



Short communication

Omega-3 fatty acids decrease protein kinase expression in human breast cancer cells

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Summary

We report that 5-day exposure to physiological concentrations of eicosapentaenoic and docosahexaenoic acids resulted in a strong decrease in expression of the RI α regulatory subunit of protein kinase A and the PKC- α isozyme of protein kinase C in the human breast cancer cell line MDA-MB-231.

Introduction

Previous studies using rat mammary carcinogenesis and nude mouse models demonstrated that dietary omega-6 polyunsaturated fatty acids (PUFAs) promote, and omega-3 PUFAs inhibit, mammary carcinogenesis [1]. The mechanisms by which omega-3 PUFAs suppress growth and metastasis are not known with certainty, but they may act to modulate second messenger systems involved in cell signaling. Protein kinases A and C (PKA and PKC) are critical intracellular components of two second messenger systems activated by the binding of extracellular ligands to cell-surface receptors.

In mammalian cells, PKA exists as two isoforms, type I and type II, distinguishable by their different R subunits, R-I and R-II [2]. PKA-I and its regulatory subunit RI α are generally overexpressed in human cancer cell lines and primary tumors, and their overexpression has been correlated with poor prognosis in breast cancer patients [3–5]. Several studies have shown that use of an RI α antisense oligodeoxynucleotide inhibited expression of RI α and induced apoptosis/differentiation in a variety of human breast cancer cell lines [6–9].

PKC is activated by free fatty acids, both *in vitro* and *in vivo* [10–12]. Recent studies using antisense oligonucleotides targeted against PKC- α reported that these constructs possess antitumor activity in patients

with ovarian cancer, and inhibit the growth of breast, bladder and lung cancer cell lines [13]. Thus, antisense inhibition of either PKA RI α or PKC- α is useful in arresting cancer cell growth. Phase 1 clinical trials testing antisense oligonucleotides against both PKA RI α and PKC- α are currently being conducted [reviewed in 14]. We evaluated the influence of PUFAs on expression of PKA RI α and PKC- α in a human breast cancer cell line.

Materials and methods

The PUFAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or linoleic acid (LA) (all from Sigma, St. Louis, MO) were prepared as stock solutions containing 0.1% butylated hydroxytoluene in an ethanol vehicle and stored at -20°C . The vehicle was added at the same concentration to controls, and the final vehicle concentration was always $< 0.2\%$. The human breast carcinoma cell line MDA-MB-231 was cultured for 5 days (with replacement of media after 3 days) in Iscove's modified Dulbecco's medium supplemented with fatty acids, 1% FBS, antibiotics, and 1.25 mg/ml delipidized bovine serum albumin (to prevent cytotoxicity of fatty acids) (Collaborative Biomedical Products, Bedford, MA). The human breast cancer cell lines ZR75-1 and MCF-7 were cultured in media recommended by the American Type Culture

Collection (Manassas, VA), with fatty acid and delipidized bovine serum albumin additions as described above for MDA-MB-231 cells.

PKA activity was measured using a colorimetric assay kit containing a fluorescent dye-labeled peptide substrate (Kemptide), according to the manufacturer's instructions (Pierce Chemical Co. Rockford, IL). For immunoblot assays, cells treated with fatty acids were washed and lysed. Equal amounts of whole cell lysate protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore Corp., Bedford, MA), blocked and incubated with antibodies against the PKA RI α , PKA RI β , PKA RII α or PKA RII β subunits, or the PKC- α isoenzyme (Transduction Laboratories, Lexington, KY). Immunoreactive bands were detected by chemiluminescence using the Immune-Star detection kit (Bio-Rad Laboratories, Hercules, CA).

Results

Treatment of cells with 5.0 μ g/ml EPA or LA over a period of 5 days resulted in a decrease in PKA activity when compared to untreated controls ($P < 0.05$) (Table 1). This effect of PUFAs on PKA activity is similar to that observed in other studies where PUFAs were found to alter PKA activity [15, 16].

PKA RI α subunit and PKC- α expression, as measured by immunoblotting and scanning densitometry, was lower in cells treated with omega-3 PUFAs (EPA and DHA at 5 μ g/ml) (Figure 1). The effect of ω -3 PUFAs on inhibition of PKA RI α expression was particularly strong. Inhibition resulting from EPA treatment was greater than from DHA treatment for both PKA RI α and PKC- α . PKA RI α expression was decreased in response to LA (an omega-6 polyunsaturated fatty acid), but expression of this subunit was inhibited to a much greater extent by omega-3 fatty acids. Expression of PKC- α was not significantly affected in cells treated with linoleic acid.

The influence of PUFAs on expression of other regulatory subunits of PKA was investigated. None of the fatty acids tested at the levels listed in Figure 1 had an influence on the expression of PKA RII α or PKA RII β in MDA-MB-231 cells. However, expression of PKA RI β was decreased when MDA-MB-231 cells were grown 5 μ g/ml of EPA or DHA, and inhibition was especially strong in the presence of 5 μ g/ml of LA (representative gel shown in Fig-

Table 1. cAMP-dependent protein kinase activity of MDA-MB-231 cells treated with polyunsaturated fatty acids*

Treatment**	PKA Activity***
Control****	27.6 \pm 0.62
EPA	23.7 \pm 0.63*****
LA	24.7 \pm 1.51*****

*MDA-MB-231 cells were treated with or without 5 μ g/ml PUFAs for 5 days. Cell lysates were assayed for PKA activity by incubating with the peptide substrate, Kemptide, which was labeled with a fluorescent dye. The reaction mixture was then applied to an affinity membrane and the phosphorylated product was eluted from the membrane with elution buffer and assayed spectrophotometrically by measuring its absorbance at 570 nm. One unit of PKA activity will transfer 1 pmole of phosphate to dephosphorylated substrate per minute per mg of kinase. EPA = eicosapentaenoic acid; LA = linoleic acid. Values represent the mean \pm SEM of three different experiments. ** $n = 6$ for each treatment.

***Units/mg protein.

****No fatty acid added.

*****Statistically significant differences between control and fatty acid-treated cells [$p < 0.05$]. Statistical significance was determined using one-way analysis of variance, followed by a least significant difference multiple comparison test.

ure 2a). Expression of the four regulatory subunits of PKA was not affected by 5 μ g/ml of EPA, DHA or LA in the estrogen-dependent cell lines ZR75-1 and MCF-7, with the exception of the PKA RII α subunit, whose expression was down-regulated by 5 μ g/ml of EPA, DHA or LA in MCF-7 cells (Figure 2b). These results suggest that estrogen receptor status of breast cancer cells may influence their susceptibility to polyunsaturated fatty acid modulation of PKA RI α subunit expression.

Discussion

The present study demonstrates that EPA, an omega-3 fatty acid, as well as LA, an omega-6 fatty acid, decrease the PKA activity in MDA-MB-231 human breast cancer cells. These results indicate a non-specific inhibitory effect of PUFAs on PKA activity, an effect also seen in brush border membranes isolated from human placental vesicles [17]. Our finding that protein kinase activity was only moderately decreased by incubation with EPA was not unexpected, since Cho-Chung et al. [18] reported that PKA RI α and RII β antisense constructs each inhibited their respective target mRNAs, and that targeting of either subunit of R

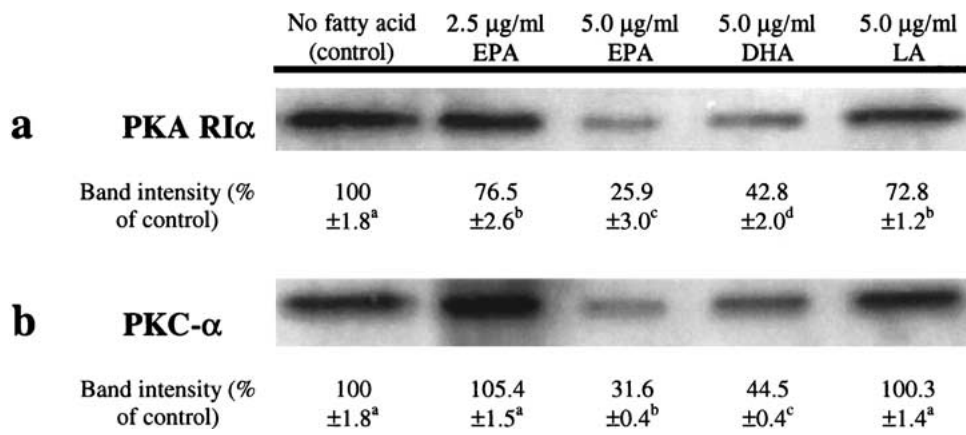


Figure 1. Expression of protein kinase A RI α and protein kinase C- α in human breast cancer cells. MDA-MB-231 human breast cancer cells were grown for 5 days in media containing the indicated concentrations of PUFAs. Cell extracts were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions, and separated proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore Corp., Bedford, MA). Membranes were incubated with antibodies against the protein kinase A RI α subunit (a) or the protein kinase C- α isoenzyme (b). Immunoreactive bands were detected by chemiluminescence using the Immune-Star goat anti-mouse IgG detection kit (Bio-Rad Laboratories, Hercules, CA). The bands were scanned with a densitometer, and band intensities are expressed as a percentage of intensities of control cells not treated with PUFAs. Values of band intensity are means \pm SEM. Values having different superscript letters are different ($p < 0.05$). Abbreviations: PKA RI α : protein kinase A RI α subunit; PKC- α : protein kinase C- α isoenzyme; EPA: eicosapentaenoic acid; DHA, docosahexaenoic acid; LA: linoleic acid.

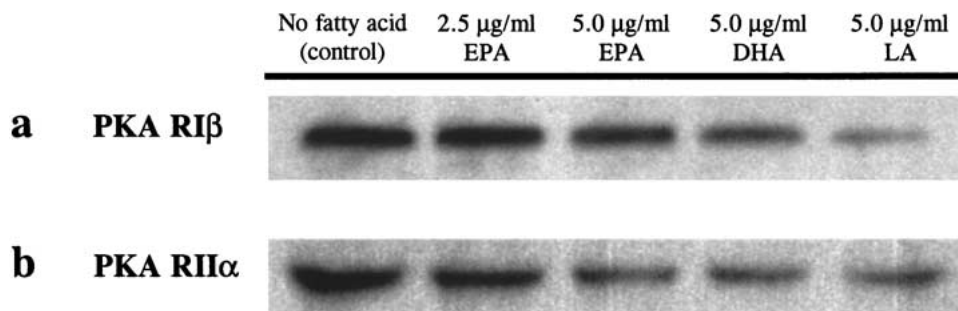


Figure 2. Expression of protein kinase A RI β and protein kinase A RII α in human breast cancer cells. MDA-MB-231 (a) or MCF-7 (b) human breast cancer cells were grown for 5 days in media containing the indicated concentrations of PUFAs. Cell extracts were immunoblotted as described in the legend of Figure 1 with antibodies against the protein kinase A RI β (a) or RII α (b) subunits. Immunoreactive bands were detected as described in the legend of Figure 1. Abbreviations: PKA RI β : protein kinase A RI β subunit; PKA RII α : protein kinase A RII α ; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid.

resulted in compensatory enhancement of expression of the other R subunit.

Our finding that omega-3 PUFAs strongly inhibit the expression of the RI α subunit of PKA and the α isozyme of PKC in MDA-MB-231 breast cancer cells suggests that inhibition of these proteins may be one mechanism whereby omega-3 PUFAs inhibit the growth and metastasis of estrogen receptor-negative cancer cells. Our results indicate that naturally occurring and relatively nontoxic omega-3 fatty acids could be used to achieve approximately the same degree of inhibition of PKA RI α and PKC- α in breast

cancer cells as was obtained with antisense constructs [19, 20].

The concentrations of PUFAs chosen for use in this study were selected because previous reports indicated that plasma concentrations of nonesterified EPA in subjects consuming a diet enriched in fish oil could reach 5–10 µg/ml [21], and that the LA concentration in plasma free fatty acids increased from 13.7 to 69.3 µg/ml after feeding a single meal of safflower oil to humans [22]. We used a maximum concentration of 5 µg/ml of PUFA in order to ensure that concentrations did not exceed maximum levels found in humans

consuming these PUFAs. The concentration of EPA necessary to achieve downregulation of PKC- α was 5 μ g/ml, which is a level that would probably be achieved in practice only by consumption of relatively large amounts of an omega-3 fatty acid supplement as a therapeutic agent. EPA enhances susceptibility to oxidation, alters membrane transport and receptor binding, and modifies eicosanoid production in cells [23], and thus might be toxic to the cancer patient. However, a recent clinical study of tolerability and dose-limiting toxicity of fish oil fatty acid capsules in patients with cancer cachexia found that a 70-kg patient can tolerate up to 21 capsules/day containing 13.1 g of EPA plus DHA [24]. These results imply that therapeutic doses of EPA and DHA may be achievable in cancer patients with only minor side effects.

The significance of our observation that PUFAs alter expression of PKA RI β in MDA-MB-231 cells and PKA RII α in MCF-7 cells is unclear, since the involvement of these subunits in the etiology of breast cancer has not been investigated in detail in previous studies. However, our results support the concept that individual PUFAs have specific effects on expression of PKA regulatory subunits in breast cancer cell lines.

It would be of interest in future studies to determine whether inhibition of protein kinase subunit or isozyme expression with omega-3 PUFAs modulates activities downstream from PKA and PKC in a manner similar to what was found using antisense constructs. For example, the finding that antisense inhibition of PKA RI α affected both serine/threonine kinase and tyrosine kinase pathways in ovarian cancer cells [9] suggests that these pathways might also be altered by omega-3 inhibition of RI α in breast cancer cells. Additionally, these fatty acids could be used in combination with chemopreventive agents that target PKA and PKC dependent and independent pathways.

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