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27-Hydroxycholesterol induces invasion and migration of breast cancer cells by increasing MMP9 and generating EMT through activation of STAT-3

Zhaoxia Shen[a,b], Dongmei Zhu[a,b], Jiao Liu[a,b], Juan Chen[a,b], Yun Liu[a,b], Chunyan Hu[a,b], Zhong Li[a,b,*,1], Yuan Li[a,b,*,1]

[a] Department of Nutrition and Food Hygiene, School of Public Health, Nanjing Medical University, Nanjing 211166, China
[b] The Key Laboratory of Modern Toxicology, Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing 211166, China

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ABSTRACT

Breast carcinoma plays a vital role in the reasons of global women's death. ER-related invasion and migration play an important part in the development and prognosis of breast cancer. Here, we found that 27-Hydroxycholesterol (27HC) could induce epithelial-mesenchymal transition (EMT) and increase the expression of the matrix metalloproteinase 9 (MMP9) at mRNA level and the active form. Meanwhile, interestingly, we found 27HC activated signal transducer and activator of transcription 3 (STAT-3) in ER positive cells except activation of ER signaling. Furthermore, inhibition of STAT-3 by siRNA attenuated the 27HC-induced improvement of MMP9 and decreased the invasion and migration ability in MCF7 and T47D cells. In addition, 27HC could also promote MMP9, vimentin and active STAT-3 in the ER negative cells MDA-MB-231. All these results not only raise a mechanism whereby 27HC enhances the invasion and metastasis, but also is helpful to realize 27HC as a potential endogenous detrimental factor in breast tumor patients.

1. Introduction

Breast cancer is the most universal cancer and a dominating health problem in women worldwide (Ahmad et al., 2012). The vast majority of breast tumors are estrogen receptor (ER) positive, and the occurrence and development of these breast cancers are estrogen-dependent (Losordo and Isner, 2001; Pequeux et al., 2012). Although it is widely recognized that the breast cancers with ER positive profile have good prognosis compared to breast cancers with negative ER profile (partly because the response to endocrine therapy), the ER plays an important role in the invasion and metastasis of breast cancer (Pequeux et al., 2012).

Exogenous estrogens exposed to human include several kinds such asenvironmental estrogen Dichloro-Diphenyl-Trichloroethane (DDT), synthetic estrogen, diethylstilbestrol phytoestrogen daidzein, lignans, coumestans in addition to other sex steroids. The sources of these factors are wide and complex. They are structurally similar to estradiol (E2), and have estrogen-like effects among women (Varinska et al., 2015; Wang et al., 2013). 27-Hydroxycholesterol (27HC) is one of the main oxysterol in the circulation, and its levels can be increased by aging, oxidative stress, and hypercholesterolemia (Hirayama et al., 2009; Nelson et al., 2013). 27HC is first identified as an endogenous selective estrogen receptor modulator (SERM) is metabolized from cholesterol by CYP27A1, and decomposed by CYP7B1 (Umetani and Shaul, 2011). As an endogenous product, function of 27HC is complex. It can induce proliferation in normal cells prostatic epithelial cells (Raza et al., 2016). Besides, 27HC promotes atherosclerosis via proinflammatory processes (Umetani et al., 2014). Recent studies indicate that the exposure of 27HC is associated with the occurrence and development of breast cancer via interacting with ER or liver X receptor (LXR) (Nelson et al., 2013; Wu et al., 2013). In our present research, we found that, 27HC enhanced the invasion and migration in breast cancer cells, during which, the signal

Abbreviations: 27HC, 27-hydroxycholesterol; MMPs, matrix metalloproteinases; E_2, estradiol; ER, estrogen receptor; EMT, epithelial-mesenchymal transition; ZEB1, zinc finger E-box-bindingprotein-1; STAT-3, signal transducer and activator of transcription 3; GREB1, growth regulation by estrogen in breast cancer 1.

* Corresponding authors at: Department of Nutrition and Food Hygiene, School of Public Health, Nanjing Medical University, Nanjing 211166, China.
E-mail addresses: lz-ny@njmu.edu.cn (Z. Li), liyuan@njmu.edu.cn (Y. Li).
[1] Zhaoxia Shen and Dongmei Zhu contributed equally to this work.

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transducer and activator of transcription 3 (STAT-3) was activated, and in turn promoted the expression of matrix metalloproteinase 9 (MMP9) and the epithelial-mesenchymal transition (EMT). All these results not only raised a novel mechanism whereby 27HC enhanced the invasion and metastasis, but also helpful to realize the 27HC as a potential endogenous detrimental factor in breast tumor patients. The reasons were: (1) 27HC, the first identified endogenous SERM is metabolized from cholesterol (Umetani and Shaul, 2011). (2) The influences of 27HC on the development of breast cancer are complex, including promoting the growth interacting with ER (Nelson et al., 2013), and inducing the invasion and migration. (3) Mechanisms involved in the 27HC-promoted progression of breast cancer are complex, such as ER, LXR (Nelson et al., 2013; Wu et al., 2013), STAT-3, and MMP9. Therefore, the potential molecular mechanisms involved in 27HC-promoted progression of breast cancer needed further studied.

2. Materials and methods

2.1. Cell culture and reagents

The ER-positive human breast cancer cell lines, MCF7 and T47D, and ER-negative cell line, MDA-MB-231were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MCF7 and T47D cells were cultured according to the ATCC's recommendations, cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12, 1:1 mixture, Life Technologies/Gibco, Grand Island, NY, USA). MDA-MB-231 cells were cultured in L-15 medium (Gibco). The medium contained 10% fetal bovine serum (FBS; Life Technologies/Gibco), 100 mg/mL streptomycin (Gibco), and 100 U/mL penicillin (Gibco). MCF7 and T47D were maintained in the presence of 5% CO_2 at 37 °C. MDA-MB-231 cells were maintained in a humidified incubator containing no CO_2 at 37 °C. Compared with ER-negative MDA-MB-231 cells, MCF7 and T47D cells have relative lower invasion and metastasis ability (Cao et al., 2016; Yoshimura et al., 2016), to remove the estrogen before experiments, MCF7 or T47D cells were cultured in Minimum Essential Medium MEM (Gibco), supplemented with 5% charcoal stripped FBS (CS-FBS, Biological Industries, Israel), but lacked phenol red for 96 h. The 27-hydroxycholesterol (27HC, purity >99.5%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and dissolved in absolute ethanol, at a stock concentration of 20 mM and stored at −20 °C. All the other reagents used in our present study were of analytical grade or the highest grade available.

2.2. Determination of cell viability

MCF7 and T47D cells were seeded in 96 well plates for 24 h, the cells were treated with 0.0–20.0 μM 27HC for 24 h, 48 h or 72 h, respectively. Then we used a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc, Kumamoto, Japan) assay solution for 4 h, as described previously (Wang et al., 2014).

2.3. Reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA from the cells was isolated with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Primers of MMP9 and STAT-3 used are listed in Supplementary Table A. RNA (2 μg) was transcribed into cDNA using AMV Reverse Transcriptase (Promega, Madison, WI, USA). For RT-PCR, the reactions were determined by analyzing the PCR products on 2% (w/v) agarose gels as we described previously (Ning et al., 2016). The qRT-PCR was performed using the Applied Biosystems 7300 Sequence Detection System (Applied Biosystems) with cycling conditions as follows: 95 °C for 30 s, 95 °C 5 s and 60 °C for 31 s for 40 cycles. Fold changes in expression of each gene were calculated by a comparative threshold cycle (Ct) method with the formula $2^{-(\Delta\Delta Ct)}$.

2.4. Western blotting

The proteins were quantitated using the BCA Kit (Beyotime Biotechnology, Haimen, China). We used SDS-polyacrylamide gels to load and separate equal amounts of proteins, and transferred them onto the nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Then we incubated the membranes with the primary antibody (Supplementary Table B) at 4° C overnight, followed by incubating them with horseradish peroxidase-conjugated secondary antibodies (Beyotime Co. Ltd, the dilution was 1: 1000) for 1 h at the room temperature. The immune complexes were detected by using an enhanced chemiluminescence kit (Cell Signaling Technology). For densitometric analyse, we used the Image-Pro-Plus 6.0 software as described previously (Si et al., 2015).

2.5. Scratch assay

After the removal of estrogen, MCF7 and T47D cells (5×10^5 cells/well) were inoculated in the six well plates, cultured in MEM (with 5% CS-FBS) for 24 h. Then we used a pipette tip to create wound and then washed with PBS for 3 time, followed by replacing to MEM with 0.0–4.0 μM 27HC. Cells were viewed and photographed under a phase-contrast microscope (Olympus) in the same spots at 0 h, 48 h, 72 h as described previously (Zhao et al., 2016).

2.6. Invasion assays

Transwell assays were performed by using growth factor-reduced, matrigel-coated filters and non-matrigel-coated filters (8-mm pore size, BD, Franklin Lakes, NJ) in 24-well plates. After the removal of estrogen, treated MCF7 and T47D cells were trypsinized and seeded onto the upper chamber of the transwells (6×10^4 cells/well) in MEM without CS-FBS. The lower chambers of the transwells were filled with MEM with10% CS-FBS. Treated MDA-MB-231 cells were trypsinized and seeded onto the upper chamber of the transwells (3×10^3 cells/well) in L-15 without FBS. The lower chambers of the transwells were filled with L-15 with 10% FBS. The chambers of ER-positive and ER-negative cells were incubated at 37 °C for 72 h and 48 h, respectively. Matrigel-coated filters were soaked in paraformaldehyde for 30 min and stained with 0.1% crystal violet for 30 min. Then using a cotton swab to remove cells on the upper surface of the filter. Migrated cells were counted with Stat Monitor in photoshop in five randomly chosen fields.

2.7. Gelatin zymography

After the removal of estrogen, MCF7 and T47D cells were cultured in MEM containing 1% CS-FBS with treatments in the presence of 5% CO_2 at 37 °C. Culture supernatants were collected and then were centrifuged. Proteins in the conditioned medium were separated, without prior boiling, by SDS-PAGE (10%) resolving gels containing 1 mg/mL gelatin (Sigma-Aldrich) under non-reducing conditions. Gels were washed three times in 2.5% Triton X-100/50 mM Tris–HCl, pH 7.6 afterelectro-phoresis, and incubated within an incubator at 37 °C in the absence of CO_2 for 1 h, in renaturation solution contained 0.15 M NaCl/10 mM $CaCl_2$/50 mM Tris–HCl, pH 7.6/0.05% NaN_3. The gels were stained with 0.005% Coomassie Blue R250 for 3 h and destained with 10% acetic acid and 10% isopropanol. MMP9 was detected as transparent bands on slab gels.