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Calcium-activated potassium channels mediated blood-brain tumor barrier opening in a rat metastatic brain tumor model

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Abstract

Background: The blood-brain tumor barrier (BTB) impedes the delivery of therapeutic agents to brain tumors. While adequate delivery of drugs occurs in systemic tumors, the BTB limits delivery of anti-tumor agents into brain metastases.

Results: In this study, we examined the function and regulation of calcium-activated potassium (K_{Ca}) channels in a rat metastatic brain tumor model. We showed that intravenous infusion of NS1619, a K_{Ca} channel agonist, and bradykinin selectively enhanced BTB permeability in brain tumors, but not in normal brain. Iberiotoxin, a K_{Ca} channel antagonist, significantly attenuated NS1619-induced BTB permeability increase. We found K_{Ca} channels and bradykinin type 2 receptors (B2R) expressed in cultured human metastatic brain tumor cells (CRL-5904, non-small cell lung cancer, metastasized to brain), human brain microvessel endothelial cells (HBMEC) and human lung cancer brain metastasis tissues. Potentiometric assays demonstrated the activity of K_{Ca} channels in metastatic brain tumor cells and HBMEC. Furthermore, we detected higher expression of K_{Ca} channels in the metastatic brain tumor tissue and tumor capillary endothelia as compared to normal brain tissue. Co-culture of metastatic brain tumor cells and brain microvessel endothelial cells showed an upregulation of K_{Ca} channels, which may contribute to the overexpression of K_{Ca} channels in tumor microvessels and selectivity of BTB opening.

Conclusion: These findings suggest that K_{Ca} channels in metastatic brain tumors may serve as an effective target for biochemical modulation of BTB permeability to enhance selective delivery of chemotherapeutic drugs to metastatic brain tumors.

Background

The blood-brain barrier (BBB), formed by the capillary endothelial cells surrounded by astrocytes, protects the

brain, but it also poses an obstacle for the delivery of therapeutic molecules into the brain. Microvessels supplying brain tumors retain some characteristics of the BBB and

form a blood-brain tumor barrier (BTB). While adequate delivery of chemotherapeutic drugs has been achieved in systemic tumors, the BTB limits such delivery to brain metastases. Therefore, understanding the biochemical modulation of BBB and BTB is critical for developing strategies to deliver therapeutic agents into metastatic brain tumors.

During the past decade, various strategies have been used to deliver therapeutic drugs selectively to brain tumors and injured brain, including, biodegradable polymers implanted into the tumor cavity [1], convection-enhanced delivery [2,3], and BBB/BTB disruption [4,5]. Our laboratory has focused on pharmacologic modulations to increase BTB permeability and increase delivery of therapeutic drugs selectively to brain tumors with little or no drug delivery to normal brain tissue [6-9]. This strategy exploits the function of certain vasomodulators that play a key role in modulation of BBB/BTB permeability. It has been demonstrated that bradykinin [10], leukotriene (LTC_4) [11-13], nitric oxide (NO) [14], c-GMP [8], and potassium channel agonists [15,16] can selectively increase capillary permeability in primary brain tumors, while leaving normal brain unaffected. These findings have already been translated into clinical studies to increase drug delivery selectively to tumor tissue in brain tumor patients [7,17-19]. Modulation of critical molecules involved in selectively increasing BTB permeability could lead to the development of effective strategy to increase chemotherapy delivery to brain tumors.

Large conductance calcium-activated potassium (K_{Ca}) channels are a unique class of ion channel coupling intracellular chemical and electrical signaling. These channels give rise to outwardly rectifying potassium currents and respond not only to changes in membrane voltage, but also to changes in intracellular calcium. Recent studies suggest that K_{Ca} channel expression levels correlate positively with the malignancy grade of glioma [20]. K_{Ca} channels are also present in cerebral blood vessels, where they regulate cerebral blood vessel tone [21] and, probably, BBB/BTB permeability [15,22]. Evidence from several studies further indicate that K_{Ca} channels play an important role in vasodilation when it is mediated by bradykinin [23,24], NO donors [25], and cyclic GMP [26]. In response to the binding of bradykinin to its type 2 receptors (B2R), intracellular Ca^{2+} is increased either by mobilization of Ca^{2+} from internal sites and influx [27] or by NO production from NO synthase activation [14]. The increase in intracellular Ca^{2+} level activates K_{Ca} channels and alters the membrane potential of cells [28]. Furthermore, previous studies have also shown that bradykinin-induced K_{Ca} channel activation in endothelial cells is potentiated by NS1619, a selective K_{Ca} channel agonist [29], and attenuated by a highly selective inhibitor, iberi-

otoxin (IBTX) [29-31]. We previously demonstrated that K_{Ca} channels are overexpressed in primary brain tumors and tumor microvessels, and such channels respond to NS1619, which selectively increases BTB permeability. The accelerated formation of pinocytotic vesicles appears to be the cellular mechanism by which K_{Ca} channels mediate increases in BTB permeability [15]. Moreover, in a rat brain tumor model, we showed that the B2R expression level on brain tumors directly correlates with bradykinin-induced BTB permeability increases [32]. Co-infusion of carboplatin with either NS1619 or a bradykinin analog, RMP-7, led to enhanced survival in intracranial tumor-bearing rats and brain tumor patients [17-19,22,33]. These data indicate that K_{Ca} channels serve as a convergence point in the modulation of BTB permeability in primary brain tumors.

Brain metastasis is a frequent complication in patients suffering from lung and breast cancer, and a significant cause of morbidity and mortality. Brain metastases are found in approximately 10% of lung cancer patients at the time of diagnosis, and up to 40% of all patients develop brain metastases during the course of their disease [34]. The prognosis of brain metastases from lung cancer is poor, with median survival of 4~5 month. Lung cancer cells that spread to the brain are generally sensitive to chemotherapeutic drug compared with primary brain tumor cells. The BTB, however, prevents the delivery of non-lipid-permeable chemotherapeutic drugs and monoclonal antibodies in sufficient amounts to achieve a therapeutic benefit [35], especially in early stage of brain metastases. Although metastatic brain tumors have ten times more than the incidence of primary brain tumors in the United States, the role and regulation of K_{Ca} channels in metastatic brain tumors to selectively open BTB have not been elucidated. As new therapeutic agents are developed which effectively treat primary tumors, an efficient delivery of these agents selectively to metastatic brain tumors across the BTB will significantly improve treatment efficacy. Here, we studied the role of K_{Ca} channel activation in BTB permeability in a metastatic brain tumor model.

Results

K_{Ca} channel mediates BTB permeability increase in a CRL-5904 metastatic brain tumor model

To determine whether K_{Ca} channels mediate BTB permeability in a metastatic brain tumor model, intracranial CRL-5904 tumor bearing-rats received intravenous infusion of NS1619 (0~120 $\mu\text{g}/\text{kg}/\text{min}$), bradykinin, IBTX, or PBS. The transport constant (K_i) was determined by radiotracer [^{14}C] sucrose uptake in the tumor core, tumor-adjacent brain tissue, and contralateral brain tissue. The data were obtained from 3~6 rats for each group. Intravenous infusion of NS1619 at 30 $\mu\text{g}/\text{kg}/\text{min}$ resulted in a significant increase of K_i values in the tumor center ($11.24 \pm 1.99 \mu\text{l}/$

g/min) as compared with PBS control ($6.58 \pm 0.75 \mu\text{l/g/min}$; $p < 0.05$, NS1619 vs PBS). A higher concentration of NS1619 at $60 \mu\text{g/kg/min}$ further increased K_i values ($12.77 \pm 1.99 \mu\text{l/g/min}$; $p < 0.01$, NS1619 vs PBS). While, increasing dose to $120 \mu\text{g/kg/min}$ did not elicit any further K_i increase. Intravenous infusion of bradykinin ($120 \mu\text{g/kg/min}$) also significantly increased BTB permeability within the tumor, with average K_i values at $13.31 \pm 2.48 \mu\text{l/g/min}$ ($p < 0.01$, bradykinin vs PBS). Furthermore, NS1619- and bradykinin- induced BTB permeability increases resulted in enhanced delivery of radiotracer [^{14}C] sucrose to the tumor center without any increase to tumor adjacent brain tissue and contralateral normal brain (Figure 1A). In a separate experiment, we found that co-administration of a specific K_{Ca} channel inhibitor, IBTX ($1 \mu\text{g/kg/min}$), blocked the increase of BTB permeability induced by NS1619 (K_i value of NS1619+IBTX is $7.58 \pm 1.31 \mu\text{l/g/min}$; NS1619+IBTX vs NS1619, $p < 0.05$). Intravenous infusion of IBTX alone did not show any effect on BTB permeability (Figure 1B). These data confirm that activation of K_{Ca} channel selectively induces BTB opening in tumor tissue, but not tumor adjacent tissue or contralateral normal brain, in a metastatic brain tumor animal model.

Expression of K_{Ca} channels and B2R in CRL-5904 cells, HBMEC and human tumor tissue of lung cancer brain metastases

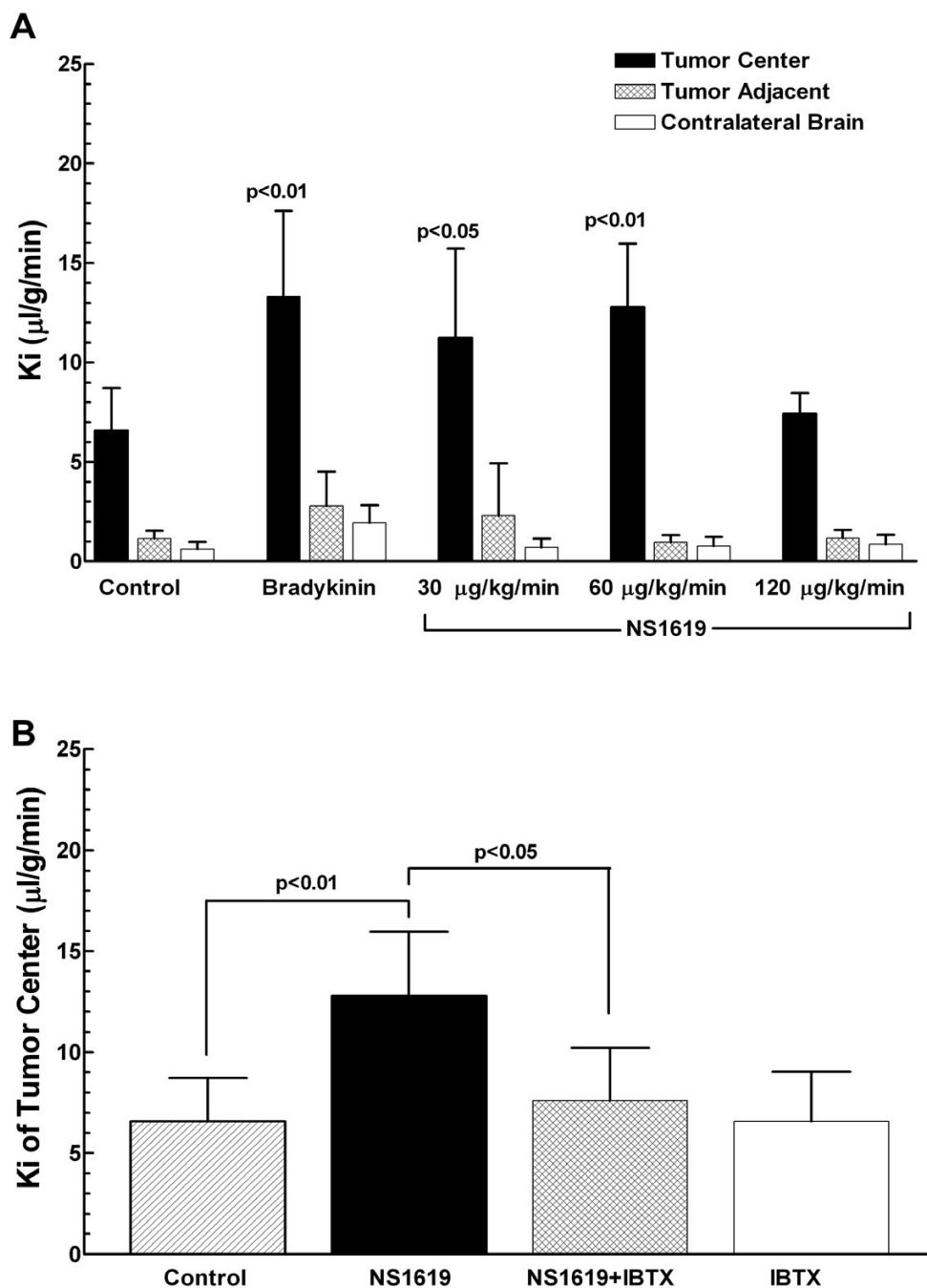
To examine whether K_{Ca} channels were present in tumor tissue, immunostaining of paraffin-embedded tissue sections from lung cancer brain metastases patients were performed. The results demonstrated that K_{Ca} channels (Figure 2A) and B2R (Figure 2B) expressed extensively in tumor masses and microvessels within the tumor. Negative control experiments of K_{Ca} channels (Figure 2C) and B2R (Figure 2D) did not show specific staining on the corresponded specimens. Elevated mRNA level of K_{Ca} channels was also detected in lung cancer brain metastases tissues from patients using real time PCR (data not shown). To further determine whether K_{Ca} channels and B2Rs are present in metastatic brain tumor and endothelial cells, we examined their expression by immunocytochemistry. Fluorescence immunostaining showed robust K_{Ca} channel expression in cultured CRL-5904 cells, which distributed on the cell membrane, cytoplasm and perinuclear components (Figure 3A~C). K_{Ca} channels were also detected in HBMEC (Figure 3G~I), but the signal intensities were lower compared with that in CRL-5904 cells. B2R expression was detected in both CRL-5904 cells (Figure 3D~F) and HBMEC (Figure 3J~L) with a higher level of expression in CRL-5904 cells. These data illustrate the presence of K_{Ca} channels in cultured metastatic brain tumor cells, endothelial cells, and most importantly, in human metastatic brain tumor tissue.

Activity of functional K_{Ca} channels in cultured CRL-5904 cells and HBMEC

Since the above data demonstrated the presence of K_{Ca} channels in metastatic brain tumors and endothelial cells as well as metastatic brain tumor tissue, we determine whether the overexpressed K_{Ca} channels were functional. Therefore we examined membrane potential activity of K_{Ca} channels in cultured tumor and endothelial cells and by using a fluorescent dye-based potentiometric assay that measures changes in membrane potential. To activate K_{Ca} channel, NS1619 ($25 \mu\text{M}$), a K_{Ca} channel agonist, was added to the cells, and membrane potential changes were monitored for up to 300 seconds. Upon activation a decrease in membrane potential was observed in CRL-5904 cells (Figure 4A) and HBMEC (Figure 4C), an effect that lasted more than 300 seconds. Furthermore, we introduced bradykinin ($25 \mu\text{M}$) to the cultured cells and observed membrane potential changes in CRL-5904 cells (Figure 4B) and HBMEC (Figure 4D) that lasted for approximately 100 seconds. Moreover, both NS1619 and bradykinin elicited greater hyperpolarization on CRL-5904 cells compared with HBMEC. IBTX (20 nM), a K_{Ca} channel antagonist, reversed the membrane potential changes on both cells caused by NS1619 and bradykinin. Furthermore, we found that NS1619 and bradykinin could induce a dose-dependent membrane potential change in CRL-5904 and HBMEC (data not shown). These data indicate that K_{Ca} channels are functional on both CRL-5904 cells and HBMEC. The K_{Ca} channels can be activated directly by NS1619 or indirectly through B2R signaling by bradykinin.

Co-culture of metastatic brain tumor and endothelial cells increases K_{Ca} Channel expression

We further investigated whether K_{Ca} channels expression is modulated by the interaction of metastatic brain tumor cells and endothelial cells. CRL-5904 cells were co-cultured with HBMEC, and protein and mRNA levels of K_{Ca} channels were examined subsequently. K_{Ca} channel were overexpressed in CRL-5904/HBMEC co-cultures compared to single cultures of either CRL-5904 cells or HBMEC by western blot assay (Figure 5A). Image quantification analysis showed an approximately 30% increase of K_{Ca} channel expression in co-culture of CRL-5904/HBMEC compared to individual cultures by normalized to β -actin as an internal control (Figure 5B). Also, individual cultures of CRL-5904 tumor cells had higher K_{Ca} channel expression than HBMEC. RT-PCR analysis also showed an increase in K_{Ca} channel mRNA levels in co-culture cells compared to individual cultures (Figure 5C). These data suggest that co-culture of metastatic tumor and brain endothelial cells results in upregulation of K_{Ca} channel.

**Figure 1**

Regional K_i values in metastatic brain tumor-bearing animals. Quantitative increases in BTB permeability after biochemical modulation are shown in dose dependent manner (A). Intravenous infusion of NS1619 (30 $\mu\text{g}/\text{kg}/\text{min}$; $n = 5$) significantly increased K_i in the tumor center compared with PBS control ($p < 0.05$). The optimal dose of NS1619 (60 $\mu\text{g}/\text{kg}/\text{min}$; $n = 5$) further increased K_i value ($p < 0.01$, NS1619 vs PBS). At higher dose (120 $\mu\text{g}/\text{kg}/\text{min}$; $n = 6$), however, no BTB permeability increased with infusion of NS1619. Intravenous infusion of BK (120 $\mu\text{g}/\text{kg}/\text{min}$; $n = 3$) also significantly increased K_i in the tumor center compared with control ($p < 0.01$). NS1619 (60 $\mu\text{g}/\text{kg}/\text{min}$)-induced BTB permeability increase was significantly attenuated by a specific K_{Ca} channels antagonist, IBTX (1 $\mu\text{g}/\text{kg}/\text{min}$; $n = 4$), ($p < 0.05$, NS1619+IBTX vs NS1619). While, intravenous infusion of IBTX ($n = 3$) alone did not show any effect on BTB permeability (B).

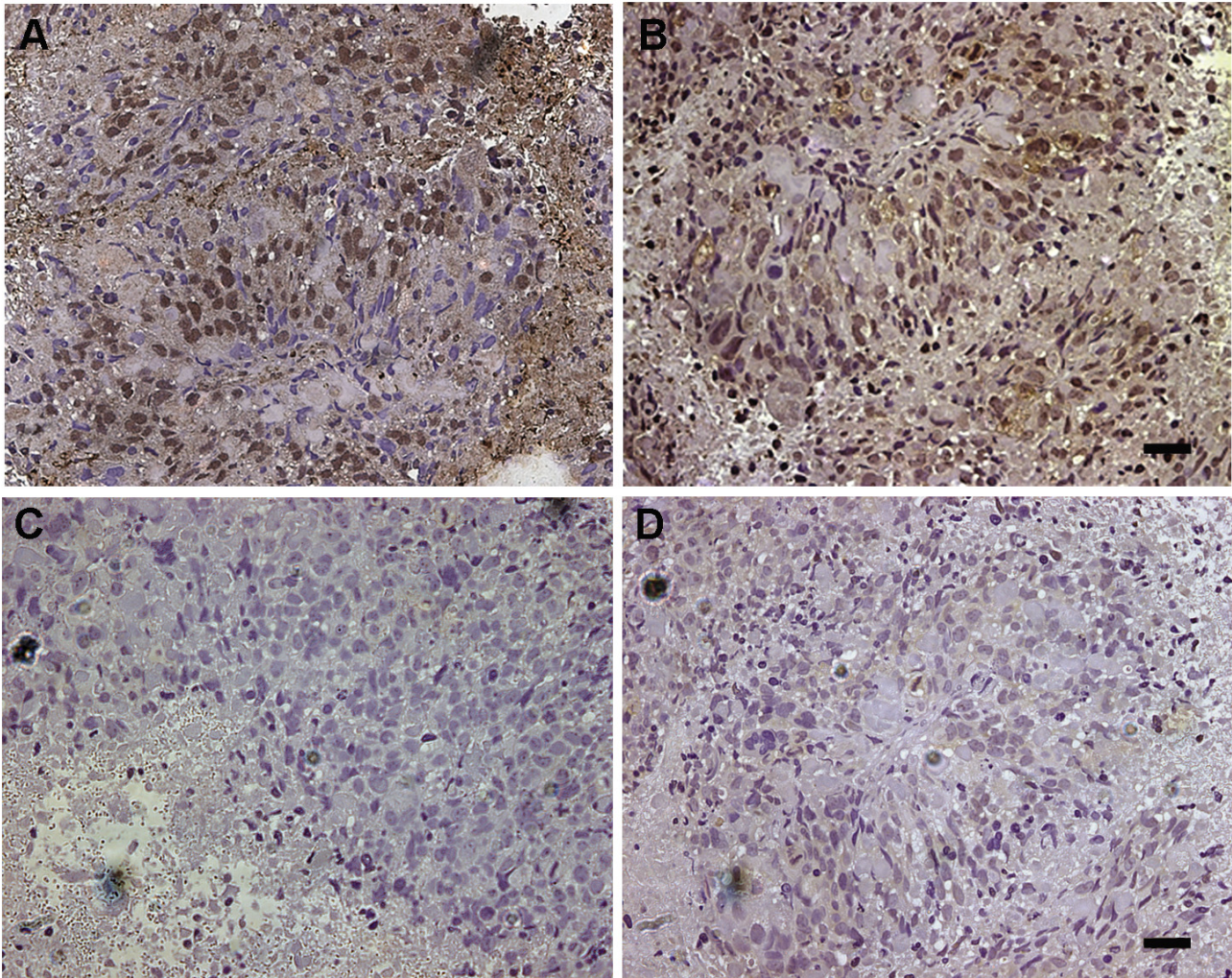
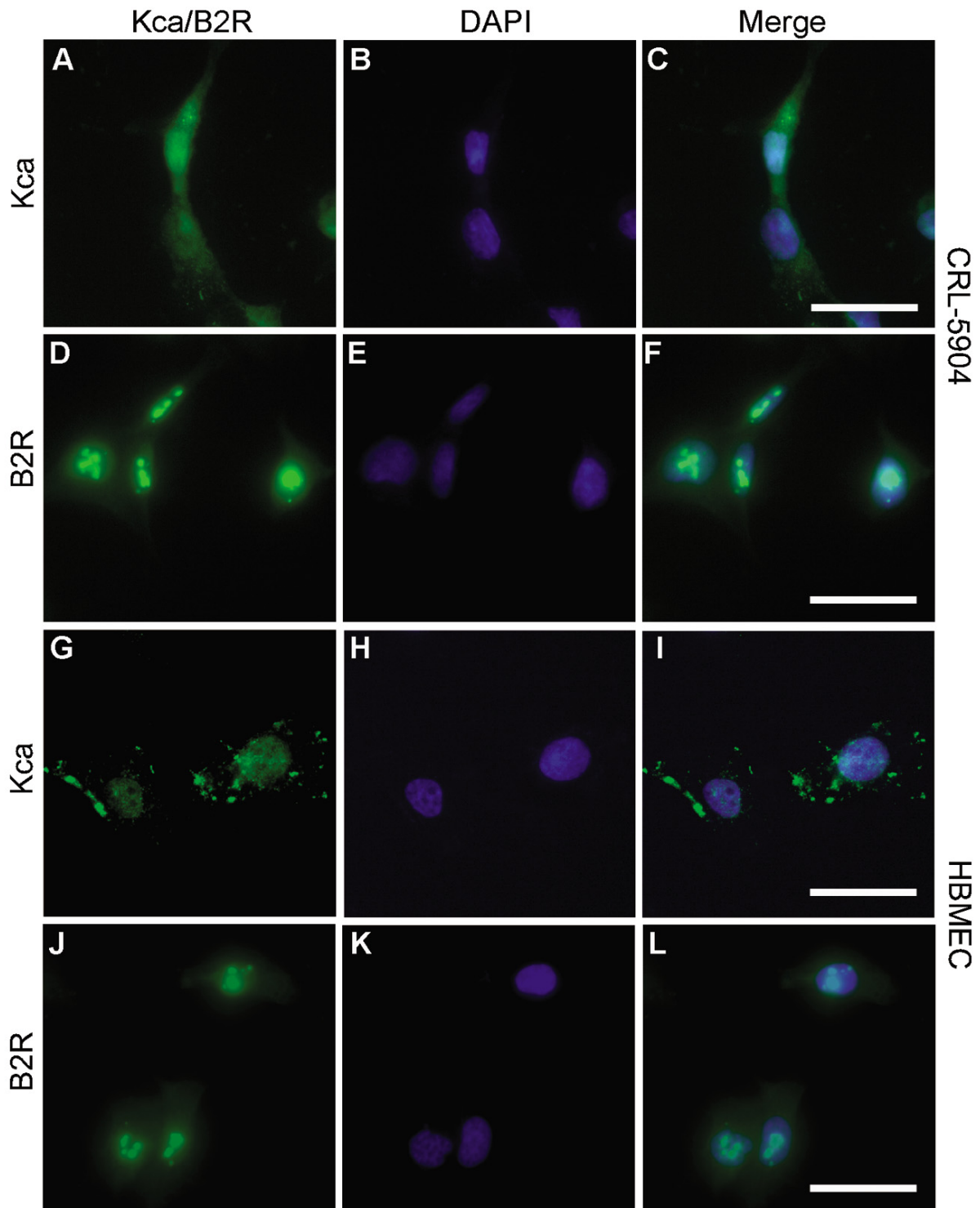


Figure 2
Expression of K_{Ca} channels and B2R in the lung cancer metastatic brain tumor tissue from patients. K_{Ca} channels (A) and B2R (B) expression were detected on the tumor cells in the tissue. Negative control experiments of K_{Ca} channels (C) and B2R (D) did not show specific staining on the corresponded specimens by deleting of primary antibodies. Scale bar = 50 μ m.

Immuno-colocalization of K_{Ca} channel expression in a CRL5904 metastatic brain tumor animal model and human lung cancer brain metastases tissue

Since K_{Ca} channel modulators can selectively increase BTB permeability without affecting normal brain, we wanted to know whether K_{Ca} channels were differentially expressed within the tumor mass compared with normal brain tissue. To address this question, we examined K_{Ca} channel and endothelial cell marker von Willebrand factor (vWF) expression in CRL-5904 tumors and human lung cancer brain metastases tissue. An abundance of K_{Ca} channel expression (green) was detected within the

tumor mass with robust microvessel formation (red) within the tumor area of CRL5904 tumor (Figure 6A). In contrast, there was less K_{Ca} channel expression in normal brain tissue adjacent to the tumor mass as well as contralateral normal brain (Figure 6B). More importantly, colocalization of K_{Ca} channels with vWF within microvessels was observed only within the tumor mass and not in normal brain. These results demonstrate elevated expression of K_{Ca} channels on endothelial cells of capillaries within the tumor mass. Immunostaining of human tissue from lung cancer brain metastases also revealed that K_{Ca} channel expression was colocalized with vWF expression in

**Figure 3**

Expression of K_{Ca} channels and B2R in CRL-5904 cells and HBMEC. K_{Ca} channels (green) distributed in CRL-5904 cells (A~C) and HBMEC (G~I). The B2R (green) were expressed in CRL-5904 cells (D~F) and HBMEC (J~L). Cells were located by counterstaining with DAPI (blue, B, E, H, K) and merged images are also shown (C, F, I, L). Scale bar = 50 μ m.

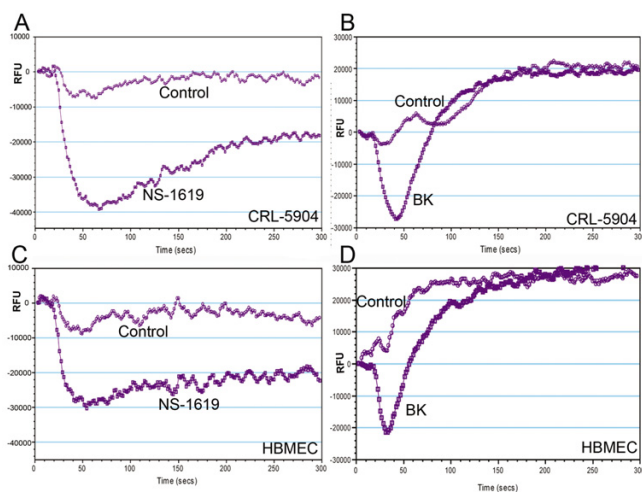


Figure 4

Measurement of functional K_{Ca} channels activity in CRL-5904 cells and HBMEC. Membrane potential changes in relative fluorescence units (RFU) were detected during a 300-second response to 25 μ M of NS1619 (A) and BK (B) respectively on the CRL-5904 cells. NS1619 and BK also elicited membrane potential changes on HBMEC (C, D). Addition of IBTX (20 nM) reversed the membrane potential to resting values.

tumor capillary endothelia (Figure 6C). These results strongly suggest that the selective BTB permeability increase induced by modulation of K_{Ca} channel in the metastatic brain tumor model is likely due to the overexpression of K_{Ca} channels on tumor cells and tumor capillary endothelia.

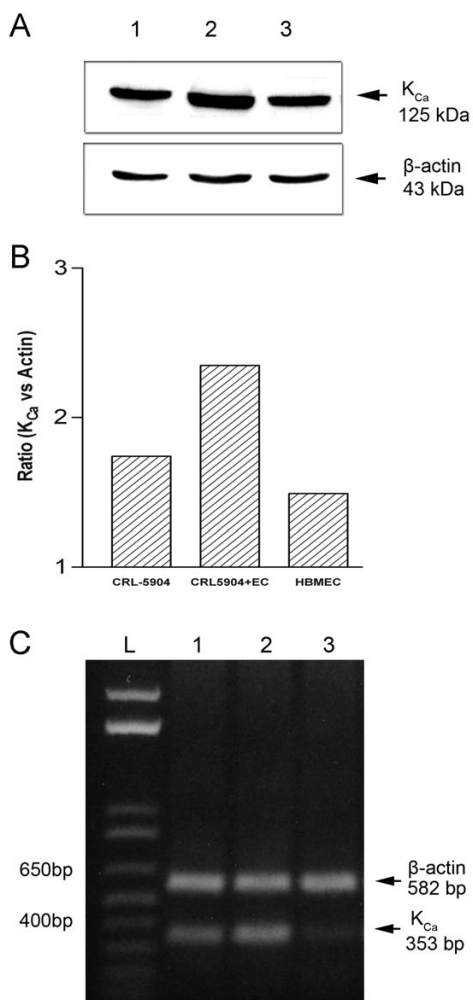
Discussion

We have studied the presence of K_{Ca} channels and B2R in primary brain tumors, however, their occurrence and function in metastatic brain tumors remained to be investigated. In this study, we detected high level expression of K_{Ca} channels in CRL-5904 tumor and brain endothelial cells, which is consistent with previous studies showing K_{Ca} channels expression in RG2 glioma and endothelial cells [15]. Other investigators have also demonstrated that the expression level of K_{Ca} channels correlates with the malignancy grade of glioma in human [36]. Therefore, these data suggest there is an intimate association between K_{Ca} channel expression and brain tumor development, which remains to be fully investigated. Additionally, we detected the presence of B2R in CRL-5904 tumor and endothelial cells. Liu *et al* also showed that B2R are expressed in cultured RG2, C6 and 9L glioma cells, more interestingly, the expression levels of B2R in tumor cells was directly correlated with the increase of BTB permeability induced by bradykinin in a rat glioma model [32]. Thus, the presence of K_{Ca} channels or B2R in metastatic

brain tumor cells or HBMEC may play a functional role in BTB permeability of metastatic brain tumors.

We examined K_{Ca} channel activity on metastatic tumor cells and capillary endothelial cells using a membrane potential assay, which is well-correlated with the patch-clamp method, used to measure changes in membrane potential (FLIPR application note, Molecular devices, CA). We found that NS1619 and bradykinin elicited greater hyperpolarization effects on CRL-5904 than on HBMEC. These findings may reflect a higher level of expression for K_{Ca} channels on metastatic tumor cells as compared to endothelial cells. Importantly, the membrane potential change induced by NS1619 lasted 3 times longer than that induced by bradykinin. These data further support the finding, from cellular level, that NS1619-elicited increases in BTB permeability in a glioma model last up to 60 minutes compared to the transient effect of bradykinin, which lasts for about 15~20 minutes [15], partially due to B2R internalization [37]. The current data illustrates that the presence of K_{Ca} channel are functional in metastatic brain tumor and endothelial cells. Similar to our findings, Reiser *et al* demonstrated that bradykinin can directly activate K_{Ca} channels in rat glioma cells [38]. Other studies have shown that bradykinin can activate K_{Ca} channels through a NO-cGMP signalling pathway [39]. Hence, our present study indicates that bradykinin-activated downstream signals, such as activation of K_{Ca} channels, may be modulated to induce membrane potential changes on brain metastatic tumor and endothelial cells.

The presence of functional K_{Ca} channels in metastatic brain tumor and brain endothelial cells suggests that biochemical modulation of K_{Ca} channels could play an important role in therapeutic BTB opening. We further investigated whether the K_{Ca} channels agonist, NS1619 and bradykinin could selectively enhance BTB permeability in a metastatic brain tumor xenograft model. These results showed that intravenous infusion of NS1619 yielded a two-fold increase the unidirectional transport of a radiotracer into metastatic brain tumors; similar to bradykinin-induced BTB permeability increase in metastatic brain tumor-bearing rats. Our previous studies have demonstrated that higher doses of intravenous bradykinin are required to increase BTB permeability compared to intracarotid infusion of bradykinin, reflecting the influence of the first pass effect with intracarotid delivery [15]. In a glioma model, it has been reported that the effects of bradykinin on BTB permeability mediated by B2R resulted in enhanced drug delivery to glioma [40]; and this effect could be attenuated by coinfusion with IBTX [15]. In this metastatic brain tumor model, we further demonstrate the presence of B2R and confirm that the bradykinin effect on permeability is mediated via K_{Ca} channels. Consistent with previous studies [41], current

**Figure 5**

Modulation of K_{Ca} channel expression in co-cultured cells (CRL-5904 cells and HBMEC). Western blot analysis for K_{Ca} channel expression on cultured cells. Lane 1, CRL-5904 cells; Lane 2, CRL-5904 and HBMEC co-culture; Lane 3, HBMEC (A). Semi-quantitative analyses of the protein bands were normalized by internal control, β-actin (B). mRNA transcription levels of different cell cultures were determined by RT-PCR. L: 1 kb ladder; Lane 1, CRL-5904 cells; Lane 2, CRL-5904 and HBMEC co-culture; Lane 3, HBMEC (C).

data confirms the selective increase of BTB permeability in brain metastatic tumors but not normal brain tissue. These results suggest that biochemical modulation of K_{Ca} channel induces a selective BTB opening in metastatic brain tumor.

Confocal images showed K_{Ca} channels overexpression in tumor tissue and tumor microvessels as compared with normal brain. More importantly, the tumor capillaries showed co-localization of K_{Ca} channels and vWF in tumor area of CRL-5904 tumor and in human metastatic brain tumor tissue. To further study the interaction between tumor and endothelial cells, we co-cultured CRL-5904 metastatic brain tumors and brain endothelial cells. We show that mRNA expression of K_{Ca} channels is upregulated in co-cultured cells compared to individual cultures. These data suggest that increased K_{Ca} channel expression and their activity in tumor endothelial cells maybe due to the tumor micro-environment or cell-to-cell communication between tumor and microvessel endothelial cells. This is consistent with reports that brain tumor cells increase K_{ATP} channel expression in endothelial cells [16].

These findings support a pivotal role for K_{Ca} channels in BTB permeability regulation. Recently clinical study showed that trastuzumab, anti-HER2 antibody, while effective in treating tumors outside the brain, fails to treat brain metastases due to its inability to cross the blood brain tumor barrier [42]. Our research has shown that K_{Ca} channel-mediated BTB permeability modulation could be a useful strategy to increase therapeutic agents, such as antibody-based therapies, delivery into metastatic brain tumors.

Conclusion

We present evidence that activation of K_{Ca} channels by a channel-specific agonist can selectively enhance BTB permeability in a metastatic brain tumor rat model. We show K_{Ca} channel and B2R are highly expressed in brain metastatic tumor cells, endothelial cells and lung cancer brain metastatic tissue. The expression level is correlated with K_{Ca} channel activity in these cells. In a metastatic brain tumor model, we demonstrate that NS1619 and bradykinin can selectively open BTB and significantly enhance the radiotracer delivery specifically to metastatic brain tumors. It is also demonstrated that K_{Ca} channels expression can be upregulated in the co-cultures of tumor cells and endothelial cells, as well as in the microvessel endothelia of brain metastases tissue. K_{Ca} channels may be exploited as specific target for selectively pharmacologic modulation of BTB to increase delivery of chemotherapeutic drugs to brain metastases.

Methods

Cell Culture

CRL-5904 cells (human non-small cell lung cancer; metastatic site: brain poorly differentiated carcinoma) and human brain microvessel endothelial cells (HBMEC) were obtained from the American tissue culture collection (ATCC, VA) and maintained in RPMI 1640 with 10% fetal bovine serum. Both cell lines were maintained in the com-

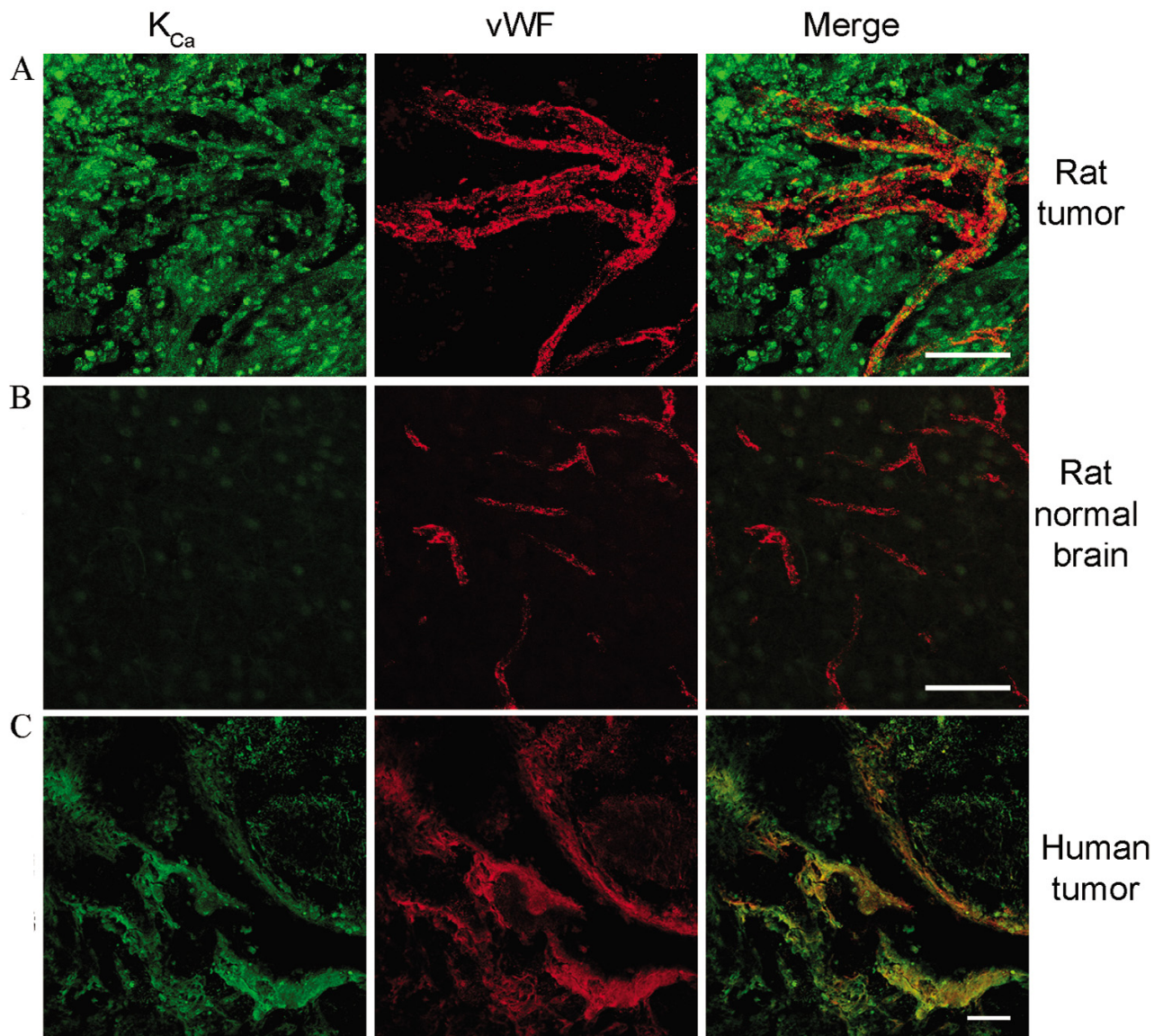


Figure 6

Localization of K_{Ca} channels in brain tumor tissues from xenogenic rat model and patients of lung cancer brain metastases. K_{Ca} channels (green) were localized in the endothelial cells of capillaries (red) in the tumor mass (A). In the contralateral side of brain, much less of K_{Ca} channel was detected in normal brain. (B). K_{Ca} channels are co-localized with von Willebrand factor, a marker of endothelial cells, in human tumor tissue of lung cancer brain metastases (C). Scale bar = 250 μ m.

mon tissue culture condition. For co-culture of CRL-5904 cells with HBMEC, the same number of CRL-5904 cells and HBMEC were co-cultured in growth medium and allowed to achieve 90% confluence. Then, the co-culture and single cultures of cells were harvested for protein or RNA extraction.

Membrane Potential Assay

The functional activity of K_{Ca} channels in CRL-5904 cells and HBMEC was measured using the FLIPR Membrane Potential Assay Kit on a FLEXstation (Molecular Devices, Sunnyvale, CA) as described previously [15]. This kit provided a fast, simple and consistent mix-and read proce-

ture. In brief, the cells were seeded in sterile, clear bottom, black 96-well plates (Corning Inc., MA) at density of 2×10^3 cells/well to achieve monolayer within 24 h. The monolayer cells were incubated with the membrane potential assay kits reagents for 30 min before loading the compounds. The anionic potentiometric dye that transverses between cells and extracellular solution in a membrane potential-dependent manner serves as an indicator of vasomodulator-induced voltage changes across the cell membrane. Dose response studies were performed with 0 to 50 μ M NS1619 or bradykinin with or without IBTX (20 nM). The FLEXstation was set up using the following parameters: excitation 530 nm, emission 565 nm, and emission cut of 550 nm wavelengths. Observations and recordings were made for 300 seconds after adding the compounds. NS1619, bradykinin and IBTX were obtained from Sigma (St. Louis, MO).

In Vivo BBB/BTB Permeability

All of the animals used were conducted in accordance with the Institutional Animal Care and Usage Committee in force at Cedars-Sinai Medical Center. A metastatic brain tumor xenograft model was established using athymic nude rats (180–200 g; Charles River Laboratories, Inc., MA) for BBB/BTB permeability studies. Athymic nude rats were anesthetized with i.p. ketamine and xylazine, and stereotactically implanted with CRL-5904 cells (2×10^5) in 4 μ l of 1.2% methylcellulose/PBS using a Hamilton syringe into the right striatum. The Coordinates were 3.4 mm lateral to bregma and 5.0 mm deep from dura. Ten days after tumor implantation, the femoral arteries of rats were cannulated to measure blood pressure and collect blood, and the femoral vein was also cannulated to administer the drugs and radiotracer. Body temperature was maintained at 37°C. Arterial blood gases, blood pressure and hematocrit were monitored. Animals with abnormal physiological parameters were eliminated from this study. In regional permeability studies, either intravenous drug or PBS was infused into the femoral vein at a rate of 66.7 μ l/min for 15 minutes. Five minutes after the start of the intravenous infusion, 50 μ Ci/kg of the radiotracer [14 C] sucrose was injected as an intravenous bolus. Arterial blood pressure was monitored throughout the experimental period with a blood pressure monitor (DigiMed, KY). The unilateral transport constant K_i (μ l/g/min), which is an initial rate for blood-to-brain transfer of radiotracer, was calculated as described by Ohno et al [43]. The K_i was determined by radiotracer [14 C] sucrose in the tumor core, tumor-adjacent brain tissue, and contralateral brain tissue using the quantitative autoradiographic (QAR) method as describe previously [44]. Quantitative analysis of the regional radioactivity was performed using a computer (Power Macintosh 7100) and Image 1.55 software (National Institutes of Health, Bethesda, MD). An optimum dose (120 μ g/kg/min, i.v.) of bradykinin estab-

lished previously was used for K_i measurements. To establish the optimal and safe dose range that would result in selective increase in BTB permeability without appreciably altering system blood pressure, various doses (0~120 μ g/kg/min, i.v.) of NS1619 were administered in metastatic brain tumor bearing-nude rats. Additional experiments were performed by coinfusing of NS1619 with IBTX (1.0 μ g/kg/min) to investigate whether inhibition of K_{Ca} channels by IBTX has any effects on NS1619-induced permeability increase.

Western Blot Analysis

The extracted protein samples were quantified to determine total protein concentrations using a protein assay kit (BioRad, CA). Same amount of each sample was fractionated on 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies anti-MaxiK α (1:200; Santa Cruz Biotechnology, CA) and β -actin (1:5000; Sigma, MO), followed by peroxidase-conjugated secondary antibodies. The signals were detected with an enhanced chemiluminescence kit (Amersham Biosciences Corp., NJ). β -actin served as an internal control.

Reverse Transcription-PCR

The extracted RNA was reverse transcribed using a Bioscript kit (Bioline) and Oligo (dT)₁₂₋₁₈ primer (Invitrogen). The resulting cDNA products were used as templates for PCR assay. The genes of the K_{Ca} channels were concurrently amplified with internal control β -actin in the same reaction tube as described previously [45]. Sequence specific primers were used for amplification of K_{Ca} channels (forward: 5'-tccaaacaaccaggctctc-3'; reverse: 5'-gggggagatgttggaagaa-3') and β -actin (forward: 5'-gcacacaccttctacaatgagc-3'; reverse: 5'-tgaaggtatgttctggatgcc-3'). PCR products were identified using agarose gel electrophoresis and ethidium bromide staining.

Immunocytochemistry Staining

CRL-5904 cells and HBMEC were fixed with 4% paraformaldehyde for 15 min, and then incubated with anti-B2R (1:200; BD Bioscience, NJ) or anti-MaxiK α (1:200; Santa Cruz, CA) antibodies. The signals were detected with FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, CA). The cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA) and cover-slipped. Paraffin-embedded, metastatic brain tumor samples from lung cancer were deparaffinized and rehydrated. The slides were incubated with primary anti-MaxiK α and anti-B2R antibodies, and followed by biotinylated secondary antibodies (1:1000; Jackson ImmunoResearch, CA). Biotinylated conjugates were detected with avidin-biotin peroxide complex (Vector Laboratories), and then developed with 3, 3'-Diaminobenzidine (DAB) method. The sections were

counterstained with hematoxylin. For the double staining, the sections were incubated with primary antibodies, anti-MaxiKá and anti-von Willebrand Factor (1:200; Chemicon, CA), and then subjected to FITC-and Tex-Red-conjugated secondary antibodies. The slides were examined under confocal microscopy. Negative control experiments were performed on all the corresponded specimens by deleting of primary antibodies.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

JH and KLB conceived the study. JH designed and performed all experiments. DY, MRS, XW, MKK, BMK, AE, KP participated in vivo BBB/BBB permeability assay. JH and XY drafted the original manuscript, and MKK, JMO, DI and KLB helped modify the final version of the manuscript.

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