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Effects of omega-3 and omega-6 fatty acids on IGF-I receptor signalling in colorectal cancer cells

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Abstract

The insulin-like growth factor (IGF) system plays a critical role in normal growth and development as well as in malignant states. Most of the biological activities of the IGFs are mediated by the IGF-IR, which is over-expressed in most tumours and cancer cell lines. Fatty acids have critical roles in both systemic physiological processes (e.g. metabolism) and cellular events (e.g. proliferation, apoptosis, signal transduction, and gene expression). Alpha-linolenic acid (ALA) and linoleic acid (LA) are essential fatty acids of the omega-3 and omega-6 families, respectively. The aim of this study was to investigate the potential interactions between fatty acids and the IGF signal transduction pathways, and to evaluate the impact of this interplay on colon cancer cells survival and proliferation. Results of Western blot analyses revealed that ALA and LA enhanced the ligand-induced IGF-IR phosphorylation and, in addition, increased receptor phosphorylation in an IGF-I independent manner. Furthermore, fatty acid treatment led to phosphorylation of downstream signalling molecules, including Akt and Erk. In addition, FACS analysis and apoptosis measurements indicated that ALA and LA have a potential mitogenic effect on HCT116 cells, as reflected by the number of cells in S phase and by a reduction of PARP cleavage, implying a reduction in apoptotic activity. In summary, our results provide evidence that omega-3 and omega-6 fatty acids modulate IGF-I action in colon cancer cells.

Keywords: *Insulin-like growth factor-I (IGF-I); IGF-I receptor; fatty acids; colon cancer*

Introduction

Colon cancer is the third most common type of cancer and is one of the leading causes of cancer-related deaths in the Western world. It has been established that lifestyle and nutrition play a crucial role in colon cancer prevention, progression, and treatment. The connection between nutrition, especially fat intake, and cancer, and the effects of specific fatty acids on cancer development has been the subject of extensive research. Alpha-linolenic acid (ALA) and linoleic acid (LA) are essential fatty acids of the omega-3 and omega-6 families, respectively, whose influence on general health and cancer prevention has been increasingly evident. Whereas nutritionists and health care providers recommend to enrich the daily diet with omega-3 fatty acids, and even to supplement them as palliative care for different types of cancer, the scientific literature regarding the potential beneficial effects of fatty acids remains controversial (Hardman, 2002; Heller *et al.*, 2004). A number of epidemiological studies reported that omega-3 fatty acids have a protective effect against the risk to develop cancer (Cave, 1991; Bartsch *et al.*, 1999; Leitzmann *et al.*, 2004; Lipkin *et al.*, 1999; Thies *et al.*, 2001; Astorg, 2004; Roynette *et al.*, 2004). Data regarding omega-6 fatty acids, however, are less consistent. Thus, whereas some studies showed no effect (Astorg, 2004; Leitzmann *et al.*, 2004), other studies showed an increased cancer risk, and even reduced cancer risk (Bartsch *et al.*, 1999; Reyes *et al.*, 2004; Thanasak *et al.*, 2005).

The insulin-like growth factor (IGF) system plays a crucial role in normal growth and development and is also thought to have a prominent role

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in tumourigenesis. The IGF system includes two mitogenic ligands (IGF-I and IGF-II), their receptors (IGF-IR and IGF-IIR, respectively), and a number of binding proteins (IGFBP1-6) that regulate ligand bioavailability. Elevated levels of IGF-I/II or IGF-IR or both are a common theme in many types of cancer. Furthermore, and consistent with a potent anti-apoptotic, pro-survival role, IGF-IR blockage was shown to inhibit tumour growth and angiogenesis and to enhance chemotherapy-induced apoptosis (Baserga, 1999; Werner and LeRoith, 2000; Adachi *et al.*, 2002; Baserga *et al.*, 2003).

In the specific context of colon cancer, both clinical and experimental evidence suggest a pivotal role for the IGF system in the initiation and/or progression of this type of neoplasm. Specifically, augmented serum levels of IGF-I were recently shown to be associated with increased occurrence of adenomatous polyps and even advanced adenomas. In addition, colon cancer cells were shown to express high levels of IGF-IR (Schoen *et al.*, 2005). Further support for the involvement of the IGF axis in colon cancer was provided by the results of epidemiological studies showing a positive correlation between circulating IGF-I levels and colon cancer risk (Ma *et al.*, 1999; Renehan *et al.*, 2004). Because of the important role of the IGFs in colon cancer development, and in view of the putative effects of omega-3 and omega-6 fatty acids on cell proliferation and signal transduction, we hypothesized that ALA and LA may modulate proliferative and signalling processes *via* mechanism/s that involve regulation of the IGF system. The results obtained show that both fatty acids enhanced the ligand-induced IGF-IR phosphorylation in colon cancer cells. In addition, fatty acids caused an increased phosphorylation of downstream cytoplasmic mediators, including PKB/Akt and MAPK/Erk, in HCT116 cells and induced IGF-IR phosphorylation in a ligand-independent manner. Consistent with activation of the IGF-I signal transduction pathway, both ALA and LA exhibited a mitogenic effect on HCT116 cells, as reflected by an increased portion of cells in S phase. Taken together, our data suggest that fatty acids of the omega-3 and omega-6 families may affect the proliferation and apoptosis of colon cancer cells *via* their ability to modulate IGF-I signalling pathways.

Materials and methods

Cell cultures

The human colorectal cancer cell line HCT116 was grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, and 50 μg/ml gentamicin sulfate. HCT116 cells were kindly provided by Dr Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD).

Fatty acid supplementation

ALA and LA fatty acids were purchased from Sigma-Aldrich Co. (Saint Louis, MO). LA was obtained as sodium linoleate powder (Cat. no. L8134) and ALA as pure oil (Cat. no. L2376). ALA oil was turned into sodium salt by adding NaOH at a molar ratio of 1:1. Stocks of fatty acids (10 mM) were prepared according to the following procedure: fatty acid salts were dissolved in ethanol to generate a light, soluble powder, and the ethanol was then evaporated under vacuum. Fatty acids were then complexed with 10% fatty acid free-bovine serum albumin (BSA) (Cat. no. 152401, MP Biomedical, Solon, OH) in saline solution. All work was performed under a nitrogen atmosphere, to prevent fatty acid oxidation. Stocks of fatty acids were kept at –80°C and were diluted in serum-free media to the indicated working dilutions.

Western blot analysis

Cells were treated with different concentrations of ALA, LA, or BSA control for 24 h, after which half of the samples were treated with 50 ng/ml IGF-I for 90 min. Cells were then harvested and lysed on ice in a buffer composed of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 (v/v), 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM PMSF. Protein concentrations were measured using the Bradford reagent (Bio-Rad Laboratories Ltd., Hercules, CA). Samples containing equal amounts of protein (60–80 μg) were subjected to 10% SDS-PAGE, followed by electrophoretic transfer of the proteins onto nitrocellulose membranes. Membranes were blocked with 3% BSA in T-TBS (20 mM Tris–HCl, pH 7.5, 135 mM NaCl, and 0.1% Tween-20) and were incubated overnight with the following antibodies: anti-human IGF-IR β-subunit (C20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-IGF-IR β (Tyr1131)/insulin receptor β (Tyr1146), anti-phospho-42/44 MAPK (Thr202/Tyr204), anti-phospho-Akt (Thr308), anti-PARP (Cell Signalling, Danvers, MA), and anti-tubulin (Sigma-Aldrich Co.). Membranes were washed extensively with T-TBS, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected using the SuperSignal West

Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Membranes were stripped using stripping buffer (Pierce), washed extensively, blocked again, and incubated with antibodies against total IGF-IR, Erk, and Akt (Cell Signalling).

Immunoprecipitation

Cells were treated with ALA, LA, or BSA control, in the absence or presence of IGF-I, for the indicated periods of time, after which they were harvested and lysed as described above. The protein lysates were precipitated overnight with a human IGF-IR β-subunit antibody, after which they were incubated with protein A/G beads (Santa Cruz Biotechnology) for 3 h. Precipitates were then washed and subjected to 10% SDS-PAGE. Western blotting was performed as described above, using an anti-phosphotyrosine antibody.

Plasmids and DNA transfections

Transient transfection experiments were performed using an IGF-IR promoter-luciferase reporter plasmid extending from nt –476 to +640 [p(–476/+640)LUC; nt + 1 corresponds to the transcription initiation site]. The basal promoter activity of this fragment, containing most of the proximal IGF-IR promoter region, has been previously described (Werner *et al.*, 1992, 1994). HCT116 cells were transfected with 1 μg of the IGF-IR promoter reporter plasmid, along with 0.2 μg of a β-galactosidase plasmid (pCMVβ, Clontech, Palo Alto, CA), using the Metafectene rea×gent (Biontex Laboratories GmbH, Munich, Germany). Twenty-four hours after transfection, fatty acids were added to the medium and cells were harvested after an additional 24 h. Luciferase and β-galactosidase activities were measured as previously described (Werner *et al.*, 1992).

Cell cycle analysis

Equal numbers of cells (15 10^5 cells/well) were seeded in 6-well plates and, after 24 h, the cultures were treated with ALA, LA, or BSA control for 6, 12, or 24 h. Cells were harvested with trypsin, centrifuged, and the pellets were re-suspended in PBS. Cells were permeabilized with 0.1% Triton-X 100, after which the DNA was labelled with propidium iodide (50 μg/ml). Samples were subjected to fluorescence-activated cell sorting (FACS) analysis, using a Becton Dickinson system (San Jose, CA). Data were analysed using the WinMDI program, version 2.8.

Cell proliferation assays

Equal numbers of cells (2×10^5 cells/well) were seeded in 6-well plates and, after 24 h, the cultures were treated with 0.01 mM ALA or BSA control. Fatty acid-containing media was replaced every 24 h. For cell counting, cells were harvested with trypsin and diluted with 0.2% Trypan-blue at a ratio of 1:1 (vol:vol). Cells were counted using a Neubauer haemocytometer counting chamber.

Statistical analysis

All results represent comparisons of specific treatments with their appropriate BSA control. Statistical significance was determined using the Student's *t*-test. A P-value (PV) less than 0.05 ($PV < 0.05$) was considered significant.

Results

Evaluation of the effects of ALA and LA on IGF-IR gene expression

ALA and LA are essential fatty acids whose potential involvement in cell proliferation and cancer progression has been the focus of significant research efforts. Because of the putative roles of omega-3 and omega-6 fatty acids in malignant transformation, and in view of the lack of information regarding the possible connection between fatty acids and the IGF system, in initial experiments we investigated the potential regulation of IGF-IR gene expression by ALA and LA. For this purpose, human colon cancer-derived HCT116 cells were treated with ALA (0.01, 0.1, and 2 mM) or LA (0.03, 0.3, and 2 mM) in serum-free, fatty acid-free media for 24 h. The rationale for the doses employed lies in the fact that, despite the difficulty in measuring free fatty acid levels in the intestine, previous studies have established that the concentrations in the lumen are in the 10-μM range (Tso *et al.*, 2004). Western blot analysis using an IGF-IR β-subunit antibody revealed that fatty acid treatment had no significant effect on total IGF-IR levels (Figures 1A, B). To further investigate the potential regulation of IGF-IR gene expression by ALA and LA, transient transfection experiments were performed in HCT116 cells using a luciferase reporter construct under the control of the proximal IGF-IR promoter [p(–476/+640/)LUC]. Twenty-four hours

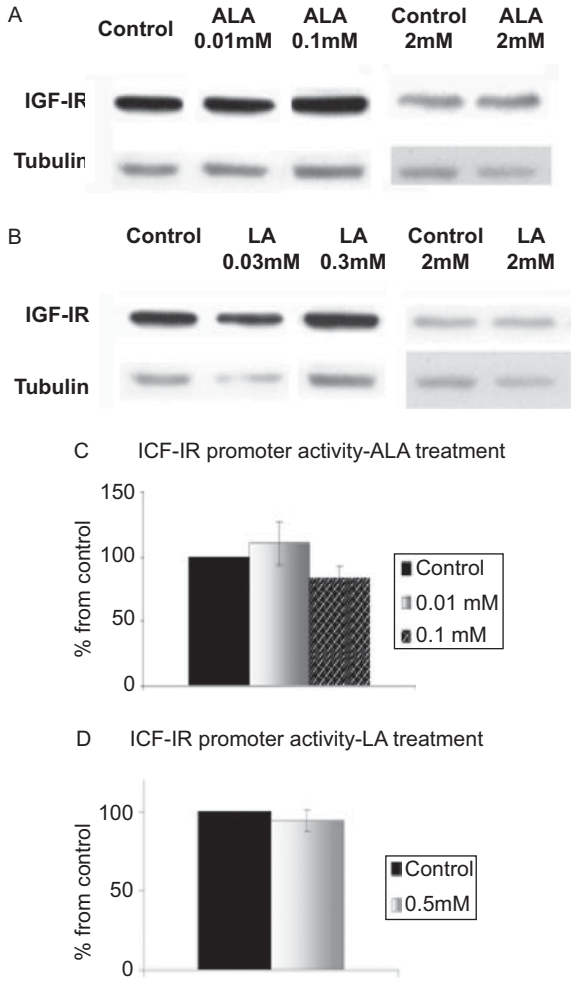

Figure 1. Effects of ALA and LA on IGF-IR expression and promoter activity. HCT116 cells were incubated with increasing concentrations of either ALA (A) or LA (B) (or BSA control) for 24 h. Cell lysates were prepared, electrophoresed through 10% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies against IGF-IR β-subunit and tubulin. In addition, HCT116 cells were transfected with an IGF-IR promoter-luciferase reporter plasmid along with a β-galactosidase expression vector. Twenty-four hours after transfection cells were treated with either ALA (C) or LA (D) at the indicated doses (or BSA, for control purposes) for an additional 24 h. Cells were collected, lysed, and luciferase and β-galactosidase activities were measured. Promoter activities are presented as luciferase normalized to β-galactosidase values. Bars are mean ± SEM (n = 3–5 independent experiments).

after transfection, the cells were treated with different concentrations of either ALA or LA (or BSA control) in serum- and fatty acid-free media for an additional 24 h, after which luciferase and β-galactosidase values were measured. The results obtained showed that at low, physiologically relevant fatty acid concentrations, no significant changes in IGF-IR promoter activities were detected in the treatment groups in comparison to controls (Figures 1C, D).

Effects of ALA and LA on IGF-I-induced IGF-IR phosphorylation

Next, the potential effect of ALA and LA on ligand-induced IGF-IR phosphorylation was examined. To this end, HCT116 cells were treated with different concentrations of ALA or LA (or BSA control) for 24 h. IGF-I (50 ng/ml) was then added to half of the samples for 90 min, after which Western blot analysis was performed using specific phospho-IGF-IR and total-IGF-IR antibodies. As expected, IGF-I treatment caused a marked phosphorylation of the receptor. ALA further enhanced the ligand-induced phosphorylation at both concentrations examined (77% increase at 0.01 mM ALA, n = 5, PV = 0.03; 70–300% increase at 0.1 mM ALA, in six independent experiments) (Figures 2A, B). LA treatment enhanced the IGF-I-induced IGF-IR phosphorylation only at a dose of 0.3 mM (70% increase, n = 6, PV = 0.019) (Figures 2C, D).

IGF-I-independent ALA- and LA-stimulation of IGF-IR phosphorylation

To evaluate the potential ligand-independent capacity of ALA and LA to activate the IGF-IR, HCT116 cells were treated with different concentrations of ALA or LA (or BSA control) for 24 h in serum- and fatty acid-free media. At the end of the incubation period, IGF-IR phosphorylation was assessed using immunoprecipitation (IP) assays. Briefly, cell lysates were immunoprecipitated with anti-IGF-IR, electrophoresed through 10% SDS-PAGE, and blotted with a phosphotyrosine antibody. Equal gel loading was assessed by re-probing with anti-total IGF-IR (not shown). As shown in Figure 3A, IP allowed detection of IGF-IR phosphorylation in cells treated with both low and high doses of ALA and LA. Similar results were seen using classical Western blots, however, given the lower sensitivity of this assay, no IGF-IR phosphorylation was seen at low fatty acid concentrations. Results of Western blots show that 0.1 mM ALA and 0.3 mM LA caused a several-fold increase of IGF-IR phosphorylation in three independent experiments (Figure 3B).

Effects of ALA and LA on downstream mediators phosphorylation

Next, we investigated the effect of ALA and LA on the phosphorylation of downstream proteins Akt/PKB and MAPK/Erk. Specifically, cells were treated with ALA and LA as described above, and Western immunoblotting was performed using specific phospho-Akt and

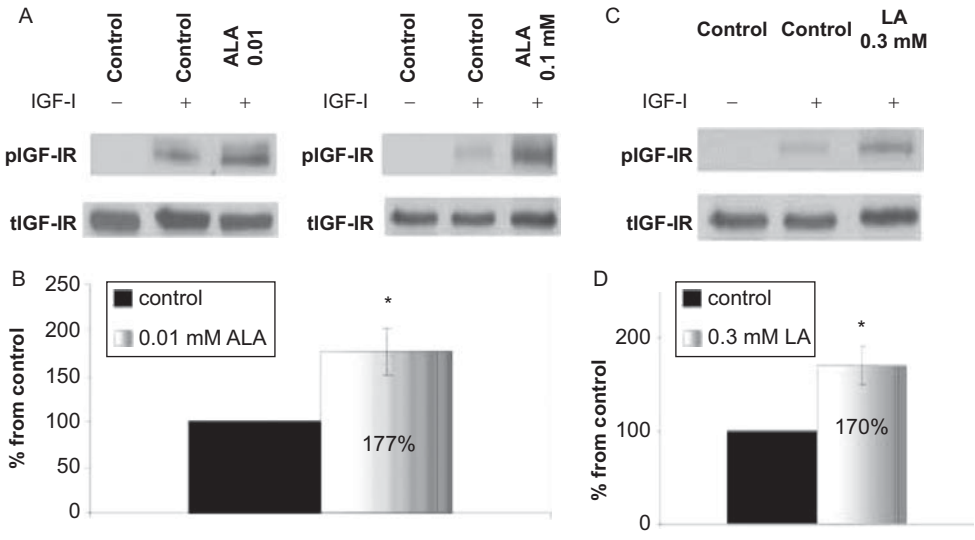

Figure 2. Effects of ALA and LA on the IGF-I-induced IGF-IR phosphorylation. HCT116 cells were incubated with 0.01 mM or 0.1 mM ALA (A) or 0.3 mM LA (C) for 24 h. Cells were then treated with 50 ng/ml IGF-I for an additional 90 min. Cell lysates were electrophoresed, transferred onto membranes, and probed with antibodies against phospho- (p)IGF-IR β-subunit and total (t)IGF-IR. (B, D) Densitometric scanning of the pIGF-IR bands normalized to the corresponding tIGF-IR bands. Bars represent the mean ± SEM of five independent experiments. *$p < 0.05$ versus untreated cells.

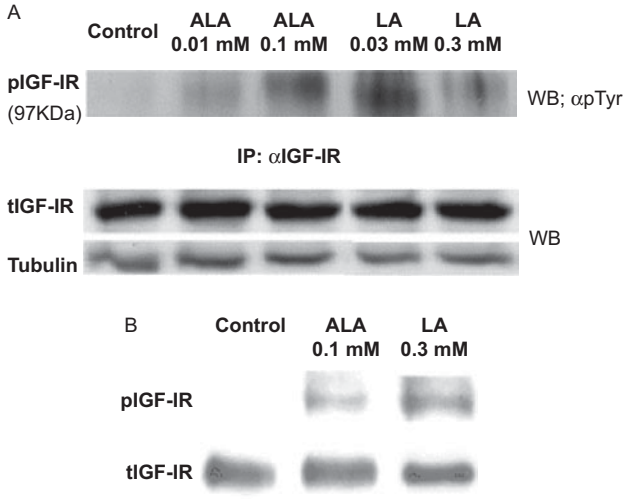

Figure 3. Effects of ALA and LA on IGF-IR phosphorylation. (A) Immunoprecipitation analysis. HCT116 cells were incubated with ALA (0.01 and 0.1 mM) or LA (0.03 and 0.3 mM LA) (or an appropriate BSA control) for 24 h. Cell lysates were precipitated with anti-IGF-IR for 20 h and precipitates were incubated with protein A/G beads for 3 h. The precipitates were washed, electrophoresed, transferred onto membranes, and probed with an anti-phosphotyrosine antibody. In a parallel experiment (bottom panel) cell lysates were analyzed for total IGF-IR content by Western blots using an anti-human IGF-IR β-subunit antibody. Blots were re-probed with a tubulin antibody as a loading control. (B) Western blot analysis. HCT116 cells were incubated with 0.1 mM ALA or 0.3 mM LA (or an appropriate BSA control) for 24 h. Cell lysates were electrophoresed, transferred onto membranes, and probed with antibodies against pIGF-IR and tIGF-IR.

phospho-Erk antibodies. Results of Western blots indicate that ALA and LA increased Akt phosphorylation at both concentrations assayed (Figure 4A). Thus, 0.01 mM ALA caused a 100% increase ($n = 6$, PV = 0.04) and 0.1 mM ALA caused an 80% increase ($n = 6$, PV = 0.023) in Akt phosphorylation. Likewise, 0.03 mM LA increased Akt phosphorylation by 50% ($n = 6$, PV = 0.04), whereas 0.3 mM LA caused a 75% increase ($n = 6$, PV = 0.03) (Figure 4B). In contrast to the enhancing effect of fatty acids on the ligand-dependent IGF-IR phosphorylation described above, neither fatty acid was able to further enhance the IGF-I-induced phosphorylation of Akt, suggesting that IGF-I treatment had already generated a maximal response (data not shown). In addition, ALA caused a marked increase in Erk phosphorylation at both doses tested: up to 1500% in seven experiments at a dose of 0.01 mM and up to 400% in five experiments at a dose of 0.1 mM ALA (Figure 4C). In contrast, the effect of LA on Erk phosphorylation, as well as the effects of both fatty acids on the IGF-I-induced Erk phosphorylation, were less consistent.

Effects of ALA and LA on proliferation and apoptosis

We next investigated the biological effects of ALA and LA on HCT116 cells. Since the IGF system is known to mediate proliferative and anti-apoptotic processes, we examined the effect of the different fatty acids on cell survival. For this purpose, cells were treated with

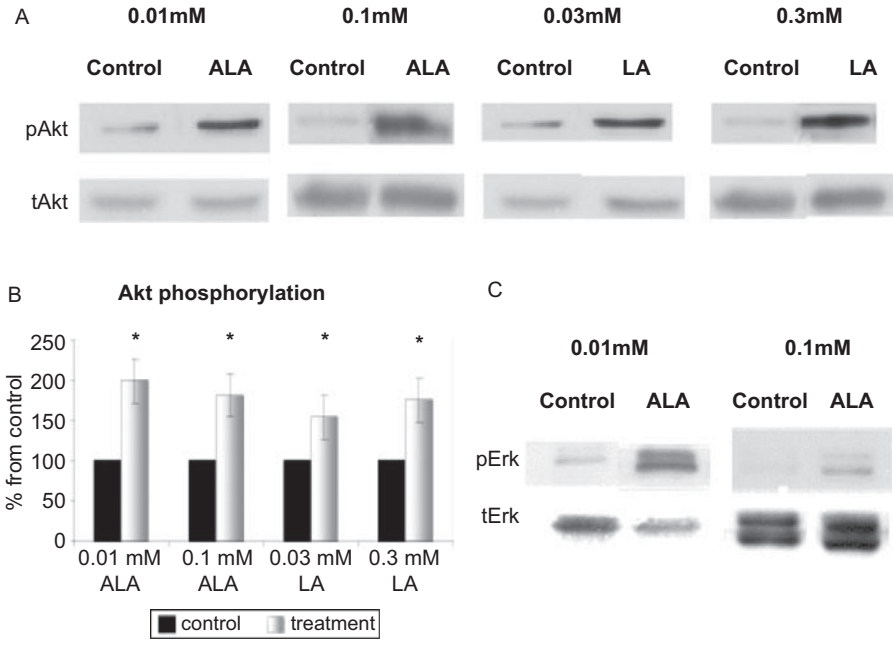

Figure 4. Effects of ALA and LA on Akt and Erk phosphorylation. HCT116 cells were incubated with ALA (0.01 and 0.1 mM) or LA (0.03 and 0.3 mM) (or BSA control) for 24 h. Cell lysates were electrophoresed, transferred onto membranes, and probed with antibodies against pAkt and tAkt (A) or pErk and tErk (C). (B) Densitometric scanning of the pAkt bands normalized to the corresponding tAkt bands. The bars represent the mean ± SEM of five independent experiments. *$p < 0.05$ versus untreated cells.

either 0.01 mM ALA or 0.03 mM LA (or BSA control) for 6, 12, or 24 h in serum- and fatty acid-free media, after which the cells were collected for FACS analysis. The results obtained revealed that ALA treatment for 6 h caused a statistically significant (5.6%, $n = 6$, PV = 0.017) increase in the portion of cells in S phase (Figure 5A). Likewise, LA caused an average increase of 5.7% in the portion of cells in S phase ($n = 5$, PV = 0.03) (Figure 5B). After 12 h of treatment, there was a slight arrest in cell growth in all groups. At this time point, the effects of ALA and LA treatments were reduced: a 2.9% increase for ALA treatment ($n = 3$) and a 4.4% increase for LA treatment ($n = 3$). These values, however, did not reach statistical significance. Cell growth was further reduced after 24 h and, at this time, ALA increased the portion of cells in S phase by 4.3% (Figure 5A) and LA increased it by 4.2% (Figure 5B). The effects of fatty acids on the portion of cells at S phase at 24 h did not reach significance. To further corroborate the proliferative effect of ALA, two independent cell-counting experiments were performed. Briefly, cells were treated daily with ALA at a dose of 0.01 mM (or BSA for control purposes), collected after 24, 48, 72, and 96 h, stained with Trypan blue and counted using a haemocytometer. In both experiments, treatment with ALA for 96 h caused a ~30% increase in cell number in comparison to untreated cells (Figures 5C and 5D).

Finally, we tested the effects of ALA and LA treatments on apoptosis by measuring Poly-ADP ribose polymerase (PARP) cleavage using Western blots. PARP is a 118-kDa protein that undergoes cleavage into 89-kDa and 24-kDa fragments at early stages of apoptosis. IGF-I treatment, as expected, reduced the intensity of the 89-kDa band (compare lanes 1 *versus* 2, Figure 6A). LA treatment (0.03 mM for 24 h) reduced PARP cleavage in a similar manner to IGF-I (compare lanes 3 vs 2, Figure 6A). At ten-fold higher doses, both ALA and LA reduced PARP cleavage in an IGF-I-independent manner (Figure 6B). These high doses of ALA and LA, in combination with IGF-I, further reduced PARP cleavage (i.e. enhanced the anti-apoptotic effect of IGF-I), compared with IGF-I treatment alone (compare lanes 2 and 3 vs lane 1, Figure 6C).

Discussion

The important role of the IGF system in colon cancer development and progression has been firmly established. Although normal colonic mucosa expresses low IGF-IR levels, these levels rise in adenoma and carcinoma and are extremely high in metastases (Hakam *et al.*, 1999). Furthermore, specific blockage of IGF-IR was shown to be associated with inhibition of tumour growth and angiogenesis, and with enhanced chemotherapy-induced apoptosis (Adachi *et al.*, 2002; Reinmuth *et al.*, 2002). In addition, high levels of circulating IGF-I were correlated with an increased colon cancer risk (Ma *et al.*, 1999), whereas

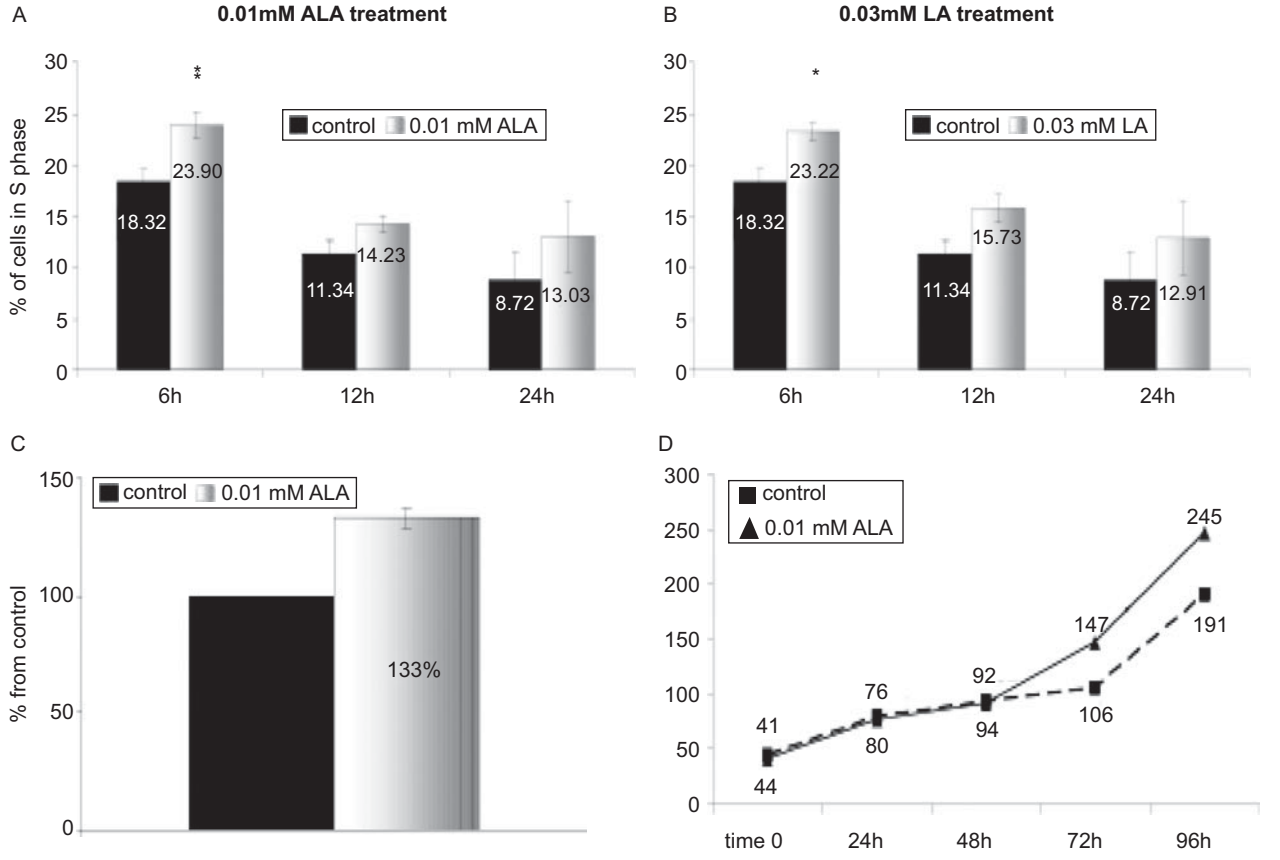

Figure 5. Effects of ALA and LA on cell cycle progression and cell proliferation. HCT116 cells were incubated with 0.01 mM ALA (A) or 0.03 mM LA (B) (or BSA control) for 6, 12, and 24 h. Cells were collected for cell cycle analysis using FACS. The bars represent the mean portion of cells in S phase ± S.E.M of at least 3 experiments. *$p < 0.05$ versus untreated cells. (C) HCT116 cells were incubated with 0.01 mM ALA or BSA control for 96 h. Cells were collected, stained with trypan blue, and counted. The bar represents the change in cell number (mean ± SEM). A value of 100% was given to the number of cells in wells incubated in the absence of fatty acids. (D) Cell proliferation in 0.01 mM ALA-treated (solid line) and control cells (dashed line) collected after 24, 48, 72 and 96 h. The graph shows the results of a typical experiment. Values are cell numbers (× 103) per well.

lowering circulating IGF-I levels in animal models induced a protective effect. Nutritional status has a profound impact on IGF-IR gene expression and activation. Furthermore, micronutrients, including folic acid, were shown to regulate IGF-IR levels in colon cancer cells by suppressing promoter activity (Attias *et al.*, 2006).

In the last few decades, there is a growing recognition of the critical impact of nutrition, especially fat intake, on prevention of different pathologies, including cancer. Fatty acids, whose metabolism is very active in the intestines, were found to influence cellular processes such as proliferation, apoptosis, signal transduction, and gene expression. Studies aimed at examining the effects of fat intake and the fatty acid composition of specific diets on the IGF system, found that the type and amount of fat in the diet influences the expression of IGF-IR in different parts of the colon (Zhang *et al.*, 1998). Furthermore, studies have shown that certain fatty acids can modulate IGF-BP secretion from the intestinal epithelia, thus regulating IGF-IR activation by IGF-I (Cave, 1991).

Our research focused on the influence of specific omega-3 and omega-6 fatty acids, ALA and LA, on the IGF-I signalling system. In view of the fact that previous studies demonstrated that fatty acids may affect gene expression, we first examined whether ALA and LA are able to regulate IGF-IR gene transcription and expression. Our results showed that neither ALA nor LA affects IGF-IR promoter activity and expression in HCT116 cells. We next examined the potential effect of ALA and LA on IGF-IR phosphorylation and on the activation of downstream signal transduction mediators. We showed that both fatty acids enhanced the IGF-I-induced IGF-IR phosphorylation and, furthermore, induced IGF-IR phosphorylation in a ligand-independent manner. Interestingly, our results also revealed that both fatty acids enhanced the activation of the IGF-IR downstream protein Akt. Consistent with these results, a number of studies have shown

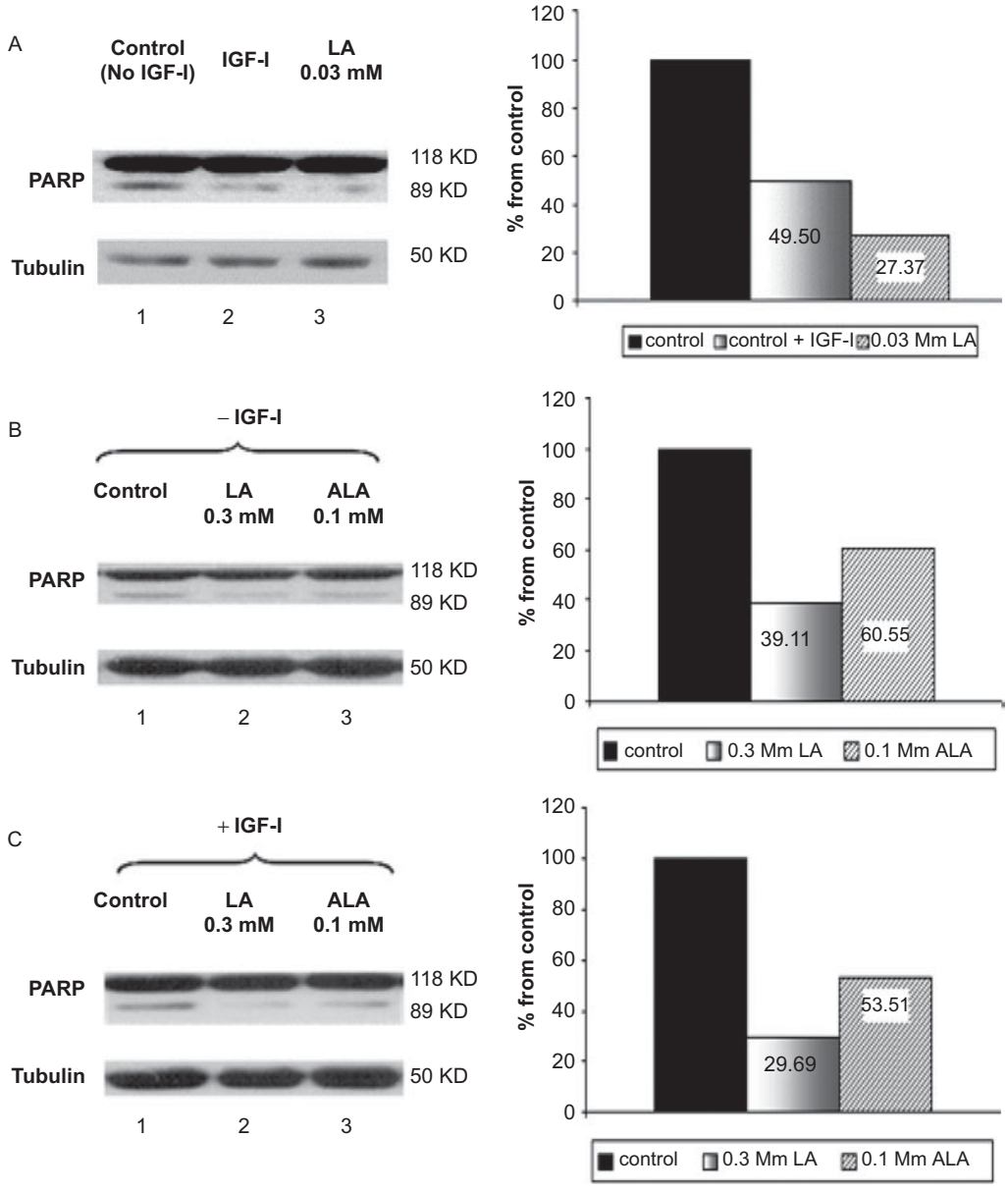

Figure 6. Reduction of PARP cleavage after ALA, LA, and IGF-I treatments. HCT116 cells were treated with either ALA, LA, or BSA control for 24 h and then supplemented with IGF-I (or vehicle) for an additional 90 minutes. Cell lysates were electrophoresed, transferred onto membranes, and probed with antibodies against PARP and tubulin. (A) PARP analysis of cells treated with 0.03 mM LA, in comparison to IGF-I treated and untreated cells. (B) PARP analysis of cells treated with 0.1 mM ALA or 0.3 mM LA, without IGF-I. (C) PARP analysis of cells treated with 0.1 mM ALA or 0.3 mM LA, followed by IGF-I treatment. The MW of the full-length (118 kDa) and cleaved (89 kDa) PARP bands are indicated. Blots were re-probed with anti-tubulin as a loading control. The bar graphs represent the scanning densitometry of the 89-kDa PARP bands shown in the corresponding gels. A value of 100% was given to the intensity of the cleaved PARP band in control cells (lanes 1).

that similar concentrations of ALA and LA increased Akt phosphorylation in vascular endothelial cells (Hennig *et al.*, 2006) and in the murine enteroendocrine cell line STC-1, and that this activation prevented the induction of apoptosis (Katsuma *et al.*, 2005). In addition, we also found that ALA induced an increase in Erk phosphorylation. LA, in contrast, had no consistent effect on Erk phosphorylation, suggesting that the effect of ALA or its metabolites is a specific one. Interestingly, experiments conducted using oleic acid (a mono-unsaturated fatty acid) and palmitic acid (a saturated fatty acid) revealed that these fatty acids did not affect IGF-IR, Akt, and Erk phosphorylation (data not shown). Therefore, our results are consistent with the notion that the effects of ALA and LA on IGF-IR signalling are fatty acid-specific, and probably occur

via metabolic processing of polyunsaturated fatty acids. Given that both Erk and Akt participate in a number of signal transduction pathways in addition to the IGF axis, the possibility that alterations in Akt and Erk phosphorylation are not a direct consequence of IGF-IR phosphorylation cannot be discarded.

In terms of the mode/s of action of ALA and LA, given that the effects on IGF-IR, Akt, and Erk phosphorylation were seen after relatively long treatments (24 h), it is reasonable to postulate that the effects of the fatty acids on signalling events were indirect, i.e. ALA and LA did not directly phosphorylate the receptor but rather, changed its sensitivity. A number of mechanisms were postulated to explain these effects, including (i) alterations in membrane phospholipids composition, leading to changes in the activities of the hormones and growth factor receptors; (ii) oxidative stress caused by fatty acid peroxidation; (iii) formation of eicosanoids, acting as 'hormone-like' mediators; (iv) interactions with different signal transduction proteins and messengers; (v) effects on hormone concentration; and (vi) effects on membrane-bound enzymes (Cave, 1991; Bartsch *et al.*, 1999; Leitzmann *et al.*, 2004; Haag and Dippenaar, 2005). Further research, including analyses of membrane composition, intracellular localization, and metabolic processing of ALA and LA, is necessary to identify the mechanism/s responsible for these effects. Of interest, HCT116 cells were shown to secrete IGF-II (Guo *et al.*, 1995). Thus, a potential mechanism of action of the fatty acids may involve their stimulation of IGF-II synthesis and secretion. In support of this mechanism, conjugated linoleic acid was shown to exhibit chemopreventive properties in the colon carcinoma cell line HT-29, and this ability was correlated with its capacity to decrease IGF-II synthesis and to down-regulate IGF-IR signalling (Kim *et al.*, 2003).

Many studies have examined the effect of dietary fatty acids, including ALA and LA, on cell proliferation, leading to contradictory results. In the present study, we examined the effects of ALA and LA treatments on HCT116 cell proliferation by comparing the portion of cells in S phase in cell cycle analysis experiments. We showed that both ALA and LA treatment for 6 h significantly increased the portion of cells in S phase. This finding cannot be attributed to a general, non-specific nutrient-related effect, since at this time point there were no differences between the control groups, held in starvation media and treated with BSA, and cells held in FBS-containing full media. Most available evidence supports the notion that omega-3 fatty acids, in contrast to omega-6 fatty acids, have a protective effect. Moreover, omega-3 and omega-6 fatty acids compete for different converting enzymes, and ALA was shown to display a greater affinity for these enzymes (Roynette *et al.*, 2004; Portolesi *et al.*, 2007). Competitive inhibition of LA metabolism may potentially explain a later effect of this fatty acid. Despite the transient nature of the ALA-induced increase in the portion of cells in S phase, our results indicate that ALA treatment for 96 h caused a 30% increase in cell number in two independent cell counting experiments. Finally, the ALA- and LA-induced reductions of PARP cleavage suggest that these fatty acids induce an anti-apoptotic effect. It remains to be established whether the biological effects of ALA and LA on cell survival and proliferation are a direct consequence of activating IGF pathways.

In summary, we provide evidence that the polyunsaturated fatty acids ALA and LA have a mitogenic effect on HCT116 cells. This effect is reflected in the number of cells in S phase and in a reduction of PARP cleavage, implying a reduction in apoptotic activity. We also showed that ALA and LA treatment increased IGF-IR phosphorylation in an IGF-I independent manner, and that these treatments affected IGF downstream pathways. Future studies will address the effects of omega-3 and omega-6 fatty acids in cell lines representing different stages of colon cancer progression. An evaluation of the beneficial versus risk effects of ALA and LA supplementation at advanced stages of colon cancer remains to be conducted.

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