Abstract. Glioblastoma is the most frequent primary tumor in the human brain. Glioblastoma cells express aromatase and the classic estrogen receptors ERα and ERβ and can produce estrogens that promote tumor growth. The membrane G protein-coupled estrogen receptor (GPER) also plays a significant role in numerous types of cancer; its participation in glioblastoma tumor development is not entirely known. The present study investigated the effect of the agonists [17β-estradiol (E2) and G1] and antagonist (G15) of GPER on proliferation and apoptosis of C6 glioblastoma cells. GPER expression was evaluated by immunofluorescence, western blotting and reverse transcription-quantitative PCR. Cell proliferation was determined using Ki67 immunopositivity. Cell viability was examined using the MTT assay and apoptosis using caspase-3 immunostaining and ELISA. C6 cells express GPER, and the immunopositivity increased after exposure to E2, G1, or their combination. GPER protein expression increased after treatment with E2 combined with G1. However, GPER mRNA expression decreased in treated cells compared with control. The percentage of Ki67 immunopositive C6 cells increased under the effect of E2 in combination with G1 or G1 alone. G15 significantly reduced Ki67 immunopositivity. Pearson’s correlation analysis revealed a positive relationship between GPER and Ki67 immunopositivity across the study conditions. Additionally, the MTT assay showed a significant reduction in C6 cell viability after G15 treatment, alone or in combination with G1. The exposure to G15 increased the percentage of caspase-3 immunopositivity cells and caspase-3 levels. Pearson’s correlation analysis demonstrated a negative correlation between GPER and caspase-3 immunopositivity across the study conditions. Glioblastoma C6 cells express GPER, and this receptor modulates cell proliferation and apoptosis. The GPER agonists E2 and G1 favored cell proliferation; meanwhile, the antagonist G15 reduced cell proliferation, viability and favored apoptosis. Therefore, GPER may be used as a biomarker of glioblastoma and as a target to develop new therapeutic strategies for glioblastoma treatment.

Introduction

Glioblastoma is a high-grade malignant tumor in the human brain (1). Glioblastoma patients have high recurrence rate and poor prognosis with only 14-18 months of survival after diagnosis (2). Current treatments include surgical tumor removal and radiotherapy followed by Temozolomide chemotherapy. These treatments only extend the survival period of patients. Therefore, new therapeutic targets to control glioblastoma development are needed. Female patients with glioblastoma have an improved outcome than males who have a higher glioblastoma incidence (3). These sex differences could be associated with estrogens and their receptors.

Glioblastoma tumor cells express aromatase and the classic estrogen receptors α and β (ERα and ERβ) and can locally produce estrogen, which promotes tumor growth (4,5). Additionally, G-protein coupled estrogen receptor (GPER) is responsible for the fast or non-genomic effects of estrogens. Activated GPER initially induces the epidermal growth factor receptor, triggering the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway. GPER also activates the phospholipase C and phosphatidylinositol 3-kinase pathways (6,7), promoting the transcription of genes related to cell survival, proliferation and apoptosis (8).
GPER expression has been reported in different types of cancer including breast, endometrial, lung, prostate, ovary and oral cancers (9-14). This receptor has been described in non-small cell lung cancer (NSCLC), and the administration of 17β-estradiol (E2) or GPER-selective agonist G1 promotes the proliferation and metastasis of these cells. By contrast, the administration of the GPER-selective antagonist G15 in the NSCLC murine model reduces the number of tumoral nodules and the tumoral index (15). However, the participation of GPER in glioblastoma development has received little attention so far, and its role in in vitro and in vivo glioma progression, at present, has not been fully elucidated.

In the present study, C6 rat glioblastoma cells were used as a research model. The C6 rat glioma model is one of the most common experimental models used in neuro-oncology (16,17). This chemical-induced glioblastoma cell line is widely used for testing therapeutics since its genetic profile resembles human glioblastomas, and it offers an accurate representation of glioblastoma characteristics. Moreover, in vivo C6 xenograft models can produce an invasive glioblastoma which allows studying the growth and the invasion of high-grade gliomas and the antitumoral potential of new therapeutic molecules (16).

Therefore, the present study aimed to assess the participation of GPER in the fate of C6 murine glioblastoma cells. Furthermore, the effect of the exposure to the agonists E2 and G1 or the antagonists G15 on the fate, proliferation, or apoptosis of C6 glioblastoma cells was also evaluated.

Materials and methods

Reagents. 17-β estradiol (E2758) (E2) was purchased from MilliporeSigma. GPER-selective agonist G1 (CAS no. 881639-98-1) or antagonist G15 (CAS no. 1161002-05-6) were purchased from Cayman Chemical Company.

Cell culture and experimental conditions. The glioblastoma C6 rat cell line was acquired from the American Type Culture Collection (cat. no. CCL-107). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12, F12-K supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.) with 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA) and 1% blotto. The cells were cultured in an incubator with 95% air and 5% CO2. The C6 cells were exposed for 48 h to the different treatments. Cells were lysed in RIPA lysis buffer supplemented with a protease inhibitor cocktail (cat. no. sc-24948A; Santa Cruz Biotechnology, Inc.). Protein concentration was determined using the Bradford microplate protocol using Coomassie Standard Set (cat. no. 5000207; Bio-Rad Laboratories, Inc.). Total protein (50 μg) was separated by SDS-PAGE (12%) and transferred onto a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% blootto, non-fat dry milk (cat. no. sc-2325; Santa Cruz Biotechnology, Inc.) dissolved in TBS-T (0.1% Tween-20) for 2 h at room temperature with the secondary antibodies; Goat polyclonal anti-Rabbit IgG (H+L) Alexa Fluor 594 antibody (1:1,000; cat. no. A-11008) and a Mouse monoclonal anti-Mouse IgG (H+L) Alexa Fluor 488 antibody (1:1,000; cat. no. A-11005) (both from Thermo Fisher Scientific, Inc.). Finally, the cells were washed with PBS and stained at room temperature for 5 min with Fluoroshield with DAPI to observe their nuclei (cat. no. F6057; Sigma-Aldrich; Merck KGaA). Fluorescence images were captured using a fluorescence microscope (Olympus Corporation). A total of 20 microphotographs at x40 magnification were captured from each condition to evaluate immunopositive cells from at least three different experiments. ImageJ software (version 1.8.0; National Institutes of Health) was used to count positively stained cells representing ~400 cells manually counted per condition.

Western blot analysis. Western blot analysis was performed as previously described (19). Briefly, C6 cells were seeded at a density of 1.4x10^6 cells/well in 100-mm Petri dishes and then exposed 48 h to the different treatments. Cells were lysed in RIPA lysis buffer supplemented with a protease inhibitor cocktail (cat. no. sc-24948A; Santa Cruz Biotechnology, Inc.). Protein concentration was determined using the Bradford microplate protocol using Coomassie Protein Assay Reagent (cat. no. 1856209; Thermo Fisher Scientific, Inc.) and the Quick Start Bovine Serum Albumin Standard Set (cat. no. 5000207; Bio-Rad Laboratories, Inc.). Total protein (50 μg) was separated by SDS-PAGE (12%) and transferred onto a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% blootto, non-fat dry milk (cat. no. sc-2325; Santa Cruz Biotechnology, Inc.) dissolved in TBS-T (0.1% Tween-20) for 1 h at room temperature. The membrane was incubated overnight at 4°C with primary rabbit polyclonal anti-GPER antibody (1:5,000) or primary rabbit polyclonal anti-GAPDH antibody (1:5,000; cat. no. ab9485; Abcam) as a loading control. Following the primary incubation, the membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG H&L (HRP-conjugated) secondary antibody (1:10,000; cat. no. ab205718; Abcam). The proteins were detected using the Western Sure Premium Chemiluminescent substrate (cat. no. 926-95000; LI-COR Biosciences). The LI-COR C-DiGit Blot Scanner was used for chemiluminescent detection (LI-COR Biosciences). Data were analyzed using the Image Studio Software 3.1.4 (LI-COR Biosciences).

Reverse transcription-quantitative (RT-q)PCR. RNA extraction was performed with TRIzol® (400 μl of TRIzol/300,000 cells/well seeded in six-well plates) according to the manufacturer's protocol (cat. no. 15596026; Thermo Scientific) and then blocked at room temperature with 1% IgG-free albumin and permeabilized with 0.25% Triton X-100 for 1 h. Subsequently, the cells were incubated overnight at 4°C either with the primary rabbit polyclonal anti-GPER antibody (1:200; cat. no. ab39742), or the mouse monoclonal anti-Ki67 antibody (1:200; cat. no. ab8191) (both from Abcam), or the mouse monoclonal anti-caspase-3 antibody (1:200; cat. no. sc-271759; Santa Cruz Biotechnology, Inc.). On the following day, the cells were washed with PBS, and incubated for 2 h at room temperature with the secondary antibodies: Goat polyclonal anti-Rabbit IgG (H+L) Alexa Fluor 488 antibody (1:1,000; cat. no. A-11008) and a Goat monoclonal anti-Mouse IgG (H+L) Alexa Fluor 594 antibody (1:1,000; cat. no. A-11005) (both from Thermo Fisher Scientific, Inc.). Finally, the cells were washed with PBS and stained at room temperature for 5 min with Fluoroshield with DAPI to observe their nuclei (cat. no. F6057; Sigma-Aldrich; Merck KGaA). Fluorescence images were captured using a fluorescence microscope (Olympus Corporation). A total of 20 microphotographs at x40 magnification were captured from each condition to evaluate immunopositive cells from at least three different experiments. ImageJ software (version 1.8.0; National Institutes of Health) was used to count positively stained cells representing ~400 cells manually counted per condition.
The integrity of total RNA was determined by 1% agarose gel electrophoresis stained with ethidium bromide and observed under UV light, and the Nanodrop One spectrophotometer (Thermo Fisher Scientific, Inc.) was used to quantify total RNA. cDNAs were synthesized from 200 ng of total RNA using oligo-dt12-18 (cat. no. 18418-012) and M-MLV reverse transcriptase (cat. no. 28025-013; both from Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The resulting cDNAs were quantified by UV-spectrophotometry and used for qPCR. The following primers were used: GPER forward, 5'-CTTCTTGCCATGC CACGCT-3', and reverse, 5'-ACATCTGACTGTCCTGGT CTG-3' (20); and GAPDH forward, 5'-GCTGTCATCAA CGGGAAAC-3' and GAPDH reverse, 5'-GACTCCAGACA TACTCAGCACC-3' (21). The primers were synthesized by Integrated DNA Technologies, Inc. qPCR was performed using Maxima SYBR-Green/ROX qPCR master mix (cat. no. K0221; Thermo Fisher Scientific, Inc.).

## Results

### Immunofluorescent staining of GPER

The immunofluorescence analyses demonstrated that C6 murine cells express GPER. Experimental data demonstrated that the treatment of these cells for 48 h with the agonists E2 or G1 and their combination (E2 and G1) significantly increased the percentage of GPER immunopositivity compared with control (P<0.05; F=237; degrees of freedom (DF)=118). By contrast, treatment of C6 cells with the antagonist G15 alone or in combination with G1 significantly reduced this percentage compared with control (P<0.05) (Fig. 1A and B).

### GPER expression in C6 cells

GPER expression in C6 cells was also evaluated by western blotting and RT-qPCR. The treatment with E2 in combination with G1 for 48 h significantly upregulated GPER protein expression compared with control (P<0.05; F=8.35; DF=17). On the other hand, the antagonist G15, alone or in combination with G1, decreased this expression compared with E2-G1 combination (P<0.05) (Fig. 2A and B). Notably, the RT-qPCR data revealed that the GPER mRNA expression decreased in C6 cells exposed to all treatments compared with control (P<0.05; F=214; DF=63). The cells exposed to G15 alone or in combination with G1 presented a higher GPER mRNA expression compared with cells exposed to the agonists (P<0.05) (Fig. 2C).

### Proliferation and viability of C6 glioblastoma cells

Ki67 immunopositivity (proliferation) was observed in C6 cells treated with E2, G1, or their combination. A decrease in Ki67 immunopositivity was observed after exposure to G15 alone or in combination with G1 (Fig. 3A). As revealed in Fig. 3B, a significant increase was observed in the percentage of Ki67 immunopositive cells under the effect of E2 in combination with G1 or G1 alone, compared with control (P<0.05; F=82.3; DF=117). However, C6 cells under the effect of the antagonist G15 alone or its combination with G1 showed a significant reduction in proliferation compared with the rest of the groups (P<0.05) (Fig. 3B). The Pearson's correlation values (r=0.86; R²=0.71; P<0.05) confirmed a positive correlation between GPER and Ki67 immunopositivity and proliferation of C6 cells (Fig. 3C). The experimental data from the MTT assay demonstrated that E2 alone or combined with G1 tended to increase the viability of C6 cells compared with the control. A significant decrease was also identified in the viability of C6 cells treated with G15 alone or in combination with G1 compared with control cells and cells exposed to agonists alone or in combination (P<0.05; F=14.65; DF=21) (Fig. 3D). It is important to mention that the Ki67 and MTT experimental data coincide in highlighting the significant effect of G15 against proliferation and viability of C6 glioblastoma cells (Fig. 3B and D).

### Apoptosis of C6 glioblastoma cells

Caspase-3 immunopositivity (apoptosis) was higher in C6 cells treated with G15 alone or in combination with G1 compared with E2, G1 and
E2 plus G1 (Fig. 4A). As demonstrated in Fig. 4B, a significant increase was observed in the percentage of caspase-3 immunopositive cells under the effect of the antagonist G15 alone or its combination with G1 compared with the rest of the groups (P<0.05; F=66.62; DF=119). Pearson’s correlation values (r=-0.97; R²=0.95; P<0.05) exhibited a negative correlation between GPER and caspase-3 immunopositivity of C6 cells (Fig. 4C). The ELISA also revealed that the G1-G15 combination treatment significantly increased caspase-3 concentration (1.66±0.302 ng/ml) in C6 cells compared with control, E2 and E2-G1 combination conditions (P<0.05; F=6.74; DF=17). Additionally, C6 cells exposed to G15 alone tended to increase their caspase-3 levels (1.26±0.176 ng/ml) compared with control (1.01±0.004 ng/ml) (Fig. 4D).

Discussion

The search for new therapeutic targets for treating glioblastoma is a priority in neuro-oncology. The present study revealed the expression of GPER in C6 murine glioblastoma cells, the effect of its agonists (E2 and G1) in increased cell proliferation, and the opposite effect of the antagonist G15, which decreased C6 cells proliferation and viability, and favored apoptosis. Therefore, GPER expression plays a crucial role in modulating the fate of glioblastoma as described for other types of tumors and represents a target to develop new therapeutic strategies against glioblastomas.

Firstly, an increase was observed in the proliferation of C6 cells exposed to E2 in combination with G1 or G1 alone. This result is consistent with Castracani et al. (24) in the
U87-MG glioblastoma cell line, who reported that E2 (5 nM) administration increased cell proliferation. MCF-7 breast cancer cell line treated with 2,000 nM of tamoxifen, a GPER agonist, also presented increased proliferation attributed to GPER activation (25). Similarly, E2 (1 nM) induced GPER activation and cell proliferation in a human seminoma cell line through ERK1/ERK2 and protein kinase A pathways (26). Additionally, the present results indicated an additive proliferative effect of G1 and E2 where G1 was predominant over E2, probably related to its selectivity for GPER.

Hirtz et al (27) recently described GPER protein expression and localization in LN229 and U251 human glioblastoma cell lines, reporting that the exposure of both cell lines to a high dose of G1 (10 µM) decreased cell proliferation in a time-dependent manner (24‑96 h), with optimal results at 72 h. The lowest dose (10 nM) induced a milder decrease in cell growth compared with untreated control cells. The present experimental data in C6 murine cells differ from the aforementioned study, as an increase was observed in proliferation of C6 glioblastoma cells exposed to 10 nM of G1 for 48 h. Therefore, the dose and time of treatment with G1 are determinant factors for the fate of glioblastoma cells, as previously described in other tumor cell lines.

Notably, the present experimental results demonstrated that C6 glioblastoma cell viability and proliferation were substantially reduced under treatment with G15 (10 µM) alone or combined with G1 (10 nM). These data are in consistency with Bai et al (13), who demonstrated a decreased cell viability of human oral squamous carcinoma cells exposed to G15 (10-20 µM) for 48 h. Collectively, the experimental data presented in the present study revealed that GPER specific-antagonist G15 affects proliferation and viability of C6 glioblastoma cells and inhibits the effects of G1. These data supported the involvement of GPER in cell proliferation and viability and the usefulness of GPER-specific antagonists in future glioblastoma treatment schemes.

The increase in GPER protein expression observed in C6 cells under E2-G1 combination treatment coincides with several previous studies. Bustos et al (28) observed an increase in GPER protein expression in HT-29 and DLD-1 colon cancer cells exposed for 24 h to 10 nM E2. Liu et al (18) also identified increased GPER protein expression in NSCLC cells (A549 and H1793) after exposure to E2 (10 nM) or G1 (10 nM) for 48 h. The same effect was reported in a urethane-induced lung adenocarcinoma murine model (15). In the present study, GPER transcript results in C6 glioblastoma cells coincide with Ariazi et al (29) in MCF-7 breast cancer cells exposed to E2 (10 nM) for 24 and 48 h, which presented a downregulation in GPER mRNA. By contrast, HT-29 and DLD-1 colon cancer cells exposed for 24 h to 10 nM E2 showed an increase in GPER mRNA (28). Thus, the effect of E2 on transcription and translation of GPER depends on treatment duration and cell type.

It was revealed that C6 cells exposed to the combination of both agonists significantly increased their GPER protein levels compared with control cells. The application of each agonist on its own presented the same tendency. Therefore, E2 and G1 probably exerted an accumulative effect on GPER protein
expression in the E2-G1 combination-treated cells, leading to excessive proliferation. However, these same cells (E2, G1, and E2-G1 combination groups) presented a downregulation of the GPER transcript compared with control, suggesting that after 48 h of exposure to the agonists, a transcriptional mechanism was involved to avoid further excessive GPER expression. Thus, the discrepancy between GPER protein and mRNA expression may correspond to a characteristic mechanism of G protein-coupled receptors (GPCR) to control their expression in the presence of high levels of agonist and avoid excessive signaling, as reported by Rajagopal and Shenoy (30). In the aforementioned study, it was described how GPCR mRNA expression is downregulated by its agonists to circumvent excessive production of GPCR proteins and undue signaling. It has also been reported that continuous stimulation by agonists may cause a redirection of the receptor to the protein degradation pathways instead of the recycling pathway (31,32). This process is long-term and is associated with receptor internalization in vesicles for degradation and decreased mRNA expression through unknown mechanisms (30).

Notably, the exposure of C6 cells to the antagonist G15 alone or in combination with G1 decreased GPER protein expression compared with the agonists-exposed cells (E2, G1 and E2-G1 combination), which also corresponds with the low proliferation of these cells. This observation confirmed that G15 can compensate the effect of E2 or G1 agonists of GPER and control GPER protein levels to that of control cells. Consequently, GPER is a target for glioblastoma treatment, and further study of GPER-specific antagonists is needed.
The role of GPER in the apoptosis of C6 cells was also investigated through the evaluation of caspase-3. The agonists E2 and G1, alone or combined, did not affect caspase-3 compared with control. However, the exposure to G15 alone or combined with G1 significantly increased caspase-3 immunopositivity percentage compared with the rest of the conditions. Similarly, the ELISA results indicated that G15 favored apoptosis and that this effect was stronger when combined with G1. Similar to the present experimental results, Wang et al. (33) showed an increase in caspase-3 activity in ovarian cancer cells exposed to G1 (2 µM) plus G15 (4 µM). The present results are also in consistency with Bai et al. (13), who reported that G15 (5-20 µM) induces G2/M phase cell arrest and apoptosis in human oral squamous carcinoma cells. By contrast, in primary astrocytes culture, GPER activation by high levels of G1 (100 nM), compared with the ones used in the present study, increased apoptosis, associated with a rise in intracellular calcium (34).

The Pearson’s correlation analyses confirmed a positive relationship between GPER expression and proliferation, but a negative association between GPER expression and apoptosis of C6 cells, indicating that high expression of GPER is linked with proliferation; meanwhile, low GPER expression may favor apoptosis. These results are consistent with several previous studies, reporting that low GPER expression is associated with a favorable prognosis in patients with cancer. Ulhaq et al. (35) showed that certain GPER single-nucleotide polymorphisms were related to cancer predisposition and that GPER expression levels were associated with higher tumor stages. Sjöström et al. (36) reported that the absence of immunohistochemical staining of GPER in breast cancer tissue is associated with an excellent long-term prognosis in these patients. Ino et al. (37) also reported that the elevated
expression of GPER is associated with poor prognosis in patients with uterine cervical adenocarcinoma. These results highlighted GPER expression as a potential biomarker in patients with glioblastoma. Therefore, the present experimental data obtained in vitro deserve further in vivo study in murine models of glioblastoma and patients with glioblastoma to assess the participation of GPER in tumor malignancy and survival.

The search for new biomarkers and therapeutic targets to improve the prognosis of patients with glioblastoma is of utmost importance. It was demonstrated that GPER expression is present in C6 murine glioblastoma cells and that its expression is regulated by its agonists of natural (E2) or synthetic origins (G1) and antagonist (G15). A low dose of G1 increased proliferation of C6 cells; by contrast, G15 had an opposite effect and favored apoptosis. Thus, the results of the present study confirmed the potential of GPER as an early detection prognosis marker and target for developing new therapeutic strategies for glioblastoma treatment. Nevertheless, based on the present results, further studies in glioblastoma murine models and tissue from glioblastoma patients with high or low levels of estrogens are needed to study the antitumoral activity of G15 or other GPER specific antagonists.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CEGA and JMDJ designed the study. CEGA performed the experiments. CEGA, LCLM, RCA and IGAG performed material preparation and data collection. AS supervised cell viability, RT-qPCR and western blotting experiments. CEGA and JMDJ analyzed and interpreted data. CEGA, LCLM, RCA and IGAG performed material preparation and data collection. AS designed the study. CEGA performed the viability, RT-qPCR and western blotting experiments. CEGA and JMDJ wrote the first draft of the manuscript. AS, JMDJ and SHDJ analyzed and interpreted data. CEGA and AS confirm the authenticity of all the raw data. CEGA, LCLM, RCA and IGAG performed

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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