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Enhancement of Glucose Transporter Expression of Brain Endothelial Cells by Vascular Endothelial Growth Factor Derived from Glioma Exposed to Hypoxia

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ABSTRACT

Increased need for glycolysis and glucose uptake for ATP production is observed in tumor cells, particularly in cells lacking of oxygen supply. Because glucose is transported from blood to tumor, glucose molecules must be delivered across glucose transporters of the vascular endothelium and tumor cells. Here we found that glioma suffered from hypoxic insults can secrete factor(s) to regulate glucose transporter expression in brain endothelium. It was found that conditioned medium from rat C6 glioma cells under hypoxia up-regulated glucose transporter type 1 (GLUT1) expression in rat brain endothelial cells, whereas conditioned medium from C6 cells under normoxia caused no significant effect. We further investigated whether the observed potentiating effect was caused by vascular endothelial growth factor (VEGF) production from C6 cells, because secreted VEGF was markedly increased under hypoxic condition. By transfection of C6 cells with VEGF small interfering RNA, it was found that conditioned medium from transfected cells under hypoxia no longer up-regulated GLUT1 expression of endothelial cells. Moreover, the addition of VEGF-neutralizing antibody to the hypoxic conditioned medium could also exert similar inhibitory effects. Furthermore, it was found that the VEGF-induced increase of GLUT1 expression in endothelial cells was mediated by the phosphoinositide-3 kinase/Akt pathway. Our results indicate that hypoxic brain glioma may secrete VEGF to increase glucose transport across blood-brain barrier.

Glucose is an essential metabolic substrate of all mammalian cells for energy demand. It is taken up into cells by energy-independent transportation down its concentration gradient, which is mediated by glucose transporter proteins (Gould and Holman, 1993). Transport of glucose across the plasma membrane of mammalian cells is the first rate-limiting step for glucose metabolism and is mediated by facilitative glucose transporter (GLUT) proteins. Rather than being mediated by a single transporter expressed in all cells, glucose transporter is mediated by a family of highly related transporters that are the products of distinct genes and are expressed in a highly controlled tissue-specific fashion (Bell et al., 1990).

The main glucose transporter isoforms in the brain are GLUT1 and GLUT3 (Leybaert, 2005). Of these, GLUT1 exists in two isoforms of different molecular masses. The 55-kDa isoform is located at the luminal and abluminal membranes of the brain endothelial cells, whereas the 45-kDa isoform is expressed in the perivascular end-feet of the surrounding astrocytes. The higher molecular mass of the 55-kDa isoform is due to the existence of a glycosylation site at the first extracellular loop of the membrane-spanning molecule of GLUT1 (Duelli and Kuschinsky, 2001). GLUT1, which is characteristically expressed in cells that serve barrier functions (Takata et al., 1990), such as the brain capillary endothelia of the blood-brain barrier (BBB) and retinal pigment epithelium of the inner and outer blood-retinal barrier, is a high-affinity transporter (1–5 mM), and transport through GLUT1 is therefore at near-saturation levels at normal physiological glucose concentrations (Sone et al., 2000).

More than any other organ, the brain is entirely dependent on a continuous supply of glucose from the circulation because glucose is almost the sole substrate for energy metabolism (Duelli and Kuschinsky, 2001). Other than that, malignant cells are known to have accelerated metabolism, high

ABBREVIATIONS: GLUT, glucose transporter; BBB, blood-brain barrier; CRE, cAMP-responsive element; CB, cytochalasin B; CM, conditioned medium; Hx, hypoxia; IGF, insulin-like growth factor; Nx, normoxia; PI3K, phosphoinositide-3 kinase; RBEC, rat brain endothelial cell; VEGF, vascular endothelial growth factor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; siRNA, small interfering RNA; PCR, polymerase chain reaction; rh, recombinant human; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one.

glucose requirements, and increased glucose uptake (Macheda et al., 2005). Elevated GLUT1 expression has been described in many cancers, such as breast cancer, colorectal cancer, and brain tumor (Yamamoto et al., 1990; Nishioka et al., 1992; Younes et al., 1995; Haber et al., 1998). GLUT1 overexpression has been correlated with a number of tumor characteristics, including enhanced invasive potential (Grover-McKay et al., 1998), proliferative activity, and decreased patient survival (Younes et al., 1995).

Increased need for glycolysis and therefore glucose uptake observed in tumor cells is particularly in hypoxic regions surrounding necrotic foci (Brown and Wahl, 1993). Because glucose is transported from blood to the tumor cells, glucose molecules must be delivered across glucose transporters on the vascular endothelium and then on the plasma membrane of the tumor cells. There is evidence that overexpression of glucose transporter proteins is observed widely in tumor cells, but there is little evidence to show that whether factors secreted by tumor can regulate glucose transporter expression in the microvessels. Here we found that glioma that suffered from hypoxic insults could release vascular endothelial growth factor (VEGF) to increase glucose transporter expression in brain endothelial cells.

Materials and Methods

Materials. Recombinant human VEGF, goat anti-rat VEGF antibody, and goat control IgG antibody were purchased from R & D Systems (Minneapolis, MN). Wortmannin, LY294002, and cytochalasin B were purchased from Sigma (St. Louis, MO). D-[3-^{3}H]Glucose (16.8 Ci/ml, 37 MBq/ml) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Cell Cultures. Immortalized adult rat brain endothelial cells (RBECs) was kindly provided by the Institute for Biological Sciences, National Research Council of Canada (Garberg et al., 2005; Yeh et al., 2007). RBECs were seeded onto 75-cm^2 flasks coated with type I rat tail collagen (50 μg/ml; Sigma) and maintained in M199 (Invitrogen, Carlsbad, CA) containing 1% D-glucose solution, 1% BME amino acid solution, 1% BME vitamin solution (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at 37°C in a humidified incubator under 5% CO_2 and 95% air.

C6 cells, originating from a rat brain glioma, were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in 75-cm^2 flasks with DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO_2 and 95% air.

Conditioned Medium Collected from Rat C6 Glioma Cells under Hypoxia. Before hypoxic insults, the medium of confluent rat C6 glioma cells seeded onto 10-cm dish was changed and covered with 10 ml of fresh M199 medium without serum. To induce hypoxia, cultures were placed into special chamber (Anaerobic System PROOX model 110; BioSpherix, Redfield, NY), which was closed and placed inside an incubator at 37°C and gassing the special chamber with a gas mixture consisting of 95% N_2/5% CO_2. After 24 h, conditioned medium (Hx CM) was collected and stored in 4°C until use. For comparison, normoxic cultures were incubated under normoxia and conditioned medium (Nx CM) was collected at the same time interval. The O_2 concentration in the medium at the end of the hypoxic and normoxic treatment was 40.9 $\pm$ 8.3 and 207.9 $\pm$ 1.8 mm Hg, respectively ($n = 3$).

D-[3-^{3}H]Glucose Uptake by Rat Brain Endothelial Cells. D-[3-^{3}H]Glucose uptake analysis was performed as described previously with minor modifications (Takakura et al., 1991). RBECs were seeded onto 24-well plate and cultured until confluence. After treating with C6 conditioned medium or recombinant human VEGF for 24 h, RBECs were washed with prewarmed extracellular fluid buffer (containing 10 mM HEPES,122 mM NaCl, 3 mM KCl, 25 mM NaHCO$_3$, 1.2 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, and 1.4 mM CaCl$_2$, pH 7.4) and balanced for 20 min with or without 20 μM cytochalasin B in parallel assay. D-[3-^{3}H]Glucose was diluted in extracellular fluid buffer (1 μCi in 500 μl/well) in the absence or presence of cytochalasin B (Yamamoto et al., 2006; Jing and Ismail-Beigi, 2007) and added to RBECs for 15 min. Incorporation of the radioactivity into the cells was linear during first 20 min ($r = 0.998$; Fig. 1A). D-[3-^{3}H]Glucose uptake was halted by removing the radioactive buffer and washed with ice-cold PBS, and then cells were lysed with 500 μl of 0.5% Triton X-100 overnight. Cell lysates at 100 μl along with 5 ml of counting solution (Ready Protein$^+$; Beckman Coulter, Fullerton, CA) were mixed in a counting vial (Wheaton Scientific, Millville, NJ), and radioactivity was measured by β-counter (Beckman LS6000TA).

Western Blot Analysis. Treated cultures were washed with cold PBS and lysed with radioimmunoprecipitation assay buffer (200 μl/dish) on ice for 30 min. After centrifugation at 18,000g for 20 min, the supernatant was used for Western blotting. Protein concentration was measured by BCA assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Equal proteins were separated on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated for 1 h with 4% dry skim milk in PBS buffer to block nonspecific binding and then incubated with rabbit antibodies against GLUT1 (1:1000; Abcam, Cambridge, UK), p-Akt, total Akt (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), or mouse antibody against α-tubulin (1:1000; Santa Cruz Biotechnology) for 1 h. After phosphate-buffered saline/Tween 20 washing, the membranes were then incubated with goat anti-rabbit or anti-mouse peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 h. The blots were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology) using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Reverse Transcriptase-PCR. Total RNA of treated cultures were extracted using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). RNA (1 μg) was used for reverse transcription by using a commercial kit (Invitrogen, Carlsbad, CA). PCR was performed using an initial step of denaturation (5 min at 95°C), 30 cycles of amplification (95°C for 30 s, 55°C for 1 min, and 72°C for 1 min), and an extension (72°C for 2 min). PCR products were analyzed on 2% agarose gels. The oligonucleotide primers used were as follows: GLUT1: forward, 5′-CCCGCTTCCTGCTCATCAA-3′, and reverse, 5′-GACCTTCT-TCTCCCGCATCATC-3′; glyceraldehyde-3-phosphate dehydrogenase: forward, 5′-GCCATCAACGCCCCTTCATT-3′, and reverse, 5′-ACGGAAGGCCATGCCAGTGAGCTT-3′; VEGF: forward, 5′-ACGAAAGCGCAAGAAATCCC-3′, and reverse, 5′-TTAACTCAA-GCTGCCTCGCC-3′; and β-actin: forward, 5′-AGGCTCTTTTCC-AGCCTTCCT-3′, and reverse, 5′-GTCTTTACGGATGTCAACGT-CACA-3′.

Transfection of Rat C6 Glioma Cells with VEGF siRNA. The transfection efficiency of C6 glioma cells was tested by using 5-carboxyfluorescein-labeled negative control siRNA before the following experiments. The transfection rate was approximately 80%. The rat VEGF Stealth siRNA duplex oligoribonucleotides (sense and antisense strands) were purchased from Invitrogen. Control siRNA with the same GC ratio was used as negative control. Different concentrations (10, 20, and 40 nM) of VEGF siRNA and Lipofectamine 2000 (10 μg/ml; Invitrogen) were premixed with Opti-MEM I (Invitrogen) separately for 5 min and then mix with each other for 25 min and then applied to the C6 glioma cells. After 24-h transfection, C6 glioma cells were put into hypoxia chamber for another 6 or 24 h to confirm the silencing effect of VEGF mRNA or protein production, respectively. The siRNA sequences were as follows: VEGF, 5′-AAAC-CGGGAUUUCUUGCGCUUUCGU-3′ and 5′-ACGAAAGCGCAAG-

AAAUCCCGGUUU-3′; negative control, 5′-UUCUCCGAACGU-GUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′.

Enzyme-Linked Immunosorbent Assay. After exposure of rat C6 glioma cells to hypoxia for 24 h, 100 μl of culture medium was collected and frozen at -80°C until measurement with Quantikine Rat VEGF Immunoassay ELISA kit (R&D Systems). After the addition of 50 μl of Assay Diluent into each microplate well, 50 μl of sample medium was then added and incubated for 2 h at room temperature on the shaker. After a brief wash, 100 μl of conjugate buffer was added and incubated for 1 h. Finally, 100 μl of substrate solution was added and incubated in dark, and 100 μl of stop solution was added 30 min later. The absorbance was measured at 450 nm by an ELISA reader (Bio-Tek Instruments, Winooski, VT).

Statistics. Values are expressed as mean $\pm$ S.E.M. of at least three experiments. Results were analyzed with one-way analysis of variance, followed by Neumann-Keuls test. Significance was defined as $p < 0.05$.

Results

Conditioned Medium from C6 Glioma Cells under Hypoxia Up-Regulated GLUT1 Expression in Rat Brain Endothelial Cells. Conditioned media collected from rat C6 glioma cells under either normoxia (Nx CM) or 24-h hypoxia (Hx CM) were added onto brain endothelial cells for 24 h. Control cultures were incubated with fresh M199 medium without serum for the same duration. Glucose uptake was linear within 20 min, and glucose transporter inhibitor cytochalasin B (20 μM) markedly antagonized the glucose uptake (Fig. 1A). As shown in Fig. 1B, treatment of Hx CM for 24 h significantly elevated glucose uptake in brain endothelial cells [2.2 ± 0.2-fold

of control ($n = 3$)], whereas Nx CM only slightly increased glucose uptake (1.2 ± 0.3-fold of control; $n = 3$). The increase of glucose uptake by Hx CM was also significantly reversed by cytochalasin B. To determine whether the increase of glucose uptake was due to alteration in glucose transporter expression, glucose transporter type 1 (GLUT1), which is widely expressed in brain endothelial cells, was examined by Western blotting. As shown in Fig. 1C, Hx CM significantly enhanced GLUT1 protein expression in brain endothelial cells (2.6 ± 0.4-fold of control; $n = 4$). Using reverse transcriptase-PCR analysis, treatment of Hx CM for 6 h also up-regulated GLUT1 mRNA expression in brain endothelial cells (2.4 ± 0.3-fold of control; $n = 4$; Fig. 1D).

VEGF Derived from C6 Glioma Cells under Hypoxia Increased GLUT1 Expression in Brain Endothelial Cells. It has been reported that hypoxic insults stimulate many kinds of cancer cell line to secrete a variety of growth factors to promote angiogenesis and tumor growth, and this phenomenon is also obvious in the tumor cells of perinecrotic regions because of insufficient oxygen supply in vivo (Plate et al., 1992; Patel et al., 1994; Kim et al., 1998; Feldkamp et al., 1999; Bos et al., 2005). Because VEGF is one of the markedly increased secreted molecules in glioma (Plate et al., 1992), we then examined whether the increased glucose uptake and GLUT1 expression in brain endothelial cells result from VEGF production in the C6 Hx CM.

Before experiments, the transfection efficiency of C6 glioma cells was tested by using 5-carboxyfluorescein-labeled negative control siRNA. The transfection efficiency was ap-
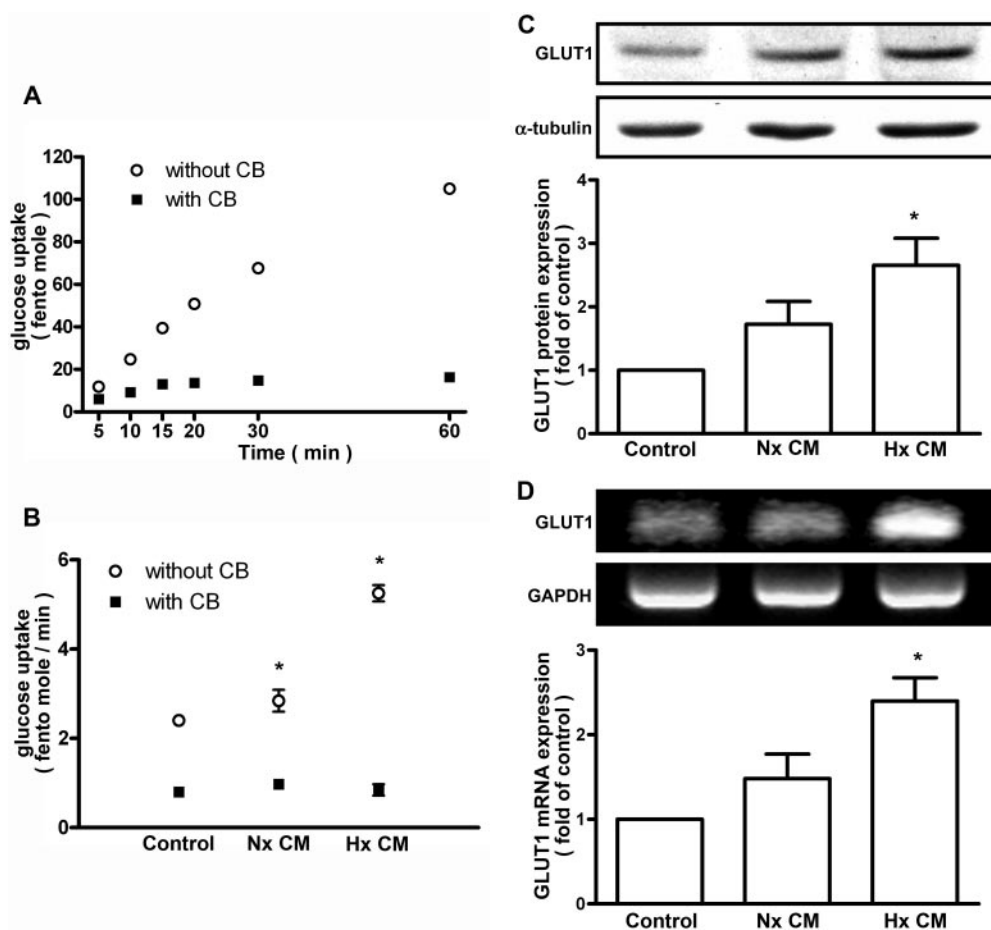

Fig. 1. Conditioned medium from rat C6 glioma cells under hypoxia up-regulates GLUT1 expression in rat brain endothelial cells. A, incorporation of D-[3-^{3}H]glucose into the cells was linear during the first 30 min. Treatment with glucose transporter inhibitor cytochalasin B (CB, 20 μM) markedly antagonized the glucose uptake. B, conditioned media from rat C6 glioma cells under either Nx CM or Hx CM for 24 h were collected. Treatment of Hx CM for 24 h significantly elevated glucose uptake in rat brain endothelial cells. Cell lysates were prepared and followed by either Western blotting for the determination of protein levels (C) or reverse transcriptase-PCR for the determination of mRNA levels of GLUT1 (D). Note that both mRNA and protein levels significantly increased by the treatment of Hx CM for 6 and 24 h, respectively. The data represent the mean $\pm$ S.E.M. from at least three independent experiments. *, $p < 0.05$ compared with control.

proximately 80%. Compared with cells transfected with negative control siRNA, transfection of VEGF siRNA in C6 glioma cells caused a reduction in VEGF mRNA expression in response to 6-h hypoxia in a dose-dependent manner. As shown in Fig. 2A, VEGF mRNA expression level was increased to 6.1 ± 0.5-fold of control under hypoxic insults, and 40 nM VEGF siRNA reduced VEGF mRNA levels by $79.8 \pm 3.3\%$ ($n = 4$). Furthermore, VEGF protein synthesis and secretion in cultured medium measured by ELISA kit revealed that there was a 5.1-fold increase after 24-h hypoxia compared with normoxia (523.3 ± 107.4 and 2681.7 ± 123.2 pg/10^5 cells, respectively), which was reduced by 40 nM VEGF siRNA transfection (839.1 ± 34.8 pg/10^5 cells, $n = 4$; Fig. 2B). Hypoxia conditioned medium collected from VEGF siRNA-transfected C6 glioma cells was then added onto brain endothelial cells. As shown in Fig. 3A, glucose uptake induced by C6 Hx CM (24 h) in brain endothelia cells was
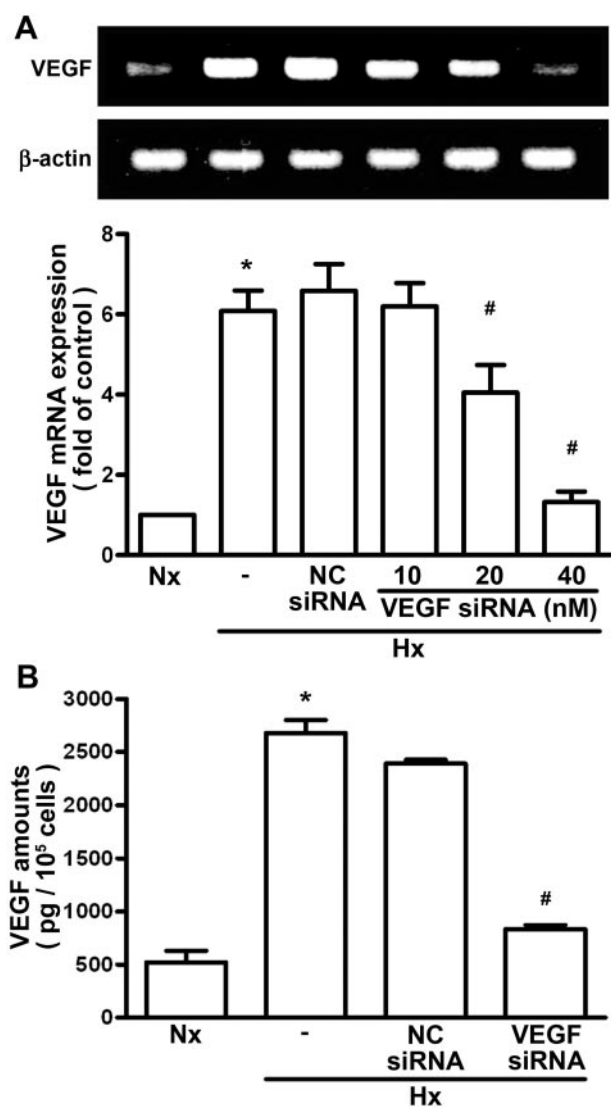

Fig. 2. VEGF secretion in response to hypoxia is inhibited by the transfection of rat C6 glioma cells with VEGF siRNA. A, VEGF siRNA dose-dependently suppressed VEGF mRNA expression of C6 glioma cells under 6-h hypoxia. B, C6 glioma cells transfected with 40 nM VEGF siRNA but not negative control siRNA (NC siRNA) significantly inhibited secreted amount of VEGF after 24-h hypoxia. The data represent the mean $\pm$ S.E.M. from four independent experiments. *, $p < 0.05$ compared with normoxia; #, $p < 0.05$ compared with hypoxia alone.
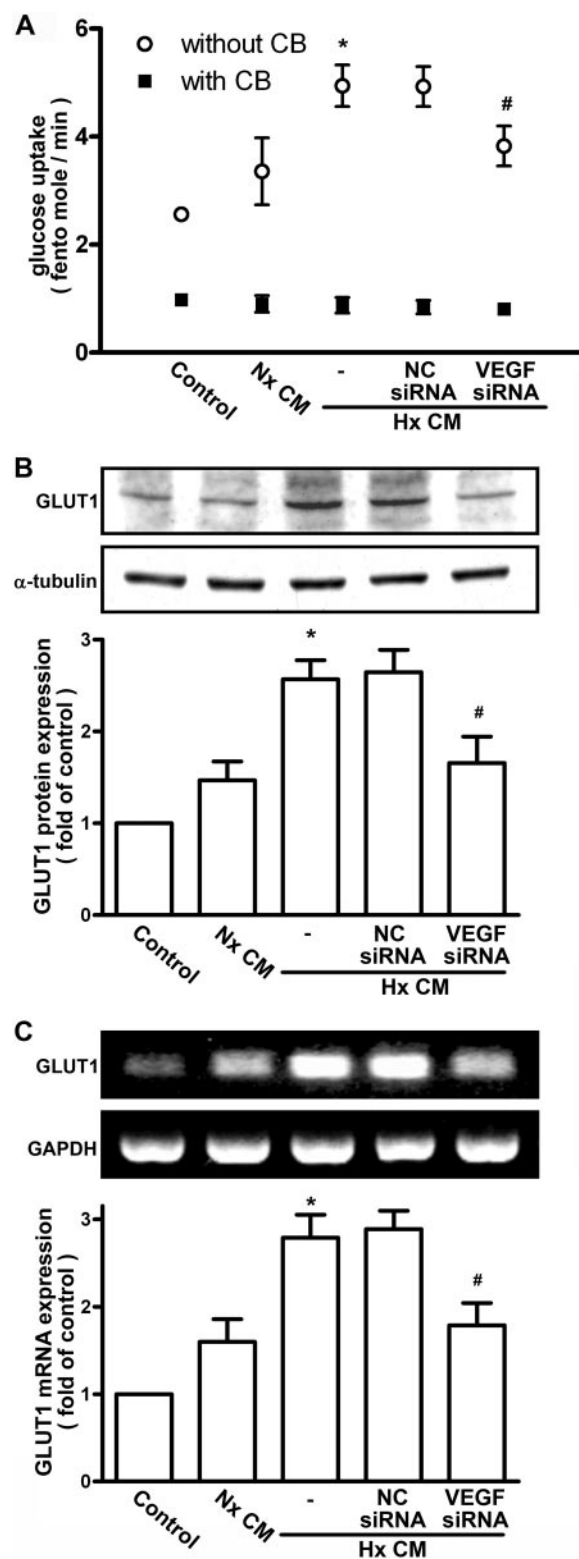

Fig. 3. Hypoxia conditioned medium-induced GLUT1 expression of brain endothelial cells is antagonized by VEGF siRNA transfection in C6 glioma cells. A, increase of glucose uptake in brain endothelial cells by Hx CM was antagonized by VEGF siRNA transfection in C6 cells. B and C, Hx CM-induced the increase of GLUT1 protein and mRNA levels was antagonized by the transfection of C6 cells with VEGF siRNA. NC siRNA was used as negative control. The data represent the mean $\pm$ S.E.M. from at least three independent experiments. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with Hx CM alone; CB, cytochalasin B 20 μM.

partially antagonized by $41.4 \pm 2.6\%$ ($n = 4$). Potentiation of GLUT1 protein and mRNA expression in brain endothelial cells was also reduced by Hx CM derived from VEGF siRNA transfected C6 glioma cells [$35.6 \pm 7.4\%$ (24 h) and $35.4 \pm 7.4\%$ (6 h), respectively ($n = 4$; Fig. 3, B and C)].

In addition, the enhanced glucose uptake and GLUT1 expression in brain endothelial cells resulting from VEGF secretion in C6 hypoxic conditioned medium were certified by the addition of VEGF antibodies to neutralize VEGF in the C6 Hx CM. As shown in Fig. 4A, the addition of 1 μg/ml VEGF neutralizing antibody partially reversed C6 Hx CM-induced glucose uptake in brain endothelial cells by $35.8 \pm 7.0\%$ (24 h; $n = 4$), whereas the addition of negative control IgG antibody caused no significant effect. The increased GLUT1 protein and mRNA expression by the treatment of C6 Hx CM was also partially reversed by the addition of VEGF antibody to the conditioned medium [$38.3 \pm 3.6\%$ (24 h) and $37.9 \pm 7.2\%$ (6 h), respectively ($n = 4$; Fig. 4, B and C)].

Exogenous VEGF Increased GLUT1 Expression in Brain Endothelial Cells. To further confirm the involvement of VEGF in glucose uptake and GLUT1 expression in brain endothelial cells, brain endothelial cells were exposed to exogenous VEGF. As shown in Fig. 5A, recombinant human VEGF (rhVEGF; 25 ng/ml, 24 h) significantly increased glucose uptake in brain endothelial cells to 1.6 ± 0.5-fold of control ($n = 3$). Recombinant human VEGF also dose-dependently up-regulated GLUT1 protein and mRNA expression levels in brain endothelial cells. Under 25 ng/ml rhVEGF treatment, GLUT1 protein and mRNA expression were increased by 1.1 ± 0.2- (24 h) and 1.5 ± 0.2-fold (6 h), respectively (Fig. 5, B and C).

Involvement of PI3K/Akt Pathway in VEGF-Induced Increase of GLUT1 Expression in Brain Endothelial Cells. Because PI3K/Akt is one of the most common downstream signaling pathways activated by growth factors and has a potential role in cancer progression (Brader and Eccles, 2004), we thus further investigated whether PI3K/Akt is involved in VEGF-induced increase of GLUT1 expression in brain endothelial cells. First of all, we examined whether rhVEGF and C6 hypoxic conditioned medium can enhance Akt phosphorylation in brain endothelial cells. As shown in Fig. 6A, 25 ng/ml rhVEGF potentiated Akt phosphorylation between 5 and 30 min, and phosphorylation was reduced after 60-min exposure. The enhanced Akt phosphorylation was inhibited by pretreatment for 30 min with 200 nM wortmannin and 10 μM LY294002 (Fig. 6B). In addition, brain endothelial cells stimulated by C6 Hx CM for 15 min also markedly increased Akt phosphorylation to 5.6 ± 0.6-fold of control, which was antagonized by wortmannin and LY294002, whereas Nx CM also increased phosphorylation of Akt (3.3 ± 0.4-fold of control; Fig. 6C). Furthermore, VEGF-induced glucose uptake in brain endothelial cells was antagonized by pretreatment for 30 min with PI3K/Akt inhibitors wortmannin and LY294002 (Fig. 6D; 24 h; Wort, 200 nM, $64.3 \pm 9.4\%$, and LY294002, 10 μM, $66.8 \pm 4.6\%$, respectively). Furthermore, wortmannin and LY294002 also inhibited rhVEGF (25 ng/ml)-induced increase of GLUT1 protein expression by 44.5 ± 7.6 and $50.3 \pm 6.0\%$, respectively (Fig. 6E). Treatment of wortmannin and LY294002 for 6 h also inhibited VEGF-induced increase of GLUT1 mRNA expression by 43.2 ± 5.3 and $44.2 \pm 5.8\%$, respectively (Fig. 6F).
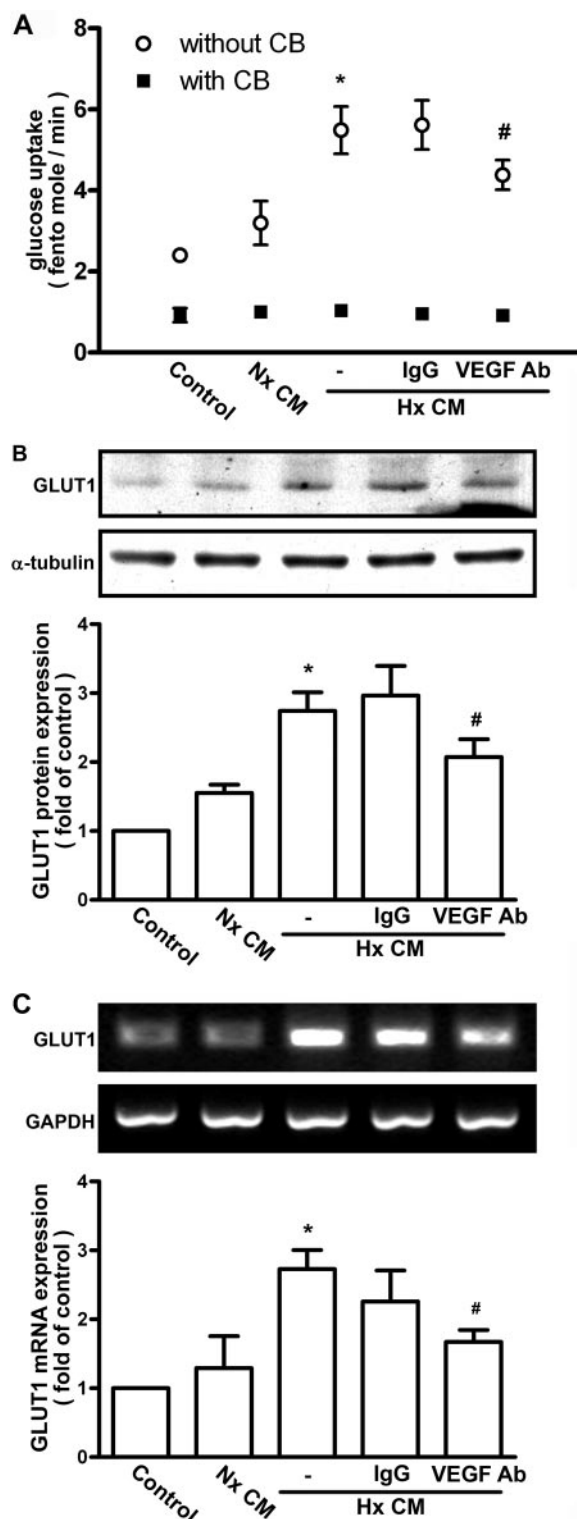
Fig. 4. Inhibition of C6 hypoxia conditioned medium-induced GLUT1 expression of brain endothelial cells by the addition of VEGF antibody. The increase of glucose uptake (A), protein expression (B), and mRNA levels (C) of brain endothelial cells induced by the treatment of C6 glioma cells Hx CM was partially reversed by the addition of VEGF antibody to the conditioned medium. IgG antibody was used as negative control. The data represent the mean $\pm$ S.E.M. from at least three independent experiments. $*$, $p < 0.05$ compared with control; #, $p < 0.05$ compared with Hx CM alone; CB, cytochalasin B 20 μM.

Discussion

Cellular proliferation requires glucose metabolism and glucose entry into cells occurs via transporter-mediated facili-

Fig. 5. Exogenous VEGF increases GLUT1 expression in brain endothelial cells. A, the addition of recombinant human VEGF (25 ng/ml; 24 h) increased glucose uptake of brain endothelial cells. B and C, treatment of 25 ng/ml rhVEGF for 24 or 6 h dose-dependently increased both protein and mRNA expression of GLUT1, respectively. The data represent the mean ± S.E.M. from at least three independent experiments. *, $p < 0.05$ compared with control; CB, cytochalasin B 20 μM.

tated diffusion. Glucose uptake is enhanced in oncogenically transformed cells, and elevated levels of glucose transporter protein often accompany this phenotype change in some transformed cells (Flier et al., 1987). Because the glucose transporter is also a proliferation-related gene and its expression is associated with the activation of signal transduction mechanisms, its expression should also be induced when a growth factor binds to its receptor (Rollins et al., 1988). VEGF exerts its mitogenic effects in various types of tumor cells, in part, by inducing the expression of genes whose products are required for endothelial cells proliferation and cancer progression (Plate and Mennel, 1995). Given evidence that VEGF may act as a survival factor in tumor (Ezhilarasan et al., 2007), the ability of VEGF to regulate endothelial cell glucose transport in conjunction with angiogenesis may serve to ensure adequate substrate delivery and blood flow during tumor progression.

Hypoxia up-regulates hypoxia-inducible factor-1α and its downstream target GLUT1 in glioma cells (Ragel et al., 2007); the increased expressed levels of GLUT1 by glioma cells bordering necrotic regions suggests that this glucose transporter protein might be induced by hypoxia, acidosis, or by a combination of both conditions (Gorin et al., 2004). However, the more energy demand for tumor growth still requires endothelium to uptake more glucose from the blood supply. Although microvessels inside the tumor do not have intact barrier function, the microvessels on the tumor periphery have intact barrier property and show strong GLUT1 expression (Harik and Roessmann, 1991). GLUT1 is used as one of the cytological markers to map tumor vascularization, and the majority of GLUT1 is expressed in endothelium of intracerebral arterioles that maintain tight junctions and high endothelial impedance. Around the tumor microenvironment, GLUT1 is expressed primarily in arterioles at the tumor periphery (Gorin et al., 2004). Here we show that BBB endothelium in the hypoxia area bordering tumor necrotic regions may be affected by secreted factor(s) such as VEGF derived from hypoxic tumor cells to increase GLUT1 expression. We demonstrated that VEGF secreted by hypoxic glioma enhanced GLUT1 expression in brain endothelial cells, resulting in the increased glucose uptake across endothelium into surrounding cells. The elevated glucose level may sufficiently support the energy demand of brain tumor cells to proliferate and survive (Patronas et al., 1985; Herholz et al., 1993; Chung et al., 2004).

VEGF has been reported previously to up-regulate glucose uptake and transporter expression in aortic endothelial cells (Pekala et al., 1990). However, Sone et al. (2000) found that VEGF enhances glucose uptake in retinal endothelial cells but has no influence on protein or mRNA expression of glucose transporter. In addition to VEGF, much evidence indicates that other growth factors can also regulate glucose transporter expression. For example, platelet-derived growth factor, fibroblast growth factor, insulin-like growth factor, and epidermal growth factor have been reported to enhance glucose uptake and increase glucose transporter mRNA or protein levels in fibroblast cell lines (Hiraki et al., 1988; Rollins et al., 1988). According to these findings, it may explain that VEGF silencing and the addition of VEGF neutralizing antibody only partially antagonized C6 hypoxic conditioned medium-induced increase of GLUT1 expression and glucose uptake (Figs. 3 and 4), which may be due to
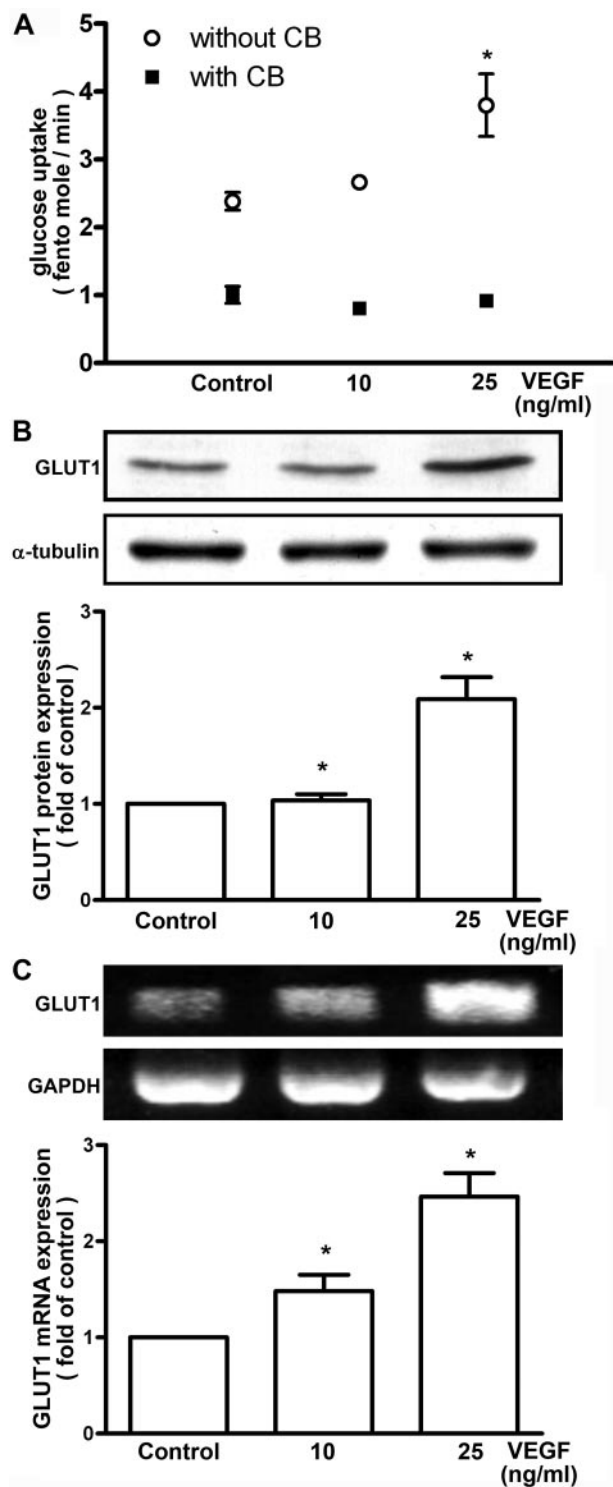
up-regulation of other growth factors by hypoxic insults in tumor cells.

Consistent with our results, Barthel et al. (1999) found that GLUT1 gene transcription is regulated by the serine/threonine kinase Akt1, and Akt may influence GLUT1 gene expression by phosphorylating a protein that binds to the
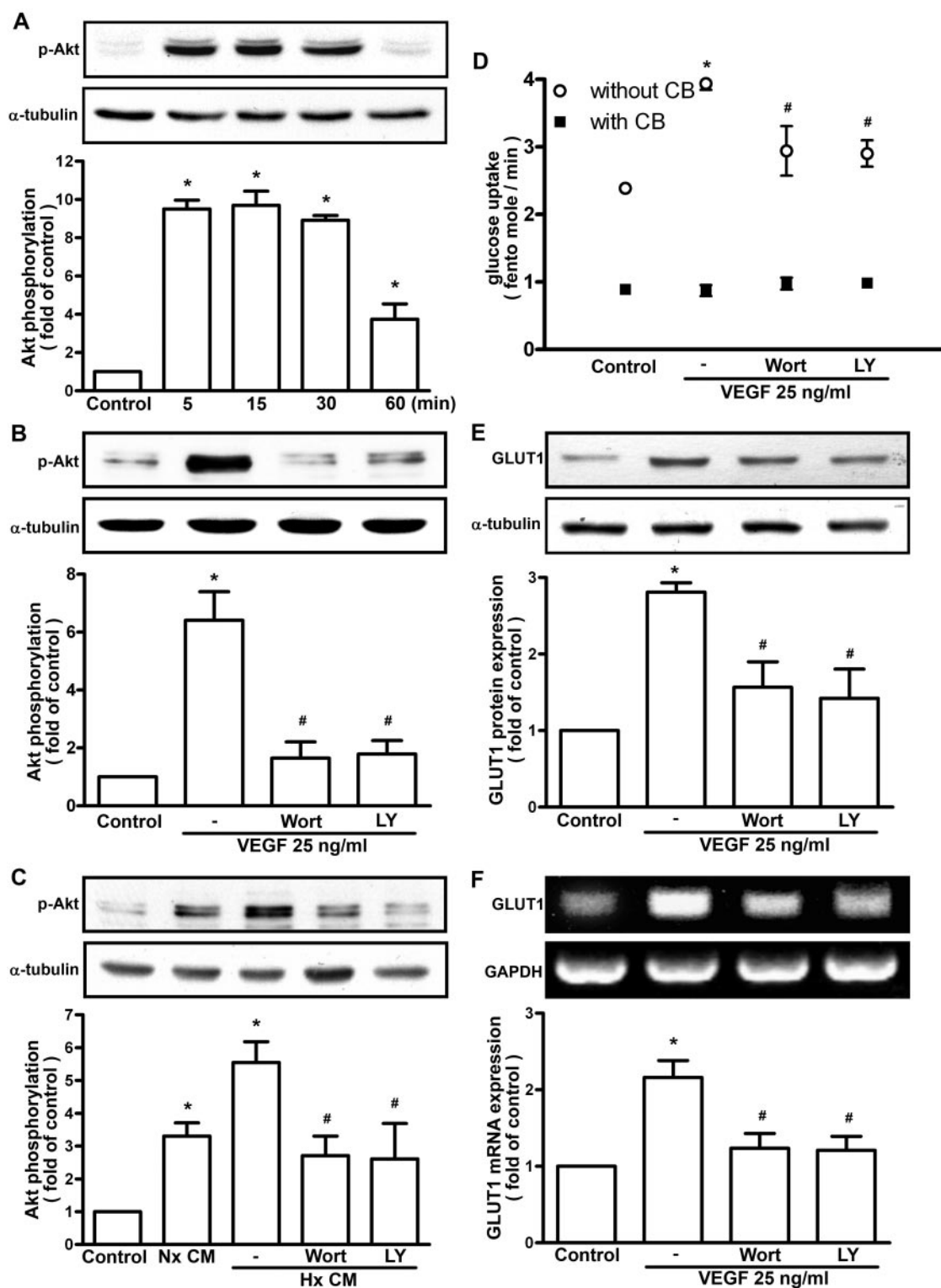

Fig. 6. Involvement of PI3K/Akt pathway in VEGF-induced increase of GLUT1 expression in brain endothelial cells. A, the addition of rhVEGF (25 ng/ml) time-dependently increased Akt phosphorylation in brain endothelial cells and returned to basal level after 60-min treatment. B, the enhanced Akt phosphorylation was inhibited by pretreatment for 30 min with 200 nM wortmannin and 10 μM LY294002. C, Hx CM collected from C6 glioma cells significantly increased Akt phosphorylation within 15 min, whereas Nx CM increased p-Akt slightly. VEGF-induced increase of glucose uptake (D), protein expression (E), and mRNA levels (F) of GLUT1 in brain endothelial cells was antagonized by PI3K/Akt inhibitors (wortmannin 200 nM, LY294002 10 μM). The data represent the mean $\pm$ S.E.M. from at least three independent experiments. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with VEGF alone.

serum-responsive element, the cAMP-responsive element, and/or the 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements (TREs) (Murakami et al., 1992; Todaka et al., 1994). Moreover, in a variety of cell types, mitogenic stimuli such as serum, platelet-derived growth factor, and epidermal growth factor rapidly induce the expression of the GLUT mRNA in parallel to the up-regulation of Jun and Fos (Rollins et al., 1988; Sistonen et al., 1989). Which transcription complex participates in the enhanced glucose uptake and GLUT1 expression in brain endothelial cells still requires further investigation.

Glucose transport is under the control of a family of related transporters. The transporter expression is tissue-specific and developmentally regulated (Pekala et al., 1990). To our knowledge, this is the first report to demonstrate that GLUT1 of brain vascular endothelium with barrier function can be regulated by secreted factor(s) from glioma. In summary, the results from this study demonstrate that hypoxic C6 glioma cells secrete factor(s) in increasing glucose uptake and GLUT1 expression in brain endothelial cells, and VEGF is partially involved in this phenomenon through PI3K/Akt pathway. These findings provide another beneficial effect in targeting VEGF for cancer therapeutic use. Furthermore, a disruption of glucose uptake via glucose transporter proteins may alter the metabolism of malignant cells, leading to reduced tumor growth. Therefore, glucose transporter proteins could be the meaningful targets for cancer treatment.

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