Selective β2 adrenergic agonist increases Cx43 and miR-451 expression via cAMP-Epac

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Abstract. It has been demonstrated that connexin 43 (Cx43) and microRNAs have significant roles in glioma. Cyclic adenosine monophosphate (cAMP) is suggested to be a regulator of connexins and microRNAs. However, it remains elusive whether cAMP and exchange protein directly activated by cAMP (Epac2), have a regulatory effect on Cx43 and microRNA-451 (miR-451) in astrocytoma cells. We treated 1321N1 astrocytoma cells with a selective β2 adrenergic agonist and a selective Epac activator with and without adenyl cyclase and protein kinase A inhibition. Cx43 and miR-451 expression were measured. Next, we evaluated the effect of miR-451 overexpression on Cx43 expression. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results demonstrated that cAMP-Epac2 increased Cx43 and miR-451 expression. However, the alteration of miR-451 expression required a higher dose of drugs. Overexpression of miR-451 had no significant effect on Cx43 expression. The MTT assay showed that cAMP-Epac stimulation and miR-451 overexpression had a synergic inhibitory effect on cell proliferation. These findings may expand our understanding of the molecular biology of glioma and provide new potential therapeutic targets.

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Introduction

Gliomas and astrocytomas are brain tumors that are associated with a high mortality rate and current treatments have not significantly improved patient survival (1). The gap junction, which is required for maintaining cell growth, has been reported to be absent in gliomas and astrocytomas. The gap junction is made of connexin proteins, which have been proposed to act as tumor suppressors (2,3). Connexin 43 (Cx43) is the major protein forming gap junction channels in astrocytes and has been proposed to have growth inhibitory effects. Expression of Cx43 is inversely correlated with the degree of malignancy (3). It is accepted that connexins not only act as critical gatekeepers of cell proliferation by controlling the intercellular exchange of essential growth regulators, but they may also affect cell cycling by non-gap junctional intercellular communication (4).

By contrast, the abnormal expression of microRNAs (miRNAs) has been linked with several types of cancer, including glioma (5). miRNAs are endogenous eukaryotic small, non-coding RNAs that negatively regulate gene expression (6). The study of the expression profile of miRNA in glioblastoma indicated that mir-451 is significantly decreased in glioblastoma compared with normal brain tissue (7). Overexpression of miR-451 in glioblastoma cells led to the inhibition of glioblastoma cell growth, invasive ability and enhanced apoptosis (8).

Another line of evidence indicated that higher grades of human brain tumors are associated with lower adenyl cyclase activity and/or cellular cAMP concentrations (9). It was stated that the β2-adrenergic receptor (β2-AR) is expressed in

glioblastomas, as well as in the human-derived 1321N1 astrocytoma cell line (10). cAMP and its related compounds inhibit the proliferation of tumor cells (11). Furthermore, studies have revealed an association between cAMP and Cx43 (12), and also between cAMP levels and the expression of miRNA (13). By contrast, other studies have demonstrated an association between miRNAs and Cx43 expression (14).

Since the data in the Oncomine cancer profiling database (http://www.oncomine.org) suggests a significant portion of gliomas and astrocytomas express the β2-AR to a greater extent than in normal brain tissue, this receptor represents a potential therapeutic target for the treatment of these tumors (15). In the present study, we analyzed the possible interaction between the cAMP-Epac signaling pathway, Cx43 and miR-451 expression in astrocytoma cells.

Materials and methods

Reagents. All drugs, including a selective β2-AR agonist, clenbuterol hydrochloride (C5423), adenylyl cyclase inhibitor (SQ 22,536; S153), PKA specific inhibitor (H-89; B1427) and Epac-specific activator 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclic monophosphate (8CPT; C8988) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies including mouse monoclonal to cx43/GJA1 (ab79010), rabbit polyclonal to Epac2 (ab21238), mouse monoclonal to β-actin (ab6276), rabbit polyclonal secondary antibody to mouse IgG (ab6728) and goat polyclonal secondary antibody to rabbit IgG (ab6721) were purchased from Abcam (Cambridge, MA, USA).

Cell culture. The human astrocytoma cell line 1321N1 was obtained from the National Cell bank of Iran, (Tehran, Iran) and maintained in DMEM containing 10% FBS supplemented with 100 unit/ml penicillin and 50 mg/ml streptomycin in a humidified atmosphere at 5% CO_2 and 37˚C. Cells were plated for RNA extraction and western blot analysis in six-well plates. Prior to drug exposure in all experiments, the medium of 70-80% confluent cells was changed with DMEM containing 1% FBS.

Cell proliferation assay. Cell proliferation was quantified using an MTT assay (Sigma-Aldrich). Mir-451 transfected and untransfected cells were seeded in 96-well plates with a density of $0.5x10^4$ cells/well containing DMEM. The attached cells were incubated for another 24 and 48 h with of 10 μg/ml ß2 agonist; 10 μg/ml ß2 agonist + 20 μg/ml AC inhibitor; and 20 μg/ml Epac activator, with DMEM as a control. Next, cells were incubated with 5 mg/ml of MTT solution at 37˚C for 3 h. We dissolved the formazan crystals in DMSO. The optical density of each well was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) measuring at a wavelength of 570 nm with background subtraction at 630 nm. The experiments were performed and repeated 3 times. We determined each treatment for mean values ± SE.

Real-time RT-PCR. Total RNA, isolated with QIAzol (Qiagen, Hilden, Germany) according to the manufacturer's instructions, was used for complementary DNA (cDNA) synthesis by reverse transcriptase (Vivantis, Oceanside, CA, USA; cat no: RTPL12). Specific primers for human Cx43 (forward: 5'-GAT GAG GAA GGA AGA GAA G-3', reverse: 5'-CGC TAG CTT GCT TGT TGT AA-3') and β-actin (forward: 5'-GCA AGA GAG GTA TCC TGA CC-3', reverse: 5'-CCC TCG TAG ATG GGC ACA GT-3') were used for this experiment using Takara SYBR Premix Ex Taq Master (Takara Bio, Inc., Shiga, Japan). Gene expression levels were quantified by Rotor-Gene 6000 (Corbett, Concorde, NSW, Australia). The relative expression ratio of Cx43 and β-actin were calculated using the relative expression software tool (REST).

Immunoblotting assay. Total cell lysates were prepared following harvesting cells in ice-cold PBS and homogenization in cold lysis buffer (RIPA buffer). Protease inhibitor cocktail (S8820; Sigma-Aldrich) was added to the lysis buffer. The lysate was centrifuged at 13,680 x g for 10 min and the supernatant was transferred to a new tube. Protein content was measured using the bicinchoninic acid assay (BCA protein kit; Pierce Biotechnology, Inc., Rockford, IL, USA). An equal amount of protein (25 μg) per well was loaded to 12.5% standard SDS-PAGE, then by using a wet system (Bio-Rad, Hercules, CA, USA), the proteins and page ruler were transferred to PVDF membranes. Membranes were blocked in 2% skimmed milk in TBST 20 (Tris-buffered saline (TBS)/Tween-20; 0.5%) for 2 h at room temperature. Membranes were incubated overnight at 4˚C with primary antibodies against cx43 and Epac2 diluted in TBST (1:1000; 0.5%). Membranes were washed three times with TBST 20 and subsequently incubated for 1 h at room temperature with the following corresponding HRP-conjugated secondary antibodies: rabbit polyclonal secondary antibody to mouse IgG and goat polyclonal secondary antibody to rabbit IgG. Following three washes with TBST 20 (0.5%), detection was performed by applying Pierce ECL Plus Western Blotting Substrate (#32134), which produced chemiluminescence from the membrane for manual X-ray film development. Levels of the analyzed proteins were normalized to β-actin levels.

Virus packaging, concentration and transduction of 1321N1 cells. For virus packaging of PB_miR-451 (contained green fluorescent protein (GFP) and puromycin resistance genes), we used a human embryonic kidney 293T cell line (HEK 293T). These cells were maintained in DMEM containing 10% FBS supplemented with 100 unit/ml penicillin and 50 mg/ml streptomycin in a humidified atmosphere at 5% CO_2 and 37˚C. Cells were co-transfected with psPAX2 plasmid containing gag/pol packaging genes, pMDG plasmid containing VSV-G and PB_miR-451 (as well as an empty vector) using the calcium-phosphate method. Following 24 h of transfection, GFP expression was assessed using an inverted fluorescence microscope (TE2000; Nikon, Tokyo, Japan). The medium containing the produced viruses were collected 24, 48 and 72 h following transfection and kept at 4˚C followed by concentration processing, including centrifuging at 21,000 rpm or 40,000 g at 4˚C for 2.5 h. The concentrated supernatants were used to infect 1321N1 human astrocytoma cells.

Puromycin treatment and total RNA extraction. Following 24 h of transduction, transducted cells were treated with puromycin (2 μg/ml) and treatment was continued for 3 days.

Cells in the negative control groups were also treated with puromycin and as expected died after 48 h. The experimental group (that was transfected with PB_miR-451) was resistant to puromycin and survived the antibiotic selection. Total RNA (containing miRNAs) was collected following 72 h puromycin treatment using QIAzol Lysis Reagent (Qiagen, Valencia, USA). An RNA pellet was dissolved in 20 μl distilled water and maintained in -70˚C until further use.

miRNA expression verification. To assess the constructs' functionality, overexpression of miR-451 was evaluated by RT-PCR in transducted 1321N1 cells. Following 72 h, transducted cells were harvested for miRNA extraction by Qiazol reagent (Qiagen). The extracted microRNAs were subjected to cDNA synthesis by Stratagene (La Jolla, CA, USA) real-time RT-PCR kit according to the manufacturer's instructions. Briefly, following poly-adenylation of total RNAs, the cDNA was synthesized by an adaptor primer provided by Stratagene. The relative quantification of miRNAs in comparison to control cells was assayed 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 miRNA in 1321N1 cells under control conditions and following 24 h of treatment with our drugs or overexpression was normalized relative to a U6 endogenous control and was then calculated using REST. Specific primers for human miR-451 and U6 were: miR-451 sense: 5'-GGA AGA TCT TGA CAA GGA GGA CAG GAG AG-3', miR-451 reverse: 5'-CCC AAG CTT GCC TTG TTT GAG CTG GAG TC-3', U6 sense: 5'-CTC GCT TCG GCA GCA CA-3', U6 reverse: 5'-AAC GCT TCA CGA ATT TGC GT-3'.

Statistical analysis. The obtained data were analyzed using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference (indicated with an asterisk *). Each experiment was repeated independently at least three times.

Results

Epac2 expression in 1321N1 astrocytoma cells. Previous studies demonstrated the presence of Epac2 in the developing and mature brain (16). To determine the putative presence of Epac2 in the 1321N1 astrocytoma cell line, we performed a western blot analysis to detect endogenous Epac2 and to test the effect of the specific β2-AR agonist and 8CPT on its expression in astrocytoma cells. As depicted in Fig. 1, we found that these cells express the Epac2 protein endogenously. Protein bands were detected at a molecular weight of 126 kDa for Epac2. The selective β2-AR agonist and Epac activator did not significantly increase Epac2 expression.

Effect of β2-AR stimulation and the Epac signaling pathway on Cx43 expression. Next, we tested the specificity of cAMP signaling on Cx43 expression in astrocytoma cells. Following 24 h treatment of cells with our drugs, selective β2-AR stimulation with clenbuterol hydrochloride (10 μg/ml) led to an increase in Cx43 protein level (Fig. 2). This effect was not dose dependent (data not shown). 1321N1 cells were pretreated with adenyl cyclase (AC) inhibitor, SQ 22,536 (20 μg/ml) and

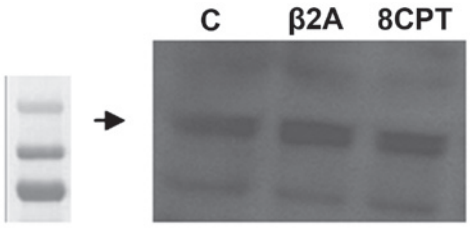

Figure 1. Detection of endogenously expressed Epac2 in 1321N1 cells using western blotting. Protein bands of Epac2 were detected at a molecular weight of 126 kDa in C, 24 h treated cells with 10 μg/ml β2A and 20 μg/ml 8CPT. 10% SDS-PAGE was used. This experiment was repeated at least three times independently. C, control or untreated; β2A, selective β2 adrenergic agonist; 8CPT, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate.

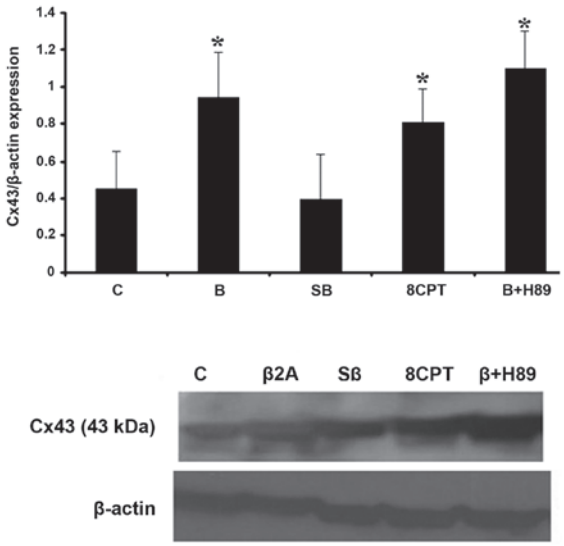

Figure 2. Expression of Cx43 protein in the 1321N1 cells. Cx43 protein levels were normalized to that of β-actin protein. Data are expressed as the means ± SEM. Each immunoblotting was duplicated to increase the reliability of the measurements. Lower panels show representative immunoblots of Cx43 (43 kDa). *$P<0.05$ vs. control group. Cx43, connexin 43; C, control or untreated; B, β2 agonist; SB, AC inhibitor + β2 agonist; 8CPT, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate; B+H89, β2 agonist + PKA inhibitor.

H-89 dihydrochloride (10 μg/ml), as a selective PKA inhibitor, for 45 min in the presence of clenbuterol. Pretreatment with SQ prevented clenbuterol upregulation of Cx43 while cells pretreated with H-89 had no effect on Cx43 upregulation, suggesting that upregulation of Cx43 observed in the presence of clenbuterol does not occur without the PKA pathway. To further investigate the possible role of Epac in the upregulation of Cx43 we used the selective Epac activator 8-pCPT-2'-O-Me-cAMP (20 μg/ml). We observed that the protein level of Cx43 was increased following 24 h treatment with this drug.

Effect of β2-AR stimulation and the Epac signaling pathway on miR-451 expression. Putative regulation of miR-451 by our drugs was verified by real time-PCR. Following 24 h treatment, a higher dose of β2-AR selective stimulation significantly increased miR-451 levels, as 10 and 20 μg/ml of clenbuterol had no significant effect on miR-451 expression. The minimum dose of clenbuterol for the modulation of

miR-451 was 40 μg/ml. Adenyl cyclase inhibition prevented these changes suggesting adenyl cyclase activation is able to increase the miR-451 expression level. To examine whether Epac activation is also capable of affecting miR-451 expression, we treated cells with 8CPT. Selective activation of Epac had the same effect of β2-AR stimulation with higher doses, as miR-451 increased significantly upon treatment with 8CPT (Fig. 3).

Evaluation of miR-451 overexpression in 1321N1 cells. For overexpression of miR-451, HEK 293T cells were used for virus packaging and these cells were transfected by the recombinant vector PB_miR-451. The fluorescent image of HEK 293T cells following transfection indicated that the majority of the cells were green due to the existence of GFP in the vector (Fig. 4A and B). Produced viruses were used for transduction. The fluorescent image of 1321N1 cells following transduction indicated that the majority of the cells were green (Fig. 4C and D). Total RNA was extracted from 1321N1 cells and real-time PCR was employed for evaluating the expression of miRNA. Results showed that miR-451 was overexpressed in comparison with the negative control (Fig. 5).

Effect of miR-451 overexpression on Cx43 and mRNA expression. Three days following puromycin treatment, the expression level of Cx43 mRNA, was evaluated using real-time PCR. Real-time PCR results demonstrated non significant alterations in the expression level of the Cx43 gene. Although the expression ratio of Cx43 mRNA was 1:2 in the overexpressed group compared with the negative control group, this difference was not significant (Fig. 6).

Synergic inhibitory effects of cAMP-Epac and miR-451 on cell proliferation. The cell proliferation assay revealed that growth was inhibited in transfected and treated cells in comparison with control cells. Maximum inhibition was observed in transfected cells that were treated with cAMP-Epac related drugs. These results demonstrate that miR-451 and the cAMP-Epac pathway may have a synergic effect on the inhibition of glioma cell growth (Fig. 7).

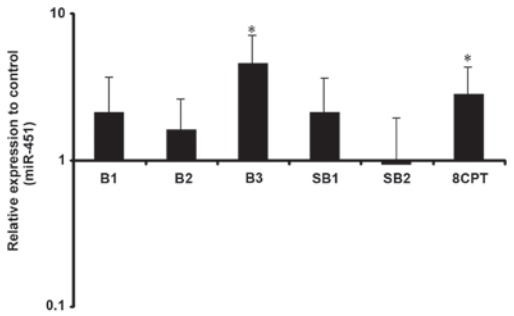

Figure 3. Logarithm of fold change of miR-451 following 24 h treatment of cells with 10 (B1), 20 (B2) and 40 μg/ml of clenbuterol (B3), 10 μg/ml of clenbuterol + 20 μg/ml of adenyl cyclase inhibitor (SB1), 20 μg/ml of clenbuterol + 20 μg/ml of adenyl cyclase inhibitor (SB2) and 8CPT (Epac activator). It was observed that the highest dose of clenbuterol (40 μg/ml) and 8CPT is able to upregulate miR-451. Error bars show the S.D. This experiment was repeated at least three times independently. *P<0.05, compared with untreated controls. 8CPT, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate.

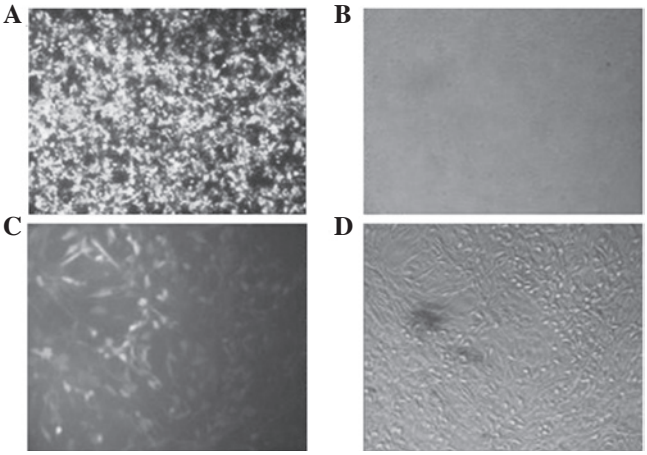

Figure 4. Confirmation of transfection and transduction. HEK 293T cells were transfected with vector PB-miR-451-eGFP [plasmid containing sequences for microRNA 451 and enhanced green flourescent protein reporter gene (eGFP)] calcium-phosphate method. (A and B) Fluorescent and phase contrast image of HEK 293T cells. Efficient transfection of HEK 293T cells was confirmed by visualizing GFP expression after 24 h by an inverted fluorescence microscope. (C and D) Fluorescent and phase contrast image of 1321N1 cells. 1321N1 cells were transfected with the virus product and following puromycin selection, cells were visualized by GFP expression. HEK 293T, human embryonic kidney cell line 293T; GFP, green fluorescent protein.

Discussion

There is widespread agreement that gap junction channels formed by the Cx43 protein are likely to play important roles (17). Cx43 has a high turnover rate with a half-life of ~1-2 h, this condition permits rapid adjustments of cell-cell communication with transcriptional regulation and degradation (18). Modulation of cAMP and its relevant signaling pathway Epac are reported to affect the level of Cx43 and its phosphorylation in the heart (19,20) and endothelial cells (21). However, the molecular nature of β2-AR stimulation and cAMP derivative induced Cx43 expression in astrocytoma cells remains unclear. We demonstrated that β2-AR stimulation through activation of the cAMP cascade upregulates Cx43 expression by increasing its mRNA and protein expression in 1321N1 astrocytoma cell lines. Investigation of the signaling pathway demonstrated that cAMP and its newly described signaling pathway, Epac2, mediates β2-AR-enhanced Cx43 expression. Previous studies which treated myocardial cells with a non selective beta adrenergic agonist stated that β adrenergic receptor and cAMP signaling pathway activation led to upregulation of Cx43 via activation of PKA and CREB (12,22). One study in 2009 stated that corticotropin-releasing hormone upregulates Cx43 expression partly via the PKA-CREB signaling pathway in cultured astrocytes (23). Since the connexin content of tumors decreases (2), the upregulation of connexins may be of therapeutic value (3). We hypothesized that selective stimulation of β2-AR and the cAMP-Epac signaling pathway results in Cx43 upregulation in astrocytoma cells. To assess this hypothesis, we evaluated the protein level of Cx43 in 1321N1 cells following our pharmacological interventions on cultured cells. Our results demonstrated that 24 h treatment with a β2-AR selective agonist increased the protein level of Cx43 and this effect was blocked by adenyl cyclase inhibitor,

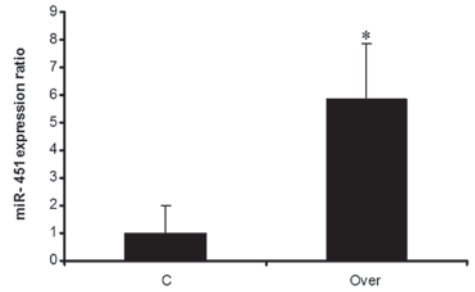

Figure 5. Expression ratio of miR-451 following transduction of 1321N1 cells. It was observed that miR-451 was upregulated 3 days following puromycin treatment compared with a control. Error bars show the S.D. This experiment was repeated at least three times independently. *P<0.01 compared with untreated controls. C, negative control; Over, transducted 1321N1 cells.

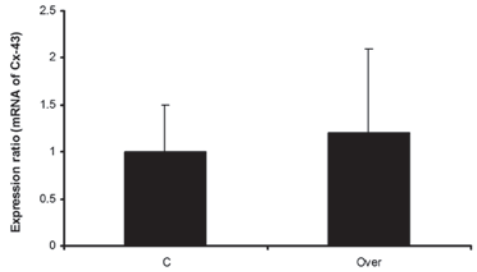

Figure 6. Expression ratio of Cx43 mRNA in 1321N1 cells following overexpression of miR-451. It was observed that following 3 days of puromycin treatment, mRNA of Cx43 in transducted cells increased, however not significantly compared with control cells. Error bars show the S.D. This experiment was repeated at least three times independently. Cx43, connexin 43; C, negative control; Over, transducted 1321N1 cells.

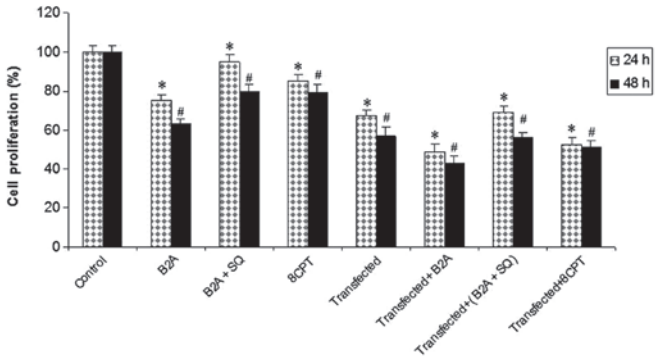

Figure 7. Synergic effects of cAMP-Epac signaling stimulation and overexpression of miR-451 on cell proliferation. MTT assay in C, 24 and 48 h treated cells with B2A, B2A+SQ, 8CPT, miR-451 transfected, transfected cells treated with B2A, B2A+SQ and 8CPT. Error bars show the S.D., n=3. *P<0.05, significantly different from control 24 h; **P<0.05, significantly different from control 48 h. C, control or untreated; B2A, 10 μg/ml β2 agonist; B2A+SQ, 20 μg/ml AC inhibitor + 10 μg/ml β2 agonist; 8CPT, 20 μg/ml Epac activator.

SQ. To investigate whether upregulation of Cx43 was working through the Epac signaling pathway, we inhibited PKA by H89 in the presence of β2-AR agonist. Selective β2-AR stimulation with simultaneous inhibition of the PKA pathway and thereby indirect stimulation of the Epac pathway had the same effect as selective β2-AR stimulation. Also, selective Epac stimulation in our cells increased the total protein level of Cx43. Toll *et al* (2011) demonstrated that selective β2-AR simulation in 1321N1 cells led to the inhibition of cell proliferation (15). By contrast, other studies indicated that connexins may affect the growth of normal or tumor cells, however the precise molecular mechanism by which connexins are capable of inducing this effect remains to be elucidated. Recent studies have demonstrated that connexins, including Cx43 exert important and extensive effects on gene expression, particularly those genes linked to growth regulation (17). Therefore, increasing Cx43 expression in astrocytoma cells may have a beneficial effect in controlling their growth and responsiveness to current treatments.

Effect of the cAMP-Epac signaling pathway on the expression of microRNAs. The profiling of miRNAs in various human disorders and their role in the etiology or treatment of them caught the attention of researchers (24). miR-451 was identified as a tumor suppressor in glioma and it was demonstrated that overexpression of miR-451 in glioma cells changed their biological behavior (8). Thus, we hypothesized that stimulation of the cAMP-Epac signaling pathway may alter miR-451 expression level in the 1321N1 astrocytoma cell line. Our results revealed a dose-dependent association between selective β2-AR stimulation and miR-451 expression level. While we have not observed any changes with 10 and 20 μg/ml of clenbuterol, increasing clenbuterol doses to 40 μg/ml, upregulates miR-451 expression level. Selective Epac signaling pathway stimulation by 8CPT upregulated miR-451 as well. Our findings are in line with other studies which demonstrated that the cAMP signaling pathway has a regulatory effect on miRNA expression (13). Our study focused mainly on the cAMP-Epac pathway and we demonstrated that Epac signaling is also involved in the alteration of miRNA biogenesis. We used astrocytoma cell lines in our study and it appears that other glioma cell lines must be studied before an accurate conclusion may be acquired. Although studying the regulation of miRNA transcription is an emerging field and further study is needed, the evidence we have presented suggests a new effect for the cAMP-Epac pathway as a regulator of miR-451 at the transcriptional level in astrocytoma cells. However, higher doses of the β2-AR agonist are needed in order to alter miR-451 expression. Administration of the β2-AR agonist and Epac selective stimulation is expected to upregulate miR-451 *in vivo*.

Functional analyses of the first identified miRNAs determined their roles in cell growth and differentiation (25). MiR-451 is significantly downregulated in glioma compared with normal brain tissue. The overexpression of miR-451 in glioma cell lines led to decreased proliferation and invasion (8). Based on our previous results, we demonstrated that cAMP has a regulatory effect on Cx43 and miR-451, therefore we speculate that these two components have an association between them. Next, we overexpressed miR-451 in the 1321N1 astrocytoma cell line and, following that, Cx43 expression level was evaluated. Our results demonstrated that overexpression of miR-451 had no effect on Cx43 mRNA expression and protein level. It seems that the effects of miR-451 and Cx43 in the progression and/or controlling of tumors were applied independently. However, further investigation is required in order to elucidate the combinational effect of these biological components in patho-physiological processes. For example, simultaneous overexpression of miR-451 and Cx43 in astrocytoma cells may uncover their cooperative effect.

This study yielded several new findings. To the best of our knowledge, we demonstrated, for the first time, that selective β2-AR and its related cAMP-Epac signaling pathway stimulation led to the increase of Cx43 expression in astrocytoma cells. Secondly, the β2-adrenoceptor-cAMP-Epac signaling pathway positively regulates miR-451 expression. Thirdly, overexpression of miR-451 had no effect on Cx43 expression. These findings not only increase our knowledge of certain aspects of the molecular biology of astrocytoma cells, but also may provide new potential therapeutic and drug targets. There are certain fundamental implications of our findings. In astrocytoma and glioma, Cx43 and miR-451 were downregulated. Upregulation of them may aid the treatment of astrocytoma and glioma, due to their specific effects since the effects of Cx43, including decreasing cell proliferation (4) and the bystander effect (26) have been proven in previous studies. MiR-451 overexpression by gene delivery or pharmacological intervention may be used with chemotherapy due to its effect in the enhancement of tumor cell sensitivity to chemotherapy agents.

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