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Molecules and Cells

Caffeine Inhibits Cell Proliferation and Regulates PKA/GSK3β Pathways in U87MG Human Glioma Cells

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Caffeine is the most commonly ingested methylxanthine and has anti-cancer effects in several types of cancer. In this study, we examined the anti-cancer effects of caffeine on gliomas, both *in vitro* and *in vivo*. *In vitro*, caffeine treatment reduced glioma cell proliferation through G_0/G_1-phase cell cycle arrest by suppressing Rb phosphorylation. In addition, caffeine induced apoptosis through caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage. Caffeine also phosphorylated serine 9 of glycogen synthase kinase 3 beta (GSK3β). Pretreatment with H89, a pharmacological inhibitor of protein kinase A (PKA), was able to antagonize caffeine-induced GSK3β^{ser9} phosphorylation, suggesting that the mechanism might involve a cAMP-dependent PKA-dependent pathway. *In vivo*, caffeine-treated tumors exhibited reduced proliferation and increased apoptosis compared with vehicle-treated tumors. These results suggest that caffeine induces cell cycle arrest and caspase-dependent cell death in glioma cells, supporting its potential use in chemotherapeutic options for malignant gliomas.

INTRODUCTION

Gliomas are the most common primary tumors arising within the brain (Nakada et al., 2007). Although surgery followed by chemotherapy is the normal treatment regimen for gliomas, most malignant gliomas are resistant to chemotherapeutic agents. Because the blood-brain barrier (BBB) restricts and regulates the delivery of chemotherapy agents, the prognosis for glioma is poor (Stupp et al., 2007; Villano et al., 2009). Therefore, it is necessary to develop highly permeable agents that cross the BBB for the chemotherapeutic treatment of patients with malignant glioma.

Caffeine (1,3,7-methylxanthine) is a common component of many popular drinks, such as tea, coffee, and soft drinks (Fredholm et al., 1999) and is the most widely ingested neuroactive substance in the world. Caffeine has a diverse range of pharmacological effects. It inhibits phosphodiesterase activity, alters intracellular calcium levels, inhibits phosphatidylinositol-3-kinase (PI3K) activity, antagonizes adenosine receptors, increases levels of cAMP, and activates cAMP-dependent protein kinase (PKA) (Daly and Fredholm, 1998; Fredholm et al., 1999; Gabrielli et al., 2007). Recently, many studies reported that caffeine has anti-cancer effects through the induction of apoptosis and suppression of cell proliferation (Bode and Dong, 2007) in several cancer types, including neuroblastoma (Jang et al., 2002), lung adenocarcinoma (Okano et al., 2008), and skin cancer (Conney et al., 2007; Hashimoto et al., 2004). Caffeine has also been reported to induce p53-independent G_1-phase arrest in lung adenocarcinoma cell lines (Qi et al., 2002). Additionally, caffeine has been shown to inhibit epidermal growth factor (EGF)-induced cell transformation in the JB6 mouse epidermal cell line (Nomura et al., 2005), inhibit the progression of lung adenoma to adenocarcinoma (Lu et al., 2006), and suppress metastasis in a transgenic mouse model of mammary tumors (Yang et al., 2004).

Although an inverse association was recently reported between caffeine and glioma risk (Holick et al., 2010), the effects of caffeine treatment on glioma have not been thoroughly investigated. Considering that caffeine penetrates the BBB and exerts multiple effects at a cellular level on the central nervous system (CNS) (Fredholm et al., 1999), caffeine might have anti-cancer effects on glioma cells. To investigate this hypothesis, we evaluated the anti-cancer effects of caffeine *in vitro* and *in vivo* using the glioma cell line, U87MG.

MATERIALS AND METHODS

Materials

All culture media and supplements were obtained from GIBCO (USA). Caffeine, 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), ribonuclease A (RNase A), and propidium iodide (PI) were purchased

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from Sigma-Aldrich (USA). Antibodies against phospho-Rb (Ser807/811), phospho-Akt (Ser473), phospho-PKA C (Thr197), p21, and cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (USA); ki-67 and active-caspase 3 antibodies were from Abcam (UK); phospho-glycogen synthase kinase 3 beta (GSK3β) (Ser9), GSK3β, and β-catenin antibodies were from Santa Cruz Biotechnology (USA); and the α-tubulin antibody was from Sigma-Aldrich.

Cell culture

The U87MG human glioma cell line was obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 incubator at 37°C.

Cell viability and proliferation assay

The effects of caffeine on human glioma cell growth were determined using the MTT colorimetric assay. Cells (3×10^3) were plated in 96-well plates and cultured overnight. After cells were treated with caffeine for 24 h, 50 μl of a 2-mg/ml MTT solution was added to each well, and the cells were incubated for 4 h. Formazan crystals were dissolved in 100 μl DMSO, and absorbance was measured at 570 nm. The effects of caffeine on cell proliferation were determined by the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay (Calbiochem, USA) following the manufacturer's protocol. After caffeine treatment, BrdU was added to the medium 4 h before the termination of the experiment. BrdU incorporation into cells was determined by anti-BrdU antibody immunostaining, and absorbance was measured at dual wavelengths of 450 and 590 nm.

Cell cycle analysis

Cells were treated with caffeine at different concentrations for 24 h, harvested, fixed in 70% ethanol, and stained with PI and RNase A. Cell cycle status was assessed using a FACSCalibur flow cytometer (Becton-Dickinson, USA) and analyzed by ModFit Cell Cycle Analysis software (Verity, USA) to determine the percentage of cells in each phase (G_0/G_1, S, and G_2/M). Ten thousand events were recorded for each sample.

Colony formation assay

Cells were plated at a density of 1×10^3 cells/100-mm dish and were incubated for 14 days to allow colonies to develop. At the end points of the colony formation assays, cells were fixed, stained with crystal violet, and photographed.

Western blot analysis

Cells were lysed in lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulphonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml aprotinin]. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., USA). Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in tris buffered saline containing 0.1% Tween-20 for 2 h at room temperature and incubated with the appropriate primary and secondary antibodies.

Animal experiments and immunohistochemical analysis

Five-week-old athymic mice (Balb/c nu/nu) were obtained from Central Lab Animal Inc. (Korea). For the xenograft tumor growth assay, U87MG cells (3×10^6 cells/150 μl phosphate buffered saline) were injected subcutaneously into the right flank of the mice (n = 9-10 mice per group). On day 7 after injection, caffeine was administered in the drinking water (1 mg/ml). The control animals were given distilled water. All protocols were approved by the Gyeongsang National University Institutional Animal Care and Use Committee (GLA-070822-M0039). Tumors were removed from mice 5 weeks after U87MG cell injection, fixed in 4% paraformaldehyde, and embedded in paraffin. Immunohistochemistry was performed on tumor tissues using the indicated antibody. Apoptotic cells were quantitatively determined using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay. TUNEL staining of tumor tissue was perfor-med with the *In Situ* Cell Death Detection Kit (Roche Applied Science, USA) according to the manufacturer's instructions. Quantification of TUNEL-positive cells was performed using Image-Pro Plus version 6.1 (Media Cybernetics, USA).

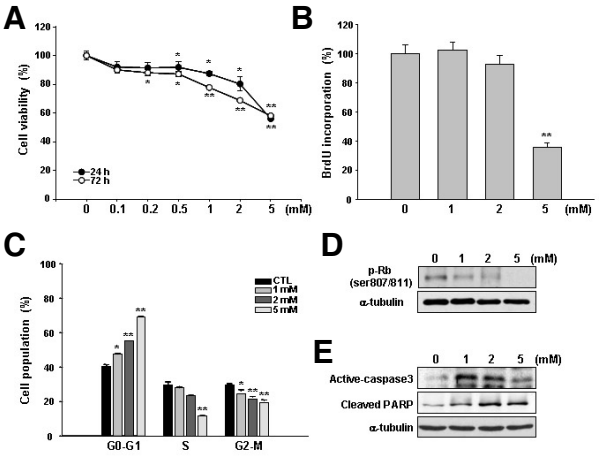

Fig. 1. Effects of caffeine on U87MG glioma cell growth. (A) Cells were treated with the indicated concentrations of caffeine for 24 to 72 h. Cell viability was determined by MTT assay. (B) Cells were treated with caffeine for 24 h, and cell proliferation was assessed by DNA synthesis using the BrdU incorporation assay. (C) Cells were harvested 24 h after caffeine treatment, and cell cycle analysis was performed by flow cytometry. Data represent the percentage of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle. (D) Cells were treated with caffeine for 24 h, and whole cell lysates were analyzed by Western blotting using specific antibodies against phospho-Rb (ser 807/811). (E) After a 12-h caffeine treatment, cleavage of caspase-3 and PARP was detected by Western blotting. Equal protein loading was confirmed by blotting with α-tubulin. Data represent the mean ± SE. *, $P < 0.05$; **, $P < 0.01$.

Statistical analysis

Data are presented as the mean ± standard error (SE). Student's *t*-test was used for all comparisons. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Caffeine decreases cell viability and causes cell cycle arrest in G_0/G_1 phase

To investigate the potential anti-cancer effects of caffeine on glioma, we used the U87MG glioma cell line. Exponentially growing cells were treated with increasing doses of caffeine, and cell viability was assessed. Caffeine treatment resulted in a dose- and time-dependent reduction of cell viability compared with control cells (Fig. 1A). Next, we examined whether caffeine

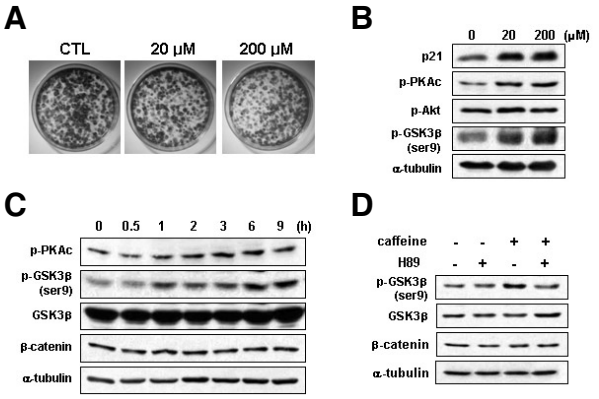

Fig. 2. Effects of caffeine on GSK3β phosphorylation. (A) Caffeine reduced colony formation after 14 days of caffeine treatment. (B) Cells were treated with caffeine for 7 days and subjected to Western blotting. (C) Cells were treated with caffeine (20 μM) for the indicated times. (D) Cells were pretreated with H89 (10 μM) for 30 min and then treated with caffeine (20 μM) for 1 h. PKA and GSK3β phosphorylation and β-catenin expression were determined by Western blotting. Equal protein loading was confirmed with α-tubulin.

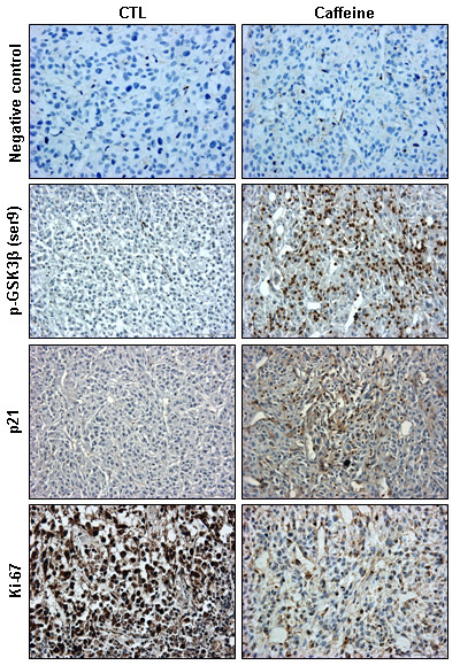

Fig. 3. Immunohistochemistry of p-GSK3β^{ser9}, p21, and ki-67. Paraffin-embedded tumor xenografts were sectioned (5-μm sections) and immunohistochemically stained with the indicated antibodies. Cells were counterstained with hematoxylin. Magnification, 400×.

could inhibit cell proliferation. In caffeine-treated cells, DNA synthesis was significantly reduced at 5 mM, but not at either 1 or 2 mM caffeine (Fig. 1B). To delineate the caffeine-induced mechanisms leading to loss of cell proliferation, cell cycle progression was determined by flow cytometry in caffeine-treated U87MG cells. As shown in Fig. 1C, treatment for 24 h with various concentrations of caffeine (1-5 mM) significantly blocked the cell cycle in the G_0/G_1 phase. However, the percentage of cells in S phase was significantly reduced at only the 5 mM dose of caffeine (Fig. 1C). To further determine the molecular basis for caffeine-induced cell cycle arrest, we examined Rb phosphorylation, which is a specific cell cycle regulatory protein whose regulation is required for the G_0/G_1-S transition. Caffeine treatment caused a dose-dependent suppression of Rb phosphorylation at Ser807/811 (Fig. 1D). In addition, caffeine induced both the activation of caspase-3 and PARP cleavage after 12 h of treatment (Fig. 1E).

Caffeine induces PKA and GSK3β phosphorylation

As the plasma concentration of caffeine in patients should be limited to 80 μg/ml (412 μM) to avoid toxicity (Nomura et al., 2005), cells were treated with 20 and 200 μM caffeine. At these concentrations, caffeine inhibited the colony formation of cells after 2 weeks in culture (Fig. 2A) and induced p21 expression (Fig. 2B). While Akt phosphorylation levels were not altered after caffeine treatment, PKA and GSK3β phosphorylation were both increased (Fig. 2B). As GSK3β is a downstream target of PKA (Fang et al., 2000; Hino et al., 2005), the effects of caffeine on these proteins were investigated. Caffeine (20 μM) increased PKA and GSK3β phosphorylation in a time-dependent manner (Fig. 2C). Pretreatment with H89, a pharmacological inhibitor of PKA, for 30 min before the addition of caffeine inhibited the phosphorylation of GSK3β (Fig. 2D). However, caffeine did not appear to directly affect β-catenin (Fig. 2C).

Caffeine inhibits tumor cell proliferation *in vivo*

Caffeine treatment induced cell cycle arrest, apoptosis, and GSK3β^{ser9} phosphorylation in U87MG cells *in vitro*. Therefore, we investigated whether caffeine also exhibits these effects on glioma cells *in vivo*. Skin xenografted tumor tissues were stained with antibodies against phospho-GSK3β^{Ser9}, p21, and Ki-67. Results showed widespread expression of ki-67-positive cells in control sections, reflecting aggressively proliferating cells. In contrast, p21 expression and GSK3β^{Ser9} phosphorylation were both increased in caffeine-treated tumor samples (Fig. 3).

Caffeine causes apoptosis *in vivo*

As caffeine induces apoptosis *in vitro*, we examined TUNEL staining on tumor sections from both vehicle-treated control and caffeine-treated groups, and the results were quantified by semiautomatic image analyses. Consistent with our *in vitro* results, we observed numerous TUNEL-positive cells in the caffeine-treated group (Fig. 4A). The cell apoptotic index was significantly increased in caffeine-treated tumors (11.6-fold, Fig. 4B). Taken together, these results indicate that caffeine causes mitotic arrest and apoptosis in a xenograft tumor model.

DISCUSSION

High concentrations (≥ 1 mM) of caffeine have been commonly used in previous *in vitro* studies (Hashimoto et al., 2004; Jang et al., 2002; Okano et al., 2008); however, treatment with these concentrations may not be physiologically applicable for the chemoprevention of cancer. It has been reported that caffeine causes adverse effects at excessively high blood concentrations (> 80 μg/ml) in humans (Nomura et al., 2005). In our previous study, oral administration of caffeine (1 mg/ml in drinking water) for 4 weeks was able to obtain an approximate 7 μg/ml serum caffeine concentration in mice. At this concentration, caffeine inhibited the migration of glioma cells and greatly increased mean survival in a mouse xenograft model of glioma (Kang et al., 2010). In present study, we demonstrate that caffeine induces cell cycle arrest at G_0/G_1 phase by inhibiting Rb phosphorylation and reduces cell proliferation through increase-

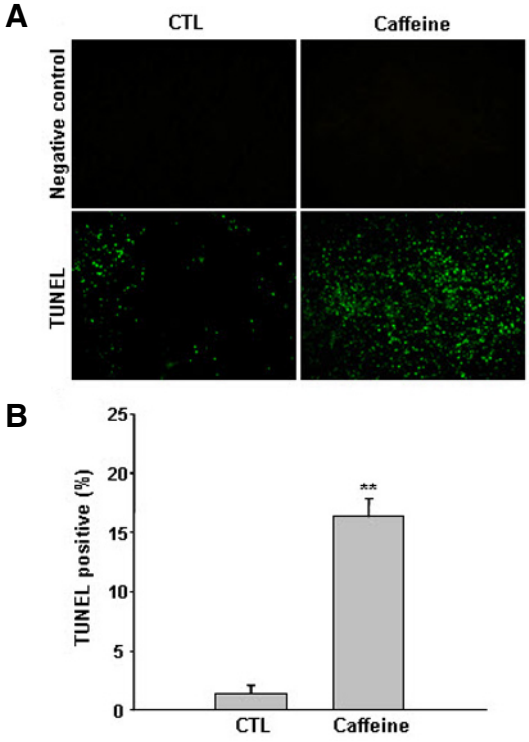

Fig. 4. Effects of caffeine on apoptosis *in vivo*. (A) Paraffin-embedded tumor xenografts were subjected to TUNEL staining. The slides were then observed by fluorescent microscopy and photographed (200×). DNA fragmentation in apoptotic cells (green) was stained using the TUNEL assay. Cell nuclei (blue) were stained with DAPI. (B) Average percentage of TUNEL-positive cells in tumors. Data are shown as the mean ± SE. **, $P < 0.01$.

ing p21 expression. In addition, caffeine induces caspase-dependent apoptosis in U87MG cells. In particular, we show that caffeine activates PKA via increasing PKA phosphorylation, thereby causing the phosphorylation (and subsequent inactivation) of GSK3β by PKA. These data suggest that caffeine has diverse effects on cell proliferation and survival mechanisms in glioma cells.

Cell cycle progression from the G_0/G_1 to the S phases is mainly controlled by two proteins, p21 and Rb (Maddika et al., 2007; Ohtani et al., 2004). The cyclin-dependent kinase (cdk) inhibitor, p21, negatively regulates cell cycle progression by inhibiting the activity of cyclin/cdk2 complexes and plays an important role in cell senescence and apoptosis (Kang et al., 2009; Lee et al., 2009). Rb phosphorylation leads to the disruption of the Rb/E2F transcription factor complex. Release of active E2F triggers the activation of a number of genes that are required for the G_0/G_1-S phase transition. In previous studies, regulation of p21 expression and Rb phosphorylation were shown to be responsible for cell cycle arrest in the G_0/G_1 phase in glioma cells (Choi et al., 2008; Tsai et al., 2006).

GSK3β is a multifunctional serine/threonine kinase that regulates various cellular pathways involved in the cell cycle, proliferation, differentiation, and apoptosis (Blomgren et al., 2007; He et al., 2003; Miyashita et al., 2009). GSK3β activity is negatively regulated by phosphorylation on Ser9 by Akt, PKA, and PKC (Blomgren et al., 2007). Although the exact role of GSK3β in malignancies is still controversial due to conflicting results from different tumor models (Miyashita et al., 2009), inactivation of GSK3β is thought to contribute to the inhibition of tumor growth via cell cycle and apoptosis regulation (Doble and Woodgett, 2003; Luo, 2009). In this study, caffeine increased GSK3β phosphorylation at Ser9; this regulation was controlled by PKA. In normal cells, constitutively active GSK3β negatively regulates the proto-oncogenic protein, β-catenin, via phosphorylation-induced degradation, limiting its expression (Blomgren et al., 2007). Interestingly, in this study, despite an induction of GSK3β phosphorylation (at Ser9), we observed no increase in β-catenin expression after caffeine treatment. This result indicates that caffeine-induced GSK3β inactivation is independent of the perturbation of β-catenin. Thus, caffeine may induce GSK3β inactivation without hyperplasia by deregulating β-catenin in glioma cells.

In conclusion, caffeine has potent anti-cancer effects against gliomas both *in vitro* and *in vivo* via the induction of cell cycle arrest and apoptosis. As brisk mitotic activity is a main feature of gliomas, caffeine may provide options for adjuvant therapies to treat patients with malignant glioma.

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