Blockage of Potassium Channel Inhibits Proliferation of Glioma Cells Via Increasing Reactive Oxygen Species

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The potassium (K⁺) channel plays an important role in the cell cycle and proliferation of tumor cells, while its role in brain glioma cells and the signaling pathways remains unclear. We used tetraethylammonium (TEA), a nonselective antagonist of big conductance K⁺ channels, to block K⁺ channels in glioma cells, and antioxidant N-acetyl-l-cysteine (NAC) to inhibit production of intracellular reactive oxygen species (ROS). TEA showed an antiproliferation effect on C6 and U87 glioma cells in a time-dependent manner, which was accompanied by an increased intracellular ROS level. Antioxidant NAC pretreatment reversed TEA-mediated antiproliferation and restored ROS level. TEA treatment also caused significant increases in mRNA and protein levels of tumor-suppressor proteins p53 and p21, and the upregulation was attenuated by pretreatment of NAC. Our results suggest that K⁺ channel activity significantly contributes to brain glioma cell proliferation via increasing ROS, and it might be an upstream factor triggering the activation of the p53/p21Cip1-dependent signaling pathway, consequently leading to glioma cell cycle arrest.

Key words: Glioma; Potassium channel blocker; Cell proliferation; Reactive oxygen species (ROS)
to the nonmalignant human cortical tissues, biopsies from patients with malignant gliomas showed dramatic overexpression of BK channels (22). Importantly, tumor malignancy grades are positively correlated with BK channel expression, suggesting an important role for the BK channel in glioma biology (23). All these suggest that BK channels, as a key player in controlling membrane potential, are critical in glioma proliferation (1,24), and there is a close link between BK channels and tumor growth (14,21). However, the role of K⁺ channels in the process of the cell cycle is poorly understood.

BK channel antagonist TEA has been reported to inhibit cell proliferation, for example, preventing mitogen-stimulated quiescent lymphocytes from becoming rapidly cycling tumor cells (1). Overexpression of BK channels in human gliomas significantly enhanced tumor sensitivity to TEA (18). Given the importance of BK channels in the glioma’s progression and TEA inhibition of BK channels in cell proliferation, we hypothesized that TEA might have an antiproliferative effect on glioma cells. So far, study about the relation between K⁺ channel activity and glioma cell proliferation has been very limited. In our previous study (25), we have shown that TEA inhibited the proliferation of C6 and 9L glioma cells and led to cell apoptosis, possibly because of an imbalance of Bcl-2/Bax expression and increased reactive oxygen species (ROS) level. ROS plays an important role in carcinogenesis via inducing apoptosis through the induction of cellular oxidative stress. Antioxidant N-acetyl l-cysteine (NAC) is a free radical scavenger and has been used as a tool investigating the role of ROS in numerous biological and pathological progresses. In this study, we used TEA to block BK channel activity in C6 or human U87 glioma cells to explore the role of BK channels in glioma cell proliferation and its underlying mechanism.

MATERIALS AND METHODS

Cell Lines and Reagents

Rat C6 and human U87 cells (glioma cell lines that express the wild-type p53 gene) were obtained from the Harbin Medical Neurosurgical Institute (Harbin, China). Glioma cells were cultured in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL) and maintained in a humidified incubator (95% air and 5% CO₂) at 37°C (Excela ECO-170). Chemicals TEA, NAC, 2',7'-dichlorofluorescein diacetate (DCFH-DA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). TEA was dissolved in phosphate-buffered saline (PBS) to make a 1-M stock solution, and 40 mM working solution was obtained by diluting with culture medium. NAC was dissolved in PBS to give a 50-mM stock solution and 500 μM working solution was obtained by diluting with medium. DCFH-DA was prepared as a 10-mM stock solution in dimethyl sulfoxide (DMSO). MTT was dissolved in PBS to obtain a 5-mg/ml work solution. All chemicals were diluted to desired concentration immediately before use. The final concentration of DMSO was less than 0.1%.

Treatments and Groups

Each glioma cell line was divided into the following four groups: 1) the control group (no treatment), 2) the TEA group, 3) the NAC group, and 4) the TEA+NAC group. Cells were treated with 40 mM TEA or 500 μM NAC for 24, 48, or 72 h and then subject to the following experimental assays. For the combination treatment, cells were pretreated with NAC for 30 min before application of TEA.

MTT Assay

The MTT assay was used to determine the number of viable cells in cultures (25). Cells were harvested in the log phase and diluted to 4×10⁵ cells/ml, and 200 μl cell suspension was then seeded in the 96-well plate. At 48 h after treatment, cells from each group were incubated with MTT for 4 h. The optical density reflecting the viable cell population was determined by spectrophotometry (Bio-Rad) at room temperature.

Cell Cycle Analysis

Cell cycle analysis was conducted using the standard method as reported by Wang et al. (26). Briefly, 4×10⁶ cells were suspended in PBS and centrifuged for 5 min at 1,500×g. The cells were resuspended and fixed in 1 ml cold 70% ethanol for 18 h. The cell pellets were washed with PBS and resuspended in 0.5 ml of 30 μg/ml propidium iodide (Sigma-Aldrich)/0.1% Triton X-100 solution containing 400 μg/ml RNaseA (Sigma-Aldrich). After 0.5-h incubation at 37°C in the dark, the fluorescence-positive cells were sorted and analyzed on a FACS Aria flow cytometer (Becton Dickinson, USA). The data were analyzed with FlowJo software, and the relative percentage of cells in each phase of the cell cycle was determined.

ROS Detection

ROS level was measured as previously described (25). After treatment, cell suspension at a concentration of 2×10⁶ cells/ml was incubated with 10μMDCFH-DAat 37°C for 30 min. In this reaction, DCFH-DA is first transported across the cell membrane and deacetylated by esterases to form the nonfluorescent 2',7'-dichlorofluorescein (DCFH). Intracellular ROS oxidizes DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF) that can be visualized at 488 nM excitation (emission at 525 nm). The cells were then harvested and washed three times with cold PBS, and the ROS levels were then determined by immunofluorescence (Leica, Germany) and FACS Aria analysis.
RNA Extraction and Quantitative
Real-time PCR (qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol® reagent (Invitrogen, USA) according to the manufacturer’s instructions. qRT-PCR was performed in triplicate in the Roche LightCycler real-time PCR System (Applied Biosystems) and normalized with β-actin endogenous control. Endogenous mRNA levels of p53 and p21Cip1 were detected using the SYBR Green PCR Master Mix kit in accordance with the manufacturer’s instructions (Clontech Laboratories, CA, USA). The real-time PCR primers for p53, p21Cip1, and β-actin are listed in Table 1. All PCR primers were synthesized by Clontech Laboratories. Relative mRNA expression was calculated by the mean value with the comparative Ct method (ΔΔCt).

Immunoblotting

Anti-p53, anti-p21Cip1, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology Biotech (CA, USA). The C6 and U87 glioma cells were treated with or without 40 mM TEA and 500 μM NAC for the indicated time periods. After treatment, the cells were harvested for the total protein extraction. The protein concentration was determined using the Bradford method. For Western blot analysis, equal amounts of protein were loaded and separated by SDS-PAGE. The gels were equilibrated in transfer buffer (50 mM Tris, 40 mM glycine, 0.375% SDS, and 20% methanol) and electrophoretically transferred to a NC membrane (Millipore, USA). The membrane was blocked with 5% skim milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and incubated overnight at 4°C with specific primary antibodies. After washing with TBST, the membrane was incubated with fluorescence-conjugated secondary antibodies (Invitrogen, USA) for 1 h. The binding of the secondary antibody was quantified using the Odyssey v1.2 software (LI-COR, USA) and normalized with β-actin.

Statistical Analysis

All values showing in the figures were obtained from at least three independent experiments, and data were presented as mean ± SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons. In those experiments where experimental values were normalized to controls, statistical difference from controls was calculated with unpaired Student’s t test. SPSS 17.0 was used for statistical analysis. A value of p < 0.05 was accepted as statistical significance.

RESULTS

K+ Channel Blockage Inhibited Proliferation of Glioma Cells

To study the role of K+ channel in the proliferation of glioma cells, TEA was used to block the K+ channel activity in cultured C6 and U87 cells. Cell viability of each group was assessed using the MTT assay at 72 h after treatment (Fig. 1). TEA-treated C6 (Fig. 1A) and U87 (Fig. 1B) cells at 72 h displayed in a dose-dependent manner. Results of the MTT assay showed that TEA treatment significantly inhibited the proliferation of C6 and U87 cells in a time-dependent manner (Fig. 1C, D), indicating that blockage of voltage-gated K+ channels inhibited proliferation of glioma cells over time. Treatment with antioxidant NAC alone showed no effect on the proliferation of glioma cells. However, pretreating cells with 500 μM NAC for 30 min reversed TEA-caused inhibition of cell proliferation. In the presence of NAC, the viability of both C6 and U87 reversed to the control level at 24 and 48 h, while it still remained significantly lower than the control level at 72 h.

Arrest of Cell Cycle in Glioma Cells by TEA

To determine the mechanisms underlying the K+ channel blockage-induced inhibition of glioma cell proliferation, flow cytometry was used to analyze the effect of TEA on the cell cycle of these cells. The representative distribution profiles of C6 and U87 cells from each group are shown in Figure 2A, B. After 48 h of TEA treatment, both C6 and U87 cells showed significantly increased cell population arrest at the G0/G1 phase (Fig. 2C, D). C6 cells from the TEA group showed significantly higher percentage of G1 phase cells (81.77 ± 0.62%) than the control group (69.79 ± 1.71%, p<0.001, n=3) (Fig. 2C).

Table 1. The Nucleotide Sequences of the PCR Primers Used to Assay Gene Expression by Real-Time Quantitative PCR

| Gene (rat) | Forward Primer | Reverse Primer |
|------------|----------------|----------------|
| p21Cip1    | 5'-CAGGGCTCAGTTGACCAAA-3' | 5'-ACTGGAGCTGCTGAGTGAG-3' |
| p53        | 5'-TGGAGCTGAGGACACCAAG-3' | 5'-GAGGTGACCCACACAAGCACA-3' |
| β-Actin    | 5'-GGAGATTCTGGCGCTGTCCT-3' | 5'-GACTCATCTTGCCCTGTCG-3' |

| Gene (human) | Forward Primer | Reverse Primer |
|--------------|----------------|----------------|
| p21Cip1      | 5'-GCTAATGTCAGCAGGCAACCT-3' | 5'-CATGCCCCTGTCCCATAGGCTC-3' |
| p53          | 5'-GCTGTTGGAGATTGGATGACAGA-3' | 5'-TGATGCTGAGGGTGAACTGAC-3' |
| β-Actin      | 5'-TGGCACCAGCACAATGAA-3' | 5'-CTAAGTCATAGTCGGCCTAGAAGCA-3' |
U87 cells from the TEA group had also increased G0/G1 cell distribution (82.56 ± 1.16%) compared to the control group (67.67 ± 1.20%, \( p < 0.001, n = 3 \)) (Fig. 2D).

In contrast, pretreatment with 500 μM NAC reversed the TEA-caused cell cycle arrest and resulted in similar G0/G1 distribution to that of the control group in both C6 and U87 cells.

**TEA-Induced Increase of ROS in Glioma Cells**

Production of intracellular ROS in glioma cells in response to treatments was measured by detecting the fluoresce intensity of DCF (488 nm). Representative fluorescent images of C6 and U87 cells from different groups are presented in Figure 3A, B. Compared to the control group, C6 and U87 cells treated with 40 mM TEA for 48 h exhibited increased fluorescent intensity. C6 and U87 cells with NAC or NAC + TEA treatment showed similar levels in the fluorescent intensity to that of the control group.

We further quantitatively analyzed the level of ROS in cells from each group using flow cytometry. Representative flow cytometry results for C6 and U87 cells are presented in Figure 4A, B. Quantitative results showed that 48-h TEA treatment significantly increased ROS production in both C6 (\( p < 0.001 \)) and U87 cells (\( p < 0.001 \)) compared to the control group (Fig. 4C, D). In contrast, NAC treatment alone decreased ROS production in C6 (\( p < 0.05 \)) and U87 (\( p < 0.01 \)) cells, suggesting the antioxidant effect of NAC consistent with previous reports. Although cells pretreated with NAC followed by TEA treatment still showed higher levels of ROS production compared to the control group, their ROS levels were significantly lower than that of the TEA group (\( p < 0.001 \)). This result indicated that TEA-induced ROS production in glioma cells...
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cells was temporally correlated with TEA-induced cell cycle arrest and inhibition of proliferation in these cells. Application of antioxidant NAC reversed the TEA-induced effect on the glioma cells, suggesting that TEA might exert its antiproliferative role through regulating intracellular production of ROS.

**Effects of Potassium Channel Blockers on Expressions of p53 and p21**

To further explore the signaling pathway involved in the TEA-mediated effect on the glioma cells, we used RT-PCR and WB to evaluate the mRNA and protein levels of p53 and p21 in cells from each group. Quantitative results of RT-PCR showed that TEA treatment increased p53 mRNA in C6 cells by 3.41-fold \((p<0.01)\) and in U87 cells by 6.42-fold \((p<0.05)\) (Fig. 5A, B). Additionally, TEA increased mRNA levels of p21 in C6 by 7.89-fold \((p<0.001)\) and in U87 by 5.79-fold \((p<0.05)\). Protein levels of p53 and p21 showed similar trends to that of mRNA expressions in response to the TEA treatment (Fig. 5C, D). There was no significant change in either mRNA or protein levels of p53 and p21 in cells treated with NAC alone. Glioma cells pretreated with NAC followed by TEA treatment showed significantly reduced mRNA and protein levels of p21 compared to the TEA group.

**DISCUSSION**

Human malignant gliomas are the most common malignant tumor in the brain. Many patients with gliomas respond poorly to the traditional radiation and chemotherapy and the recurrence rate of human malignant gliomas is 100\% (27). Therefore, understanding the mechanism involved in glioma cell proliferation is critical for developing an efficient and specific strategy to inhibit the progression of this tumor.

TEA is a nonspecific BK channel blocker. The result that TEA inhibited proliferation of glioma cells indicates an involvement of BK channels in the growth of tumor cells. This result is consistent with previous reports about the antitumor effect of TEA on other types of tumor cells. Importantly, because TEA cannot penetrate the cell membrane and blocks the K+ channels specifically from the extracellular part of the channel (28), using TEA excludes the possibility of blockage of other intracellular signal transduction, suggesting the specific effect evoked by TEA on the K+ channels of glioma cells.

Cellular generation of ROS is central to redox signaling. Cells generate ROS as products or byproducts that act as either signaling molecules (29) or cellular toxicants (30). Increasing glioma cell sensitivity to oxidative stress not only inhibits tumor growth but also enhances the...
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Efficacy of chemotherapeutic drug-induced ROS. Many anticancer drugs exert their effects through this characteristic and induce apoptosis in cancer cells. On the other hand, ROS can induce the formation of carcinogens. As an antioxidant and free radical scavenger, NAC can reduce the ROS-caused damage to DNA and antagonistic apoptosis induced by cytotoxic substances.

In our previous study, we have proved that TEA significantly inhibited the proliferation of C6 and 9L glioma cells with the concentrations at 40 mM and 60 mM. Furthermore, TEA induced inhibition of glioma cell growth via the induction of apoptosis because both TUNEL and annexin V staining were positive following treatment with 40 mM TEA, but not with 20 mM TEA, indicating that TEA only induced apoptosis of glioma cells at high concentrations (>40 mM) but not at low ones (<20 mM). Cell viability of each group was assessed using MTT assay at 72 h after treatment in this study, and TEA-treated C6 and U87 cells at 72 h displayed in a dose-dependent manner. It also had significant inhibition effect at 40 mM. So in this study we choose the concentration of 40 mM for further research.

In this study, we reported that treatment of TEA significantly reduced the proliferation of rat and human glioma cells. The antiproliferative effect of TEA was associated with arrested cell cycle at the G0/G1 phase checkpoint and increased intracellular production of ROS. Application of antioxidant NAC attenuated TEA-mediated inhibition on the growth of glioma cells. The blockage of K+ channels might result in activation of downstream signaling pathway that causes upregulation of tumor-suppressor proteins p53 and p21, ultimately leading to cell cycle arrest or programmed cell death of glioma cells. These findings disclose a novel role of K+ channel in regulating the cell cycle of glioma cells and suggest that ROS is one of the important signal pathways involved in glioma progression.

Studies have revealed that ROS can act as an upstream signal that triggers p53 activation (31). Activation of p53 induces transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor p21^CIP1^, leading to cell cycle arrest (32). In this study, we observed that TEA-induced increase of ROS was accompanied by upregulation of p53 and p21.

Additionally, this study provides a novel insight into developing a strategy for the treatments of human malignant glioma. However, more in-depth study is needed to understand the machinery underlying how ROS regulates p53-targeted gene selection.

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