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The activation of the G protein-coupled estrogen receptor (GPER) inhibits the proliferation of mouse melanoma K1735-M2 cells

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ABSTRACT

The activation of the G protein-coupled estrogen receptor (GPER) by its specific agonist G-1 inhibits prostate cancer and 17β-estradiol-stimulated breast cancer cell proliferation. Tamoxifen (TAM), which also activates the GPER, decreases melanoma cell proliferation, but its action mechanism remains controversial. Here we investigated the expression and the effects of GPER activation by G-1, TAM and its key metabolite endoxifen (EDX) on melanoma cells. Mouse melanoma K1735-M2 cells expressed GPER and G-1 reduced cell biomass, and the number of viable cells, without increasing cell death. Rather, G-1 decreased cell division by blocking cell cycle progression in G2. Likewise, TAM and EDX exhibited an antiproliferative activity in melanoma cells due to decreased cell division. Both G-1 and the antiestrogens showed a trend to decrease the levels of phosphorylated ERK 1/2 after 1 h treatment, although only EDX, the most potent antiproliferative antiestrogen, induced significant effects. Importantly, the targeting of GPER with siRNA abolished the cytostatic activity of both G-1 and antiestrogens, suggesting that the antitumor actions of antiestrogens in melanoma cells involve GPER activation. Our results unveil a new target for melanoma therapy and identify GPER as a key mediator of antiestrogen antiproliferative effects, which may contribute to select the patients that benefit from an antiestrogen-containing regimen.

1. Introduction

Emerging evidences suggest that the G protein-coupled receptors (GPCRs) play a central role in cancer development and progression [1]. In fact, several GPCRs are overexpressed in diverse types of cancer tissues and the interference with GPCRs and their downstream targets thus represents an attractive strategy for cancer treatment. Namely, some GPCRs have been shown to be involved in tumorigenesis and metastatic progression of melanoma [1].

The G protein-coupled receptor 30 (GPR30), now officially designated G protein-coupled estrogen receptor 1 (GPER), was identified between 1996 and 1998 by four different laboratories as a new 17β-estradiol-binding protein structurally distinct from the classical estrogen receptors (ER) [2–5]. It was demonstrated that GPER mediates the proliferative effects of estrogen on some cancer cells [6,7] and that its cellular activation involves the transactivation of the epidermal growth factor receptor (EGFR), the mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) signaling pathways [8–10]. The identification of G-1, the first GPER-selective agonist [11], with no notable activity towards ERα or on 25 other important GPCRs [12], provided an important tool for studying the GPER-mediated signaling. While G-1 decreases endothelial and urothelial cell proliferation [13,14] and inhibits the proliferation of prostate cancer [15] and 17β-estradiol-

Abbreviations: BCA, bicinchoninic acid; BrdU, 5-bromo-2′-deoxyuridine; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ECF, enhanced chemifluorescence; EDX, endoxifen; ER, estrogen receptor; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GPER, G protein-coupled estrogen receptor; GPR30, G protein-coupled receptor 30; IGF-1R, insulin-like growth factor-1 receptor; LDH, lactate dehydrogenase; MAPK, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PMSF, phenylmethanesulphonyl fluoride; PVDF, polyvinyldiene fluoride; siRNA, small interfering RNA; SRB, sulforhodamine B; TAM, tamoxifen; TBS, tris-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; WB, western blot.

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stimulated breast cancer cells [16], it might increase the proliferation of other cells. In fact, in ovarian and endometrial cancer cells, G-1 induced an increase in cell proliferation [17,18], suggesting that the effects resulting from the activation of the GPER may vary according to the cell type.

Besides the specific agonist G-1 and 17β-estradiol, the selective ER modulators tamoxifen (TAM) and 4-hydroxytamoxifen have also been shown to behave as agonists for GPER [7,19–21]. TAM, an anticancer drug often used in breast cancer treatment, inhibits melanoma cell proliferation [22–25], invasion and metastasis [26] and thus it has also been used in the clinical management of melanoma as a single agent or, more often, in combination with other chemotherapeutic agents [27,28]. However, the mechanisms underlying the antitumor activity of TAM in melanoma are poorly understood and the clinical trials performed to clarify the benefit of TAM in therapeutic regimens for advanced and metastatic melanoma have produced contradictory results [29]. Based on these findings, we hypothesize that the antitumor activity of TAM in melanoma might involve GPER activation. Thus, we investigated the expression of GPER and the effects of G-1 and antiestrogenic compounds on the proliferation of a highly invasive melanoma cell line (K1735-M2), as well as the activation of the extracellular signal-regulated kinases (ERK) 1/2 and Akt signaling pathways, since theses kinases regulate cell proliferation and were shown to be activated downstream of GPER [8,30].

We show, for the first time, that K1735-M2 cells express GPER and that G-1 decreases melanoma cell proliferation due to decreased cell division, similar to the action of TAM and its active metabolite, endoxifen (EDX). The silencing of GPER inhibits the antiproliferative effects of G-1 and antiestrogens, thus implicating the GPER in the antitumor activity of antiestrogens in melanoma cells.

2. Materials and methods

2.1. Reagents

G-1, TAM and EDX were obtained from SIGMA-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and antibiotic/antimycotic solution (10,000 units penicillin, 10 mg streptomycin, 25 μg amphotericin B per mL) were purchased from SIGMA-Aldrich (St Louis, MO, USA). Fetal Bovine Serum (FBS) and trypsin were obtained from Gibco, Invitrogen Life Technologies (Carlsbad, California, USA). All of the other chemicals were purchased from SIGMA-Aldrich (St Louis, MO, USA) and were of the highest grade of purity commercially available. TAM stock solutions were prepared in absolute ethanol. G-1 and EDX were kept in dimethyl sulfoxide (DMSO). Primary specific rabbit polyclonal antibody to GPER and anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Abcam (Cambridge, UK); primary specific rabbit polyclonal antibodies to phospho-Akt, total-Akt and phospho-ERK 1/2 were obtained from Cell Signaling Technology (MA, USA); primary specific rabbit polyclonal antibody to total-ERK 1/2 was purchased from Millipore (MA, USA); mouse monoclonal antibody to β-actin was purchased from SIGMA-Aldrich (St Louis, MO, USA).

2.2. Cell culture

K1735-M2 mouse melanoma cells were cultured in DMEM, supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution, and kept in a humidified incubator with 5% CO_2/95% air, at 37 °C. Cells were plated at a 6.1 × 10^4 cells/cm^2 density and the drugs added 24 h later, when a confluency of 30–40% was observed, ensuring that the experiments were conducted under conditions of exponential proliferation. The volumes added did not exceed 0.5% (v/v). Vehicle controls were performed.

2.3. Western-blot (WB) analysis

For the preparation of total cell extracts, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and placed on lysis buffer, containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 1 mM dithiothreitol (DTT), pH 7.4, supplemented with 1 mM phenylmethanesulphonyl fluoride (PMSF) and a cocktail of protease inhibitors (P8340, Sigma). Extracts were submitted to three freeze/thaw cycles and centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatants were recovered and the protein quantified by the bicinchoninic acid (BCA) assay kit (Pierce, as part of Thermo Fisher Scientific, IL, USA). Equal amounts of protein from total cell extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using polyacrylamide gels of 10% and then transferred into a polyvinyldiene fluoride (PVDF) membrane. The membranes were blocked with tris-buffered saline (TBS)-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween) with 5% nonfat milk, for 1 h at room temperature, and then incubated overnight at 4 °C with the primary antibody in TBS-T with 5% nonfat milk. After extensive washing, membranes were incubated with the secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature. After additional washes, the membranes were developed using the enhanced chemifluorescence (ECF) substrate, and scanned on the Typhoon 9000 scanner (Amersham Biosciences). Appropriate controls were used to ensure equal protein loading as indicated in the figure legends. The bands were analyzed using the ImageQuant TM software from Amersham Biosciences. For subsequent reprobing, the membranes were stripped of antibody with 0.2 M NaOH, blocked again and incubated with the appropriate antibodies.

2.4. Sulforhodamine B (SRB) assay

The effects induced by the drugs on melanoma cells were assessed by the SRB assay [31]. At designated time points, the cultures were fixed with absolute methanol containing 1% acetic acid, and stored at −20 °C overnight. The methanol was decanted and the plate air-dried. The labeling solution, containing 0.5% SRB in 1% acetic acid, was added to each well and the plate incubated for 1 h, at 37 °C. Plates were rinsed with 1% acetic acid, air-dried, and the bound dye eluted with 10 mM Tris buffer, pH 10. The absorbance was measured in a Synergy HT plate reader at 540 nm. As SRB binds to basic amino acid of proteins of adherent cells, the absorbance at 540 nm provides an estimate of total protein mass (biomass), which is related to cell number. The absorbance obtained in control cultures was considered 100%. The number of independent experiments is indicated in figure legends.

2.5. Lactate dehydrogenase (LDH) assay

The LDH assay allows to determine the cytotoxic effects of drugs based on the release of this cytosolic enzyme into the extracellular medium following the loss of cellular membrane integrity [32]. The culture medium was collected after 72 h of incubation with the drugs and centrifuged at 14,000 rpm for 10 min at 4 °C. An aliquot of supernatant (100 μL) was collected and incubated with substrate mixture, containing 40 μM lactate in perchloric acid 3%, and 3.6 mM NAD^+, in tris-hydrazine buffer (80 mM tris, 400 mM hydrazine, 5 mM EDTA, pH 9.5). The enzymatic reaction that occurs leads to the reduction of NAD^+ to NADH by oxidation of lactate to pyruvate. Thus, the amount of NADH is directly related to LDH activity in the