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Short Communication

Non-classical estrogen receptors action on human dermal fibroblasts

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Abstract

Objective: To study the possible non-genomic effect of selective estrogen receptor modulators on human dermal fibroblasts (HDF).
Materials and Methods: WS1 cells were used to test the effect of raloxifene. The mRNA expressions of estrogen receptor (ER) α and β and G protein-coupled ER 1(GRP30) were examined by reverse transcription polymerase chain reaction. Apoptosis was identified by TUNEL assay and FACS analysis. MAPK and PI3 K/Akt pathways were determined by immunoblotting analysis.
Results: Neither ERα nor ERβ, but GPR30 was detected in WS1 cells. Raloxifene increased apoptosis, which was blocked by pertussis toxin, an inhibitor of G protein, or by LY294002. Phosphorylated p38 MAPK and Akt were also increased after raloxifene treatment.
Conclusion: SERMs could induce apoptosis of HDF through G protein and PI3 K/Akt signaling, which may help understand the role of SERMs on the skin.

Keywords: Apoptosis; Estrogen; Human dermal fibroblasts; Non-genomic; Selective estrogen receptor modulator

Introduction

A number of studies have shown the important roles of estrogen on the skin, based on the changes that are seen in postmenopausal women [1]. However, despite the knowledge that estrogens have such important effects on skin and estrogen receptors (ERs) have been detected in the skin, the cellular and subcellular sites and mechanisms of estrogen action are still poorly understood [2]. In addition, there is very limited data available addressing the effects of selective estrogen receptor modulators (SERMs) on skin [2]. Due to the potential risk of the use of estrogen after menopausal status, SERMs have been considered as a possible alternative in postmenopausal women [3–6]. Classically, estrogen action is mediated through ER dimers, including ERα and ERβ [7, 8]. However, non-genomic or ER-independent estrogen action is shown. Therefore, it is reasonable to suppose that these molecules, such as SERMs, might act on the skin through a non-genomic effect. An orphan receptor G protein-coupled estrogen receptor 1 (GPR30), which belongs to one of several guanine nucleotide-binding protein (G-protein)-coupled receptors [9, 10], is also reported to be one of the estrogen-mediated non-genomic effects.

The aim of this study was to determine the possible non-genomic effect of estrogen, and SERMs on skin; therefore,

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WS1 cells, human dermal fibroblasts (HDFs) without expression of both ERα and ERβ, were tested.

Materials and methods (Supplementary materials and methods)

Reagents

Reagents for the treatment of HDF were prepared as follows: raloxifene (RAL), G protein inhibitor pertussis toxin (from Sigma, St Louis, MO, USA), PI3 K inhibitor LY294002, ERK inhibitors PD98059, and p38 MAPK inhibitor SB203580 (from Calbiochem; San Diego, CA, USA) were dissolved in dimethylsulfoxide (DMSO; Sigma).

Cell culture

The normal human fetal skin fibroblast cell line, WS1, was isolated from the midscapular skin of a 12-week-old African-American female embryo; this line has a doubling potential of 67. The cells were cultured in minimum essential medium (MEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM L-glutamine (Sigma), 100 units/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma). Cells were maintained in 100-mm tissue culture dishes in a humidified chamber at 5% CO_2 and 37°C, and subcultured every 2 to 3 days by trypsin-EDTA (Gibco, Grand Island, NY, USA). For all subsequent experiments, WS1 cells were seeded in MEM Alpha medium without phenol red (α-MEM without phenol red; Gibco). After 18 hours of incubation, the medium was replaced and the cells were subjected to various drug treatments.

WST1 assay

WST1 cell proliferation reagent (Roche, Mannheim, Germany) measures the metabolic activity of viable cells. Cells were seeded in 96-well plates at 5×10^3 cells/100 μL/well. Following 18 hours of incubation, various drugs were added to the cell culture. After the indicated time, the supernatants were discarded and the cells were washed with phosphate buffered saline (PBS). Then, WST1 was added to each well at a 1:20 dilution, and the cells were incubated at 37°C for a further 2 hours. The supernatants were quantified spectrophotometrically at 450 nm, with a reference wavelength at 640 nm. Data were presented as the percentage of survival relative to that in a vehicle-treated control culture. All WST1 assays were performed in duplicate.

RNA isolation and RT-PCR

Total RNA was collected with the RNeasy kit (Qiagen, Milan, Italy) as described by the manufacturer. For reverse transcription polymerase chain reaction (RT-PCR) studies, first-strand cDNA was synthesized from 1–5 μg of total RNA with an oligo(dT) primer and Super Script II reverse transcriptase (Invitrogen, Life Technologies, Inc., Carlsbad, CA, USA). Primers for human ERα, ERβ, GPR30, and GAPDH genes are as follows (5′ to 3′): ERα, forward, CTA CTG CAT CAG ATC CAA GG; reverse, GTC ATT GGT ACT GGC CAA TCT; ERβ, forward, TGG TCA GGG ACA TCA TCA TGG; reverse, TCA AAG AGG GAT GCT CAC TTC TG; GPR30, forward, GGC TTT GTG GGC AAC ATC; reverse, CGG AAA GAC TGC TTG CAG G; GADPH, forward, ATT GTT GCC ATC AAT GAC CC; reverse, AGT AGA GGC AGG GAT GAT GT. The conditions used for amplification were as follows: 94°C for 30 seconds followed by 52°C for 1 minute and 72°C for 30 seconds in a 25-μL reaction buffer containing cDNA generated from 1 μL of cDNA, 0.2 mM of each dNTP, 0.2 μM of each primer, and 2.5 units of *Taq* polymerase. Detection of the amount of PCR product after 35 cycles was performed after electrophoresis on 1.5% agarose gels and ethidium bromide staining.

Detection of apoptosis by TUNEL analysis

Apoptosis of the single cell is detected by fluorescent labeling of DNA strand breaks (a hallmark of apoptosis). Cells were plated in 35-mm culture dishes at 5×10^5 per dish and cultured overnight, followed by the administration of test reagents. After an indicated interval of drug treatments, the supernatant was discarded and the cells were collected by trypsinization. The cells were washed twice with PBS and fixed by incubating in 4% paraformaldehyde at room temperature for 1 hour. After another PBS wash, the cells were permeabilized by resuspension in 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate (Sigma) on ice for 2 minutes, followed by a PBS wash. Then a 50 ml TUNEL reaction mixture (*In Situ* Cell Death Detection Kit; Roche), which contained terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides, was added and the cells were incubated at 37°C for 1 hour in the dark. After a further PBS wash, the cells were subjected to fluorescence-activated cell scanning analysis (FACS; BD, Palo Alto, CA, USA) with excitation at 488 nm and emission at 525–550 nm.

FACS analysis for activated caspases in apoptotic cells

CaspACE FITC-VAD-FMK in situ marker (Promega, Madison, WI, USA) is a fluoroisothiocyanate (FITC) conjugate of the cell-permeable caspase inhibitor VAD-FMK. This structure allows delivery of the inhibitor into the cell, where it binds to activated caspases, serving as an in situ marker for apoptosis. Cells cultured in 35-mm dishes were treated with various drugs. At the end of cell culture, the supernatant was removed and the cells were detached by trypsin-EDTA. After two consecutive PBS washes, the cells were stained with 10 μM CaspACE FITC-VAD-FMK *In Situ* Marker, followed by incubation at room temperature in the dark for 20 minutes. Cells were then washed with PBS and subjected to FACS analysis with excitation at 488 nm and emission at 525–550 nm.

Immunoblotting assay

Immunoblotting assays were performed to measure the synthesis of procollagen type I and to detect the activation of