Induction of apoptosis by lipophilic activators of CTP:phosphocholine cytidyltransferase α (CCT α)

Thomas A. LAGACE¹ and Neale D. RIDGWAY²

Atlantic Research Centre, Departments of Pediatrics and Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS, Canada B3H 4H7

Farnesol (FOH) inhibits the CDP-choline pathway for PtdCho (phosphatidylcholine) synthesis, an activity that is involved in subsequent induction of apoptosis. Interestingly, the rate-limiting enzyme in this pathway, CCT α (CTP:phosphocholine cytidyltransferase α), is rapidly activated, cleaved by caspases and exported from the nucleus during FOH-induced apoptosis. The purpose of the present study was to determine how CCT α activity and PtdCho synthesis contributed to induction of apoptosis by FOH and oleyl alcohol. Contrary to previous reports, we show that the initial effect of FOH and oleyl alcohol was a rapid (10–30 min) and transient activation of PtdCho synthesis. During this period, the mass of DAG (diacylglycerol) decreased by 40 %, indicating that subsequent CDP-choline accumulation and inhibition of PtdCho synthesis could be due to substrate depletion. At later time points (>1 h), FOH and oleyl alcohol promoted caspase cleavage

and nuclear export of CCT α , which was prevented by treatment with oleate or DiC8 (dioctanoylglycerol). Protection from FOH-induced apoptosis required CCT α activity and PtdCho synthesis since (i) DiC8 and oleate restored PtdCho synthesis, but not endogenous DAG levels, and (ii) partial resistance was conferred by stable overexpression of CCT α and increased PtdCho synthesis in CCT α -deficient MT58 cells. These results show that DAG depletion by FOH or oleyl alcohol could be involved in inhibition of PtdCho synthesis. However, decreased DAG was not sufficient to induce apoptosis provided nuclear CCT α and PtdCho syntheses were sustained.

Key words: apoptosis, CTP:phosphocholine cytidyltransferase (CCT), diacylglycerol, farnesol (FOH), lipophilic activator, phosphatidylcholine.

INTRODUCTION

The duplication of cellular components is required for proliferating cells to progress through the cell cycle. PtdCho (phosphatidylcholine), the major phospholipid in eukaryotic cell membranes, is one such component that displays a periodic accumulation during G₁/S phase [1,2]. The fundamental role of PtdCho in cell survival is underscored by the observation that genetic [3,4], pharmacological [5] or metabolic [6] disruption of PtdCho synthesis leads to decreased cell proliferation and apoptosis. The synthesis of PtdCho by the CDP-choline pathway is controlled by the rate-limiting enzyme CCT (CTP:phosphocholine cytidyltransferase), an amphitrophic enzyme that is regulated by reversible translocation to membranes [7]. The three human isoforms of CCT are encoded by two genes: CCT α , the major isoform ubiquitously expressed in all tissues, and CCT β 1 and CCT β 2, which display tissue-specific distribution [8,9]. CCT isoforms have conserved catalytic and membrane-binding domains, suggesting shared regulatory mechanisms. However, unlike CCT β isoforms that are localized to the ER (endoplasmic reticulum)/cytoplasm [8,9], CCT α is localized to the nucleus and nuclear envelope in many cell types by virtue of an N-terminal NLS (nuclear localization signal) [10–12]. An exception is lung-derived cells where CCT α is localized primarily in cytoplasmic and ER compartments [13].

CCT α is involved in important nuclear functions such as coordinating PtdCho synthesis with cell-cycle regulation [1,2] and

regulating proliferation of the nucleoplasmic reticulum [14], an intranuclear membrane network implicated in calcium signalling and nuclear–cytoplasmic transport [15,16]. Increased PtdCho biosynthesis during G₁/S phase of the cell cycle was correlated with elevated CCT α transcription [17] and enzyme activation by dephosphorylation [1]. Another study that investigated CCT α localization and activation during cell-cycle progression of serum-stimulated quiescent IIC9 fibroblasts demonstrated that enzyme phosphorylation did not change, but instead CCT α was exported from the nucleus and bound to the ER during the G₀–G₁ transition, a period of elevated PtdCho synthesis [18]. This apparent lack of requirement for nuclear CCT α agrees with an earlier study in which expression of a partially extranuclear form of CCT α missing the NLS supported PtdCho synthesis and growth in CCT α -deficient MT58 cells [11]. In contrast, CCT α fused to green fluorescent protein was constitutively nuclear in nine asynchronously growing cell types, and at all phases of the cell cycle in synchronized CHO-K1 cells (Chinese-hamster ovary K1 cells) [12]. The reasons for these discrepancies are unclear but could be cell-specific and/or related to CCT α overexpression.

Another link between cell proliferation and PtdCho synthesis was established by the finding that various apoptotic agents, including the isoprenoid FOH (farnesol), disrupt PtdCho synthesis [19–22]. FOH-induced apoptosis could be prevented by supplementation with PtdCho, lyso-PtdCho or DAG (diacylglycerol), suggesting that reduced PtdCho synthesis, or a signalling molecule derived from PtdCho, was a primary mechanism [19,23,24].

Abbreviations used: CCT, CTP:phosphocholine cytidyltransferase; CEPT, choline/ethanolaminephosphotransferase; CHO-K1 cells, Chinese-hamster ovary K1 cells; CPT, cholinephosphotransferase; DAG, diacylglycerol; DiC8, dioctanoylglycerol; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, fetal calf serum; FOH, farnesol; LPDS, lipoprotein-deficient serum; MT58-CCT α cells, MT58 cells stably expressing rat CCT α ; NLS, nuclear localization signal; PARP, poly(ADP-ribose) polymerase; PtdCho, phosphatidylcholine.

¹ Present address: Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390, U.S.A.

² To whom correspondence should be addressed, at Rm C306, CRC Bldg, Dalhousie University, 5849 University Avenue, Halifax, NS, Canada B3H 4H7 (email nrldgway@dal.ca).

On the basis of the accumulation of CDP-choline in [^3H]choline-labelling experiments and inhibition of CPT (cholinephosphotransferase) and/or CEPT (choline/ethanolaminephosphotransferase) by FOH *in vitro* [23–25], the site of inhibition by FOH appeared to be the terminal step in the CDP-choline pathway. However, this mechanism was questioned based on the inability of CEPT overexpression and restoration of PtdCho synthesis to prevent apoptosis in CHO-K1 cells, and the lack of *in vitro* inhibition of CEPT by FOH [26]. This suggested that inhibition of CPT/CEPT by FOH was indirect and could result from limiting levels of the substrate DAG [27] and/or cellular acidification [19].

Since CPT/CEPT appeared to be secondarily affected, other mechanisms were investigated to explain how FOH altered the CDP-choline pathway and induced apoptosis. Interestingly, FOH rapidly activated CCT α *in vivo* and *in vitro* and promoted translocation of CCT α to the nuclear envelope followed by export from the nucleus [28]. FOH and other apoptotic stimuli caused caspase-mediated proteolysis of the N-terminal NLS of CCT α , suggesting that activation and disruption of CCT α nuclear localization was important in suppression of PtdCho synthesis and/or induction of apoptosis. To test this hypothesis further, we examined whether CCT α expression, activity and localization were involved in susceptibility of CHO-K1 cells to two lipophilic apoptotic activators of CCT α , namely FOH and oleyl alcohol. PtdCho synthesis was rapidly and transiently stimulated by FOH and oleyl alcohol, concurrent with a 40% decrease in DAG mass. However, cells could be rescued from FOH-induced apoptosis by 'natural' lipid activators of CCT α , which acted by maintaining CCT α in the nucleus and by sustaining PtdCho synthesis without restoring endogenous DAG levels. This shows that PtdCho synthesis is important for cell survival and that cellular DAG levels are linked to reduced PtdCho synthesis and to altered lipid homeostasis.

EXPERIMENTAL

Cell culture

CHO-K1 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% (v/v) FCS (fetal calf serum) and proline (medium A) at 37°C. MT58-CCT α (MT58 cells stably expressing rat CCT α) and a mock-transfected control (MT58) were maintained in medium A at 33°C [29]. Cells were subcultured in medium A and then switched to DMEM with 5% LPDS (lipoprotein-deficient serum; medium B) for 16–18 h before the start of experiments. All experiments were performed when cell monolayers were approx. 50% confluent, since induction of apoptosis by FOH and oleyl alcohol diminished at higher confluence. FOH and oleyl alcohol (Sigma–Aldrich) stock solutions (60 mM) were prepared in ethanol, which did not exceed 0.1% (v/v) in the culture medium. DiC8 (dioctanoylglycerol) and triacsin C (Biomol, Plymouth Meeting, PA, U.S.A.) were prepared in ethanol, and added to a warm medium to facilitate dissolution prior to addition to cells. Oleate (Matreya, Pleasant Gap, PA, U.S.A.) was prepared as a 10 mM stock complexed to 10% (w/v) BSA.

Analysis of PtdCho synthesis and DAG mass

The effects of FOH and oleyl alcohol on synthesis of PtdCho and its precursors, phosphocholine and CDP-choline, was determined by [^3H]choline labelling. Briefly, cells were incubated with [^3H]choline (1–2 $\mu\text{Ci}/\text{ml}$) in choline-deficient medium A or B supplemented with FOH, oleyl alcohol and/or other lipids or drugs (refer to Figure legends for specific details). At the end of the [^3H]choline-labelling period, cells were harvested in methanol/water (5:4, v/v) and [^3H]choline-labelled PtdCho and then water-

soluble intermediates were extracted, separated by TLC and quantified as described previously [30]. [^3H]Choline incorporation was normalized relative to total cellular protein [31].

DAG and ceramide masses were quantified by the *Escherichia coli* DAG kinase method [32]. Following various treatment regimes, cells were harvested and a total lipid extract was prepared [30]. DAG and ceramide in aliquots of the lipid extract were phosphorylated with [γ - ^{32}P]ATP (Mandel–NEN, Guelph, ON, Canada) and DAG kinase (Calbiochem, San Diego, CA, U.S.A.). Phosphatidic acid and phosphoceramide were resolved by TLC, and quantified by scintillation counting and comparison with a dioleoylglycerol or ceramide standard curve.

Immunofluorescence microscopy

CHO-K1 cells cultured on glass coverslips were fixed in 3% (v/v) formaldehyde in phosphate buffer (10 mM potassium phosphate, pH 7.4, 225 mM NaCl and 2 mM MgCl_2) and permeabilized with 0.05% (w/v) Triton X-100 for 10 min at -20°C . Endogenous CCT α was detected with a polyclonal antibody directed against the C-terminus [33] and an AlexaFluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Chromatin was visualized by staining with Hoechst 33258 (Riedel-de Haen, Seelze, Germany) as described previously [28]. Cells were viewed on a Zeiss Axioplan 2 fluorescence microscope with a Planapo X100 oil immersion objective and equipped with an AxioCam HRm camera. Within experiments, parameters such as exposure time, lamp intensity and image manipulation in Adobe Photoshop were kept constant.

Analysis of apoptosis

Native and caspase-cleaved forms of CCT α and PARP [poly(ADP-ribose) polymerase] were extracted from cells in the following manner. Cells were harvested in cold PBS (10 mM potassium phosphate, pH 7.4, and 150 mM NaCl), collected by centrifugation (1000 g for 1 min) and the pellet was solubilized in high-salt Triton X-100 buffer (10 mM Tris, pH 7.4, 1% Triton X-100, 500 mM NaCl, 1 mM dithiothreitol, 0.1 mM PMSF and protease inhibitor cocktail). Equal amounts of cell protein (15 or 25 μg) were resolved by SDS/PAGE, transferred on to nitrocellulose and immunoblotted with CCT α or PARP (Santa Cruz Biotechnology) polyclonal antibodies.

Apoptosis was also measured by a sensitive sandwich ELISA that detected the presence of cytoplasmic mono- and oligonucleosomes (Cell Death Detection Elisa Plus; Roche, Laval, QC, Canada). Results were expressed as fold increase in absorbance at 405 nm relative to untreated controls. A comparison with other measures of apoptosis (chromatin condensation and caspase cleavage of PARP) indicated that a 30–40-fold increase in nucleosome release following FOH or oleyl alcohol treatment corresponded to approx. 50–60% apoptotic cell death.

RESULTS

Stimulation of PtdCho synthesis and depletion of DAG by lipophilic CCT activators

In CHO-K1 cells, FOH caused rapid nuclear membrane translocation and activation of CCT α *in vivo* and *in vitro* [28], as well as inhibition of PtdCho synthesis and accumulation of CDP-choline [25]. Since it was unclear why these seemingly paradoxical events would occur simultaneously, we re-examined the early effects of FOH on PtdCho synthesis. In previous studies [19,25,28], the syntheses of PtdCho and CDP-choline pathway intermediates were measured by simultaneous addition of [^3H]choline and FOH.

Figure 1 Transient stimulation of PtdCho synthesis by FOH in CHO-K1 cells

CHO-K1 cells were cultured in medium B for 24 h before the start of experiments. (A) Cells were cultured in choline-free medium B containing [³H]choline (2 μCi/ml) for 30 min prior to the addition of FOH (0–60 μM) for an additional 30 min. Cells were harvested and [³H]choline incorporation into PtdCho (▼), phosphocholine (■) and CDP-choline (▲) was measured. Results are the means ± S.D. for triplicate determinations from a representative experiment. Abbreviations: P-choline, phosphocholine. (A–C) Cells were preincubated with [³H]choline (2 μCi/ml) for 30 h prior to the addition of 60 μM FOH (▲) or ethanol (■). At the indicated times, cells were harvested and isotope incorporation into PtdCho (B), phosphocholine (C) and CDP-choline (D) was quantified. Results are the means ± S.D. for three independent experiments. **P* < 0.05 compared with ethanol control.

This method would not accurately measure early effects of FOH on the pathway due to limiting incorporation of [³H]choline into the pathway. Thus experiments were performed in which CHO-K1 cells were prelabelled with [³H]choline for 30 min prior to the addition of increasing concentrations of FOH for an additional 30 min (Figure 1A). Using this method, it was evident that 10 and 20 μM FOH stimulated PtdCho synthesis. Concentrations of FOH that were previously shown to induce apoptosis (40 and 60 μM) caused a dramatic decrease in [³H]phosphocholine and elevation of CDP-[³H]choline, indicating activation of CCTα and inhibition of CPT/CEPT. However, this resulted in no net change in PtdCho synthesis.

Results shown in Figure 1(A) suggested that concentrations of FOH that induce apoptosis could increase PtdCho synthesis prior to 30 min. To test this, CHO-K1 cells were prelabelled with [³H]choline for 30 min, FOH (60 μM) was added to cells and [³H]choline incorporation into PtdCho and water-soluble metabolites was measured at intervals up to 30 min (Figures 1B–1D). FOH treatment for 10 and 15 min caused a significant 30–40% increase in PtdCho synthesis (Figure 1B) that occurred while [³H]phosphocholine (Figure 1C) and CDP-[³H]choline (Figure 1D) levels were decreasing and increasing respectively. The increase in PtdCho synthesis was transient and returned to control values by 30 min apparently due to the increasing block in CDP-[³H]choline conversion into PtdCho. Extension of the time course in Figure 1(B) to 120 min resulted in 70–80% inhibition of PtdCho (results not shown).

Reduced PtdCho synthesis and CDP-choline accumulation could have resulted from depletion of the CPT/CEPT substrate

Figure 2 DAG depletion in FOH-treated CHO-K1 cells

CHO-K1 cells were cultured in medium B for 24 h before the start of experiments. (A) Cells were treated with increasing concentrations of FOH (0–60 μM) in medium B for 30 min and DAG mass was determined in lipid extracts as described in the Experimental section. Results are the means ± S.D. for triplicate determinations from a representative experiment. (B) Cells were treated with FOH (60 μM) in medium B for the indicated time periods and masses of DAG (■) and ceramide (▲) were quantified in lipid extracts. Results are the means ± S.D. for three independent experiments. **P* < 0.05 compared with control.

DAG. Indeed, cells can be rescued from FOH-induced apoptosis by addition of exogenous DAG [23], implying that endogenous DAG levels were limiting. To determine whether DAG depletion contributed to inhibition of PtdCho synthesis, CHO-K1 cells were treated with FOH and cellular DAG mass was measured by the *E. coli* DAG kinase method. There was a dose-dependent 35–50% decrease in the mass of DAG when CHO-K1 cells were treated with 20–60 μM FOH for 30 min (Figure 2A). In cells treated with 60 μM FOH, DAG depletion was detected 5 min after FOH treatment and was significantly reduced by 35% after only 15 min (Figure 2B). A 40–50% reduction in DAG mass was maintained even after FOH treatment for 2 or 3 h (Figure 8A and results not shown). Ceramide mass did not change in response to FOH (Figure 2B).

To determine if other lipophilic CCTα activators had a similar effect on the CDP-choline pathway, CHO-K1 cells were treated with oleyl alcohol. Oleyl alcohol promoted CCT translocation to membranes and stimulated PtdCho synthesis in HeLa cells *in vivo* [34], but a closer examination of its effects on the CDP-choline pathway and apoptosis was not reported. Preliminary results indicated that 60 μM oleyl alcohol was the optimal dose for disrupting the CDP-choline pathway (results not shown) and for inducing apoptosis in CHO-K1 cells (see Figure 5). Preincubation of CHO-K1 cells with [³H]choline for 30 min prior to the addition of oleyl alcohol (60 μM) resulted in a transient 1.7-fold increase in PtdCho synthesis at 30 and 60 min (Figure 3A) that returned to control values by 2 h. Increased [³H]PtdCho synthesis was paralleled by a rapid reduction in [³H]phosphocholine, indicative of CCTα activation (Figure 3B). The developing block in PtdCho synthesis at 2 h was explained by a sharp increase in CDP-[³H]choline (Figure 3C) and a 40% decline in DAG mass. Oleyl alcohol had no effect on ceramide mass (Figure 3D). While these results were almost identical with those observed with FOH (Figures 1 and 2), it is notable that maximal effects of oleyl alcohol occurred at later times (1–2 h). These results show that activation of CCTα and PtdCho synthesis, and reduction of DAG mass, by the apoptotic agents FOH and oleyl alcohol is rapid and precedes inhibition of the pathway.

Rescue of FOH- and oleyl alcohol-induced apoptosis by lipid activators of CCTα

Exogenous lipids, including DAG and PtdCho, could attenuate FOH-induced apoptosis by re-establishing DAG-dependent

Figure 3 Transient stimulation of PtdCho synthesis by the CCT α activator oleyl alcohol

(A–C) CHO-K1 cells were cultured in medium B for 24 h before the addition choline-free medium B with [3 H]choline (1.5 μ Ci/ml) for 30 min. Cells were then treated with oleyl alcohol (60 μ M; $\blacktriangle$) or ethanol vehicle ($\blacksquare$) for the indicated time periods and harvested, and [3 H]choline incorporation into PtdCho (**A**), phosphocholine (**B**) and CDP-choline (**C**) was measured. Results are the means $\pm$ S.D. for triplicate determinations from a representative experiment. **(D)** CHO-K1 cells were cultured in medium B and treated with oleyl alcohol (60 μ M) for up to 2 h. Masses of DAG ($\blacksquare$) and ceramide ($\blacktriangle$) were quantified in lipid extracts. Results are the means $\pm$ S.D. for three independent experiments. * $P < 0.005$ compared with solvent control.

signalling pathways or restoring PtdCho levels. Since DAG is an activator of CCT α , protection could also be mediated by specific effects on activity or localization of the enzyme. While examining the mechanism of rescue in more detail, we noted that CHO-K1 cells cultured in a medium containing 5 % FCS were relatively resistant to FOH-induced apoptosis, measured by appearance of the 85 and 35 kDa caspase cleavage products of PARP and CCT α [28] respectively compared with cells cultured in 5 % LPDS (Figure 4). Inhibition of FOH-induced caspase cleavage of PARP and CCT was also evident in cells cultured in LPDS supplemented with oleate or the short-chain DAG analogue DiC8. On occasion, a cross-reactive protein of approx. 75 kDa (indicated by asterisks in Figure 4) was detected with PARP antibody that was not related to generation of the 85 kDa PARP caspase cleavage product. A trivial explanation for the results shown in Figure 4 could be DiC8- or oleate-mediate inhibition of FOH uptake into cells. This was not the case, however, since uptake of [3 H]FOH over a 4 h period was either unaffected or slightly enhanced in cells supplemented with DiC8 or oleate (results not shown). Similar to FOH, oleyl alcohol caused a dose-dependent induction of apoptosis in CHO-K1 cells cultured in 5 % LPDS based on caspase proteolysis of CCT α (Figure 5A) and release of nucleosomes into the cytoplasm (Figure 5B). Both of these parameters returned to control levels when cells were preincubated with DiC8 for 30 min prior to the addition of oleyl alcohol to the culture medium.

CCT α is also exported from the nucleus during FOH-induced apoptosis by a mechanism that does not require caspase cleavage but occurs after translocation to the nuclear envelope [28]. To determine whether lipid supplementation also prevented export of CCT α from the nucleus, immunofluorescence microscopy was

Figure 4 FOH-induced caspase cleavage of PARP and CCT α is prevented by oleate or DAG

CHO-K1 cells were cultured in medium A (FCS) for 16 h, medium B (LPDS) for 16 h or in medium B for 16 h followed by supplementation with oleate or DiC8 (500 μ M) for 30 min prior to the addition of FOH (60 μ M). Cells were harvested at the indicated times after FOH addition, total cellular extracts (15 μ g of protein) were resolved by SDS/PAGE, and CCT α and PARP were detected by immunoblotting. Similar results were obtained in two independent experiments. Asterisk indicates a protein that cross-reacts with the PARP antibody but is not related to the 85 kDa PARP caspase cleavage product.

Figure 5 Oleyl alcohol induces DAG-dependent apoptosis

CHO cells were cultured in medium B for 24 h before the start of experiments. **(A)** Cells (in medium B) were incubated for 4 h with increasing concentrations of oleyl alcohol (0–89 μ M) or preincubated for 30 min with DiC8 (500 μ M) prior to the addition of oleyl alcohol (60 μ M). Total cellular extracts (15 μ g of protein) were separated by SDS/PAGE and immunoblotted for CCT α . **(B)** Cytoplasmic nucleosomes were measured in cells cultured in medium B and treated with oleyl alcohol (60 μ M) for 4 h in the absence or presence of a 30 min preincubation with DiC8 (500 μ M). Results are expressed as fold increase in apoptosis relative to ethanol-treated control cells and are the averages of duplicate determinations from a representative experiment. Abbreviation: OIOH, oleyl alcohol.

used to monitor the localization of CCT α in CHO-K1 cells treated with FOH (Figure 6A) or oleyl alcohol (Figure 6B). CHO-K1 cells cultured in a medium with LPDS and FOH for 2 h displayed significant chromatin condensation and release of CCT α from

Figure 6 Oleate and DiC8 prevent FOH- and oleyl alcohol-mediated export of CCT α from the nucleus

(A) CHO-K1 cells cultured on glass coverslips were incubated in medium A (FCS) or medium B (LPDS) for 24 h, or cultured in medium B supplemented with oleate (500 μ M), DiC8 (500 μ M) or TPA (200 nM) for 30 min prior to addition of FOH (60 μ M) or ethanol (no addition, NA) for 2 h. CCT α was localized by immunofluorescence using an Alexa-488 conjugated secondary antibody and chromatin was visualized with Hoechst 33258 as described in the Experimental section. (B) CHO-K1 cells cultured in medium B for 24 h were treated with oleyl alcohol (60 μ M) in medium B for the indicated time periods. One set of cells was preincubated for 30 min with DiC8 (500 μ M) prior to addition of oleyl alcohol for 4 h. Cells were processed for immunofluorescence localization of CCT α and chromatin as described above.

the nucleus to cytoplasmic or perinuclear regions (Figure 6A). In cells cultured in a medium containing FCS, CCT α was primarily at the nuclear envelope but with evidence of extranuclear staining in some cells. In CHO-K1 cells cultured in LPDS + FOH and supplementation with oleate or DiC8, CCT α was primarily in the nucleoplasm or nuclear envelope. This was not the result of protein kinase C activation since PMA did not prevent nuclear export. While FCS, DiC8 and oleate prevented CCT α export from the nucleus, translocation to the nuclear envelope in response to FOH was not affected. Oleyl alcohol also promoted the initial translocation of CCT α from the nucleoplasm to the nuclear envelope after 1 h, and by 2 and 4 h the enzyme was exported outside of the nucleus in > 50 % of the cells (Figure 6B). Similar to FOH, DiC8 prevented oleyl alcohol-mediated CCT α export but not translocation to the nuclear envelope. Thus supplementation of cells with lipid activators of CCT α , which are also substrates for the CDP-choline pathway, prevented CCT α proteolysis and mislocalization in response to FOH and oleyl alcohol.

Following conversion into a CoA ester, oleate is a precursor for a variety of complex lipids (including DAG) that could be responsible for attenuation of apoptosis. To determine whether oleoyl-CoA synthesis, and potential conversion into a complex lipid, was required for inhibition of FOH-induced apoptosis, cells were pretreated with the acyl-CoA synthase inhibitor triacsin C [35,36]. As expected, oleate prevented FOH-induced apoptosis in CHO-K1 cells based on inhibition of caspase cleavage of PARP and CCT α (Figure 7A) and release of cytosolic nucleosomes (Figure 7B). When oleoyl-CoA synthesis was prevented by treat-

Figure 7 Triacsin C prevents oleate-mediated rescue from FOH-induced apoptosis

CHO-K1 cells were cultured in medium B for 24 h prior to the start of experiments. (A, B) Cells (in medium B) were preincubated for 30 min with oleate (500 μ M) or DiC8 (500 μ M) in the absence or presence of triacsin C (9.6 μ M) prior to treatment with FOH (60 μ M) for 2 h. Total cellular extracts (15 μ g of protein) were resolved by SDS/PAGE, and CCT α and PARP were detected by immunoblotting. (C, D) Levels of cytoplasmic nucleosomes were measured in cells treated as described for (A, B) using the Cell Death ELISA, and results are expressed relative to ethanol-treated controls. Results are the averages of duplicate determinations from a representative experiment.

ment with triacsin C, apoptosis was re-established to levels observed in cells treated with FOH alone, suggesting that inhibition of apoptosis by oleate required activation and/or conversion into a complex lipid. The specificity of this effect was demonstrated by the fact that triacsin C treatment did not bypass the protective effect of DiC8 (Figures 7B and 7D), or on its own promotes apoptosis (Figure 7A).

Rapid depletion of DAG preceded FOH-induced caspase activation, nuclear export of CCT α and disruption of the CDP-choline pathway. These effects were prevented by preincubation with exogenous DAG or oleate, suggesting that restoration of endogenous DAG levels and/or PtdCho synthesis was involved. To determine which was required, endogenous DAG mass and PtdCho synthesis were measured in CHO-K1 cells treated with FOH in combination with oleate and DiC8 to prevent apoptosis (Figure 8A). None of the agents on their own significantly affected DAG levels. FOH caused a 40 % reduction in DAG mass that was not prevented by pretreatment with oleate. Pretreatment of cells with DiC8 prior to FOH addition partially restored endogenous DAG to a level that was not significantly different from cells treated with DiC8 alone. DiC8 mass in these cells was approx. 2.5-fold that of endogenous DAG in control and FOH-treated cells (19.5 and 22.3 pmol/mg respectively). PtdCho synthesis was also monitored under the same conditions described in Figure 8(A) by pulse-labelling cells for 1 h prior to harvest (Figure 8B). Treatment with oleate or DiC8 activated PtdCho synthesis 2-fold compared with untreated cells, and maintained PtdCho synthesis in FOH-treated cells at a rate similar to untreated cells. The production of PtdCho species containing DiC8 accounted for only 6–10 % of the total PtdCho in DiC8-treated cells in the presence

Figure 8 Effects of oleate and DiC8 on PtdCho synthesis and DAG mass in FOH-treated CHO-K1 cells

(A) CHO-K1 cells were cultured for 16 h in medium B followed by the addition of medium B supplemented with oleate (250 μ M), DiC8 (200 μ M) or solvent control (no addition; 'NA'). After 30 min, cells received FOH (60 μ M) (black bars) or no treatment (white bars). After FOH treatment for 2 h, cells were harvested and total lipids were extracted for DAG quantification. Results are the means $\pm$ S.D. for 3–6 independent experiments. * P < 0.001 compared with untreated or oleate-treated cells; ns, not significant compared with DiC8-treated cells. (B) PtdCho synthesis was measured by pulse-labelling cells (treated as described for A) with [3 H]choline for the final 1 h of FOH treatment (60 μ M). Results are the means $\pm$ S.D. for three independent experiments.

Figure 9 Overexpression of CCT α in MT58 cells partially prevents apoptosis in response to FOH

MT58 or MT58-CCT α cells were cultured in medium B for 16 h prior to the addition of medium B containing FOH (60 μ M). (A) Cells were incubated with FOH (0–6 h) or solvent (zero time control), and cytoplasmic nucleosomes were measured as an index of apoptosis. Results are presented as fold increase in nucleosome release relative to solvent-treated control, and are the means $\pm$ S.D. for five independent experiments. (B) Cells received choline-deficient medium B for 6 h followed by staggered addition of FOH for the indicated time periods. For the final hour of each treatment, cells were pulse-labelled with [3 H]choline (2 μ Ci/ml) and incorporation into PtdCho was quantified. Results are the means $\pm$ S.D. for three independent experiments. * P < 0.005.

or absence of FOH (results not shown). Thus restoration of endogenous DAG levels by DiC8 could be involved in protection against apoptosis, but this is clearly not the mechanism in the case of oleate. Rather, activation of CCT α and PtdCho synthesis by both DiC8 and oleate correlated with maintenance of nuclear CCT α and protection against apoptosis.

CCT α overexpression delays FOH-induced apoptosis

Since CCT α activation and increased PtdCho synthesis were correlated with protection against apoptosis, we determined whether the same effect was achieved by increased PtdCho synthesis due to CCT α overexpression. Stable overexpression of CCT α in MT58 cells harbouring a temperature-sensitive CCT α allele resulted in a 3-fold increase in PtdCho synthesis at 33 $^{\circ}$ C relative to mock-transfected controls (Figure 9A). Treatment with FOH progressively inhibited PtdCho synthesis in both cell lines, mea-

sured by [3 H]choline incorporation during the final hour of each treatment. While initial PtdCho synthesis rates were increased in the CCT α -overexpressing cells, PtdCho synthesis in both cell lines was virtually undetectable after 4–6 h. Induction of apoptosis in both cell lines, measured by the release of nucleosomes into the cytoplasm, was evident by 4–6 h and was enhanced 2-fold in MT58 controls relative to the MT58-CCT α cells (Figure 9B). Similar results were observed when cells were treated with FOH at 37 $^{\circ}$ C (results not shown). Altered sensitivity to FOH was not correlated with DAG mass since MT58 and MT58-CCT α cells displayed a similar 27 and 25 % decrease in DAG respectively after exposure to FOH for 1 h (mean for two experiments).

DISCUSSION

In addition to inducing apoptosis in cultured cells, cytotoxic agents directed against a variety of cellular targets also caused accumulation of CDP-choline and inhibition of PtdCho synthesis [19]. In the case of FOH, these effects were linked to inhibition of the terminal step in the CDP-choline pathway catalysed by CPT/CEPT [19,25]. The finding that FOH specifically activated CCT α *in vitro* and *in vivo* [28], but did not inhibit CEPT activity *in vitro* [26], led us to re-examine this proposed mechanism. In the present study, we demonstrate that activation of CCT α , stimulation of PtdCho synthesis and DAG depletion by FOH and oleyl alcohol are early events that precede maximal CDP-choline accumulation and inhibition of PtdCho synthesis. Although DAG levels were reduced by FOH and oleyl alcohol, this was not a prerequisite for apoptosis since this effect was maintained under conditions of cell survival. The primary factors affecting sensitivity to FOH and oleyl alcohol were the activity of CCT α at the nuclear envelope and PtdCho synthesis.

Under normal conditions, the product of the CCT α reaction, CDP-choline, is rapidly converted into PtdCho by CPT/CEPT. In FOH-treated cells, the accumulation of CDP-choline, and resultant inhibition of PtdCho synthesis, was interpreted as inhibition of CPT/CEPT. However, inhibition of CEPT activity in membranes from overexpressing CHO-K1 cells was time-dependent and not evident in a mixed micelle assay, suggesting that FOH perturbed the membrane environment or substrate delivery [26]. Previous studies also suggested that FOH inhibited CPT/CEPT activity by an indirect mechanism [25], further casting doubt that CPT/CEPT was a direct target for FOH *in vivo*. Based on the results of our study, an alternative mechanism for FOH- and oleyl alcohol-mediated inhibition of PtdCho synthesis and accumulation of CDP-choline can be evoked that involves uncoupling of the last two steps in the pathway by (i) depletion of DAG and (ii) activation and nuclear export of CCT α . Endogenous DAG mass was reduced by 40 % after FOH or oleyl alcohol treatment for 15 or 60 min respectively, which coincided with the early stages of CDP-choline accumulation but PtdCho synthesis was still unaffected. This argues that CPT/CEPT becomes substrate-limited with respect to DAG, resulting in decreased PtdCho synthesis at later times. This concept is supported by the reported sensitivity of PtdCho synthesis to DAG levels [27,37]. Since neither FOH nor oleyl alcohol is used as a precursor for DAG in the CDP-choline pathway, decreased DAG mass could have resulted from increased consumption due to transient activation of PtdCho synthesis (Figure 1A). However, on the basis of isotope-labelling experiments, it was concluded that the rate of PtdCho synthesis controlled partitioning of DAG between triacylglycerol and phospholipid biosynthetic pathways but did not affect DAG levels [38]. This equilibrium is clearly not maintained under conditions of pharmacological CCT α activation when flux is diverted to PtdCho synthesis or possibly altered triacylglycerol

metabolism. Alternatively, FOH or oleyl alcohol could reduce DAG mass by inhibition of its release from phospholipids by phospholipase C and/or D [24]. This seems unlikely since the extent of inhibition of PtdCho turnover in that report is not consistent with the rapidity and magnitude of DAG depletion observed here. In yeast, a similar rapid decrease in DAG mass occurred after FOH treatment that coincided with mitochondrial malfunction and generation of reactive oxygen species [39]. However, effects of FOH on the yeast CDP-choline pathway are unknown.

Elevated levels of CDP-choline, in addition to indicating CPT/CEPT inhibition by limiting the DAG substrate, could also be the result of CCT α activation and/or inefficient substrate transfer or 'channelling' of CDP-choline to CPT/CEPT [40]. The rapid and potent activation of CCT α by FOH and oleyl alcohol could produce a situation where CPT/CEPT becomes the rate-limiting reaction in the CDP-choline pathway. This situation could also deplete CTP levels by sequestering nucleotide in a metabolically inert CDP-choline pool. In CHO-K1 and other cultured cells, CCT α is localized to the nucleoplasm or inner nuclear envelope, while CEPT and CPT are localized to the ER/nuclear envelope and the Golgi apparatus respectively [41]. This native topology facilitates efficient channelling of CDP-choline to CPT/CEPT by an unknown mechanism. FOH and oleyl alcohol disrupt this topology as a result of CCT α nuclear export after 1–2 h (Figure 6) [28], potentially contributing to CDP-choline accumulation and inhibition of PtdCho synthesis. Disruption of the CDP-choline pathway by this mechanism is finalized later in the apoptotic programme by virtue of caspase cleavage of the CCT α NLS and prevention of import [28]. It is important to note that extranuclear CCT α is not associated with decreased PtdCho synthesis under all conditions. CCT α has been shown to reside partially or completely in extranuclear compartments in hepatocytes [42] and alveolar cells [13]. In these cells, PtdCho synthesis is required for secretion of lipoproteins and surfactant, perhaps necessitating that CCT α be associated with sites of synthesis in the ER or Golgi apparatus.

Prevention of FOH-induced cell death by exogenous DAG, together with our observation of rapid depletion, indicated a primary role for this lipid in apoptosis. However, the lack of restoration of DAG during oleate-mediated rescue, and only partial recovery by DiC8, suggested that this was not the case. Instead, rescue by oleate and DiC8 was correlated with activation of CCT α at the nuclear envelope, prevention of nuclear export and sustained PtdCho synthesis. Oleate did not rescue FOH-treated cells in the presence of the acyl-CoA synthase inhibitor triacsin C, indicating that a fatty acid-derived lipid was required. Oleate can potentially activate PtdCho synthesis by serving as a precursor for DAG synthesis and as a direct activator of CCT α . On the other hand, DiC8 was a poor substrate for PtdCho synthesis but activated CCT α [43]. Therefore the lack of effect of triacsin C on DiC8 suggests that the likely mechanism for rescue involves supply of an endogenous lipid activator. We and others have shown that triacsin C prevented an increase in PtdCho synthesis in response to elevated fatty acid synthesis, and this was not correlated with changes in DAG mass or synthesis [38,44]. In the context of FOH treatment, prevention of apoptosis by increased PtdCho synthesis was also not accompanied by recovery of DAG mass, indicating that DAG is an indicator of altered lipid biosynthesis rather than the apoptotic status of the cell and is not the oleate-derived lipid activator of CCT α .

It is well established that exogenous purified lipids alter the sensitivity of cells to FOH-induced apoptosis [23,45]. However, protection was also afforded by whole serum, which contains PtdCho in the form of lipoproteins, as well as fatty acids. Com-

pared with cells cultured in LPDS, CHO-K1 cells cultured in 5 % FCS were resistant to 60 μ M FOH based on the lack of caspase cleavage of PARP and partial nuclear export of CCT α (Figure 6A). This suggests that previous studies examining FOH-induced apoptosis in cultured cells, many of which were performed in media containing 5–10 % FCS, could have been influenced by the excessive amounts of FOH required to overcome the protective effects of serum lipids. While other cell-specific features or bio-availability due to serum factors could affect sensitivity to FOH, it is now apparent that intrinsic serum lipids are a confounding factor that should be eliminated.

Unlike the complete suppression of FOH-induced apoptosis by oleate or DiC8 supplementation, overexpression of CCT α in MT58 cells caused a delay in apoptosis and had no effect on suppression of PtdCho synthesis or DAG mass. Evidently, increased PtdCho synthesis following membrane translocation and activation of CCT α is necessary for full protection against FOH-induced apoptosis. Establishing CCT α and the CDP-choline pathway activity as primary determinants of sensitivity to FOH and oleyl alcohol appears to be at odds with a previous study from our group that dissociated FOH-induced apoptosis from PtdCho synthesis by overexpression of CEPT [26]. In light of our current findings, there are two explanations to account for that observation. First, CHO-K1 cells overexpressing CEPT were treated with 80 μ M FOH in medium containing 5 % FCS [26]. In our hands, 5 % FCS delayed CCT α export from the nucleus (Figure 6A) and prevented caspase cleavage of CCT α and PARP (Figure 4). Thus a combination of the increased concentration of FOH and protective effects of serum lipids could have obscured the cell-survival response in CEPT-overexpressing cells. Secondly, in contrast with MT58-CCT α cells, basal PtdCho synthesis was not increased in CHO-K1 cells overexpressing CEPT since it is not the rate-limiting enzyme [26]. Thus increasing PtdCho synthesis prior to FOH treatment could be the factor that provides resistance to FOH.

Our results show that lipophilic, non-substrate activators of CCT α disrupt PtdCho synthesis by a combined reduction in DAG mass and mislocalization of CCT α . This leads to substrate depletion of the terminal enzymes CPT/CEPT, inhibition of PtdCho production and apoptosis. Since rapidly dividing tumour cells require abundant PtdCho for membrane synthesis, pharmacological disruption of CCT α and PtdCho synthesis with agents such as FOH or oleyl alcohol could be an approach to block cell proliferation and promote apoptosis.

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