

# Cyclooxygenase inhibitor induces the upregulation of connexin-43 expression in C6 glioma cells

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**Abstract.** The present study was performed to determine whether aspirin, a cyclooxygenase (COX) inhibitor, has an effect on the expression of connexin 43 (Cx43) in C6 glioma cells. Using an *in vitro* glioma invasion model, the expression of Cx43 protein in C6 cells was significantly increased following aspirin treatment at a dose of 8 mmol/l for 30, 60 and 120 min via western blot analysis. The peak value of the Cx43 expression was observed in C6 cells after 120 min of aspirin treatment, which was significantly reduced by prostaglandin E2 (PGE2). In addition, aspirin also significantly increased the gap junction intercellular communication (GJIC) activity and reduced glioma invasion, which was induced by PGE2. This led to the conclusion that the aspirin-induced glioma invasion decrease may be associated with the increased expression of Cx43 protein and formation of GJIC.

## Introduction

Brain glioma is one of the most common central nervous system diseases, which is invariably associated with a high mortality (1). Gliomas are characterized by an intense local invasiveness that limits the usefulness of preferred surgical treatment (2,3). Therefore, reducing the invasiveness of glioma will allow for a significant treatment option for brain tumors.

Lan *et al* (4) demonstrated that aspirin is a potent antitumor agent through the inhibition of the  $\beta$ -catenin signaling pathway in glioma cells. However, the mechanism of aspirin-induced glioma invasion decrease remains to be elucidated.

Glioma, an aggressive form of adult brain tumor, is difficult to treat due to its invasive nature. The current treatment for glioma is resection of the tumor, followed by chemotherapy and radiation therapy (5,6). Even with such radical treatments,

patients with glioma suffer from recurring tumors that arise due to the invasive nature of glioma cells. In addition to the histological changes, several molecular changes occur in the process of glioma genesis (7-9). Previous studies have shown a decrease the expression of the gap junction protein connexin 43 (Cx43) in gliomas (10-13). Cx43 is the major gap junction protein in astrocytes; gap junctions directly link the cytoplasm of adjacent cells, thus establishing a glial syncytium.

Prostaglandin (PG) has been shown to promote tumor angiogenesis and induce cell proliferation, suggesting that glioma invasion may be associated with PG. In addition, several experimental and human tumors synthesize prostanooids (14-16), which can be increasingly produced during tumor development. These cyclooxygenase (COX) metabolites may influence the physiopathological processes associated with tumor development. The capacity of tumors to grow, disseminate and influence host homeostasis has, in certain cases, been associated with the production of elevated amounts of specific prostanoids.

Aspirin, a non-steroidal anti-inflammatory drug, is used widely to relieve pain, fever and peripheral inflammation. Low-dose aspirin (75-150 mg/day) is recommended for long-term prophylaxis of thrombotic events such as heart attacks and strokes, while a higher dose (1 g) has analgesic and antipyretic effects (17). Aspirin irreversibly inhibits COX-1, which converts arachidonic acid (20:4n-6) to PG endoperoxides, and thus reduces PG formation (18).

Based on the aforementioned results, we hypothesize that aspirin could reduce the glioma invasion through regulating the expression of Cx43, and this process is mediated by PGE2 production. To test the hypothesis, we utilized an *in vitro* glioma invasion model and investigated the effects of aspirin to reduce the glioma invasion. In addition, whether aspirin had an effect on the expression of Cx43 at a protein level was examined by western blot analysis, and the function of gap junction intercellular communication (GJIC) was tested by the scrape-loading dye transfer technique method in C6 cells.

## Materials and methods

**C6 cell culture.** Rat C6 glioma cells (obtained from the Cell Center Department of the Chinese Academy of Medical Sciences, Beijing, China) were grown in Dulbecco's modified

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Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Inc., Dreieich, Germany) supplemented with 15% heat-inactivated fetal bovine serum (FBS; cat. no. 10270-106; Hyclone, Thermo Fisher Scientific, Inc., Shanghai, China), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin under standard culture conditions. When the cells reached confluency, the medium was aspirated and fresh serum-free medium was added to the cells for 12 h. The cells were subsequently washed once with sterile phosphate-buffered saline (PBS), and fresh serum-free medium was added. The following experiments were carried out for the cells treated with 8 mmol/l aspirin (Sigma Resources and Technologies, Inc., Santa Clara, CA, USA) for 30, 60 and 120 min.

**Measurement of PGE2.** An enzyme-linked immunosorbent assay was performed to measure the level of PGE2 expression using the appropriate kits from HyCult Biotechnology (Uden, The Netherlands) and R&D Systems, Inc. (Minneapolis, MN, USA), following the manufacturer's protocol. All the assays were performed in triplicate, and data are shown as mean  $\pm$  standard error of the mean.

**Cx43 protein extraction and western blot analysis.** The effect of aspirin on Cx43 protein expression was analyzed using western blot analysis. Protein homogenates of the C6 cells samples were prepared by rapid homogenization in 10 volumes of lysis buffer. Samples were centrifuged at 17,000  $\times$  g for 1 h. The protein concentration of soluble materials was determined by the Coomassie G250 binding method. The protein lysates (12  $\mu$ g per lane for each sample) were fractioned on 12% SDS-polyacrylamide gels, followed by transfer to nitrocellulose membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked in blocking buffer (5% non-fat dairy milk dissolved in Tris-buffered saline with Tween-20) overnight at 4°C. The blots were subsequently incubated with rabbit polyclonal antibody anti-Cx43 (dilution 1:400; cat. no. sc-9059; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h. The Cx43 protein bands on these immunoblots were visualized using the enhanced chemiluminescence kit (Boster Inc., Wuhan, China). The Cx43 protein bands and  $\beta$ -actin bands were scanned using the Bio-Rad Gel Doc<sup>TM</sup> XR+ Gel imaging system (Bio-Rad, Berkeley, CA, USA), and integrated density values (IDVs) were calculated by the Quantity One software and normalized with that of  $\beta$ -actin.

**GJIC activity.** The scrape-loading dye transfer technique is a method to evaluate GJIC activity by calculating the number of cells containing the dye or measuring the distance of dye permeation through the gap junctions. Glioma cells were digested into single cells in 10 mg/ml collagenase I at 37°C overnight and 0.25% trypsin-EDTA at room temperature for 2 min. Glioma cells were cultured in DMEM/medium supplemented with 10% FBS and an antibiotic mixture (penicillin 100 U/ml and streptomycin 100  $\mu$ g/ml) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Aspirin was added to the culture medium for 30, 60 and 120 min when the cell densities had reached ~70% confluence. Subsequent to washing the cells with PBS three times, 10 scrapes were made through the confluent cells with a sterile pipette tip in the presence of warm PBS containing 1 mg/ml lucifer yellow. The cells were further

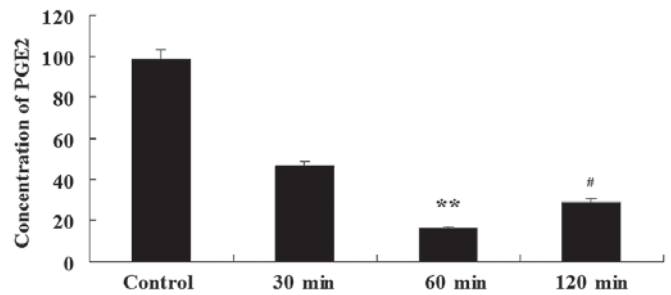


Figure 1. Concentration of PGE2 assessed by an enzyme-linked immunosorbent assay in the control and aspirin-treated groups (8 mmol/l for 30, 60 and 120 min). Data are presented as mean  $\pm$  standard deviation (n=15, each). \*\*P<0.01 and #P<0.05 vs. the control group. PGE2, prostaglandin E2.

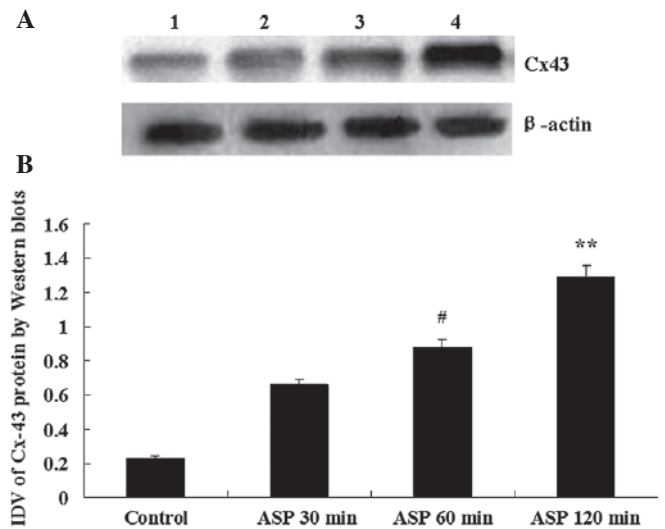


Figure 2. Effects of aspirin treatment on the Cx43 protein expression levels in the C6 cells at 30, 60 and 120 min. (A) Representative western blots illustrating the differences in the bands of Cx43. Lane 1, control group; lane 2, 30 min aspirin-treated group; lane 3, 60 min aspirin-treated group; lane 4, 120 min aspirin-treated group. (B) Changes of the relative IDVs of Cx43 (n=15, each). \*\*P<0.01 and #P<0.05 vs. the control group. Cx43, connexin 43; ASP, aspirin; IDV, integrated density value.

incubated at 37°C and with 5% CO<sub>2</sub> for 5 min. Following this, lucifer yellow was removed, the cells were washed with PBS three times and they were fixed with 4% paraformaldehyde in PBS. The diffusion length of fluorandiol in gliomas was measured under a laser scanning confocal microscope (Olympus, Tokyo, Japan). GJIC activity was expressed as the mean diffusion length.

**In vitro invasion assay.** The C6 cells were resuspended in DMEM containing 15% fetal calf serum to obtain a concentration of 10<sup>7</sup> cells/ml and seeded (10<sup>6</sup> cells/well) in the upper compartments for 120 min; the C6 cells that migrated to the lower compartment were counted under a light microscope (Olympus). The experiments were performed in triplicate, and migration was determined by calculating the number of migrating cells.

**Statistical analysis.** Results are presented as the mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) was used to compare the group differences in the measurements of

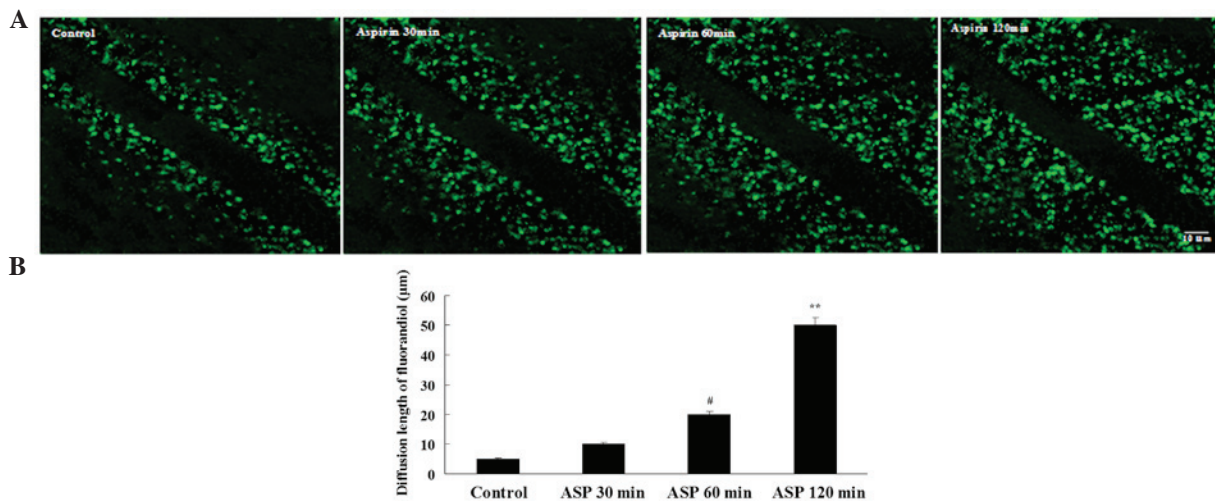


Figure 3. ASP treatment enhances GJIC function in C6 cells. Cultured C6 cells were treated with aspirin for 30, 60 and 120 min, following which aspirin was replaced with 1 mg/ml lucifer yellow and the scrape-loading dye transfer assay was performed as described. (A) Lucifer yellow transfer through the gap junction was enhanced significantly following aspirin treatment. (B) Quantitative analysis of GJIC function of C6 cells. \*\* $P < 0.01$  and # $P < 0.05$  vs. the control group. ASP, aspirin; GJIC, gap junction intercellular communication.

Cx43 protein. Dunnett's post hoc tests were applied to compare specific group differences when the ANOVA revealed a significant difference. For other measurements, the data were assessed using paired Student's t-test.

## Results

**Relative levels of PGE2 from C6 cells.** The content of PGE2 in the experimental groups was 2.12-6.05-fold lower compared to the control group; therefore, aspirin significantly enhanced the downregulation of PGE2. Specifically, aspirin decreased PGE2 after 60 min of treatment. The relative levels of PGE2 in the control group, and the 30, 60 and 120 min aspirin groups were  $98.24 \pm 1.23$ ,  $46.21 \pm 1.32$ ,  $16.47 \pm 2.31$  and  $29.05 \pm 1.98$   $\mu\text{g/l}$ , respectively (Fig. 1). This demonstrated that aspirin has a direct inhibitory effect on the PGE2 level in glioma.

**Aspirin induces overexpression of Cx43 proteins in C6 cells.** In C6 cells infused with sterile saline (control group), the level of Cx43 expressed was low. Compared with the control group, the expression of Cx43 protein was markedly increased at three time-points following aspirin treatment. The IDVs of Cx43 at control group, 30, 60 and 120 min group were  $0.238 \pm 0.058$ ,  $0.669 \pm 0.055$ ,  $0.886 \pm 0.065$  and  $1.292 \pm 0.048$ , respectively. The largest IVD was at 120 min after aspirin treatment (Fig. 2).

**Aspirin enhances GJIC activity in C6 Cells.** Dye transfer is a commonly used method to evaluate GJIC activity by calculating the number of dye-labeled cells or measuring the gap junction dye permeation. To determine if the increases in Cx43 protein are associated with the changes in GJIC activity, the cells were treated with aspirin for 30, 60 and 120 min, respectively, and the dye transfer between neighboring cells was evaluated. Aspirin increased the fluorescent yellow diffusion distance and the peak value at 120 min, suggesting that aspirin can enhance the communication function of the gap of glioma cells (Fig. 3).

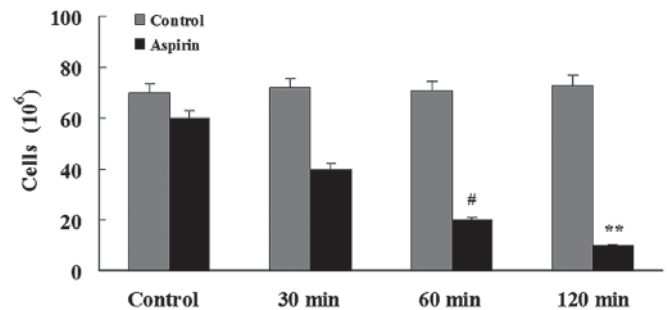


Figure 4. ASP induces a reduction in glioma invasiveness. C6 cells were subjected to aspirin treatment for different times and were compared with the control. Data are mean  $\pm$  standard deviation. \*\* $P < 0.01$  and # $P < 0.05$  vs. the control group. ASP, aspirin.

**Glioma invasion.** The percentage of migrating cells was 1.6- to 4.1-fold lower in the aspirin-treated groups compared to the control group. Aspirin significantly reduced the number of migrating C6 cells after 120 min (Fig. 4).

## Discussion

Inflammation has emerged as a major factor promoting cancer development and supporting cancer progression. However, inflammation can also have cancer-inhibitory effects (19-22). Inflammatory mediators can be produced by the stroma, by tumor-infiltrating leukocytes or directly by the cancer cells themselves. Prominent among tumor-sustaining mediators is PGE2, a prostanoid lipid associated with enhancement of cancer cell survival, growth and migration (23). COX-1 and -2, enzymes that are critical for the production of PGE2, are often overexpressed in colorectal, breast, stomach, lung and pancreatic cancers (24). Whether glioma similarly express abnormal levels of COX-2 remains to be elucidated. Castelli *et al* (25) reported that glioma can secrete PGE2. The present results showed that the expression of PGE2 and COX-2 in C6 cells were significantly higher compared to those in astrocytes.



The C6 glioma cell line has been widely used in the cellular and molecular characterization of glial cells. One of the well-known characteristics of astrocytes concerns the aspect of intercellular coupling via gap junctions (26). Numerous studies have reported the presence of gap junctions between astrocytes morphologically, electrophysiologically and immunohistochemically (27-30). Gap junction proteins are encoded by a family of genes, and two of the most characterized proteins are Cx32 (31,32) and Cx43 (33). The study by Naus *et al* (34) showed that the mRNAs encoding these two proteins are readily detectable in the neonatal and adult brain (35,36). In addition, primary cultures of astrocytes express only the Cx43 mRNA, and the level of this mRNA is significantly reduced in the glioma cells (35).

Dysregulation of gap junction coupling is a phenotypic alteration commonly observed in neoplastic cells. The studies by Bodenstein *et al* and Saunders *et al* (37-39) demonstrated a specific loss of homotypic and heterotypic GJIC in metastatic cells. While the dysregulation of GJIC in neoplastic cells is apparent, the specific signaling events and the mediators of those signaling events between malignant cells or between malignant cells and the surrounding stromal compartment appear to be largely context dependent (40,41). Furthermore, restoration of GJIC appears to reduce the metastatic ability of cancer cells in certain cases (42,43). The present study used the transfer dye method to detect the function of GJIC in glioma cells, and it was found that the fluorescent yellow diffusion distance was short, indicating that the cell gap junction communication function is weak, which may be one of the reasons for its aggressive nature.

The COX-2 inhibitor aspirin was used to treat the C6 cells. The results showed that the expression of COX-2 and PGE2 were significantly decreased, and the 30 and 60 min treatments reduced the levels the most, respectively, suggesting that aspirin may inhibit the synthesis of PGE2 by inhibiting of COX-2. The expression of the CX43 protein was shown to be the highest at 120 min of treatment, while the GJIC function of the glioma cells was the strongest, and the fluorescent yellow diffusion distance increased. At this time, the glioma invasion was the lowest. This indicates that aspirin can enhance the function of GJIC by inhibiting the expression of PGE2, which can decrease the invasion of glioma.

In conclusion, the biochemical mechanism of the aspirin-induced glioma invasion decrease is complex. The present results showed that one of the possibilities may be through the PGE2/GJIC signal pathway. This study further proved that if the ability of the glioma cells to synthesize PEG2 is eliminated, then the GJIC function can effectively be enhanced. Treatment of patients with a COX inhibitor that is similar to aspirin at surgery may provide more potential benefits. Although the present study is only a preliminary investigation, it indicates that the use of aspirin may make glioma therapy more effective, and may increase the survival and quality of life of the patients.

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