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Journal of Nutritional Biochemistry 28 (2016) 70–82

Journal of Nutritional Biochemistry

Treatment with kaempferol suppresses breast cancer cell growth caused by estrogen and triclosan in cellular and xenograft breast cancer models

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Received 5 May 2015; received in revised form 25 September 2015; accepted 30 September 2015

Abstract

As a phytoestrogen, kaempferol (Kaem) is one of bioflavonoids, which are found in a variety of vegetables including broccoli, tea and tomato. In this study, the antiproliferative effects of Kaem in triclosn (TCS)-induced cell growth were examined in MCF-7 breast cancer cells. TCS promoted the cell viability of MCF-7 cells via estrogen receptor α (ERα) as did 17β-estradiol (E2), a positive control. On the other hand, Kaem significantly suppressed E2 or TCS-induced cell growth. To elucidate the molecular mechanisms of TCS and Kaem, alterations in the expressions of cell cycle, apoptosis and metastasis-related genes were identified using western blot assay. The treatment of the cells with TCS up-regulated the protein expressions of cyclin D1, cyclin E and cathepsin D, while down-regulated p21 and bax expressions. Kaem reversed TCS-induced gene expressions in an opposite manner. The phosphorylation of IRS-1, AKT, MEK1/2 and ERK was increased by TCS, indicating that TCS induced MCF-7 cell proliferation via nongenomic ER signaling pathway associated with IGF-1R. Kaem presented an antagonistic activity on this signaling by down-regulating the protein expression of pIRS-1, pAkt and pMEK1/2 promoted by E2 or TCS. In an *in vivo* xenografted mouse model, tumor growth was induced by treatment with E2 or TCS, which was identified in the measurement of tumor volume, hematoxylin and eosin staining, bromodeoxyuridine and immunohistochemistry assay. On the contrary, E2 or TCS-induced breast tumor growth was inhibited by co-treatment with Kaem, which is consistent with *in vitro* results. Taken together, these results revealed that Kaem has an anticancer effect against procancer activity of E2 or TCS, a xenoestrogen, in breast cancer and may be suggested as a prominent agent to neutralize breast cancer risk caused by TCS.
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Keywords: Triclosan; Kaempferol; Breast cancer; Estrogen receptor; Metastasis; Apoptosis

1. Introduction

Due to diverse inherited and environmental factors, normal cells transform into cancer cells that can grow excessively and spread to other parts of the body by interrupting normal cellular functions such as DNA repair, cell cycle and apoptosis, leading to lethal diseases [1,2]. Surgical removal, radiation treatment and chemotherapy are main treatments to cure cancers, along with adjuvant therapies including biological gene or hormonal therapies [3]. As natural substances, phytoestrogens that are derived from fruits and vegetables have been proposed for substitute treatments against human diseases for a long time [4,5]. Due to the similar molecular structures between phytoestrogens and endogenous hormones, phytoestrogens may have similar or competitive functions with hormones in human body [6,7]. Especially in estrogen responsive cancers including breast cancer, phytoestrogens have been used as hormone replacement therapy (HRT) in prolonged treatments for its prevention [8,9]. *In vitro* and *in vivo* studies suggest that polyphenolic compounds, flavonoids, may inhibit breast cancer cell growth by competing 17β-estradiol (E2) to estrogen binding sites for estrogen receptor (ER) [10–12]. As interests in beneficial effects of phytoestrogens grow, more detailed underlying mechanism needs to be investigated [13].

Kaempferol (Kaem), which is a phytoestrogen belonging to the flavonoids, is mostly found in fruits and plants such as apple, tomato, broccoli, green tea, angelica decursiva and ginkgo leafs [5,14,15]. Current studies described that Kaem may have an anticancer effect and osterosarcoma [16–20]. There have been also substantial studies to reveal the underlying mechanisms about anticancer effect of Kaem in connection with cell cycle, apoptosis, angiogenesis, inflammation and generation of reactive oxygen species [21]. Furthermore, Kaem has been known to down-regulate several signaling pathways to inhibit cancer development. For instance, Kaem inhibited cancer cell growth and induced cell death in A549 lung cancer cells through

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http://dx.doi.org/10.1016/j.jnutbio.2015.09.027
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mitogen-activated protein kinase (MEK)-MAPK pathway and inhibited the phosphorylation of MAPK pathway and the activation of c-Fos and NFATc in bone marrow cells [22]. Kaem also down-regulated the expression of ERK, JNK and p38 in U-2 OS human osteosarcoma cells [23]. Kaem suppressed PI3K/Akt signaling pathways by directly binding with PI3K and the subsequent inhibition of nuclear factor kappa B (NF-κB) and AP-1 activities, which impact a number of cellular processes, including proliferation, angiogenesis and apoptosis [24].

Triclosan [TCS;5-chloro-2-(2,4-dichloro-phenoxy)-phenol] is a synthetic antimicrobial agent that has been usually found in household and personal care products, such as soaps, deodorants, toothpastes and cosmetics [25]. Concerns about the possible risks of TCS on human health have been arisen since it has been extensively used and detected in liver, human breast milk, urine and blood [26–29]. Recent studies reported that TCS has estrogenic and androgenic activity as an endocrine-disrupting chemical (EDC) [30,31]. Regarding estrogenic activity and endocrine disruption of TCS, an *in vitro* assay demonstrated that TCS can amplify the growth of BG-1 ovary cancer cells by regulating the expressions of cell cycle- and apoptosis-related genes via ER-dependent pathways [32]. Our previous study also showed that two EDCs such as TCS and octylphenol may promote breast cancer progression via an ER-mediated signaling cascade [33]. In addition, current evidence suggested that exposure of TCS has relevance to cancer risk by showing the development of liver tumors by TCS in male and female mice, even though human studies are lacking in number [34,35].

In the present study, we investigated whether TCS may have cancer risk inducing breast cancer proliferation as an EDC as well as Kaem as a natural anticancer compound may have a chemopreventive effect itself as well as on TCS-induced breast cancer progression. As an appropriate breast cancer model, MCF-7 human breast cancer cell line was used in cellular and *in vivo* animal experiments. Since MCF-7 breast cancer cell line is a typical estrogens-responsive cell line expressing ER and its viability and proliferation are influenced by endogenous estrogen as well as xenoestrogens, it is considered to be useful for examining each role of E2, TCS and Kaem and their relationship in breast cancer progression. In addition, this study focused on the elucidation of underlying mechanisms of TCS and Kaem in MCF-7 breast cancer proliferation and metastasis via ER and IGF-1R signaling pathways.

2. Materials and methods

2.1. Reagents and chemicals

E2 and TCS were purchased from Sigma Aldrich, Corp (St. Louis, MO, USA). Kaem was purchased from Abcam, Corp (ab120357, Cambridge, UK Cambridge, UK). ICI 182,780, Propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) were purchased from Tocris, Corp (Ellisville, MO, USA). All chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Junsei Chemical Corp., Tokyo, Japan).

2.2. Cell culture

MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories Corp., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Corp.), 1% of antifungal HEPES (Invitrogen Life Technologies, Corp., Carlsbad, CA, USA) and 1% of penicillin G and streptomycin (Cellgro/Mediatech, Corp., Manassas, VA, USA) at 37°C in a humidified atmosphere of 5% with CO_2-95% air. To prevent the effects of estrogenic components in DMEM and FBS and to measure intrinsic estrogenicity of EDCs, MCF-7 cells were also cultured in phenol red-free DMEM supplemented with 5% charcoal-dextran stripped FBS (CD-FBS). Cells were detached with 0.05% trypsin/0.02% EDTA in Mg^{2+}/Ca^{2+}-free Hank's balanced salt solution (PAA Laboratories, Corp., Pasching, Austria) before the treatment of E2, TCS or Kaem.

2.3. Cell viability assay

To evaluate the effect of E2, TCS or Kaem on the proliferation of MCF-7 cells, MTT assay was performed. MCF-7 cells were seeded at a density of 4000 cells/100 μl in phenol red-free DMEM with 5% CD-FBS medium per well of 96-well plates (SPL Life Sciences, Gyeonggi-do, Republic of Korea). After preincubation in phenol red-free DMEM with 5% CD-FBS medium for 48 h, the cells were treated with various concentrations of TCS or Kaem (TCS: 10^{-6}–10^{-9} M, Kaem: 50–100 μM) in phenol red-free DMEM with 5% CD-FBS supplemented with 0.1% DMSO for 4 days. DMSO as a vehicle was used as a control and E2 as a positive control. Cell viability was detected with the addition of MTT (Sigma Aldrich, Corp.) solution. MTT (10 μl of 5-mg/ml solution) was added to each well, and the plates were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. Supernatants were removed, and 100 μl of DMSO was added to each well to dissolve the resultant formazen crystals. The optical density of each well was measured at 540 nm using an ELISA reader (VERSA man, Molecular Devices, Sunnyvale, CA, USA) and used to calculate the number of viable cells. In addition, to demonstrate the connection between E2 or TCS action and ER signaling pathway, MCF-7 cells were co-treated with E2 or TCS along with ICI 182,780, PPT and DPN for 6 days. The concentrations of ICI 182,780, PPT and DPN were 10^{-9} M. To evaluate the effect of Kaem in TCS or E2-induced breast cancer cell proliferation, Kaem was added at concentrations of 50 μM and 100 μM in the presence of 10^{-6} M of TCS or 10^{-9} M of E2. After treating these reagents, the identical experimental procedure was performed using MTT assay. These experiments were performed in triplicates for statistical analysis.

2.4. Western blot analysis

To measure protein expression of pIRS1, pAkt, pMEK1/2, pERK1/2, cyclin D1, cyclin E, p21, bax and cathepsin D, MCF-7 cells were cultured to a density of 1.0×10^6 cells per of 60-mm dish (SPL Life Sciences, Corp.) and then treated with DMSO, E2, TCS, Kaem, combinations of Kaem and TCS or E2 for a period of time. The concentrations of E2, TCS and Kaem were 10^{-9} M, 10^{-6} M and 50 μM, respectively. After treatment, the cells were suspended in 80 μl of 1×RIPA buffer (50-mM Tris–HCl, pH 8.0; 150-mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS). Total protein concentrations were determined using bicinchoninic acid (Sigma Aldrich, Corp.), and 50 μg of total protein was then separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidenedifluoride membrane (Bio-Rad Laboratories, Corp.), and the membranes were blocked with 5% bovine serum albumin (Sigma Aldrich Corp.) for 1.5 h at room temperature. The membranes were then incubated with rabbit polyclonal anti-IRS1 (1:5,000; Abcam, Corp.), anti-pAkt (1:400; Santa Cruz Biotechnology, Corp., CA, USA), anti-pMEK1/2 (1:2,000; Cell Signaling Technology, Corp., Danvers, MA, USA), anti-pERK1/2 (1:1,000; Cell Signaling Technology, Corp.), anti-cyclin E (1:500; Santa Cruz Biotechnology, Corp.), anti-cathepsin D (1:10,000; Abcam, Corp.) antibodies and mouse monoclonal anti-cyclin D1 (1:500; Abcam, Corp.), anti-p21 (1:2,000; Cell Signaling Technology, Corp.), anti-bax (1:1,000; Antibodies-online, Corp., Atlanta, USA) and anti-GAPDH (1:1,000; Santa Cruz Biotechnology, Corp.) antibodies for overnight at room temperature. Target proteins were detected with a West-Q Chemiluminescent Substrate Plus kit (GenDEPOT, Corp., Barker, TX, USA). These experiments were performed in triplicates for statistical significance.

2.5. Animal experiments

A total of 36 female BALB/c nu/nu mice, 6-week old, were purchased from Orient bio Corp. (Seongnam, Republic of Korea), and the experiment progressed according to the protocols approved by the Animal Care Committee of Chungbuk National University (CBNUA-627-13-01). The room was kept at 24°C under a 12-h light–dark cycle. Mice were acclimated for at least 1 week prior to the experiments. To manufacture animal models xenografted with MCF-7 breast cancer cell line, MCF-7 cells (5×10^6) were mixed with Matrigel (BD Biosciences, Corp., Bedford, MA, USA) at 1:3 volume ratio of Matrigel to PBS in 140 μl, and injected subcutaneously (s.c.) into the middle of the back of the mice. Mice were monitored for tumor growth every week, and the tumor volumes were measured using a vernier caliper and expressed by length×width×high×0.5236 (mm^3). Once tumor sizes reached 55 mm^3, the mice were surgically ovariectomized under anesthesia using avertin (Sigma Aldrich, Corp.). The mice were reinstated for 1 week after surgery, divided into five groups and injected s.c. with corn oil (vehicle, n=5), E2 [n=5; 20-μg/kg body weight (b.w.)], TCS (n=5; 100-mg/kg b.w.), Kaem (n=5; 100-mg/kg b.w.), E2+Kaem (n=5; E2 20-μg/kg b.w.+Kaem 100-mg/kg b.w.) and TCS+Kaem (n=5; TCS 100-mg/kg b.w.+Kaem 100-mg/kg b.w.) two or three times a week for 6 weeks. During the administration period, the tumor volumes were also measured every week. After 6 weeks, the mice were euthanized according to the university protocols. Especially, in case of conducting bromodeoxyuridine (BrdU) incorporation assay, 200-μl BrdU (Sigma Aldrich, Corp.) at 10 mg/ml in PBS was injected ip 2 h before sacrifice. After sacrifice, tumor masses of the mice were collected, fixed in 4% paraformaldehyde and embedded in paraffin for histopathology and immunohistochemistry (IHC) analysis.

2.6. Hematoxylin and eosin (H&E) staining

Paraffin-embedded MCF-7 xenografted tumors were sliced into 4-μm sections using a Leica microtome (Model RM2145). Tissue sections were deparaffinized by immersion in xylene. Prepared slides were stained using H&E (Sigma Aldrich, Corp.) staining method. All sections of tumor masses were investigated under a CKX41 light microscope (Olympus, Japan) for digital photography.

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