

Effects of docosahexaenoic acid and eicosapentaenoic acid on androgen-mediated cell growth and gene expression in LNCaP prostate cancer cells

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There is some epidemiological support for a protective influence of ω -3 fatty acids against prostate cancer. We wanted to explore whether ω -3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can affect androgen receptor function in prostate cancer cells. Our study showed that both DHA and EPA inhibit androgen-stimulated cell growth. Androgenic induction of prostate-specific antigen (PSA) protein was repressed by DHA and EPA in a dose-dependent manner. The mRNA levels of five androgen up-regulated genes, PSA, ornithine decarboxylase, NKX 3.1, immunophilin fkbp 51 and Drg-1, were decreased with DHA treatment in the presence of androgens. Transfection experiments using a DNA vector containing androgen-responsive elements demonstrated that both DHA and EPA could interfere with transactivation activities of the androgen receptor (AR). However, western blot analysis of AR protein showed that DHA and EPA treatments did not change AR expression levels. Interestingly, the proto-oncoprotein c-jun was increased by DHA treatment. A transient transfection found that forced expression of c-jun inhibited AR transactivation activity. Thus, this study found that the inhibitory effects of ω -3 polyunsaturated fatty acids on AR-mediated actions are due, at least in part, to an increase in c-jun protein.

Introduction

Prostate cancer is the most commonly diagnosed cancer in the USA after non-melanoma skin cancer and it is the second leading cause of cancer death in American men. Although prostate cancer is just as common in Japan as in the USA, death rates from prostate cancer are significantly lower in Japan (1). It is unlikely that these differences are all genetic, because Japanese men who migrate to the USA die of prostate cancer with increasing frequency as a function of the number of years they reside in the USA (2). It is possible a portion of this paradox could be explained by dietary factors.

Dietary intake of essential fatty acids, including both ω -3 and ω -6 fatty acids, is crucial for many important cellular processes, including cell proliferation and differentiation (3).

Abbreviations: AR, androgen receptor; ARE, androgen-responsive element; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; β -gal, β -galactosidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Mib, mibolerone; ODC, ornithine decarboxylase; PSA, prostate-specific antigen.

Epidemiological studies demonstrated a correlation between high consumption of animal fat and death from prostate cancer (4). Controlled case studies added support to a positive association between dietary fat, particularly saturated animal fat, and prostate cancer (5). Note that animal fat contains high amounts of ω -6 fatty acids. A large prospective study of American men showed a positive association between α -linoleic acid (an ω -6 fatty acid) in the diet and prostate cancer (6). *In vitro* studies (7,8) showed that the growth of PC-3 human prostate cancer cells is stimulated in the presence of linoleic acid. Diets high in ω -6 polyunsaturated fatty acids can stimulate prostate cancer development (9,10). On the other hand, a nested case-control study on plasma lipid levels and the development of prostate cancer suggested that low plasma levels of α -linoleic acid might be associated with a reduced risk of prostate cancer (11). Wang *et al.* showed that lowering the proportion of fat in the diet decreased the growth rate of human prostate adenocarcinoma cells in nude mice (12).

Conversely, long-chain ω -3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can inhibit *in vitro* and *in vivo* cell growth of prostate cancer cells (13–16). There is epidemiological support for a protective influence of ω -3 fatty acid against prostate cancer (17–19). Japanese and Alaskan Eskimo men who eat large quantities of fish have a low risk for prostate cancer. Fish traditionally provide the major source of animal proteins and fat for these people. Fish oil is rich in ω -3 fatty acids. It has been suggested that external factors such as fish oil may have a role in repressing the development and growth of prostate cancer (19). Moreover, Mishina *et al.* reported that Japanese men that consumed low quantities of seafood were associated with increased prostate cancer risk (18). Therefore, long-chain ω -3 polyunsaturated essential fatty acids found in fish oil and other dietary factors may be beneficial for prostate cancer chemoprevention.

Androgens play an important role in proliferation, differentiation, maintenance and function of the prostate (20). Evidence shows that androgens are also involved in the development and progression of prostate cancer (21). The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily (22) and is the essential mediator for androgen action. In addition to its physiological functions, the AR plays a critical role in the development of prostate cancer. The LNCaP cell line is a well-established, androgen-responsive prostate cancer cell line obtained from a lymph node metastasis of a prostate cancer patient (23). LNCaP cells express the AR and a number of androgen-inducible genes, such as prostate-specific antigen (PSA) and hK2 (23,24). This study explored whether DHA and EPA can inhibit the function of the AR in LNCaP prostate cancer cells.

Materials and methods

Cell culture

Human prostate cancer cell line LNCaP (American Type Culture Collection) was grown in RPMI 1640 medium supplemented with 5% fetal bovine serum

(FBS) at 37°C and 5% CO₂ until reaching ~50–70% confluence. The medium was changed to serum-free RPMI 1640 to deplete undesired steroids for 24 h prior to experiments. Cells were then treated with RPMI 1640 containing 5% charcoal-stripped FBS and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) or *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) (Sigma, St Louis, MO) dissolved in ethanol at designated concentrations with or without mibolerone (Mib) (NEN, St Louis, MO), a non-metabolizable synthetic androgen, dissolved in ethanol. Equivalent amounts of solvent were added to control cells.

Cell proliferation and PSA quantification assays

LNCaP cells were seeded at 2×10^4 cells/well in 24-well plates. After 72 h they were treated with serum-free RPMI 1640 for 24 h and then incubated with varying amounts of DHA or EPA with or without 1 nM Mib in medium containing 5% charcoal-stripped serum. Six days after treatment 400 μ l of spent medium was collected for total PSA protein measurement by the Tandem-E PSA kit (Hybritech). An MTS assay was performed (Promega, Madison, WI) to measuring cell viability. Four wells per treatment were used for the above assays and they were repeated twice. PSA levels in spent medium were normalized to cell number.

Transient transfections

LNCaP or PC-3 cells were plated in 60 mm dishes. Cells were transiently transfected as previously described (25) with either three copies of the hK2 ARE in a pGL3 promoter plasmid, CMV β -gal, a human AR expression vector or c-jun expression vector as indicated. The AR and c-jun constructs are driven by the SV40 promoter (26). LNCaP cells were transfected with liposomes containing dimethyldioctadecyl ammonium bromide (Sigma) and L- α -lecithin (Sigma) (4:10 w/w) and PC-3 cells were transfected with lipofectamine (Gibco BRL, Grand Island, NY). After 24 h LNCaP cells were treated with 150 μ M DHA or EPA with or without 3.2 nM Mib. Whole cell extracts were prepared for luciferase assay according to the manufacturer's instructions (Promega). A CMV β -galactosidase (β -gal) expression vector and a parental vector (pGL3) were included as controls in the above transfections. β -Gal activity and total protein were assayed using the Bradford assay (Bio-Rad, Hercules, CA) for normalization purposes. Each transfection was done three times and standard deviations were calculated.

Northern blots

After steroid depletion as mentioned above, LNCaP cells were treated with varying amounts of DHA and 1 nM Mib as indicated and RNA was collected by the guanidine isothiocyanate method (27). A denaturing RNA gel was run and transferred to a nylon membrane (Bio-Rad) according to the GeneScreen protocol of New England Nuclear. Fifteen micrograms of total RNA were loaded in each lane. cDNAs for PSA, ornithine decarboxylase (ODC) (28), NKX 3.1 (29), fkbp 51, Drg-1 (30) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were labeled with [³²P]dCTP by random priming and used as probes. Hybridization was performed according to Clontech protocols with ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The films were autoradiographed at -70°C.

Western blot analysis

LNCaP cells were plated in 10 cm dishes at 9×10^5 cells/dish in RPMI 1640 (Mediatech, Herndon, VA) and 5% FBS (Biofluids, Rockville, MD). After steroid depletion as mentioned above the cells were treated with 1 nM Mib and varying concentrations of DHA and EPA. Cells were collected at designated times for whole cell protein preparation according to the Santa Cruz Biotechnology protocol. Protein levels were measured by a DC protein assay (Bio-Rad). Fifteen micrograms of protein were loaded into precast 4–12% NuPage gels (Novex, San Diego, CA), run with MOPS buffer and transferred according to the manufacturer's instructions to a nitrocellulose membrane (Bio-Rad). Ponceau S was added to the membranes and they were visualized with a digital camera. The membranes were blocked overnight at 4°C in TBST (20 mM Tris-HCl, pH 8.0, 137 mM NaCl and 0.1% Tween 20) and 5% dry milk. The membranes were washed three times for 10 min each with TBST. Primary antibody for the AR (Pharmingen, San Diego, CA) at a 1:1000 dilution or c-jun (Calbiochem, La Jolla, CA) at a 1:500 dilution were incubated at room temperature for 1 h. The membranes were washed three times for 10 min each with TBST. The appropriate horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ) at a 1:10 000 dilution was also incubated for 1 h at room temperature. The membranes were washed again and Renaissance chemiluminescence (NEN, Boston, MA) was used according to the manufacturer's instructions. β -Tubulin detected by specific β -tubulin antibody (1:1000) (Sigma) was also used as a control for protein loading and transfer efficiency.

Statistics

Results were analyzed by Student's *t*-test. $P < 0.05$ was accepted as the level of significance.

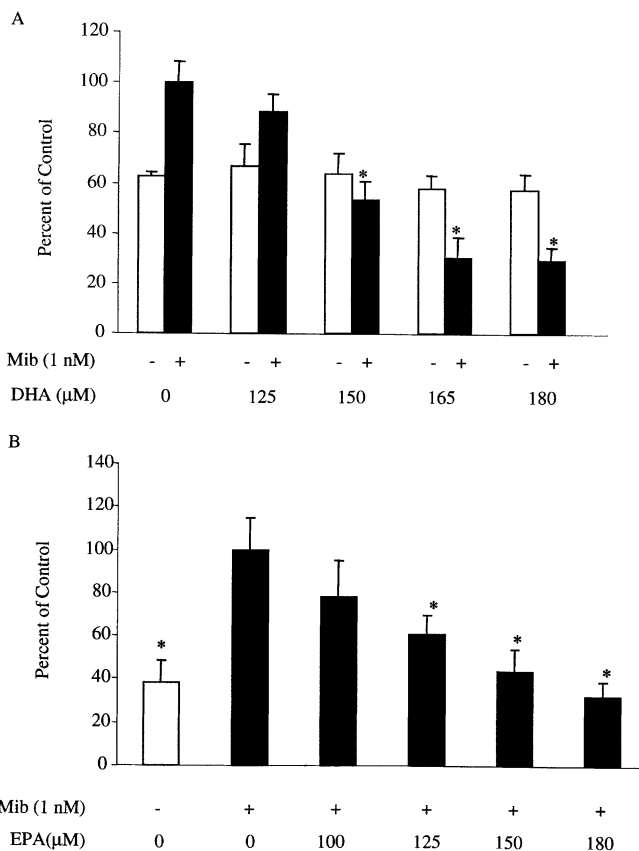


Fig. 1. Effects of DHA and EPA on androgen-stimulated growth responses in LNCaP cells. LNCaP cells were incubated with DHA (A) or EPA (B) as indicated for 6 days with or without Mib followed by a MTS assay performed in quadruplicate (bars indicate standard deviation). *, Significant inhibition compared with the no treatment control in (A) and with Mib treatment in (B).

Results

We examined the effects of DHA and EPA on androgen-stimulated growth in LNCaP cells. DHA-treated cells in the presence Mib showed a dose-dependent decrease in cell growth with a statistically significant P value of <0.05 . However, DHA-treated cells without androgen stimulation showed little response. This suggests that DHA inhibits the androgen-mediated growth response. Interestingly, androgens seemed to further potentiate the inhibitory effect of DHA when compared with that without androgens (Figure 1A; 31). EPA treatment showed a similar effect, decreasing androgen-stimulated cell growth to the same level as cells with no androgen treatment (Figure 1B).

To ascertain that DHA and EPA indeed affect androgen action in LNCaP cells we then examined the effect of these lipids on androgen-regulated expression of a well-known androgen-regulated gene, PSA. The normalized data in Figure 2A showed a dramatic decrease in androgen-stimulated PSA secretion by DHA. Figure 2B, for EPA treatment, demonstrates a similar pattern to Figure 2A, except that EPA treatment required higher concentrations for significant inhibition. Similar results were seen for another androgen-regulated protein, hK2 (data not shown).

Northern analysis was performed to see whether different androgen-regulated genes are affected by DHA treatment. The androgen-responsive genes NKX 3.1, ODC, the immunophilin fkbp 51, Drg-1 and PSA were all tested to ascertain the effects

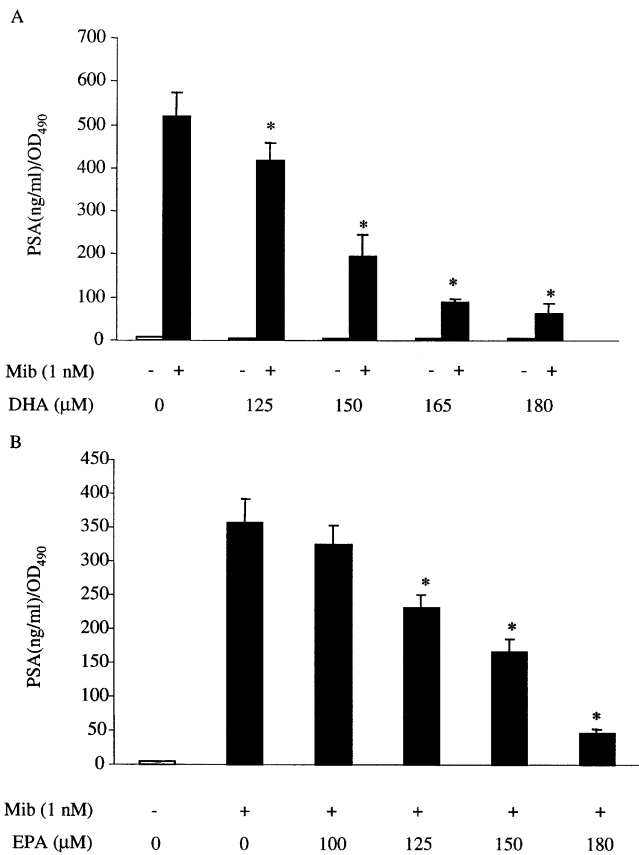


Fig. 2. Androgen-induced expression of PSA protein is affected by DHA and EPA in LNCaP Cells. Total PSA quantification was performed on the spent medium from cells treated with DHA (A) or EPA (B). These protein levels were normalized to the MTS measurements shown in Figure 1. *, Significant inhibition compared with the no treatment controls.

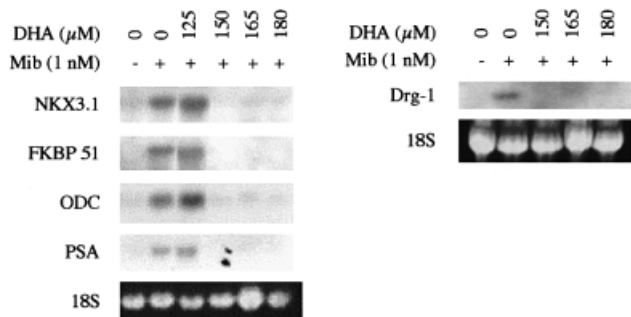


Fig. 3. Effects of DHA on androgen-induced expression of androgen-regulated genes. Northern blot analysis of PSA, ODC, NKX 3.1, fkbp 51, Drg-1 and GAPDH mRNAs in LNCaP cells treated with DHA and Mib for 24 h. 18S rRNA is shown as a loading control.

of the ω-3 fatty acids. Figure 3 shows that all mRNAs probed were up-regulated by androgens and that treatment with DHA at 150 μM or higher concentrations greatly inhibited the induced response.

Given that all the androgen-inducible genes tested were inhibited at the mRNA level, the next step was to determine if DHA and EPA had any effect on AR-mediated transactivation of androgen-regulated genes. Therefore, a construct containing three copies of an androgen-responsive element (ARE) in front of a luciferase reporter gene and an empty vector were transfected into LNCaP cells with or without Mib for 24 h to test whether DHA or EPA can directly affect AR-mediated

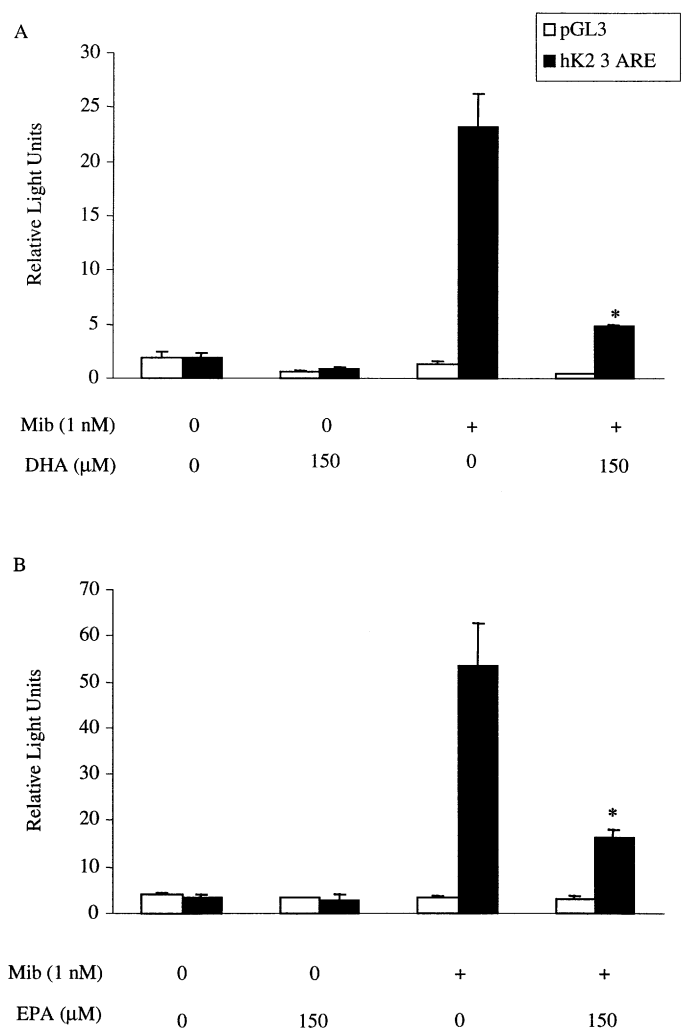


Fig. 4. Effects of DHA and EPA on AR-mediated transcription of a heterologous reporter gene. LNCaP cells were transiently transfected with pGL3 SV40 or pGL3 SV40-3 ARE. After 24 h cells were treated with DHA (A) or EPA (B) in the presence or absence of Mib. Cell extracts were prepared for luciferase and β-gal assays. Luciferase activities were normalized to β-gal activities and are presented as relative light units/mU β-gal. Transfection was repeated three times (bars indicate standard deviation). *, Significant inhibition compared with the no treatment controls.

transcriptional activity. Figure 4 shows the activity of cells treated with or without Mib. In Mib-treated cells the ARE gives a strong androgenic induction of luciferase activity. However, DHA and EPA treatments inhibited this androgenic response ($P < 0.05$). This assay strongly suggests that these lipids can affect transcriptional function of the AR.

Furthermore, western blot analysis was performed to determine whether the lipids have an effect on expression of AR protein. As shown in Figure 5, AR protein levels were increased by androgens, which could not be changed by DHA treatment for 24 or 36 h. Similarly, EPA did not alter AR expression.

In order to ascertain what is inhibiting AR function the levels of c-jun and c-fos were checked. Figure 6 shows that the level of c-jun increased with DHA treatment up to 3.5 times the control levels. The graph depicts the normalized data. This experiment was repeated twice and representative data are shown. However, the levels of c-fos were unaltered by DHA treatment (data not shown).

Previous studies by us and others showed that stimulated

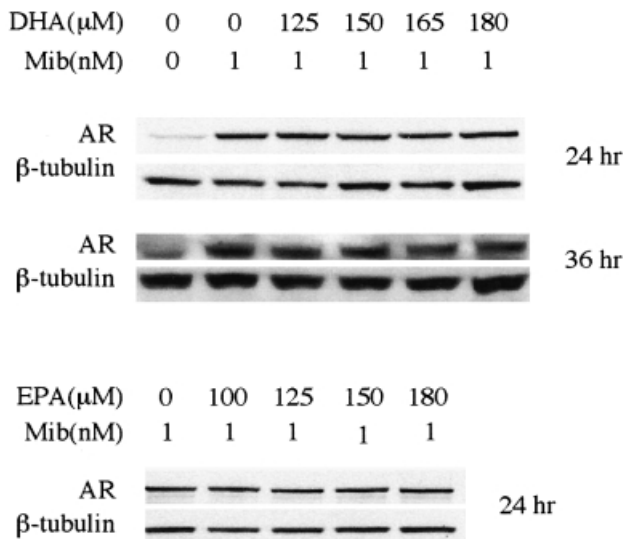


Fig. 5. Effects of DHA and EPA on expression of AR protein. A representative western blot analysis of the AR in LNCaP cells treated with varying amounts of DHA or EPA for the indicated times is shown. β -Tubulin was used as an internal control.

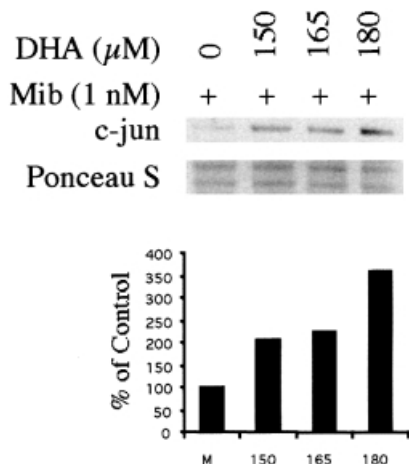


Fig. 6. Effect of DHA treatment on expression of c-jun protein. A representative western blot analysis showing c-jun protein levels in LNCaP cells treated with varying amounts of DHA and EPA for 24 h. Ponceau S staining is included as a loading and transfer control. The graph depicts protein levels normalized to Ponceau S staining as a percentage of the control.

overexpression of c-jun protein can inhibit function of the AR. Transient transfection was performed to determine the effects of c-jun expression on AR function. The hK2 3 ARE construct was transfected into PC-3 cells lacking the AR along with an AR expression vector and a c-jun expression vector (26,32–34). Figure 7 demonstrates that increasing amounts of c-jun cause decreases in androgen-induced activities of the ARE.

Discussion

Latent, non-infiltrating prostate cancer prevalence throughout the world varies little, however, mortality rates from prostate cancer differ greatly among industrialized countries (35). Asian men have a much lower mortality rate from prostate cancer than do American men. However, the rate increases when Asian men emigrate to the USA (36). The epidemiology suggests that dietary factors are the main cause of this increased risk of prostate cancer in the west (37). One aspect

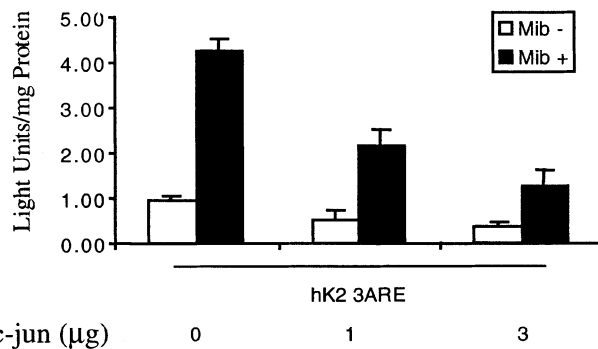


Fig. 7. Effects of c-jun on transcriptional activity of the AR. A transient transfection was performed using the hK2 3 \times ARE construct in PC-3 cells co-transfected with an AR and c-jun expression vector. The various concentrations of c-jun are indicated. The cells were treated with or without Mib for 24 h then cell extracts were prepared for luciferase assay and total protein was measured. The open bars represent no Mib treatment and the closed bars denote Mib treatment. The results are shown as light units/mg protein. The experiment was performed in triplicate (bars indicate standard deviation).

of the diet that differs greatly between Asia and the USA is the consumption of fish oil enriched in long-chain ω -3 fatty acids. Laboratory and epidemiological studies suggest that these ω -3 fatty acids may be useful for cancer prevention.

Previous studies have shown that ω -3 fatty acids inhibit prostate cancer cell growth *in vitro*, however, the mechanism of inhibition was not completely elucidated (7,8). Our data show for the first time that the ω -3 fatty acid DHA decreases androgen-stimulated LNCaP cell growth. Furthermore, androgenic induction of five androgen-regulated genes was significantly repressed by DHA at steady-state mRNA levels. Gene transfer experiments demonstrated that DHA repressed androgenic up-regulation of at least the PSA and hK2 genes at the transcriptional level. Similarly, EPA was able to reduce both the translational and transcriptional levels of these two androgen-regulated genes. The above results strongly suggest that EPA and especially DHA treatment inhibit androgen action, including the cell growth response.

One observation in our study requires further discussion. Northern blots showed that 125 μ M DHA had no inhibitory effects on mRNA levels of the genes tested, including PSA, after 24 h treatment. One possibility as to why PSA and hK2 protein levels but not their mRNA levels were decreased by 125 μ M DHA is that these cells were incubated for 6 days as opposed to 24 h. Therefore, it is possible that the extended treatment time is responsible for the concentration change. In addition, expression or secretion of both PSA and hK2 protein may potentially be more sensitive to DHA treatment than that of their mRNAs. Further study will be required to clarify the mechanism for the above concentration effect.

Our results certainly demonstrate that the ω -3 fatty acids exhibit repressive effects on androgenic induction of gene expression. Both PSA and ODC are well-known genes as direct targets of the AR (24,38). The lipids inhibit expression of the prostate-specific genes PSA and NKX 3.1 (29) and the ubiquitous genes ODC (28), fkbp 51 (39) and Drg-1 (30). Fkpb 51 has for the first time found to be up-regulated by androgens in this laboratory (submitted for publication). Together with the transient transfection study, the data suggest that the ω -3 fatty acids can impair the transactivation ability of the AR.

The inhibition of expression of ODC could explain, in part,

the decrease in cell growth. Polyamines are known to be involved in the processes of proliferation and differentiation of normal and neoplastic cells (38). ODC is the key enzyme in the biosynthetic pathway of polyamines (40). Forced over-expression of ODC can induce transformation of mammalian cells (41). It has been suggested that overexpression of ODC may be involved in the oncogenic process. Inhibitors of ODC have been used in cancer chemoprevention (42). Therefore, it is possible that repression of expression of ODC may be involved in the growth inhibitory effect of DHA in LNCaP cells.

The function of nuclear receptors like the AR can be affected by expression level (43), phosphorylation (44), dimerization (45), nuclear localization (46), ligand binding, interaction capability with various proteins such as heat shock proteins (e.g. hsp70 and hsp90), co-activators and other cross-talking factors (47,48). The ω -3 fatty acids do not seem to be able to reduce AR protein levels, although AR function is inhibited. Androgens can stabilize the AR and hence increase AR levels (49). Western blot analysis of the AR shows that the lipids do not interfere with the androgen-mediated stabilizing effect. In addition, we have examined the effects of the ω -3 fatty acids on the levels of hsp70 and hsp90 as well as the AR-specific co-activator ARA70 and did not find any inhibitory effects (data not shown).

Since AR protein levels were unaffected by DHA and EPA treatment and AR function was clearly affected, the levels of c-jun and c-fos were examined. The AP-1 transcription factors are composed of c-fos and/or c-jun nuclear proteins and can act as cross-talking factors for the AR. There is increased evidence that AR function could be affected by interaction with AP-1 proteins (26,32–34) in prostate cells. Previous studies have shown that c-jun alone could inhibit the formation of AR–ARE complexes (34). Our study found that c-jun expression is increased by DHA treatment and transcriptional activity of the AR is decreased with increasing c-jun expression. The gene transfer experiments performed in this study further confirm that inhibition of AR function is the result of increased levels of c-jun induced by DHA.

In this study we have shown a novel aspect of DHA and EPA in that they can attenuate AR-mediated action in androgen-responsive prostate cells. We show that inhibition of LNCaP cell growth is the result of a reduction in androgen action, possibly via impairment of AR function due to increased c-jun protein. Thus this study provides an additional mechanism for the anti-prostate cancer activities of the lipids. These lipids may have the potential to become chemopreventive and/or chemotherapeutic agents for prostate cancer.

Acknowledgements

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