10 μg protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 1% western blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 1 h and exposed to rabbit polyclonal anti-GADD153 (CHOP), anti-phospho-ATF2, both diluted 1:250, anti-JNK/SAPK kinases, anti-phospho-JNK/SAPK kinases or anti-phospho-p38 MAPK, all diluted 1:1000, mouse monoclonal anti-phospho-c-Jun, dilution 1:250, or goat polyclonal anti-actin, dilution 1:500. Following four washing steps with TBS-Tween (50 mM Tris; 150 mM NaCl; 1 % Tween20, pH 7.5), blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were washed four times with TBS-Tween

and the respective proteins were detected by a reaction on Supersignal chemiluminescent substrate (Pierce, Cheshire, UK) and exposure to x-ray films.

Cell rescue experiments and quantification of apoptosis

Cells were plated at 3 x 10^5 cells in 60 mm dishes containing 5 ml of medium and incubated at 33 ˚C or the non-permissive temperature of 40 ˚C. At indicated time-points 20 μM SB203580 p38 MAPK inhibitor or 40 μM SP600125 JNK/SAPK kinase inhibitor were added to the cell cultures. At the indicated time-points culture medium was removed, and cells were washed twice with PBS and fixed in methanol, prior to staining with propidium-iodide (final concentration; 2.4 μM). Stained cells were observed using a LEICA DMR fluorescence microscope. Cell numbers were established by counting total population of nuclei. Live and apoptotic cells were differentiated from each other on the basis of definitive nuclear fluorescence and distinct morphological changes, including nuclear condensation and nuclear fragmentation. Condensed or fragmented nuclei were scored as apoptotic. A minimum of 200 cells was counted in five random fields per dish.

Results

Inhibition of CHOP expression in MT58 cells postpones the onset of apoptosis.

In a previous study we have shown that inhibition of PC synthesis results in a strong increase of CHOP protein after 16 h, preceding apoptosis (22). Therefore CHOP could play an important role in the transcriptional activation of apoptotic downstream targets during PC depletion. To investigate this, MT58 cells were stably transfected with a control plasmid or a plasmid containing anti-sense CHOP cDNA. The transfected cells were exposed for 24 h to the non-permissive temperature of 40 °C. The anti-sense CHOP over-expressing MT58 cells did not show the increase in CHOP expression, observed in control transfected MT58 and previously (22) in non-transfected MT58 cells (see Fig. 1A).

Next, we assessed the effect of over-expression of anti-sense CHOP mRNA in MT58 on apoptosis caused by PC depletion. As shown in Figure 1B, MT58 cells with anti-sense CHOP mRNA still go into apoptosis, but the onset of apoptosis is delayed with 8-10 h, compared to vector control cells. Similar results were observed for two independent anti-sense CHOP expressing MT58 clones (data not shown). The effect of the prevention of CHOP expression on apoptosis in MT58 cells was not caused by an effect on cell growth, as

the anti-sense CHOP and the vector control transfected cells showed similar growth curves at the permissive temperature, as assessed by cell counting (data not shown).

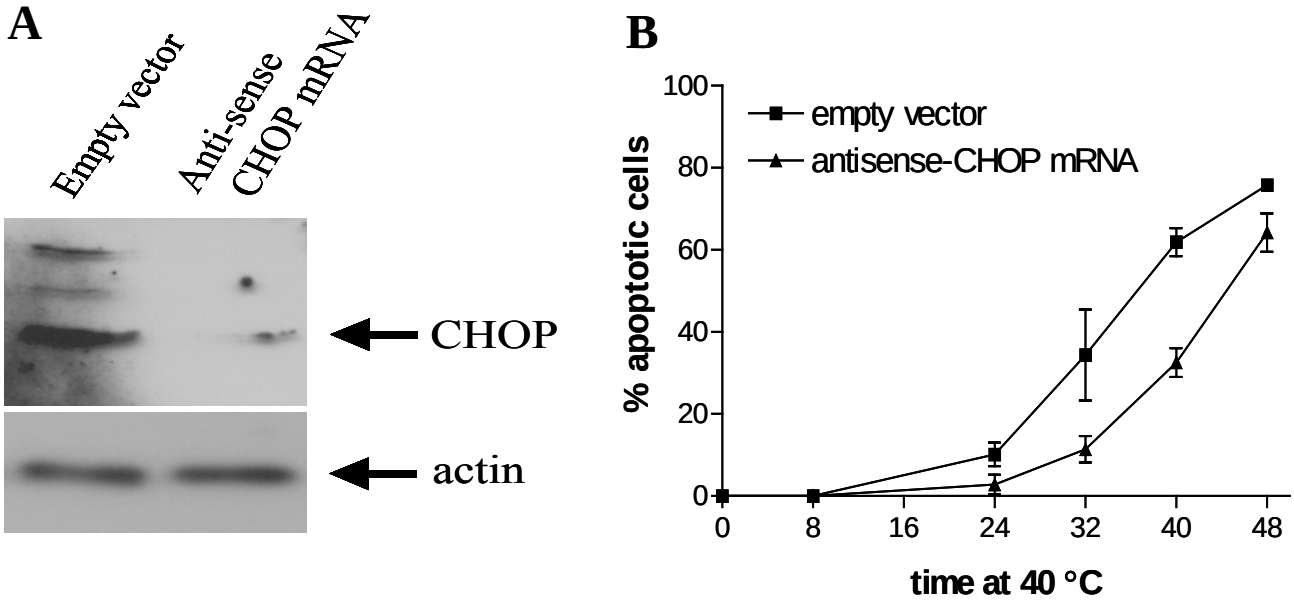

Figure 1. Inhibition of CHOP expression delays the onset of apoptosis.

Panel A, Immunoblot of CHOP expression in control vector pcDNA 3.1 transfected (empty vector) and anti-sense CHOP stably transfected MT58 cells. Cells were cultured for 24 h at 40 ˚C to induce the expression of CHOP, before lysis in SDS-PAGE sample buffer. Lysates containing equal amounts of protein (10 µg) were separated by SDS-PAGE and immuno-blotted with rabbit polyclonal antibodies against CHOP. Actin was used to confirm that the slots were equally loaded.

Panel B, Empty vector (squares) or anti-sense CHOP (triangles) expressing MT58 cells were plated on 60-mm dishes in 5 ml Ham's F12 medium at a density of 5 X 10^4 cells/dish. After 24h at 33 ˚C, cells were incubated at 40 ˚C for various time periods. At the indicated times cells were fixated and stained with Propidium Iodide (PI). Apoptotic cells were quantified as described in Experimental procedures. A minimum of 100 cells from 5 different areas of each plate was evaluated for quantification of apoptosis. Shown are the means ± S.E. of three independent experiments and similar results were observed for two independent anti-sense CHOP expressing MT58 clones.

Transcriptional activation of CHOP by PC depletion

To elucidate the regulation of CHOP expression during PC depletion, we analysed the CHOP promoter. It has been shown that regulation of CHOP expression by various kinds of stress is mediated through the promoter sequence between nucleotide position –954 and +91 of the 5' upstream region (30, 41). Serial deletion mutants of the CHOP promoter, fused to the coding region of the luciferase (LUC) reporter gene, were used to identify regions responsive to PC

depletion. These constructs were transiently transfected into MT58, the parental CHO-K1 and in MT58 cells, stably transfected with the CTα gene. The latter cells were used to exclude differences between MT58 and CHO-K1 cells not related to the inactivation of CT. The transfected cells were incubated for 24 h at 40 °C and the response to PC depletion was determined by a LUC assay (see Fig. 2). MT58 cells showed increased levels of LUC expression from the –442 and –649 deletion constructs, compared to CHO-K1 or CT-expressing MT58. Upon transfection with the –442 and -649 CHOP promoter constructs, the luciferase level in the MT58 cells incubated at the non-permissive temperature was comparable to the level in CHO-K1 cells treated with tunicamycin, a potent inducer of ER stress (see Fig. 2).

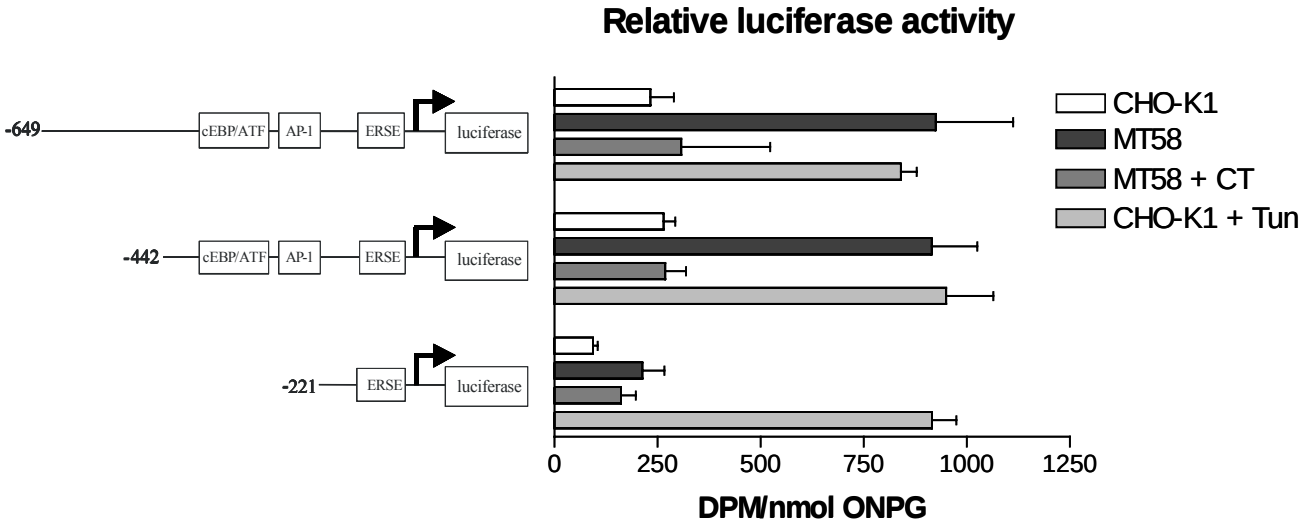

Figure 2. Deletion analysis on the activation of the CHOP promoter by PC depletion. The plasmids pCHOP-LUC –649, –442 and –211, correspond to the human CHOP promoter region from nucleotide –649, –422, –211 to +91 fused to the luciferase (LUC) gene. CHO-K1, MT58, CT-expressing MT58 were transiently transfected with these plasmids along with the plasmid pCMV-βgal as described in Experimental procedures. 48 h after transfection, cells were shifted to 40 °C for 24 h, and in case of CHO-K1 incubated without or with 1 μM tunicamycin (as a positive control). Subsequently, cells were harvested and assayed for luciferase and β-galactosidase activity. Results are given as relative luciferase activity per nmol ONPG hydrolysed. Shown are the means ± S.E. of five independent experiments.

This suggests that PC depletion increased transcription of the CHOP gene to a similar extend as ER stress. Deletion to -211 decreased the activation of the CHOP promoter in MT58 to

similar levels found in CT-expressing MT58. A shorter fragment (-119) of the CHOP promoter did not result in a further decrease of activity in all three cell lines (data not shown). To verify that the observed increase in CHOP promoter activity was caused specifically by CT inhibition, CHO-K1 and MT58 were transfected with the –442 deletion construct and incubated for 24 h at the permissive temperature of 33 ºC. No significant difference in luciferase activity was observed between the two cell lines at 33 ºC (data not shown). These results suggest that the regulatory element(s) present between nucleotide –442 and nucleotide –221 of the CHOP promoter are responsible for mediating CHOP gene activation in response to PC depletion.

In the region between –422 and –221 two known stress-responsive elements are present, an AP-1 site (40) and a C/EBP-ATF site within an amino acid response element (41-43). Inactive mutants of these elements were made by site-directed mutagenesis to investigate their role in the activation of CHOP transcription during PC depletion. Furthermore, the ERSE element between –104 and –75 was also mutated as a negative control. CHO-K1, MT58 and CT expressing MT58 cells were transfected with these constructs and exposed to the non-permissive temperature for 24 h. Mutation of the ERSE or AP-1 site did not result in any significant difference in LUC expression (see Fig. 3A). By contrast, mutation of the C/EBP-ATF element resulted in a decrease in LUC expression to levels comparable to that of wild-type CHO-K1 or CT-expressing MT58 cells transfected with the wild-type –422 CHOP promoter construct. However, CHO-K1 and CT-expressing MT58 cells, transfected with the C/EBP-ATF mutant did also show a reduction in CHOP promoter activity, compared to their counterparts transfected with wild-type –422 CHOP promoter construct, suggesting that the C/EBP-ATF is also involved in basal expression of CHOP at 40 °C.

To further investigate the role of the AP-1 and C/EBP-ATF site, luciferase constructs were used containing two copies of the AP-1 (jun2TRE), E4-ATF, or C/EBP-ATF site placed immediately upstream of a thymidine kinase (TK) promoter (29). These constructs were transfected into CHO-K1, MT58 and CT-expressing MT58 cells and incubated at 40 ºC for 24 h. The adenovirus E4ATF binding site did not induce an increase in LUC expression in response to PC depletion (see Fig. 3B). A very low level of inducible promoter activity was observed with the distal AP-1 binding site, compared to the controls, but this response was several fold lower than the induction of luciferase from the C/EBP-ATF construct in response to PC depletion. Together, these findings suggest that the up-regulation of CHOP transcription when cells are faced with a shortage in PC is mediated, at least in part, by the C/EBP-ATF site.

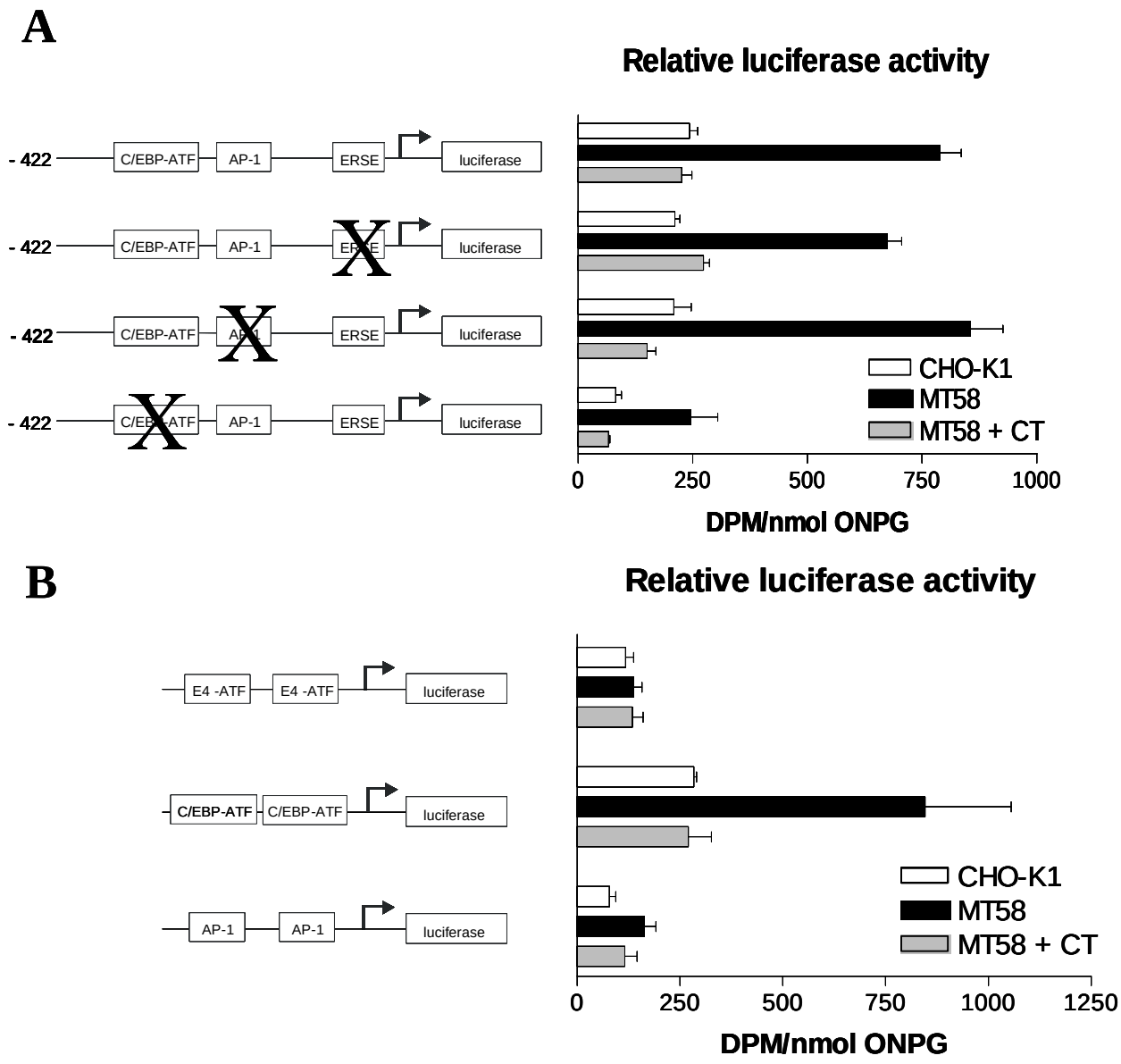

Figure 3. Identification of a C/EBP-ATF site as the active element in the CHOP promoter during PC depletion.

Panel A, Wild-type K1, MT58 and CT-expressing MT58 were transiently transfected with 5' inactive mutans of the C/EBP-ATF, AP-1 or ERSE site in the CHOP promoter region –442 till +91, as described in Experimental procedures. The experiments were performed as described in the legend of Fig. 2. Shown are the means ± S.E. of three independent experiments.

Panel B, Wild-type K1, MT58 and CT-expressing MT58 were transiently transfected with luciferase constructs containing two copies of ATF binding site in the adenovirus E4 promoter (E4-ATF), a C/EBP-ATF binding site in the AARE (C/EBP-ATF) or the distal AP-1 binding site in the c-jun gene promoter (AP-1). The experiments were performed as described in the legend of Fig. 2. Shown are the means ± S.E. of three independent experiments.

The transcription factor ATF2 is activated by phosphorylation during PC depletion

A transcription factor that was identified to bind C/EBP-ATF during CHOP activation is ATF2 (50). To identify whether this transcription factor is activated during PC depletion, CHO-K1 and MT58 cells were incubated for various time points at 40 ˚C and whole cell extracts were prepared for western blotting using phosphor-specific antibodies against ATF2. The results demonstrate that the temperature shift in MT58 led to an increase of phosphorylated ATF2 after 8 hours till 24 h (see Fig. 4). Incubation of wild-type CHO-K1 at 40 ˚C for 24 h had no effect on the level of phosphorylated ATF2. The AP-1 binding transcription factor c-Jun was only increased after 24 h in MT58 when cultured at 40 ˚C (data not shown).

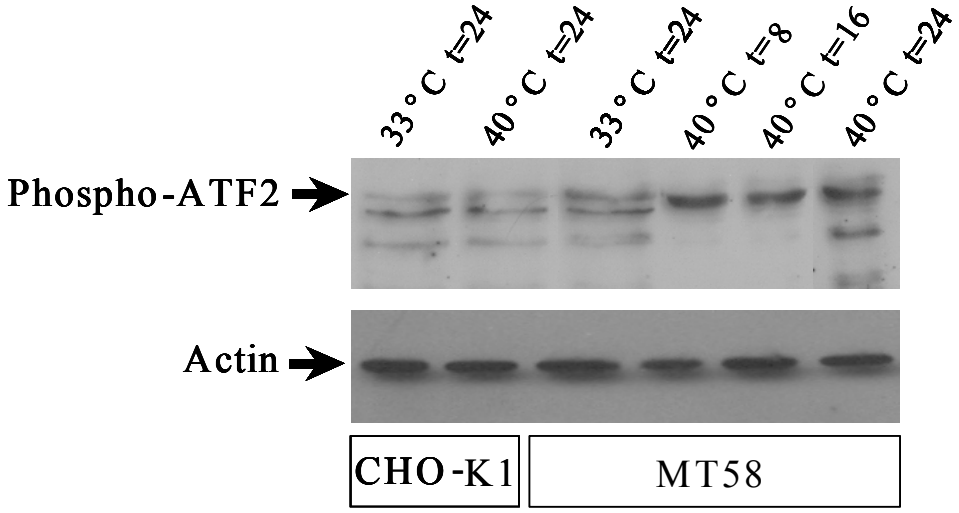

Figure 4. ATF2 is phosphorylated during inhibition of PC synthesis. The role of a potential regulatory transcription factor of CHOP expression was investigated by analysis of the phosphorylation status of the transcription factor ATF2. CHO-K1 and MT58 cells were cultured in 60 mm dishes at 33 ˚C or switched to 40 ˚C for the indicated time periods. Cells were harvested and aliquots were taken to determine the protein concentration. Total cell homogenates (10 μg of protein) were separated on SDS-PAGE and the amount of phospho-ATF2 and actin (the internal control) were detected by western blotting using rabbit polyclonal α-phospho-ATF2 antibody, and a goat polyclonal α-actin antibody, respectively. Results shown are representative for three independent experiments.

Inhibition of PC synthesis induces the phosphorylation of JNK kinase.

JNK and p38 MAPK were described to be responsible for the phosphorylation of c-jun during CHOP transcription activation. JNK was also suggested to play a role in ATF2 phosphorylation. To determine if JNK or p38 MAPK are involved in the CHOP induction, caused by PC depletion, we assessed the effect of inhibition on PC synthesis on the

phosphorylation status of these kinases. Phosphorylation of JNK and p38 MAPK is necessary for their activity in signaling cascades in response to cellular stresses. Culturing MT58 cells at 40 °C resulted in an increase in the phosphorylation state of JNK at 16 and 24 h (see Fig. 5A)

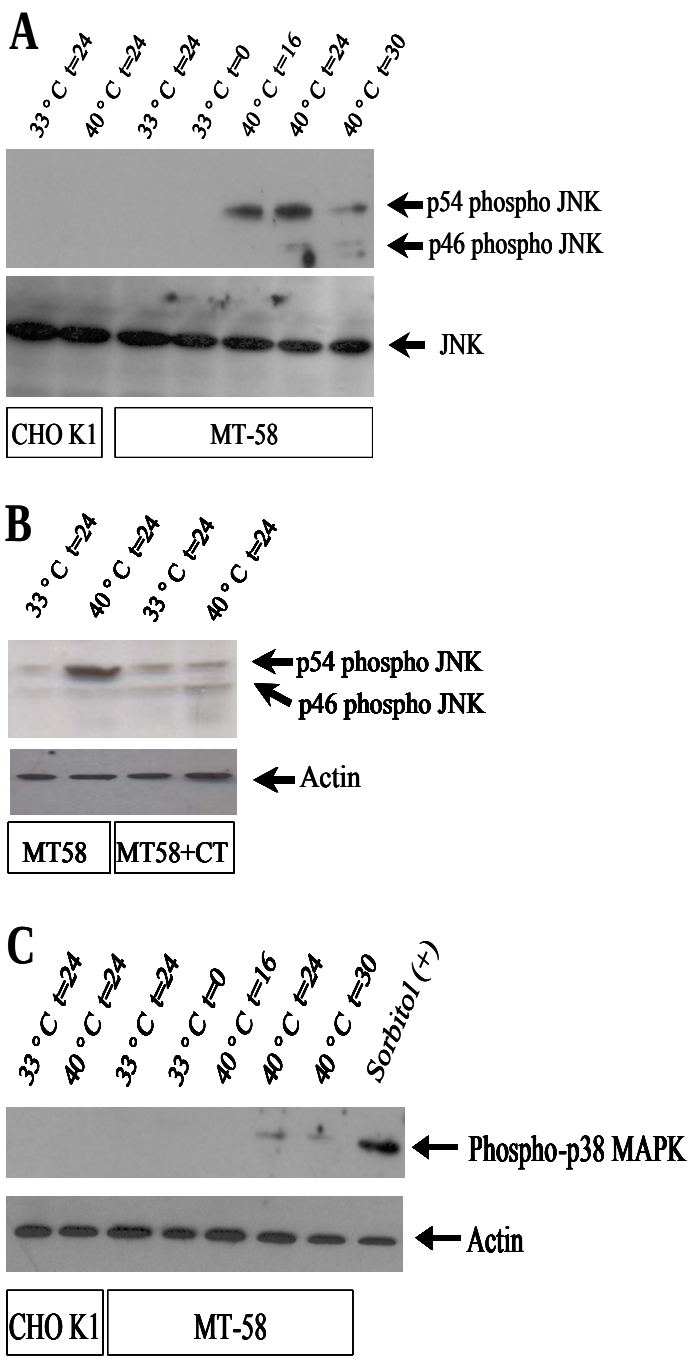

Figure 5. JNK is phosphorylated during inhibition of PC synthesis. To identify a kinase possible responsible for the phosphorylation of ATF2, we investigated the phosphorylation status of the stress-induced kinases JNK (Panel A + B) and p38 MAPK (Panel C). CHO-K1 and MT58 cells were cultured in 60 mm dishes at 33 ˚C or switched to 40 ˚C for the indicated time periods. As positive control for p38 MAPK CHO-K1 cells were treated with sorbitol. Cells were harvested and aliquots were taken to determine the protein concentration. Total cell homogenates (10 μg of protein) were separated on SDS-PAGE and the amount of phospho-JNK, phospho-p38 MAPK, JNK and actin (the internal controls) were detected by western blotting using rabbit polyclonal α-phospho-JNK antibody, a rabbit polyclonal α-phospho-p38 MAPK antibody, rabbit polyclonal α-JNK antibody and a goat polyclonal α-actin antibody, respectively. Results shown are representative for three independent experiments.

In contrast, CHO-K1 cells did not contain any phosphorylated JNK. The phosphorylation of JNK at 40 ºC observed in MT58 cells was clearly correlated with the defect in PC synthesis as MT58 cells over-expressing CTα showed no increase in phosphorylated JNK (see Fig. 5B). Incubation of CHO-K1 or MT58 cells at the non-permissive temperature did not result in the

phosphorylation of p38 MAPK, although these cells were capable of phosphorylating p38 in response to sorbitol treatment (see Fig 5C). Taken together these results show that active JNK is present in MT58 cells incubated at the non-permissive temperature, and therefore may play a role in the response of these cells to PC depletion.

JNK is involved in the apoptotic process of PC depleted cells, but not in the induction of CHOP.

It has been shown that the JNK inhibitor, SP600125, specifically inhibits activation of JNK in response to stress stimuli (47, 48). To investigate the role of JNK in the apoptosis of PC depleted cells, we determined if MT58 cells, incubated at the non-permissive temperature, could be rescued by adding SP600125 to the medium at various time points. In the presence of SP600125 only 10% ± 2% of the MT58 cells became apoptotic after 48 h at the non-permissive temperature (see Fig. 6; upper panel). In contrast, 60% ± 5% of the MT58 cells died in the absence of the JNK inhibitor. MT58 cells grown at 40 ˚C could be rescued almost completely if SP600125 was added within 8 hours and partly when added within 16 h (see Fig. 6; upper panel). Addition of the inhibitor after 24 h or later did not prevent the apoptotic process. In contrast, addition of p38 MAPK inhibitor SB203580 was not capable to rescue

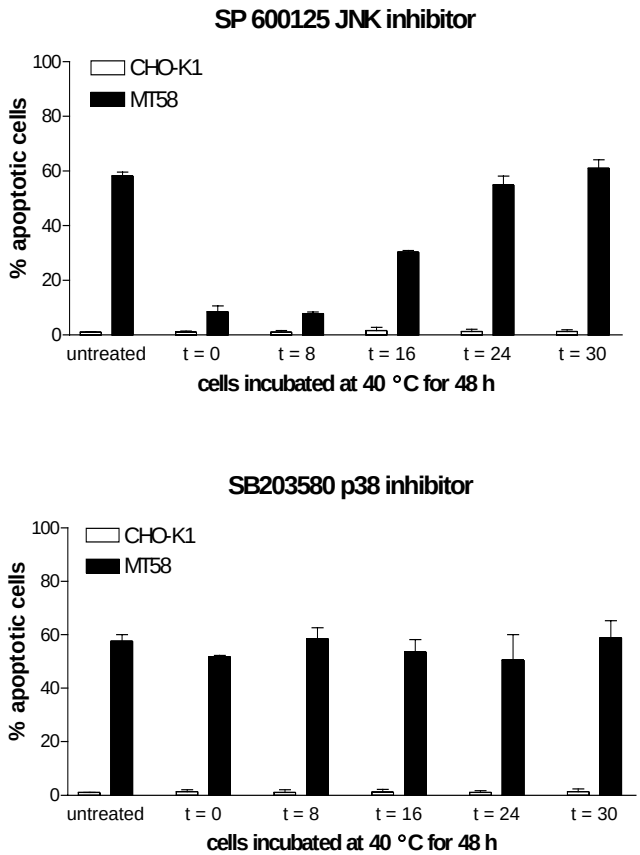

Figure 6. SP600125 JNK inhhibitor rescues cells from PC depletion-induced apoptosis. MT58 cells were treated with the JNK inhibitor SP600125 *(upper panel*) or the p38 MAPK inhibitor SB203580 (*lower panel*). To investigate if these inhibitors can rescue MT58 cells from apoptosis, sub-confluent cells were shifted to 40 ˚C. SP600125 (final concentration 40 μM) and SB203580 (final concentration 20 μM) were added at the indicated time-points to the cells and refreshed every 24 h afterwards. Cells were harvested 48 h after shifting cells to 40 ˚C, and apoptosis was assessed as described in Experimental procedures. Open bars, wild-type K1; black bars, MT58. Shown are the means ± S.E. of three independent experiments.

MT58 cells from apoptosis during incubation at 40 ˚C (see Fig. 6; lower panel). These results suggest that the activation of JNK kinase takes place before 16 h and its effect is an early event in the apoptotic process of MT58 cells, depleted of PC.

To elucidate the role of JNK in CHOP expression, MT58 cells were pre-treated with SP600125 before shifting to 40 ˚C. JNK inhibitor-treated MT58 cells showed an elevated CHOP expression at 16 and 24 h, comparable to CHOP expression levels of untreated MT58 cells (see Fig. 7A). The CHOP induction observed in MT58 cells is not an effect of the inhibitor, because SP600125 treated MT58 cells, incubated at 33 ˚C or SP600125 treated wild-type K1 cells, incubated at 33 ˚C or 40 ˚C did not show induction of CHOP expression. Furthermore ATF2 phosphorylation is also not depending on JNK. JNK inhibitor-treated MT58 cells showed an elevated level of phospho-ATF2 at 8, 16 and 24 h, comparable to phospho-ATF2 levels of untreated MT58 cells (see Fig. 7B). In conclusion we have evidence that JNK plays a role in the apoptotic processes of PC depleted cells but is not required for the induction of CHOP or the phosphorylation of ATF2.

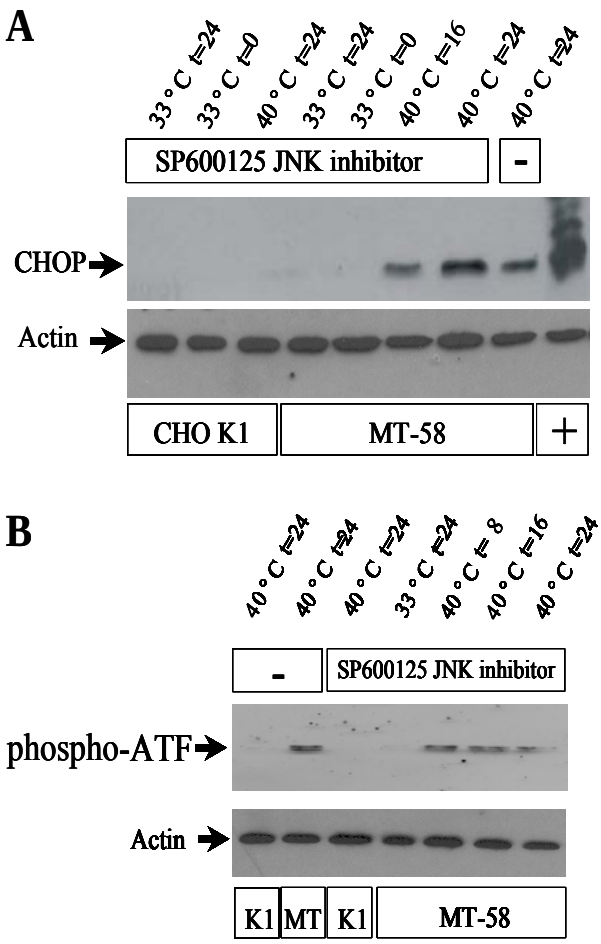

Figure 7. CHOP expression and ATF2 phosphorylation is not dependend on JNK during PC depletion. CHO-K1 and MT58 cells were shifted to 40 °C and incubated in the absence or presence of 40 μM SP600125 for the indicated time periods. *Panel A:* CHOP expression was estimated by western blotting of equal amounts of proteins (10 μg) from whole cell extracts, using specific rabbit polyclonal αCHOP as first antibody, and goat-anti-rabbit horseradish peroxidase-conjugated antibody as secondary antibody. Identical blots were probed for actin as internal control. These finding were reproduced in four independent experiments. *Panel B:* Phospho-ATF2 levels were estimated by western blotting on equal amounts of proteins (10 μg) from whole cell extracts, using rabbit polyclonal α-phospho-ATF2 antibody, and a goat polyclonal α-actin antibody antibody, respectively. Results shown are representative for two independent experiments.

Discussion

Inhibition of PC biosynthesis induces apoptosis by an unknown pathway. In previous work we reported that inhibition of PC synthesis induces the expression of the pro-apoptotic, ER stress-related protein CHOP, but does not result in a canonical ER stress response (22). We demonstrate here that the induction of CHOP plays a significant role in the induction of apoptosis, caused by PC depletion, since inhibition of CHOP expression by anti-sense CHOP mRNA significantly delays the onset of the terminal execution phase of the apoptotic process. This suggests that CHOP expression is an early upstream event in the signaling cascade initiated in response to PC depletion, eventually leading to apoptosis. However, prolonged inhibition of PC synthesis still results in apoptosis in CHOP anti-sense MT58 cells implying that other stress pathways not involving CHOP are activated as well.

CHOP is a transcription factor that is induced by cellular stress, especially ER stress. However, CHOP induction has also been observed in apoptotic pathways, independent of the ER stress response. For instance, amino acid limitation, especially leucine and arginine, up-regulates CHOP expression via a response element, known as the AARE (29, 41). Here we report that a *cis* DNA sequence located upstream from the transcription start site (-442 to –211) is involved in the activation of CHOP expression during PC depletion. Evidence is provided that a C/EBP-ATF site in this region is responsible for the increased CHOP expression during PC depletion. The C/EBP-ATF composite site present in the CHOP promoter is highly conserved between human and hamster (49). The C/EBP-ATF composite site in the CHOP promoter is a part of the AARE and can interact with specific members of the C/EBP and ATF/CREB transcription factor families (29, 43, 50). All members of these families contain a DNA binding domain consisting of a cluster of basic amino acids and a leucine zipper region (b-ZIP domain; 51). They can form homodimers or heterodimers through their leucine zipper regions (52, 53). The group of Fafournoux reported that binding of ATF2 to the C/EBP-ATF composite site of the AARE is essential for the transcriptional activation of CHOP by amino acid deprivation (29). Our present results demonstrate that the transcription factor ATF2 is phosphorylated at an early stage in MT58 cells at the non-permissive temperature, preceding CHOP expression. In contrast, the transcription factor c-jun is phosphorylated after 24 h, indicating that it is not involved in the regulation of CHOP expression. Therefore, it is tempting to speculate that CHOP expression during PC depletion might be mediated by binding of ATF2 to the C/EBP-ATF site.

The trans-activating capacity of ATF2 is depending on phosphorylation of N-terminal residues Thr-69, Thr-71 and Ser-90 by stress-activated protein kinases (54-56). JNK and p38 MAPK were implicated in the phosphorylation of transcription factors involved in CHOP expression (27, 44). JNK is present in an active, phosphorylated state in MT58 cells incubated at 40 °C and treatment of MT58 cells with the specific JNK inhibitor SP600125 can rescue these cells from apoptosis. Therefore, JNK activity could be responsible for the phosphorylation of ATF2 during PC depletion and the consequent induction of CHOP. However, we still observed CHOP induction and ATF2 phosphorylation in MT58 cells at the non-permissive temperature that were treated with SP600125. These results suggest that JNK is not essential for the induction of CHOP expression and ATF2 phosphorylation. Other kinases, besides JNK and p38 MAPK, have been identified to be capable of ATF2 activation. Induction of growth arrest by treatment of cells with the tumor promoters 12-*0*-tetradecanoyl-phorbol 13-acetate (TPA) and Saikosaponin *a* depends on ATF2 phosphorylation by ERK, another MAPK family member (57). ATF2-dependent positive regulation of the human insulin gene requires Ca^{2+}/calmodulin dependent protein kinase IV (CaM kinase IV). This kinase phosphorylates ATF2 also on Thr73, in combination with Thr69 and Thr71 (58). Protein kinase A is known to phosphorylate ATF2 on Ser62 in response to cyclic AMP (59). Moreover, a recently identified, nuclear Ser-Thr kinase, vaccinia-related kinase-1 (VRK1) is described to phosphorylate ATF2 on Ser62 and Thr73, thereby stabilizing the ATF2 protein which results in its activation (60). ATF2 is likely to integrate many types of cellular signals that might reach the transcription factor by different kinases. The group of Lazo reported that phosphorylation of ATF2 by two kinases, VRK1 and JNK on different amino acids can have an additive effect on the activation of transcription (60). This dual activation may cooperate different signals if all of them are acting at sub-optimal conditions, or be exclusive if any of them reaches a maximum effect. Therefore, we cannot exclude that in this study ATF2 might also be phosphorylated by JNK in combination with another kinase. In that case, inhibition of JNK with SP600125 would still result in ATF2 phosphorylation and CHOP induction by this other kinase. Furthermore, JNK could be involved in phosphorylation of CHOP, rather than its induction. Both JNK and p38 MAPK are able of phosphorylating CHOP on Ser78 and Ser81 in response to stress (26, 44). Phosphorylation of CHOP is required for enhanced transcriptional activation of its downstream targets (45). On the other hand, the JNK signaling could also be independent of CHOP signaling. As MT58 will be faced with several insults during prolonged PC depletion by the reduction of membranes, these insults will evoke early and late stress responses. CHOP induction seems to play a role in the early response, whereas

JNK could be responsible for late events. This is in agreement with the fact that we observe phosphorylation of c-jun after 24 h.

In conclusion, we have shown that the induction of the pro-apoptotic CHOP gene by PC depletion is more similar to its induction in the response to amino acid limitation compared to glucose deprivation as it involves the C/EBP-ATF response element, and presumably activation of ATF2, rather than the ER stress/UPR pathway. The latter (ER stress) pathway was shown to be involved in the apoptotic response to cholesterol loading in macrophages, a treatment that was suggested to cause a misbalance between cholesterol and phospholipids in biological membranes (61). Since PC depletion is also likely to cause a change in the phospholipid-to-cholesterol ratio, it is interesting to note that both membrane-disturbing treatments cause an induction of CHOP, but by different mechanisms. Defining the precise upstream cascade of molecular events that result in CHOP expression, ATF2 phosphorylation and JNK activation by inhibition of PC synthesis will be an important contribution to the understanding of phospholipid homeostasis in mammalian cells.

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CHAPTER 5

Inhibition of phosphatidylcholine synthesis is not the primary pathway in hexadecylphosphocholine-induced apoptosis

Michiel H.M. van der Sanden, Martin Houweling, Daniël Duijsings, Arie B. Vaandrager and Lambert M.G. van Golde

Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, and Institute of Biomembranes, University of Utrecht, The Netherlands

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Synopsis

The anticancer drug hexadecylphosphocholine (HePC), an alkyl-lysophospholipid analog, has been shown to induce apoptosis and inhibit the synthesis of phosphatidyl-choline (PC) in a number of cell lines. We investigated whether inhibition of PC synthesis plays a major causative role in the induction of apoptosis by HePC. We therefore directly compared the apoptosis caused by HePC in CHO cells to the apoptotic process in CHO-MT58 cells, which contain a genetic defect in PC synthesis. HePC-provoked apoptosis was found to differ substantially from the apoptosis observed in MT58 cells, since it was (i) not accompanied by a large decrease in the amount of PC and diacylglycerol, (ii) not preceded by induction of the pro-apoptotic protein GADD153/CHOP, and (iii) not dependent on the synthesis of new proteins. Furthermore, lysoPC as well by lysophosphatidylethanolamine could antagonize the apoptosis induced by HePC, whereas only lysoPC was able to rescue MT58 cells. HePC also induced a rapid externalisation of phosphatidylserine. These observations suggest that inhibition of PC synthesis is not the primary pathway in HePC-induced apoptosis.

Introduction

A number of alkyl-lysophospholipid analogs (ALPs) possess a potent anti-neoplastic activity. These anticancer lipids include 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3) and hexadecylphosphocholine (HePC), which were both tested with encouraging results in clinical settings as a purging agent in autologous bone marrow transplantation and in the treatment of skin metastases of breast cancer, respectively (1). The anticancer effect of these ALPs is believed to be caused by their apoptotic effects on transformed cells. The mechanism by which these lipids induce apoptosis however is not known. ALPs were shown to affect the activity of various enzymes in biological membranes, which may contribute to their apoptotic action (1, 2). Especially the inhibition of CTP:phosphocholine cytidylyltransferase (CT), the rate limiting enzyme in the biosynthesis of phosphatidylcholine (PC), is considered a key target in this respect (3-6). The subsequent inhibition of PC synthesis was suspected to cause the induction of apoptosis, as addition of lysoPC prevented the cytotoxic effect of both HePC and ET-18-OCH_3, presumably by providing a source for PC synthesis independent of CT activity (3, 5). In case of HePC treatment, lysoPC could even restore normal cell proliferation. In contrast lysoPC could not reverse the cytostatic effect of ET-18-OCH_3 (3). Likewise, overexpression of CT could prevent the apoptosis induced by ET-18-OCH_3, but could not restore proliferation of the HeLa cells in the presence of this ether lipid (4). The choline head group was shown to be essential for the inhibition of CT and the antiproliferative effect of HePC, because hexadecylphosphoserine and hexadecylphosphoethanolamine did not act as inhibitors of PC synthesis and did not induce apoptosis (6). In line with a causative relationship between inhibition of PC synthesis and apoptosis, choline deficiency was shown to induce apoptosis in cell lines as well as *in vivo* (7). Further evidence that inhibition of PC synthesis can induce apoptosis comes from a cell line with a temperature sensitive defect in CT (8). The MT58 cell line is a Chinese hamster ovary (CHO) derived cell line with a mutation in CT-α, which renders the enzyme unstable at elevated temperatures. MT58 cells can grow and sustain normal PC levels at the permissive temperature of 33 ^{o}C. However, shifting these cells to 40 ^{o}C leads to a more than 50 % drop in PC levels and a subsequent growth arrest and apoptosis (8-10). Addition of exogenous PC or lysoPC could rescue the MT58 cells at the non-permissive temperature (11), suggesting that indeed a lack of PC or a PC derived compound caused the observed apoptosis. As expected transfection with recombinant wild type CT-α or CT-β also allowed MT58 cells to

grow normally at the non-permissive temperature (12, 13). Recently it was shown that one of the earliest events in the PC depleted MT58 cells was the induction of the pro-apoptotic ER-stress-related protein GADD153/CHOP (10).

To investigate whether inhibition of PC synthesis plays a major causative role in the apoptosis induced by the anti-neoplastic ether lipids, we directly compared the induction of apoptosis by HePC in wild type CHO-K1 to the apoptosis in MT58 cells grown at the non-permissive temperature.

Here we report that the HePC-provoked apoptosis differs substantially from the apoptosis observed in cells with a genetic defect in PC synthesis in that, (i) no depletion of PC was observed, (ii) it is not preceded by an induction of CHOP and does not require the synthesis of new proteins and (iii) it can be rescued by both lysoPC and lysophosphatidylethanolamine. These observations argue against a major causative role of CT inhibition in the HePC-induced apoptosis.

Materials and Methods

Materials

HAM's F12 nutrient mixture and foetal bovine serum (FBS) were from Gibco BRL, Paisley, Scotland and culture dishes and flasks from Nunc Inc. (Rochester, NY, USA). The radio-chemicals [^{3}H-methyl]choline chloride (83 Ci.mmol^{-1}), [1-^{3}H]ethanolamine hydrochloride (17.9 Ci.mmol^{-1}) and [(-^{32} P]ATP (3000 Ci.mmol^{-1}) were from Amersham International, Bucks, United Kingdom. Prefab Silica Gel G60 thin-layer chromatography plates were purchased from Merck (Darmstad, Germany), and lysoPC and lysoPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA). The Supersignal chemiluminescent substrate kit (ECL) to detect proteins on immunoblots was supplied by Pierce (Cheshire, UK). All other, not specified chemicals were of analytical grade.

Cell culture

CHO-K1 and CHO-MT58 cells were cultured in HAM's F12 containing 7 % FBS, 100-units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in 80 cm^{2} culture flasks at 33 ^{o}C, 5% CO_2 and 90% relative humidity and were sub-cultured twice a week and media changed every 2-3 days

Incorporation of radioactive precursors into phosphatidylethanolamine and phosphatidylcholine

Cells were plated at a density of 3 x 10^5 cells in 60 mm dishes containing 5 ml of medium and incubated at 33 ^oC. 24 h after plating the cells were pre-incubated for 30 min at 40 ^oC in the presence of various concentrations HePC and subsequently pulsed with [^{3}H]ethanolamine and [^{3}H]choline for 1 h. Incubations were stopped by removing the medium, washing the cells three times with ice-cold phosphate buffered saline (PBS) and addition of methanol. Lipids were extracted from the cells by the method of Bligh and Dyer (14) and phospholipids were separated by thin-layer chromatography (TLC) on prefab silica G60 plates in a solvent system of chloroform:methanol:water 65:35:4 (v/v/v). Phospholipids were visualised with iodine vapour and identified by comparison to known standards. The silica was scraped off the plate and the amount of radioactivity incorporated was determined by liquid scintillation counting.

Quantification of apoptosis

Cells were plated at a density of 3 x 10^5 cells in 60 mm dishes containing 5 ml of medium and incubated at 33 ^oC. In the experiments with CHO-K1 cells, 24 h after plating incubations were started by adding various agonists or their solvent (control) and cells were shifted to 40 ^oC. In case of the MT58, cells were incubated at 33 ^oC or at the non-permissive temperature of 40 ^oC. At the indicated time-points culture medium was removed, and cells were washed twice with PBS and fixed in methanol, prior to staining with propidium-iodide (final concentration; 2.4 μM). Stained cells were observed using a LEICA DMR fluorescence microscope. Cell numbers were established by counting total population of nuclei. Live and apoptotic cells were differentiated from each other on the basis of definitive nuclear fluorescence and distinct morphological changes, including nuclear condensation and nuclear fragmentation. Condensed or fragmented nuclei were scored as apoptotic. A minimum of 200 cells was counted in five random fields per dish.

Determination of sn-1,2-diacylglycerol and ceramide levels

The different cell lines were incubated under various conditions, lipids were extracted and the amount of diacylglycerol (DAG) and ceramide was determined enzymatically with DAG-kinase exactly as described by Preiss *et al.* (15) except for the TLC procedure. [(-^{32}P]phosphatidic acid and [(-^{32}P]ceramide-phosphate were separated on prefab silica G60 plates with chloroform:pyridine:formic acid (60:30:7; v/v/v) as the developing solvent. Spots

were visualized using autoradiography and identified using known standards. The silica containing [(-^{32}P]phosphatidic acid and [(-^{32}P]ceramide-phosphate was scraped off the plates and the amount of radioactivity was measured by liquid scintillation counting.

Immunoblotting

Cells were washed with ice-cold PBS and scraped off the plate in 100 µl PBS. Aliquots were taken to measure protein content and 50 µl of cell suspension in PBS was lysed with 50 µl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentration; 62.5 mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.003% bromophenol blue, pH 6.8). After boiling samples for 10 min, 4 µg protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 1% western blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 1 h and exposed to rabbit polyclonal anti-GADD153 (CHOP), dilution 1:750. Following four washing steps with TBS-Tween (50 mM Tris; 150 mM NaCl; 1 % Tween20, pH 7.5), blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were washed four times with TBS-Tween and CHOP protein was displayed by a reaction on Supersignal chemiluminescent substrate and exposure to x-ray films.

Detection of plasma membrane phosphatidylserine externalisation.

CHO-K1 cells were inoculated into Delta T4 dishes (BiopTechs, Butler, PA) in DMEM/Ham's F12 medium containing 7% FBS. After 24 hours, when cells had reached a density of approx. 30-50%, adherent cells were washed twice with Hanks' Buffered Saline Solution (HBSS; Invitrogen) and incubated for 30 min in 900 µl Annexin V-FLUOS incubation buffer (Annexin V-FLUOS staining kit; Roche Applied Science, Almere, The Netherlands) containing 2 µg/ml Annexin V-fluorescein, 0.25 µg/ml propidium iodide and 2 µg/ml Hoechst 33342 (Sigma-Aldrich) at 37 ºC and 5% CO_2. After this incubation period, the dish was mounted on a Bioptechs Delta T4 dish incubator (Bioptechs) set at 37 ºC under a Radiance 2100 MP multiphoton microscope (Biorad, Veenendaal, The Netherlands). Samples were examined and at different time points, 100 µl of Annexin V-FLUOS incubation buffer was added containing 100 µM HePC (final concentration 10µM). Subsequently, images were taken with interval periods of 1 min. Propidium iodide fluorescence was imaged using a He/Ne laser (λ_{ex} 543 nm) and a 575-625 nm filter; Annexin V-fluorescein fluorescence was imaged using an Ar laser (λ_{ex} 488 nm) and a 500-530 nm filter; Hoechst 33342 fluorescence

was detected using a Tsunami multiphoton laser (λ_{ex} 780 nm) and a 410-490 nm filter. Laser intensities were kept as low as possible to prevent possible irradiation damage to the cells. Leakage of the plasma membrane was detected by loading the cells with 5 μM BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 30 min in HBBS at 37 ºC and 5% CO_2. After removal of unincorporated dye, BCECF fluorescence was imaged with the multiphoton microscope using an Ar laser (λ_{ex} 488 nm) and a 500-530 nm filter.

Other methods

Cell protein was measured using the Coomassie^R Plus protein assay reagent kit (Pierce) according to the manufacturer's instructions, using BSA as a standard. The amount of phosphate in phospholipids was quantified by phosphorus analysis according to Rouser [16]. Data are expressed as mean ± S.D. All statistical analyses were done using unpaired *t*-testing.

Results

HePC inhibits PC synthesis and induces apoptosis in CHO-K1 cells.

The CHO-mutant cell line MT58 is used as a genetic model to study the effects of inhibition of PC synthesis, because it has a temperature sensitive mutation in the CT-α gene (17). When grown at the non-permissive temperature of 40 ^{0}C, PC synthesis is decreased by more than 80% and the MT58 cells undergo apoptosis between 30 and 72 hours (10). So in order to investigate whether inhibition of PC synthesis is important in the mechanism by which the anti-neoplastic drug HePC induces apoptosis, we compared under the apoptosis induced by HePC in CHO-K1 wild type cells to the apoptosis occurring in MT58 cells grown at 40 ^{o}C. As described for other cell lines, HePC inhibited in a dose-dependent manner the synthesis of PC, but not of phosphatidylethanolamine (PE), and induced apoptosis in CHO-K1 cells grown at 40 ^{o}C (Fig. 1). However, from Table 1 it is clear that this inhibition of PC synthesis is transient. Prolonged (12 h) exposure of CHO-K1 cells to HePC totally abolished its inhibitory effect on PC synthesis. At our conditions, 75 μM HePC was required to induce a massive apoptotic response in CHO-K1 cells, although at 50 μM HePC a small increase in apoptosis was already observed. MT58 cells overexpressing CT-α showed the same sensitivity towards HePC-induced apoptosis (data not shown). At the dose of 75 μM HePC, PC synthesis was inhibited by approximately 60 %, whereas 50 μM HePC caused an inhibition of less than 50 %, as measured by [^{3}H]choline incorporation into PC. In general the observed apoptosis by

HePC occurred earlier (the first apoptotic signs were observed within 12-16 h), than in case of the genetic inhibition of CT in the MT58 cells, which showed no signs of apoptosis until 30 h (10).

HePC effects on PC mass and DAG:ceramide ratio differ from those induced by a genetic defect in PC synthesis.

In the MT58 cells, incubation at 40 ^{o}C causes a rapid depletion of PC levels. After a 12 h incubation at the non-permissive temperature the PC pool was approximately reduced by 50 %, and after 24 h only 20 % of the PC was left [10]. However, as shown in Fig.2, incubation of the parent CHO-K1 cells under similar conditions in the presence of 50-100 μM HePC did affect the amount of PC to a large extent, despite the considerable inhibition of choline incorporation observed at those dosages (see Fig. 1A).

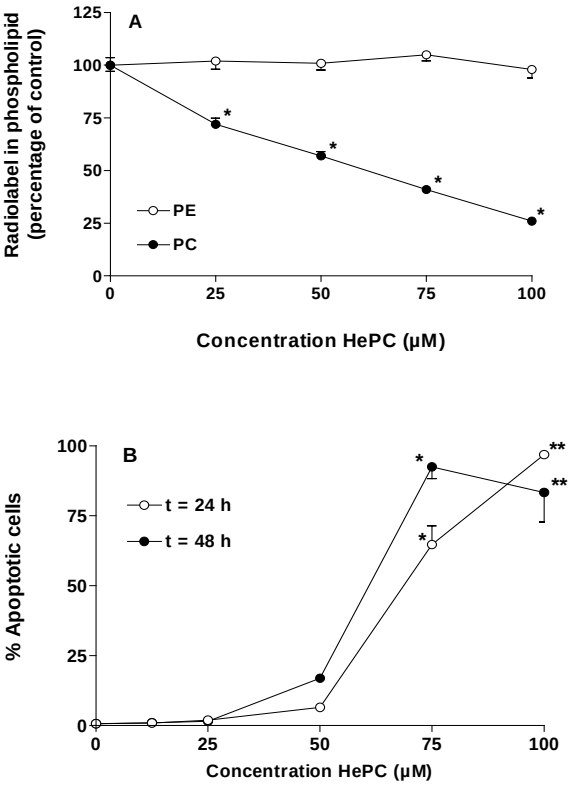

Figure 1. Effect of hexadecylphosphocholine (HePC) on the incorporation of labelled precursors into phosphatidylcholine and phosphatidylethanolamine and apoptosis. CHO-K1 cells were pre-incubated for 30 min at 40 ^{o}C in the absence or presence of variable concentrations HePC and the incorporation of [^{3}H]choline into PC (*closed circles*) and [^{3}H]ethanolamine into PE (*open circles*) was determined after a 60 min pulse with the respective labels (A). Another set of cells was incubated for 24 (open circles) or 48 h (closed circles) with the same concentrations of HePC and the amount of apoptotic cells was scored (B). Values are means ± S.D. of triplicate incubations of one representative experiment, which was repeated twice (n = 3). Significantly different from control: * $P < 0.001$; ** $P < 0.01$

Table 1. Transient effect of HePC on the rate of phosphatidylcholine synthesis

Concentration HePC (μM)	Rate of [^{3}H]choline incorporation into PC (dpm x 10^{-3}/mg protein)	
	Zero time	12 h
0	17.3 ± 0.5	15.3 ± 0.6
25	14.4 ± 0.2	14.9 ± 0.4
50	11.9 ± 0.6	14.5 ± 0.8
100	6.2 ± 0.2	16.3 ± 0.7

CHO-K1 cells were pre-incubated for 30 min at 40 ^{0}C in the absence or presence of variable concentrations HePC (zero time) and the incorporation of [^{3}H]choline into PC was determined after a 60 min pulse. In parallel, dishes were pre-treated for 12 h at 40 ^{0}C in the presence of various concentrations HePC prior to the 30 min pre-incubation with fresh HePC and the subsequent a 60 min pulse with [^{3}H]choline (12 h).

Although the HePC-induced inhibition of PC synthesis did not lead to large changes in PC pool size, it might still affect the levels of lipid signaling molecules whose formation is dependent on PC synthesis. The lipid second messenger DAG, which is generally thought to enhance cell survival pathways, can be directly formed from PC, and PC is required for the conversion of the pro-apoptotic lipid ceramide into sphingomyelin (SM) by donating the phosphocholine head group. Therefore, we next investigated the effects of HePC on DAG and ceramide levels. As shown in Fig. 3, HePC at a concentration known to induce apoptosis (100 μM), increased the levels of both the DAG and ceramide approximately two-fold, causing only a small change in the ratio of these signaling lipids. This ratio is considered to be an important parameter in inducing apoptosis (18). In contrast, incubation of the MT58 cells at 40 ^{o}C resulted in a four-fold decrease in the DAG to ceramide ratio, due to a two-fold increase in ceramide accompanied by a two-fold decrease in DAG levels (Fig. 3).

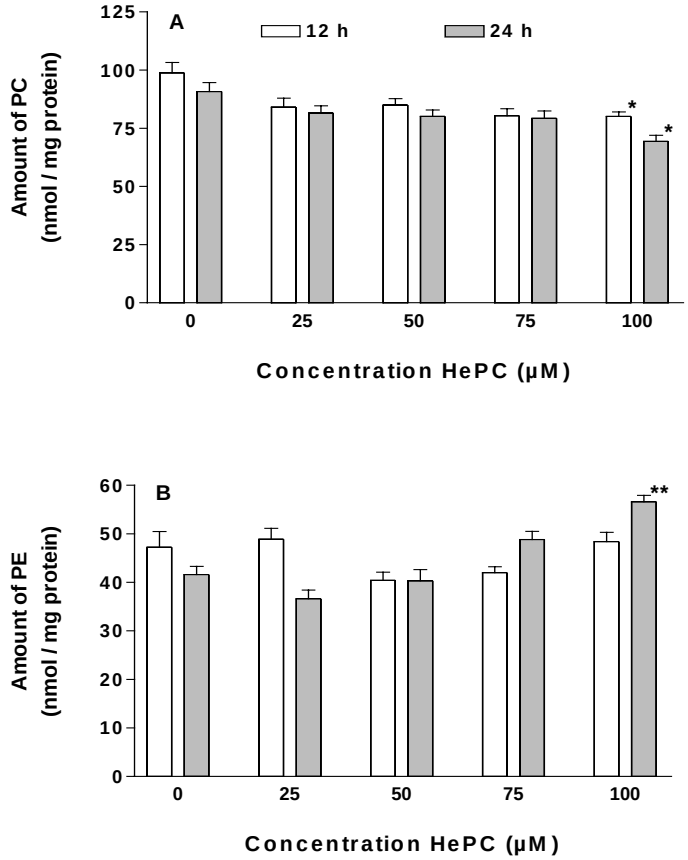

Figure 2. Effect of HePC on the amount of phosphatidylcholine and phosphatidylethanolamine in CHO-K1 cells. CHO-K1 cells were incubated in the absence or presence of different concentrations HePC. After 12 (open bars) and 24 h (closed bars) the incubations were stopped and the amount of PC (A) and PE (B) was determined by phosphor analysis as described in Material and methods. Values are means ± S.D. of triplicate incubations of one representative experiment, which was repeated twice (n = 3). Significantly different from control: * $P < 0.02$; ** $P < 0.01$

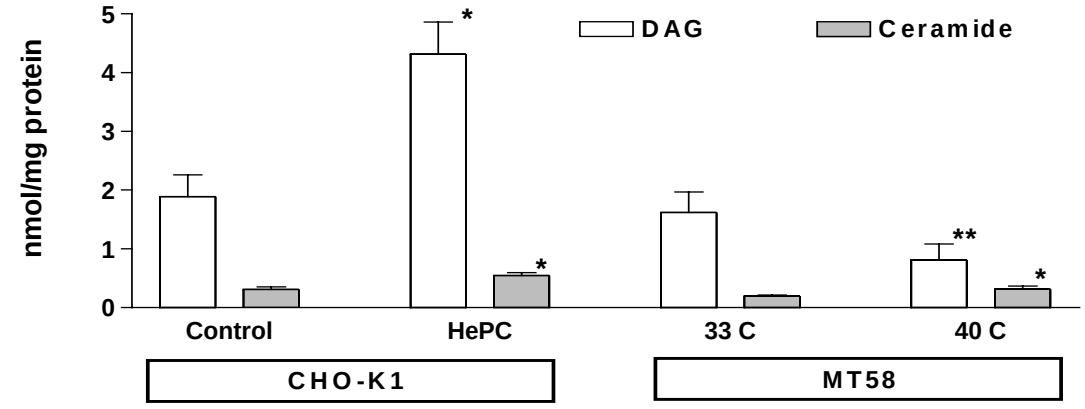

Figure 3. Inhibition of phosphatidylcholine synthesis: Effect on diacylglyderol and ceramide levels in CHO-K1 and MT58 cells. CHO-K1 and MT58 cells were grown at 33 °C on 100-mm dishes till approx. 50% confluency Subsequently, CHO-K1 cells were shifted to 40 °C and incubated in the absence or presence of 100 :M HePC for 16 h. At the same time half of the MT58 samples were shifted to 40 °C, and the remaining cultures were left at 33 °C as control. After 16 h the incubations were stopped, lipids extracted and DAG (open bars) and ceramide (solid bars) levels were measured by the diacylglycerol kinase assay, as described in Materials and methods. The results represent the means ± S.D. of three independent experiments, each performed in triplicate. * $P < 0.05$ *versus* control; ** $P < 0.005$ *versus* control.

HePC does not induce the expression of CHOP

We have shown previously, that PC depletion in MT58 cells causes the induction of the transcription factor CHOP/GADD153, prior to the onset of the execution phase of apoptosis (see Fig. 4), or after the start of apoptosis (10). However, HePC at apoptotic concentrations, did not induce the expression of CHOP in CHO-K1 cells either before (8 h) or after the onset (24 h) of apoptosis (Fig. 4).

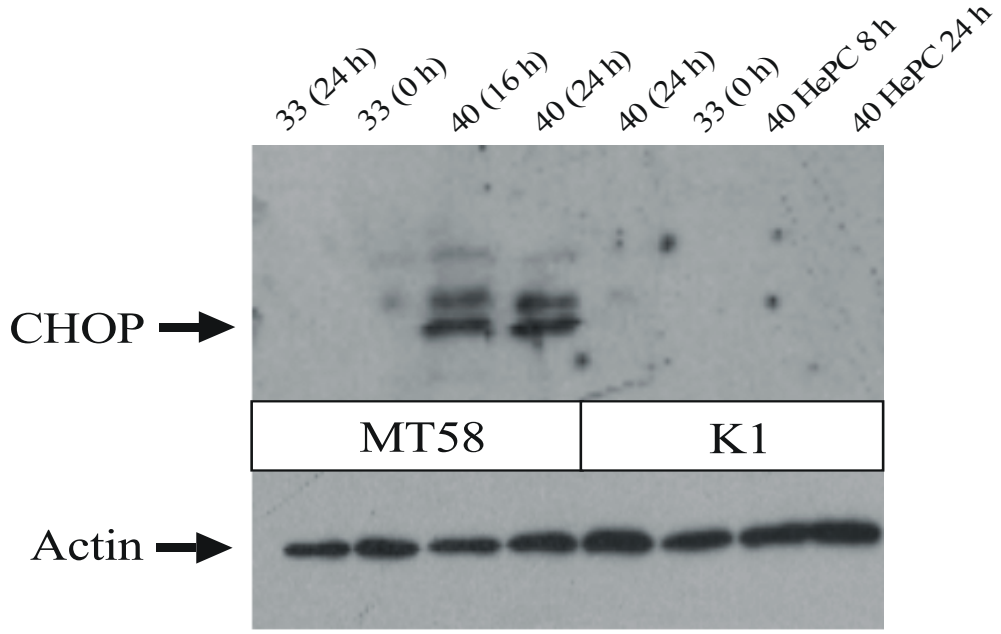

Figure 4. CHOP levels in phosphatidylcholine-depleted MT58 and HePC-treated CHO-K1 cells. CHO-K1 cells were shifted to 40 °C and incubated in the absence or presence of 75 :M HePC for the indicated time periods. At the same time MT58 cells were shifted to 40 °C for 16 and 24 h, whereas some cultures were left at 33 °C as control. CHOP expression was estimated by Western blotting on equal amounts of proteins (4 μg) from whole cell extracts, using specific rabbit polyclonal α-GADD153/CHOP as first antibody, and goat-anti-rabbit horseradish peroxidase-conjugated antibody as secondary antibody. Identical blots were probed for actin as internal control. These finding were reproduced in three independent experiments.

We next investigated whether the induction of a pro-apoptotic factor like CHOP was required for the induction of apoptosis by incubating the cells with the translation inhibitor cycloheximide. As shown in Fig. 5, the induction of apoptosis by HePC did not depend on the induction of newly synthesized proteins as cycloheximide stimulated the apoptosis in combination with HePC. In contrast, cycloheximide almost completely prevented the

apoptosis of MT58 cells incubated at the non-permissive temperature. Since cycloheximide did not affect the decrease in PC levels in the MT58 cells incubated at 40 ^{o}C (data not shown), this suggests that PC depletion leads to apoptosis by induction of a newly synthesized factor, possibly CHOP.

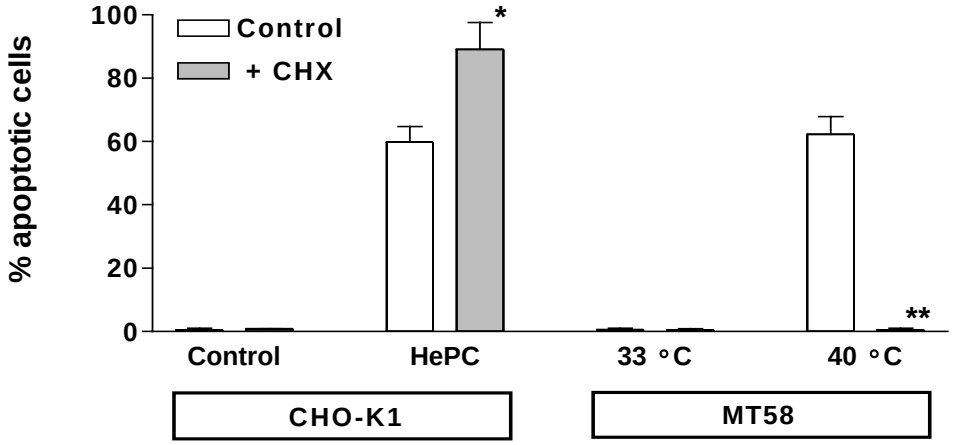

Figure 5. Effect of cycloheximide (CHX) on HePC- or phosphatidylcholine depletion-induced apoptosis. CHO-K1 and MT58 cells were plated on 60-mm dishes at a density of 3 X 10^5 cells/dish. After 24 h at 33 °C, CHO-K1 cells were shifted to 40 °C and to half of the samples HePC (75 :M) was added. MT58 cells were shifted to 40 °C or left at 33 °C. CHX (20 :g/ml) was added to the cells 30 min before exposure to HePC (CHO-K1) or shift to the non-permissive temperature (MT58). Cell viability was assessed as described in Materials and methods. Open bars, control; solid bars, CHX-treated. Shown are the means ± S.E. of two independent experiments. *, $p < 0.05$ *versus* control; **, $p < 0.005$ *versus* control

LysoPE and lysoPC can protect CHO-K1 cells against HePC-provoked apoptosis

Compelling evidence for implicating the inhibition of CT as a causative factor in HePC-, or ALP-induced apoptosis, was the observation that exogenous lysoPC could protect against the apoptotic effects of the ALPs in a number of cell lines (3, 5). On the other hand, based on the experiments described above, we have no indication that CT-inhibition plays a large role in the HePC-induced apoptosis. Therefore, we examined whether lysoPC could protect the CHO-K1 cells against HePC treatment. As shown in Fig. 6, lysoPC was able to protect both the MT58 cells grown at 40 ^{o}C and the HePC-treated CHO-K1 wild type cells from apoptosis. To confirm that the rescue by lysoPC was specific, we also tested lysoPE. As expected for a singular defect in PC synthesis, lysoPE was unable to prevent the apoptosis in MT58 cells. Despite the fact that lysoPE was unable to attenuate the effect of HePC on PC synthesis (data

not shown), it was as potent as lysoPC in rescuing CHO-K1 cells treated with HePC (see Fig. 6). This suggests that a more general disturbance in membrane structure rather than a specific disturbance in PC metabolism underlies the HePC–induced apoptosis.

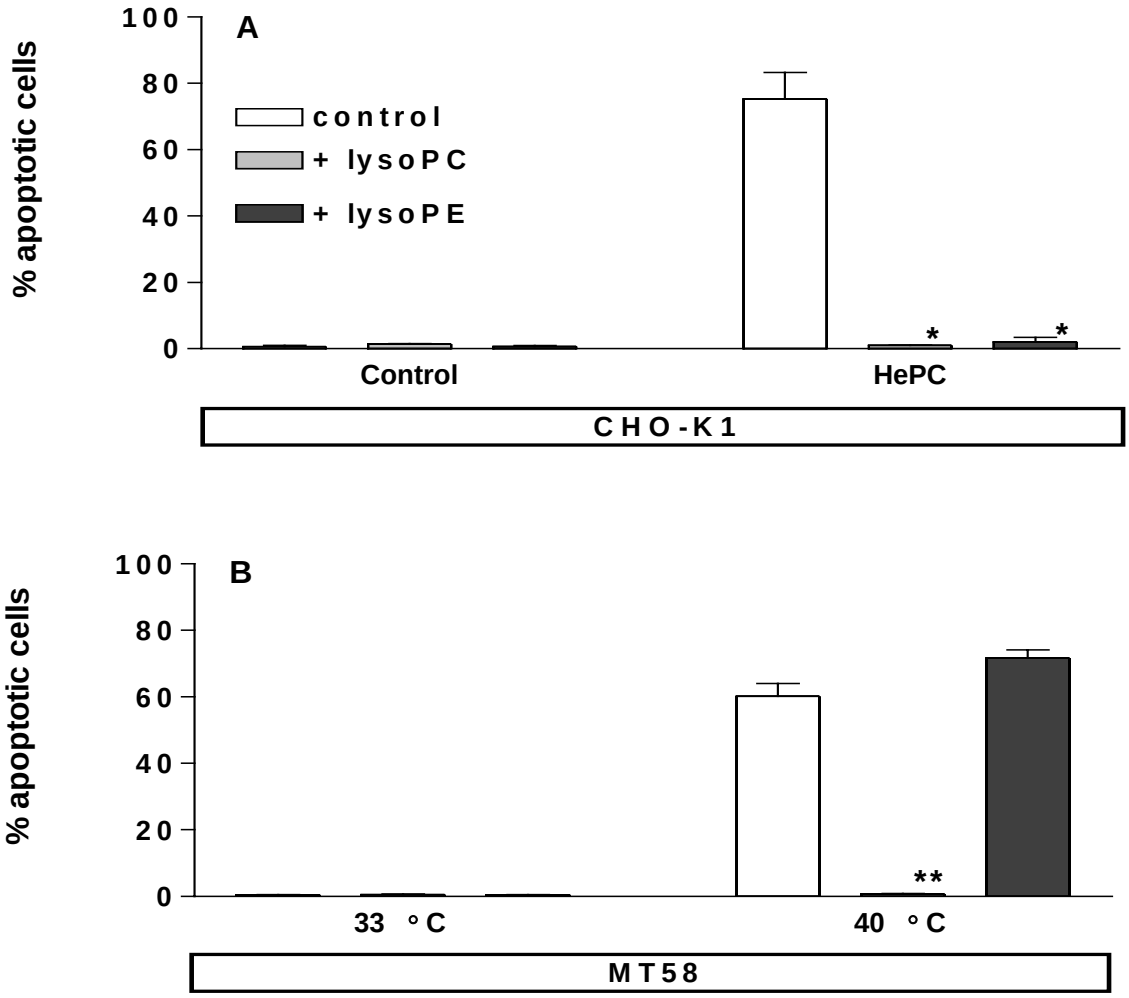

Figure 6. HePC-treated CHO-K1 cells can be rescued from apoptosis by adding lysoPC and lysoPE. CHO-K1 and MT58 cells were grown to approx. 50% confluency and subsequently shifted to 40 °C. *Panel A*, CHO-K1 cells were supplemented with 75 :M HePC or left untreated as control. LysoPC and LysoPE were added to a final concentration of 25 :M and 75 :M, respectively, 30 min after addition of HePC. Cells were harvested 24 h after addition of HePC, and apoptosis was assessed as described in experimental procedures. *Panel B*, MT58 cells were shifted to 40 °C or left by 33 °C. Prior to the temperature shift LysoPC and LysoPE were added to a final concentration of 25 :M and 75 :M, respectively and refreshed every 24 h afterwards. MT58 cells were harvested 48 h after the temperature shift, and apoptosis was assessed as described in experimental procedures. Open bars, control; grey bars, LysoPC-treated and black bars, LysoPE-treated. Shown are the means ± S.E. of three independent experiments.*, significantly different from control at $p < 0.005$.

HePC has rapid effects on PS externalization

The capacity of HePC to affect the structure of membranes was also noted in experiments directed to detect the exposure of phosphatidylserine (PS) to the outer leaflet of the plasma membrane. In general, the preferential localization of PS in the inner leaflet is disturbed by the activation of a scramblase during the early stage of the execution phase of apoptosis (19, 20). However, as shown in Fig. 7, we observed the externalization of PS, as detected by binding of fluorescently labeled annexin V, as early as 2 – 5 min after addition of HePC (10 μM in the absence of serum), i.e. long before the onset of apoptosis. The staining of annexin V was not caused by permeabilization of the plasma membrane as no internal membranes were stained by annexin V, and no influx of the cell-impermeable nuclear dye propidium iodide was noted (Fig. 7). Also no efflux of the cytosolic dye BCECF was observed after addition of HePC in parallel experiments (data not shown). These data indicate that HePC can rapidly affect the composition and thus the properties of the membrane bilayer.

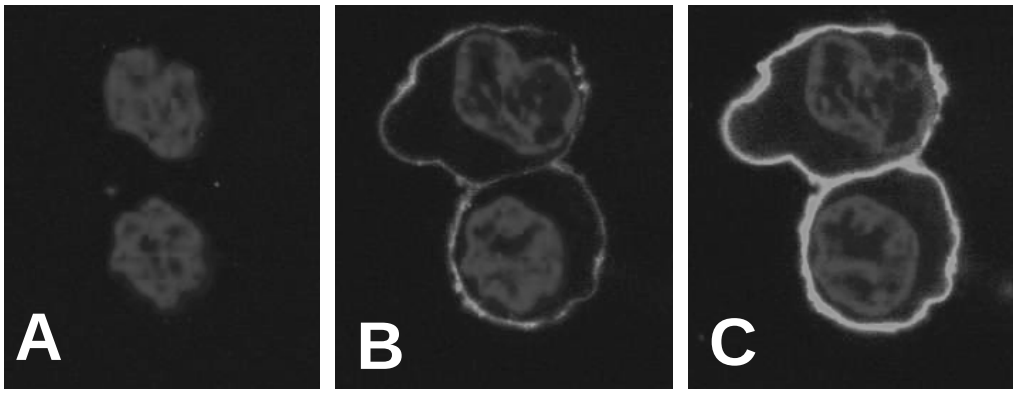

Figure 7. HePC induces a rapid exposure of phosphatidyserine to the outside of CHO-K1 cells. CHO-K1 cells were incubated under a multiphoton microscope in the presence of 2 μg/ml Annexin V-fluorescein, 0.25 μg/ml propidium iodide, and 2 μg/ml Hoechst 33342 at 37 ºC and 5% CO_2. At various time points fluorescence was recorded as described in the method section before (A), or 2 min (B) and 5 min (C) after addition of 10 μM HePC.

Annexin V-fluorescein (green) stains PS. Propidium iodide (red) and Hoechst 33342 (blue) are membrane impermeable and permeable stains for nuclei, respectively.

Discussion

Several alkyl-lysophospholipid analogs (ALPs), like HePC, have a profound cytotoxic and cytostatic effect, especially on transformed cell lines, concurrently with an inhibition of CT, the rate-limiting enzyme in PC synthesis. Since addition of lysoPC rescues cells from the effects of ALPs, these lipid analogs are thought to mediate their effects by inhibiting PC synthesis (3-6). To further examine the role of inhibition of PC synthesis in the mechanism of action of ALPs we choose to study the effects of HePC on the CHO-K1 cell line, of which a mutant MT58 is available that exhibits an isolated defect in PC synthesis, when incubated at 40 ^{o}C. The CHO-K1 cells may well serve as a model for other cell lines used in previous studies, as we demonstrated that HePC induces apoptosis in these cells at concentrations that also causes a robust inhibition of PC synthesis and that lysoPC can prevent its apoptotic effect.

However, we noticed that the HePC-induced apoptosis in CHO-K1 cells differed in a number of ways from the apoptosis observed in the genetically CT deficient MT58 cell, making it very unlikely that inhibition of PC synthesis is a major causative factor in HePC induced apoptosis.

In the first place, the apoptosis induced by HePC occurs ealier (starting at 12-16 h), than the apoptosis caused by PC depletion in the MT58 cells (starting at 30-40 h; (10)), although the remaining PC synthesis is higher in HePC-treated cells compared to that in MT58 cells grown at 40 ^{o}C.

Secondly, HePC at apoptotic concentrations caused only a relatively minor depletion of PC mass when compared to the MT58 cells grown at the non-permissive temperature. The reason for this relatively small HePC-mediated decrease in PC, despite the robust inhibition of PC synthesis (60 %-70 % inhibition by 75-100 μM HePC), might be that the inhibition of PC synthesis is transient (see Table 1). Another possibility is, that HePC not only inhibited the synthesis of PC, but simultaneously diminished its degradation. It was found previously that MT58 cells, grown at the permissive temperature of 33 ^{o}C, can maintain their PC content at the same level as CHO-K1 cells, despite a 50-60 % lower rate of PC synthesis (10). At the same time, PC breakdown in MT58 cells at 33 ^{o}C is lower than in the parent CHO-cells, and much lower than observed in both cell lines at 40 ^{o}C (A.B. Vaandrager, unpublished observations).

Thirdly, HePC induced a rise in the level of the PC-derived lipid-signaling molecule DAG, whereas a decrease was observed in MT58 cells grown at 40 ^{o}C. This latter observation suggests that DAG is in equilibrium with the amount of PC, as both lipids were decreased to a similar extent. The surplus of DAG, which cannot enter the PC synthesis pathway in MT58 cells, is presumably converted into TAG (21). So the HePC-induced increase in DAG is presumably not related to the inhibition of PC synthesis, but may be caused by a stimulation of phospholipase D, as reported previously for several other cell lines (22, 23). The above mentioned difference in effect on DAG levels led to a change in the ratio of DAG to ceramide, as the level of this latter, pro-apoptotic, lipid was increased to a similar extent in both the HePC-treated CHO cells and in MT58 cells grown at 40 ^{o}C. The dynamic cellular ratio of DAG and ceramide is suggested to be a mechanism to regulate apoptosis (18, 24). In MT58 cells the increase in ceramide levels might be a direct consequence of the drop in the amount of PC, since PC serves as the donor of the phosphocholine head group required to convert ceramide into SM (25). In HaCaT cells, HePC was also shown to increase ceramide levels. In these cells this increase in ceramide formation was correlated with an inhibition of SM and PC synthesis (26). Furthermore, in CHO-K1 cells the HePC-induced disturbance of the membrane asymmetry (Fig. 7) may lead to a relocalization of SM to the inner leaflet, where it might be a target for sphingomyelinases (c.f. (27)).

Fourthly, no synthesis of new proteins seems required for the HePC-induced apoptosis, in contrast to the apoptosis observed in MT58 cells at the non-permissive temperature. Likewise, induction of the pro-apoptotic ER stress- related factor CHOP was noticed in PC depleted MT58 cells, but not in HePC treated CHO cells. The faster time course of the HePC-provoked apoptosis may relate to the activation of an existing apoptotic machinery, without the necessity of synthesis of additional proteins as in case of PC depletion-induced apoptosis.

Finally, the HePC-induced apoptosis could be prevented by addition of either lysoPE or lysoPC, whereas only lysoPC, but not lysoPE, could rescue MT58 cells grown at the non-permissive temperature. This suggests that the rescue of the HePC-induced apoptosis by lysoPC was not specific for this class of lipids and questions the previously hold notion that lysoPC specifically counteracts the loss of PC due to the HePC-provoked inhibition of CT.

As inhibition of CT and the subsequent inhibition of PC synthesis is clearly not the sole pathway by which HePC induces apoptosis, HePC must affect other processes in biological membranes besides CT. These other targets may include: stimulation of the cellular stress-related, apoptosis inducing, SAP/JNK pathway (28, 29), stimulation of FAS clustering

(30); inhibition of the Akt/PK-B survival pathway; inhibition of the MAP/ERK (31); inhibition of phospholipase C (32) and protein kinase C activation (33); and as mentioned before, stimulation of ceramide formation (22); and phospholipase D (22, 23). Furthermore, HePC may induce a general disturbance in the membrane structure or bilayer packing, which may lead to membrane rupture or leakage, if HePC is accumulated at sensitive sites. When this occurs in lysosomal or mitochondrial membranes, apoptosis will be a direct consequence (34-36). Recently, the apoptotic properties of ceramides were also hypothesized to involve its capacity to modulate membrane structures and dynamics (37).

Besides ALPs, other lipids, like short-chain ceramides, farnesol, geranylgeraniol, and chelerythrine, were shown to induce apoptosis and to inhibit PC synthesis (25, 38-42). For farnesol, it was recently shown that inhibition of PC synthesis was not absolutely required for the induction of apoptosis (43). In the light of our observations with HePC it may be questioned also, whether the inhibition of PC plays a major role in the apoptosis induced by these lipid drugs.

Acknowledgements

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CHAPTER 6

Summarizing Discussion

Summarizing discussion

PC has an essential role in the cell as a major structural element of cellular membranes as well as a signaling molecule. Inhibition of PC synthesis, by chemical treatment or in a genetic model, results in the induction of apoptotic processes. In this thesis studies were initiated to characterize the underlying regulatory pathway of PC depletion-induced apoptosis by using a CHO mutant MT58, containing a thermo-sensitive mutation in CT, as a model system. Shifting MT58 cells to the non-permissive temperature of 40 ˚C resulted in PC depletion of more than 50 % within 24 h. Early apoptotic features appeared in several cells around 32 h, and most cells were apoptotic within 48 h. However, MT58 cells can be rescued from PC depletion-induced apoptosis if they are supplemented with a PC analogue, LysoPC. The ultimate time point that cells could be rescued from apoptosis was 30 h. Although that MT58 cells have a drastic decreased cellular PC content after 24 at 40 ˚C, they are still viable and can even proliferate if PC homeostasis is restored by LysoPC addition.

The effect of a prolonged inhibition of PC synthesis on PC breakdown, composition of PC species, and morphological changes in MT58 cells are described in **chapter 2**. Despite the inhibition of PC synthesis in MT58 cells, the degradation of PC remained normal. This resulted in a decrease of the total PC content of the cell with more than 75 % after 24 h. Mass spectrometry analysis of the PC species revealed that PC species with a mono-unsaturated acyl-chain at the sn-1 position, especially 18:1/18:1 and 18:1/18:2 species, are preferentially reduced by the inhibition of PC synthesis. In contrast, the cellular PC content of MT58 cells incubated at 40 °C is enriched with saturated 16:0/16:0 PC, but also with poly-unsaturated PC species. The physiological relevance of this differences in species profile in PC-depleted MT58 cells, and the molecular mechanism causing these differences remain to be established. The differences in PC species might be a result of increased PC synthesis via an alternative pathway in MT58 cells, like base-exchange, or a preferential breakdown or remodelling of specific PC species in MT58 cells in comparison to CHO-K1 cells.

Inhibition of PC synthesis at the step of CT may also inhibit the consumption of DAG as this molecule is used for the formation of PC in the last step of the Kennedy pathway. The surplus of DAG is presumably converted into TAG to reduce the cellular levels of DAG in PC depleted MT58 cells. This idea is enforced by the observation of an increased number of lipid droplets in PC depleted MT58 cells. These lipids droplets are storage organelles for the accumulated TAG during PC depletion. Furthermore, inhibition of PC synthesis is expected to

influence the synthesis of new membrane structures and the composition of existing membranes. After 24 h of PC depletion, structures like the ER and the Golgi were disrupted in MT58 cells, whilst the plasma membrane, mitochondriae and the membrane of the nucleus seem to be intact. Membranes of the secretory pathway that are in a dynamic equilibrium are affected the most. The drastic changes on the ER could be a possible trigger for the induction of stress pathways that eventually result in apoptosis. The ER has been identified to mediate several perturbations in the cell, like glucose starvation, Ca^{2+} overload and accumulation of unfolded or misfolded proteins. When faced with one of these stresses, the ER induces growth arrest to restore homeostasis or to induce apoptosis after prolonged and severe stress. This process is known as the ER stress response. A possible relationship between ER stress and PC metabolism is supported by the fact that most of enzymes of PC synthesis, CT and CPT, are located at the membrane of the ER.

The role of the ER stress response in the apoptosis of PC depleted MT58 cells was investigated in **chapter 3**. Apoptosis of MT58 cells induced by depletion of PC, is preceded by an increase of pro-apoptotic C/EBP-homologous protein (CHOP/GADD153) after 16 hours, to a maximum at 24 h. CHOP encodes a bZip transcription factor and is either not expressed or present at low levels under normal physiological circumstances, but it is induced strongly in response to ER stress. Over-expression of CHOP has been shown to induce growth arrest and apoptosis. In contrast, other characteristics of the specific ER stress response such as expression of the ER chaperone BiP/GRP 78, attenuation of protein translation or induction and activation of caspase 12 (a protease specifically involved in apoptosis that results from stress in the ER), did not differ between MT58 and CHO-K1 in time when cultured at 40 ˚C. These results suggest that PC depletion in MT58 specifically induces the ER stress-related protein CHOP, without raising a general ER stress response.

Although CHOP is often implicated with ER stress response, several studies have observed CHOP induction in ER-stress response-independent apoptotic pathways. The group of Fafournoux reported that amino acid limitation, especially leucine and arginine, up-regulates CHOP expression, but not BiP expression. Binding of Fas ligand to the Fas receptor, results in a signaling cascade that leads to an increase and activation of CHOP and consequently in Fas-regulated apoptosis. Cell death mediated by C6-ceramides also involves increased expression of CHOP. In conclusion, there are several mechanisms leading to the induction of CHOP by PC depletion in the cell. Therefore, determining the effect of inhibition of PC synthesis on the various cis-acting elements of the CHOP promoter might be helpful to elucidate the mechanism of how PC depletion is sensed by a cell.

In **chapter 4** the importance of CHOP expression in apoptosis, induced by PC depletion, was investigated with anti-sense CHOP mRNA over-expressing stable MT58 cell lines. These cells were relatively less susceptible to PC depletion-induced apoptosis, resulting in a delay of the onset of the terminal execution phase of the apoptotic process with at least 8-12 h. This result indicate that CHOP expression is an early upstream event in the signaling cascade and responsible for the early response to PC depletion. However, prolonged inhibition of PC synthesis still results in the apoptosis of MT58 cells suggesting that other apoptotic pathways are induced under these severe conditions.

To elucidate which regulatory mechanism is responsible for the expression of CHOP during PC depletion-induced apoptosis, CHOP promoter studies were performed. With the help of 5'end-deletion mutants of the CHOP promoter a specific region in the CHOP promoter located upstream from the transcription start site (-442 to –211) was identified to be responsible for the induction of CHOP expression in MT58 cells during PC depletion. Closer analysis of this promoter region indicated that two stress-induced regulatory elements are present, a C/EBP-ATF element and an AP-1 element. We have provided evidence that the C/EBP-ATF site in this region is responsible for the increased CHOP expression. Mutations in this DNA element resulted in a loss of responsiveness to PC depletion in MT58 cells. However, a slight decrease in CHOP transcription activity has also been observed in the control cell lines transfected with the C/EBP-ATF mutant and incubated at 40 ˚C. This decrease might represent the fraction of basal activity that is already evoked by the temperature shift and thus potential heat stress. The C/EBP-ATF composite site present in the CHOP promoter is highly conserved among species, including human and hamster. The C/EBP-ATF composite site in the CHOP promoter is also shown to be an element of the AARE. The AARE element is responsible for the induction of CHOP during amino acid deprivation and activation depends on the binding of a phosphorylated transcription factor known as ATF2. Phosphorylated ATF2 levels are already observed after 8 h in MT58 incubated at the non-permissive temperature and remained elevated until 24 h. This event precedes the induction of CHOP expression. Therefore we suggest that CHOP expression during PC depletion is mediated via a C/EBP-ATF element in the CHOP promoter and might depend on the binding of ATF2 to this element. These findings might also indicate that the AARE is not an exclusive element for amino acid deprivation, but also involves other stress events such as PC depletion or an exposure to arsenite.

The transactivating capacity of ATF2 is depending on phosphorylation of the N-terminal residues Thr-69, Thr-71 and Ser-90 by stress-activated protein kinases. In addition,

the role of two SAPKs, JNK and p38 MAPK in PC depleted cells as well as possible ATF2 phosphorylation was investigated. JNK is present in an active, phosphorylated state in MT58 cells incubated at 40 °C and treatment of MT58 cells with the specific JNK inhibitor SP600125 can rescue these cells from apoptosis. However, JNK inhibitor SP600125 treated cells still have an elevated CHOP expression, comparable to non-treated cells. Furthermore, JNK inhibitor SP600125 treatment does not result in a decrease in phosphorylated ATF2 in PC depleted MT58 cells. These results suggest that JNK is not involved in the induction of CHOP expression and ATF2 phosphorylation. Nevertheless, inhibition of JNK can rescue PC depleted MT58 cells from apoptosis. An explanation could be that JNK is responsible for post-translational events of CHOP, like phosphorylation, rather than its induction. Besides JNK and p38 MAPK, several other kinases, have been identified, capable of ATF2 activation. ATF2 has been described to be phosphorylated by two kinases on different amino acids. This dual activation may cooperate different signals if all of them are acting at sub-optimal levels, or be exclusive if any of them reaches a maximum effect. Therefore, we cannot exclude that under our conditions ATF2 might also be phosphorylated by JNK in combination with another kinase. In that case, inhibition of JNK with SP600125 would still result in ATF2 phosphorylation and CHOP induction mediated by this other kinase. Another possibility is that JNK is involved in a parallel stress event or in a late stress event that also results in apoptosis. It can be suggested that JNK might be involved in an alternative pathway that replaces signaling pathways depending on CHOP expression.

Next we investigated whether inhibition of PC synthesis also plays a major causative role in the induction of apoptosis by HePC, a pharmacological drug of ALP family and capable of inhibiting CT. We therefore directly compared apoptosis caused by HePC in CHO-K1 cells to the apoptotic process in CHO-MT58 cells. The results are described in **chapter 5**. We described that HePC-provoked apoptosis differed substantially from the apoptosis observed in MT58 cells. In the first place, HePC-induced apoptosis occurs earlier (starting at 12-16 h), than the apoptosis caused by PC depletion in the MT58 cells (starting at 30-40 h). Second, HePC at apoptotic concentrations caused only a relatively minor depletion of total PC mass when compared to the MT58 cells grown at the non-permissive temperature. The reason for this relatively small HePC-mediated decrease in PC, despite the robust inhibition of PC synthesis (60 %-70 % inhibition by 75-100 μM HePC), might be that inhibition of PC synthesis is transient. Another possibility is that HePC not only inhibited the synthesis of PC, but simultaneously diminished its degradation. A third difference is that HePC treatment does not result in a decrease in DAG like observed in MT58 cells incubated at 40 °C. Fourthly, the

apoptotic events in HePC treated cell do not require de novo synthesis of proteins, and do not show any CHOP induction. In contrast, pretreatment with cycloheximide, a translation inhibitor can rescue MT58 cells from apoptosis induced by PC depletion. Furthermore, one of the main characteristics in MT58 cells during PC depletion is the increase of CHOP expression after 24 h. Finally, HePC treated cells can be saved from apoptosis with either lysoPE or lysoPC, whereas only lysoPC, but not lysoPE, could rescue MT58 cells grown at the non-permissive temperature. Together, all these results suggest that the rescue of the HePC-induced apoptosis by lysoPC was not specific for this class of lipids and argue against the hypothesis that inhibition of PC synthesis is the primary target of HePC during apoptosis. As inhibition of PC synthesis is clearly not the sole pathway by which HePC induces apoptosis, HePC must affect other processes in biological membranes besides CT inactivation. Possible other targets described to be influenced by HePC include; SAPK/JNK pathway, stimulation of FAS clustering, inhibition of the Akt/PK-B survival pathway; inhibition of the MAP/ERK, inhibition of phospholipase C, protein kinase C activation and stimulation of ceramide formation. Furthermore, HePC may induce a general disturbance in the membrane structure or bilayer packing, which may lead to membrane rupture or leakage of cellular organelles like ER, Golgi and mitochondria. This would result in the release of cytotoxic molecules like cytochrome C, Ca^{2+} and ROS. The accumulation of these molecules in the cytosol will unavoidable lead to apoptosis.

Besides ALPs, other lipids, like short-chain ceramides, farnesol, geranylgeraniol, and chelerythrine, were shown to induce apoptosis and to inhibit PC synthesis. For farnesol, it was recently shown that inhibition of PC synthesis was not absolutely required for the induction of apoptosis. In the light of our observations with HePC it may be questioned also, whether the inhibition of PC plays a major role in the apoptosis induced by these lipid drugs.

Future prospects

Considering the underlying regulatory pathway of PC depletion-induced apoptosis, we have identified CHOP/GADD153, ATF2 and JNK as key players in cellular stress responses to inhibition of PC synthesis. Internal structures as Golgi and ER are disrupted in PC depleted cells and this event could be a trigger for the observed cellular stress pathways that eventually result in apoptosis. Although the results from the studies described in this thesis have

addressed several topics concerning the regulation of apoptosis during PC depletion, but many issues remain to be solved.

The main question that has to be clarified is the identification of upstream mechanisms that result in ATF2 phosphorylation and the induction of CHOP via the C/EBP ATF element. Important in this aspect is the role of JNK. As mentioned earlier, JNK does not affect CHOP induction, but is essential for the apoptotic processes during PC depletion. Future research should identify whether JNK activation is independent of cellular stress responses that require CHOP or plays a role in the activation of CHOP. Upstream events that result in the activation of ATF2 and CHOP induction could be an oxidative stress response or the efflux of Ca^{2+} from internal stores of ER and Golgi to the cytosol. Under normal circumstances cytosolic levels of Ca^{2+} and ROS are kept low by storage of Ca^{2+} in compartments in ER and Golgi or by storage and detoxifying of ROS in the mitochondria, respectively. However, PC depletion in MT58 might evoke a change in membrane permeability of these organelles and consequently results in leakage or rupture of the storage compartments, thereby releasing Ca^{2+} and ROS into the cytosol. These events induce stress pathways including MAPK kinases like JNK and eventually result in apoptosis. In this respect investigating the precise role of Ca^{2+} and ROS in connection to PC depletion could provide insight in downstream events during PC depletion that result in ATF2 activation and CHOP induction. Other important key players in upstream signalling of ATF2 and CHOP could be ceramides. Ceramides are naturally occurring membrane sphingolipids that have emerged to as important second messengers in apoptotic signaling. A number of diverse apoptosis-promoting agents, including tumour necrosis factor α, chemotherapeutic drugs, ischemia/reperfusion, irridation and FAS antigen activation can all induce the generation of ceramide by SM hydrolysis by sphingomyelinase (SMase). Under normal circumstances ceramide is used for the synthesis of SM by transferring phosphorylcholine from PC to ceramide by SM synthase. However, in PC depleted MT58 cells this reaction does hardly occur and results in an increased level of cellular ceramide. In lipid metabolism, ceramide and DAG are closely linked by residing opposite of the spingomyelin cycle. The balance between these molecules is important to cellular processes like proliferation and cell death. High levels of DAG result in cell proliferation, whereas an increase in ceramide along with a decrease in DAG will lead to a shift of the DAG-ceramide ratio towards ceramide. This will result in the induction of apoptosis via inhibition of Bcl-2, activation of PP2A and the activation of MLKs. MLKs are a part of stress-induced signaling pathways that will eventually activate the transcription factors c-jun, c-fos and ATF2. Therefore, MLKs could be a connection between increased ceramide levels in MT58 cells and

the induction of CHOP by ATF2. Future research using dominant negative mutants of MLKs or using the MLK inhibitor CEP-1347 might elicudate the role of ceramides and MLKs in CHOP induction in PC depleted MT58 cells.

Another issue that remains to be addressed whether a specific pathway exists that regulates PC homeostasis or whether apoptosis induced by PC depletion might be the result of a more non-specific, general stress response. In other words, are cells capable of reacting to an insult of PC depletion in an active manner and adapt to the disturbed homeostasis? Such a specific response pathway has been shown to exist for the regulation of cholesterol homeostasis. This pathway depends on a family of transcription factors designated sterol regulatory element binding proteins (SREBP). These proteins are bound to membranes of the ER and form a complex with SREBP cleavage-activating protein (SCAP), which escorts the SREBPs to the Golgi in the absence of cholesterol. SREBPs are sequentially cleaved by two Golgi specific proteases, site-1 protease (S1P) and site-2 protease (S2P), releasing a soluble transcription factor fragment from the amino terminus. This transcription factor migrates to the nucleus, where it activates the expression of genes involved in the synthesis of cholesterol and fatty acids. Homeostasis is achieved by a negative feedback loop in which high cholesterol levels block the proteolytic release of SREBPs from the Golgi membranes by retention of SREBPs to the ER evoked by SCAP. Interestingly, high levels of cholesterol in macrophages also lead to the activation of CT and an accumulation of PC. Therefore it seems that an active signalling mechanism exists between cholesterol synthesis and PC synthesis. In this aspect it would be interesting in future research to investigate the effect of PC depletion in MT58 cells on cholesterol homeostasis and the possible role of SREBPs in this event. Glucose deprivation on the other hand is not mediated via a specific pathway but is thought to be one of the insults that results in the activation of the ER stress response. Low levels of glucose in the cell will affect the glycosylation of proteins in the ER. This will result in an accumulation of non-glycosylated proteins which can not be properly folded to their usually conformation anymore. These unproperly folded proteins are eventually the signal for activation of the UPR in the ER. Future research should elucidate whether the effects of PC depletion could be non-specific. As mentioned earlier, a decrease in PC content could result in a higher susceptibility to leakage of internal membranes and the released molecules could evoke stress cascades. Alternatively, PC synthesis inhibition might lead to an accumulation of ceramides and this could be the trigger for the activation of stress pathways. CHOP induction during PC depletion is mediated via a C/EBP ATF response element in the CHOP promoter. The fact that this element is also responsible for CHOP induction during amino acid

deprivation, 15-d-prostaglandin-J2 treatment and arsenite-induced apoptosis, suggests that it mediates several types of stresses and that it is not specific for one type of insult. This favors the idea that PC depletion is regulated by a non-specific stress response but further research is absolutely necessary to confirm this hypothesis.

A third point we have to raise is the role of CT. Although it is thought that PC depletion is the signal that result in the induction of cellular stress pathways and apoptosis, we can not rule out that CT itself also plays a role in signaling pathways. Thus it seems unlikely because LysoPC addition can rescue MT58 cells grown at the non-permissive temperature from apoptosis while these cells have no functional CT.

A concluding remark concerns the inhibition of PC synthesis by treatment with lipid drugs like ALPs that are used as common tools to study apoptotic pathways depending on PC depletion. Several of these lipid drugs possess a potent anti-neoplastic activity and are used for the treatment of skin metastases of breast cancer. To place the results found in PC depleted MT58 cells in a broader context, we compared this model with a pharmacological model of PC synthesis inhibitor HePC, which is currently used in cancer therapy. However, we found that PC synthesis inhibition is not the main pathway by which HePC induces apoptosis. Further research should be done to elucidate if this would also be the same for others PC synthesis inhibitors like ET-18-OCH_3, farnesol, geranylgeranol and chelerythrine. And if it is the case, the next step would be to investigate what is the pathway that is responsible for apoptosis, induced by these inhibitors and why treatment with lysoPC or lysoPE could reverse the apoptotic process.

Nederlandstalige Samenvatting

Verantwoording

Door ervoor te kiezen om naast een summarizing discussion (Hoofdstuk 6) een samenvatting in het Nederlands te schrijven, heb ik de mogelijkheid mijn werk op een andere wijze toe te lichten. Omdat ik mij hiermee wil richten tot een bredere doelgroep, heb ik gekozen om een uitgebreide inleiding te schrijven ter introductie van het onderwerp van mijn onderzoek. Daarin heb ik getracht specialistische termen te omzeilen en enige noodzakelijke begrippen uitgebreider te beschrijven. Vervolgens beschrijf ik in het overzicht de gevonden resultaten van mijn onderzoek en de conclusies die we daaruit hebben getrokken. Ik zal eindigen met mijn slotconclusie en de mogelijke toekomstperspectieven van dit onderzoek. Met deze samenvatting hoop ik een aantal geïnteresseerden een plezier te doen.

Inleiding

Alle levende wezens zijn opgebouwd uit cellen. De kleinste organismen bestaan uit slechts één cel. Enkele voorbeelden hiervan zijn bacteriën en het pantoffeldiertje. Zoogdieren, inclusief de mens, bestaan uit miljarden cellen. Een groep gelijkvormige cellen met dezelfde functie vormt een weefsel en verschillende soorten weefels vormen weer een orgaan, zoals het hart.

De term “cel” is voor het eerst gebruikt door Robert Hooke in 1665. Hij bestudeerde dunne plakjes plantaardig weefsel. De lege kamertjes die hij in kurk zag, noemde hij “cellulae”. Het belang van de cel als elementaire bouwsteen voor alle levende organismen werd in 1840 beschreven door Jacob Schleiden en Theodor Schwann. Zij vergeleken de microscopische bouw van planten en dieren en vonden grote overeenkomsten tussen beiden met betrekking tot de cellulaire opbouw en groei. In 1855 stelde de Duitser Virchow de celtheorie op: “Omnis cellula e cellula; oftewel alle planten en dieren zijn opgebouwd uit cellen. De vermenigvuldiging van cellen geschiedt door deling van bestaande cellen. Deze celtheorie, waarbij de cel als eenheid van leven wordt beschouwd, is tot op de huidige dag nog steeds geldig.

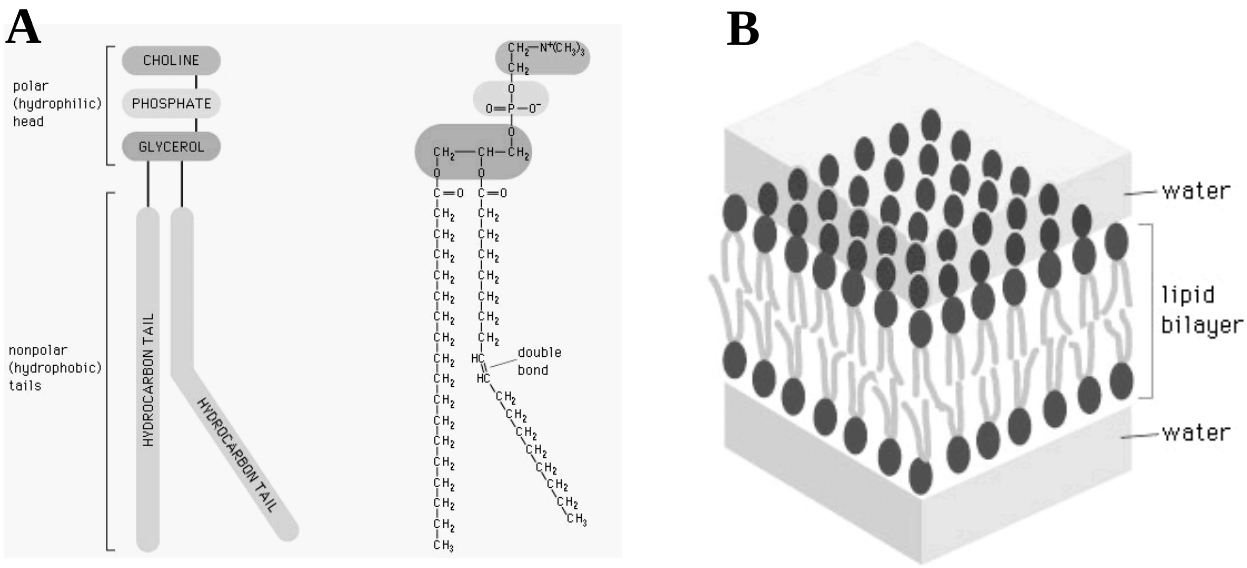
De precieze bouw van een cel is afhankelijk van de functie, maar alle cellen zijn omgeven door een celwand, ook wel celmembraan genoemd. Hierbinnen bevinden zich de celkern (nucleus), het cytoplasma (ook wel bekend als protoplasma) en de celorganellen. De celkern bevat chromosomen die opgebouwd zijn uit DNA ketens, het bouwplan van het

organisme. Op deze chromosomen bevinden zich de genen, die de dragers zijn van de erfelijke eigenschappen. Het cytoplasma is een waterige substantie waarin ondere andere eiwitten, vetten, suikers, vitaminen en elektrolyten zoals kalium, natrium, fosfor en calcium aanwezig zijn. Daarnaast bevinden zich in het cytoplasma allerlei structuren, die men samenvat onder de naam celorganellen. De belangrijkste celorganellen zijn de mitochondriën, de lysosomen, het endoplasmatisch reticulum (ER) en het Golgi-apparaat. De mitochondriën zorgen voor de energievorming uit voedingstoffen en zijn de "energiebedrijven" van de cel. De lysosomen zorgen voor de afbraak van afval- en niet- verteerbare stoffen. Daarna worden deze overbodige stoffen uit de cel verwijderd. De lysosomen kan men beschouwen als de afvalverwerkingsbedrijven van de cel. Het ER is een netwerk van kanalen dat samen met het Golgi-apparaat verantwoordelijk is voor het transport van eiwitten. Het ER en het Golgi-apparaat vormen samen de infrastructuur van de cel.

Elke cel wordt begrensd door een dun, flexibel membraan, namelijk het celmembraan en is ook wel bekend als celwand. Dit membraan is van essentiëel belang voor het goed functioneren van een cel. Raakt het celmembraan beschadigd dan betekent dit tevens het einde van de cel. De belangrijkste taak van het celmembraan is om het karakteristieke chemische en fysische inwendige milieu (intracellulaire ruimte) van de cel te beschermen tegen de buitenwereld (extracellulair milieu). Naast een afschermende rol en transport van specifieke stoffen hebben membranen ook een belangrijke signaalfunctie zodat de cel kan reageren op veranderingen in de buitenwereld. Verder bezitten de meeste cellen inwendige of interne membraanstructuren. Deze interne membraanstructuren omgeven de verschillende organellen en waarborgen het specifieke interne milieu van het betreffende organel.

Celmembranen zijn opgebouwd uit fosfolipiden, eiwitten en koolhydraten. Essentieel voor de structuur van het membraan zijn de fosfolipiden. Fosfolipiden bestaan uit twee vetzuurstaarten gekoppeld aan een kopgroep met een negatief geladen fosforzuurgroep (zie Fig. 1A). Dit verklaart de naam fosfolipiden. Deze geladen kopgroep heeft een sterke affiniteit voor water en wordt daarom hydrofiel genoemd. De vetzuurstaarten hebben echter een geringe affiniteit voor water en worden daarom hydrofoob genoemd. Moleculen met zowel hydrofiele als hydrofobe regio's noemen we amfipathische moleculen. Individuele fosfolipide-moleculen zijn niet of nauwelijks in water oplosbaar. Op de juiste wijze gerangschikt, namelijk naast elkaar gelegen met de kopgroepen naar buiten (zie Fig. 1B), zijn fosfolipiden wel in staat om stabiele structuren te vormen. Door deze rangschikking ontstaat een zelfsluitende dubbellaag tussen het waterige cytoplasma en het buitenmilieu: het celmembraan. Deze dubbellaag van fosfolipiden vormt een barrière, waardoor sommige

stoffen snel worden doorgelaten, en anderen slechts zeer langzaam of in het geheel niet. De snelheid waarmee een molecuul het membraan kan passeren hangt af van zijn molecuulgrootte, lading en lipofiliteit (vetminnendheid). Fosfolipiden zijn dus essentieel als structurele bouwstenen van membranen. Daarnaast functioneren fosfolipiden als signaalmolecuul in allerlei reacties van de cel. De vetzuurstaarten van fosfolipiden kunnen eventueel dienen als uiterste reservebron van energie .



Figuur 1. Structuur van de fosfolipide phosphatidylcholine (A) en de opbouw van het celmembraan, de fosfolipide dubbellaag (B).

Het meest voorkomende en belangrijkste fosfolipide in zoogdiercellen is fosfatidylcholine (PC). Van het totale fosfolipide gehalte in de cel is meer dan 50 % PC. Dit molecuul werd voor het eerst beschreven door Gobley in 1847 als een essentieel bestanddeel van de eierdooier. Hij noemde het gevonden molecuul “lecithin” naar het griekse equivalent voor eierdooier (*lekithos*). In 1860 beschreven Diakonow en Strecker de specifieke structuur van PC, bestaande uit twee vetzuurstaarten verbonden met een choline groep door een fosfodiesterverbinding (zie Fig. 1A). PC is het belangrijkste structuurelement van membranen van zoogdiercellen. PC dient tevens als basis voor de aanmaak van twee andere fosfolipiden, fosfatidylserine (PS) en sfingomyelin (SM). Daar deze twee fosfolipiden ook deel uitmaken van de structuur van het membraan, levert PC, zij het indirect, een nog grotere bijdrage aan de opbouw van het membraan. PC wordt ook gebruikt als grondstof voor de aanmaak van signaalmoleculen die allerlei biochemische processen in de cel regelen, zoals celdeling en het handhaven van de cellulaire stabiliteit (homeostase).

PC wordt gemaakt uit choline in drie aparte, enzymatische stappen. Dit proces is bekend als de Kennedy synthese. Choline is een stof die we binnenkrijgen via onze voeding en vervolgens de cel binnenkomt via specifieke transport-eiwitten in het membraan. In de eerste reactie van de Kennedy synthese wordt een fosfaatgroep van ATP overgedragen aan choline. Hierdoor ontstaat fosfocholine en deze reactie is afhankelijk van het enzym choline kinase. De tweede stap van de reactie is afhankelijk van het enzym CTP-fosfocholine cytidylyltransferase (CT). Hierbij wordt fosfocholine omgezet in CDP-choline. De snelheid van deze reactie is bepalend voor het verloop van de hele PC synthese en daarom wordt CT ook wel het snelheidsbepalende enzym genoemd. Zolang er voldoende PC in de cel aanwezig is, blijft CT in het cytoplasma maar is het niet actief. Bij een tekort aan PC in membraanstructuren, begeeft CT zich naar deze membraanstructuren en gaat daarmee een binding aan. Door deze membraan-associatie wordt CT dus actief en gaat PC aanmaken om het tekort aan te vullen. In de laatste stap van de PC synthese wordt CDP-choline omgezet in PC door het enzym CDP-choline:1,2-diacylglycerol cholinephospho-transferase (CPT). In deze reactie wordt CDP omgewisseld voor het molecuul diacylglycerol (DAG). Elke cel is in staat om PC te maken via de Kennedy synthese.

PC speelt een centrale rol in de cel. Een goed functioneerde synthese van PC is essentieel voor het welzijn van elke levende cel. De juiste opbouw van de celmembranen is van deze synthese afhankelijk. Ook het proces van celdeling vereist een goed functionerende PC synthese voor de aanmaak van nieuwe membranen van de dochtercellen. Verstoring van de PC synthese zou al deze processen ingrijpend beïnvloeden. Cellen waarbij de synthese van PC kunstmatig wordt geremd door chemische stoffen, groeien niet meer en gaan uiteindelijk dood via een proces dat we geprogrammeerde celdood noemen.

Geprogrammeerde celdood oftewel apoptose is het verschijnsel dat een cel zichzelf doodt, wanneer hij niet meer nodig is of wanneer hij een bedreiging kan vormen voor andere cellen. Apoptose van overbodige cellen is een verschijnsel dat vaak optreedt tijdens de embryonale ontwikkeling. Een voorbeeld hiervan is het verdwijnen van de staart van het kikkervisje tijdens de ontwikkeling naar volwassen dier. Infectie met een virus of mutaties in het DNA die kunnen leiden tot de ontwikkeling van een potentiële kankercel (transformatie), zijn situaties waarbij een cel een gevaar voor omliggende cellen (en dus het gehele organisme) kan worden. Om deze dreiging weg te nemen, maakt de cel zichzelf dood voordat het virus zich kan repliceren en andere cellen kan infecteren of voordat de potentiële kankercel oneindig gaat delen waardoor gezonde cellen worden verdrongen. Apoptose is dus een essentiëel proces zowel tijdens de embryonale ontwikkeling als in het handhaven van de

homeostase en bij immuunregulatie. Het proces van apoptose kan worden verdeeld in drie stadia: (I) de beslissing om te sterven of te blijven leven; (II) Het afsterven van de cel; (III) Het afbreken van de restanten van de gestorven cel door macrofagen of naburige cellen. Alle componenten van het zelfmoordprogramma zijn in elke cel aanwezig en kunnen elk moment worden geactiveerd. Het apoptotisch proces bestaat uit een aantal checkpoints. Het is een samenspel is van overlevings- en dood signalen en regelmechanismen in de cel. Afwijkingen in deze apoptotische regelmechanismen kunnen leiden tot het ontstaan van kanker. In een aantal gevallen van kanker zijn genen, die verantwoordelijk zijn voor de vorming van de eiwitten van dit zelfmoordprogramma niet meer actief. Ook virussen (b.v. het AIDS virus) proberen het apoptose mechanisme te onderdrukken.

Veel soorten van chemotherapie zijn gebaseerd op het activeren van het apoptotische proces in kankercellen. Kankercellen zijn eigenlijk niets anders dan cellen die ongeremd blijven delen. Voor deling van cellen is de aanmaak van nieuw PC nodig. Remming van PC synthese met bepaalde chemische stoffen leidt ertoe dat cellen niet meer delen en zelfs in apoptose gaan. Het behandelen van kankercellen met deze stoffen zou een tweeledig effect hebben. Ten eerste remmen ze het delingsproces van de kankercel, waardoor deze zich niet verder ongeremd kan vermenigvuldigen. Ten tweede stimuleren deze stoffen het apoptotisch proces, waardoor de kankercel afsterft. Deze stoffen zijn mogelijk te gebruiken als chemotherapie, maar een beter inzicht in hun werking is noodzakelijk voor toepassing in de praktijk.

Resultaten beschreven in dit proefschrift

PC bepaalt voor een groot deel de eigenschappen van membranen en speelt tevens een rol in signaaloverdracht. Als er iets fout gaat in het fosfolipidenmetabolisme kan dit ernstige gevolgen hebben voor het functioneren van de cel en van het organisme als geheel. Dit onderzoek beschrijft de effecten van langdurige remming van PC synthese op de cel. In het eerste hoofdstuk wordt beschreven wat er in de literatuur al bekend is over de relatie tussen de remming van PC synthese en de stimulatie van celdood. Kort samengevat zijn er al tal van stoffen beschreven die de PC synthese remmen en daardoor apoptose opwekken. Het onderliggende mechanisme is echter nog niet bekend. Het doel van dit onderzoek is om de gevolgen van een tekort aan PC in de cel vast te stellen en te ontdekken wat het mechanisme kan zijn dat verantwoordelijk is voor de celdood van de cellen. Hierdoor hopen we een beter

inzicht te krijgen in de rol van PC synthese remming en de activatie van het apoptotisch proces.

In hoofdstuk 2 wordt beschreven wat er met de cel gebeurt bij een langdurig tekort aan PC. Een tekort aan PC in cellen kan tot stand komen door ze te behandelen met chemische stoffen die PC synthese remmen. Het grote nadeel van het gebruik van remmers is dat ze niet specifiek zijn en ook andere reacties in de cel beïnvloeden. Daarom is voor deze studie gebruik gemaakt van een genetisch gemanipuleerde cellijn die bekend is als MT58. Deze cellijn heeft een verandering (mutatie) in het CT enzym van de PC synthese. Deze mutatie is temperatuurgevoelig. Cellen die groeien bij 33 °C hebben een goed functionerend CT enzym en kunnen voldoende PC maken. De mutatie wordt effectief als cellen worden blootgesteld aan een temperatuur van 40 °C of hoger. Het gemuteerde CT enzym is dan instabiel en wordt direct afgebroken. Door de remming van CT in de PC synthese wordt de aanmaak van nieuw PC voorkomen en dit leidt tot een tekort aan PC in MT58 cellen die groeien bij 40 °C. Door deze cellen 24 uur bloot te stellen aan een temperatuur van 40°C kunnen we het PC niveau in deze cellen aanmerkelijk verminderen en de effecten van een tekort aan PC bestuderen.

De resultaten beschreven in hoofdstuk 2 laten zien dat een langdurige remming van de PC synthese ingrijpende gevolgen heeft voor de cel. Het gehalte van PC in de cel is dramatisch gedaald doordat de afbraak gewoon doorgaat, terwijl de aanmaak wordt geremd. De afname van PC in de cel wordt gevolgd door een soortgelijke afname van DAG terwijl een toename van DAG was verwacht. Dit verschijnsel is te verklaren doordat DAG ook fungeert als signaalmolecuul in de cel. Hoge niveaus van DAG zouden daardoor allerlei reacties in de cel kunnen beïnvloeden en zelfs verstoren. Daarom wordt DAG omgezet in triacylglycerol (TAG) om het DAG niveau in de cel laag te houden. De toename van TAG zal leiden tot de vorming van vetdruppeltjes in de cel. Dit is inderdaad het geval, want MT58 cellen die geïncubeerd zijn bij 40 °C, bevatten een verhoogd aantal vetdruppeltjes. Daarnaast tast het tekort aan PC de aanmaak van nieuwe membraanstructuren en ook de samenstelling van de huidige membranen aan. Dit komt omdat membranen dynamische structuren zijn, waarvan de fosfolipiden continue worden vervangen door de wisselwerking tussen aanmaak en afbraak. Met name de membraanstructuren van het ER en het Golgi apparaat zijn ingrijpend veranderd in MT58 cellen met een PC tekort. Deze afname van ER- en Golgi membraan kan erop duiden dat de cel prioriteiten stelt en als eerste zijn interne membranen inkrimpt en de cel- en kernmembraan worden zo lang mogelijk in stand gehouden. De veranderingen in het ER

kunnen leiden tot een stress reactie van de cel, die bekend staat als de ER stress respons. Deze ER stress respons probeert in eerste instantie de homeostase van de cel te herstellen, maar als dat niet lukt gaat de cel in apoptose.

In hoofdstuk 3 is onderzocht of de ER stress response inderdaad verantwoordelijk is voor het apoptotisch proces van cellen met een PC tekort. Het ER is verantwoordelijk voor de bewerking van eiwitten en enzymen. Het ER zorgt ervoor dat eiwitten in de juiste vorm zijn gevouwen. Na een kwaliteitskeuring worden de eiwitten naar de plek van bestemming getransporteerd. Het ER fungeert ook als opslagplaats voor calcium (Ca^{2+}). Dit is nodig omdat een te hoog gehalte aan vrij Ca^{2+} in het cytoplasma giftig is voor de cel. Het ER is er mede verantwoordelijk dat de homeostase van een cel gehandhaafd blijft. Wanneer er verstoringen (stress) plaatsvinden in de cel wordt de ER stress respons geactiveerd. De ER stress respons wordt gekenmerkt door vier aparte fases: (I) Verhoogde aanmaak (expressie) van specifieke hersteleiwitten zoals BiP en HSP70;(II) Remming van de algemene eiwitsynthese; (III) Afbraak van beschadigde eiwitten; (IV) Activering van het apoptotisch proces door verhoging van pro-apoptotische eiwitten en activering van een ER specifieke caspase. Caspases zijn een specifieke groep van eiwitten die aan het eind staan van het apoptotisch proces. Deze caspases zijn verantwoordelijk voor de uiteindelijke executie van de cel tijdens het apoptotish proces en kunnen worden beschouwd als de beulen van de cel. De eerste drie fases zijn gericht op herstel van de cellulaire homeostase. Bij voortdurende stress zal de ER stress response uiteindelijk leiden tot de laatste fase, waarbij de cel wordt vernietigd voordat hij schadelijk kan worden.

In de experimenten beschreven in hoofdstuk 3 is allereerst onderzocht of MT58 cellen met een PC tekort ook werkelijk sterven via een apoptotisch proces. 32 uur nadat ze bij 40 °C waren gezet, vertonen MT58 cellen de eerste apoptotische verschijnselen en de meeste cellen waren apoptotisch na 48 uur. Reddingsexperimenten met LysoPC, een molecuul dat via een andere synthese route in PC kan worden omgezet, bewezen dat deze cellen pas na 30 uur niet meer te redden waren. Na een blootstelling van 24 uur hebben MT58 cellen al wel een ernstig tekort aan PC, maar deze cellen blijken nog steeds levensvatbaar. De homeostase kan dus nog worden hersteld door toevoeging van lysoPC. Vervolgens is onderzocht of specifieke kenmerken van de ER stress repons zijn geactiveerd in MT58 cellen met een tekort aan PC. Deze cellen hebben na 24 uur een extreem hoog niveau van het pro-apoptotisch eiwit CHOP. CHOP is een van de eiwitten die in de vierde fase van de ER stress respons worden geïnduceerd. Alle andere kenmerken van de ER stress respons werden echter niet aangetroffen in MT58 cellen bij 40 °C. Hieruit kan worden geconcludeerd dat een tekort aan

PC leidt tot een verhoogde expressie van het eiwit CHOP, waarna de cel sterft via apoptose. Dit proces is echter onafhankelijk van de ER stress respons.

In hoofdstuk 4 is de rol van CHOP in de apoptose van MT58 cellen met een PC tekort verder onderzocht. CHOP, ook wel bekend als GADD153, is een transcriptie factor. Transcriptie factoren zijn eiwitten die de expressie van andere eiwitten reguleren. Eiwitten worden gecodeerd door genen in het DNA. Van een gen wordt eerst het zogenaamde boodschapper RNA of messenger RNA (mRNA) gemaakt. Dit mRNA dient vervolgens als boodschapper voor eiwitsynthese, hetgeen gebeurt met behulp van speciale eiwitten die ribosomen heten. De expressie van mRNA wordt gereguleerd door een klein stukje DNA, de zogenaamde promoter, die voor het gen ligt. Aan kleine gebiedjes in de promoter, de regulatoire elementen, kunnen zich transcriptie factoren binden, waardoor een gen tot expressie komt. De binding van de transcriptie factor CHOP aan het DNA leidt tot de expressie van eiwitten die betrokken zijn bij de regulatie van apoptose. Meestal wordt CHOP geassocieërd met apoptotische processen van de ER stress respons, maar CHOP is ook betrokken bij ER onafhankelijk processen.

In hoofdstuk 4 is aangetoond dat CHOP een essentiële rol speelt in de apoptose van MT58 cellen met een tekort aan PC. Dit blijkt uit het feit dat MT58 cellen, waarin de expressie van CHOP wordt onderdrukt, pas 10 uur later in apoptose gaan. Dit suggereert dat de onderdrukking van CHOP de cellen niet van apoptose kan redden, omdat een ander mechanisme het waarschijnlijk overneemt. CHOP is echter wel bij de snelle stress respons betrokken. De regulatie van CHOP is bestudeerd aan de hand van de CHOP promoter om het mechanisme, verantwoordelijk voor de verhoogde CHOP expressie, te identificeren. In de CHOP promoter zijn verschillende regulatoire elementen aanwezig, die door verschillende soorten stress geactiveerd kunnen worden. In dit onderzoek is één bepaalde regio in de CHOP promoter gevonden die betrokken is bij de verhoogde expressie van CHOP, veroorzaakt door een tekort aan PC. In deze regio zijn twee regulatoire elementen aanwezig, namelijk een C/EBP-ATF element en een AP-1 element. Hieraan kunnen respectievelijk de transcriptie factoren ATF2 en c-jun binden, wat leidt tot de verhoogde expressie van het CHOP gen. Door deze twee regulatoire elementen zó te veranderen (muteren), dat de transcriptie factoren niet meer konden binden, kon de rol van de afzonderlijke elementen in de regulatie van CHOP expressie door PC tekort worden bestudeerd. Uit deze experimenten bleek dat het C/EBP-ATF element verantwoordelijk is voor de expressie van CHOP tijdens de stress van een tekort aan PC. Dit wordt nog eens bevestigd door de aanwezigheid van de actieve vorm van de transcriptie factor ATF2 na 8 uur in MT58 cellen. De opkomst van actieve transcriptie factor

ATF2 gaat vooraf aan de verhoogde expressie van CHOP. ATF2 wordt geactiveerd door het overdragen van een fosfaatgroep van ATP aan ATF2 (dit proces heet fosforylering). Kinases zijn verantwoordelijk voor de fosforylering van eiwitten. In deze studie is ook nog gekeken welke stress gevoelige kinase verantwoordelijk is voor de activering van ATF2 en dus de expressie van CHOP. JNK kinase is aanwezig in de actieve vorm in MT58 cellen met een tekort aan PC. Daarnaast kunnen MT58 cellen bij 40 °C worden gered van apoptose als ze van tevoren zijn behandeld met een specifieke JNK remmer. Maar MT58 cellen behandeld met JNK remmer hebben nog steeds CHOP expressie. Daarom kan worden geconcludeerd dat JNK kinase niet verantwoordelijk is voor de fosforylering van ATF2 en de expressie van CHOP. Maar CHOP zelf moet ook nog eerst worden gefosforyleerd voordat het kan binden aan regulatoire elementen van de betrokken genen. Het zou kunnen zijn dat JNK verantwoordelijk is voor de fosforylering van CHOP. De gevonden resultaten suggereren dat CHOP expressie een belangrijke rol speelt in het apoptotische proces van MT58 cellen met een PC tekort. Deze expressie hangt waarschijnlijk af van binding van de transcriptiefactor ATF2 aan het regulatoire C/EBP-ATF element van de CHOP promoter. JNK speelt hierbij geen rol, maar zou nog verantwoordelijk kunnen zijn voor de activering van CHOP via fosforylering.

In hoofdstuk 5 is er een vergelijking gemaakt tussen remming van de PC synthese in ons genetisch model van de cellijn MT58 en remming met een chemische stof hexadecylphosphcholine (HePC). HePC behoort tot de groep van alkyllysophospholipids (ALPs). HePC remt de PC synthese door remming van het enzym CT. HePC is een middel dat al wordt gebruikt voor de experimentele behandeling van borsttumoren. HePC induceert apoptose in getransformeerde cellen, en alhoewel het mechanisme hierachter niet bekend is, gaat men ervan uit dat de remming van PC synthese hierbij een belangrijk rol speelt. Dit is met name gebaseerd op het feit dat HePC behandelde cellen van apoptose gered kan worden door toevoeging van LysoPC. Om te bepalen of de remming van PC inderdaad verantwoordelijk is voor de inductie van apoptose in HePC behandelde cellen, hebben we dit model met de kenmerken van het apoptotisch proces in ons genetisch model vergeleken. In beide modellen zagen we een remming van de PC synthese, die leidt tot apoptose. Dit apoptotisch proces was in beide modellen omkeerbaar door het toevoegen van lysoPC aan de cellen. Maar waar remming van PC synthese in MT58 wel leidt tot een tekort in PC, was dit niet waarneembaar in HePC behandelde cellen. Daarnaast zagen we ook geen verhoogde expressie van het eiwit CHOP in het HePC model en het apoptotisch proces in HePC behandelde cellen is ook niet afhankelijk van verhoogde expressie van nieuwe eiwitten. HePC

behandelde cellen zijn ook te redden met lysoPE, een stof die lijkt op de fosfolipide fosfatidylethanolamine. Deze verschillen met het genetische model MT58 duiden erop dat de remming van PC synthese niet de primaire target is van HePC. HePC beïnvloedt waarschijnlijk nog andere reacties in de cel en de remming van PC synthese is niet het primaire mechanisme waardoor HePC apoptose induceert.

In hoofdstuk 6 zijn alle bevindingen in een breder perspectief geplaatst. In dit hoofdstuk is geprobeerd om te speculeren over wat de mogelijke achterliggende mechanismen zijn, die leiden tot de expressie van CHOP tijdens remming van PC synthese. Verder wordt hier uitgewijd welke effecten van het tekort aan PC het signaal kunnen zijn voor de start van het gehele apoptotische proces.

Eindconclusie en toekomstperspectieven

In deze studie hebben we het effect van langdurige PC synthese remming op de cel onderzocht. Een structureel tekort aan PC leidt tot een ophoping van vetdruppels, verstoring van de membraanstructuren van het ER en Golgi, geremde groeisnelheid en uiteindelijk tot celdood via apoptose. Bij dit apoptotisch proces speelt het enzym CHOP een belangrijke rol. De verhoogde expressie van CHOP in deze cellen wordt gereguleerd door het C/EBP-ATF element van de CHOP promoter en hangt waarschijnlijk af van binding van de transcriptie-factor ATF2 aan dit element. JNK speelt hierbij geen rol, maar zou verantwoordelijk kunnen zijn voor de activering van CHOP via fosforylering. Deze resultaten geven een indicatie hoe apoptose door een tekort aan PC wordt gereguleerd. Verder onderzoek zal moeten ophelderen welk mechanisme verantwoordelijk is voor de activatie van ATF2 in dit proces. Een mogelijk kandidaat hiervoor is het oxidatieve stress mechanisme. Oxidatieve stress zou kunnen ontstaan doordat de membranen van met name de celorganellen van structuur veranderen waardoor reactieve zuurstofradicalen kunnen vrijkomen. Deze zuurstofradicalen zijn schadelijk voor cellen en kunnen leiden tot de activatie van apoptose.

De resultaten beschreven in dit proefschrift zullen een beter inzicht verschaffen in de werking van PC synthese remmers die mogelijk toepasbaar zijn in chemotherapie. Daarom zijn de effecten op de cel van de PC synthese remmer HePC vergeleken met de resultaten van ons genetisch model. Hieruit bleek echter dat apoptose veroorzaakt door HePC niet het gevolg is van de remming van PC synthese maar via een ander effect van deze stof. Aanvullende studies zullen nodig zijn om vast te stellen of dit ook het geval is voor andere PC synthese remmers.

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Michiel

Curriculum vitae

Michiel van der Sanden werd geboren op 8 januari 1975 te Oosterhout, Noord-Brabant. Het VWO werd doorlopen op het St. Oelbert gymnasium te Oosterhout, waar in 1994 het diploma werd behaald. Hetzelfde jaar begon hij aan de studie Biologie aan de Universiteit van Utrecht. Zijn eerste afstudeerstage liep hij bij de vakgroep Vergelijkende Endocrinologie van de faculteit Biologie te Utrecht onder begeleiding van Dr J. Bogerd. In dit onderzoek werd een alternatieve manier gezocht om gonatroop hormoon (GTH) te produceren. Hiervoor werden de twee subunits van GTH in een expressievector gekloneerd en in gist tot expressie gebracht. Zijn tweede afstudeerstage voerde hij uit bij de onderzoeksgroep signaaltransductie van het Hubrecht Laboratorium, Nederlands instituut voor Ontwikkelingsbiologie (NIOB) onder begeleiding van Dr. J. Den Hertog en Drs Blanchetot. Het onderzoek betrof een studie naar de rol van de Receptor Protein Tyrosine Phosphatase α (RPTPα) in cellen. Daarvoor werd bestudeerd welke eiwitten een mogelijke interactie aan kunnen gaan met RPTPα via specifieke domeinen. Het doctoraal diploma werd behaald in januari 2000. Aansluitend in februari 2000 werd een aanstelling verkregen als Assistent in Opleiding bij de hoofdafdeling Biochemie en Celbiologie van de faculteit Diergeneeskunde aan de Universiteit Utrecht. Tijdens deze aanstelling is promotie-onderzoek verricht onder begeleiding van Prof. Dr. L.M.G. van Golde, Prof. Dr. J.B. Helms en Dr. A.B. Vaandrager. De resultaten van dit promotie-onderzoek staan beschreven in dit proefschrift. Vanaf mei 2004 is hij werkzaam als post-doc onderzoeker bij de vakgroep Signaaltransductie van het Deutsches Krebsforschungszentrum (DKFZ) in Heidelberg, Duitsland.

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DOKTER.. 'T IS DE HAMSTER!!
HIJ DOET 't BIJNA NIET MEER!!
Ach Gut.. ARM ARM BEEST..
CHOP!!
BLAF

Einde