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Inhibition of constitutive Akt (PKB) phosphorylation by docosahexaenoic acid in the human breast cancer cell line MDA-MB-453

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ABSTRACT

Many breast cancer cells express aberrantly activated receptor tyrosine kinases and are associated with deregulated phosphorylation of Akt (PKB). They are also often associated with a high level of free monounsaturated (MUFA) and saturated (SFA) fatty acids. We studied the effect of DHA and other polyunsaturated fatty acids (PUFAs) on these anomalies in a human breast cancer cell line, MDA-MB-453. Inhibitors of the Akt T308 kinase (PDK1) or S473 kinase (mTORC2, DNA-dependent protein kinase and integrin-linked kinase) and combinations of two of them incompletely inhibited, or even enhanced, the phosphorylation in this cell line. In contrast, it was found that DHA as well as other PUFAs inhibited Akt phosphorylation on T308 after 24 h. These PUFAs also blocked phosphorylation of S473, although certain omega-6 PUFAs were ineffective. After 48 h, only DHA inhibited Akt phosphorylation on the both residues. DHA, and other PUFAs though less efficiently, also elevated the expression of a mitochondrial enzyme, 2,4-dienoyl-CoA reductase, which catalyzes process necessary for β-oxidation of PUFAs. These PUFAs were present in the cells at high concentrations and reduced the amount of free and phospholipid-bound MUFAs. DHA most efficiently blocked deregulated cell proliferation while the effects of other PUFAs were moderate. These results suggest that DHA suppressed the growth of the cancer cell through its specifically persistent block of Akt phosphorylation in conjunction with modulation of fatty acid metabolism.

1. Introduction

Many breast cancer cells are dependent on aberrant signaling through the PI3-k/Akt pathway for deregulated growth and survival [1–6]. This anomaly is often due to the constitutively active receptor tyrosine kinases (RTKs) such as ErbBs, FGFR, or IGFR [7–9]. Activation of PI3-k by these receptors leads to generation of PIP_3 that can recruit Akt to the membrane where it is phosphorylated on T308 by PDK1. RTK signaling also activates mTOR complex 2 (mTORC2), which fully activates Akt-pT308 by phosphorylation on S473 [1,3,5,6]. Akt S473 also can be phosphorylated by non-canonical kinases, e.g., DNA-dependent protein kinase (DNA-PK) and integrin-linked protein kinase (ILK), and mutation of these kinases induces cancer phenotypes [10,11]. The regulation mechanism of this pathway further contains a negative feedback loop, in which mTOR complex 1 (mTORC1) downregulates the PI3-k adaptor insulin receptor substrate 1 (IRS1) through phosphorylation of Grb10 [12,13]. Thus, inhibition of mTOR inhibits phosphorylation on S473 but enhances that on T308 [14,15]. Although inhibition of PI3-k/Akt pathway as well as upstream RTKs is often considered in therapeutics, this is often only partially effective because of the multiple mutations in these and other multilayered controls.

Polyunsaturated fatty acids (PUFAs), in particular docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$), reportedly play beneficial roles against progression of breast cancer [16–18]. Tested on this and various other cancers, cell culture studies have shown that DHA contributes to reduction of growth rate and/or induction of apoptosis. DHA has been suggested to affect gene expression such that controlled by PPARγ and is also proposed to change membrane functions that are linked to cell growth/survival. It induces ER stress [19] and also disturbs distribution of ligand-activated EGFR and Ras in lipid rafts [20–22]. Involving peroxidation reaction, DHA also impairs integrity of organelles such as mitochondria [17,18]. While DHA thus affects functions that are also necessary for normal cells, it remains to be determined whether DHA could affect cancer-specific chronic RTK activation or aberrant Akt phosphorylation. It is also not clear whether DHA could affect cancer-specific energy metabolism, e.g., aerobic glycolysis [23] and association of high levels of free saturated and monounsaturated fatty acids (SFA and MUFA, respectively).

Abbreviations: PUFA, polyunsaturated fatty acid; RTK, receptor tyrosine kinase; FGFR, fibroblast growth factor receptor; IGFR, insulin-like growth factor receptor; PDK1, phosphoinositide-dependent kinase 1; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; DNA-PK, DNA-dependent protein kinase; ILK, integrin-linked kinase; FFA, free fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; VE, vitamin E; PLD, phospholipase D; DAG, diacylglycerol

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In this study, we addressed the effect of DHA on the human breast cancer cell line MDA-MB-453. This cell line overexpresses ErbB-2 by transactivation of the gene [24,25]. It also expresses a constitutively active FGFR4 dimer due to a missense mutation [26]. It is also mutated in p53 and other tumor suppressor genes [27,28]. We observed that the cells expressed constitutively phosphorylated Akt and Erk1/2. Free SFAs and MUFAs were also accumulated. We found that DHA as well as many other PUFAs suppressed the phosphorylation of Akt in this cell line. They were accumulated in free and phospholipid-bound forms and modified fatty acid metabolism. They lead to a reduction in the amount of MUFAs, among other effects. Although PUFAs affected various cancer phenotypes, only DHA could inhibit the phosphorylation of Akt for 48 h after treatment. These distinct effects may correlate to the uniquely efficient reduction of growth of this cell line by DHA.

2. Materials and methods

2.1. Fatty acids and other reagents

Free fatty acids (FFAs), 14:0, 16:0, $16:1^{\Delta 7}$, $18:1^{\Delta 9}$, $18:2^{\Delta 9,12}$, $18:3^{\Delta 6,9,12}$ and $20:4^{\Delta 5,8,11,14}$ (Wako Pure Chemicals, Osaka, Japan), $18:3^{\Delta 9,12,15}$ and $22:6^{\Delta 4,7,10,13,16,19}$ (Cayman Chemicals, Ann Arbor, MI), $20:5^{\Delta 5,8,11,14,17}$, $22:4^{\Delta 7,10,13,16}$, $22:5^{\Delta 4,7,10,13,16}$ and $22:5^{\Delta 7,10,13,16,19}$ (Nu-Chek Prep, Elysian, MN) and $22:3^{\Delta 13,16,19}$ (Enzo Life Sciences, Plymouth Meeting, PA) were purchased from indicated distributors. All the double bonds are in *cis* configuration. They were dissolved in ethanol and stored at −80 °C. BX-912 and NU7441 (Axon Medchem, Groningen, The Netherland), Ku-0063794 (Chemdea, Ridgewood, NJ), QLT0267 (Valocor Therapeutics, Vancouver, BC) and Akt inhibitor VIII (Wako) were purchased from indicated distributors, respectively. Other reagents not specified were obtained from Wako.

2.2. Cell culture, treatment with fatty acids and growth determination

Human breast cancer cell line MDA-MB-453 (ATCC; HTB-131) was maintained in DMEM (high glucose) containing 10% FBS and antibiotics in 5% CO_2 at 37 °C. Before experiments, cells were precultured with 20 μM vitamin E (VE) for 24 h. Ethanol solutions of free fatty acids (FFAs) were dried under N_2 and suspended in the complete medium (0.5 ml) by extensive vortexing. They were applied to the cell culture for up to 72 h. Cells were collected by trypsin treatment before and after the incubation. Live cell numbers were determined by using trypan blue.

2.3. Immunoblotting analysis

Cells ($0.5–1\times10^6$/6 cm dish) treated with PUFAs were scraped in ice-cold TBS (10 mM Tris–HCl, 150 mM NaCl pH7.4) containing 1 mM NaF and 100 μM Na_3VO_4. After centrifugation, resuspended cells were aliquoted and stored frozen in liquid N_2. The same protein amounts (8 μg except for 1 μg for probing β-actin) were repeatedly probed for specific proteins after separation by SDS-PAGE in the presence of 2-mercaptoethanol. Blots on PVDF sheets (immobilon-P, Millipore, Billerica, MA) were blocked with 5% defatted milk in TBS containing 0.1% Tween 20, NaF and Na_3VO_4. The following antibodies were used: anti-Akt1/2/3 (rabbit polyclonal), anti-phospho Akt (p-Akt, p-T308, 244 F9 rabbit monoclonal), anti-p-PDK1 (p-S241, C49H2 rabbit monoclonal), anti-p-P38 MAPK (p-T180/p-Y182, rabbit polyclonal), anti-p-Erk5 (p-T218/p-Y220, rabbit polyclonal) (Cell Signaling, Danvers, MA), anti-p-Akt1/2/3 (p-S473) (rabbit polyclonal), anti-p-Erk1/2 (p-T202/p-Y204, 22A, mouse monoclonal), anti-PTEN (A2B1, mouse monoclonal), anti-β-actin (AC-15, mouse monoclonal), anti-2,4-dienoyl-CoA reductase (30-P, mouse monoclonal) (Santa Cruz, Santa Cruz, CA), anti-Erk1 (mouse monoclonal, BD Biosciences, Franklin Lakes, NJ) and anti-rabbit IgG HRP (Donkey) and anti-mouse IgG HRP (Goat) (Promega, Madison, WI). ECL system (GE healthcare, Buckinghamshire, UK) or Immunostar LD (Wako) was used for detection. Results were recorded as 16-bit grayscale images by using LAS3000 image analyzer (Fuji, Tokyo, Japan). Densitometric analysis was done on 16-bit images by using ImageJ software.

2.4. Analysis of fatty acids

Cells (3×10^6/6 cm dish) incubated with PUFAs (500 fmol/cell) for 24 or 48 h were scraped, washed and resuspended in 0.5 ml TBS. FFAs were extracted by the published methods using acidic hexane/*tert*-butyl methyl ether (1:1) [29]. The amount of FFAs was determined by conversion of them in acyl-CoA in conjunction with quantitation using acyl-CoA oxidase (using NEFA C-test, Wako). The intrinsic acyl-CoA in tissue cells shares 1/1000 times lower than that of FFAs [30]. The cell number and the protein amount for the cell samples were determined. Portions of the FAs were esterified by using BF_3/CH_3OH at 100 °C for 5 min [31]. The samples were analyzed by GC–MS. AutoSystem XL Gas Chromatograph equipped with a capillary column HR-1 (Shinwa Chemical Industries, Ltd., Kyoto, Japan) and interfaced with TurboMass Mass Spectrometer (Perkin Elmer, Wellesley, MA) were used. The amounts of individual FFAs per cell were determined from the fractional area intensities of GC–MS results.

For analysis of phospholipids, cells were treated with acetone at 4 °C for two times to remove FFAs. They were then treated with $CHCl_3/CH_3OH$/1 N HCl (2:1:0.8) or $CHCl_3/CH_3OH/H_2O$ (2:1:0.8). Phospholipids were recovered in $CHCl_3$ by adding $CHCl_3$/1 N HCl (1:1) or $CHCl_3/H_2O$ (1:1). The $CHCl_3$ phase was taken, evaporated to dryness and stored after re-dissolution in $CHCl_3$ at −80 °C. FAs in the phospholipids were esterified by BF_3/CH_3OH at 100 °C for 15 min.

3. Results

3.1. Akt is constitutively phosphorylated in MDA-MB-453 cells

The human breast cancer cell line MDA-MB-453 shows a mixture of epithelial and mesenchymal-like morphologies. The cells grew rapidly even on non-treated plastic surfaces. Looking downstream of the aberrant receptor tyrosine kinases, i.e., ErbB-2 and FGFR4, western blotting analysis indicated that Akt is constitutively phosphorylated on both T308 and S473 (Fig. 1). Phosphorylation of Akt on both

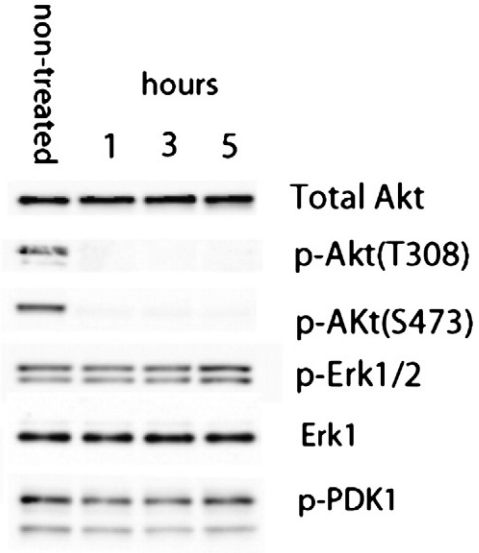

Fig. 1. Constitutive phosphorylation of Akt, Erk1/2, and PDK1 in MDA-MB-453 cells and the effect of Akt inhibitor VIII. Cells were treated with Akt inhibitor VIII (1 μM) for the indicated hours and probed for Akt (total, p-T308 and p-S473), Erk (p-Erk1/2 and Erk1) and p-PDK1 by immunoblotting. A typical result from three independent experiments is shown.