Cell-cycle arrest in Jurkat leukaemic cells: a possible role for docosahexaenoic acid

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Docosahexaenoic acid (DHA) is known to have anti-cancer activities by mechanisms that are not well understood. In the present study, we test one possible pathway for DHA action in Jurkat leukaemic cells. Low doses of DHA (10 μM) are shown to induce cell-cycle arrest, whereas higher doses are cytotoxic. However, when cells that were pre-treated with 10 μM DHA are given an additional 10 μM DHA dose, cell viability rapidly decreases. Immunoblotting reveals that repeated low doses of DHA results in activation of caspase 3, implying induction of apoptosis. DHA (10 μM) is shown to increase ceramide levels after 6 h of incubation and, after 24 h, the cells appear to be arrested in S phase. With DHA, the amount of phosphorylated retinoblastoma protein (pRb) decreases significantly. Western blot analysis also shows that DHA greatly reduces the level of cyclin A, while increasing the level of p21 WAF1, a cellular inhibitor of cyclin A/cyclin-dependent kinase 2 (cdk2) activity. Furthermore, the observed DHA-induced doubling of the ratio of hypophosphorylated pRb (hypo-pRb) to total pRb is inhibited by tautomycin and phosphatidic acid (PA), known inhibitors of protein phosphatase 1 (PP1), and by the PP2 inhibitor okadaic acid. The present study demonstrates one possible connected pathway for DHA action. By this pathway, low doses of DHA increase ceramide levels, which leads to inhibition of cdk2 activity and stimulation of PP1 and PP2A. The net effect of cdk2 inhibition and protein phosphatase activation is an inhibition of pRb phosphorylation, consequently arresting Jurkat cell growth.

Key words: ceramide, cyclin A, cyclin-dependent kinase 2 (cdk2), protein phosphatase, retinoblastoma protein, S phase.

INTRODUCTION

Epidemiology, dietary studies in humans and animals, and experiments in laboratory tissue cultures have shown that fish oils have anti-cancer properties against a variety of carcinomas [1–3]. Many studies have attributed the beneficial effects to fish oil's high content of $\omega-3$ fatty acids, particularly docosahexaenoic acid (DHA). However, despite intense investigation, the exact mechanism behind the anti-cancer properties of DHA remains unclear. Although there have been many reports of DHA affecting the activity of specific proteins (e.g. protein kinase C [4], rhodopsin [5], Na^+,K^+-ATPase [6] and Na^+ channel [7]), numerous membrane properties (e.g. permeability [8], 'fluidity' [9], lipid packing [5], fusion [9], deformability [10] and lipid microdomain formation [11,12]) and eicosanoid biosynthesis [13], there have been no attempts to connect possible pathways that link various aspects of DHA's mode of action from the fatty acid's initial incorporation into cells to its final outcome of cytotoxicity.

It is well documented that a number of drugs exert their anti-cancer effects via the inhibition of cell-cycle progression. For example, Brefeldin inhibits growth of DU-145 (prostate) cells by modulating specific G1 cell-cycle regulators and arresting cells in G_1 phase [14]. Similarly, resveratrol has also been shown to disrupt the G_1/S transition in human prostate cancer cell lines [15], whereas 15-deoxy-Δ12,14-prostaglandin J2, an activator of peroxisome-proliferator-activated receptor γ, causes accumulation of prostate cancer cells in S phase [16]. A synthetic retinoid, CD437, also induces S-phase arrest and apoptosis in LNCaP and PC-3 prostate cancer lines [17]. Studies have shown that ciprofloxacin causes cell growth arrest in S phase in bladder carcinoma HTB9 cells [18]. The effect of this drug was associated with dephosphorylation of cyclin-dependent kinase 2 (cdk2), which forms complexes with cyclin A. Another drug, camptotactin, induces S-phase arrest in MDA-MB231 and G1 phase in 101A breast carcinoma cells [19]. Similarly, an increased delay in S-phase progression and subsequent induction of apoptosis was also observed in carbonyloxycamptothecin-treated A549/p16-1 cells, with specifically down-regulated cyclin A levels [20]. It was also shown that non-apoptotic doses of ceramide arrest MCF-7 and T44D cells in S phase. 5-Fluorouracil, a well-known anti-cancer drug, also causes the arrest of human colorectal carcinoma cells in S phase [21]. These are just a few examples of studies clearly demonstrating that many anti-cancer drugs affect the early onset of the cell cycle and block progression from G_1 to S phase and that many of the drugs that arrest cells in S phase do so by modulating the expression of cyclin A and the activity of cdk2.

Previously, we demonstrated that DHA prolongs the S phase in cultured spleen lymphocytes [22]. Subsequently, other investigators demonstrated that DHA arrests malignant cells in S phase [23] and prevents G_1/S progression in human HT-29 colonic cells [24], vascular smooth muscle cells [25], and urothelial cells [26]. These observations indicate that DHA can exert its anti-cancer

Abbreviations used: CAPP, ceramide-activated protein phosphatase; cdk, cyclin-dependent kinase; DAG, diacylglycerol; DHA, docosahexaenoic acid; DTT, 1,4-dithio-DL-threitol; hypo-pRb, hypophosphorylated pRb; PA, phosphatidic acid; PE, phenylethanolamine; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; pRb, retinoblastoma protein; SMYase, sphingomyelinase.

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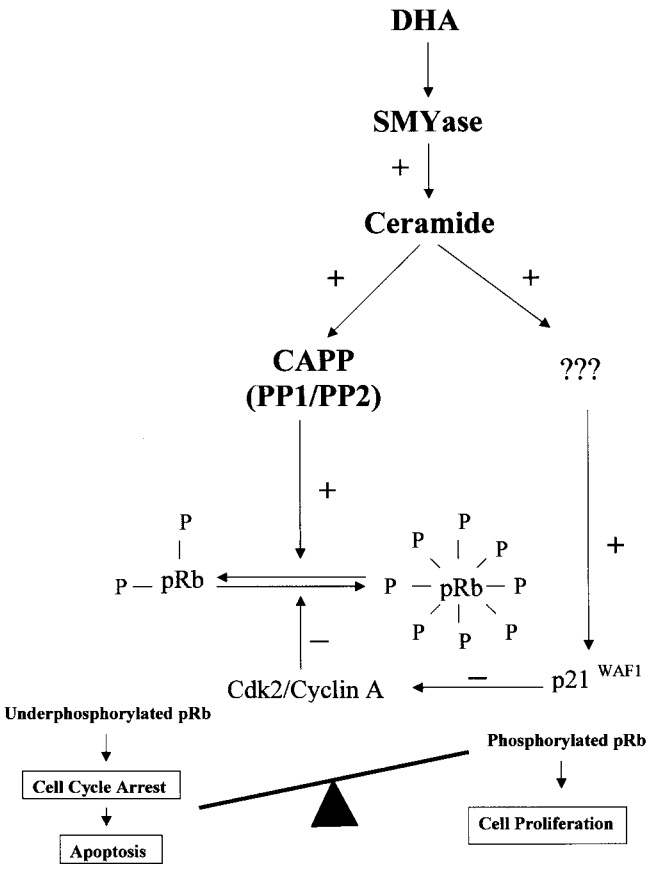

Scheme 1 Proposed action of DHA on cell-cycle arrest in Jurkat leukaemic cells

Incorporation of DHA into the cell membrane fractions causes activation of SMYase and generation of ceramide. Ceramide mediates its effects via activation of CAPPs. Activation of CAPP (PP1 and/or PP2A) results in dephosphorylation of pRb phosphorylation. Ceramide also causes increased expression of $p21^{WAF1}$ and, subsequently, inhibition of cyclin A–cdk2 activities. The overall effect of DHA results in hypophosphorylation of pRb protein and cell-cycle arrest.

effects by arresting cell-cycle progression. At the present time, however, little is known about the molecular and cellular events that lead to DHA-mediated cell cycle arrest. Previously, we demonstrated that DHA-induced apoptosis in Jurkat cells is mediated by protein phosphatases 1 and 2A (PP1 and PP2A) [2,27]. These enzymes are known to be involved in the regulation of the phosphorylation status of retinoblastoma protein (pRb), a key cell-cycle regulator [28]. The present study was therefore undertaken to investigate the cellular events that mediate DHA-induced cell-cycle arrest in Jurkat leukaemic cells. Our initial hypothesis was that DHA might cause activation of sphingomyelinase (SMYase), generating ceramide, which in turn activates ceramide-activated protein phosphatase (CAPP) [29]. Activation of CAPP (PP1 and/or PP2A) would result in hypophosphorylation of pRb. Furthermore, pRb phosphorylation could also be inhibited by ceramide-mediated enhanced expression of the protein $p21^{WAF1}$ [30]. Therefore, the effect of DHA-mediated ceramide formation might cause cell-cycle arrest through hypophosphorylation of pRb. The proposed pathway linking DHA to cell cycle arrest and inhibition of cell proliferation is outlined in Scheme 1. It should be emphasized that DHA's effects on cells are undoubtedly complex and the experiments discussed here test each step in only one of the possible pathways.

EXPERIMENTAL

Materials

Human Jurkat (clone E6-1) leukaemic cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.). Foetal calf serum was purchased from HyClone Laboratories (Logan, UT, U.S.A.), and RPMI 1640 was purchased from BioWhittaker (Walkersville, MD, U.S.A.). DHA was obtained from Nu-Chek-Prep, Inc. (Elysian, MN, U.S.A.). Monoclonal anti-procaspase 3 antibody came from Transduction Laboratories (Lexington, KY, U.S.A.), and monoclonal anti-cyclin A antibodies were supplied by Santa Cruz Laboratories (Santa Cruz, CA, U.S.A.). Propidium iodide came from Becton Dickinson (San Jose, CA, U.S.A.). BSA, 0.4 % (w/v) Trypan Blue solution, potassium hydroxide, monoclonal anti-actin antibodies, and all other reagents and chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Cell culture

Jurkat leukaemic cells (clone E6-1) were routinely cultured in 2 % foetal calf serum in RPMI 1640 supplemented with 100 units/ml of penicillin/streptomycin and 2 mM glutamine, and were incubated in a humidified atmosphere at 37 °C with 5 % CO_2. Cells (1×10^6) were washed once with serum-free RPMI 1640 and then treated with varying concentrations of DHA. The fatty acids were freshly prepared each day from sealed stocks by dissolving them in ethanol; the final concentration of ethanol in the culture medium did not exceed 0.05 %. Control experiments were carried out by adding ethanol to Jurkat cells. The Trypan Blue exclusion assay, a widely used procedure in cytotoxicity and apoptosis studies, was used to determine cell viability, and the live and dead cells were enumerated using a haemocytometer.

Cell-cycle analysis

Jurkat cell samples (1×10^6 cells/ml) were treated with 10 μM DHA and incubated for 24 h at 37 °C. Cell-cycle analysis was performed using a Becton Dickinson FACStar^{PLUS} flow cytometer. Cells were labelled with propidium iodide and then excited with an argon, water-cooled laser emitting at 488 nm. Propidium iodide was detected using a 630 ± 20 nm band pass filter. Cell-cycle analyses were performed using Modfit LT software (version 2.0, Becton Dickinson). A doublet discriminatory gate was established to ensure only authentic targeted events were permitted for analysis.

Measurement of ceramide

Ceramide in lipid extracts of DHA-treated cells was quantified using the diacylglycerol (DAG) kinase assay as described in [31]. Briefly, stimulated cells were extracted with $CHCl_3/CH_3OH/HCl$ [100:100:0.6 (by vol.)]. The lipid extracts (organic phase) were dried under N_2, and DAG was digested by mild alkaline hydrolysis in 0.1 M methanolic potassium hydroxide for 1 h at 37 °C. The samples were re-extracted, dried, and solubilized by sonication in a 50 μl reaction mixture containing 5 mM cardiolipin (Avanti Polar Lipids, Alabaster, AL, U.S.A.), 1 mM diethylenetriaminepenta-acetic acid (Sigma Chemical Co.), 7.5 % octyl-β-glucopyranoside (Calbiochem, San Diego, CA, U.S.A.) and 10 mM imidazole, pH 6.6. The reaction was started by adding 100 μl of reaction buffer (100 mM imidazole/HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$ and 2 mM EGTA), 20 μl of 20 mM 1,4-dithio-DL-threitol (DTT), 19 μl of cold 20 mM ATP, 1 μl of [γ-^{32}P]ATP (10 μCi) and 10 μl of *Escherichia coli* DAG

kinase (Calbiochem). The reaction was incubated for 1 h at room temperature (22 °C) and then stopped by adding 0.5 ml of $CHCl_3$/methanol/HCl [100:100:0.6 (by vol.)]. The lipids were extracted into the organic phase as described above, and ^{32}P-labelled ceramide 1-phosphate was resolved by TLC using chloroform/acetone/methanol/acetic acid/water [10:4:3:2:1 (by vol.)]. The spots for ceramide 1-phosphate were identified using an authentic standard and scraped and counted in a Beckman LS6000IC liquid scintillation counter (Beckman Instruments Inc., Arlington Heights, IL, U.S.A.).

Western blotting

After incubation, the cells were treated with 50 μl of lysis buffer [20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10 % (v/v) glycerol, 1 % Nonidet P40, 2 mM PMSF, 1 mg/ml leupeptin, 0.15 unit/ml aprotinin and 2.5 mM di-isopropyl fluorophosphate] for 10 min on ice. Protein concentrations in each cell lysate were measured using a bicinchoninic acid ('BCA') protein assay system (Pierce, Rockford, IL, U.S.A.). An equal amount of detergent-solubilized protein extracts was analysed by SDS-PAGE (8 % or 12 % gels). The separated proteins were transferred electrophoretically on to a PVDF membrane. TTBS [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05 % (v/v) Tween 20] containing 2 % (v/v) BSA was used to block residual binding sites on the membrane. The membranes were then incubated with primary antibody [anti-cyclin A antibody, anti-actin antibody, anti-phospho-pRb antibody or anti-caspase 3 antibody, 1:1000 in TTBS containing 1 % (v/v) BSA] for 1 h at room temperature. The membranes were then washed three times with TTBS for 10 min each and incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG [1:1000 dilution in TTBS containing 1 % (v/v) BSA] for 1 h. Blots were developed using an enhanced chemiluminescence kit and X-ray film (Amersham Biosciences, Little Chalfont, Bucks, U.K.). Quantification of bands was performed by densitometric analysis using a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY, U.S.A.).

Analysis of cdk2 activities

cdk2 activities in cells that were treated with DHA (10 μM) or vehicle (ethanol) were determined as described in [32]. Cell lysates containing equal amounts of proteins (500 μg/ml) were precleared by incubating for 2 h at 4 °C with 2 μg of normal mouse IgG (Santa Cruz Biotechnology) and 20 μl of protein A/G agarose beads (Oncogene Research Products, San Diego, CA, U.S.A.). The beads were removed by centrifugation at 800 ***g*** for 5 min at 4 °C, and the lysates were incubated with 5 μg of monoclonal anti-cdk2 antibodies (Santa Cruz Biotechnology) overnight at 4 °C on a rocker. The immunoprecipitated cdk2 was isolated using 20 μl of protein A/G agarose beads that had been washed five times in lysis buffer (see above) and, finally, washed once with kinase buffer (20 mM Tris/HCl, pH 7.4, 7.5 mM $MgCl_2$ and 1 mM DTT). The agarose beads containing immunoprecipitated cdk2 were then suspended into 20 μl of kinase buffer, supplemented with 20 μM ATP, 5 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham International, Arlington Heights, IL, U.S.A.), and 5 μg of histone H1 protein (Roche Applied Science, Indianapolis, IN, U.S.A.). The mixture was incubated at 30 °C for 30 min before the reaction was stopped by boiling the samples in 20 μl of Laemmli SDS loading buffer for 5 min. The samples were then separated by SDS/PAGE (12 % gel). The gel was dried and subjected to autoradiography. Quantification of bands was performed by densitometric analysis using the Kodak Image Station 440CF.

Analysis of hypo-pRb to total pRb ratios

Ratios of hypo-pRb to total pRb were analysed by flow cytometry as described previously in [24]. Jurkat cells were incubated with 10 μM DHA in the presence or absence of tautomycin (10 nM) or okadaic acid (10 nM) for 24 h. After incubation, the cells were washed once with PBS and then fixed with 1 % (w/v) formaldehyde in PBS for 15 min at 4 °C. Cells were washed once with PBS and then suspended in ice-cold 80 % (v/v) ethanol and incubated for 2 h at −20 °C. The ethanol-fixed cells were washed again with cold PBS and then suspended in 1 ml of 0.25 % (v/v) Triton X-100 in PBS on ice for 5 min. The cells were then isolated after centrifugation at 800 ***g*** for 5 min at 4 °C and suspended in 100 μl of PBS containing 1 % (v/v) BSA and 0.5 μg of FITC-conjugated anti-(hypo-pRb) (clone G99-549 BD, Biosciences Pharmingen, San Diego, CA, U.S.A.), which specifically recognizes hypo-pRb, and 0.5 μg of phenylethanolamine (PE)-conjugated anti-pRb (clone G3-245 BD, Biosciences Pharmingen), which specifically recognizes total pRb. These antibodies do not interfere with each other's binding and therefore allow for the quantification of ratios of hypo-pRb to total pRb in the same set of samples [23]. Quantification of hypo-pRb to total pRb ratios was performed using a FACStar^{PLUS} Flow Cytometer equipped with a water-cooled argon laser emitting at a wavelength of 488 nm. FITC-conjugated antibody was detected through a 530 ± 30 nm bandpass filter, whereas PE-conjugated antibody was detected through a 575 ± 26 nm bandpass filter. Subsequent analysis of data was performed using Cell Quest Software (Becton Dickinson). A gate was established to ensure that cellular debris and necrotic cells were excluded from analysis. Arbitrary mean fluorescence units were determined based on gated events in order to calculate the ratios of hypo-pRb to total pRb.

Analysis of p21^{WAF1} expression

Expression of p21^{WAF1} was analysed by flow cytometry. Briefly, cells (1×10^6/ml) were incubated with DHA, PA, or DHA + PA, as described above. Cells were then harvested and fixed in Reagent A (Fix and Perm Kit, Caltag Laboratories, Burlingame, CA, U.S.A.) for 10 min at room temperature. The fixed cells were washed with 5 % (v/v) BSA in PBS and then suspended in 95 % (v/v) methanol for 30 min on ice. Cells were washed once as described above and then suspended in Reagent B (Fix and Perm Kit, Caltag Laboratories) and 5 μg/ml FITC-conjugated anti-p21^{WAF1} antibodies (Oncogene Research Products, San Diego, CA, U.S.A.) for 1 h at room temperature in the dark. Finally, cells were washed and suspended in 5 % (v/v) BSA solution and analysed by flow cytometry for FITC labelling, as described above.

RESULTS

DHA induces cell-cycle arrest

Cellular growth and cytotoxic effects of various concentrations of DHA were measured by manually counting total (dead and alive) Jurkat cells using the Trypan Blue exclusion assay. At 0 to 10 μM DHA, no significant difference in cell viability was observed; however, concentrations of DHA > 10 μM caused increased cell death in a concentration-dependent manner (Figure 1a). Figure 1(b) shows the effect of DHA on cell growth and indicates that treatment with 5 μM DHA had no effect, and that the cells continued to grow at a rate similar to the control (vehicle-treated) cells. However, higher concentrations of DHA inhibited cell growth in a concentration-dependent manner. DHA at 10 μM arrested cell growth, and concentrations greater than this induced cell death, with the majority of cells dying within 4 to 6 h.

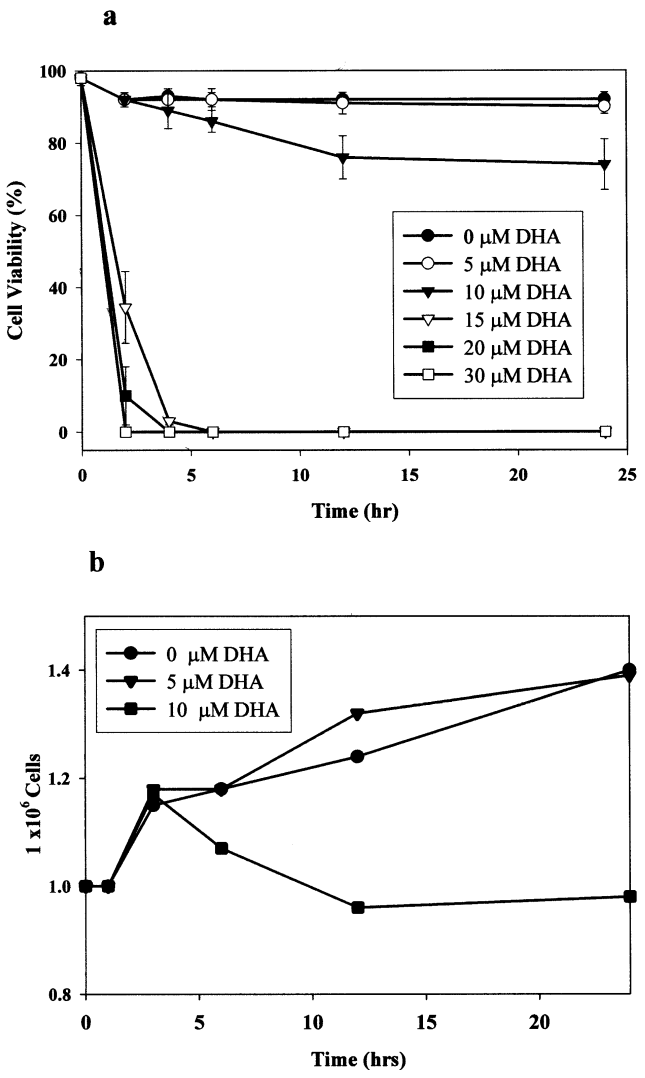

Figure 1 Effect of DHA on Jurkat leukaemic cell viability and growth

Cells (1×10^6/ml) were incubated in the presence of various concentrations of DHA for different time periods. In (**a**), the percentage of viable cells was calculated by counting live and dead cells using the Trypan Blue exclusion assay. Results are means ± S.E.M. for three experiments. In (**b**), cell growth was assessed by calculating the mean number of total live cells after each incubation period.

We studied further the effect of repeated treatment of low doses of DHA on Jurkat cell survival. Cells that had been pre-incubated in the absence or presence of either 5 or 10 μM DHA for 24 h were then incubated further in the presence or absence of an additional dose of DHA (5 or 10 μM) for another 24 h. In the absence of prior treatment with DHA, 5 or 10 μM DHA did not have a significant effect on cell viability (Figure 2a). Addition of DHA (5 or 10 μM) to Jurkat cells that had been pretreated with a dose of 5 μM DHA also had no significant effect on cell viability (Figure 2b). However, cells that were pretreated with 10 μM DHA exhibited a progressive decline in cell viability with additional DHA. Their viability was decreased to 50 % ($P = 0.002$) and 40 % ($P < 0.001$) upon incubation for 24 h with 5 and 10 μM DHA respectively (Figure 2c). These results indicate that cells that are pretreated with low doses of DHA are primed to die upon subsequent exposure to additional DHA. We also examined the effect of repeated low-dose DHA exposure on induction of

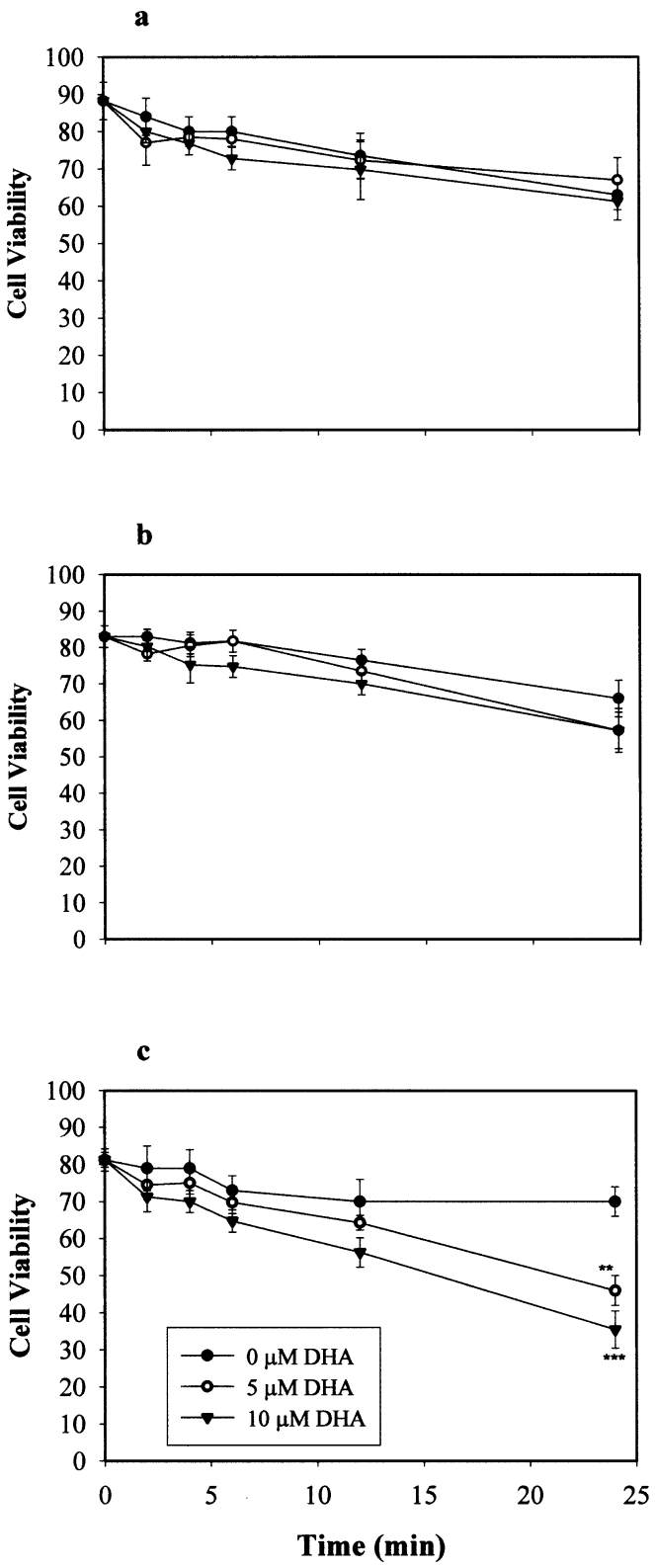

Figure 2 Effect of repeated treatment with DHA on Jurkat cell viability

Cells (1×10^6/ml) were pre-incubated with (**a**) ethanol (control), (**b**) 5 μM DHA or (**c**) 10 μM DHA for 24 h at 37 °C, as described in the Experimental section. The cells from each DHA initial treatment group were then further subdivided into three groups and subsequently retreated with ethanol (●), 5 μM DHA (○), or 10 μM DHA (▼) and incubated for another 24 h. Percentage cell viability was determined by the Trypan Blue exclusion method. Results are means ± S.E.M. for three experiments and were analysed by ANOVA. Significant differences compared with the control are indicated: **, $P = 0.002$; ***, $P < 0.001$.

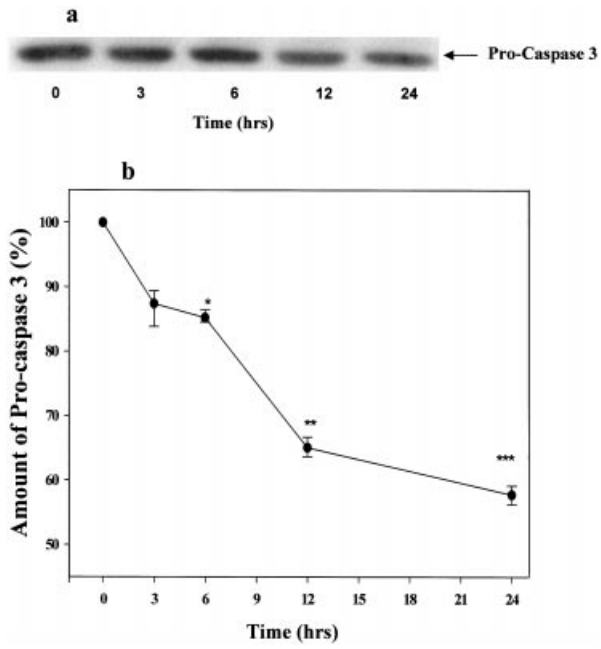

Figure 3 Effect of repeated treatment with DHA on caspase 3 proteolysis

Cells (1×10^6/ml) that were pre-incubated with 10 μM DHA for 24 h at 37 °C were subsequently treated with 10 μM DHA and incubated for various time periods. Caspase 3 bands were detected by Western immunoblotting as described in the Experimental section. Panel (**a**) is a typical representation of the results from three experiments, whereas the analysed data in panel (**b**) shows the means $\pm$ S.E.M. of three experiments. Results were analysed by ANOVA. Significant differences relative to zero-time treatment are indicated: *, $P = 0.01$; **, $P = 0.002$; ***, $P < 0.001$.

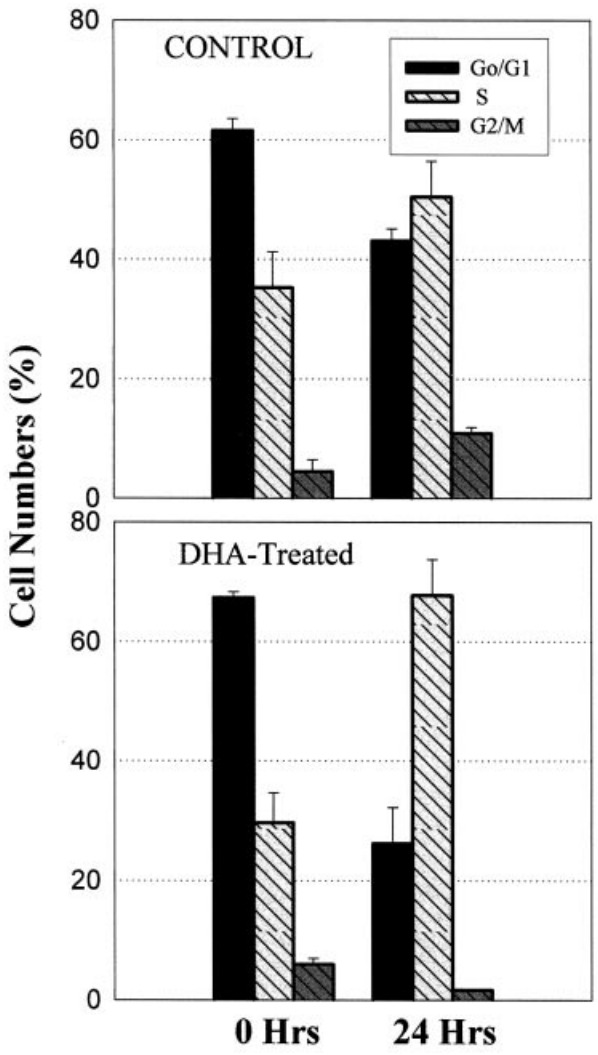

Figure 4 Effect of DHA on cell-cycle progression

Cells (1×10^6/ml) were treated with either vehicle (control) or 10 μM DHA (DHA-treated) and incubated for 24 h at 37 °C. Cell-cycle analysis was performed as described in the Experimental section. Cells in G_0/G_1 phase are represented by solid black bars, cells in S phase are represented by grey hatched bars and cells in G_2/M phase are represented by white hatched bars.

apoptosis. The results demonstrated that 3 or 6 h of DHA treatment slightly reduces procaspase 3 enzyme (an indicator of apoptosis); however, further incubation for 12 and 24 h caused a significant decrease of 45% to 50% in the amounts of procaspase in growth-arrested Jurkat cells (Figure 3). These observations suggest that the initial 24 h treatment with 10 μM DHA primed cells, which subsequently underwent apoptotic cell death upon further treatment with a second dose of 10 μM DHA. We investigated further the priming effect of 10 μM DHA by analysing its effects on cell-cycle progression as discussed below.

DHA causes cell cycle-arrest in S phase

The growth-inhibitory effects of 10 μM DHA were examined by analysing cell-cycle progression using flow cytometry. In the control experiments under serum-free conditions, approx. 15% more cells accumulated in S phase [35.5 ± 6% (0 h) compared with 50.5 ± 5% (24 h)]. However, when cells were treated with 10 μM DHA, approx. 38% more of the cells accumulated in S phase [29.7 ± 5% (0 h) compared with 67.7 ± 6% (24 h)] (Figure 4, shaded areas). Concomitant with these changes, the percentage of cells in the G_0/G_1 phase was decreased from 61.6 ± 2% to 43.1 ± 2% in the control cells, whereas cell accumulation in the G_0/G_1 phase in DHA-treated cells decreased from 67.3 ± 1% to 26.2 ± 6% (Figure 4, filled areas). Furthermore, it appeared that, in the presence of 10 μM DHA, only a small fraction of the Jurkat cells were progressing into G_2/M phase (1.65 ± 0.1% DHA-treated compared with 10.9 ± 1% control). These observations indicate that 10 μM DHA has inhibited the growth of Jurkat cells by arresting cells in S phase and blocking their progression into G_2/M phase.

Effect of DHA on ceramide formation

Our hypothesis proposes that DHA increases ceramide levels, which then reduces pRb phosphorylation, resulting in cell-cycle arrest. We therefore examined ceramide levels in DHA-treated cells as a possible cause of DHA-induced growth arrest in Jurkat leukaemic cells. DHA (10 μM) induced a several-fold increase in ceramide formation, with a maximum 4-fold ($P < 0.001$) increase noted as early as 3 h after treatment (Figure 5). After this time, ceramide formation decreased slightly.

DHA effects on cell-cycle arrest are mediated by pRb hypophosphorylation

Phosphorylation of pRb is essential for progression through the cell cycle. Therefore, we assessed the time course for the

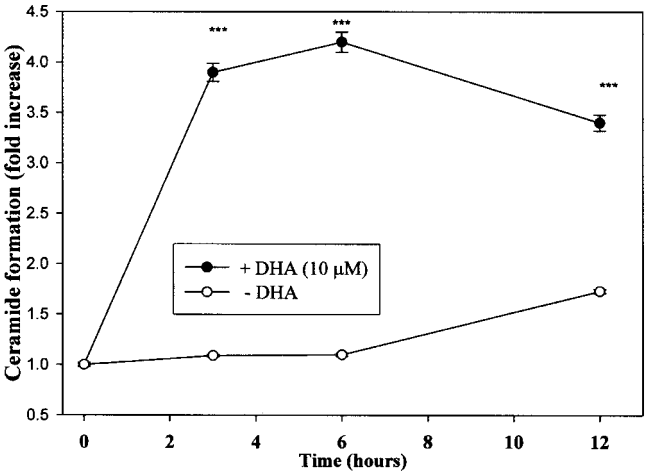

Figure 5 DHA effect on ceramide formation

Cells (1×10^6/ml) were incubated with ethanol or 10 μM DHA for various time periods and then extracted with $CHCl_3/CH_3OH/HCl$ [100:200:0.6 (by vol.)]. Ceramide levels relative to the DHA-free (ethanol) control were determined as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments. Results were analysed by Student's *t* test, relative to ethanol treatment (control) at each time point. ***, $P < 0.001$.

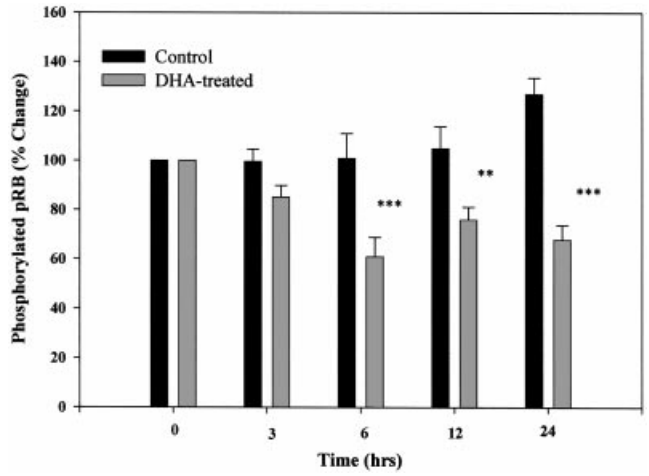

Figure 6 Phosphorylation of pRbs

Cells (1×10^6/ml) were grown in the presence of either vehicle (control) or 10 μM DHA (DHA-treated). The phosphorylation status of pRb was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments and were analysed by Student's *t* test, relative to the control at each time point. **, $P < 0.01$; ***, $P < 0.001$.

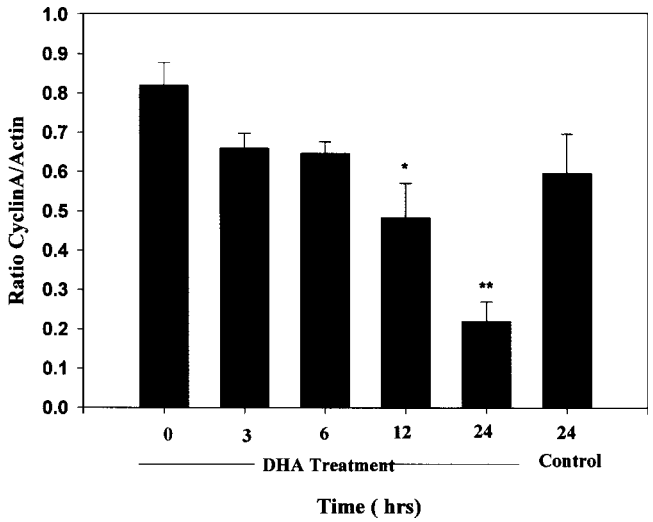

Figure 7 DHA effect on cyclin A

Cells (1×10^6/ml) were treated with 10 μM DHA and incubated for various time periods. Cyclin A levels were detected and normalized for actin content by Western immunoblotting analysis as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments and were analysed by ANOVA. Significant differences relative to zero-time treatment are indicated: *, $P = 0.008$; ***, $P < 0.001$.

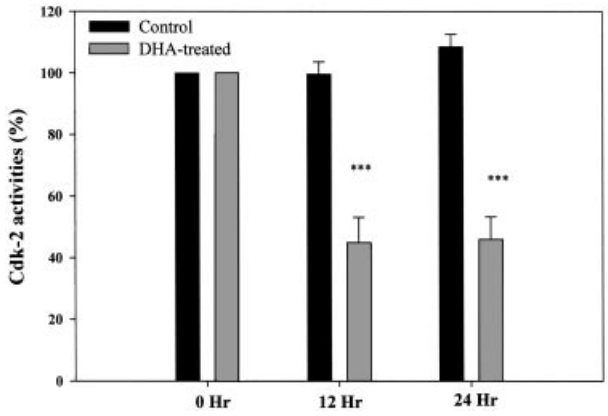

Figure 8 DHA effects on cdk2 activity

Cells (1×10^6/ml) were treated with ethanol (control) or 10 μM DHA (DHA-treated) and incubated for 0, 12 and 24 h at 37 °C as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments. Results were analysed by Student's *t* test, relative to control treatment at each time point. ***, $P < 0.001$.

phosphorylation of pRb in the presence or absence of DHA. The results presented in Figure 6 demonstrate that phosphorylation of pRb was not changed significantly in control cells over 24 h, whereas it was decreased by 40 % ($P < 0.001$) as early as 6 h after DHA treatment and remained significantly lower ($P < 0.01$ to $P < 0.001$) during the 24 h incubation.

DHA modulates cyclin A expression and cdk2 activity in Jurkat cells

Cyclin A, a mediator of S-phase progression, binds to cdk2 in S phase and regulates phosphorylation of pRb. Cellular levels of cyclin A were therefore assessed by Western blot analysis. The results presented in Figure 7 indicate that even 3 h of DHA treatment significantly decreased the expression of cyclin A in Jurkat cells. By 12 h of incubation with 10 μM DHA, cyclin A expression was decreased to 55 % ($P = 0.008$) and was further decreased to 26 % ($P < 0.001$) after 24 h. In control cells after 24 h of incubation, cyclin A expression was decreased to 70 %.

Cellular levels of cyclin A are essential for the activity of cdk2 in S phase. Therefore, we also compared cdk2 activity of DHA-treated Jurkat cells with that of non-treated Jurkat cells using an *in vitro* kinase assay. The results shown in Figure 8 indicate that, in control cells, cdk2 activities were not significantly decreased,

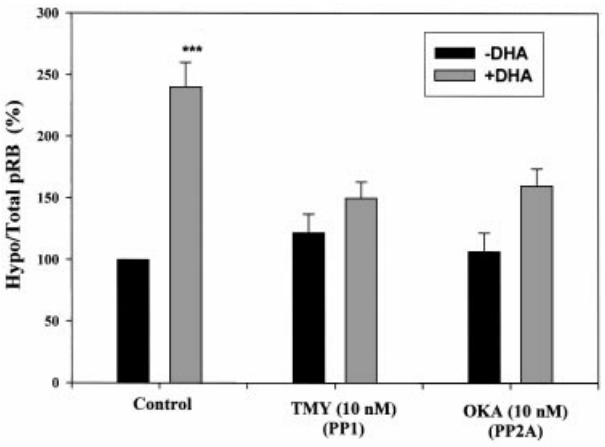

Figure 9 Effects of DHA and protein-phosphatase inhibitors on phosphorylation of pRb

Cells (1×10^6/ml) were treated with ethanol or 10 μM DHA in the presence or absence of 10 nM tautomycin (TMY) or 10 nM okadaic acid (OKA) for 24 h at 37 °C as described in the Experimental section. Results means ± S.E.M. for three experiments. Results were analysed by Student's *t* test, relative to no DHA treatment in each group. ***, $P < 0.001$.

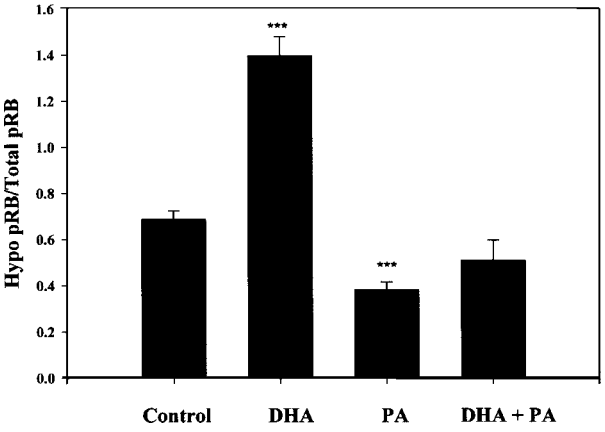

Figure 10 Effects of PA on phosphorylation of pRb

Cells (1×10^6/ml) were treated with ethanol (control), 10 μM DHA, 25 μM PA or 10 μM DHA + 25 μM PA and incubated for 24 h at 37 °C. Cells were labelled with FITC-conjugated anti-(hypo-pRb) and PE-conjugated anti-pRb (total pRb) antibodies as described in the Experimental section. Results are means ± S.E.M. for three experiments. Results were analysed by ANOVA. Significant differences relative to ethanol treatment (control) are indicated: ***, $P < 0.001$.

whereas, in DHA-treated cells, cdk2 activities were diminished by 50 % ($P < 0.001$).

These results indicate that DHA can arrest Jurkat cells in the S phase of the cell cycle by inhibiting cyclin A expression and consequently inhibiting cdk2 activity.

Effect of protein-phosphatase inhibitors on pRb phosphorylation

We also investigated the effect of protein phosphatases on the phosphorylation of pRb. The results presented in Figure 9 clearly indicate that hypo-pRb more than doubled in the presence of 10 μM DHA. Tautomycin and okadaic acid, known inhibitors of PP1 and PP2A respectively, were then tested on pRb phosphorylation. These compounds inhibited DHA-induced hypo-phosphorylation of pRb (Figure 9). The involvement of PP1 in pRb phosphorylation was further examined using PA, a highly potent and selective inhibitor of PP1 [33]. Figure 10 demonstrates that phosphorylation of pRb by DHA was also effectively inhibited by PA.

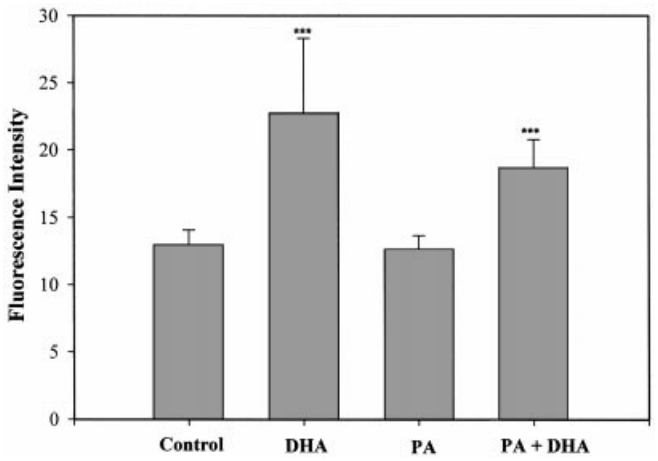

Figure 11 Effect of DHA and PA on p21^{WAF1} levels

Cells (1×10^6/ml) were treated with ethanol (control), 10 μM DHA, 25 μM PA or 10 μM DHA + 25 μM PA and incubated for 24 h at 37 °C. p21^{WAF1} was detected by FITC-conjugated anti-p21^{WAF1} antibodies as described in the Experimental section. Results are means ± S.E.M. for three experiments. Results were analysed by ANOVA. Significant differences relative to ethanol treatment (control) are indicated: ***, $P < 0.001$.

DHA increases p21^{WAF1} expression

The cellular levels of p21^{WAF1} also affect phosphorylation of pRb by regulating cyclin A/cdk2 activities. We therefore assessed the effect of DHA on p21^{WAF1} levels and investigated whether protein phosphatases are involved in p21^{WAF1} modulation. The results presented in Figure 11 indicate that DHA (10 μM) caused a 2-fold ($P < 0.001$) increase in p21^{WAF1} expression after 24 h of incubation. The PP1 inhibitor, PA, had no effect on p21^{WAF1} by itself and also did not inhibit the DHA-induced increase in p21^{WAF1} expression.

DISCUSSION

Long-chain polyunsaturated $\omega-3$ fatty acids inhibit the growth of cancer cells both *in vivo* and *in vitro*. For example, studies demonstrate that dietary supplementation with DHA (as a pure agent or as a component of fish oils) increase apoptotic cell death in normal rat colonic cells [34], HT-29 colon cancer cells [35] and Morris hepatocarcinoma 3924A cells [36]. It has also been reported that DHA induces apoptosis in cultures of lung carcinoma A427 cells [37], Hep2 human larynx tumour cells [38], pancreatic Mia-Pa-Ca-2 cells [39] and embryonic carcinoma Tera-2 cells [40]. $\omega-3$ fatty acids also suppress growth of MDA-MB-231 human breast cancer cells in athymic nude mice [41], increase survival time in dogs with lymphoma [42] and reduce the risk of prostate cancer in humans [3]. It is obvious from these and many other similar studies that DHA can be cytotoxic to cancer cells. Some studies have suggested that DHA may affect cell growth by altering cell-cycle progression [22–26]. In the present study, we investigated some aspects of the molecular mechanism by which DHA may modulate the cell cycle in leukaemic cells.

Our earlier studies using a model lipid bilayer suggest that DHA incorporation into membranes containing sphingomyelin

and cholesterol affects the formation of a type of lipid microdomain known as 'lipid rafts' [12]. These microdomains also contain sphingomyelinase, an enzyme that generates ceramide, a potent second messenger involved in cell-cycle arrest and apoptosis [43,44]. Ceramide levels also change during the progression of the cell cycle [45]. We therefore examined levels of ceramide in DHA-induced growth-arrested cells. As predicted, our results demonstrate that DHA causes increased ceramide formation (Figure 5), probably resulting from DHA-induced activation of SMYase in the plasma membranes. Ceramide is also known as a potent activator of a protein phosphatase specific for cdk2 [30] and also functions as a modulator of pRb phosphorylation [45].

Progression through each phase of the cell cycle is tightly regulated and involves the expression and rapid degradation of the cyclin A–cdk2 complex. In general, the levels of cdks are relatively constant throughout the cell cycle, whereas the cyclin levels vary substantially [46]. Cyclin A appears in S phase with the onset of DNA synthesis [47]. Cyclin A associates initially with cdk2 and later with cdc2 [47]. The association, and hence the activities of cdk2 and cdc2, are essential for progression through S phase to G_2 phase. Many of the effects of cyclin A–cdk2 are mediated through phosphorylation of the protein pRb. pRb controls the progression of the cell cycle by regulating the activities of transcription factors, most importantly E2F2 and E2F3. In a hypophosphorylated state, pRb physically associates with these transcription factors and blocks their ability to activate gene expression of products necessary for cell-cycle progression. Once phosphorylated, pRb loses much, if not all, of its growth-inhibitory capability and permits the advance into late G_1, and hence into the remainder of the cell cycle. It has also been suggested that cyclin A–cdk2 contributes to the sustained phosphorylation of pRb in S phase [48], and hence in the progression of the cell cycle. Since our studies indicate that DHA-treated cells exhibit cell-cycle arrest in S phase, we therefore explored the activities of cyclin A–cdk2. The results of the present study indicate that the effect of DHA on cell-cycle arrest may be mediated through diminished expression of cyclin A (Figure 7) and inhibition of cdk2 activity (Figure 8), resulting in hypophosphorylation of pRb (Figure 6). In the present study, the role of other cyclin–cdk complexes that regulate G_1 and G_2/M phases were not examined. However, it is possible that DHA may also effect expression of other cyclins, including cyclins D, E and B, and therefore may also alter cdk4 activity.

Furthermore, PP1 has also been implicated in the dephosphorylation of pRb [28]. Our research has demonstrated that DHA treatment of Jurkat cells leads to activation of PP1 and PP2A (Figures 9 and 10; [2]). Therefore, activation of PP1 and PP2A by DHA may contribute further to hypophosphorylation of pRb. Our studies also indicate that DHA induces elevated levels of $p21^{WAF1}$ (Figure 11). Ceramide has been shown to enhance expression of p21 [30], a cellular inhibitor of cdk2. Through the p21 mechanism, it is possible that elevated levels of ceramide lead to inhibition of cdk2. It therefore appears that DHA-induced ceramide may regulate phosphorylation of pRb by direct activation of protein phosphatases and perhaps by inhibition of cyclin A–cdk2 activities via increased expression of $p21^{WAF1}$. Although in the present study, we did not examine the molecular mechanism by which ceramide leads to increased expression of $p21^{WAF1}$, it is clear that PP1 is not involved upstream of $p21^{WAF1}$ expression. A role for ceramide in the induction of p21 via activation of nuclear factor κ-B (NFκB) and/or p53 has been established by various studies [49,50].

Our results also suggest that DHA's effect on cell-cycle arrest occurs when incorporation of DHA in the membranes reaches a certain threshold level (Figure 2b). Any further addition of DHA then results in a cytotoxic effect, possibly due to its detergent-like properties. Furthermore, in the experiments presented here, DHA was added in serum-free conditions. In other experiments, we reported that, in the presence of serum, concentrations of DHA approx. 4 times higher are required to observe similar effects [2,27]. The results presented in Figures 2 and 3 also indicate that growth-arrested cells undergo apoptosis upon repeated treatment with low doses of DHA. Apoptosis appears to be mediated via caspase 3 activation (Figure 3). Our previous results [2] suggest that activation of caspase 3, and hence induction of apoptosis by DHA, is mediated through activation of protein phosphatases. Our studies are consistent with several others that have shown apoptosis can be mediated by activation of protein phosphatases. For example, Wolf and Eastman [51] demonstrated that activation of PP1 plays an important role in Fas-induced apoptosis by stimulating the mitochondrial release of cytochrome *c* and caspase activation in HL-60 and Jurkat cells. Similarly, activation of a PP2A-like phosphatase has been demonstrated to have a key role in the induction of apoptosis in a neuronal cell line [52]. Other studies have shown that CAPP, a member of the PP2A family, is involved in receptor-mediated induction of apoptosis in various cell lines [29]. These studies suggest that protein phosphatase activation may be a common feature of cells that undergo apoptosis. However, at the present time, it is not clear how DHA activates protein phosphatases and how activation of protein phosphatases is linked to cell-cycle arrest and induction of apoptosis.

In summary, the present study indicates that low doses of DHA may induce cell-cycle arrest in Jurkat leukaemic cells through hypophosphorylation of pRb by inhibiting cdk2 activity and stimulating protein phosphatase activities. Furthermore, repeated low doses of DHA induce apoptosis via activation of caspase 3. Although these studies probe one possible pathway for DHA's effect on cell growth and viability, many other, often overlapping, possible modes of action undoubtedly exist. Indeed, unravelling the complex molecular mechanisms for DHA action will keep investigators busy for a long time.

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