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ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 106 – 114
Journal of Nutritional Biochemistry

Docosahexaenoic acid induces apoptosis in colorectal carcinoma cells by modulating the PI3 kinase and p38 MAPK pathways☆

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Received 26 July 2007; received in revised form 7 November 2007; accepted 10 December 2007

Abstract

Numerous studies have shown that long-chain polyunsaturated fatty acids can kill cancer cells in vitro as well as in vivo, while normal cells remain unaffected. Unfortunately, the cellular and molecular mechanisms responsible for this phenomenon are still poorly understood. The aim of this study was to investigate the potential chemopreventative/antiproliferative potential of docosahexaenoic acid (DHA) in an adenocarcinoma cell line ($CaCo_2$ cells) and to evaluate the signalling pathways modulated by it. DHA (5-50 μM) significantly inhibited cell viability in a dose-dependent manner in $CaCo_2$ cells, while the viability of normal colon cells (NCM460 cells) was not compromised. DHA also induced apoptosis in $CaCo_2$ cells, as indicated by increases in caspase-3 activation and poly-ADP-ribose polymerase cleavage. Signalling proteins, which include extracellular signal-regulated kinase, p38 mitogen-activated protein kinase (MAPK), Akt and p53 were analysed by Western blotting using phosphospecific and total antibodies. The protein inhibitors wortmannin (phosphoinositide 3 kinase inhibitor), PD 98059 (MEK inhibitor) and SB 203580 (p38 inhibitor) as well as silencing RNA [small interfering RNA (siRNA)] of the p38 MAPK protein, were used to investigate cross-talk between signalling pathways. DHA supplementation significantly suppressed Akt phosphorylation, which also correlated with decreased cell viability and increased apoptosis in $CaCo_2$ cells. Furthermore, siRNA experiments suggested a possible role for p38 MAPK in the phosphorylation of p53 at Ser^{15}, a site which is associated with DNA damage. DHA might thus exert its beneficial effects by means of increased apoptosis and suppression of the important survival-related kinase, Akt.
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Keywords: Docosahexaenoic acid; Colon cancer; p38 MAPK; Akt/PKB, p53; Signalling

1. Introduction

Cancer is a major health problem in both industrialized and developing nations [1]. Worldwide, cancer rates are expected to increase by 50% by the year 2020 [2]. The role of dietary factors, especially lipids, in the aetiology and progression of various cancers has been under intense investigation the past 3 decades. Epidemiological studies of Alaskan natives have demonstrated a reduced incidence of cancers, including cancers of the lung, larynx, bladder, prostate, breast and colon, and investigators have correlated this with the large quantities of polyunsaturated fatty acids (PUFAs) present in their diet [3]. In both animal and cell culture models, this phenomenon was successfully reproduced, indicating that long-chain PUFAs can significantly reduce tumour growth in vivo and suppress cell viability and induce apoptosis of cancer cells in vitro [4–7]. Nevertheless, despite intense investigation, the precise molecular mechanisms responsible for the anti-tumourigenic properties of PUFAs remain elusive. Recent studies suggested that, at this stage, most proposals of possible mechanisms are only speculative. According to one of these proposed mechanisms, certain long-chain n-3 PUFAs can change membrane organisation in such a way that they can influence the activities of various downstream signalling pathways [8]. This proposal is of particular interest, since the modulation of the activities of signalling proteins by fatty acids has been previously demonstrated in different cell lines, including smooth muscle and liver cells [9,10]. However, this has rarely, if ever, been investigated with regard to the outcome of cell death in cancer cells.

☆ This work was funded by research grants from the National Research Foundation and the Medical Research Council of South Africa.

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doi:10.1016/j.jnutbio.2007.12.005

Extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) are Ser/Thr kinases that are activated in a cascade-like fashion that is typical of members of the MAPK family. ERK is mostly associated with cellular survival and proliferation brought on by mitogenic inputs, and p38 MAPK is activated by different types of cellular stress and is mostly associated with apoptotic cell death and differentiation [11,12].

Akt, also a Ser/Thr kinase, activated via a specific enzyme known as phosphoinositide 3 (PI3) kinase [13,14] is involved in cellular processes such as proliferation, differentiation and metabolism and is fundamental to cellular survival by means of the suppression of apoptosis [15].

The *p53* gene, which encodes the p53 transcription factor, is widely implicated in carcinogenesis and has been found to be mutated in more than 50% of human tumours [16]. Target genes which are also regulated by p53 include the proapoptotic bcl-2 family genes *puma*, *noxa*, *bid* and *bax*, the death receptor genes *Fas* and *DR5* and the mitochondrial pathway genes *APAF-1* and caspase-6. Furthermore, p53 also prevents the induction of survival pathways by blocking the activation of Akt via PTEN [17]. Overall, p53 serves to protect the cell from the potentially damaging consequences of various stresses by means of cell cycle arrest to allow DNA repair or the initiation of apoptosis [13].

Despite various studies on fatty acid-induced apoptosis in cancer cells, the signalling mechanisms in docosahexaenoic acid (DHA)-mediated apoptosis are still poorly elucidated. In this study, we investigated the cytotoxic effect of DHA on human colon adenocarcinoma cells ($CaCo_2$ cells) and compared its effects with a normal colon cell line (NCM460 cells). The phosphorylation and activity of two MAPKs, ERK and p38 MAPK, as well as on Akt (also known as protein kinase B) and the transcription factor p53 were investigated, and cross-talk between signalling pathways was assessed using specific inhibitors and RNA interference [small interfering RNA (siRNA)]. To further evaluate the significance of our findings obtained in our cell model, the effect of DHA was correlated with the induction of apoptosis.

2. Materials and methods

2.1. Materials

$CaCo_2$ cells, Eagle's Minimum Essential medium, foetal calf serum (FCS), trypsin and penicillin/streptomycin were obtained from Highveld Biological (Lyndhurst, RSA). NCM460 cells were purchased from InCell (San Antonio, TX, USA). Cell culture plastics were purchased from Greiner Bio-one (Frickenhausen, Germany). DHA (n-3, 22:6), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and protease inhibitors were obtained from Sigma Chemicals (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). PD 98059 (MEK inhibitor), and water-soluble SB 203580 (p38 MAPK inhibitor) were obtained from Calbiochem (La Jolla, CA, USA). Wortmannin (PI3 kinase inhibitor), rabbit polyclonal antibodies, rabbit monoclonal antibodies and the SignalSilence p38 MAPK siRNA kit were purchased from Cell Signaling Technology (Beverly, MA, USA). All electrophoresis equipment was from Bio-Rad (Herculus, CA, USA). Polyvinylidene fluoride (PVDF; 0.45 μm) membranes for Western blots were obtained from Millipore (Bedford, MA, USA). The chemiluminescence system (ECL Plus and ECL), autoradiography film as well as antirabbit horseradish peroxidase (HRP)-linked secondary antibody (from donkey) were obtained from Amersham Biosciences (Arlington Heights, IL, USA). Other standard chemicals were obtained from commercial sources.

2.2. Preparation of fatty acid stock solutions

Fatty acid stock solutions (100 mM) were prepared in 99% ethanol as described previously [6]. The stock solutions were then stored under nitrogen gas in opaque microcentrifuge tubes at −80°C to prevent their degradation.

2.3. Cell culture

$CaCo_2$ and NCM460 cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in MEM supplemented with 10% FCS and 1% penicillin/streptomycin (standard medium). Cells were routinely subcultured before reaching confluency and maintained for up to 20 passages. Cell numbers were determined using a haemocytometer following trypsinization. For MTT assays, $CaCo_2$ and NCM460 cells were seeded in 35 mm Petri dishes (120 000 and 350 000 cells/dish, respectively). For Western blotting, $CaCo_2$ cells were seeded in 35 mm (120 000 cells/dish) and 60-mm Petri dishes (240 000 cells/dish), respectively. $CaCo_2$ cells were seeded in six-well plates for RNA interference experiments (120 000 cells/well).

Prior to fatty acid treatment, standard media were replaced by MEM supplemented with 2% FCS and 1% penicillin/streptomycin (serum-poor medium). Working fatty acid solutions (1 mM) were also prepared in serum-poor medium. Cells were treated with the working fatty acid solutions at a final concentration of 10 μM. Cells treated with the ethanol vehicle only were used as controls.

2.4. Treatment with kinase inhibitors

Stock solutions of kinase inhibitors were prepared in DMSO (wortmannin and PD 98059) or in water (SB 203580) and stored at −20°C. $CaCo_2$ cells were treated with the inhibitors wortmannin (500 nM), PD 98059 (10 μM) or SB 203580 (10 μM) in standard medium for 30 min. Media were then replaced with serum-poor medium, treated with DHA for 30 min and prepared for Western blot analysis.

2.5. Cell viability assessment

The MTT assay was employed to determine the effect of fatty acids and the different vehicles on viability (or cell functionality). Following treatment with fatty acids or

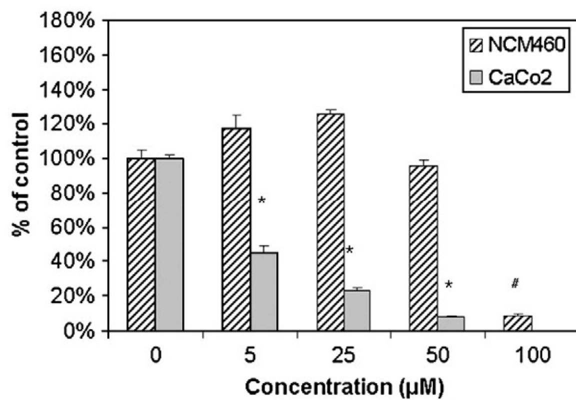

Fig. 1. The effect of supplementation with different concentrations of DHA on NCM460 and $CaCo_2$ cell viability. Cells were cultured in standard medium until 70–80% confluency and supplemented with fatty acids (10 μM) in serum-poor medium for 48 h. The MTT assay was performed as described in the Methods section. Values are expressed as percentages of controls (100%); $^{*}P<.001$; $^{\#}P<.05$ vs. control (n=3).

vehicle for 48 h, cells were washed with phosphate-buffered saline (PBS) and incubated with MTT (0.2% in PBS) for 2 h at 37°C. The crystals formed were then dissolved in 0.002% Triton X-100 in 1% HCl-isopropanol. Each sample was assayed spectrophotometrically (540 nm). Viability of samples is expressed relative to control values (100%).

2.6. SiRNA knockdown of p38 MAPK

$CaCo_2$ cells were plated in six-well plates in standard medium. The following day, siRNA was freshly prepared in serum-free medium containing the transfection agent. The cells were then transfected with 15 nM siRNA in standard medium. After 24 h, the siRNA-containing medium was taken off, and cells were treated with DHA in serum-poor medium for 30 min. Cells were then lysed and prepared for Western blot analysis.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

Cells were washed twice with ice-cold PBS and lysed on ice with 120-μl lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P40 substitute, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 4 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 1 mM Na_3VO_4 and 1 mM NaF). The lysates were sonicated for short bursts and centrifuged at 4°C for 15 min at 6000*g*. Aliquots of the supernatants (20 μg/lane) were then resolved on 7%, 10% or 12% sodium dodecyl sulfate-polyacrylamide gels at 200 V for 50 min. Resolved proteins were then transferred onto PVDF membranes in a Bio-Rad Trans-Blot SD semidry transfer cell (15V, 1 h). The PVDF membranes were routinely stained with Ponseau Red for visualization of proteins and stripped and reprobed with anti-actin antibody to ensure equal loading. Residual binding sites on the membrane were blocked in 5% nonfat milk in Tris-buffered saline (TBS)-T [1× TBS, pH 6.8, 0.1% Tween-20] for 1 h. Blots were then incubated overnight at 2–4°C with anti-rabbit primary antibodies (1:1000) in TBS-T (for polyclonals) or 5% milk (for monoclonals). Blots were washed in TBS-T (3×5 min) and incubated with the HRP-conjugated secondary antibody (1:8000) in TBS-T at room temperature for 1–2 h and washed (3×15 min). Bands were finally visualized with ECL or ECL Plus. Densitometric data of autoradiographs were determined with the Un-Scan-It software package (version 5.1, Silk Scientific, Orem, UT, USA), and phosphorylated protein values were corrected for minor differences in protein loading, if required. All blots were scanned at a resolution of 150 dpi. The exact outline of each band was demarcated in the Un-Scan-It programme, which takes all aspects of density and distribution into account. The full experimental range was analyzed on a particular blot. These analyses were performed under conditions where autoradiographic detection was in the linear response range.

2.8. Statistical analysis

All experiments were carried out in triplicate, except where stated otherwise. Results were analysed by one-way analysis of variance for comparison of multiple groups, followed by a post hoc test with the Bonferroni correction using Prism version 2.01 (GraphPad Software, San Diego, CA, USA). Data are presented as mean±S.E.M.

3. Results

3.1. Cytotoxicity of DHA in normal and cancer cells

To assess the effect of DHA on viability of the cells, different concentrations were tested on both cell lines (Fig. 1). In $CaCo_2$ cells, decreased viability was observed with increased concentrations of DHA, although the dose efficacy can only be evaluated in conjunction with its effect on NCM460 cells. Low and medium concentrations (5–50 μM) seem to be most efficient because NCM460 viability is not compromised. The high dose (100 μM) was toxic to both cell lines.

3.2. Effect of DHA on the phosphorylation status of different signalling molecules

The short-term as well as long-term effects of DHA on $CaCo_2$ cells were assessed by Western blotting using phosphospecific and total antibodies directed against the proteins Akt, ERK, p38 MAPK and p53. $CaCo_2$ cells were cultured until 70–80% confluency and then supplemented with DHA (10 μM) in serum-poor medium for varying time periods ranging from 2 min to 6 h (short-term treatment) and from 6 to 48 h (long-term treatment).

To investigate Akt phosphorylation, both phosphorylation sites are required for full activity, Ser^{473} and Thr^{308} were inspected separately. Short-term DHA supplementation induced phosphorylation of Akt at Ser^{473} as early as 2 min

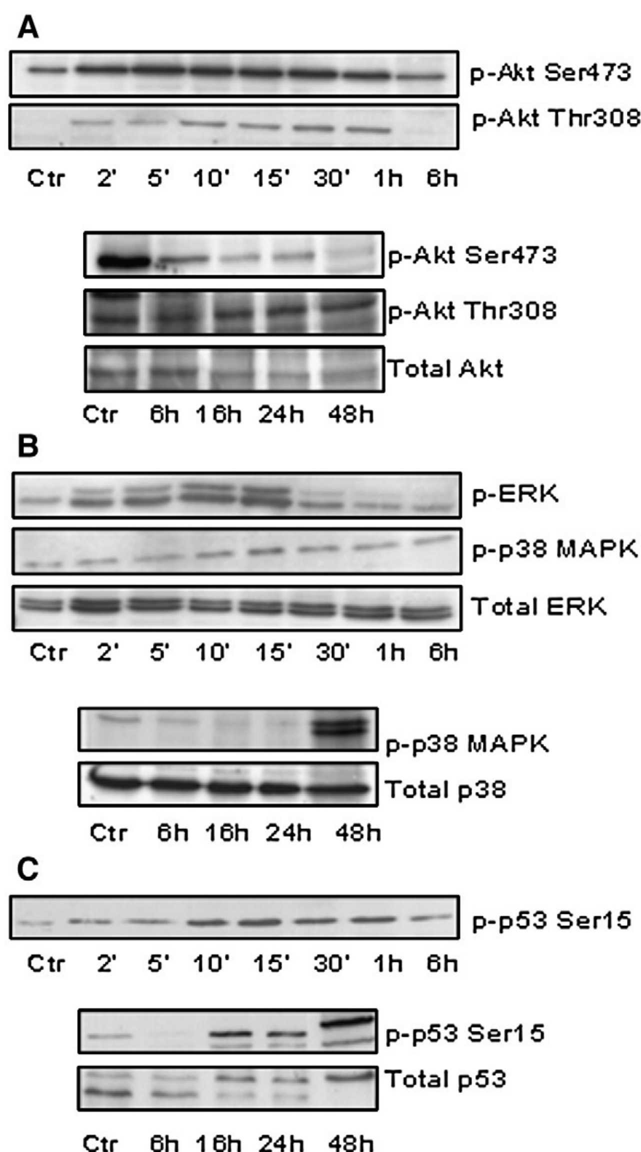

Fig. 2. The effects of short- and long-term DHA supplementation on phosphorylation of signalling molecules. $CaCo_2$ cells were cultured in standard medium until 70–80% confluency and supplemented with DHA (10 μM) in serum-poor medium for up to 6 h (short-term treatment) or 48 h (long-term treatment). Cells were lysed at the indicated times and prepared for Western blotting. (A) Phosphorylation of Akt at Ser^{473} and Thr^{308}. Total Akt is shown following long-term DHA treatment. (B) Phosphorylation of ERK and p38 MAPK on dual sites required for activation. Total ERK and total p38 MAPK are shown following short- and long-term treatment, respectively. (C) Phosphorylation of p53 at Ser^{15}. Total p53 is shown following long-term treatment. Data presented are indicative of at least three experiments.

following treatment (Fig. 2A). The increased intensity of Ser^{473} phosphorylation continued up to 10 min after addition of the fatty acid but declined thereafter to its basal intensity. Short-term DHA treatment also induced a modest increase in phosphorylation of Akt Thr^{308}, which was present up to 1 h. After 6 h, phosphorylation intensity had returned to its basal state. Unlike with the Ser^{473} residue, long-term treatment did not have any effect on the intensity of Thr^{308} phosphorylation. Total protein levels of Akt were unaltered.

For ERK and p38 MAPK phosphorylation, the antibodies used recognised these proteins only when dually phosphorylated on specific Thr and Tyr residues. With short-term DHA supplementation, a distinct trend was observed toward increased phosphorylation of ERK1/2, which decreased toward its basal intensity before 1 h (Fig. 2B). Regarding the phosphorylation of p38 MAPK, short-term DHA supplementation did not seem to have any effect, although an increase was observed after 48 h. The total protein levels of ERK1/2 and p38 MAPK were unaltered.

For p53, we used an antibody which only recognizes p53 when phosphorylated at Ser^{15}. This phosphorylation site is associated mainly with DNA damage [18]. Short-term DHA supplementation seemed to induce phosphorylation of p53 at Ser^{15}, which was maximal at 10–15 min (Fig. 2C). After 6 h, phosphorylation intensity had returned to its basal state. With long-term supplementation, increased phosphorylation was observed after 48 h. The total protein level of p53 did not change.

3.3. Apoptosis

To test whether long-term supplementation with DHA induces apoptotic cell death in cancer cells, we investigated the cleavage of the apoptotic proteins caspase-3 and poly ADP-ribose polymerase (PARP). Procaspase-3 can be cleaved by an initiator caspase into 20-, 19- or 17-kDa active fragments [19]. A monoclonal antibody which detects the 17- and 19-kDa subunits was used. In normal conditions, equal amounts of these subunits were detected, which was taken to represent the basal level of apoptosis for $CaCo_2$ cells (Fig. 3A). Long-term DHA supplementation significantly altered the cleavage pattern of caspase-3 after 16h when the level of the larger subunit decreased significantly, although this was not accompanied by an increase of the smaller subunit. Forty-eight hours following the addition of DHA, the 17-kDa subunit had nearly disappeared, together with a return of the 19-kDa subunit to its normal intensity. The disappearance of the smaller fragment of caspase-3 at 48 h was associated with PARP cleavage to yield a 89-kDa fragment (Fig. 3B). This was detected with the use of a monoclonal antibody, which detects uncleaved PARP as well as its subunits following cleavage.

3.4. The influence of DHA on relationships between signalling molecules

The pharmacological kinase inhibitors wortmannin, PD98059 and SB203580 were used to inhibit the activities of PI3 kinase, MEK and p38 MAPK, respectively. Because the specificity of SB203580 has been questioned [20], we also used siRNA to suppress the expression of the p38 MAPK protein. $CaCo_2$ cells were cultured until 70–80% confluence and treated with the inhibitors (wortmannin, 500 nM; PD98059, 10 μM; SB203580, 10 μM) for 30 min in

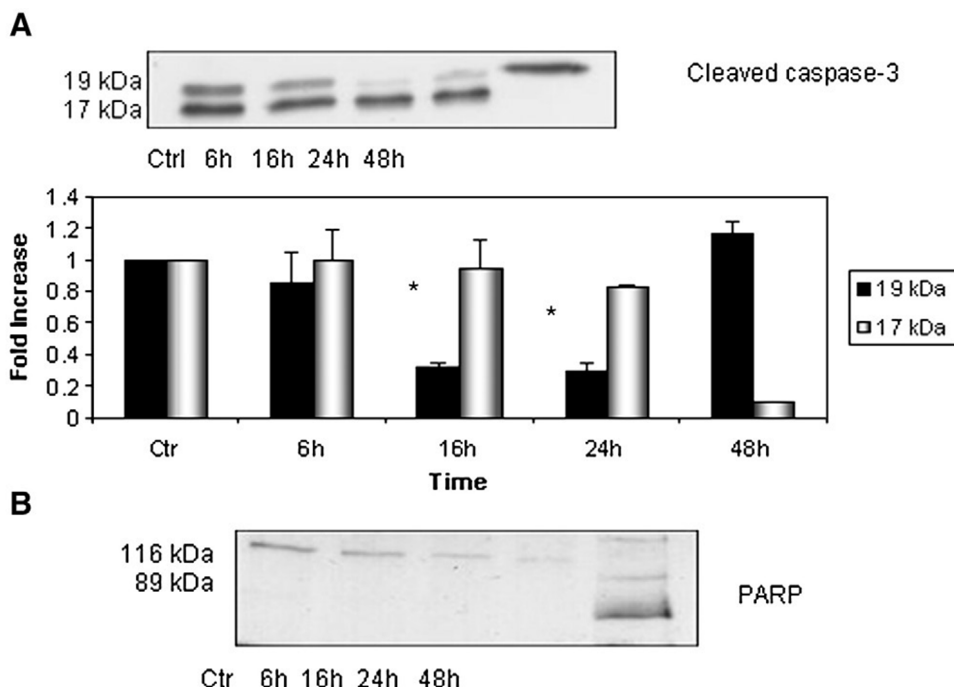

Fig. 3. The effect of long-term DHA supplementation on the cleavage of procaspase-3 and PARP. $CaCo_2$ cells were cultured and treated with DHA for up to 48 h as described earlier. Cells were lysed at the indicated times and prepared for Western blotting. (A) Subunits, 19-kDa and 17-kDa, of caspase-3 following cleavage. The graph represents densitometric data of caspase-3 subunits; *P<.01 vs. control (n=3). (B) Full-length PARP (116 kDa) and the 89-kDa subunit following cleavage. Data are indicative of three separate experiments.

serum-poor medium or transfected with siRNAs against p38 MAPK in standard medium for 24 h. Thereafter, cells were supplemented with DHA (10 μM) in serum-poor medium for 30 min prior to lysis. In an MTT assay, the possibility of any vehicle effects (DMSO and ethanol) was ruled out.

The phosphorylation status of Akt (Ser^{473} and Thr^{308}), ERK (Thr^{202}/Tyr^{204}), p38 (Thr^{180}/Tyr^{182}) and p53 (Ser^{15}) was measured with phosphospecific antibodies. Treatment with wortmannin reduced the phosphorylation of Akt at Ser^{473} in both the presence and absence of DHA (Fig. 4A). PD98059 and SB203580 did not have any effect on Ser^{473} phosphorylation in the absence of DHA; however, DHA caused a significant increase in Ser^{473} phosphorylation in the presence of SB203580 (P<.05). PD 98059 suppressed the dual phosphorylation of ERK1/2, both in the presence and absence of DHA (Fig. 4B). Wortmannin also reduced ERK1/2 phosphorylation in both cases. Although SB203580 did not have an inhibitory effect in the absence of DHA, DHA seemed unable to alter ERK1/2 phosphorylation following SB203580 treatment. However, short-term experiments indicated that ERK1/2 phosphorylation had already returned to a basal state by 30 min following DHA addition.

In these experiments, 30-min DHA supplementation suppressed p53 (Ser^{15}) phosphorylation significantly (P<.01). This suppression was also seen in the presence of SB203580 (P<.05), although wortmannin and PD98059 attenuated this.

Samples treated with p38 MAPK-siRNA showed decreased band intensity when probed for p38 MAPK (Fig. 4C). Phosphorylation of p53 at Ser^{15} was suppressed by DHA when p38 MAPK was knocked down in this manner, although the knockdown by itself did not affect phosphorylation. Phosphorylation of Akt at Ser^{473} was not suppressed by the knockdown of p38 MAPK.

4. Discussion

In order for a therapeutic agent to be truly effective, it should be toxic to tumour cells without harming normal cells. From the literature, it seems as though fatty acids fulfill this criterion. Also, fatty acids have been shown to augment standard cancer therapeutics in two ways: (*a*) by enhancing efficacy of chemotherapeutic drugs and (*b*) by reducing the side effects associated with chemotherapy [21]. Our results have shown that low DHA concentrations are appropriate, as normal cell viability is not compromised at these concentrations. A high dose, on the other hand, is cytotoxic to both normal and cancer cells.

DHA has been shown to modulate the activities of various signalling proteins and transcription factors in various cell types. These include ERK1/2 in human T lymphocytes [22], Akt in neurons [23], p38 MAPK in vascular smooth muscle [9], adenylyl cyclase in liver cells [10], nuclear factor κB in macrophages [24] and Ras and

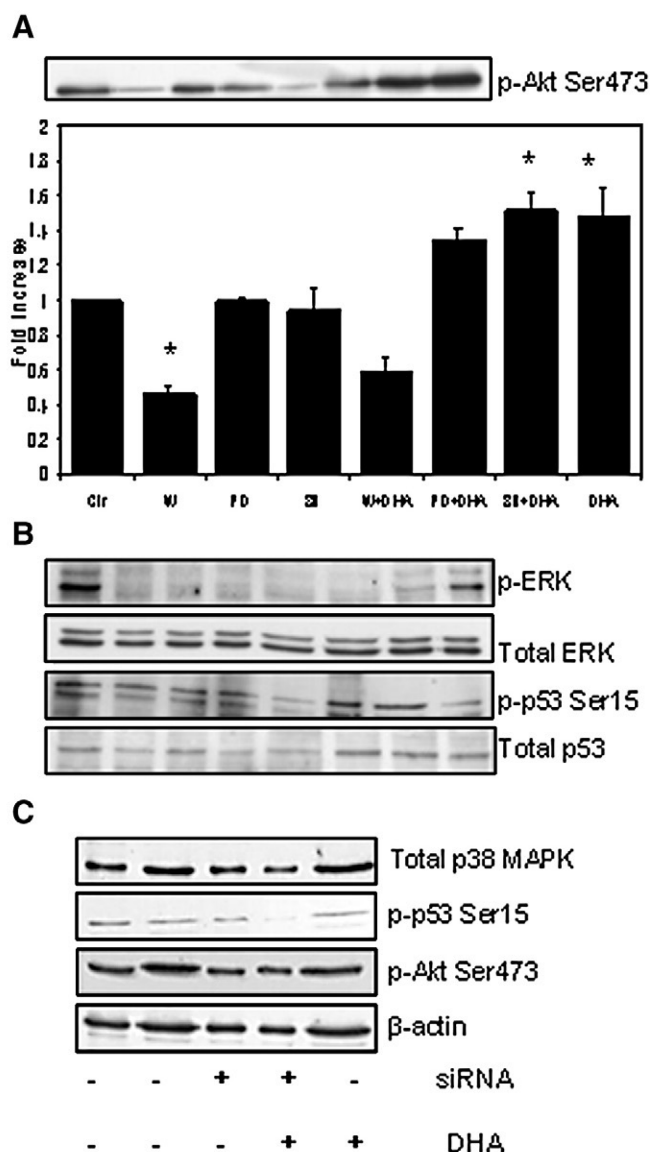

Fig. 4. The effect of kinase inhibitors and p38 MAPK-siRNA on the relationships between signalling molecules. $CaCo_2$ cells were cultured as described earlier and treated with either wortmannin (500 nM), PD98059 (10 μM) or SB203580 (10 μM) for 30 min. Alternatively, $CaCo_2$ cells were treated with siRNA in standard medium for 24 h. Following treatment with inhibitors/siRNA, cells were also treated with DHA (10 μM) for 30 min in serum-poor medium. (A) Phosphorylation of Akt at Ser^{473}. The graph represents densitometric data; **P*<.05 vs. control (*n*=3). (B) Phosphorylation of ERK and p53 (Ser^{15}), together with total ERK and p53. (C) In each blot, Lanes 1 and 2 contain controls with 10% and 2% FCS, respectively. The effect of siRNA directed against p38 MAPK on total p38 protein and the phosphorylation of p53 at Ser15 and Akt at Ser473. Lanes 1 and 2 contain controls with 10% and 2% FCS, respectively. The total protein level of β-actin was used to control for equal sample loading.

protein kinase C in colon cells [25,26]. However, these studies were not done in the context of colon cancer research. An exception is PKC, which has been studied extensively in tumour cells and is believed to be involved in the beneficial effects of PUFAs [25,27]. We therefore investigated whether well-known signalling molecules can be influenced by DHA in our colon cancer model. Since time course studies have shown that such changes can be induced as soon as 1 min following DHA addition [9], we decided to compare the effects of short (2 min–6 h) and long (up to 48 h) incubation times.

The mechanism of the activation of Akt is still controversial, even though it is a widely researched field. It is clear that the sites required for activation are Ser^{473} and Thr^{308} (in Akt1), and it is mostly believed that phosphorylation occurs at Thr^{308} before phosphorylation at Ser^{473} takes place [14]. Our results have shown that following short-term DHA supplementation, an increase in phosphorylation occurs at both these residues and returns to a basal state within less than 6 h. Whether this transient increased phosphorylation, which is indicative of activation, has any effects on gene expression is questionable. However, long-

term DHA supplementation suppressed Ser^{473} phosphorylation, while Thr^{308} phosphorylation remained unaltered. The decreased Ser^{473} phosphorylation might be associated with decreased activity of Akt, as the phosphorylation of this residue is associated with full activation. Because interaction with membrane phospholipids is essential for Ser^{473} phosphorylation [14], the way in which DHA modifies phospholipids could be responsible for the decreased Ser^{473} phosphorylation observed. Moreover, as Akt signalling is associated with cell survival, the restriction of full Akt activity could lead to apoptosis [28]. For most of the proteins investigated, the short-term response was different from the effects seen with long-term incubation. The short-term phosphorylation changes should probably not be associated with changes in the gene expression profile of the cells but rather be regarded as nongenomic effects that could be related to the initial rearrangement and modification of the phospholipids in the cell membrane.

Wortmannin, an irreversible inhibitor of PI3 kinase [29], suppressed the phosphorylation of Akt at Ser^{473} and also inhibited the increased phosphorylation induced by DHA. In the presence of PD98059, DHA was also unable to induce the increased phosphorylation that was seen with the use of DHA (alone or following SB203580 treatment). This supports a possible upstream role for MEK or perhaps ERK1/2 in the phosphorylation of Akt at Ser^{473}. Although it has been suggested that p38 MAPK could be responsible for this phosphorylation following receptor stimulation by certain agonists [30], our results did not reflect this. The use of siRNAs to inhibit the expression of p38 MAPK did not diminish phosphorylation at the Ser^{473} residue, whether treatment with DHA followed or not. It was not surprising to see that Thr^{308} phosphorylation was abolished by wortmannin, since PDK1 requires interaction with phosphoinositides [30]. Interestingly, DHA was unable to induce phosphorylation at Thr^{308} following treatment with either PD98059 or SB203580. This could suggest upstream signalling roles of MEK, ERK1/2 or p38 MAPK in the activation of PDK1, the kinase responsible for Thr^{308} phosphorylation.

The increased phosphorylation of ERK1/2 observed with DHA supplementation could actually reflect activation of PKC, as it is known that PKC can activate the ERK cascade via Raf-1 [31,32] and that DHA can influence PKC activity [25]. PD98059 successfully inhibited the phosphorylation of ERK1/2, in the presence and absence of DHA. Wortmannin also reduced ERK1/2 phosphorylation in both cases. Previous reports have stated that PI3 kinase is an upstream mediator of ERK1/2 activity [33], which would explain this effect. Furthermore, short-term experiments have shown that ERK1/2 phosphorylation had already returned to basal levels after 30 min following DHA addition, which partially explains the lack of phosphorylation in those groups that were treated with an inhibitor and DHA.

Although fatty acids have been shown to induce rapid p38 MAPK phosphorylation in other systems [9], this effect was not seen in the $CaCo_2$ cells. A change in phosphorylation following DHA supplementation was observed only at 48 h. This could be indicative of a delayed stress response, and might also contribute, together with suppression of Akt signalling, to induce apoptosis via ASK1 [34]. It has been speculated repeatedly that the cytotoxicity of PUFAs in cancer cells can be ascribed to their peroxidation and the generation of free radicals [35,36]. Oxidants are known to activate ASK1 upstream of p38 MAPK, and this pathway can be associated with apoptosis [34]. It is possible that ASK1 is not activated by DHA supplementation directly but, instead, by other proapoptotic proteins such as Fas and TRAF2 [37]. This could explain the delayed response that was observed in p38 MAPK activation following DHA supplementation and why no short-term effects were observed.

The protein p53 has been described as a substrate for many kinases in vitro, and numerous phosphorylation sites have been described for it. Nevertheless, relatively little is known concerning which sites are phosphorylated by which kinases [38]. In the current study, phosphorylation at Ser^{15}, which is primarily associated with DNA damage [18], has been examined. This site is known to be phosphorylated by ATR and DNA-PK in vivo [39]. Short-term DHA supplementation seemed to increase p53 (Ser^{15}) phosphorylation transiently, as this effect was already abolished in less than 6 h. However, phosphorylation at Ser^{15} was strongly increased within 16 h and remained elevated. This increase can be associated with a stress response associated with loss of DNA integrity. Once again, increased oxidative stress that could be associated with PUFA supplementation could be responsible for damage to DNA and Ser^{15} phosphorylation. In the kinase inhibitor experiments, Ser^{15} phosphorylation was lower with DHA than the control value after 30 min. This effect was also observed with SB203580 and DHA treatment, whereas it was attenuated by both wortmannin and PD98059. A possible role for p38 MAPK in the phosphorylation of p53 at Ser^{15} was investigated with siRNA. It was shown that following knockdown of p38 MAPK, DHA is unable to induce phosphorylation of Ser^{15}. This implicates a role for p38 MAPK in the phosphorylation of p53 at Ser^{15} and also for p53 activation.

Caspase-3 is an important effector caspase in the apoptotic pathway. It is responsible for the cleavage of various substrates, including PARP, a DNA repair enzyme. We have demonstrated that DHA significantly induced caspase-3 and PARP cleavage in the $CaCo_2$ cells. However, differential cleavage of the inactive enzyme was apparent. At 48 h, which was also when cleavage of PARP first became apparent, the cleaved caspase-3 subunit was 19 kDa in size. Although it is known that caspase-3 can be cleaved at more than one site to yield active enzymes of different molecular masses [19], our results implicate a possible differential role for these different active enzymes. Furthermore, it was also previously demonstrated in HT-29 cells that a low dose of another long chain n-3 PUFA (polyunsaturated fatty acid), EPA (eicosapentaenoic acid), could induce caspase-3 cleavage after 24 and 48 h of exposure [40].

In conclusion, our results have clearly shown that DHA, the longest chain and most unsaturated fatty acid in biological systems [41], has multiple cellular effects. Our results indicate that the induction of apoptosis in cancer cells by DHA is associated with an inhibition of the survival-related kinase, Akt Ser^{473} phosphorylation and an increase in p38 MAPK phosphorylation. Furthermore, DHA also decreased viability of cancer cells while normal cells remain unaffected. From our findings with pharmacological inhibitors and siRNA, we propose novel mechanisms for DHA-induced apoptosis and cytotoxicity. Firstly, we suggest a role for ERK in the activity of PDK1, the kinase responsible for Thr^{308} phosphorylation of Akt. Secondly, we propose that p38 MAPK is responsible for the phosphorylation of Ser^{15} in p53.

Our results confirmed the antitumourigenic efficacy of DHA in cancer cells, together with its safety with regard to normal cells. We therefore believe that the clinical use of n-3 fatty acid therapy, whether as a preventative agent or as an adjuvant to conventional therapy, is long overdue.

Acknowledgments

Our sincere gratitude to Incell, America from whom the normal colon cell line (NCM460 cells) was obtained.

References

[1] Laurier J. WHO report: alarming increase in cancer rates. Retrieved 21/02/2006, 2006, from http://www.wsws.org/articles/2003/apr2003/canc-a26.shtml.

[2] Increase in global cancer rates: the World Cancer Report. Oncol Forum 2003:2–4.

[3] Lanier AP, Bender TR, Blot WJ, et al. Cancer incidence in Alaska natives. Int J Cancer 1976;18:409–12.

[4] Rose DP, Connolly JM. Effects of dietary omega-3 fatty acids on human breast cancer growth and metastasis in nude mice. J Natl Cancer Inst 1993;85:1743–7.

[5] Connolly JM, Coleman M, Rose DP. Effects of dietary fatty acids on DU145 human prostate cancer cell growth in athymic nude mice. Nutr Cancer 1997;29:114–9.

[6] Nano JL, Nobili C, Girard-Pipau F, et al. Effects of fatty acids on the growth of Caco-2 cells. Prostaglandins Leukot Essent Fatty Acids 2003;69:207–15.

[7] Chen ZY, Istfan NW. Docosahexaenoic acid is a potent inducer of apoptosis in HT-29 colon cancer cells. Prostaglandins Leukot Essent Fatty Acids 2000;63:301.

[8] Wassall SR, Brzustowicz MR, Shaikh SR, et al. Order from disorder, corralling cholesterol with chaotic lipids. The role of polyunsaturated lipids in membrane raft formation. Chem Phys Lipids 2004;132:79–88.

[9] Diep QN, Touyz RM, Schiffrin EL. Docosahexaenoic acid, a peroxisome proliferator-activated receptor-alpha ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. Hypertension 2000;36:851–5.

[10] Flier J, Lokesh BR, Kinsella JE. Increased 5' nucleotidase activity in plasma membranes from rat liver following ingestion of fish oil. Nutr Res 1985;5:277–83.

[11] Shi Y, Gaestel M. In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. Biol Chem 2002;383:1519–36.

[12] Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–83.

[13] Krauss G. Biochemistry of Signal Transduction and Regulation. 3rd ed. Weinheim: Wiley-Vch; 2003.

[14] Scheid MP, Woodgett JR. Unravelling the activation mechanisms of protein kinase B/Akt. FEBS Letters 2003;546:108.

[15] Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002;14:381–95.

[16] Nigro JM, Baker SJ, Preisinger AC, et al. Mutations in the p53 gene occur in diverse human tumour types. Nature 1989;342:705.

[17] Watson AJM. An overview of apoptosis and the prevention of colorectal cancer. Crit Rev Oncol Hematol 2006;57:107–21.

[18] Shieh SY, Ikeda M, Taya Y, et al. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 1997;91:325–34.

[19] Uetsuki T, Takemoto K, Nishimura I, et al. Activation of neuronal caspase-3 by intracellular accumulation of wild-type alzheimer amyloid precursor protein. J Neurosci 1999;19:6955–64.

[20] Godl K, Wissing J, Kurtenbach A, et al. An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. Proc Natl Acad Sci U S A 2003;100:15434–9.

[21] Hardman WE. Omega-3 fatty acids to augment cancer therapy. J Nutr 2002;132:3508S–12S.

[22] Denys A, Hichami A, Khan NA. Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase (ERK1/ERK2) signaling in human T cells. J Lipid Res 2001;42:2015–20.

[23] Akbar M, Calderon F, Wen Z, et al. Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. Proc Natl Acad Sci U S A 2005;102:10858–63.

[24] Novak TE, Babcock TA, Jho DH, et al. NF-kappa B inhibition by omega-3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. Am J Physiol Lung Cell Mol Physiol 2003;284:L84–9.

[25] Holian O, Nelson R. Action of long-chain fatty acids on protein kinase C activity: comparison of omega-6 and omega-3 fatty acids. Anticancer Res 1992;12:975–80.

[26] Collett ED, Davidson LA, Fan YY, et al. n-6 and n-3 polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes. Am J Physiol Cell Physiol 2001;280:C1066–75.

[27] Blobe GC, Obeid LM, Hannun YA. Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev 1994;13:411–31.

[28] Kennedy SG, Wagner AJ, Conzen SD, et al. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 1997;11:701–13.

[29] Wymann MP, Bulgarelli-Leva G, Zvelebil MJ, et al. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol Cell Biol 1996;16:1722–33.

[30] Dong LQ, Liu F. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. Am J Physiol Endocrinol Metab 2005;289:E187–96.

[31] Hausser A, Storz P, Hubner S, et al. Protein kinase C mu selectively activates the mitogen-activated protein kinase (MAPK) p42 pathway. FEBS Lett 2001;492:39–44.

[32] Kolch W, Heidecker G, Kochs G, et al. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 1993;364:249–52.

[33] Fuller G, Veitch K, Ho LK, et al. Activation of p44/p42 MAP kinase in striatal neurons via kainate receptors and PI3 kinase. Brain Res Mol Brain Res 2001;89:126–32.

[34] Matsukawa J, Matsuzawa A, Takeda K, et al. The ASK1-MAP kinase cascades in mammalian stress response. J Biochem (Tokyo) 2004;136:261–5.

[35] Conklin KA. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. Altern Med Rev 2002;7:4–21.

[36] Sagar PS, Das UN. Cytotoxic action of *cis*-unsaturated fatty acids on human cervical carcinoma (HeLa) cells in vitro. Prostaglandins Leukot Essent Fatty Acids 1995;53:287–99.

[37] Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807–69.

[38] Ashcroft M, Kubbutat MHG, Vousden KH. Regulation of p53 function and stability by phosphorylation. Mol Cell Biol 1999;19:1751–8.

[39] Bulavin DV, Saito S, Hollander MC, et al. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J 1999;18: 6845–54.

[40] Latham P, Lund EK, Brown JC, et al. Effects of cellular redox balance on induction of apoptosis by eicosapentaenoic acid in HT29 colorectal adenocarcinoma cells and rat colon in vivo. Gut 2001;49:97–105.

[41] Stillwell W, Wassall SR. Docosahexaenoic acid: membrane properties of a unique fatty acid. Chem Phys Lipids 2003;126:1.