Inhibition of phosphatidylcholine synthesis induces expression of the endoplasmic reticulum stress and apoptosis-related protein CCAAT/enhancer-binding protein-homologous protein (CHOP/GADD153)

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Inhibition of *de novo* synthesis of phosphatidylcholine (PC) by some anti-cancer drugs such as hexadecylphosphocholine 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 (ER) and result in ER stress responses, which can lead eventually 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 CCAAT/enhancer-binding protein-homologous protein (CHOP; also known as GADD153) after 16 h, to a maximum at 24 h. Incubation of wild-type CHO-K1 or CT-expressing MT58 cells at 40 °C did not induce differences in CHOP protein levels in time. In contrast, expression of the ER chaperone BiP/GRP78, induced by an increase in misfolded/unfolded proteins, and caspase 12, a protease specifically involved in apoptosis that results from stress in the ER, did not differ between MT58 and CHO-K1 cells in time when cultured at 40 °C. Furthermore, heat-shock protein 70, a protein that is stimulated by accumulation of abnormal proteins and heat stress, displayed similar expression patterns in MT58 and K1 cells. These results suggest that PC depletion in MT58 induces the ER-stress-related protein CHOP, without raising a general ER stress response.

Key words: BiP/GRP78, CHO mutant MT58, CTP:phosphocholine cytidylyltransferase.

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 and of phosphatidic acid in several signal-transduction pathways [1]. PC synthesis may also influence the levels of the signal molecule ceramide, since sphingomyelin 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 into CDP-choline, which is catalysed by the enzyme CTP:phosphocholine cytidylyltransferase (CT) [5]. CT localizes mainly to the endoplasmic reticulum (ER) and nucleus [6]. CT activity in cells is regulated primarily by association with membrane lipids, translocation between ER, cytoplasm and nucleus [7–11], and by gene expression [9,10,12,13]. An important role for PC biosynthesis has been implicated in both the control of cell proliferation and cell death [14–17]. Inhibition of CT by hexadecylphosphocholine, an alkylphosphocholine, or inhibition of CDP-choline:1,2-diacylglycerol cholinephosphotransferase by geranyl geraniol 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 ER, and might lead to an induction of ER stress, the so-called ER stress response [25–28]. The ER can respond to different insults in several ways. 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 GRP78 [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 the unfolded protein response [28,29,31]. BiP shares great homology with the cytosolic heat-shock protein (HSP) 70 stress protein [29,31]. HSP 70 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 which is involved in mediating apoptosis is CCAAT/enhancer-binding protein (C/EBP)-homologous 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

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; CHO, Chinese hamster ovary; CHOP, CCAAT/enhancer-binding protein-homologous protein; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; FMK, t-butoxycarbonyl-D-fluoromethylketone; HSP, heat-shock protein; lysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; PERK, double-stranded-RNA-regulated protein kinase-like ER kinase.

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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 post-translational 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 double-stranded-RNA-regulated protein kinase-like ER 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.

MATERIALS AND METHODS

Materials

Ham's F12 medium, fetal bovine serum and calcium-free PBS were purchased from Gibco-BRL (Grand Island, NY, U.S.A.), and culture dishes and flasks were from Nunc (Rochester, NY, U.S.A.). [*methyl*-^{3}H]Choline chloride (83.0 Ci/mmol) was obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.), t-butoxycarbonyl-D-fluoromethylketone (FMK) was purchased from Alexis (Läuflingen, Switzerland), and penicillin, streptomycin, trypsin/EDTA solution and all other chemicals were from Sigma (Poole, Dorset, U.K.). Prefabricated silica gel G TLC plates were purchased from Merck (Darmstadt, Germany). Polyclonal anti-actin, anti-GRP78 (BiP), anti-caspase-12 and anti-GADD153 (CHOP) antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). 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 (Tattenhall, Cheshire, U.K.).

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% fetal bovine serum, 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 5×10^4 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 and 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 the total population of nuclei, and cells that contained condensed or fragmented nuclei were scored as apoptotic.

Cell-rescue experiments

Cells were plated at 5×10^4 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 or 33 °C. Cell numbers and apoptosis were established as described above.

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

Dishes (100 mm) were incubated at 33 or 40 °C for the indicated time periods, prior to labelling with 1 μCi/ml [*methyl*-^{3}H]choline in complete medium for 1 h. Incorporation of label was quenched by removing the medium, washing the cells twice 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 gel G plates in a solvent system of chloroform/methanol/water (65:35:4, by vol.). PC spots were scraped off and radioactivity in the spots was measured by liquid-scintillation counting.

Determination of PC pool size

Cells (5×10^4) were plated in 60 mm dishes and incubated for various time periods at 40 or 33 °C. Cells were collected in 1 ml of Tris buffer (150 mM, pH 7.5). The protein content was determined using the Coomassie® Plus protein assay reagent kit 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), using two solvent-delivery pumps and an external solvent mixer (Kontron Instruments). The flow rate was 1 ml/min and the column temperature was kept at 40 °C during all runs. Elution was performed with a gradient of hexane/isopropanol/acetone (82:17:1, by vol.) to isopropanol/chloroform/acetone (85:12:3, by vol.). A Varex MKIII light-scattering detector was used for the detection of (phospho)lipids. The detector signal was recorded and integrated by EZ Chrom™ chromatography DATA system software (Scientific Software). 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 into 100 μl of PBS. Aliquots were used to measure protein content, and the remaining 50 μl of cell suspension in PBS was lysed with 50 μl of SDS sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.003% Bromophenol Blue, pH 6.8, final concentrations). After boiling samples for 10 min, 4 μg of 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, U.S.A.) for 1 h and exposed to rabbit polyclonal anti-GADD153 (CHOP; dilution, 1:750), goat polyclonal anti-GRP78 (BiP; 1:1000) or mouse monoclonal anti-HSP 70 (1:2500). Following four washing steps with TBS-

Tween (50 mM Tris, 150 mM NaCl and 1 % Tween 20, 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) and exposure to X-ray film.

Measurement of protein synthesis

Cells were washed twice in PBS and incubated in methionine-free RPMI 1640 medium/10 % dialysed fetal calf serum (1 h, 40 °C) prior to labelling (1 h, 40 °C, 100 μCi/ml [^{35}S]methionine). Cells were washed with cold PBS and scraped off the plate into 200 μl of PBS. A 100 μl suspension was used for trichloroacetic acid precipitation of the proteins, and incorporation of radioactivity into trichloroacetic acid-precipitated proteins was measured with a scintillation counter. The remaining 100 μl of cell suspension was lysed with 100 μl of SDS 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 a 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 MT58 cells, stably transfected with wild-type CT-α gene, at 33 and 40 °C. As shown in Figure 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 MT58 cells was decreased even further to only approx. 15 % of that in the parental CHO-K1 cells or the CT-expressing MT58 cells. In contrast, the temperature shift to 40 °C only marginally affected the [^{3}H]choline incorporation into PC in wild-type CHO or CT-expressing MT58 cells (Figure 1).

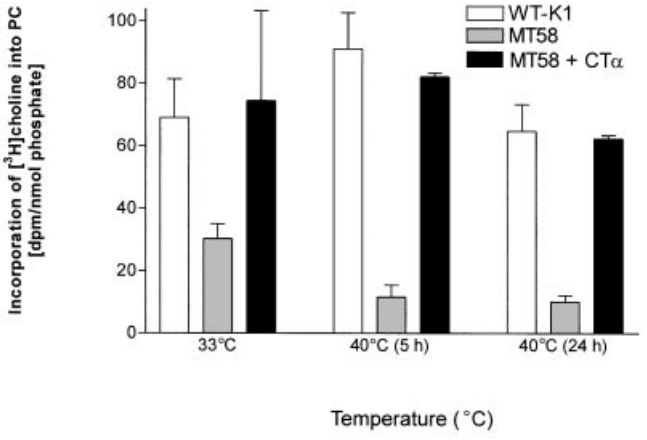

Figure 1 Effect of the non-permissive temperature on PC biosynthesis in CHO cells

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

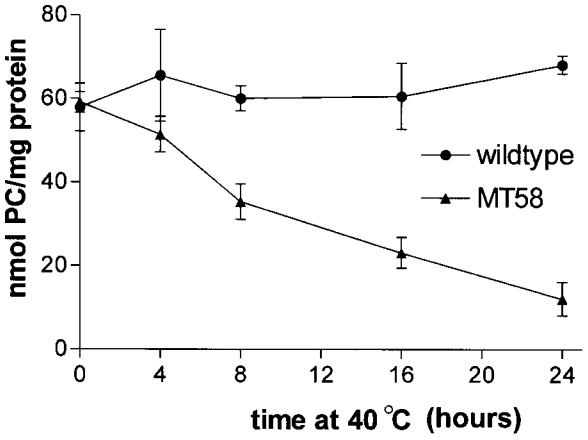

Figure 2 PC biosynthesis inhibition leads to reduction in PC pools

CHO-K1 and MT58 cells were grown in 60 mm dishes at 33 or 40 °C. At the indicated time points, cells were harvested and aliquots were taken to determine protein concentrations of samples (in μg/ml). After lipid extraction PC mass was determined by HPLC as described in the Materials and methods section. The PC concentration per μg of protein is plotted for CHO-K1 (●) and MT58 (▲) cells over time. The results represent the means ± S.E.M. from three independent experiments.

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 (Figure 2). After just 8 h a 40 % decrease in PC content was observed, and after 24 h at 40 °C only 20 % of the PC was left in the MT58 cells. The decrease in PC mass was apparently not compensated by other phospholipids, as the total amount of phosphorus in the lipid fraction also decreased from 135 to 75 nmol/mg of protein after 24 h of incubation at 40 °C (results not shown). As shown in Figure 2, incubation of the control CHO-K1 cells at 40 °C did not lead to changes in PC content.

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 MT58 cells. As shown in Figure 3(A), MT58 cells grow at 33 °C at a slightly reduced rate compared with wild-type CHO-K1 cells. However, when shifted to 40 °C, MT58 cells grew normally up to 24 h, but started dying by apoptosis between 24 and 48 h (Figure 3). This apoptotic process was found to depend on caspases for the final execution, because MT58 cells pretreated with the broad-spectrum caspase inhibitor FMK showed increased survival after incubation at 40 °C. In the presence of FMK only 13.2 ± 1.5 %

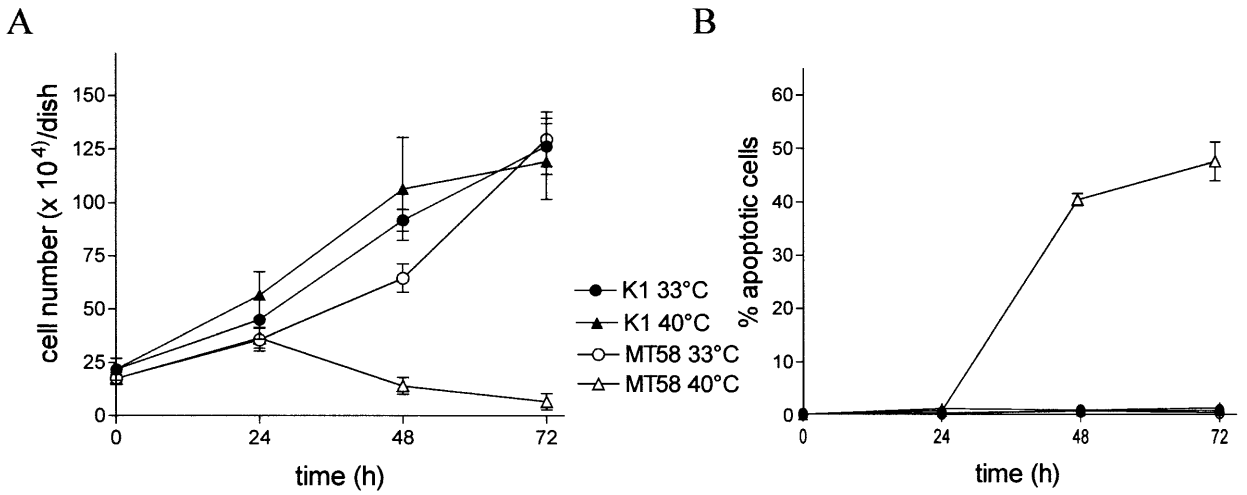

Figure 3 Inhibition of cell proliferation and induction of apoptosis in MT58 at 40 °C

CHO-K1 and MT58 cells were plated on 60 mm dishes with 5 ml of Ham's F12 medium at a density of 5×10^4 cells/dish. After 24 h at 33 °C, cells were then incubated at either 40 °C (△, ▲) or 33 °C (○, ●). At the indicated times some dishes were fixed and stained with propidium iodide. Total cell populations (**A**) and the percentage of apoptotic cells (**B**) were quantified as described in the Materials and methods section. A minimum of 100 cells from five different areas of each plate was evaluated for quantification of apoptosis. Shown are the means ± S.E.M. from three independent experiments.

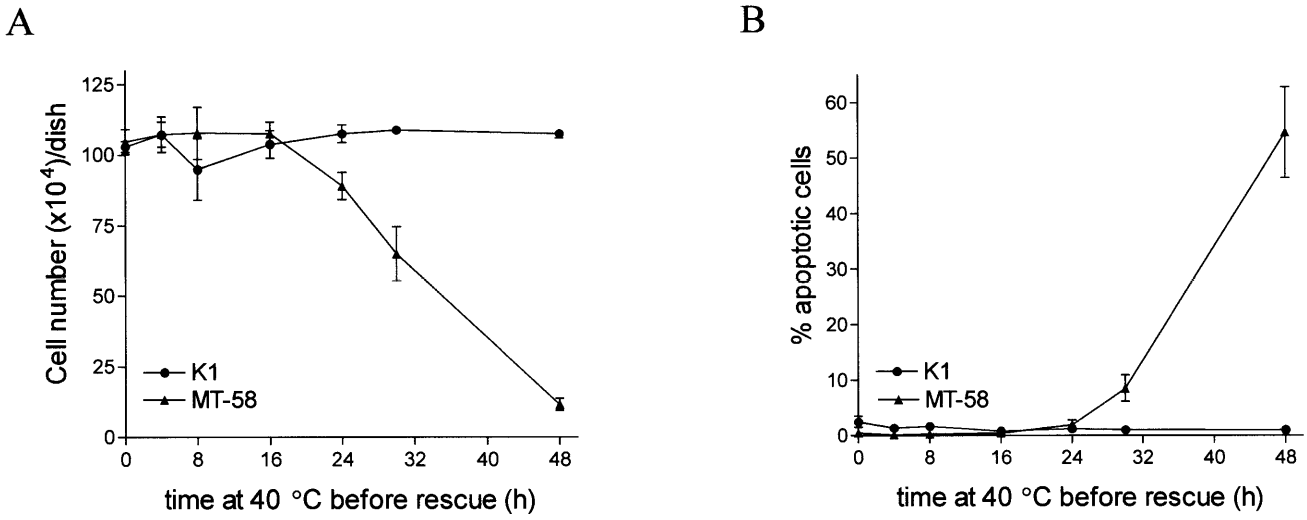

Figure 4 Rescue from apoptosis, caused by PC depletion, with lysoPC

To investigate whether 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 being shifted to 40 °C, and viability and apoptosis were assessed. ●, Wild-type K1 cells; ▲, MT58 cells. Total cell populations (**A**) and the percentages of apoptotic cells (**B**) were quantified as described in the Materials and methods section. Shown are the means ± S.E.M. from three independent experiments.

of the MT58 cells became apoptotic after 48 h at the non-permissive temperature (results not shown). In contrast, 45–50% of the MT58 cells died in the absence of the caspase inhibitor (Figure 3B).

To investigate at which time point the PC depletion 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 h (Figure 4). However, addition of lysoPC at 48 h could not reverse the apoptotic process. These results suggest that the point at which 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.

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

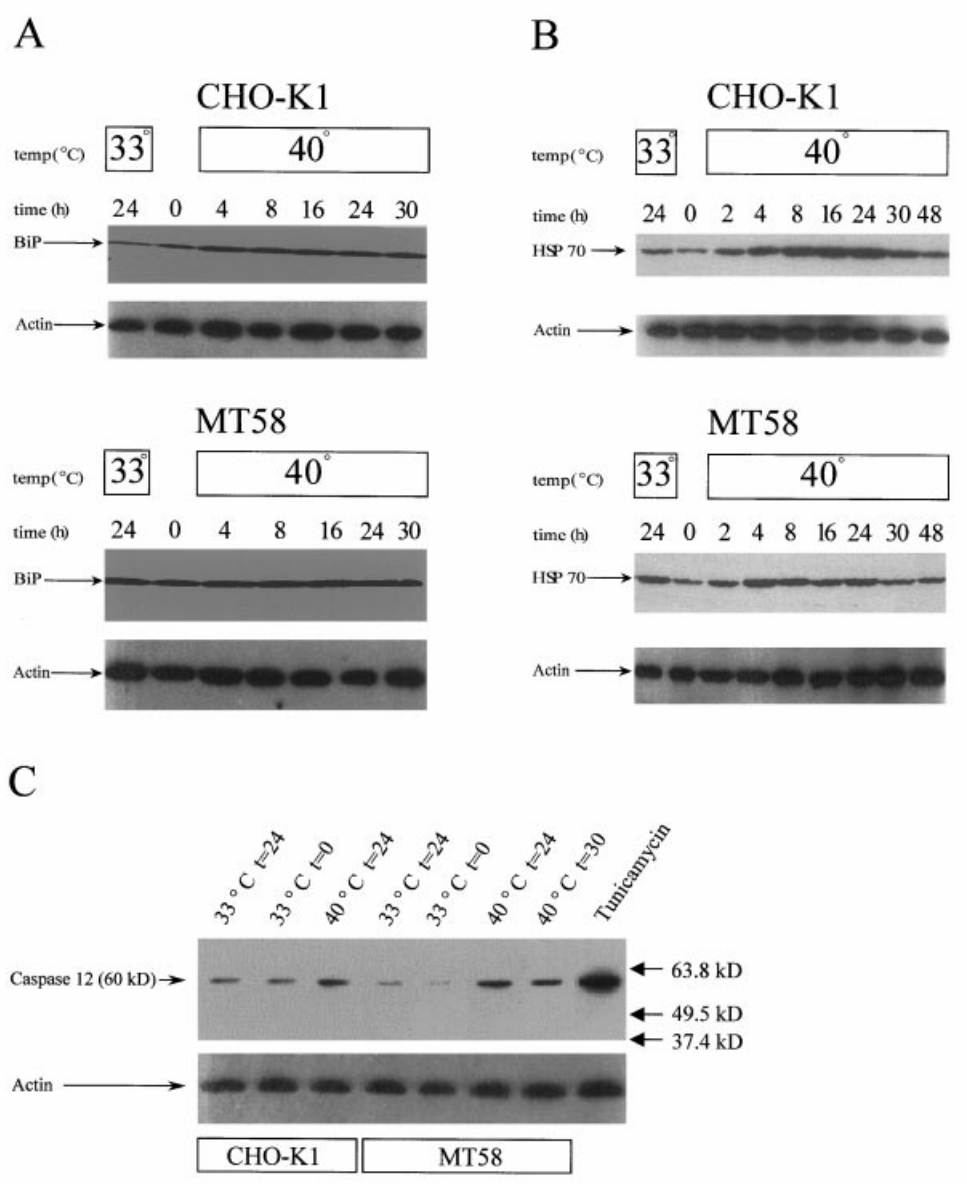

Figure 5 Effect of PC depletion on BiP, HSP 70 and caspase-12 response

The role of the ER stress response in PC depletion was investigated by analysis of two ER-related proteins, BiP (**A**) and caspase-12 (**C**). Furthermore, the expression of HSP 70, induced by stressors such as malfolded proteins and heat, was investigated (**B**). 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 (results not shown). Total cell homogenates (4 μg of protein) were separated on SDS/PAGE and the amounts of BiP, caspase-12, HSP 70 and actin (the internal control) were detected by Western blotting using goat polyclonal α-GRP78/BiP, rabbit polyclonal α-caspase-12, mouse monoclonal α-HSP 70 and goat polyclonal α-actin antibodies, respectively. Results shown are representative of three independent experiments.

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/GRP78. BiP is a protein responsible for the proper folding of proteins; over-expression of BiP can lead to cell survival [28] and high levels of BiP protein expression are indicative of ER stress [28,40,42]. However, no differences in expression levels of BiP were observed between CHO-K1 and MT58 cells cultured at 40 °C (Figure 5A). Like BiP, HSP 70 is induced by the 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 Figure 5(B), HSP 70 levels are increased in both MT58 and CHO-K1 cells as early as 2 h after shifting the cells to 40 °C, presumably as a 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 MT58 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 CHO-K1 and MT58 cells after 24 h, when compared with the robust induction of caspase-12 by tunicamycin, a typical ER-stress-inducing agent (Figure 5C).

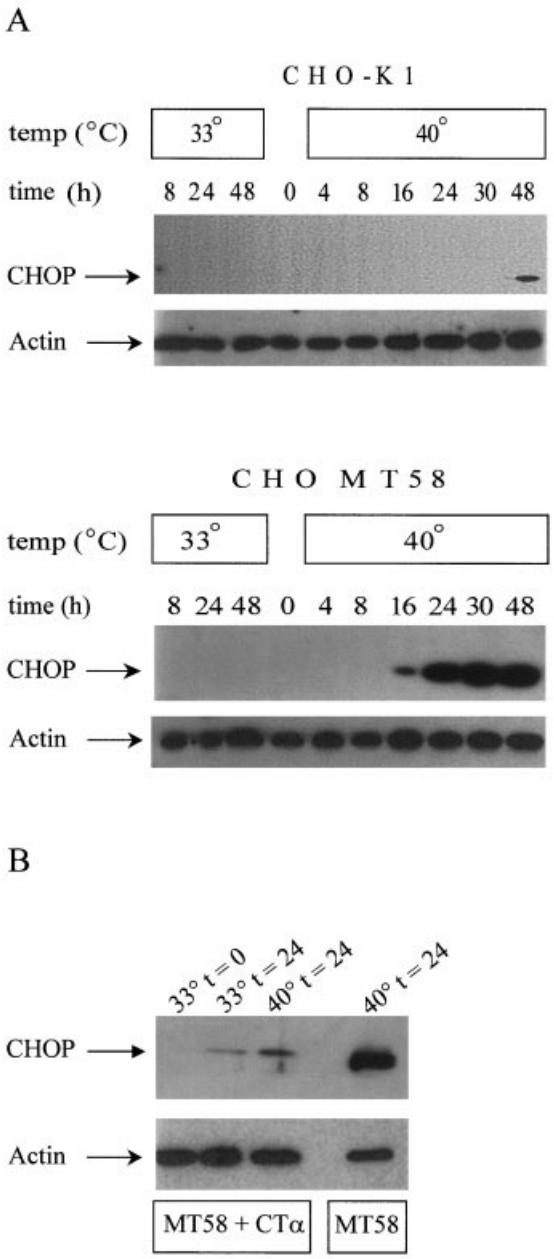

Figure 6 CHOP induction in PC-depleted MT58 cells

(**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 the primary antibody, and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Identical blots were probed for actin as the internal control. These findings were reproduced in five independent experiments. (**B**) Western-blot analyses on homogenates of MT58 or CT-expressing MT58 cells were performed as described for (**A**). MT58 + CT cultures (MT58 + CT) were incubated for 24 h at 33 or 40 °C and compared with MT58 at 40 °C for 24 h. These findings were reproduced in two independent experiments.

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 (Figure 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 MT58 cells was clearly correlated with the defect in PC synthesis, as MT58 cells stably expressing recombinant CT-α showed only a slight increase in CHOP expression (Figure 6B). Furthermore, the increase in CHOP expression in MT58 cells at 40 °C is not the result of a small difference in confluency between MT58 and CHO-K1 cells (results not shown) or of an enhanced sensitivity of the MT58 cells to ER stress. In both cell lines incubation with tunicamycin (10 μg/ml) or dithiothreitol (1 mM) increased the expression of CHOP to a similar level, starting at 6 h, with a maximum after 24 h (results not shown). Also, no difference in the apoptotic response to tunicamycin between CHO-K1 and MT58 cells was observed (results 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, and 118 ± 10 % after 24 h, at 40 °C). In contrast, induction of ER stress in wild-type and MT58 cells by incubation with tunicamycin (10 μg/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 MT58 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).

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 CHO cell line containing a thermo-sensitive mutation in CT, we were able to confirm that PC depletion induces apoptosis [16]. Although MT58 cells 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 to the non-permissive temperature, and precedes the onset of the 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 signalling cascade, which eventually leads to apoptosis. CHOP is suggested to be pro-apoptotic, as mouse embryonic fibroblasts derived from CHOP$^{-/-}$ animals exhibited significantly less programmed cell death when challenged with ER-disrupting agents, compared with 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 unfolded protein response [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 (which inhibits the ER Ca^{2+}-ATPase), brefeldin A (an inhibitor of vesicle transport between ER and Golgi) and AIF4 (which inhibits 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 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 DOCs (for downstream of CHOP), has been identified as targets of the CHOP heterodimer [37], it has been suggested that there are more unidentified targets. The tight linkage between the 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 co-ordinately regulated with the ER chaperone BiP [42,55]. Both proteins have a shared upstream signalling 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 °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 observe 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 eIF2α, resulting in a general inhibition of protein synthesis and a preferential translation of the transcription factor ATF4, 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, another protein often associated with protein misfolding and stress, is not significantly different between CHO-K1 and MT58 cells when grown at the non-permissive temperature.

Although CHOP is often implicated in the ER stress response, its induction has also been observed in apoptotic pathways, independent of the ER. The group of Fafournoux [57] reported that limitation of amino acids, especially leucine and arginine, up-regulates CHOP expression, but not BiP expression. 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 c-Jun N-terminal kinase/p38 kinase leads to an increase and activation of CHOP, resulting eventually in Fas-regulated apoptosis [41]. Cell death mediated by C_6-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 element [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 in elucidating the mechanism of how PC depletion is sensed by a cell.

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