Activation of β-adrenergic receptor promotes cellular proliferation in human glioblastoma

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Abstract. Glioblastoma multiforme is the most common and aggressive form of primary malignant brain tumor. Previous evidence demonstrates that β-adrenergic receptors (β-ARs) are closely associated with the occurrence and development of brain tumors. However, the functional role of β-ARs in human glioblastoma and the underlying mechanisms are not fully understood. In the present study, by using the MTT assay, western blotting, and the reverse transcription polymerase chain reaction, it was revealed that isoproterenol (ISO), an agonist of β-ARs, promoted the proliferation of U251 cells but not U87-MG cells, and that this effect was blocked by the β-ARs antagonist propranolol. It was also demonstrated that ISO transiently induced extracellular signal-related kinase 1/2 (ERK1/2) phosphorylation, and that blocking the mitogen-activated protein kinase pathway by U0126 inhibited ERK1/2 phosphorylation and suppressed U251 cell proliferation. In addition, β-ARs activation increased the expression of matrix metalloproteinase (MMP) family members MMP-2 and MMP-9 mRNA through ERK1/2 activation. In conclusion, these data suggest that β-ARs induce ERK1/2 phosphorylation, which may in turn increase MMPs expression to promote U251 cell proliferation. These results provide additional insight into the specific roles of β-ARs in glioblastoma.

Introduction

Glioblastoma multiforme (GBM), or grade IV astrocytoma as classified by the World Health Organization (1), is the most common and malignant primary brain tumor in humans, with an incidence rate of 3.19 cases per 100,000 person/year in the United States 2005-2009 (1). The important characteristics of GBM are the high cellular proliferation rate, genetic instability, diffuse infiltration and high angiogenesis, conferring high levels of aggression and drug resistance in GBM (2). In spite of previous significant improvements in treatment, including surgery, radiotherapy and chemotherapy, the overall median survival rate remains only 12-16 months at present (3). This poor prognosis suggests that therapeutic resistance is a significant problem of GBM. Thus, it is urgent to identify novel candidate factors involved in glioma proliferation and angiogenesis that may assist to develop effective targeted therapies for GBM.

A growing number of studies have demonstrated that exposure to stress may promote tumor progression in many types of cancers (4-6) and the adrenergic system has been widely recognized to serve a significant role in stress signaling (7). Exposure to stressful events induces the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn results in the release of glucocorticoids and catecholamines, including norepinephrine (NE) and epinephrine (E), from the adrenal gland and from the brain and sympathetic nerve terminals (8). Concurrently, the secretion of dopamine, which assists to control the reward and pleasure centers of the brain, is reduced. This alteration in homeostasis leads to a microenvironment that is beneficial to tumor growth and progression in experimental models of disease (5). The functions of NE and E are primarily mediated by the activation of adrenergic receptors (ARs) including α-ARs and β-ARs (9). There are 3 subtypes of β-ARs, specifically β1-AR, β2-AR and β3-AR, which have been identified to date (10). β1-ARs and β2-ARs are widely expressed in the majority of mammalian cell types, while β3-ARs are almost exclusively expressed in adipocytes that have seldom been studied (11).

β-ARs are members of the superfamily of G protein-coupled receptors (GPCR), which activate the Gs protein to initiate multiple signaling cascades and therefore lead to numerous pathological conditions such as cardiac, psychiatric, immunological and endocrine disorders (12). In previous decades, numerous studies have demonstrated that β-ARs may also regulate different processes of cancer initiation and progression, through activating the classical cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and
mitogen-activated protein kinases (MAPK) pathway (13,14). For example, in pancreatic ductal adenocarcinomas cells, it has been suggested that β-AR agonists promote cell growth by activating signaling via adenyl cyclase (AC) and its downstream effectors cAMP, PKA and nuclear transcription factor cAMP-responsive element-binding protein (15). In addition, the extracellular signal-related kinase 1/2 (ERK1/2) signaling pathway, activated by transactivating epidermal growth factor receptor (EGFR), is also associated with this process (16,17). Previously, the potential involvement of β-ARs in the modulation of astrocytoma cancer cell proliferation has been suggested (18). However, the detailed underlying mechanisms by which these events occur remain unknown, but are becoming characterized.

The present study was designed to investigate the function and mechanism of β-ARs in human glioblastoma U251 cells. The results demonstrated that the β1-AR and β2-AR subtypes were expressed in U251 cells but not U87-MG cells. In addition, the activation of the β-ARs by isoproterenol (ISO) significantly enhanced the rate of cell proliferation in U251 cells via activation of the ERK1/2 pathway and matrix metalloproteinase (MMP)-2 and MMP-9 mRNA expression. These results may provide additional insight into the specific roles of β-ARs in glioblastoma.

Materials and methods

Antibodies and reagents. Primary antibodies including phospho-ERK1/2 (Thr202/Tyr204) (cat no. 9101; 1:2,000), ERK1/2 (cat no. 9120; 1:2,000) and β-actin (cat no. 4970; 1:4,000), the secondary antibody horseradish peroxidase (HRP)-linked goat anti-rabbit IgG antibody (cat no. 7074; 1:4,000) and U0126 (a specific ERK1/2 inhibitor) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). ISO and propranolol (PRO) were purchased from Tocris Bioscience (Bristol, UK). The culture medium and other solutions used for cell culture were purchased from Invitrogen, Thermo Fisher Scientific, Inc. (Waltham, MA, USA). MTT was purchased from Carl Roth GmbH & Co., KG (Karlsruhe, Germany).

Cell culture. Human glioblastoma cell lines U87-MG and U251 were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 mM glutamine, 50 µg/ml streptomycin and 50 IU/ml penicillin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Reverse transcription polymerase chain reaction (RT-PCR). The total cellular RNA from the U251 and U87-MG cells was extracted using TRIzol reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The RNA concentration was measured using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). Then the RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). PCR amplification was performed using 5 µl cDNA, 12.5 µl 2X Taq PCR Master Mix (Promega Corporation, Madison, WI, USA), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer and 6.5 µl ddH2O. The specific primers used are summarized in Table I. The thermocycling protocol was as follows: Amplification step, 94°C for 5 min; 30 cycles of denaturation for 1 min at 95°C; 1 min of annealing at 55°C; elongation at 72°C for 1 min; and extension at 72°C for 1 min. The PCR products were electrophoresed on a 1.5% agarose gel and the bands in the gels that were stained with ethidium bromide were visualized under ultraviolet light transilluminators (Chemidoc XR® System; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gene β-actin was used as a control.

RT-quantitative PCR (qPCR). The total RNA and cDNA were obtained as aforementioned. The PCR primers used for the analysis are summarized in Table I. The 20 µl PCR system was composed of 2 µl cDNA, 10 µl SYBR® Premix Ex Taq II, 0.4 µl ROX Reference Dye II (Takara Bio, Inc., Otsu, Japan), 0.8 µl 10 µM forward primer, 0.8 µl 10 µM reverse primer and 6 µl double-distilled H2O. The RT-qPCR amplification was performed using the ViiA7 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycler conditions: 95°C for 30 sec; 40 cycles of denaturation for 5 sec at 95°C; 34 sec of annealing at 60°C; elongation at 95°C for 15 sec; and extension at 60°C for 1 min. The quantification method for qPCR data was the 2ΔΔCt method (19). At least three independent experiments were conducted and samples were assessed in triplicate for each experiment. The same PCR system as above was used without template cDNA was used as a negative control.

Western blot analysis. Cells were cultured in serum-free DMEM overnight at 37°C prior to drug treatment. To determine the time course effect of ISO on ERK1/2 activation, cells were treated with ISO (10 µM) for 1, 2, 5, 10, 20 and 30 min at 37°C. To determine the effects of propranolol (PRO) and U0126 on ISO-mediated ERK1/2 activation, cells were pretreated with PRO (10 µM) or U0126 (20 µM) for 30 min and then with ISO (10 µM) for 5 min at 37°C. Following treatment, cells were washed with ice-cold PBS twice, and lysed in ice-cold lysis buffer. The lysate was sonicated and centrifuged at 12,000 x g at 4°C for 5 min. The protein concentration of extracts was determined by using the Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein (20 µg/lane) were loaded and separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA), and blocked in 5% non-fat dry milk for 1 h at room temperature. Subsequently, the membrane was incubated with different primary antibodies including phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 and β-actin overnight at 4°C and then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:20,000) for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) and semi-quantified using ImageJ software (version 1.47t; National Institutes of Health, Bethesda, MD, USA).

MTT assay. Cellular viability was measured using the MTT assay as described previously (20). Briefly, cells were seeded in flat-bottomed 24-well plates (5x104 cells/well) and grown for 24 h in DMEM supplemented with 10% FBS. Following
24 h, cells were starved overnight at 37°C and incubated with different drugs. To determine the dose course effect of ISO on the proliferation of U251 and U87-MG cells, cells were treated with 0.1, 1, 5, 10, 30 or 50 µM ISO for 24 h at 37°C. To determine the time course effect of ISO on the proliferation of U251 cells, cells were treated with ISO (10 µM) for 24, 48, 72 and 96 h at 37°C. To determine the effects of PRO and U0126 on ISO-mediated proliferation, cells were pretreated with PRO (10 µM) or U0126 (20 µM) for 30 min and then with ISO (10 µM) for 48 h at 37°C. Following drug treatment, 20 µl MTT (5 mg/ml) was added to each well, and the cells were allowed to grow in complete media at 37°C for 3 h. The supernatant was removed, then 500 µl dimethyl sulfoxide was added to each well and mixed for 10 min to dissolve the crystal. Subsequently, the absorbance was determined using a microplate spectrophotometer assay reader at 570 nm.

**Statistical analysis.** Data are indicated as the mean ± standard error of the mean from at least three independent experiments. Statistical analysis was performed using the unpaired two-tailed Student's t-test (comparison of two groups) or one-way analysis of variance followed by the Tukey's test (comparison of >2 groups). P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**Expression of β-ARs in human glioblastoma cell lines.** To detect whether β-ARs were expressed in cultured glioblastoma cells, the two cell lines U251 and U87-MG were profiled for β₁-AR and β₂-AR mRNA expression. The RT-qPCR analysis revealed that β₁-AR and β₂-AR transcripts were expressed in U251 cells; however, they were undetectable in U87-MG cells (Fig. 1A). These results were confirmed by RT-qPCR analysis (Fig. 1B). The β₂-AR transcript levels were increased compared with β₁-AR in U251 cells (Fig. 1B; P=0.0135). These data suggest that β-ARs may be functional and serve a role in the development process of U251 cells.

**Isoproterenol promotes the proliferation of U251 cells.** Based on the expression profile of β-ARs in U251, but not U87-MG cells, whether β-ARs activation was involved in U251 cell proliferation was then investigated. Firstly, the dose-dependent effect of ISO, an agonist of β-ARs, on the proliferation of U251 cells was analyzed. The cells were treated with 0.1, 1, 5, 10, 30 or 50 µM ISO for 24 h and then proliferation was measured by MTT assay. These results revealed that ISO significantly increased the proliferation of U251 cells, which peaked at 10 µM ISO and decreased at higher concentrations of ISO (Fig. 2A). Notably, no effect of ISO-mediated cell proliferation was detected in U87-MG cells (Fig. 2B). This is in accordance with the results demonstrating no expression of β-ARs in U87-MG cells (Fig. 1A). Secondly, the effect of different time treatment of ISO on U251 cell proliferation was detected. The cells were incubated with 10 µM ISO for 24, 48, 72 or 96 h. As indicated in Fig. 2C, ISO significantly increased the proliferation of U251 cells, with the most marked effect at 48 h. Thus, 10 µM ISO was chosen as the reference concentration, and 48 h as the treatment time for the following studies unless specifically indicated.

To additionally confirm that ISO-induced proliferation was specifically mediated by β-ARs, the U251 cells were pretreated with β-AR antagonist PRO and then stimulated by ISO. As expected, PRO pre-treatment to block endogenous β-ARs activity significantly suppressed ISO-induced U251 cell proliferation (Fig. 2D). Taken together, these results indicate that specific activation of β-ARs is able to promote the cell proliferation in U251 cells.

**Table I. Primers used for reverse transcription PCR and quantitative PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1-AR</td>
<td>5'-GGGAGAGCGATTTAGGG-3'</td>
<td>5'-CAAGGAAAAAGCTGGG-3'</td>
<td>270</td>
</tr>
<tr>
<td>β2-AR</td>
<td>5'-CAGGAAAAGCAGGGTTG-3'</td>
<td>5'-AAATGCTGAAGTTGAC-3'</td>
<td>334</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-ATCGAGGGGCTGACATTAGAGG-3'</td>
<td>5'-AGGAAAGGAAGCTGGAAG-3'</td>
<td>179</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5'-CCGTCGCCCATCATCAATTC-3'</td>
<td>5'-GCAGGCACTAGAGGTTCAGG-3'</td>
<td>90</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-TGGTCTCTGGCTCTGTTG-3'</td>
<td>5'-GCTGCTGTTCGGTGAATGG-3'</td>
<td>111</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; β-AR, β-adrenergic receptors; MMP, matrix metalloproteinase.
**β-ARs activation induces ERK1/2 phosphorylation in U251 cells.** The aforementioned results indicated that ISO treatment may significantly promote U251 cell proliferation. The underlying molecular mechanisms of ISO-induced cell proliferation in U251 cells were then explored. MAPK pathways have been widely recognized to regulate a variety of physiological processes including cell proliferation, differentiation and apoptosis in cancer cells (21). Thus, to define the role of MAPK cascades in the effect elicited by ISO, cells were treated with 10 µM ISO for 1, 2, 5, 10, 20 and 30 min, and then ERK1/2 phosphorylation levels were detected using western blotting. ISO induced ERK1/2 phosphorylation in a rapid and transient manner, which peaked at 5 min and decreased following 20 min, while ERK1/2 expression levels remained unchanged (Fig. 3A and B). Additionally, ERK1/2 phosphorylation was significantly blocked by PRO pretreatment (Fig. 3C and D), suggesting β-ARs activation is able to induce ERK1/2 phosphorylation in U251 cells.

**ISO-induced proliferation is mediated by ERK1/2 pathway.** Whether ISO-mediated ERK1/2 activation was involved in cell proliferation of U251 cells was investigated. The effects of U0126, a specific MAPK/ERK (MEK)1/2 inhibitor, on ISO-induced ERK1/2 phosphorylation were first explored. As demonstrated in Fig. 4A and B, pretreating U251 cells with U0126 effectively abolished ISO-induced ERK1/2 phosphorylation. Next, the MTT assay showed that the proliferative effects of ISO were also significantly suppressed by U0126 (Fig. 4C). These results indicate that ERK1/2 pathway is essential for ISO-mediated proliferation in U251 cells.

**β-ARs activation increases MMP-2 and MMP-9 mRNA expression through ERK1/2 activation.** MMPs, particularly
the gelatinases MMP-2 and MMP-9, have been documented as key factors to the proliferation and invasion of cancer cells (22,23). Whether β-ARs activation regulates the expression of MMP-2 and MMP-9 mRNA in U251 cells was then examined. As demonstrated in Fig. 5A, the results indicated that MMP-2 and MMP-9 mRNA expression was significantly upregulated following ISO treatment. In addition, the pretreatment of U251 cells with U0126 inhibited ISO-induced MMP-2 and MMP-9 mRNA expression (Fig. 5B and C). These results suggest that β-ARs-mediated MMP-2 and MMP-9 mRNA expression occurs through the ERK1/2 pathway.

**Discussion**

The main results of the present study concern the mechanism of β-ARs-mediated cell proliferation of glioblastoma cells. It was demonstrated that: i) β₁-AR and β₂-AR are expressed in U251 cells but not in U87-MG cells; ii) activation of β-ARs by ISO promoted the proliferation of U251 cells through the ERK1/2 pathway; and iii) β-ARs activation increased MMP-2 and MMP-9 mRNA expression, which may be important for U251 cell proliferation.

Previous studies have demonstrated that β-ARs are expressed in human clinical glioblastoma specimens obtained from operated patients (24,25). Additionally, studies have indicated that β-ARs are also expressed in glioblastoma cell lines and primary cultures derived from human biopsies (26) and in the human-derived 1321N1 astrocytoma cell line (27). In addition, studies with the human-derived U118 glioma cell line indicate that there is a low but significant expression of β-ARs in these cells, but to a lesser extent compared with 1321N1 cells (18). However, the function of β-ARs in U87-MG cells remains debated. Previous studies demonstrate that in vitro U87-MG cells do not express functional...
β-ARs (18,24). However, all three subtypes of β-ARs appear to be detectable in U87-MG tumors in vivo, indicating that β-ARs expression processes may take place and may be functional during tumor development (24). Additionally, (R,R')-4-methoxy-1-naphthylfenoterol, a selective β₂-AR agonist, was identified to inhibit cellular proliferation of U87-MG cells (28). All these data suggest that β-ARs may also be functional, by an unknown mechanism. Nevertheless, the exact function of β-ARs in U251 glioma cells and the underlying mechanism are not fully understood. In the present study, the expression of β-ARs in U251 and U87-MG cells was first measured by RT-PCR and RT-qPCR. The results demonstrated that β₁-ARs and β₂-ARs were expressed in U251 cells, but not in U87-MG cells, which was consistent with a previous study (24).

In previous years, evidence has suggested that β-AR agonists and antagonists affect cell growth and function, which may lead to the inhibition or induction of malignant diseases (29). The effect of β-AR activation is cell-specific as β₂-AR antagonists have been demonstrated to attenuate cell growth. For example, β-ARs activation may promote cell proliferation of certain cancer cells including lung cancer cells (20), ovarian carcinoma (5), human hepatocellular carcinoma cells (30), pancreatic (31), prostate (32), gastric (33) and colorectal cancer cells (34). By contrast, several studies have demonstrated that the β-AR agonist ISO suppresses the proliferation of MDA-MB-231 human breast cancer cells (35,36). In addition, in U118 glioma cells and 132N1 astrocytoma cells, treatment with β₂-AR agonists (R,R')-fenoterol or isoproterenol inhibited cell proliferation (18). However, the results of the present study indicated that the activation of β-ARs by ISO promoted U251 cell proliferation. How this difference occurs, and the underlying mechanism, requires additional investigation. As aforementioned, obtaining β-AR expression in U87-MG tumors in vivo would imply that β-AR may facilitate tumor formation by promoting cellular proliferation. Another potential probability is that this cell type-specific divergence on cellular proliferation may be due to cross-talk between β-ARs and other GPCR-linked signaling cascades including gamma-aminobutyric acid B receptors (GABA_B receptors) (37), α₂-AR (38), bradykinin B1 receptors (39), oxytocin (40), and cannabinoid receptors (28). For example, the ISO-induced signaling cascade, cellular proliferation and cellular migration in pancreatic ductal adenocarcinoma cells may be potently inhibited following stimulation of GABA_B receptor signaling (37).

The MMPs are a family of proteolytic enzymes that regulate various cell behaviors that are relevant in cancer biology through the degradation of the extracellular matrix surrounding tumors (41). Although MMPs, particularly MMP-2 and MMP-9, have been considered to be important factors in facilitating invasion and metastases through the degradation of type IV collagen, a major component of the basement membrane, additional evidence also demonstrates that MMPs may promote cancer cell proliferation (23). Previous, studies have suggested that NE may affect the progression of ovarian cancer by modulating the expression of MMPs and the angiogenic cytokine, vascular endothelial growth factor, in ovarian cancer cells (42). Additionally, β-ARs inhibition suppressed the expression of MMP-2 and MMP-9 in human brain microvascular endothelial cells (24) thus increasing the proliferative, invasive and metastatic potential of these cells. In the present study, it was revealed that MMP-2 and MMP-9 mRNA expression was significantly increased upon β-ARs activation. Consistently, the proliferation of U251 cells was also enhanced through the activation of β-ARs. In addition, pretreatment with U0126 to block ERK1/2 activity significantly reduced the upregulation of MMP-2 and MMP-9. Previous studies have demonstrated that the activation of the EGFR-MEK-ERK signaling pathway causes the overexpression of MMP-2 and MMP-9 in prostate cancer cells (43). However, whether EGFR transactivation pathway or other signaling cascades are involved in β-ARs-induced cell proliferation in glioblastoma cells requires additional study.

Acknowledgements

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