Docosahexaenoic acid induces proteasome-dependent degradation of β-catenin, down-regulation of survivin and apoptosis in human colorectal cancer cells not expressing COX-2

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n-3 Polyunsaturated fatty acids have been shown to powerfully inhibit the growth of colon cancer cells, mainly acting as pro-apoptotic agents through inhibition of cyclooxygenase-2 (COX-2) expression. Since dysregulation of β-catenin expression is frequently found at early stage of colorectal carcinogenesis, we analyzed whether docosahexaenoic acid (DHA) may modify the expression of β-catenin in colon cancer cells (SW480 and HCT116) over-expressing this protein, but lacking COX-2. Furthermore, we investigated if alterations in β-catenin expression may be associated with apoptosis induction. Treatment of cells with increasing concentrations of DHA induced a dose- and time-dependent inhibition of β-catenin protein expression which, however, was not accompanied by modifications in β-catenin transcription. Conversely, the proteasomal inhibitors MG132 and lactacystin prevented DHA-induced β-catenin decrease, suggesting that DHA may regulate the proteasomal degradation of β-catenin. The reduced levels of β-catenin were accompanied by decreased translocation of β-catenin into the nucleus, where it acts as a transcription factor in concert with T-Cell Factor (TCF). DHA, at the same range of concentrations, was also able to induce apoptosis by a caspase-3-dependent mechanism and to cause a dose- and time-dependent increase of survivin, an apoptosis inhibitor undetectable in normal tissues and expressed in colorectal cancer through TCF–β-catenin stimulation. Several other proteins regulated by the TCF–β-catenin pathway and involved in regulation of tumor growth were down-regulated by DHA, including peroxisome proliferator-activated receptor-6, membrane type 1 (MT1)-matrix metalloproteinase (MMP), MMP-7 and vascular endothelial growth factor. The present study, thus, raises the possibility that DHA may exert pro-apoptotic and antitumoral effects through proteasomal regulation of β-catenin levels and alterations in the expression of TCF–β-catenin target genes.

Introduction

Dietary fatty acids have been shown to influence the incidence and growth of colorectal cancer (1–3), the most common cause of non-smoking-related cancer deaths in the Western world (4). Particularly, many epidemiological data suggest an inverse relationship between colorectal cancer risk and consumption of diets rich in n-3 polyunsaturated fatty acids (PUFAs) (5–7). Moreover, in our laboratory it was demonstrated that dietary supplementation with the main components of fish oil, eicosapentaenoic acid and docosahexaenoic acid (DHA) inhibited the abnormal proliferation observed in the epithelial cells of colon crypts of patients with sporadic colorectal adenomas at high risk of colon cancer (8, 9). Conversely, we found that the homeostasis of normal colon epithelium was not altered by treatment in vivo with these fatty acids, leaving the structural and numerical organization of epithelial cells unchanged in rat colon mucosa (10). These findings are supported by experimental data showing the beneficial effects of a dietary n-3 PUFA treatment in animals exposed to chemical carcinogenesis in order to develop colon cancer or bearing transplanted colonic tumors (11–13). Plenty of data are also available to show the growth inhibitory effects of n-3 PUFAs on colon cancer cells in culture (12, 14, 15). In particular, recently, we have reported the antiangiogenic effects of DHA in human HT-29 colon cancer cells growing in vitro or injected in nude mice (12). We suggested that the DHA-driven inhibition of the cyclooxygenase-2 (COX-2)–prostaglandin E2 (PGE2)–phosphorylated extracellular regulated protein kinase (pERK)–hypoxia-inducible factor-1 (HIF-1) pathway was involved in the antiangiogenic effect of this fatty acid. DHA also caused induction of apoptosis in HT-29 cells and in other strains of human colorectal cancer cells (14–16). Recently, it has been suggested that DHA may exert pro-apoptotic effects in cancer cells by inhibiting the expression of the enzyme COX-2 (12, 14), whose ability to confer resistance to apoptosis has been reported in a variety of studies (see 17 for a review). However, it is possible that DHA may also act via mechanisms independent of COX-2 inhibition. In particular, in the present work, we studied the regulation of β-catenin and downstream TCF–β-catenin target gene expression by DHA. Dysregulation of Wnt signaling and β-catenin expression is believed to be central to the early stages of sporadic carcinogenesis in humans, and recent works suggest that the Wnt–β-catenin pathway regulates the apoptotic process in colorectal cancer cells (18–19). In particular, it was demonstrated that different drugs, such as exisulind and indomethacin, are able to circumvent defective regulation of β-catenin accumulation, reducing nuclear β-catenin levels and increasing apoptosis in colon cancer cells. Recently, Narayanan et al. (14) showed that DHA reduces the expression of β-catenin protein in Caco-2 cells, simultaneously decreasing also the levels of COX-2 and inducing apoptosis. In the present work we used two strains of human colorectal cells lacking COX-2 in order to investigate the involvement of β-catenin and TCF–β-catenin target genes in the pro-apoptotic effect of DHA. Moreover, we attempted to discriminate whether DHA acts on β-catenin synthesis or degradation.

Materials and methods

Cell lines

The human colon adenocarcinoma cell lines, HCT116 and SW480, were obtained from the American Type Culture Collection (Rockville, MA). HCT116 cells carry activating mutation of the β-catenin gene (CTNNB1) and SW480 cells are mutant for adenomatous polyposis coli (APC) and wild type for CTNNB1 (20). Both cell lines have been studied and characterized extensively (21). They have been described as COX-2 negative, since they did not express COX-2, either at mRNA or at protein level (22). In agreement, we did not observe any detectable expression of COX-2 protein in either cell lines, through preliminary western blot analysis (data not shown). HCT116 cells were cultured in McCoy’s 5a medium supplemented with 5% fetal calf serum, 2 mM L-glutamine without antibiotics; SW480 cells were cultured in L-15 medium supplemented with 5% fetal calf serum and 2 mM glutamine without antibiotics. Cells were maintained in log phase by seeding twice a week at a density of 3 × 10⁵ cells/ml in a humidified atmosphere at 37°C under 5% CO₂. The experiments were performed 1 day after trypsinization.
DHA was purchased from Sigma–Aldrich (Sigma, St Louis, MO). DHA was added at the beginning of the experiments from an absolute ethanol stock solution and control cells were treated with the same amount of vehicle alone. The final ethanol concentration never exceeded 0.5% (vol/vol). In order to prevent oxidation, DHA was stocked under nitrogen and stored at –80°C.

The ubiquitin–proteasome pathway inhibitors lactacystin and MG132 were purchased from Sigma–Aldrich and Calbiochem (San Diego, CA), respectively. Lactacystin was prepared as a 3 mM stock solution in sterile, distilled water and MG132 as a 5.0 mM stock solution in dimethylsulfoxide. Caspase inhibitor I (z-VAD-fmk) was purchased from Calbiochem.

Western blot analysis
Total cell protein extracts were obtained as described previously (14). Briefly, cells (10 × 10⁶) were collected and lysed with cold lysis buffer (1 mM MgCl₂, 350 mM NaCl, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.5 mM ethylenediaminetetraacetic acid, 0.1 mM ethyleneglycol-bis(aminohexamethylene)-tetraacetic acid, 1 mM diethiothreitol, 1 mM Na₃P₂O₇, 1 mM phenylmethylsulfonlfuoride, 1 mM aprotinin, 1.5 mM leupeptin, 20% glycerol, 1% NP-40). The protein levels were quantified spectrophotometrically by Bradford method using the Bio-Rad assay (Hercules, CA). Total cell proteins (100 µg) were subjected to electrophoresis on 10% polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% dried milk in [phosphate-buffered saline (PBS) plus 0.05% Tween-20] and probed with the primary antibodies to β-catenin (clone H-102, catalog no. sc-1745, Santa Cruz Biotechnology, Santa Cruz, CA), survivin (clone D-8, catalog no. sc-17779, Santa Cruz Biotechnology) and cyclin D1 (clone HD-11, catalog no. sc-246, Santa Cruz Biotechnology). Following incubation with secondary antibodies, the immunocomplexes were visualized by the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech Italia, Milan, Italy) and quantitated by densitometric scanner. As a loading control, the blots were re-probed with an anti-β-actin antibody (Amersham, Pharmacia Biotech Italia) at a 1:1000 dilution.

Real-time polymerase chain reaction
Total RNA was extracted from tissue samples using Trizol according to the manufacturer’s protocols (Invitrogen Life Technologies, Paisley). RNA was eluted in diethylpyrocarbonate-treated water (0.01% DEPC) and stored at –80°C until reverse transcription–polymerase chain reaction (RT–PCR) analysis was performed. Nucleic acid concentrations were measured by absorbance spectrophotometry (Hewlett-Packard HP UV/VIS spectrophotometer 8450). To eliminate genomic DNA from each sample, total RNA (500 ng) was treated with DNase I amplification grade (Invitrogen Life Technologies) following manufacturer’s instructions, and afterwards RNA was employed for cDNA synthesis. RT–PCR assay was performed using the two-step method. For the first-step RT reaction, we used Superscript III first-strand synthesis system for RT–PCR (Invitrogen Life Technologies), as described in the manufacturer’s procedure for cDNA synthesis with oligo(DT). For the second-step PCR, we employed Quantitect SYBR® Green Kits (Qiagen, Hilden, Germany) and Quantitect® Primer Assays (Qiagen) for human β-actin and β-catenin, according to manufacturer’s protocol described for the real-time thermalcycler LightCycler (Roche, Mannheim, Germany). PCR data were analyzed by Relative Quantification Software (Roche) and expressed as target:reference ratios. The final ethanol concentration never exceeded 0.5% (vol/vol). In order to prevent oxidation, DHA was stocked under nitrogen and stored at –80°C.

For immunofluorescence analysis, cells were plated in four-well chamber slides (Nunc, Rochester, NY) for 24 h before treatment with 10 µM DHA for 48 h. Afterwards, cells were fixed in 2% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in 0.1 M PBS pH 7.4 containing 0.2% bovine serum albumin, air dried and re-hydrated in PBS. Then cells were incubated with a rabbit polyclonal antibody against β-catenin (Santa Cruz, Biotech, Santa Cruz, CA), diluted 1:500 in PBS containing 3% normal goat serum for 2 h at room temperature. Negative controls were performed by omitting the primary antibody. After two washings in PBS for 10 min, an anti-rabbit IgG Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in PBS was added for 1 h at room temperature. Cells were then washed in PBS and mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) to counterstain DNA. Cells were observed using a Zeiss Axioshot (Germany) fluorescence microscope. Microphotographs were acquired using a digital video camera (Axiocam MRC, Zeiss, Germany) and an Axiosvision Zeiss software.

Apoptosis detection
The percentage of apoptotic cells was determined morphologically by staining the cells with acidine orange (100 µg/ml) and analyzing them by fluorescence microscopy at high power magnification (×400), as described previously (23).

To confirm the results of morphological analysis, caspase-3 activity was evaluated. The activity of caspase-3 was measured by a fluorometric assay as described previously (23). Briefly, cells were incubated for the indicated times and harvested. Cells (2 × 10⁶) were lysed in 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM ethylenediaminetetraacetic acid, 0.5% octylphenyl-polyethylene glycol (Sigma–Aldrich) and 150 mM NaCl. Cell lysates were incubated with 50 µM fluorogenic substrate, N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Alexis Biochemicals, San Diego, CA), in a reaction buffer (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.5, containing 50 mM NaCl and 2.5 mM dithiothreitol) for 120 min at 37°C. The release of AMC was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

Apoptosis induction was further analyzed by a double staining method using fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide. Annexin V, a calcium- and phospholipid-binding protein, binds preferentially to a negatively charged inner membrane phospholipid, phosphatidylserine, on exposure to cell surface and detects early cellular apoptotic changes, whereas the normally impermeable vital dye propidium iodide detects cells undergoing necrotic changes. Apoptosis was detected with an Annexin V–FITC kit (Oncogene Research, Cambridge, MA) according to the manufacturer’s instructions. Briefly, at the times indicated, cells (0.5 × 10⁶) were collected, washed with ice-cold PBS and centrifuged (3000 g for 5 min). The cell pellet was re-suspended in ice-cold binding buffer. After that, the Annexin V–FITC solution was added (1.25 µl of 50 µM) and the samples were incubated for 15 min in room temperature in the dark. After centrifugation (3000 g for 5 min), the cell pellet was re-suspended in 0.5 ml ice-cold binding buffer, propidium iodide solution (10 µl of 0.5 µl) was added and samples were immediately analyzed by flow cytometry (Coulter Epics XL-MCL, 620 nm filter).

Statistical analysis
The results were expressed as the means ± SEs. One-way analysis of variance (ANOVA) was used to determine significant differences among groups. Tukey’s honestly significant difference post hoc test was used for pairwise comparisons after analysis of variance. Statistical significance was accepted at P < 0.05.

Results
Figure 1 (A–D) shows that HCT116 and SW480 colon cancer cells expressed high levels of β-catenin protein in control conditions. The treatment of both the cell types with increasing concentrations of DHA (2.5–10 µM) for 48 h reduced the levels of β-catenin in a concentration-dependent manner (Figure 1A–D). DHA was added to the cells in a range of concentrations (2.5–10 µM) which did not induce necrotic changes after 48 h, as measured using the membrane-permeable ethidium bromide and propidium iodide (data not shown). Moreover, we found that the DHA-induced β-catenin decrease was time dependent. After a 24 h treatment with DHA (10 µM), the β-catenin:actin protein ratio decreased from 1.22 ± 0.09 to 0.94 ± 0.06 (23.3 ± 1.1% decrease) in HCT116 cells and from 1.28 ± 0.08 to 0.96 ± 0.07 (P < 0.05, 25.0 ± 1.4% decrease). The reduction became much more conspicuous at 48 h in both the cells examined and the β-catenin:actin ratio decreased from 1.26 ± 0.07 to 0.50 ± 0.05 (P < 0.05, 60.3 ± 3.4% decrease) in HCT116 cells and from 1.32 ± 0.09 to 0.52 ± 0.04 (P < 0.05, 60.6 ± 2.7%) in SW480 cells. Furthermore, we examined β-catenin mRNA levels in order to ascertain if β-catenin protein reduction was due to an altered transcription of its gene. We measured β-catenin mRNA levels by real-time quantitative RT–PCR after 6 and 12 h treatment and normalized them to β-actin mRNA levels amplified in the same experiment, using a dedicated relative quantification software (Figure 1E–F). The final data (expressed as β-catenin/β-actin mRNA ratio) are shown. Beside a small transient decrease, observed after 6 h in SW480 cells, the ratio remained unmodified in both the cell types, suggesting that β-catenin transcription is not altered by DHA.

We next investigated whether the DHA-induced decrease of β-catenin could be ascribable to modifications in the degradation of the protein. Since, dependently phosphorylation, ubiquitination and degradation by the proteasome, we next investigated whether the decrease of β-catenin protein induced by DHA could be mediated by proteasome-dependent degradation using the ubiquitine–proteasome inhibitor MG132 (Figure 2A–D). We found that 10 µM MG132 inhibited...
the DHA-induced reduction of β-catenin protein in both the cell types. This finding suggests that DHA decreases the cellular levels of β-catenin through an ubiquitin–proteasome-dependent process. The results obtained with MG132 were also confirmed (Figure 3E) treating HCT116 cells with another proteasome inhibitor, lactacystin (100 μM).

We next examined the modification of β-catenin localization following DHA treatment by indirect immunofluorescence detection of the protein in SW480 cells (Figure 3). β-Catenin may localize in nucleus or in cytoplasm, especially in peri-plasmamembrane position where it binds to E-cadherin. In particular, in cells over-expressing β-catenin, as the majority of colon cancer cells, a high amount of protein is in a dephosphorylated form and translocates into the nucleus activating the transcription of a variety of target genes with growth-promoting effects (24). In control conditions, we observed that β-catenin is present in all cellular localizations, but preferentially accumulates in the nucleus (Figure 3A). We counterstained cells with 4′,6-diamidino-2-phenylindole (Figure 3B) to confirm the preferential nuclear localization of the protein in control conditions. On the other hand, cells treated with DHA showed a preferential cytoplasmic localization of β-catenin (Figure 3C and D).

Previously, it has been shown that various NSAIDs (19, 25) and other unrelated compounds (25–29) induce apoptosis in colon and in other strains of cancer cells by a mechanism involving the decrease of β-catenin expression. For this reason, we investigated whether the reduced expression of β-catenin induced by DHA could be associated with apoptosis stimulation. In Figure 4, the pro-apoptotic effects of DHA are shown. The morphological examination of acridine orange-stained cells (Figure 4A and B) revealed that DHA exerts pro-apoptotic effects in a concentration-dependent manner both in HCT116 (A) and SW480 cells (B). In particular, after 48 h treatment, the pro-apoptotic effect became significant at 5 μM DHA in HCT116 cells (Figure 4A) and at 2.5 μM DHA in SW480 cells (Figure 4B), and it reached a maximum in both cell types at 10 μM DHA. The parallel
effects of DHA (2.5–5 μM) on both β-catenin expression and apoptosis may be suggestive of a role for β-catenin in the DHA-induced apoptotic response. We confirmed the pro-apoptotic effect of DHA performing the cytofluorimetric analysis of HCT116 cells marked with Annexin V–FITC (Figure 4C), which allows the identification of cells in both early- and late-apoptotic phases. In agreement with the results obtained with the morphological examination of acridine orange-stained cells, the percentage of all apoptotic cells (in early and late apoptosis) increased by ~3-folds after 48 h treatment. In particular, a large increase in the portion of early-apoptotic phase cells (by 360%) and a smaller, but significant one in late-apoptotic phase cells was noticed (by 213%). In HCT116 cells, we studied the timing of apoptosis appearance and observed that apoptosis was induced as early as 24 h after DHA treatment, and the number of apoptotic cells further increased after 48 h (Figure 4D). Furthermore, we observed that the activity of caspase-3 increased with a similar temporal pattern. At 12 h, no caspase-3 activation was detected, while a moderate, but significant increase in the activity was observed after both 24 and 48 h treatment with DHA (Figure 4E).

Since caspases have been shown to cleave β-catenin during apoptosis (30), we evaluated whether the decrease in β-catenin protein carried out by DHA treatment could be ascribable also to DHA-induced caspase activation. HCT116 cells were treated simultaneously with DHA and the z-VAD-fmk, caspase-3 inhibitor (31) for 48 h (Figure 5). Caspase inhibitor, used at 25 μM, a concentration able to block completely DHA-induced apoptosis (data not shown), did not modify significantly the β-catenin reduction induced by DHA, suggesting that the DHA-induced down-regulation of β-catenin levels did not depend on caspase activation. Then, DHA-induced apoptosis, probably by β-catenin down-regulation, appears to be caspase-3 dependent while DHA-induced β-catenin reduction does not depend on caspase-3 activity.

Recently, it has been shown that in colorectal cancer cells TCF–β-catenin pathway stimulates powerfully the expression of survivin (32), an apoptosis-inhibiting protein that is thought to contribute to tumor cell immortality (33). On this basis, we evaluated the modifications in the expression of survivin induced by DHA (Figure 6A–D).

We found that DHA, used at the same concentrations which induce apoptosis and reduce β-catenin expression (2.5–10 μM), was also able to inhibit survivin expression in a concentration-dependent manner, suggesting a possible role for survivin in the development of DHA-induced apoptosis. Figure 6E shows that other products of TCF–β-catenin target genes that are involved in tumorigenesis are significantly (P < 0.05) down-regulated by DHA in both the cell lines. In particular, we have observed the reduced expression of the matrix metalloproteinases (MMPs), matrix metalloproteinase-7 (MMP-7) and membrane type 1 (MT1)-MMP and the angiogenic factor vascular endothelial growth factor (VEGF), which are over-expressed in human colorectal cancer, and whose elevated levels correlate with a poor clinical outcome (34–36). Also the expression of peroxisome proliferator-activated receptor-δ, another protein recently involved in the regulation of apoptosis and whose expression is regulated by β-catenin (37, 38), was significantly (P < 0.05) decreased after 48 h treatment with 10 μM DHA.

Discussion

In this study, we demonstrate that DHA is able to induce apoptosis and circumvent defective regulation of β-catenin by reducing the total and nuclear levels of this protein in HCT116 and SW480 human colon cancer cells. The levels of the anti-apoptotic protein survivin and other products of TCF–β-catenin target genes, such as peroxisome proliferator-activated receptor-δ, MT1-MMP, MMP-7 and VEGF, involved in regulation of apoptosis, tumor invasion and neoangiogenesis were also diminished by DHA treatment. We previously found that DHA exerts pro-apoptotic and antiangiogenic effects in colonic cancer cells modulating the COX-2–PGE2 pathway (12). Moreover, the pro-apoptotic effect was also confirmed in the present paper by preliminary experiments showing that DHA, at 10 μM (the maximal concentration used in the present work), increased the number of apoptotic LS-174 and HT-29 cells by 357.1 ± 21.1% and 328 ± 23.9%, respectively (data not shown).

However, DHA exerts its pro-apoptotic effect also in COX-2-negative HCT116 and SW480 colon cancer cells, suggesting that this
effect is COX-2 independent. As the majority of colorectal cancer cells in humans, these cells constitutively over-express β-catenin protein (39, 40). In particular, the increased levels of β-catenin derive from the presence of a mutated inactive APC in SW480 cells and from an activating mutation of β-catenin gene (CTNNB1) in HCT116 cells (21). All these genotypic features make these cells genotypically very similar to the most dysplastic mucosal epithelial cells in human sporadic colorectal adenomas that, as early lesions with potential to progress towards malignancy, may represent the possible target of dietary DHA chemopreventive treatment in tumorigenesis of colon cancer (39). It has been shown that it is possible to revert accumulation of β-catenin in human colorectal cancers containing endogenous inactive APC alleles by re-establishing β-catenin degradation through neo-expression of APC (41). This situation resulted in a substantial diminution of cell growth due to induction of apoptosis. Moreover, it has been reported that reduced β-catenin expression is involved in the induction of apoptosis of colon cancer cells and other strains of cells by different drugs and nutritional factors, including various NSAIDs drugs (22), butyrate (25, 26), trefoil factor 3 (27), curcumin (28) and chlorofillin (29). For this reason, we evaluated the capability of DHA to alter β-catenin expression and to induce apoptosis in HCT116 and SW480 colon cancer cells. We found that, in both the cell lines, DHA reduced the levels of the β-catenin protein in a concentration-dependent manner. This phenomenon was evident at relatively low DHA concentrations (2.5–10 μM) and was accompanied by a parallel dose-dependent induction of apoptosis, which was caspase-3 dependent. Furthermore, the temporal pattern of β-catenin reduction by DHA was coincident with the onset and development of apoptosis. All these data suggest a possible link between the decrease of β-catenin and the induction of apoptosis observed after DHA treatment. Our results are in agreement with those recently reported by Narayanan et al. (14) in Caco-2 cells using low DHA concentrations (5 μM). They observed that DHA induced apoptosis and down-regulated several molecular targets, among which β-catenin and COX-2, highly expressed in Caco-2 cells.

The DHA-induced decrease of β-catenin was not related to reduced transcription of its gene, since the levels of mRNA were not modified by DHA treatment in both the cell types. On the contrary, we found that modifications of proteasomal degradation of β-catenin were involved in the DHA effect. This was proven by the use of the ubiquitin–proteasome inhibitors MG132 and lactacystin, which inhibited at a large extent the DHA-induced decrease of β-catenin in both cells. The observation that this phenomenon also takes place in SW480 cells, which are APC mutant, and therefore unable to carry out an APC-dependent ubiquitin–proteasomal degradation of β-catenin, suggests that, in these cells, DHA is able to induce a proteasomal degradation of β-catenin in an APC-independent way. In agreement with our data, it was recently found that different NSAID compounds (indomethacin and sulindac sulfide) are able to induce ubiquitin-dependent degradation of β-catenin in these APC mutant cells (19, 42). In particular, in these cells, the activation of a protein kinase G-dependent mechanism of proteasomal β-catenin degradation by the NSAID sulindac sulfone was recently demonstrated (43). Moreover, our finding that DHA may stimulate proteasomal-dependent degradation in cells is in keeping with the recent observations of Botolin et al. (44). They found that DHA is able to accelerate the disappearance of G.Calviello et al.
nuclear sterol regulatory element binding protein-1 in hepatocytes stimulating a 26S proteasome-dependent process. 

Another possibility to explain the DHA-induced reduction of β-catenin levels could be its processing by the caspases activated during the DHA-induced apoptosis, since recently it has been reported that β-catenin can be cleaved by caspase-3 activation (30). However, in our experimental conditions, the concomitant treatment of cells with the inhibitor of caspase-3, z-VAD-fmk and DHA for 48 h did not prevent the DHA-induced reduction of β-catenin, ruling out the possibility that apoptosis could contribute to the observed reduction of β-catenin levels.

The decrease in cellular β-catenin protein levels was accompanied by lower levels of β-catenin in nuclear location, as demonstrated by immunofluorescent staining of β-catenin. DHA effect on β-catenin cellular location appears crucial in explaining the antitumoral effect of n-3 PUFA demonstrated in colon cancer, since nuclear β-catenin, binding to TCF family of transcription factors, can activate the transcription of genes involved in tumor growth (45). The diminished function of β-catenin as a nuclear transcription factor in the presence of DHA was demonstrated by the decreased levels of products of genes notably activated by TCF–β-catenin (32). Other gene products whose transcription is activated by TCF–β-catenin (34, 35, 48), such as VEGF, MMP-7 and MT1-MMP, are implicated in the regulation of cell growth, invasion and angiogenesis in colorectal cancer. In particular, MMP-7 has shown to be over-expressed in 75% of human colorectal cancers (34) and its degree of over-expression correlates with stages of disease and/or prognosis (49). Similarly, MT1-MMP is over-expressed in different tumors, including colon cancer (50) and it has been associated with invasive cancer and metastasis (51, 52). Finally, VEGF, whose levels are elevated and correlate with a poor clinical outcome in colon cancer, is self-produced by neoplastic cells and represents a factor strictly related to the induction of neovascularization in these cancers (36). We have shown that all these gene products, whose transcription is controlled by the TCF–β-catenin pathway, were down-regulated by DHA in both the colon cancer cell lines. It is possible to suggest that the ability of DHA to negatively control the expression of these factors closely associated to growth and progression of tumors may contribute to explain the antiangiogenic, antiinvasive and antimetastatic effects of n-3 PUFAs observed in in vivo models of colon cancer (12, 53). We previously demonstrated that n-3 PUFAs are able to decrease VEGF expression in colon cancer cells through the negative regulation of COX-2–PGE2–pERK–HIF-1 pathway (12). The observation that DHA is able to reduce VEGF levels also in colon cancer cells lacking COX-2

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Fig. 6. DHA modulation of the expression of different TCF–β-catenin target genes. Survivin expression in HCT116 (A and C) and SW480 (B and D) cells treated with increasing DHA concentrations (2.5–10.0 μM) for 48 h. Survivin protein expression was evaluated by western blotting. (A and B) Results represent the means ± SEs of at least four experiments. Values with different letters are significantly different (P < 0.05, one-way ANOVA, followed by Tukey’s test); (C and D) one representative of four similar β-catenin western blot analyses is shown. (E) Cells were exposed to 10 μM DHA for 48 h and expression of peroxisome proliferator-activated receptor-δ, MMP-7, MT1-MMP and VEGF proteins was evaluated by western blotting. One representative of four similar β-catenin western blot analyses is shown.
suggests that both the molecular pathways able to activate VEGF transcription in colon cancer cells (TCF–β-catenin and COX-2–PGE$_2$–PERK–HIF-1) may be negatively influenced by n-3 PUFA.

The finding that DHA inhibits the expression of β-catenin and of TCF–β-catenin-related gene products in colon cancer cells supports the hypothesis that DHA may act as chemopreventive and/or chemotherapeutic agent against colon cancer. In particular, in colon tumors, especially in the early pre-malignant lesions, many cells may over-express β-catenin, but lack COX-2 expression (39). For this reason, it would seem very appropriate the combination of DHA, able to inhibit both COX-2 and β-catenin expression in colon cancer, with selective COX-inhibitors, such as celecoxib, which recently has received much attention in chemotherapy of colon cancer. In fact, recently, it was found that an in vitro combined treatment of DHA and celecoxib is able to synergistically inhibit HCA-7 colon cancer cell growth (54). This combination of chemopreventive agents appears particularly appropriate to arrest the growth of cancer cells in colonic tumors, even when the cells do not express COX-2.

In conclusion, our results show that DHA reduces β-catenin protein levels in colon cancer cells over-expressing this protein. Since transcription of β-catenin is not modified by DHA, the fatty acid seems to act mainly through the induction of ubiquitin-dependent proteasomal degradation of the protein. It is possible that the negative modulation exerted by DHA on the level of transcriptionally active β-catenin present in the nucleus may counteract the dysregulated activity of Wnt–β-catenin transduction pathway of colon cancer cells, inhibiting the expression of tumorigenic β-catenin-regulated genes. This property of DHA could further support its role as a suitable chemopreventive and chemotherapeutic agent.

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