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Omega-3 free fatty acids attenuate insulin-promoted breast cancer cell proliferation

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ABSTRACT

High insulin levels in obese people are considered as a risk factor to induce breast carcinogenesis. And consumption of fish oils which mainly contain omega-3 fatty acids is associated with a reduced risk of breast cancer. However, whether omega-3 free fatty acids (FFAs) modulate insulin signaling pathway to prevent breast cancer is poorly understood. The current study tested the hypothesis that omega-3 FFAs attenuate insulin-induced breast cancer cell proliferation and regulate insulin signaling pathway. We show here that omega-3 FFAs attenuate MCF-7 cell proliferation and Akt and Erk1/2 phosphorylation levels stimulated by insulin. Knockdown Shp2 by siRNA resulted in significantly elevated omega-3 FFAs–activated Akt phosphorylation but failed to change insulin-stimulated Akt and Erk1/2 phosphorylation. And viable cell number was not affected by either downregulation of Shp2 expression or Erk1/2 inhibitor U0126 treatment. These observations indicated that omega-3 FFAs attenuate insulin-promoted breast cancer cell proliferation and insulin-activated Akt phosphorylation.

1. Introduction

High insulin level is significantly associated with an increased risk of breast cancer in women. Insulin acts as a traditional hormone to promote human tumor growth by stimulating cell proliferation, which was considered to enhance the risk of breast cancer [1,2]. Epidemiological data also showed that high serum insulin level in women was positively associated with an increase in frequency of postmenopausal breast cancer [3]. Type 2 diabetic mellitus patients exposed to exogenous insulin were more subjected to cancer [4]. Insulin deficiency was associated with less aggressive cancer proliferation in vivo [5].

Omega-3 polyunsaturated fatty acids consist of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linolenic acid (ALA). Omega-3 free fatty acids (omega-3 FFAs) cannot be synthesized by mammals themselves, so they have to be supplied by diet intake. Meta-analysis of data from 21 independent prospective cohort studies has demonstrated that intake of marine omega-3 FFAs was associated with a lower risk of breast cancer [6]. Omega-3 FFAs, particularly DHA, suppress cell proliferation and promote cell apoptosis [7,8]. Moreover omega-3 FFAs attenuated obesity-related inflammation which was associated with lower risk of breast cancer. Chung et al [9] showed omega-3 FFAs–enriched high-

Abbreviations: ALA, α-linolenic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; TBST, Tris-buffered saline with Tween 20.

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fat diet reduced inflammation in the obese mammary fat pad in the absence of tumor cells and inhibited Py230 tumor growth in vivo. It has also been revealed that omega-3 FFAs could modulate Akt signaling pathway and the expression of cell apoptotic genes BAD, Bcl-2, and caspase-9 to play an anti–breast cancer role [10,11].

Shp2 is a nonreceptor tyrosine phosphatase type 11 (PTPN11) that activates signaling through Ras and Erk pathways, which are involved in mesenchymal to epithelial transition and cell apoptosis in breast cancer cells [12,13]. The expression of Shp2 in breast cancer cells was dramatically higher than that in normal cells, and upregulation of Shp2 significantly increased the tumorigenesis of breast carcinoma [14,15]. Importantly, acting immediately downstream of HER2, Shp2 positively promotes the receptor activation and signal transformation [16]. Ras/Erk and PI3K/Akt also participate in Shp2-mediated signaling pathways [17].

As mentioned earlier, high blood insulin level increased the risk of breast cancer, and omega-3 FFAs reduced breast cancer development. In this study, we hypothesized that omega-3 FFAs modulate insulin signaling pathway to prevent insulin-induced breast cancer cell proliferation. Here, MCF-7 breast cancer cells with or without insulin were stimulated with omega-3 FFAs; MTT and viable cell number counting results showed that omega-3 FFAs promoted breast cancer cell apoptosis and suppressed insulin-induced breast cancer cell proliferation. We further observed the effect of omega-3 FFAs on insulin signaling pathway; immunoblotting results suggested that Erk1/2 phosphorylation was dramatically reduced by high-dose omega-3 FFAs without insulin. The insulin-induced Erk1/2 and Akt phosphorylation was significantly decreased by EPA and DHA addition. However, p38MAPK phosphorylation was similar in cells with combination of insulin and omega-3 FFAs and either treatment alone. Knockdown Shp2 of Erk1/2 and Akt signals upstream by siRNA resulted in elevated Akt phosphorylation by FFAs stimulation, whereas the cell number and Erk1/2 activation remained unchanged with or without insulin treatment. These observations indicated that omega-3 FFAs might modulate Akt signaling though Shp2.

2. Methods and materials

2.1. Cell culture and cell counting

MCF-7 cells were obtained from ATCC and incubated in high-glucose Dulbecco modified Eagle medium (HyClone) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin 100× (Beyotime Biotechnology, China). MCF-7 cells were starved in serum-free medium for 6 hours before treatment. Omega-3 free fatty acids (EPA, DHA, and ALA) were purchased from NU-CHEK PREP INC. USA and dissolved in ethanol. Insulin (Novo Nordisk, Novolin, Isophane Protamine Biosynthetic Human Insulin Injection) was purchased from Wuxi Fifth People Hospital for human injection. MCF-7 cells were treated with 50 μmol/L Erk1/2 inhibitor U0126 (Sigma, St Louis, MO, USA). MCF-7 cells were counted with a cell counting chamber according to manufacturer's introduction.

2.2. Proliferation assay and DAPI staining

MCF-7 cells were cultured in a 96-well plate with 5000 cells/well and incubated with either insulin or FFA. Culture medium was replaced with 200 μL of fresh medium containing 20 μL of MTT solution (2 mg/mL) and incubated for 4 hours at 37°C. Cells were lysed with 200 μL of DMSO, and the absorbance was measured at 490 nm. MCF-7 cells were fixed for 15 minutes with 4% paraformaldehyde and incubated with DAPI solution (300 nmol/L) for 30 minutes. Cell proliferation and cytotoxicity assay kit (MTT) and DAPI staining solution were purchased from Beyotime Biotechnology, China.

2.3. Immunoblotting

MCF-7 cells were lysed with RIPA lysis buffer and briefly sonicated to break chromosomal DNA. Cell lysates were separated on 10% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to polyvinylidene fluoride membranes. Nonspecific binding was blocked by 1-hour incubation of the membranes, at room temperature, in 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; 0.1% Tween 20 in Tris-buffered saline). The blots were then incubated overnight at 4°C with the primary antibodies. After this, the membranes were washed 3 times with TBST and finally incubated for 2 hours at room temperature with goat anti-rabbit horseradish peroxidase–conjugated secondary antibody at 1:2000 dilutions in the buffer containing 5% nonfat dry milk in TBST. The blots were developed for visualization using enhanced chemiluminescence detection kit. The densities of the bands were quantified using a UVP GelDoc-It Imaging Systems.

Akt, p-Akt^{ser473}, Erk1/2, p-Erk1/2, p38, p-p38, and shp2 antibodies (Cell Signaling Technology) at a 1:1000 dilution in the buffer containing 5% nonfat dry milk or 2.5% bovine serum albumin in TBST were used to recognize each corresponding protein, respectively.

2.4. RNA interference

JetPRIME transfection reagent was purchased from Polyplus-transfection Biotechnology Company (**City, State, Country**). Shp2 siRNA was used for transfection assay according to manufacturer's introduction. A total of 100 000 to 150 000 cells were seed per well of 6-well plate with 2 mL of growth medium for 24 hours. Dilute 110 pmol siRNA was diluted into 200 μL of jetPRIME buffer and mixed with 4 μL jetPRIME reagent, vortex for 10 seconds, incubate for 15 minutes at room temperature, then add the transfection mix to the cells, incubate for 24 hours. The siRNA transfection results were detected by Western blot.

Shp2 siRNA forward primer is 5′-ATGACATCGCGGAGAT GGTTT-3′; reverse sequence is 5′-GGGTTACTCTTACTGGGCCTT-3′.

2.5. Statistical analyses

Statistical analyses were performed by independent *t* test or, in case of multiple comparisons, were made by 1-way analysis of variance (ANOVA) using GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA, USA). Data are shown as the means ± SEM. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, $^{*}P < .05$ was