The Role of mPR δ and mPR ϵ in Human Glioblastoma Cells: Expression, Hormonal Regulation, and Possible Clinical Outcome

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Abstract

Glioblastomas (GBM) are the most frequent and aggressive primary tumor of the central nervous system. In recent years, it has been proposed that sex hormones such as progesterone play an essential role in GBM biology. Membrane progesterone receptors (mPRs) are a group of G protein–coupled receptors with a wide distribution and multiple functions in the organism. There are five mPRs subtypes described in humans: mPR α , mPR β , mPR γ , mPR δ , and mPR ϵ . It has been reported that human-derived GBM cells express the mPR α , mPR β , and mPR γ subtypes, and that progesterone promotes GBM progression in part by mPR α specific activation; however, it is still unknown if mPR δ and mPR ϵ are also expressed in this type of tumor cells. In this study, we characterized the expression and hormonal regulation of mPR δ and mPR ϵ in human GBM cells. We also analyzed a set of biopsies from TCGA. We found that the expression of these receptors is dependent on the tumor's grade and that mPR δ expression is directly correlated to patients' survival while the opposite is observed for mPR ϵ . By RT-qPCR, Western blot, and immunofluorescence, the expression of mPR δ and mPR ϵ was detected for the first time in human GBM cells. An *in silico* analysis showed possible progesterone response elements in the promoter regions of mPR δ and mPR ϵ , and progesterone treatments downregulated the expression of these receptors. Our results suggest that mPR δ and mPR ϵ are expressed in human GBM cells and that they are relevant to GBM biology.

Keywords Membrane progesterone receptors · Glioblastoma · Brain tumors · Progesterone · Astrocytoma

Introduction

Glioblastoma multiforme (GBM) is the most frequent and aggressive central nervous system (CNS) tumor that arises from the malignization of glial cells, glial progenitor cells, or transformed neural stem cells [1]. These tumors are characterized as fast-growing due to their high proliferative and invasive capabilities and for the low survival time of the patients

after diagnosis, which goes from 12 to 16 months [1–3]. Therefore, it is crucial to elucidate the molecular mechanisms involved in the progression of GBM. Moreover, it is known that these tumors are more frequent in men than in women, in a proportion of 3:2; however, the factors involved in this gender bias have not been elucidated [1]. Regarding this issue, in recent years, it has been proposed that sex hormones, such as progesterone (P4), play an essential role in GBM biology [4].

P4 is a steroid hormone produced in ovaries, adrenal glands, and placenta, and is involved in the regulation of critical reproductive functions, including menstrual cycle and maintenance of pregnancy [5]. Also, it is synthesized and metabolized in various regions of the CNS [6], where it regulates sexual behavior, mood, neuroprotection, myelination, learning, and memory, as well as tumor growth in pathological conditions [6, 7]. P4 exerts its effects on target cells by two mechanisms: the genomic and the non-genomic ones. Through the genomic pathway, P4 permeates the plasmatic membrane to attach to its intracellular progesterone receptor (PR), which functions as a transcription factor that binds to specific DNA sequences named progesterone response

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elements (PRE), which are commonly localized in various promoter regions, thus regulating the expression of several genes [8–10]. Meanwhile, the non-genomic pathway of P4 is associated with rapid cell signaling changes induced via PR ligand-independent activation by membrane-associated kinases, or via the activation of different G protein-coupled membrane receptors to progesterone (mPRs) [11–13].

mPRs are G protein-coupled surface receptors that belong to the progestin and AdipoQ receptor family (PAQR). There are five mPRs subtypes described in humans: mPR α (PAQR7), mPR β (PAQR8), mPR γ (PAQR5), mPR δ (PAQR6), and mPR ϵ (PAQR9); the mPR α , mPR β , and mPR γ subtypes are paired to inhibitory G proteins while the mPR δ and mPR ϵ are associated to stimulatory G proteins [14–17]. Activation of these mPRs by P4 or by some of its metabolites such as dihydroprogesterone and allopregnanolone initiates intracellular signaling cascades linked to the modulation of cAMP levels, mobilization of intracellular Ca²⁺, or the activation of kinases such as MAPKs, PI3K, Akt, and c-Src [11, 13, 14]. These mPRs regulate physiological processes involved in reproduction, development, immunological, and neuroendocrine responses [16, 18–22], and its participation in breast and ovarian cancer progression has also been demonstrated [23–25].

Previous works have suggested that P4 contributes to the tumor progression of GBM since human-derived GBM cells express several PR; plus, this hormone increases proliferation, migration, and invasion of GBM cells in both in vitro and in vivo models [26, 27, 28]. Additionally, it has been demonstrated that P4 enhances the infiltration of GBM cells from the tumor area into healthy tissue in the cerebral cortex [2930]. However, the use of the pharmacological antagonist of the PR mifepristone (RU486) partially blocks P4 effects on GBM cells [27282930], suggesting the participation of other mechanisms triggered by P4 in these cells, such as those activated by mPRs.

Our group has demonstrated that human-derived GBM cell lines U87 and U251 express the mPR α , mPR β , and mPR γ subtypes [31]. Furthermore, it was demonstrated that mPR α activation by the specific mPR agonist 10-ethenyl-19-norprogesterone (Org OD 02-0) promotes the proliferation, migration, and invasion in these cells, and that these effects are mediated by the phosphorylation of Akt and Src kinases [32]. Despite this, it is still unknown if the mPR δ and mPR ϵ subtypes are expressed in GBM and if these receptors could have any role in this type of cancer.

For this reason, in the present study, we characterized the mPR δ and mPR ϵ subtypes expression in human GBM by using a set of biopsies data obtained from The Cancer Genome Atlas (TCGA), and in experiments carried out in the cell lines U87 and U251 derived from human GBM. The hormonal regulation of these receptors by P4 was also studied in the mentioned cell lines. Our results showed that the

expression of mPR δ is positively correlated to the patients' survival, while mPR ϵ levels are negatively correlated to clinical outcome. We also found out that both receptors are expressed in GBM cell lines and that their expression is down-regulated by P4.

Material and Methods

TCGA Data Analysis

RNA-Seq counts from normal tissue (5), and human astrocytoma primary tumors (55 grade II, 112 grade III, 155 grade IV) were obtained from the Glioblastoma and Low-Grade Glioma projects of The Cancer Genome Atlas (TCGA) repository (<https://portal.gdc.cancer.gov/>). The data were downloaded and processed using TCGAbiolinks package version 2.12.6 [33] for R. DESeq2 version 1.34.1 [34] was used for the differential expression analysis and data normalization. Samples were stratified according to gene expression into three groups: “low” corresponding to the first quartile, “medium” for the second and third quartile, and “high” for the upper. Survival plots were generated with TCGA_analyze_survival tool from TCGAbiolinks.

Cell Culture and Treatments

U87 and U251 human GBM cell lines (purchased from ATCC, Georgetown, WA, USA) were cultured in phenol red and high glucose Dulbecco's modified Eagle's medium (DMEM, In vitro, Mexico City, Mexico) supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, and 0.1 mM non-essential amino acids, at 37 °C in a humidified 5% CO₂ atmosphere. Both cell lines were tested for mycoplasma contamination using the Universal Mycoplasma Detection Kit (ATCC, Georgetown, WA, USA); all samples were negative (Supplementary Material 1). 2×10^5 cells were plated into 6-well plates. Twenty-four hours before treatments, the medium was changed for phenol red-free and high glucose DMEM (In vitro, Mexico City, Mexico), supplemented with charcoal-stripped FBS (HyClone, PA, USA). Treatments for RT-qPCR and Western blot were P4 (coupled to cyclodextrin, 1 nM, 10 nM, 100 nM, and 1 μ M) and vehicle (V, 0.02% cyclodextrin) for 12 and 24 h; P4 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

RNA Isolation and RT-qPCR

After treatments, total RNA was extracted using TRIzol LS Reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. RNA concentration and purity were assessed through the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

The integrity of the samples was determined with a 1.5% agarose gel electrophoresis. Healthy human astrocyte (HA) total RNA was obtained from ScienCell (cat: 1805, ScienCell, CA, USA). One microgram of total RNA was reverse transcribed to cDNA using the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT_{12–18} as primers. Two microliters from the previous reaction was subjected to qPCR using the FastStart DNA Master SYBR Green I reagent kit for LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The relative abundance of PAQR6 (mPR δ) and PAQR9 (mPR ϵ) mRNA was calculated using 18S mRNA as an endogenous reference. Relative expression levels were calculated by the Δ Ct method. Three biological replicates for each experiment were done. Primers used and the sequences of the fragments amplified are addressed in Supplemental Material 2.

Western Blot

After treatments, cells were detached from culture plates with PBS-EDTA and centrifuged. Cell pellets were lysed with RIPA buffer plus protease inhibitors (1 mM EDTA, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM PMSF). Total protein was obtained by centrifugation at 14,000 rpm (4 °C for 5 min). The concentration of each sample was determined using Pierce 660 nm Protein Assay reagent (Thermo Fisher Scientific, MA, USA) and NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA) according to the manufacturer's protocol. Thirty micrograms of protein were subjected to SDS-PAGE in a 12% acrylamide gel. Proteins were transferred to a PVDF membrane for 2 h (60 mA at semi-dry conditions). Membranes were blocked with 5% bovine serum albumin (BSA; In Vitro, Mexico City, Mexico) and then incubated at 37 °C for 3 h with one of the following antibodies: mPR δ (cat: NBP1-59428; Novus Biologicals, CO, USA) in dilution 1:1000 or mPR ϵ (cat: ab185466; Abcam, USA) in dilution 1:400. Blots were then incubated with a 1:7500 dilution of goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (cat: sc-2004 Santa Cruz Biotechnology, TX, USA) at room temperature for 45 min. mPR content was normalized to γ -tubulin. Blots were stripped with glycine (0.1 M, pH 2.5, 0.5% SDS) at room temperature for 30 min, and incubated with anti- γ -tubulin antibody (cat: T3195-2ML, Sigma-Aldrich, MO, USA) at 4 °C overnight. Membranes were incubated with a 1:7500 dilution of goat anti-rabbit secondary antibody as previously described. Bands were detected exposing blots to Kodak Biomax Light Film (Sigma-Aldrich, MO, USA) after incubation with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, MA, USA). Densitometry of the bands was performed with ImageJ 1.45S software (National Institutes of

Health, WA, USA). Three independent cultures for each experiment were done.

Immunofluorescence

U87 and U251 cells were fixed using 4% paraformaldehyde solution at room temperature for 20 min, followed by three washes in PBS. Then, for intracellular detection of the mPRs, fixed cells were permeabilized and blocked with 1% BSA and 0.2% Triton X-100 (Sigma-Aldrich, MO, USA) in PBS at room temperature for 30 min, while for cell membrane detection, fixed cells were just blocked with 1% BSA in PBS at room temperature for 30 min. Next, permeabilized and non-permeabilized cells were incubated with primary antibody rabbit anti-mPR δ (cat: NBP1-59428; Novus Biologicals, CO, USA) or anti-mPR ϵ (cat: ab185466; Abcam, USA) in dilution 1:100 at 4 °C for 24 h. Cells were washed three times in PBS and then incubated with secondary antibody goat anti-rabbit Alexa Fluor 568 (Thermo Fisher Scientific, MA, USA) in dilution 1:500 at room temperature for 60 min. Cells were rinsed three times in PBS, and nuclei were stained with 1 mg/ml Hoechst 33342 solution (Thermo Fisher Scientific, MA, USA). Finally, cells were coverslipped using fluorescence mounting medium (Polysciences Inc., PA, USA), and visualized in an Olympus Bx43 microscope. Immunofluorescence negative controls consisted of cells incubated without the primary antibody.

Bioinformatic Analysis of Progesterone Response Elements

Gene sequences were obtained from the Human Genome Resources at NCBI (https://www.ncbi.nlm.nih.gov/genome/gdv/browser/?context=genome&acc=GCF_000001405.38). The promoter regions and transcription start site (TSS) were determined through the Ensembl database [35]. Putative binding sites for PR were searched with JASPAR [36], Uniprot UGENE software v.1.26.3 [37], and TRANSFAC [38] algorithms. For all analyzed genes, the binding sites predicted by two or more databases with a matrix similarity score higher than 0.8 or *P* value < 0.05 were established as potential progesterone response element (PRE).

Statistical Analysis

Data from TCGA were plotted and analyzed using R version 3.5.2. To examine the relation between tumor grade and the level of expression of each mPR, a chi-square test of independence was performed. Experimental data were analyzed and plotted using the GraphPad Prism 5.0 software (GraphPad Software, CA, USA). Statistical analysis between groups was performed using a one-way ANOVA with a Tukey post-test. A *P* value < 0.05 was considered statistically significant.

Results

mPR δ and mPR ϵ Expression in GBM Is Dependent of Tumor Grade and Correlates to Clinical Outcome

Expression data from TCGA were analyzed in order to find out the mPRs that are differentially expressed in GBM with respect to healthy tissue. In total, expression profiles from 5 normal tissues and 155 GBM were compared. Differential expression analysis shows that mPR β , mPR δ , and mPR ϵ are downregulated in GBM (Fig. 1a). As mPR β expression and hormonal regulation in GBM have been previously described [31], we decided to focus on mPR δ and mPR ϵ . In order to find out if their expression is dependent on the astrocytoma grade, data from 55 grade II, 112 grade III, 155 grade IV tumors (GBMs), and 5 normal tissue samples were stratified by quartiles according to the expression level of mPR δ and mPR ϵ , where quartile 1 has the samples with the lowest levels of expression and quartile 4 the highest (Fig. 1b). A chi-square test of independence was performed to examine the relation between the quartile the samples belong to and the tumor's

grade. We found that mPR δ expression is negatively correlated to the tumor grade χ^2 (9, $N = 327$) = 86.454, P value < 0.0001, while mPR ϵ expression is independent of the tumor grade, χ^2 (9, $N = 327$) = 17.388, P value = 0.043.

To further analyze the possible clinical outcome of mPRs expression, samples were classified into *low* (first quartile), *medium* (second and third), and *high* (fourth quartile). Low expression of mPR δ was correlated to poor prognosis, while the opposite is observed regarding mPR ϵ , where patients with low expression live longer than those with higher levels (Fig. 1c). Since gender bias in GBM incidence is well known and expression levels of mPR δ and mPR ϵ are relevant to patient clinical outcomes, samples were stratified by gender in order to test for differences in survival time. No differences were detected for mPR δ since both females and males have a better prognosis if they have a high expression of this gene (Fig. 2a, Supplementary Material 3); on the contrary, males with high levels of mPR ϵ have a significantly lower survival time after diagnosis, while women's prognosis is independent of mPR ϵ expression. (Fig. 2b, Supplementary Material 3).

Fig. 1 mPRs expression in human astrocytoma primary tumor biopsies. RNA-Seq data from 322 astrocytoma primary tumors (55 grade II, 112 grade III, 155 grade IV, or GBM) and 5 normal tissues were obtained from TCGA repository. **a** Log2 fold change of mPRs in GBM compared with normal tissue, significant changes ($P < 0.05$) are denoted in red. **b** Samples were stratified in quartiles according to expression of mPR δ or

mPR ϵ and plotted by tumor grade, and a chi-square test of independence was performed in order to evaluate the correlation between the quartile the samples belong to and the tumor grade. **c** Survival plots of patients with *low* (first quartile, blue), *medium* (second and third, black), and *high* (fourth quartile, red) expression of mPR δ or mPR ϵ

Fig. 2 Differences in survival by patients' gender. Samples from TCGA repository were classified into high and low levels of **a** mPR δ or **b** mPR ϵ expression and stratified by gender in order to test for differences in

survival time. P values were obtained by a log-rank test performed with the TCGAbiolinks package for R version 3.5.2

mPR δ and mPR ϵ Are Expressed in Human GBM Cells Under Basal Conditions

In order to confirm the observations made, basal expression levels of mPR δ (PAQR6) and mPR ϵ (PAQR9) were analyzed by RT-qPCR and Western blot in human GBM cell lines and compared with those in healthy human astrocytes (HA). Interestingly, mPR δ expression was lower in U251 and U87 cells as compared with that in HA. In

contrast, mPR ϵ expression was higher in both GBM cell lines with respect to that in HA. Furthermore, mPR δ levels were 100 times higher than those of mPR ϵ in both cell lines (Fig. 3a). It is important to address that this is the first time that the expression of mPR δ and mPR ϵ is demonstrated in human GBM cells. Western blot experiments showed a band of around 35 kDa for both receptor subtypes (Fig. 3b) in U87 and U251 cells. Both proteins had similar expression patterns in U87 and U251 cells; no significant

Fig. 3 mPRs mRNA and protein expression in human astrocytoma cell lines. **a** RNA extraction followed by RT-qPCR was carried out in U87 and U251 cells cultured under basal conditions. mPRs relative expression (normalized to 18S ribosomal RNA by ΔC_t method) is shown. Healthy human astrocytes total RNA (HA) was used as normal tissue control. **b**

U251 and U87 cells were lysed, and mPRs were evaluated by Western blot. Densitometry of each band was plotted. Three independent cultures for each experiment were done. Results are expressed as the mean $\pm$ S.E.M. (*) P value < 0.001 vs all groups. (#) P value < 0.001 vs U87 and HA, (%) P value < 0.001 vs U251 and HA

differences were found between the mPR δ and mPR ϵ protein content (Fig. 3b).

Intracellular and cell membrane localization of mPR δ and mPR ϵ was also analyzed by immunofluorescence in GBM permeabilized and non-permeabilized cells (Fig. 4). For both receptors, U87 and U251 permeabilized cells showed positive

staining distributed in the cytoplasm and the nucleus, while in non-permeabilized cells, positive staining resulted in punctuated marks distributed across the plasmatic membrane. Permeabilized and non-permeabilized GBM cells incubated without primary antibody were used as negative controls (Supplementary Material 4).

Fig. 4 Cellular localization of mPR δ and mPR ϵ in GBM cells. Intracellular and cell membrane localization of mPR δ and mPR ϵ was detected in U87 and U251 cells by immunofluorescence. Nuclei (blue), mPR positive stain (red), and merge are shown in permeabilized and non-permeabilized cells. All images were captured with a $\times 600$ amplification

P4 Regulation of mPR δ and mPR ϵ in U87 Cells

As previously described by Valadez-Cosmes et al. [30], mPRs expression can be regulated by sex hormones such as P4. In order to predict a possible regulation of mPR δ and mPR ϵ by this hormone, an *in silico* analysis was performed to find putative PRE. Promoter sequences of PAQR6 (mPR δ) and PAQR9 (mPR ϵ) genes were obtained from ENSEMBL (core promoter region, as indicated in the database) and analyzed with several bioinformatic tools (JASPAR, TRANSFAC, UGENE). Only PRE predicted by two or more algorithms (identity score > 0.8 or *P* value < 0.05) were considered as positive hits. In both promoters, potential PRE were found; according to the analysis, PAQR6 has three possible PR binding sites, whereas PAQR9 presents only one (Fig. 5).

RT-qPCR assays were performed in order to evaluate the P4 regulation of mPR δ and mPR ϵ expression in U87 cells. Cells were treated with P4 at different concentrations (1 nM, 10 nM, 100 nM, and 1 μ M) or vehicle (V, 0.02% cyclodextrin) for 12 or 24 h. mPR δ mRNA levels were reduced by P4 100 nM and 1 μ M after 12 h of treatment as compared with V; this inhibition was maintained after 24 h of treatment only by P4 100 nM (Fig. 6). mPR ϵ expression was inhibited by P4 10 nM, 100 nM, and 1 μ M after 12 h of treatment. At 24 h, all the concentrations of P4 inhibited mPR ϵ expression (Fig. 6).

Western blot analysis in U87 cells showed that P4 (1 nM and 100 nM) decreased mPR δ content after 12 h of treatment

compared with V; however, only P4 (1 μ M) significantly decreased mPR δ content after 24 h. No significant changes in mPR ϵ protein content were observed after 12 h with any of the P4 concentrations; however, after 24 h, P4 1 nM, 100 nM, and 1 μ M decreased mPR ϵ content as compared with the V (Fig. 7).

Discussion

GBMs are the most aggressive primary tumors of the CNS due to their poor prognosis. Recent studies have suggested that P4 and its receptors are relevant for GBM progression [26–29]. It has been demonstrated that P4 enhances the infiltration of GBM cells from the tumor area into healthy tissue in the cerebral cortex [29, 30] and that increases proliferation, migration, and invasion of human GBM cell lines [26–28]. However, the use of the pharmacological antagonist of the PR mifepristone (RU486), only partially blocks P4 effects on GBM cells [27, 28, 30], suggesting the participation of other mechanisms triggered by P4 in these cells such as those activated by mPRs.

In particular, our group has focused on mPRs, which are a group of G protein-coupled receptors associated with rapid cell signaling changes induced by P4. There are five mPRs subtypes described in humans: mPR α , mPR β , mPR γ , mPR δ , and mPR ϵ [12, 15, 17]. By analyzing the data from the

Fig. 5 *In silico* analysis of promoter sequences of PAQR6 (mPR δ) and PAQR9 (mPR ϵ). Promoter sequences were obtained from the ENSEMBL database (core promoter region) and analyzed for putative PRE with several bioinformatic tools: JASPAR, TRANSFAC, and

UGENE. For each gene, a white rectangle indicates the promoter region. Black arrows indicate the transcription start site and the gene transcription direction. The putative PRE are denoted with a blue square. A scale bar of 500 bp is included

Fig. 6 P4 effects on mPR δ and mPR ϵ expression. U87 GBM cells were treated with P4 at different concentrations (1 nM, 10 nM, 100 nM, and 1 μ M) or V (0.02% cyclodextrin) for 12 or 24 h. PAQR6 and PAQR9 mRNA levels were quantified by RT-qPCR. Three independent experiments were performed for each sample. mPRs relative expression (normalized to 18S ribosomal RNA by Δ Ct method) is shown. Results are expressed as the mean $\pm$ S.E.M. (*) P value < 0.05 vs V, (#) P value < 0.05 vs V and P4 1 nM, (%) P value < 0.05 vs V, P4 1 nM and P4 10 nM, (&) P value < 0.05 vs P4 100 nM

TCGA, it was observed that mPR β , mPR δ , and mPR ϵ are downregulated in GBM with respect to healthy tissue, which suggests that these receptors are relevant to tumor progression. Previous studies from our group have demonstrated that human-derived GBM cell lines U87 and U251 express mPR α , mPR β , and mPR γ [31] and that mPR α activation increases cell proliferation and invasion by Akt and Src phosphorylation [32]. However, no research was conducted before on mPR δ and mPR ϵ subtypes in GBM.

It was found that mPR δ and mPR ϵ expression is dependent on the tumor grade. There is evidence that mPR δ mRNA is upregulated in prostate cancer cells and this correlates with lower survival rates, suggesting that mPR δ expression in prostate cancer should be considered as a predictor of malignancy [39]. However, in this study, it was shown that patients with high expression of mPR δ have a better prognosis than those with lower levels regardless of gender. On the contrary, high levels of mPR ϵ are associated with poor prognosis, and this effect depends on the patients' gender, which suggests a possible hormone regulation.

By using RT-qPCR and Western blot approaches, we detected the expression of both receptors at mRNA and protein levels in U87 and U251 cell lines with predominant expression of the PAQR6 mRNA in both cell lines (100 times greater) as seen by RT-qPCR. This differential expression had been previously described by Pang et al. in several regions of the human brain such as the forebrain, corpus callosum, hypothalamus, and spinal cord [14]. It is also important to denote that this is the first time that the expression of these receptors is described in GBM cell lines. Expression levels of both receptors were compared in GBM cell lines and normal tissue. mPR δ expression was

lower in U251 and U87 cells compared with HA. However, mPR ϵ expression was higher in both GBM cell lines with respect to HA, which suggests that these receptors have a different role in GBM biology. Analysis of TCGA data of the low-grade glioma and GBM projects confirmed that mPR ϵ expression is lower than that of mPR δ in both astrocytoma and normal tissue, and that the expression of both receptors is correlated to the tumor grade. However, Western blot assays revealed no significant differences in the expression of both receptors. It is essential to mention that mPR δ and mPR ϵ signals detected in Western blot under basal conditions appeared as a single band in the expected molecular weight (~35 kDa); interestingly, the Western blot for the P4 treatments shows a double band which has already been observed for the mPR β in glioma spheroids by Hiavaty et al. [40].

Meanwhile, by immunofluorescence, we detected in non-permeabilized U87 and U251 cells positive marks of mPR δ and mPR ϵ in the cell membrane of both cell lines. As reported before, mPR δ is mainly detected in membrane preparations, and it is associated with Gs α -subunits [14]. Remarkably, we also detected positive marks of both mPRs expression in cytoplasm and nucleus which is not unexpected since mPR α and mPR β intracellular localization has been reported in GBM cells due to the synthesis, transport to membrane, or degradation pathways that occur in membrane receptors [31].

P4 downregulates mPR δ and mPR ϵ in U87 GBM cells after 12 h of exposure; furthermore, we observed by an *in silico* analysis that mPR δ and mPR ϵ gene promoter regions contain PRE sequences, which explains their regulation by P4. One of the molecular functions of P4 in its target cells is to regulate the expression of its own receptors; previously we

Fig. 7 P4 effects on mPR δ and mPR ϵ protein content in U87 human GBM cells. Cells were treated with P4 (1 nM, 10 nM, 100 nM, and 1 μ M) or V (0.02% cyclodextrin) during 12 or 24 h. Cells were lysed, and proteins (30 μ g) were separated by electrophoresis on 12% SDS-

PAGE gel, transferred to a PVDF membrane, and incubated with an antibody against mPR δ or mPR ϵ . Each experiment was performed three times. Results are expressed as the mean $\pm$ S.E.M. * P value < 0.05 vs V; ** P value < 0.01 vs V

have reported that P4 downregulates the expression of the PR and the mPR α , whereas it upregulates the expression of the mPR β in human GBM cells [31, 41]. Gonzalez-Orozco et al. demonstrated that the activation of mPR α in U87 and U251 cells by the specific mPR agonist Org OD 02-0 increases cell proliferation, migration, and invasion [32].

Respecting the mechanisms of action of mPR δ and mPR ϵ , it has been shown that these receptors mediate the neuroprotective effects of P4 and its metabolite allopregnanolone in neurons of the hippocampus in a model of starvation, by increasing the intracellular levels of cAMP, indicating that both membrane receptors are coupled to stimulatory G proteins. Interestingly, in that study, it was also observed that recombinant human mPR δ and mPR ϵ expression in transfected MDA-MB-231 breast cancer cells diminished starvation-induced apoptosis [14].

Both mPR δ and mPR ϵ have high affinity for P4 ($K_d \sim 3$ nM) [14], and it has been shown that allopregnanolone increases proliferation of GBM cells, which suggests that

this metabolite should exert its effects by mPR δ binding, since it has been reported that allopregnanolone has a higher affinity for this receptor subtype compared with PR [42]. These data suggest that P4 and allopregnanolone should induce rapid P4 signaling in GBM cells through the activation of mPR δ or mPR ϵ .

Conclusion

mPR δ and mPR ϵ are relevant to GBM biology. An analysis of TCGA data revealed that mPR δ expression is directly correlated to patients' survival, while the opposite is observed for mPR ϵ . We also demonstrated that human GBM cell lines U87 and U251 express the mPR δ and mPR ϵ subtypes, showing that mPR δ exhibits high content and membrane localization. Moreover, in the U87 cell line, both mPRs are downregulated by P4.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Aylin del Moral-Morales, Juan Carlos González-Orozco, and José Moisés Capetillo-Velázquez. The first draft of the manuscript was written by Aylin del Moral-Morales and Juan Carlos González-Orozco, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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