cAMP-Epac Pathway Stimulation Modulate Connexin-43 and MicroRNA-21 Expression in Glioma Cells

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Article info:
Received: 23 May 2014
First Revision: 25 June 2014
Accepted: 11 October 2014

Key Words:
cAMP, Epac, Cx43, miR-21, Glioma, 1321N1, U87MG, Beta adrenergic receptor

ABSTRACT

Introduction: Malignant astrocytic gliomas are the most common and lethal brain malignancies due to their refractory to the current therapies. Nowadays, molecular targeted therapy has attracted great attention in treatment of glioma. Connexin 43 (Cx43) and micro ribonucleic acid-21(miR-21) are among molecules that are involved in glioma development and progression. These molecules showed potential to be as target molecules with regard to glioma. Some studies have reported that cyclic adenosine monophosphate (cAMP) signaling could be effective on Cx43 and miR-21 in tissues other than in brain. We investigate possible relationship between β-adrenergic receptor and its newly described downstream, exchange protein directly activated by cAMP (Epac) signaling pathway and expression of Cx43 and miR-21 in low (1321N1) and high grade (U87MG) glioma cell lines.

Methods: We treated cells with β-adrenergic agonist and Epac activator with and without adenyl cyclase inhibitor. Cx43 and miR-21 expression were measured with real-time PCR.

Results: Our data showed that in 1321N1 cells, β-adrenergic-Epac pathway stimulation up and down-regulated Cx43 and miR-21 expression respectively. Whereas, in U87MG cells these interventions had no effect on Cx43 and miR-21 expression.

Discussion: These findings demonstrate that low grade astrocytoma cells have better response to our pharmacological interventions.

1. Introduction

Brain tumors including glioma and glioblastoma are diverse groups of malignancies that remain unresponsive to conventional treatment approaches, including radiotherapy and cytotoxic chemotherapy. So these tumors are coupled with a high mortality rate and poor survival. Molecular neuro-oncology has now started to elucidate signaling pathways that may be amenable to molecular targeted therapy (Furnari et al., 2007). Previous studies demonstrated that some glial markers including Cx43 that down-regulated in this pathophysiology changed in glioma (Huang et al., 1999). Cx43, the primary protein forming gap junction channels in astrocytes, has been probed to a great degree owing to its growth inhibitory effects (Kardamia et al.,

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2007). Reduction or loss of Cx43 expression which mediated cellular interconnection is commonly observed in glioma. Suggesting that the expression of Cx43 have inverse correlation with the degree of malignancy (Huang et al., 1999). Over-expression of Cx43 led to reduction of proliferation and formation of tumor (Huang et al., 1998). Phosphorylation of Cx43 by protein kinase C (PKC) decreases dye coupling in many cell types (Bao et al., 2004).

MicroRNAs are small non-coding RNAs, which function in RNA silencing and post-transcriptional regulation of gene expression. Alteration in expression of microRNAs (miRNAs) has correlation with several cancers, including brain tumors and specifically glioma (Lawler et al., 2009). They can play both role of oncogenes or tumor suppressors by binding to 3' un-translated region (3'UTRs) of tumor-suppressor genes and oncogenes respectively (Novakova et al., 2009). MiR-21 is one of the miRNAs that up-regulated in glioma. One study suggested that aberrantly expression of miR-21 may block expression of apoptotic genes and thereby exert anti-apoptotic effect (Chan et al., 2005). Down-regulating of miR-21 inhibited epidermal growth factor receptor (EGFR) pathway and glioma growth. In combinational therapies with S-Trail, cytotoxic effect enhanced (Corsten et al., 2007; Zhou et al., 2010) and human glioblastoma cells become more sensitive to chemotherapy agents (Ren et al., 2010).

Given that the records in the Oncoming cancer profiling database (http://www.oncomine.org) proposes that a major portion of gliomas express the beta 2 adrenergic receptor (ß2-AR) to a greater extent than in normal brain tissue. So this receptor stands for potential therapeutic target for treatment of these tumors (Toll et al., 2011). Some studies showed relationship between cAMP and Cx43 expression and gap junction gating in tissues such as heart (Xia et al., 2009). Also different lines of reports have showed relationship between cAMP level and miRNAs expression (Lu et al., 2009; Keller et al., 2012). Previous works have demonstrated that some effects of cAMP depend on its newly described downstream andEpac pathway. In other words, besides protein kinase A (PKA), cAMP has another intracellular downstream pathway, (Epac), that mediate some effects of cAMP in mammalian cells (Bos, 2006).

Considering the roles of cAMP signaling pathway, Cx43 and miR-21 in the pathogenesis of glioma we evaluate the effect of β-adrenergic receptor (βAR) and Epac signaling pathway stimulation on Cx43 and miR-21 expression in glioma cells.

2. Methods

2.1. Materials

All drugs in this study including non-selective β adrenergic agonist, Isoproterenol hydrochloride (Iso) [16504], selective β2-AR agonist, Clenbuterol hydrochloride, (C5423), adenyl cyclase inhibitor, SQ 22,536 (S 153), PKA specific inhibitor (H-89) [B1427], Epac-specific activator 8-(4-chlorophenylthio)-2-O-methyl-adenosine-3,5-cyclic monophosphate (8CPT) [C8988] were purchased from Sigma-Aldrich (USA). Qiazol and reverse transcriptase were purchased from Qiagen and vivantis respectively. Treatment condition wasthe same as what we performed in our previous study (Mostafavi et al., 2014).

2.2. Cell culture

The human glioblasoma cell line U87MG and human astrocytoma cell line 1321N1 were obtained from the Stem Cell Technology Research Center, (Tehran, Iran) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) supplemented with 100 unit/ml penicillin and 50 mg/ml streptomycin under a humidified atmosphere at 5% CO_2 and 37˚C temperature. Confluent monolayers were passaged routinely by trypsinization. Cells were plated for RNA extraction analysis in six-well plates. Cells were treated with 70-80% confluence following medium change with DMEM containing 1% FBS.

2.3. Real-time RT PCR

The relative quantification of CX43 gene expression was carried out on treated cells and controls 24 hours after treatment. Total cellular messenger RNA (mRNA) was isolated using the Qiazol (Qiagen) according to manufacturer's protocol and then used for complementary deoxyribonucleic acid (cDNA) synthesis by reverse transcriptase (vivantis Cat No: RTPL12). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) served as the reference gene. The primers used in the reactions had the following sequences: Cx43 forward: 5′- GAT GAG GAA GGA AGA GAA G -3′, Cx43 reverse: 5′- CGC TAG CTT GCT TGT TGT AA -3′, Epac2 forward: 5′- CGA TTC ACT GAC TCC CTT AC -3′, Epac2 reverse: 5′- CTT CCA AAT GTG TGA TAG ATT AG -3′, HPRT1 forward: 5′- CCT GGC GTC GTG ATT AGT G -3′, HPRT1 reverse: 5′- TCA GTC CTG TCC ATA ATT AGT CC -3′. Gene expression levels were quantified by Rotor Gene 6000 (Corbett, Concorde, NSW, Australia). The relative expression ratio of Cx43 and HPRT1 in

U87MG and 1321N1 cells under control conditions and after 24h of treatment with our drugs were calculated using the relative expression software tool (REST).

2.4. miRNA expression quantification

To evaluate miR-21 expression, real-time reverse transcription polymerase chain reaction (RT PCR) was done for treated and control cells. After 24h, treated cells were collected for miRNA extraction by Qiazol reagent (Qiagen). cDNA synthesis was done using Stratagene real-time PCR kit and according to manufacturer's protocol. Briefly, after poly-adenylation of total RNAs, the cDNA was synthesized by adaptor primer provided by company.

The relative quantification of miRNAs in comparison with control cells was assessed by real-time RT PCR with Stratagene SYBR green master mix (Stratagene), according to the manufacturer's instructions in a Rotor-Gene 6000 system (Corbett, Concorde, NSW, Australia) [n=3]. The relative expression ratio of miRNAs under control conditions and after 24h of treatment with our drugs was normalized relative to U6 endogenous control and then was calculated using the REST. The primers used in the reactions had the following sequences: miR-21 sense: 5'-GAA TTC CGA TCT TAA CAG GCC AGA AAT GC-3', miR-21 reverse: 5'-AGA TCT CCA CCA GAC AGA AGG ACC AGA GT-3', U6 sense: 5'-CTC GCT TCG GCA GCA CA-3', U6 reverse: 5'- AAC GCT TCA CGA ATT TGC GT-3'.

2.5. Statistical analysis

The obtained data were analyzed by a statistical software package (SPSS for Windows) and P-values<0.05 were considered statistically significant. P-values less than 0.05 were considered as statistically significant. Asterisk (*) indicates that the result is significant. Each experiment repeated independently at least three times.

3. Results

3.1. EPAC2 expression in glioma cell lines (U87MG and 1321N1) under different conditions

Based on previous reports EPAC2 is present in developing and mature brain (Bos et al., 2006). To check expression of EPAC2 in U87MG and 1321N1 cell lines, we performed RT-PCR analysis. As illustrated in Figure 1, we found that these glioma cell lines express EPAC2 mRNA. However, our treatments did not change significantly the level of EPAC2 mRNA (Figure 1).

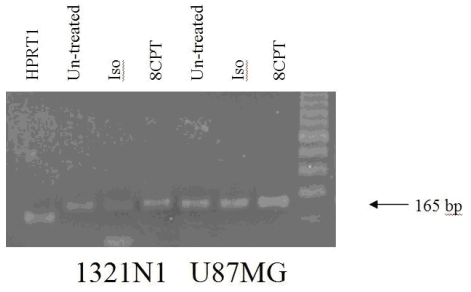

Figure 1. Detection of endogenously expressed Epac2 in 1321N1 and U87MG cell lines (165 base pairs) in un-treated and treated cells with 20 µg/ml Iso and 25µg/ml selective Epac activator (8CPT). HPRT1 was used as internal control (100 bp).

3.2. Effect of non-selective βAR stimulation and Epac signaling pathway on Cx43 mRNA expression

In 1321N1 cells, Iso significantly upregulates the Cx43 mRNA level (P<0.01). Pretreatment of cells with SQ for 45 minutes before adding the Iso, showed Adenylate Cyclase (AC) inhibition and blocked Iso effect on the Cx43 mRNA expression. Selective Epac stimulation using 8CPT did not change the Cx43 mRNA expression compared to un-treated group (Figure 2A). In U87MG cells, beta agonist treatment had no significant effect on the Cx43 expression. As expected, when these cells pretreated with AC inhibitor, no effect was detected. Finally, U87MG cells were treated with 25 µg/ml of 8CPT, no significantly change in Cx43 mRNA was detected (Figure 2B). These data suggested that non-selective βA stimulation only increases Cx43 mRNA level in low grade astrocytoma cell line (1321N1) and Epac stimulation had no effect on Cx43 expression in both low and high grade glioma cell lines.

3.3. Effect of selective β2AR stimulation and Epac signaling pathway on miR-21 expression

To evaluate the selective β2AR stimulation effect on miR-21 expression, we inevitably used only 1321N1 cells, because previous studies reported that U87MG cells do not express β2AR (Toll et al., 2011). After 24 hours treatment, 10 µg/ml of selective β2-AR agonist significantly decreased miR-21 expression level in 1321N1 cells (P<0.05). Treating cells with Clenbuterol 20 µg/ml, decreased miR-21 expression (P<0.05), but this effect was not dose dependent. Pre-treatment with AC inhibitor, reversed selective β2AR stimulation effect. Application of Epac selective agonist decreased

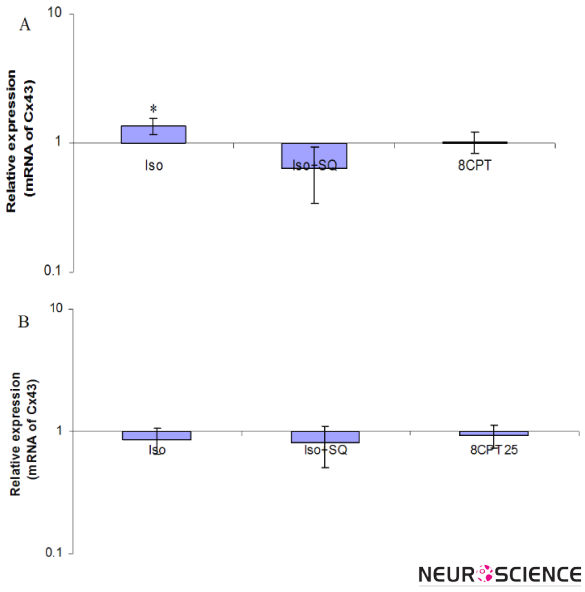

Figure 2. Non- selective βAR and Epac pathway stimulation effects on Cx43 mRNA expression. 1321N1 (A) and U87MG (B) cells treated with Iso (20 µg/ml) [βAR agonist], Iso (20 µg/ml) + AC inhibitor (20 µg/ml) [Iso +SQ] and 8CPT (25 µg/ml) [Epac activator] for 24 hours and changes in transcript amount were determined by real-time quantitative PCR (qPCR). It was revealed that only Iso increased Cx43 expression in 1321N1 cells. (*P< 0.01, Comparison were done relative to untreated controls).

significantly miR-21 expression in this cell (P<0.05) (Figure 3).

3.4. Effect of non-selective βAR stimulation and Epac signaling pathway on miR-21 expression in U87MG cells

Based on previous reports that showed functional relationship between cAMP and some miRNAs expression (Lu et al., 2009; Keller et al., 2012), here we evaluated putative regulation of miR-21 by stimulation of non-selective βAR and Epac signaling pathway in U87MG cells by real time-PCR. After 24 hours treatment, although the amount used (20 µg/ml of Iso and 25 µg/ml of 8CPT) decreased miR-21 expression, but these effects were not significant (Figure 4).

4. Discussion

4.1. cAMP-Epac effects on Cx43 expression

In our study, βAR stimulation increased Cx43 mRNA level in 1321N1 astrocytoma cells, but Epac activator did not boost the βAR effect. In addition, in U87MG cells Isoproterenol treatment and Epac signaling pathway stimulation had no effect on Cx43 mRNA level significantly. These results suggest that Epac signaling pathway has no role on βAR stimulation, and malignancy severity may change the rate of response to treatment.

Cx43 is one of the brain and astrocytes markers. Alteration of this marker resulted in progression of glioma due to its physiological roles (Huang et al., 1999). Previous studies demonstrated that Cx43 controls cell growth and has tumor suppressor and growth inhibitory effects (Iacobas et al., 2012). Decreasing levels of Cx43 and gap junction interconnection are commonly observed in glioma (Huang et al., 1999) and up-regulation of Cx43 seem to had suppressor effect on tumor (Sanchez-Alvarez et al., 2006). In order to find a treatment solution for malignant brain tumors based on targeted molecular agents, researches have used differentiation agents such as cAMP and its related signaling components (Toll et al., 2011,Kurino et al., 2002). Change in cAMP and its down-stream signaling pathway including PKA and Epac have demonstrated to affect connexin level. This signaling pathway has also been shown to be a major modulator of Cx43 in the heart (Somekawa et al., 2005). However, the molecular nature of βAR stimulation and cAMP derivative-induced effect on Cx43 expression in glioma cells were not evaluated. Previous researches showed that Cx43 expression level was changed in some pathophysiologic conditions including glioma (Huang et al., 1999). Studies on peripheral organs like heart showed that activation of cAMP and its down-stream pathways may affect Cx43 expression (Somekawa et al., 2005). These results suggest that cAMP signaling only up-regulate Cx43 in low grade astrocytoma cells but not in malignant glioma.

We also found that βAR stimulation enhanced Cx43 expression via cAMP activation in low grade astrocytoma cell line. However, Epac selective agonist did not

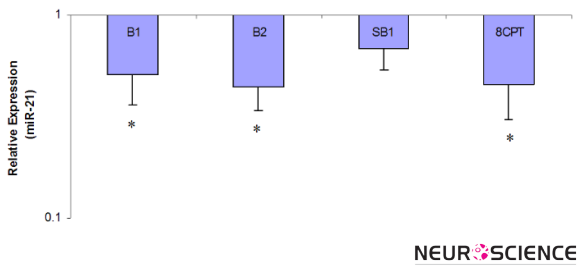

Figure 3. Selective β2-AR and Epac pathway stimulation effects on miR-21 expression. 1321N1 cells treated with 10 (B1) and 20 (B2) µg/ml of Clenbuterol, 10 µg/ml of Clenbuterol + 20 µg/ml of adenyl cyclase inhibitor (sterol biosynthesis inhibitor; SB1) and 25 µg/ml of 8CPT (Epac activator). It was observed that both doses of Clenbuterol and 8CPT could down regulate miR-21 expression. (*: P< 0.05, Comparisons were done relative to untreated controls).

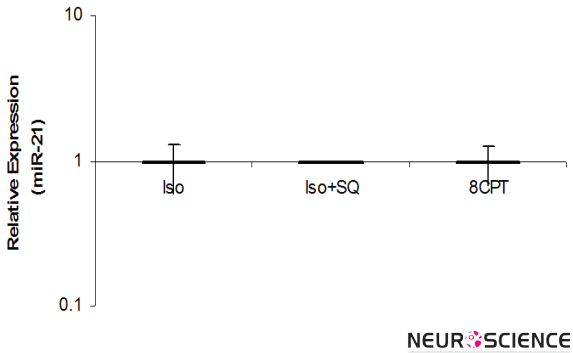

Figure 4. Non- selective βAR and Epac pathway stimulation effects on miR-21 expression. U87MG cells treated with Iso (20 µg/ml) [βAR agonist], Iso (20 µg/ml) + AC inhibitor (20 µg/ml) [Iso +SQ] and 8CPT (25 µg/ml) [Epac activator] for 24 hours and changes in miRNAs were determined by real-time qPCR. It was revealed that none of treatments had significant effects. (Comparisons were done relative to untreated controls).

change Cx43 mRNA expression, in our previous study (Mostafavi et al., 2014), 25 µg/ml selective Epac agonist led to enhancement of Cx43 protein but not Cx43 mRNA in 1321N1 cells. Thus, we concluded that this amount of Epac agonist presumably had post-translational effect instead of transcriptional effect.

4.2. cAMP-Epac effects on miR-21 expression

According to our findings, lower doses of Clenbuterol (10 and 20 µg/ml) down-regulated miR-21 expression significantly, although it was without dose dependency. As high grade U87MG cells do not express β2-AR, so we evaluated cAMP signaling pathway stimulation effects with Isoproterenol and selective Epac activator on miR-21 expression and the results showed that none of our treatments had significant effect on this miRNA expression.

MiRNA genes are located in fragile sites of the genome and this provides an adequate testimony for the roles of miRNAs in tumors including glioma (Farazi et al., 2011). Several reports have shown the potential use of miRNA-based approach in glioma therapy (Silber et al., 2009). MiR-21 being one of the most up-regulated miRNAs in gliomas, with anti-apoptotic and pro-invasive functions, appears to be an automatic choice for therapeutic interventions (Chan et al., 2005; Papagiannakopoulos et al., 2008). Our previous data (Mostafavi et al., 2014), showed that selective β2-AR stimulation with the highest dose of Clenbuterol (40 µg/ml) led to up-regulation of miR-451 in low grade 1321N1 astrocytoma cells. It seems that pharmacological interventions are effective only in low grade astrocytoma and for molecular target therapy in high grade glioma, other strategies such as interference RNA may be more effective.

In conclusion, this study revealed that Cx43 and miR 21 expression in high grade glioma did not modulated with cAMP-Epac signaling pathway. However, pharmacological interventions in low grade astrocytoma could be effective on Cx43 and miR 21 expression. Modulation of mentioned molecules may open interesting new therapeutic approaches for glioma, although further studies are needed to expose complex relationship and their long term regulation and to confirm these findings for the eventual applications in clinics.

Acknowledgment

This study was supported by grant #12290 from Deputy of Research, Tehran University of Medical Sciences. We also want to thank Stem Cell Technology Research Center, Tehran, Iran, for their support in supplying materials.

Conflict of Interest statement

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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