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Bradykinin-Induced Cell Migration and COX-2 Production Mediated by the Bradykinin B1 Receptor in Glioma Cells

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ABSTRACT

Bradykinin is produced and acts at the site of injury and inflammation. Recent reports have also shown that bradykinin selectively modulates blood–tumor barrier permeability. However, the molecular mechanisms and pathologic roles underlying bradykinin-induced glioma migration remain unclear. Glioma is the most common primary adult brain tumor, with a poor prognosis because of the ease with which tumor cells spread to other regions of the brain. In this study, we found that bradykinin increases the cell migration and expression of cyclo-oxygenase-2 (COX-2) in glioma cells. Bradykinin-mediated migration was attenuated by the selective COX-2 inhibitor NS-398. Moreover, increased motility of glioma cells and expression of COX-2 were mimicked by a bradykinin B1 receptor (B1R) agonist and markedly inhibited by a B1R antagonist. Bradykinin-mediated migration was attenuated by phosphoinositide 3-kinase (PI-3 kinase)/AKT inhibitors LY 294002 and wortmannin. Bradykinin stimulation also increased the phosphorylation of the p85 subunit of PI-3 kinase and serine 473 of AKT. Treatment of bradykinin with AP-1 inhibitors Tanshinone IIA and curcumin also reduced COX-2 expression and glioma cell migration. Moreover, treatment of bradykinin also induced phosphorylation of c-Jun in glioma cells. AP-1 promoter analysis in the luciferase reporter construct showed that bradykinin increased AP-1 transcription activity and was inhibited by LY 294002 and wortmannin. One mechanism underlying bradykinin-directed migration is transcriptional up-regulation of COX-2 and activation of the B1R receptor, PI-3 kinase, AKT, c-Jun, and AP-1 pathways. J. Cell. Biochem. 110: 141–150, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: BRADYKININ; MIGRATION; COX-2; AKT; AP-1

Glioblastoma is one of the most common primary central nervous system tumors and its biology presents significant problems for successful treatment. One of the most significant hurdles relating to its treatment is the aggressive local invasion of malignant cells from the original tumor. Invasion into the surrounding normal brain renders complete surgical resection impossible. Similarly, chemotherapy and ionizing radiation alone or in combination have produced only a modest increase in median survival due to problems both with the effective targeting of the invading cells and their innate resistance to conventional radiotherapy and chemotherapy [Stupp et al., 2005; Koul et al., 2006]. Despite commonly used treatment procedures, such as surgery, radiation, and chemotherapy [Boiardi et al., 1991], the survival of patients with such tumors has not been improved [Kondo et al., 2004]. Effective treatment will ultimately require more understanding of the signaling pathways that stimulate glioma cell migration as well as the identification and specific targeting of the critical effectors.

Bradykinin is raised during trauma and stroke, leading to an increase in blood–brain barrier (BBB) permeability, and is therefore regarded as a mediator of neurogenic inflammation in the brain [Kamiya et al., 1993; Abbott, 2000; Lehmberg et al., 2003]. It has been reported that bradykinin induces astrocytic and microglial cell migration [Ifuku et al., 2007; Hsieh et al., 2008]. Recent reports have

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also shown that bradykinin is implicated in head and neck squamous cancer cell tumorigenesis, wherein it also promotes cell migration and proliferation [Thomas et al., 2006; Zhang et al., 2008b]. In addition, two types of bradykinin receptors, the bradykinin B1 and B2 receptors, have been detected in glioma, gastric, duodenal, lung, and hepatic cancers [Wu et al., 2002; Taub et al., 2003; Zhao et al., 2005]. It has previously been determined that the bradykinin B1 receptor (B1R) is present in microglia [Ifuku et al., 2007] and contributes to microglia migration. Concentrations of B1R also significantly increase in neurons and astrocytes after ischemia-reperfusion [Su et al., 2009b]. We therefore investigated the role of B1R in bradykinin signaling in the migration of glioma cells.

PI-3 kinase is a heterodimeric protein consisting of a catalytic subunit (p110) and a regulatory subunit (p85). AKT, a direct downstream effector of PI-3 kinase, is a serine/threonine kinase [Franke et al., 1997]. AKT can be modulated by multiple intracellular signaling pathways and it acts as a transducer for many pathways initiated by the PI-3 kinase, activated by the growth factor receptor complex. In addition, activation of PI-3 kinase/AKT is involved in bradykinin stimulation [Pan et al., 1999; Cohen et al., 2007]. Numerous reports have shown that the PI-3 kinase/AKT pathway plays a critical role in cyclo-oxygenase-2 (COX-2) expression [Hsieh et al., 2006; Tang et al., 2006; Chen et al., 2009]. In this study, we hypothesized that the PI-3 kinase/AKT signaling pathway is regulated by tumor migration.

COX-2 is thought to be responsible in different types of inflammation and tumorigenesis. Numerous reports have shown that COX-2 is involved in growth, migration, and angiogenesis in numerous cancer cells [Murata et al., 1999; Liu et al., 2000; Mukherjee et al., 2005; Wang et al., 2005; Cohen et al., 2006; Khor et al., 2007]. Therefore, COX-2 is an important target for chemoprevention in numerous tumors [Dannenberg et al., 2005]. Evidence has also demonstrated that bradykinin is capable of inducing COX-2 expression in artery smooth muscle cells, airway cells, and head and neck squamous cell carcinoma [Brian et al., 2001; Bradbury et al., 2002; Bradbury et al., 2004], and that bradykinin induces COX-2 expression and increases cell migration in head and neck squamous cell carcinomas [Zhang et al., 2008b].

In this study, we investigated how bradykinin increases glioma cell migration and the underlying signaling cascade. Our data show that bradykinin acts through the B1R receptor to activate the PI-3 kinase, AKT, c-Jun, and AP-1 pathways to cause COX-2 induction, leading to tumor migration.

MATERIALS AND METHODS

MATERIALS

Bradykinin was purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and OPTI-MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA). Isopropyl-β-D-thio-galactopyranoside (IPTG) was purchased from Sigma–Aldrich. Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG, primary antibodies against c-Jun, phospho-c-Jun (Ser^{63}), PI-3 kinase (p85), β-actin, AKT, and phospho-AKT (Ser^{473}) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against PI-3 kinase (p85) phosphorylated at Tyr^{458} was purchased from Cell Signaling and Neuroscience (Danvers, MA). Wortmannin was purchased from Calbiochem (San Diego, CA). Tanshinone IIA was purchased from Biomol (Plymouth Meeting, PA). The COX-2 IPTG-inducible expression vector (IPTG-COX-2) was a gift from Dr. W. M. Fu (Department of Pharmacology, National Taiwan University, Taipei, Taiwan). AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). All other chemicals were obtained from Sigma–Aldrich.

CELL CULTURES

C6 and U251 cells, originating from a rat and human brain glioma, respectively, were purchased from the American Type Culture Collection (Manassas, VA). C6 and U251 cells were maintained in 75-cm^2 flasks with F12 medium and DMEM (Invitrogen Life Technologies), respectively, and supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air.

TRANSFECTION AND REPORTER GENE ASSAY

Cells were transiently transfected with 1 μg IPTG-COX-2 plasmid, or 0.8 μg pAP-1-luciferase plasmid and 0.4 μg β-galactosidase expression vectors. C6 cells were grown to 80% confluent in 6-well (for IPTG-COX-2) or 12-well (for pAP-1-luciferase) plates and were transfected by Lipofectamine 2000 (LF2000; Invitrogen) on the following day. Plasmid DNA and LF2000 were premixed in OPTI-medium for 20 min and then applied to the cells. An equal volume of medium containing 20% FBS was added 6 h later. After transfection for 24 h, LF2000-containing medium was replaced with fresh serum-free medium and treated with bradykinin for another 24 h.

To prepare reporter gene assay lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from the plates. The supernatant was collected after centrifugation at 10,000*g* for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (30 μg) were placed into wells of an opaque black 96-well microplate. Luciferase activity was determined by a dual-luciferase reporter assay system (Promega), and activity value was normalized by a β-galactosidase expression vector.

REVERSE TRANSCRIPTASE-PCR AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using the oligo(dT) primer, then amplified using the following oligonucleotide primers:

B1R [481 base pairs (bp)] 5′-GTGGTCAGCGGGGTCATCAAGG-3′ and 5′-GGAAAGCGAAGAAGTGGTAAGG-3′; GAPDH (452 bp) 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGC-TGTA-3′.

Each PCR cycle was carried out for 30 s at 95°C, for 30 s at 55°C, and for 1 min at 68°C. PCR products were then separated electrophoretically in a 2% agarose gel and stained with ethidium

bromide. The band intensity was quantified with a densitometric scanner and presented as the relative level of GAPDH.

Quantitative real-time PCR using SYBR Green I Master Mix was analyzed with a model 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). After preincubation at 50°C for 2 min and 95°C for 10 min, the PCR was performed as 40 cycles of 95°C for 10 s and 60°C for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as C_T). The oligonucleotide primers were as follows: B1R: 5′-GCAGCGCTTAACCATAGCGGAAAT-3′ and 5′-CCAGTTGAAACGGTT CCCGATGTT-3′; COX-2: 5′-TGTA-TGCTACCATCTGGCTTCGG-3′ and 5′-GTTTGGAACAGTCGCTCGT-CATC-3′; GAPDH: 5′-CTCAACTACATGGTCTACATGTTCCA-3′ and 5′-CTTCCCATTCTCAGCCTTGACT-3′.

WESTERN BLOT ANALYSIS

Cells were treated with bradykinin for various time periods and then washed with cold PBS that had been lysed for 30 min on ice with radio-immunoprecipitation assay buffer. Protein samples were separated by SDS (sodium dodecyl sulphate)–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then probed overnight with primary antibody at 4°C. After undergoing three PBS washes, the membranes were incubated with secondary antibodies. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The blots were subsequently stripped through incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 0.1 M β-mercaptoethanol) and reprobed for β-actin as a loading control. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

MIGRATION ASSAY

In vitro migration assays were performed using Costar Transwell inserts (Costar, NY; pore size, 8 μm) in 24-well plates. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including LY294002, wortmannin, curcumin, Tanshinone IIA, or vehicle control. According to a cell viability assay, the various concentrations of inhibitors did not affect the death of glioma cells (data not shown). Approximately 1×10^4 cells in 100 μl of serum-free medium were placed in the upper chamber, and 400 μl of the same medium containing bradykinin was placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, then the cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of bradykinin treatment (corrected migration cell number = counted migration cell number/percentage of viable cells) [Lu et al., 2009; Su et al., 2009a].

MEASUREMENT OF CELL VIABILITY

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells cultured in 24-well plates were treated with various concentrations of bradykinin for 24 h. After incubation, MTT (0.5 mg/ml) was added for 60 min, the culture medium was then removed, and cells were dissolved in dimethyl sulfoxide and shaken for 10 min. The absorbency (OD) values at 550 nm were immediately measured in a microplate reader. The absorbance indicates the enzymatic activity of mitochondria and provides information on cell viability.

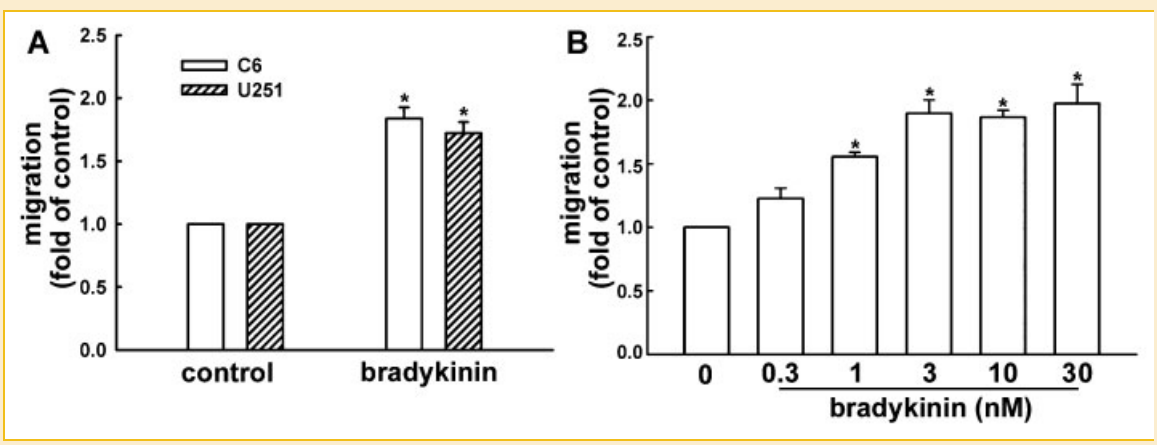

Fig. 1. Bradykinin induces the migration activity of glioma cells. A: C6 and U251 cells were incubated with bradykinin (10 nM). In vitro migration activities measured with the Transwell assay after 24 h showed that all bradykinin concentrations supported the cell migrations. B: C6 cells were treated with various concentrations of bradykinin. In vitro migration activity measured after 24 h showed that bradykinin induced cell migration in a concentration-dependent manner. Results are expressed as the mean ± SEM of at least four independent experiments.

STATISTICAL ANALYSIS

The values given are mean ± SEM. The significance of difference between the experimental group and control groups was assessed by the Student's *t*-test. The difference was significant if the *P* value was < 0.05.

RESULTS

BRADYKININ/B1 RECEPTOR DIRECTS THE MIGRATION OF GLIOMA CELLS

Bradykinin is produced and acts at the site of injury and inflammation. Previous reports have shown that bradykinin stimulates the directional migration of microglia [Ifuku et al., 2007] and astrocytes [Hsieh et al., 2008] in the central nervous system. However, whether bradykinin affects the migration of glioma cells remains unclear. In this study, bradykinin-regulated glioma cell migration was examined using the Transwell assay with correction of bradykinin-induced proliferation effects [Lu et al., 2009; Su et al., 2009a]. Bradykinin directed the migration of both rat glioma C6 and human malignant glioma U251 cells (Fig. 1A). Furthermore, bradykinin increased the migration of C6 glioma cells in a concentration-dependent manner (Fig. 1B). Bradykinin mediated the increase of cell migration and reached its maximum effect at the concentration of 3 nM. Stimulation of cells with bradykinin at a concentration of up to 30 nM did not affect cell viability, as assessed by the MTT assay (data not shown). The interaction of bradykinin with the B1 receptor in microglia has been reported to induce microglial migration [Ifuku et al., 2007]. To investigate the role of the B1R receptor in the bradykinin-mediated increase of cell migration, we assessed the B1R receptor expression in C6 cells. The mRNA level of the B1R receptor after bradykinin stimulation in C6 cells was assessed by RT-PCR (Fig. 3B) and quantified by real-time PCR (Fig. 2A). The mRNA level of the B1R receptor was significantly increased in response to bradykinin treatment. We then examined whether the bradykinin/B1R interaction is involved in the signal transduction pathways leading to bradykinin-induced glioma cell migration. Bradykinin-induced C6 glioma cell migration was antagonized by treatment with des–Arg^9–[Leu^8]–bradykinin (1 μM), a B1R antagonist (Fig. 2B). In addition, treatment with a B1R agonist ([Lys–des–Arg^9]–bradykinin, 1 μM) effectively increased the migration of C6 glioma cells (Fig. 2C). These data suggest that bradykinin-induced glioma cell migration may occur via the B1R.

BRADYKININ/B1 RECEPTOR–DIRECTED GLIOMA CELL MIGRATION INVOLVES COX-2 EXPRESSION

It has been reported that COX-2 expression is involved in cancer cell migration and metastasis. A recent report has also shown that bradykinin directed the head and neck squamous cell carcinoma migration through COX-2 up-regulation [Zhang et al., 2008b]. We therefore hypothesized that COX-2 may be involved in bradykinin/B1R-directed migration of glioma cells. As shown in Figure 3A,B, bradykinin increased COX-2 protein expression in a time- and concentration-dependent manner. The mRNA level of COX-2 expression after bradykinin stimulation was assessed by RT-PCR

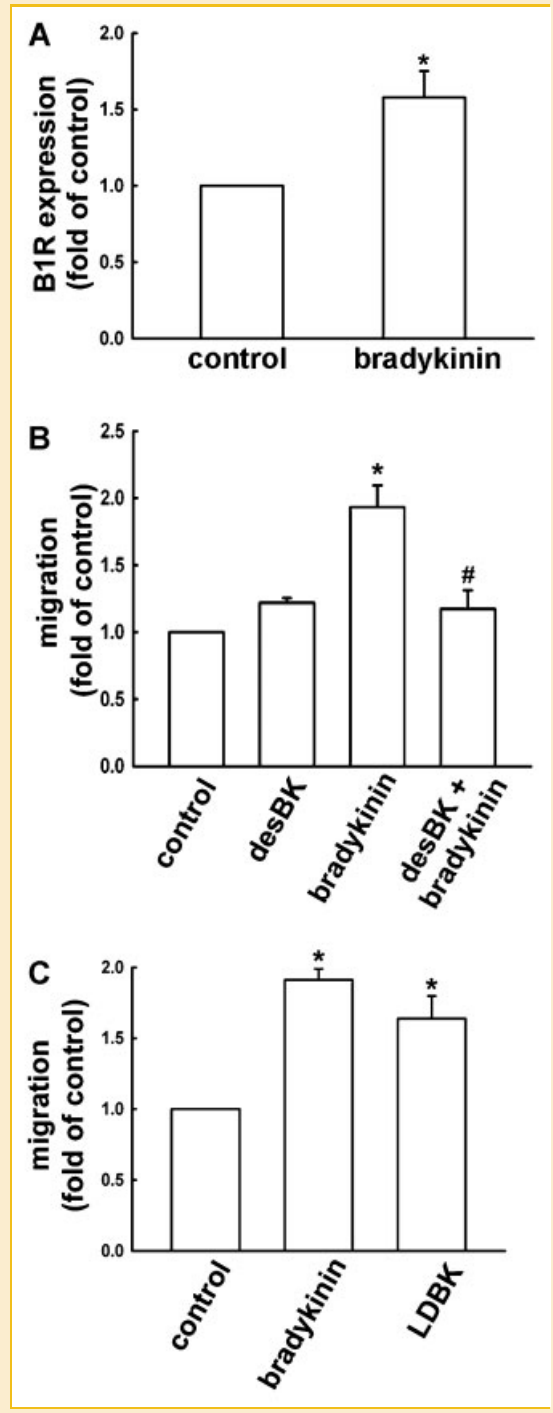

Fig. 2. The B1R receptor is involved in bradykinin-directed migration of C6 glioma cells. A: B1R mRNA level was quantified by real-time PCR. B: Cells were pretreated with the B1R antagonist des–Arg^9–[Leu^8]–bradykinin (desBK) for 30 min followed by treatment with bradykinin (10 nM) for 24 h. C: Cells were incubated with bradykinin or the B1R agonist [Lys–des–Arg^9]–bradykinin; LDBK and in vitro migration activities were measured after 24 h with the Transwell assay. Results are expressed as the mean ± SEM of four independent experiments. $^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the bradykinin treatment group.

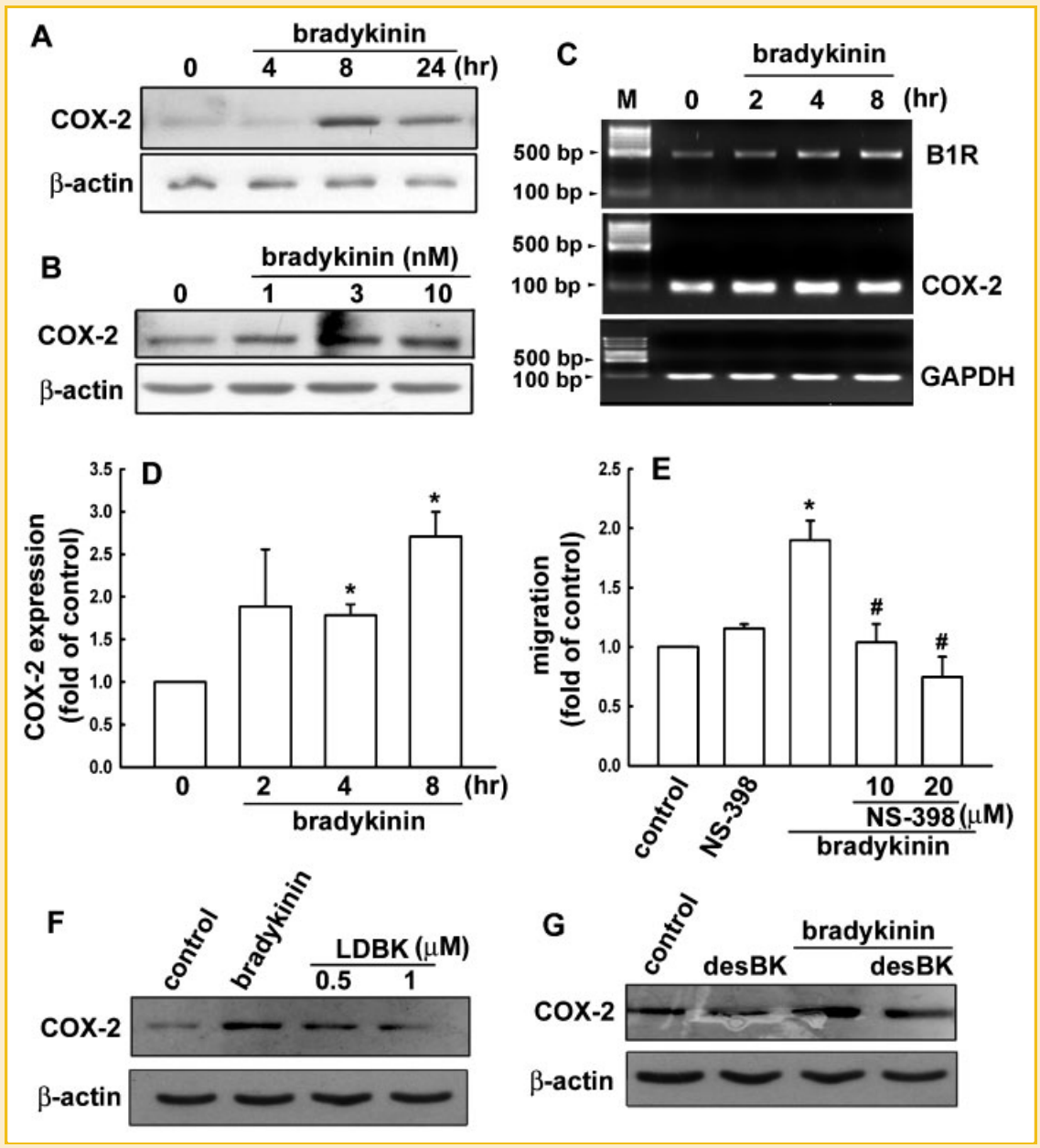

Fig. 3. Bradykinin-directed migration activity involves up-regulation of COX-2. A: C6 cells were incubated with bradykinin (10 nM) for indicated time periods, and the protein extracts were collected and COX-2 protein levels determined using Western blot analysis. B: Cells were treated with various concentrations of bradykinin for 24 h, and the COX-2 protein expression was analyzed. C: Cells were treated with bradykinin for indicated time periods, and total RNA was collected. Levels of B1R, COX-2 and GAPDH mRNA were determined by RT-PCR. M, marker. D: The mRNA level of COX-2 expression after bradykinin stimulation was quantified by real-time PCR. E: Cells were pretreated with the selective COX-2 inhibitor NS-398 (10 or 20 μM) for 30 min followed by stimulation with bradykinin. In vitro assessments after 24 h showed that NS-398 is capable of inhibiting migration activity. F: Cells were incubated with bradykinin or LDBK ([Lys-des-Arg^9]-bradykinin) for 24 h, and COX-2 protein expression was determined using Western blot analysis. G: Cells were pretreated with desBK (des-Arg^9-[Leu^8]-bradykinin) for 30 min followed by stimulation with bradykinin for 24 h, and COX-2 protein expression was analyzed. Results are expressed as the mean ± SEM of four independent experiments.

(Fig. 3C) and was further quantified by real-time PCR (Fig. 3D). Pretreatment of cells with the selective COX-2 antagonist NS-398 for 30 min markedly inhibited bradykinin-induced cell migration (Fig. 3E). Furthermore, treatment of cells with the B1R agonist [Lys-des-Arg^9]-bradykinin induced COX-2 over-expression (Fig. 3F). In addition, the increase in bradykinin-induced COX-2 expression was abolished by the B1R antagonist des-Arg^9-[Leu^8]-bradykinin (Fig. 3G). On the other hand, treatment of IPTG for 24 h enhanced COX-2 protein expression in glioma cells pretransfected with IPTG-COX-2 plasmid (Fig. 4A). Pretransfection with the IPTG-COX-2 plasmid and stimulation with IPTG also increased glioma cell migration (Fig. 4B). These data suggest that bradykinin/B1R-induced cancer cell migration may occur via activation of COX-2 over-expression.

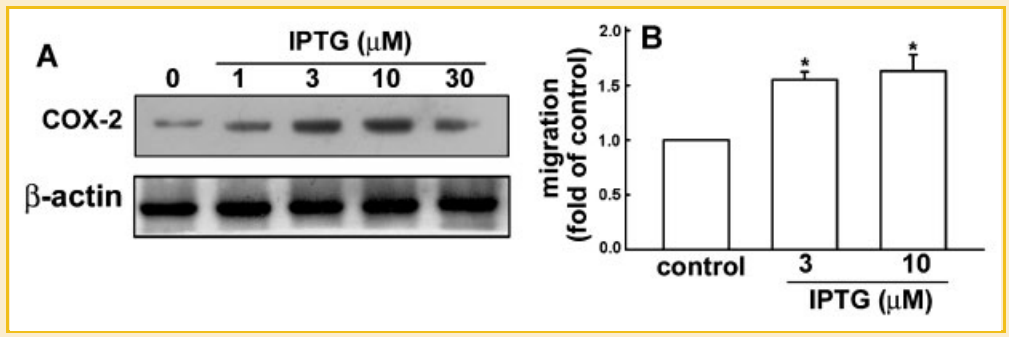

Fig. 4. Involvement of COX-2 expression increases migration activity in C6 glioma cells. C6 cells were transfected with the IPTG-COX-2 plasmid for 24 h and stimulated with IPTG for another 24 h. COX-2 protein expression and in vitro migration activity were determined by Western blot analysis (A) and the Transwell assay (B), respectively. Results are expressed as the mean ± SEM of four independent experiments.

PI-3 KINASE/AKT SIGNALING PATHWAY IS INVOLVED IN BRADYKININ-MEDIATED COX-2 UP-REGULATION AND GLIOMA CELL MIGRATION

The PI-3 kinase/AKT signaling pathway may be activated by a variety of growth factors or chemoattractants [Lu et al., 2009]. To examine the role of PI-3 kinase/AKT in cancer migration and COX-2 up-regulation, we determined PI-3 kinase and AKT phosphorylation in response to bradykinin. Stimulation of cells with bradykinin increased the phosphorylation of PI-3 kinase (p85), which peaked at about 10 min and was sustained to 120 min (Fig. 5A). Bradykinin also increased AKT phosphorylation, starting at 5 min and peaking at 30 min, which was sustained to 120 min (Fig. 5B). Bradykinin-induced migration activity of glioma cells was greatly reduced by treatment with specific PI-3 kinase inhibitors LY294002 or wortmannin (Fig. 5C). Moreover, the bradykinin-induced increase in COX-2 expression was reduced by the PI-3 kinase inhibitors

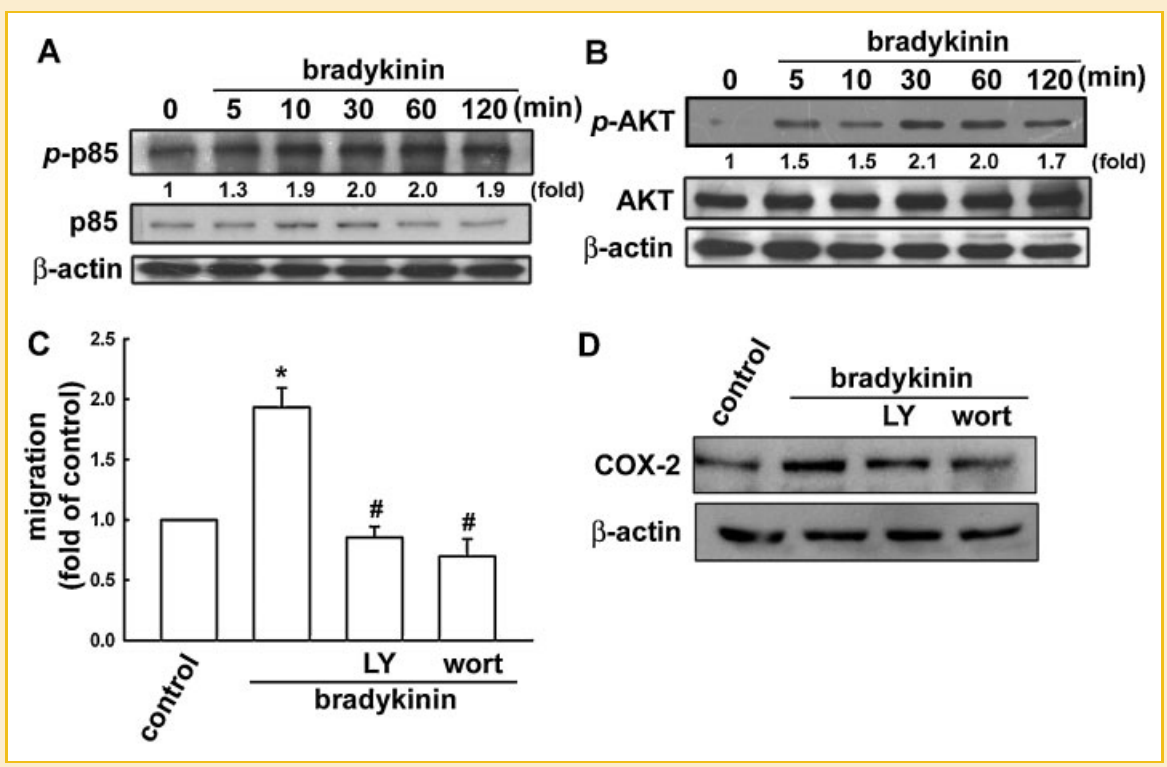

Fig. 5. The PI-3 kinase/AKT signaling pathway is involved in bradykinin-induced cell migration. C6 cells were incubated with bradykinin (10 nM) for indicated time periods. The phosphorylation of PI-3 kinase (p85) (A) and AKT (B) were determined by Western blot analysis. Note that bradykinin activated the PI-3 kinase and AKT pathways. Cells were pretreated with PI-3 kinase inhibitors LY294002 (10 μM) or wortmannin (100 nM) for 30 min and then stimulated with bradykinin (10 nM). In vitro migration was measured with the Transwell assay after 24 h (C). COX-2 protein expression was determined by Western blot analysis (D). Results are expressed as the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the bradykinin-treated group.

(Fig. 5D). These results indicate that the PI-3 kinase/AKT pathway is involved in bradykinin-induced migration activity and COX-2 up-regulation in glioma cells.

INVOLVEMENT OF AP-1 IN BRADYKININ-INDUCED CELL MIGRATION AND COX-2 EXPRESSION

As previously mentioned, AP-1 activation is a necessary transcription factor of bradykinin in cancer cells [Fredholm and Altiok, 1994]. To examine whether AP-1 activation is involved in bradykinin-induced cancer cell migration, the AP-1 inhibitors Tanshinone IIA and curcumin were used. Our results showed that both Tanshinone IIA and curcumin inhibited bradykinin-induced glioma cell migration (Fig. 6A) and COX-2 expression (Fig. 6B). In addition, stimulation of bradykinin increased c-Jun phosphorylation in a time-dependent manner and peaked at 60 min (Fig. 6C). To directly determine the AP-1 activation after bradykinin treatment, glioma cells were transiently transfected with AP-1-luciferase. As shown in Figure 6E, bradykinin treatment of glioma cells for 24 h

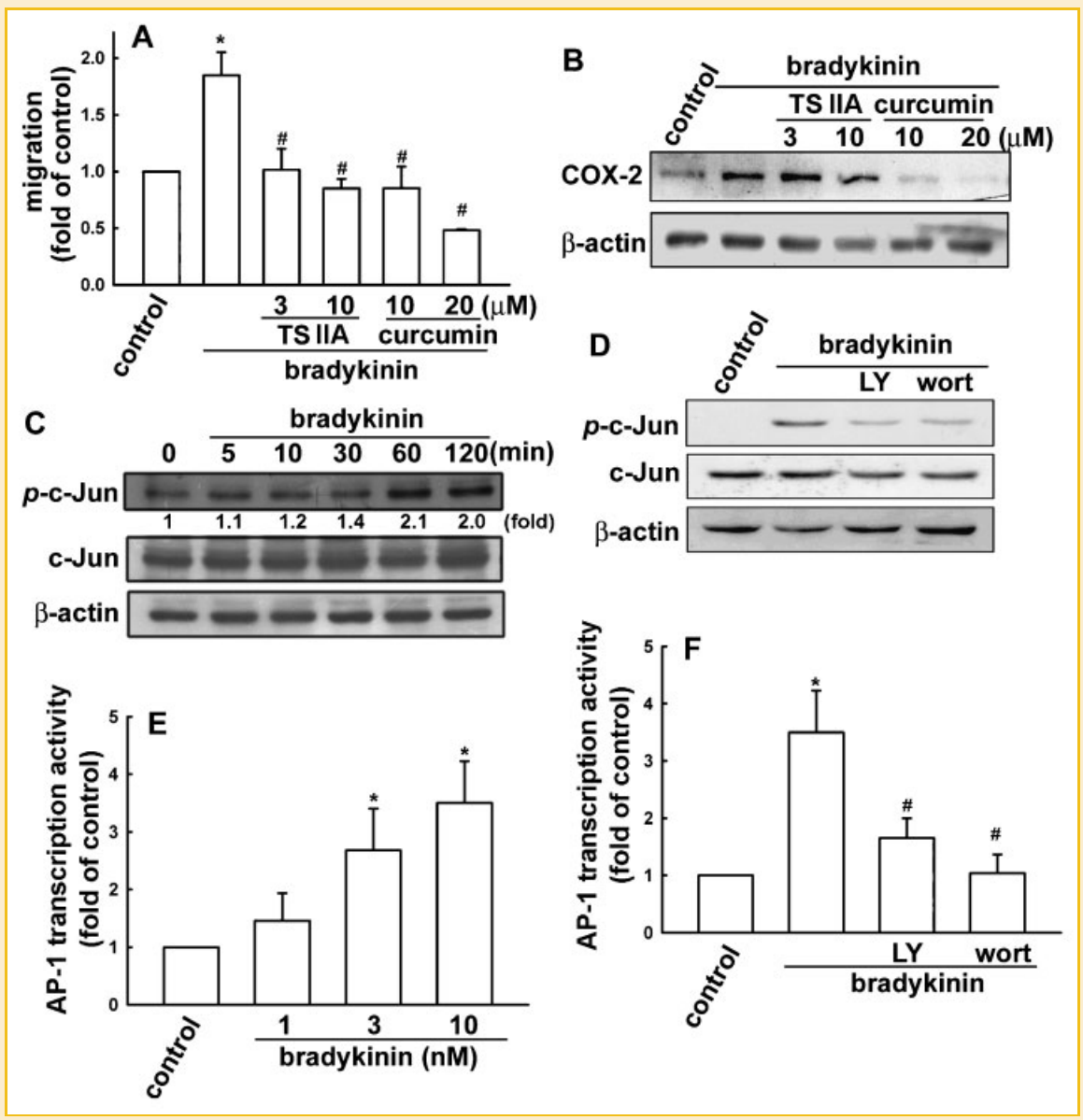

Fig. 6. Bradykinin induces cell migration and COX-2 up-regulation through AP-1. C6 cells were pretreated with the AP-1 inhibitor Tanshinone IIA (TS IIA) or curcumine for 30 min followed by stimulation with bradykinin (10 nM). In vitro migration was measured with the Transwell assay after 24 h (A). COX-2 protein expression was determined by Western blot analysis (B). Cells were treated with bradykinin (10 nM) for the indicated time periods, and the phosphorylation of c-Jun was determined by Western blot analysis (C). Cells were pretreated with PI-3 kinase inhibitor LY294002 (LY) or wortmannin (wort) for 30 min, the phosphorylation of c-Jun was determined by Western blot analysis (D). Cells were transfected with AP-1 luciferase plasmid for 24 h and stimulated with bradykinin for another 24 h (E). Cells were transfected with the AP-1 luciferase plasmid for 24 h and pretreated with LY294002 (LY) or wortmannin (wort) for 30 min before incubation with bradykinin (10 nM) for another 24 h (F). Luciferase activity was measured and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the bradykinin-treated group.

enhanced AP-1-luciferase activity. We further examined the role of PI-3 kinase/AKT in bradykinin-induced c-Jun and AP-1 activation. As shown in Figure 6D, treatment of cells with LY294002 or wortmannin antagonized the bradykinin-induced expression of c-Jun phosphorylation. Furthermore, pretreatment with LY294002 or wortmannin also markedly inhibited the increase in bradykinin-induced AP-1 transcription activity (Fig. 6F). These data suggest that activation of PI-3 kinase, AKT, c-Jun and AP-1 is important for bradykinin-induced glioma cell migration and COX-2 expression.

DISCUSSION

The elucidation of the molecular biology of cancer cells in recent years has identified alterations in various molecular pathways in different cancers. This information is currently being exploited to develop potential therapeutic targets. It has been reported that bradykinin induces cell migration of astrocytes, microglia, and neutrophils [Lo et al., 1999; Ifuku et al., 2007; Hsieh et al., 2008]. Several studies have reported mechanisms for the neuronal effects of bradykinin in the central nervous system. Moreover, bradykinin receptors are expressed in all cell types in the brain, including neurons, astrocytes, microglial cells, and oligodendrocytes [Stephens et al., 1993a,b; Delmas et al., 2002; Ifuku et al., 2007]. In this study, we found that bradykinin induces malignant rat C6 and human U251 glioma cell migration.

Two subtypes of bradykinin receptors have been distinguished, termed the B1 and B2 receptors. Numerous reports have shown that bradykinin exerts many biological functions through the B2 receptor in the central nervous system. Interestingly, the B1 receptor is expressed at low levels in neuronal cells under normal conditions, but is upregulated after application of bradykinin [Noda et al., 2003, 2007]. On the other hand, the BBB is the most important factor limiting the efficacy of chemotherapeutic agents for the treatment of brain tumors. It has been reported that bradykinin selectively acts through the B2 receptor to increase the permeability of the BBB [Inamura and Black, 1994; Nomura et al., 1994]. Previous reports have also shown that bradykinin induces COX-2 expression via B2 receptors in astrocytes [Hsieh et al., 2007]. However, a recent study indicated that both motility and chemotaxis of bradykinin-induced microglial migration is mediated by the B_1 receptor [Ifuku et al., 2007]. Furthermore, bradykinin has anti-inflammatory effects in the brain mediated by glial cells via the B1 receptor [Noda et al., 2007]. In this study, we found that bradykinin induces glioma cell migration and COX-2 expression through the B1R. This response may be mimicked by a B1-specific agonist and blocked by a B1-specific antagonist.

It has been reported that COX-2 transcription may be activated in cells responding to tumor promoters, growth factors, oncogenes, and cytokines via AP-1 [Zhang et al., 2008a]. Previous studies have suggested that signaling through the B1 receptor may contribute to activation of AP-1 [Naraba et al., 1998; Christiansen et al., 2002]. It has been reported that PI-3 kinase/AKT and c-jun signals induce the activation of AP-1 transcription factors [Yamaguchi et al., 2003; Hou et al., 2009]. Phosphorylation of the p85 subunit is required for the activation of the p110 catalytic subunit of PI-3 kinase. In this study, we found that bradykinin enhanced the p85 subunit phosphorylation in glioma cells. Pretreatment of cells with PI-3 kinase inhibitors LY294002 or wortmannin antagonized increases in cell migration and COX-2 expression in response to bradykinin stimulation. We also found that bradykinin increases AKT Ser^{473} phosphorylation. In addition, c-Jun is a major component of AP-1 complex in many cells. In most cells, c-Jun is a moderately labile protein, subject to polyubiquitination on multiple lysine residues and phosphorylation of Ser^{63} in c-Jun, leading to increased transcriptional activity. Here, we showed that bradykinin induces phosphorylation of c-Jun (Ser ^{63}), but the PI-3 kinase inhibitors LY294002 and wortmannin reduced bradykinin-induced phosphorylated c-Jun expression. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that bradykinin induces an increase in AP-1 activity. In addition, LY294002 and wortmannin also reduced bradykinin-increased AP-1 promoter activity.

These results indicate that bradykinin directed the migration of glioma cells through the B1R receptor and via upregulation of COX-2 expression. Moreover, bradykinin also increased the activation of the PI-3 kinase/AKT and the downstream transcription factor c-Jun, resulting in the activation of AP-1 on COX-2 expression and contributing to tumor migration.

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