Original Paper

Caffeine Inhibits Migration in Glioma Cells through the ROCK-FAK Pathway

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Key Words

Caffeine • Rho associated kinase • Focal adhesion kinase • Glioma

Abstract

Aims: Glioma is the most malignant brain tumor that has the ability to migrate and invade the CNS. In this study, we investigated the signaling mechanism of caffeine on the migration of glioma cells. **Methods:** The effect of caffeine on cell migration was evaluated using Transwell and wound healing assays. The expression of the focal adhesion complex as it related to cell migration was assayed using Western blotting and immunostaining. **Results:** Caffeine decreased the migration of rat C6 and human U87MG glioma cells and down-regulated the expression of phosphorylated focal adhesion kinase (p-FAK) and p-paxillin. Caffeine also decreased p-FAK staining at the edge of glioma cells and disassembled actin stress fibers. Additionally, caffeine elevated expression of phosphorylated myosin light chain (p-MLC), an effect that could be blocked by Y27632, a rho-associated protein kinase (ROCK) inhibitor, but not myosin light chain kinase inhibitor, ML-7. Y27632 also inhibited the caffeine-reduced expression of p-FAK and p-paxillin as well as cell migration. **Conclusion:** Caffeine decreased the migration of glioma cell through the ROCK-focal adhesion complex pathway; this mechanism may be useful as part of clinical therapy in the future.

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Introduction

Gliomas are common malignant brain tumors derived from glia cells in the central nervous system. Glioma invasion into other brain tissue makes the disease difficult to treat, and despite surgery and chemotherapy, the mean survival rate for glioma patients is usually less than a year [1]. According to the World Health Organization, gliomas can be classified

into the following three groups, based on the differentiation of astrocytomas: diffuse astrocytoma, anaplastic astrocytoma, and undifferentiated glioblastoma multiform (GBM) [2]. It is important to determine therapeutic solutions that may work in combination with the traditionally clinical therapy for treating gliomas.

Coffee, tea, or caffeine-containing beverages have been reported to reduce the risk of gliomas in adults [3]. Caffeine can penetrate the blood-brain barrier, which normally restricts chemotherapeutic agents or drugs [4]. By increasing phosphorylation of p21 and glycogen synthase kinase 3 beta (GSK-3 β), caffeine reduces the proliferation of human U87MG glioma cells both *in vitro* and in subcutaneous xenograft models [5]. Moreover, caffeine increases ionizing radiation-induced G1 arrest in several human glioblastoma cells [6]. High concentrations of caffeine reduce migration and invasion *in vitro* and in brain slices through inositol-1, 4, 5-triphosphate receptor (IP3R)-calcium cascades in human glioblastoma cells [7]. Unfortunately, the mechanism by which caffeine reduces glioblastoma cell migration and invasion remains unclear.

Adhesion, migration, and invasion are important for glioma cell metastasis. The focal adhesion complex is responsible for cell adhesion to the extracellular matrix (ECM) through integrin [8]. The activity of integrin β 1 and focal adhesion kinase (FAK) are both related to the formation of the focal adhesion complex, which controls cell adhesion and migration in human glioblastoma [9]. Moreover, the expression of FAK, a protein kinase involved in the focal adhesion complex, is elevated in human anaplastic astrocytoma and glioblastoma [10]. Down-regulation of integrin- α v β 3, which adheres to fibronectin, decreases the migration of human glioblastoma cells [11]. Furthermore, *rho*-associated protein kinase (ROCK) and myosin light chain kinase (MLCK) both contribute to the phosphorylated myosin light chain (MLC), decreasing the turnover of focal complexes and cell migration in fibroblasts [12]. In human glioblastoma cells, ROCK activation decreases cell migration and impairs the balance of F- and G-actin [13]. The focal adhesion complex may be a potential treatment target for glioblastoma.

The aim of this study was to determine the effects of caffeine on cell migration and the underlying mechanisms for these effects in glioma cells. This research suggests that the ROCK-FAK cascade plays an important role in the effect of caffeine in glioma cells.

Materials and Methods

Cell culture

Rat C6 and human U87MG glioblastoma cell lines were purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 100 IU/ml penicillin and streptomycin (pH 7.4) (all obtained from Gibco BRL, Grand Island, NY) in a humidified atmosphere of 5% CO₂ at 37 °C.

Drugs

The 4', 6-diamidino-2-phenylindole diacetate (DAPI) and 2,3,3'-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) used in this study were obtained from Sigma-Aldrich (St. Louis, MO). Caffeine was purchased from TOCRIS (TOCRIS Cookson Inc., Bristol, UK). Y27632 and ML-7 were obtained from Enzo Life Sciences (Plymouth Meeting, PA).

Cell survival assay

C6 and U87MG glioblastoma cells were plated at 2×10^4 cells per well in a 24-well plate. Different concentrations of caffeine or vehicle control H₂O were then added to the culture medium for 24 h. After the cells were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, and pH 7.4), 500 μ l of DMEM containing 0.5 mg/ml of MTT was added and incubation continued for another 4 h. The cells were then lysed with 500 μ l of DMSO. Absorbance at 590 nm was measured.

Migration assay

Cell migration was assayed using Transwell and wound healing assays. The Transwell migration assay was prepared by seeding 5×10^4 C6 or U87MG glioma cells in the upper chamber of a Transwell (Costar, Acton, MA, USA). After cell attachment, growth medium either with or without caffeine was added for 24 h. The cells on the lower side were fixed in 10% formalin and stained with Coomassie Brilliant Blue G250 (Sigma, St. Louis, MO). The number of migrated cells was counted in three randomly selected fields from each membrane, with the membranes examined three times in each experiment group. The wound healing assay was prepared by growing cells to confluence to form a monolayer. The cells were then scratched using a P200 pipette tip and photographed as previously described [14]. After 24 h of treatment with caffeine, wound healing was photographed again. Images are representative of three different experiments.

Adhesion assay

After being treated with either caffeine or H_2O for 24 h, the U87MG glioma cells were suspended and then plated on fibronectin-coated or non-coated dishes as described previously [15]. Cells were left to adhere for 15 min, after which the medium and non-adhered cells were discarded. After being washed with PBS, the adhered cells were examined by microscope and counted per field.

Western blotting

After the various treatments, the C6 or U87MG glioma cells were washed once with PBS and then homogenized in lysis buffer (10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES, 25 mM HEPES, 0.15% Triton X-100, 1 μ g/ml of pepstatin A, 1 μ g/ml of leupeptin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). Protein samples (60 μ g per lane) were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad Life Science). Strips from the membrane were blocked with 5% non-fat milk in Tris-buffered saline, pH 8.2, containing 0.1% Tween (TBS-Tween) and incubated overnight at 4 °C with a 1:500 dilution of rabbit antibodies against phosphorylated FAK (p-FAK), phosphorylated paxillin (p-paxillin), GAPDH (BioVision Research Products, Mountain View, CA), phosphorylated MLC (p-MLC), MLC, and ROCK (Cell Signaling) or mouse antibody against β -actin (Sigma-Aldrich), integrin $\beta 1$, FAK, and paxillin (BD Biosciences Pharmingen, San Diego, CA). After the washings, the strips were incubated with a 1:7500 dilution of alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibodies (Promega Corp., Madison, WI). Peroxidase-conjugated secondary antibody was used for chemiluminescence detection. After buffer wash, the blots were reacted in ECL substrate developing solution (Millipore). The density of the bands on the nitrocellulose membrane was quantified by densitometry using Gel Pro 3.1 (Media Cybernetics, Silver Spring, MD), taking density of the control sample as 100% and expressing density of the test sample relative to the expression of internal control as a relative value. Phosphorylated proteins were normalized to the total protein first.

Immunocytochemistry

After caffeine treatment, the cells were rinsed with PBS and fixed with 10% formalin. Primary antibody dissolved in PBS with blocking solution (5% non-fat milk in 0.1% Triton X-100) was added at 4 °C overnight. After rinsing with PBS, FITC-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) were introduced. Stress fibers were stained using TRITC-conjugated-phalloidin (Sigma), while DAPI was counterstained to observe the nucleus. Eventually, the cells were mounted with mounting medium (Gel mount Aqueous, Sigma), observed using a fluorescent microscope (DM2500, Leica, Germany), and recorded with a Nikon D1X digital camera (Luca-R, Andor, Northern Ireland).

Statistical analysis

All experiments were performed at least three times and the results were expressed as the mean $\pm$ SEM for the total number of experiments. Significant differences between means were assessed using the Kruskal-Wallis test. The Mann-Whitney test was used for post analysis. Statistical significance was set at $p < 0.05$.

Fig. 1. The effect of caffeine on cell viability and migration. (A) Human U87MG glioma cells and (B) rat C6 glioma cells were treated with H₂O or different concentrations of caffeine for 24 hours. After treatment, the survival rate was analyzed using MTT tests. Values are expressed relative to the control group. * $p < 0.05$; ** $p < 0.01$ compared to the control group. (C) Rat C6 and human U87MG glioma cells were seeded in the upper chamber of a Transwell. After 24 hours of incubation with or without caffeine, the number of cells in the lower chamber was calculated. (D) Rat C6 and U87MG glioma cell monolayers were scratched and incubated with or without

0.5 mM caffeine for 24 h treatment. The wound area was analyzed using ImageJ software and expressed relative to 0 hours. * $p < 0.05$; ** $p < 0.01$ compared to the control group in 24 h.

Results

Caffeine reduced cell migration in glioma cells

The effect of caffeine on the survival rate of glioma cells was first tested using MTT assays. The cell viability of human U87MG and rat C6 glioma cells was unaffected when the cells were given doses of caffeine under 0.5 mM (Figs. 1A and 1B). Caffeine at 1 mM reduced

Fig. 2. Caffeine reduced activity of the focal adhesion complex. (A) C6 glioma cells were treated for the indicated time with H₂O or 0.5 mM caffeine. Cell lysates were then analyzed for integrin β 1, FAK, p-FAK, paxillin, and p-paxillin using western blotting of C6. (B) U87MG glioma cells were treated with H₂O or 0.5 mM caffeine for 24 h, and the cell lysates were then analyzed for the aforementioned proteins. The results were representative of those

obtained in five experiments. GAPDH was used as the loading control. The lower panels show quantitative analyses of the levels of the aforementioned proteins ($n=5$); $*p<0.05$ compared to the control group. (C) The adhesion ability of human U87MG glioma cells was assayed. After 24 hr of treatment with H₂O or 0.5 mM caffeine, cells were detached from the culture dish and replated on fibronectin-coated or non-coated dishes for 15 min. $***p<0.001$ compared fibronectin-coated control group after 24 h. (D) Cell lysates were analyzed for FAK, p-FAK, paxillin, and p-paxillin using western blotting in U87MG glioma cells after caffeine treatment in fibronectin-coated dishes for 24 h. $*p<0.05$ when compared to the control group.

the survival rate of C6 glioma cells to 70%. To avoid any effects on cell viability, doses of 0.5 mM caffeine were used throughout this study.

The effect of caffeine on glioma cell migration was investigated using Transwell migration and wound healing assays. The migration of C6 glioma cells was significantly

Fig. 3. Effects of caffeine on the distributions of FAK and stress fibers in C6 glioma cells. After 24 h treatment, cells were immunostained with FAK (A, B) and counter stained with TRITC-phalloidin (C, D) and DAPI (E, F). Graph G and H are merged images. Bar = 20 μ M.

reduced after treatment with 0.5 or 1 mM caffeine for 24 h (Fig. 1C). Although C6 glioma cell survival is more sensitive to caffeine than U87MG cell survival, treatment with 0.1 and 0.5 mM caffeine reduced U87MG glioma cell migration (Fig. 1C). The results of the wound healing assay indicated that the wound area of the control group was larger than that of the caffeine group after 24 h both in C6 and U87MG glioma cells (Fig. 1D). These results indicated that caffeine decreased glioma cell migration.

Caffeine reduced the phosphorylation of focal adhesion proteins and cell adhesion

Focal adhesion proteins were studied in glioma cells after treatment with caffeine. A time-course study revealed that p-FAK and p-paxillin levels were decreased in C6 cells after 24 h of treatment with caffeine (Fig. 2A). Caffeine also led to downregulation of p-FAK and p-paxillin in U87MG cells (Fig. 2B). Cell adhesion ability was tested using fibronectin-coated dishes after caffeine treatment. Caffeine reduced the adhesion ability of glioma cells when replated on dishes coated with fibronectin (Fig. 2C). Furthermore, caffeine decreased p-FAK and p-paxillin on fibronectin-coated dishes (Fig. 2D). These results indicated that caffeine decreased activity of the focal adhesion complex, which contributed to the reduction in the adhesion ability of glioma cells.

Immunostaining was performed to observe the distribution of FAK and actin stress fibers. In the control group, FAKs were detected as puncta at the cell edge and ventral

Fig. 4. Effect of caffeine on the phosphorylation of MLC. (A) C6 glioma cells were treated for different time intervals, and cell lysates were collected and analyzed for levels of phosphorylated MLC (p-MLC), MLC, β -actin and GAPDH (internal control). (B) U87MG glioma cells were treated with 0.5 mM caffeine for 24 h, and cell lysates were collected and analyzed for phosphorylated MLC (p-MLC), MLC and GAPDH (internal control). (C) Cells were treated with Y27632 (10 μ M) and ML-7 (2 μ M) for 1 h before and during incubation with 0.5 mM caffeine for 24 hours, after which cell homogenates were analyzed for p-MLC. * p <0.05 compared to the control group; # p <0.05 compared to the caffeine-treated group.

adhesion site (Fig. 3A). After caffeine treatment, the FAK distribution and the structure of stress fibers were impaired and accumulated at the cell edges, which was disadvantageous for cell migration (Fig. 3B and 3D). Moreover, few micronuclei (MN) were observed after caffeine treatment (Fig. 3F), which is a consequence of the conversion of TOP2II/DNA complexes to permanent DNA damage [16].

Caffeine regulated cell migration through rock-fak cascade

The phosphorylation of MLC, a substrate for both ROCK and MLCK [12], was two times higher than the level of the control group after caffeine treatment in non-coated dishes and fibronectin-coated dishes (Fig. 4A and 4B). As a result, a ROCK inhibitor, Y27632, and MLCK inhibitor, ML-7, were chosen to confirm the upstream activation of MLC by caffeine. Only Y27632 significantly prevented caffeine-induced MLC phosphorylation (Fig. 4C), suggesting that caffeine caused MLC phosphorylation via ROCK activation. Moreover, Y27632 blocked caffeine-reduced FAK and paxillin phosphorylation in C6 and U87MG glioma cells (Fig. 5). Although ML-7 inhibited the reduction of paxillin phosphorylation caused by caffeine in C6 glioma cells, only Y27632 prevented caffeine-decreased cell migration in C6 glioma cells (Fig. 6A) and U87MG glioma cells (Fig. 6B). Taken together, these results suggest that caffeine attenuated the phosphorylation of focal adhesion proteins and migration through ROCK in glioma cells.

Fig. 5. Caffeine reduced the activity of focal adhesion proteins by ROCK. Cells were treated with Y27632 or ML-7 were pre-treated for 1 h before and during, 24 h incubation with 0.5 mM caffeine, and then the cell homogenates were analyzed for p-FAK and p-paxillin in C6 (A) and U87MG (B) glioma cells (GAPDH as internal control). * $p < 0.05$ compared to the control group; # $p < 0.05$ compared to the caffeine-treated group.

Discussion

Although caffeine has been reported to reduce cell migration and invasion, the mechanism responsible for these effects remains unclear [7, 17]. Caffeine alters IP3R and subsequently, intracellular calcium levels, which reduce migration and invasion in human glioma cells [7]. In non-glioma cells, caffeine decreases invasion of human leukemia U937 cells through the Ca^{2+} /ROS-mediated suppression of ERK/c-fos and activation of p38 MAPK/c-jun pathways [17]. Furthermore, caffeine inhibits the motility of extra-villous trophoblasts by blocking the mammalian target of rapamycin complex 2 and Akt [18]. Here, caffeine treatment impairs focal adhesion protein activity that is blocked by ROCK activation, which leads to the down-regulation of both adhesion ability and migration in glioma cells.

The Rho-family pathway and calcium signaling are two basic systems that regulate the cytoskeleton and cell motility [19]. ROCK inhibition leads to Rac1 activation, which results in increased migration and invasion in human glioma cells [20]. Treatment with

Fig. 6. Caffeine reduced cell migration by ROCK. C6 (A) and U87MG (B) glioma cells were treated with Y27632 or ML-7 for 1 h before and during incubation with 0.5 mM caffeine for 24 h, and then analyzed for cell migration. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ compared to the control group; # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$ compared to the caffeine-treated group; +++ $p < 0.001$ compared to the Y27632+caffeine-treated group.

Y27632 resulted in a two-fold increase in cell migration in glioma cells. Moreover, ROCK activation causes a shift from filamentous to monomeric actin, leading to the disappearance of stress fibers in human U87MG glioma cells [13]. In the present study, caffeine induced MLC phosphorylation and stress fiber disassembly, indicating the involvement of ROCK in caffeine's effects on glioma cells.

Caffeine has been reported to increase MN formation in mouse bone marrow cells, while also reducing the number of MN induced by an antineoplastic drug, methotrexate [21]. Caffeine leads to MN formation in human lymphocytes, which is related to apoptosis and necrosis [22]. However, Alves et al. note that there is no correlation between MN formation and cell viability [23]. As shown in the present study, although few MN were observed after caffeine treatment, there was no difference in cell viability between the control-group and caffeine-treated group.

ROCK has been correlated with protein phosphatases as subsequently facilitating downstream signaling pathway. Phosphatase and tensin homolog (PTEN), a substrate

of ROCK, dephosphorylates FAK and paxillin and contributes to the regulation of cell migration in mouse pre-osteoblast MC3T3-E1 cells [24]. Moreover, the ROCK signaling pathway coordinates protein phosphatase 1 (PP1) with cofilin activity to regulate skeletal dynamics in enteric neural crest cells [25]. However, both C6 and U87 glioma cells lack PTEN expression [15, 26]. Therefore, the role that regulation of phosphatases by ROCK plays in controlling FAK and paxillin phosphorylation after glioma cells are treated in caffeine warrants further investigation.

Intake of caffeine is estimated to be 200-250 mg/person per day in the USA [27], and 260 mg/person per day in Japan [28]. The dose used in this study (10^{-4} M in U87MG cells) corresponds to an intake of 250 mg caffeine, or 2-3 cups of coffee by a 70 kg human [29, 30]. Hence, the concentrations of caffeine applied in this study are physiologically relevant.

Fig. 7. The signaling pathway of caffeine decreased migration in glioma cells.

Conclusions

Caffeine reduces the adhesion and migration of glioma cells by impairing the focal adhesion complex through ROCK (Fig. 7). In the future, caffeine may be helpful as part of adjunct therapy for glioma in addition to surgery, chemotherapy, and radiotherapy.

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Disclosure Statement

The authors report no conflicts of interest.

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