Increase in Brain Tumor Permeability in Glioma-Bearing Rats with Nitric Oxide Donors

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Abstract

Purpose: The blood-brain tumor barrier (BTB) significantly limits the delivery of chemotherapeutics to brain tumors. Nitric oxide (NO) is involved in the regulation of cerebral vascular permeability. We investigated the effects of NO donors, L-arginine and hydroxyurea, on BTB permeability in 9L gliosarcoma-bearing Fischer rats.

Experimental Design: The rats implanted with 9L gliosarcoma were dosed orally with hydroxyurea and L-arginine. BTB permeability, defined by the unidirectional transport constant, K_i, for [^{14}C]sucrose was measured. The expression of neural and endothelial NO synthase (NOS) in tumors and normal brain tissue was examined. Further, the levels of NO, L-citrulline, and cGMP in the tumor and normal brain tissue were measured.

Results: Oral administration of L-arginine or hydroxyurea significantly increased BTB permeability when compared with the nontreated control. The selective effects were abolished by iberiotoxin, an antagonist of calcium-dependent potassium (K_{Ca}) channel that is a cGMP pathway effector. The expression of endothelial NOS, but not neural NOS, was higher in tumor vessels than in those of normal brain. Moreover, the levels of NO, L-citrulline, a byproduct of NO formation from L-arginine, and cGMP were enhanced in the tumor tissue by oral administration of L-arginine and/or hydroxyurea.

Conclusions: Oral administration of L-arginine or hydroxyurea selectively increased tumor permeability, which is likely mediated by alteration in cGMP levels. The findings suggest that use of oral NO donors may be a strategy to enhance the delivery of chemotherapeutics to malignant brain tumors.

The prognosis for patients with malignant glioma remains poor despite combined therapeutic modalities, including surgery, radiation, and chemotherapy (1, 2). Although it is an important therapy for malignant glioma, chemotherapy has not consistently achieved clinical benefits, with overall response in 10% to 30% of patients (3, 4). Poor delivery of chemotherapeutics into brain is a major limit in the treatment of malignant glioma (4, 5). The blood-brain tumor barrier (BTB), which includes the microvessels supplying brain tumors, retains many characteristics of the normal blood-brain barrier that significantly impedes adequate delivery of chemotherapeutics into brain tumors (4–6).

BTB, however, has unique characteristics that differentiate it from normal blood-brain barrier. These include receptors, ion channels, and enzymes that are overexpressed in the tissue and microvessels of brain tumors (6–10). One approach to improve chemotherapy of brain tumors has taken advantage of these unique characteristics to pharmacologically modulate BTB permeability. We have identified previously certain vasoactive molecules including bradykinin, nitric oxide (NO), cGMP, and calcium-dependent potassium (K_{Ca}) channel agonists that act on a receptor, ion channel, and/or enzyme to selectively increase delivery of compounds to brain tumors (11). In particular, clinical attempts have been taken to improve chemotherapy by adjunctive administration of bradykinin and its analogue, RMP-7, agonists to the bradykinin-2 receptor, in brain tumor patients (10). Due to the large variability and side effects, however, the clinical attempts were not very successful (12). The molecular pathways underlying the regulation of BTB permeability by the vasoactive molecules have been elucidating, and there are clinically used drugs that have been shown to modulate these pathways. With quick translation to clinical use in mind, we therefore sought to identify one or more clinically approved drugs that could be used to selectively modulate transport of chemotherapeutics across the BTB.

NO is unstable free radical gas that serves as a novel message molecule in the central nervous system (13, 14). Studies have suggested a role for NO in regulation of tumor permeability and cerebral blood flow (15–18). The actions of NO are mediated by the activation of soluble guanylate cyclase and the consequent increase in the concentration of cGMP, which has also been shown to modulate BTB permeability (19–21). L-Arginine, a semiessential amino acid, is the main source

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Received 7/26/07; revised 12/15/07; accepted 2/7/08.

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doi:10.1158/1078-0432.CCR-07-1826

for the generation of NO via NO synthase (NOS; ref. 19). Hydroxyurea is a cycle-specific drug, which has been used to treat myeloproliferative diseases (22, 23) and sickle cell anemia (24). Hydroxyurea has been reported to be oxidized by heme group to produce NO via a series of intermediate reactions in rats and in humans (25–27). The drug has also been found to produce NO in an endothelial NOS (eNOS)-dependent manner (27).

NOS is a cell type–specific enzyme that catalyzes the synthesis of NO. Several isoforms of NOS exist, two of which are constitutive (28, 29). The constitutive NOS were found in vascular endothelium and the neurons (eNOS and nNOS, respectively; refs. 28, 29) and is activated by agonists that increase intracellular Ca^{2+} concentrations and enhance calmodulin binding (28). NOS catalyzes the oxidation of L-arginine to yield equimolar amounts of NO and L-citrulline (19).

The current study was to investigate whether NO donors L-arginine and hydroxyurea, which are clinically approved for patients, enhance brain tumor permeability in 9L gliosarcoma-bearing rats. The effects of oral L-arginine and hydroxyurea on the transport of a radioactive tracer across the BTB into tumor tissue were studied. The expression of neural NOS (nNOS) and eNOS in implanted intracerebral tumors and normal brain tissue was examined. Further, we measured the levels of NO, L-citrulline, and cGMP in the tumor and normal brain tissue after orally treating the animals with hydroxyurea or L-arginine. The findings from this study support clinical implication of NO donors in improving chemotherapy for malignant brain tumors.

Materials and Methods

Materials. All animal experiments were conducted in accordance with policies set by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center and by NIH guidelines. Female Fischer rats, weighting 150 to 180 g, were used (Harlan). L-Arginine was obtained from Major Pharmaceuticals and hydroxyurea was obtained from Par Pharmaceutical. [^{14}C]sucrose (363 mCi/mmol) was obtained from ICN Biomedicals (Dupont New England Nuclear).

Intracerebral tumor implantation. 9L gliosarcoma cells were kept frozen until use and then thawed and maintained in a monolayer culture in DMEM with 10% fetal bovine serum. The rats were anesthetized with i.p. injections of ketamine (50 mg/kg)/xylazine (6 mg/kg) and immobilized in a stereotactic frame. A Hamilton syringe was lowered into the right basal ganglia to a depth of 4.5 mm from the dural surface, 3 mm lateral and 1 mm anterior to the bregma, and was then withdrawn 0.5 mm to create a space for tumor accumulation. Intracerebral injections of 1×10^5 9L glioma cells in 5 μL medium with 1.2% methylcellulose were then administered over a period of 10 min. The skin incision was closed using sterile surgical staples.

Animal preparation. For K_i study described below, the tumor-implanted rats were divided into three groups according to treatments: (*a*) i.v. infusion of saline, (*b*) oral L-arginine, and (*c*) oral hydroxyurea. Six days after tumor implantation, the rats were anesthetized with ketamine/xylazine. One femoral vein was cannulated for administration of drugs and radiotracer. One femoral artery was cannulated to withdraw arterial blood, and the other femoral artery was cannulated to monitor systemic blood pressure. Body temperature was maintained at 37°C, and arterial blood gases, blood pressure, and hematocrit were monitored. Animals with abnormal physiologic variables were eliminated from this study.

Regional tumor permeability study. [^{14}C]sucrose was used as the tracer to measure tumor permeability as described previously (7, 8, 11). In brief, saline was infused into the femoral vein at a rate of 66.7 μL/min for 15 min. Five minutes after the start of the i.v. infusion, 50 μCi/kg [^{14}C]sucrose was injected as an i.v. bolus. Immediately after the injection of the tracer, a peristaltic withdrawal pump was used to withdraw femoral arterial blood at a constant rate of 0.083 mL/min for the determination of serum radioactivity. After the completion of the experiments, the animals were euthanized by decapitation, and the brains were rapidly removed and frozen.

Quantitative autoradiography and K_i calculation. The frozen brains were mounted onto pedestals with M1 embedding matrix, after which 20 μm coronal sections were cut with a cryostat. The sections were thawed and mounted onto slides, and autoradiographs were generated by exposing the sections along tissue-calibrated ^{14}C standards on a phosphor screen for 5 days. Quantitative analysis of the regional radioactivity for tumor and other brain areas was done using a computer (Power Macintosh 7100) and Image 1.55 software (NIH). The initial rate for blood-to-brain transfer (K_i) was calculated as described previously (8, 10, 30).

Dose-response and time course of drug treatment. To establish an optimal dose that selectively increases BTB permeability without appreciably altering system blood pressure, varying doses of L-arginine (10-600 mg/kg) or hydroxyurea (40-320 mg/kg) were administered in a separate study. To study the duration of permeability increase by L-arginine or hydroxyurea, K_i was measured at different time points from 30 to 120 min after the oral treatments (200 mg/kg L-arginine and 80 mg/kg hydroxyurea). Previous studies suggest an important role of K_{Ca} channels in regulation of BTB permeability (11). Additional rats were infused with iberiotoxin (0.26 μg/kg/min), a K_{Ca} channel antagonist, to investigate whether inhibition of K_{Ca} channels by iberiotoxin attenuates permeability increases induced by NO donors.

Measurement of nitrate and nitrite in tumor tissues. The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-). Sum of nitrate and nitrite is an index of total NO production. Six days after tumor implantation, the rats bearing 9L tumors were decapitated. The rats received single dose of hydroxyurea (80 mg/kg), L-arginine (200 mg/kg) for 60 min, or no treatment. The tumors and contralateral normal tissues were carefully dissected and homogenized. Nitrate and nitrite were measured by using a fluorometric assay kit (Cayman Chemical).

Immunohistochemistry. To detect expression of nNOS and eNOS in implanted 9L tumors, the rats were decapitated 6 days after tumor implantation. Additional rats were decapitated 30, 60, 90, and 120 min after L-arginine treatment for measurement of the levels of cGMP and L-citrulline in tumor and normal brain tissues. Serial coronal section (20 μm) of the brains were cut on a cryostat, mounted to gelatin-coated slides, and processed for immunohistochemistry. Sections were incubated with primary mouse anti-nNOS antibody, mouse anti-eNOS antibody, rabbit anti-L-citrulline antibody, or rabbit anti-cGMP antibody (all from Chemicon International) diluted in 1:200, 1:200, 1:200, and 1:1,000 in blocking buffer (5% goat serum and 0.01% Triton X), respectively. Secondary anti-mouse or anti-rabbit IgG conjugated with fluorescent was used to detect the primary antibodies. The negative controls were done without any primary antibody.

To determine whether nNOS or eNOS is present on capillary endothelial cells, the sections were also incubated with polyclonal anti-factor VIII primary antibody and subsequently secondary anti-rabbit IgG conjugated with rhodamine (tetramethylrhodamine isothiocyanate). Immunohistochemistry and confocal laser scanning microscope analysis were used to detect colocalization of factor VIII and nNOS or eNOS.

The images for expression of L-citrulline and cGMP were obtained by Zeiss fluorescent microscopy (Carl Zeiss MicroImaging). The immunofluoresence intensity of cGMP staining was measured at four randomly chosen areas of tumor, brain surrounding tumor (areas within 2 mm of the border of the tumor), contralateral cortex, respectively, using Zeiss AxionVersion software. Pictures of the tumor tissue were taken with a magnification of ×20 under same conditions.

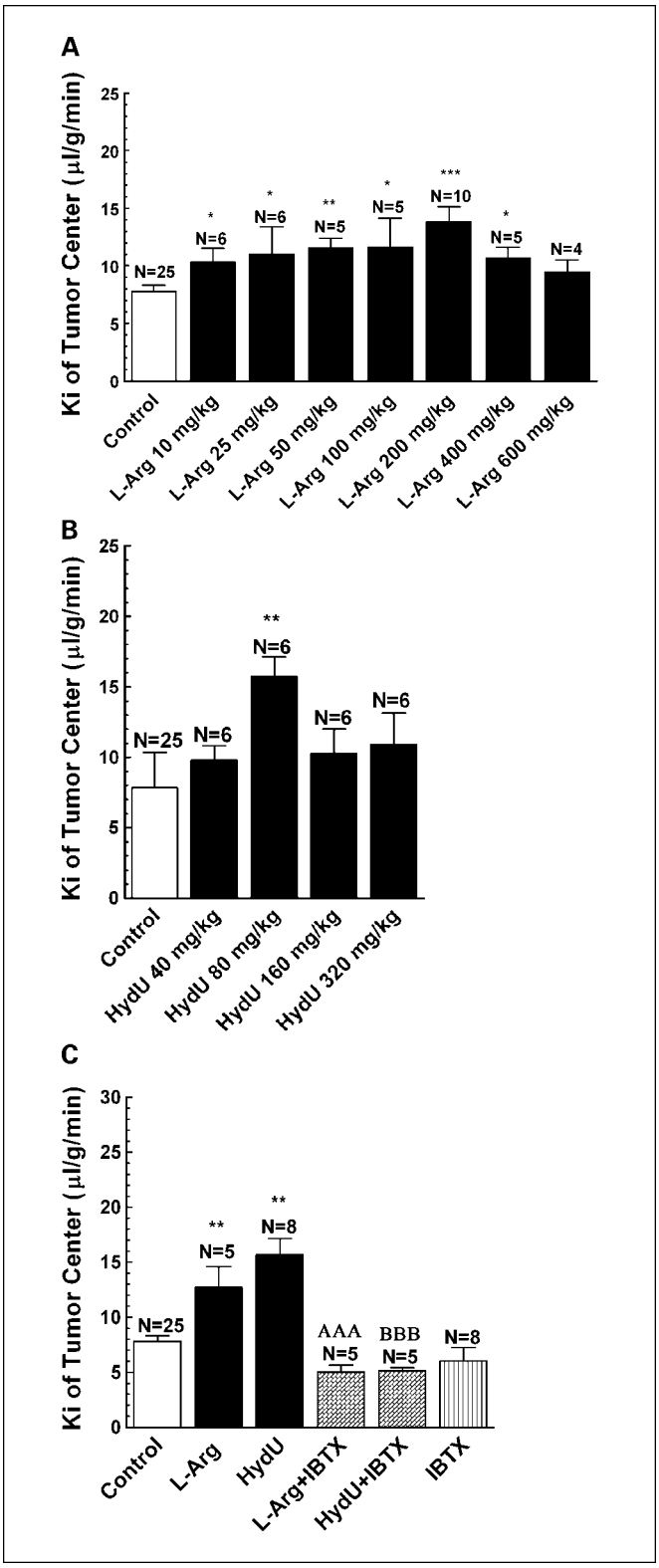

Fig. 1. Effect of oral NO donors on tumor permeability. The regional K_i values (μL/g/min) in 9L tumors was shown after different treatments. *Columns,* mean; *bars,* SE. *L-arg,* L-arginine; *HydU,* hydroxyurea. L-Arginine or hydroxyurea was administered orally at various doses followed by permeability determination at the 50- to 60-min time interval. *A,* L-arginine treatment. *B,* hydroxyurea. *C,* effect of iberiotoxin on tumor permeability induced by L-arginine or hydroxyurea. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different from saline control group. AAA, $P < 0.001$, significantly different from L-arginine-treated group; BBB, $P < 0.001$, significantly different from hydroxyurea-treated group.

Confocal microscopy. Immunoreactive visualization of nNOS or eNOS and factor VIII was done. In brief, confocal images were captured using a Leica True Confocal Scanner Spectrophotometer laser scanning confocal microscope (inverted) equipped with argon (488 nm) and krypton (568 nm) lasers. Fluorescent signal for nNOS or eNOS expressed on brain tumor-bearing rat brain sections were visualized using the 488 nm argon laser line and that for factor VIII using the 568 nm krypton laser line. Fluorescence signals for fluor-488 or fluor-568 were displayed individually as green and red pseudocolor projections, respectively, or merged as overlay projections to visualize possible colocalization.

Western blot analysis. Tumor tissue and normal brain were rapidly homogenized and lysated in 200 μL lysis buffer [1% SDS, 1.0 mmol/L sodium vanadate, 10 mmol/L Tris (pH 7.4)]. After centrifugation, the supernatant was separated by electrophoresis on a 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane (Immobilon; Millipore). Nonspecific binding sites were blocked in TBS with 5% bovine serum albumin and 0.1% Tween 20 for 1 h. Membranes were rinsed for 30 min in buffer (0.1% Tween 20 in TBS) and then incubated with mouse anti-nNOS (1:1,000), eNOS (1:1,000), or actin (1:1,000) followed by anti-mouse IgG horseradish peroxidase conjugate. All primary and secondary antibodies were obtained from Santa Cruz Biotechnology. After rinsing with buffer, the immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Biotech) according to the manufacturer's instructions. The film signals were digitally scanned and quantified using NIH Image software. Actin was used as the internal control.

Statistical analysis. Results are expressed as mean ± SD, where applicable. The statistical analyses of K_i and cGMP among different groups, with or without drug treatment, were done using ANOVA followed by either unpaired parametric analysis of Student's t test or by nonparametric analysis of the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

Effect of L-arginine and hydroxyurea on BTB permeability. To determine the effect of NO donors on BTB permeability, we experimentally induced brain tumors in Fischer rats using the 9L gliosarcoma cell line, a syngeneic rat brain tumor model. Six days after implantation, brain tumor regional permeability studies were done as described in Materials and Methods. Effect of oral administration of various concentration of L-arginine (10-600 mg/kg) on tumor permeability was shown in Fig. 1A. L-Arginine significantly ($P < 0.05$) increased the tumor permeability with all the tested doses, except 600 mg/kg, when compared with the saline-treated controls. L-Arginine (200 mg/kg) was found to have the maximum effect on the BTB permeability.

Effect of oral administration of various concentration of hydroxyurea (40-320 mg/kg) on tumor permeability was shown in Fig. 1B. The tumor permeability was significantly increased with a dose of 80 mg/kg ($P < 0.01$) compared with the controls. We also sought to determine if L-arginine or hydroxyurea significantly altered arterial blood pressure at the doses used. No significant changes in arterial blood pressure at the optimal doses for either L-arginine (200 mg/kg) or hydroxyurea (80 mg/kg) were observed (data not shown). The followed studies were then done with a dose of 200 mg/kg for L-arginine or 80 mg/kg for hydroxyurea.

The permeability of normal brain and the brain tissue within 2 mm surrounding tumor was not increased by treatment of L-arginine or hydroxyurea (data not own). Iberiotoxin, a selective K_{Ca} channel antagonist, abolished L-arginine- or

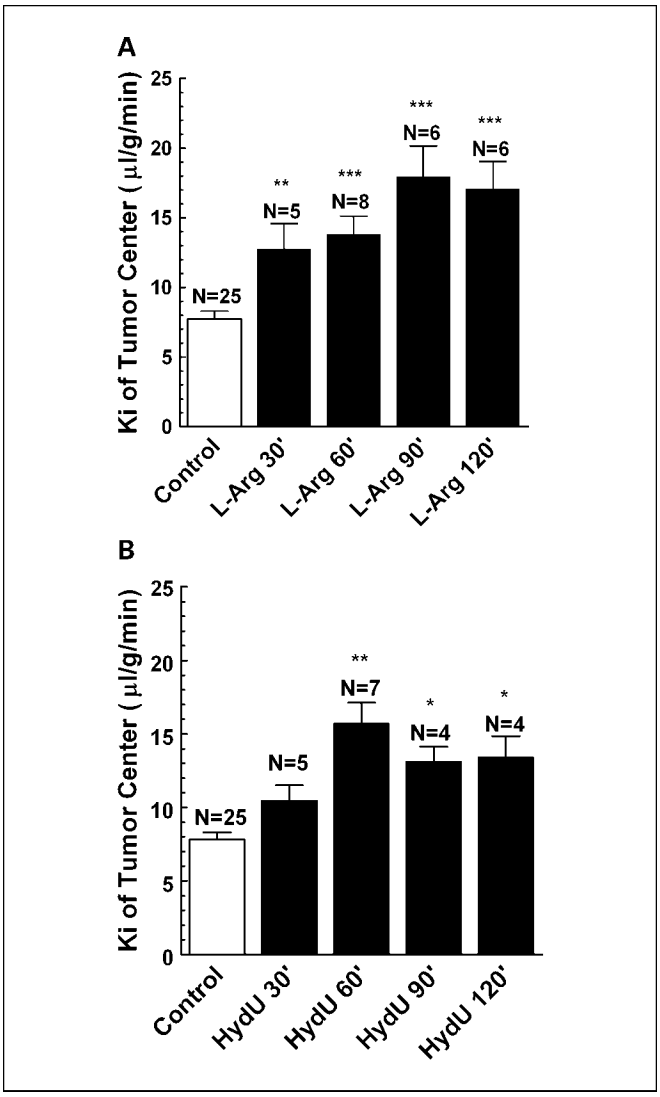

Fig. 2. Time course of effect of oral NO donors on tumor permeability. NO donors were administered orally followed by permeability determination at varying time points. *Columns,* mean; *bars,* SE. *A,* L-arginine treatment. *B,* hydroxyurea treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different from saline control group.

hydroxyurea-induced BTB permeability (Fig. 1C), consistent with involvement of K_{Ca} channel as an effector in regulating BTB permeability (11).

To determine whether L-arginine- or hydroxyurea-induced BTB permeability increase in 9L tumor-bearing rats was transient or could be sustained over a period, we measured the K_i for tumor permeability at different time points. L-Arginine (200 mg/kg) or hydroxyurea (80 mg/kg) was given 30, 60, 90, and 120 minutes before K_i determination. Tumor permeability remained elevated between 30 and 120 minutes after oral administration of L-arginine (Fig. 2A) or hydroxyurea (Fig. 2B). The permeability reached its maximum at 90 and 60 minutes for L-arginine and hydroxyurea, respectively.

Expression of nNOS and eNOS in implanted 9L tumors of Fischer rats. The expression of nNOS and eNOS in experimentally induced 9L tumors of Fischer rats was examined by using immunohistochemistry and Western blot analysis. Higher levels of eNOS were expressed in the tumor than that in the normal brain. As expected, eNOS was coexpressed in cells that were factor VIII immunopositive, a marker for vascular endothelia (Fig. 3A). Lower levels of eNOS-immunopositive staining were found in the brain parenchyma surrounding the tumor or in normal brain tissue. In contrast, higher expression of nNOS was found in the brain parenchyma surrounding the tumor or in normal brain tissue, whereas very few nNOS expression was found in tumor tissue (Fig. 3B). Consistent with the immunochemistry results, Western blot analysis showed protein expression levels for eNOS were higher in tumor tissue when compared with normal brain, whereas those for nNOS were higher in normal brain when compared with tumor tissue (Fig. 3C).

Effect of L-arginine and hydroxyurea on NO levels in 9L tumors of rats. The final products of NO *in vivo* are nitrite and nitrate. We used the sum of nitrite and nitrate levels to reflect NO production in tissues. Both hydroxyurea and L-arginine enhanced NO levels in 9L tumor tissues when compared with no treatment (Fig. 4). Interestingly, hydroxyurea also increased NO levels in normal contralateral tissue, whereas L-arginine showed no effect. It seemed that the tumor tissues had a basal higher level of NO when compared with normal tissues. The results provide direct evidence that NO levels in 9L tumors can be increased by oral administration of hydroxyurea and L-arginine.

Effect of L-arginine treatment on L-citrulline levels in 9L tumors of rats. L-Citrulline is a byproduct of NO formation from L-arginine; therefore, L-citrulline production is considered a reliable indicator of NOS enzymatic activity and NO production (31–33). As an alternative approach to localize NOS and measure NO levels, immunohistochemical staining of L-citrulline was done (Fig. 5). Low levels of L-citrulline expression in tumor tissue were observed in the absence of L-arginine treatment. More intense immunostaining for L-citrulline in tumor tissue was found 30 and 60 minutes after L-arginine treatment compared with untreated animals, with a peak at 60 minutes, and it decreased at 90 minutes. However, very little immunoreactivity of L-citrulline was observed in normal brain after L-arginine treatment. These results show that L-arginine treatment specifically augmented L-citrulline levels in the 9L tumor.

Effect of L-arginine treatment on cGMP levels 9L tumors of rats. To support the hypothesis that cGMP signaling is involved in the effect of NO donors on BTB permeability, the levels of cGMP in tumor tissue in 9L tumor-bearing rats with or without L-arginine treatment were measured by using immunohistochemistry. Semiquantitative measurement of cGMP immunohistochemistry in the untreated tumor-bearing rats showed that normal brain contralateral to the tumor had very low levels of cGMP, whereas tumor tissue had relatively higher levels of cGMP-immunopositive staining (Fig. 6A and B). L-Arginine treatment further increased cGMP immunostaining in the tumor tissue. cGMP staining in tumor tissue was increased at 30 and 60 minutes and returned to baseline at 90 minutes after L-arginine treatment compared with untreated tumor-bearing rats (Fig. 6A and B). We did not observe any increase in immunofluorescent signal for cGMP in the normal brain contralateral to the tumors in L-arginine-treated rats (data not shown). These results suggest that the selective BTB permeability effects of NO donor are related to the increased levels of cGMP within the tumor compared with normal brain.

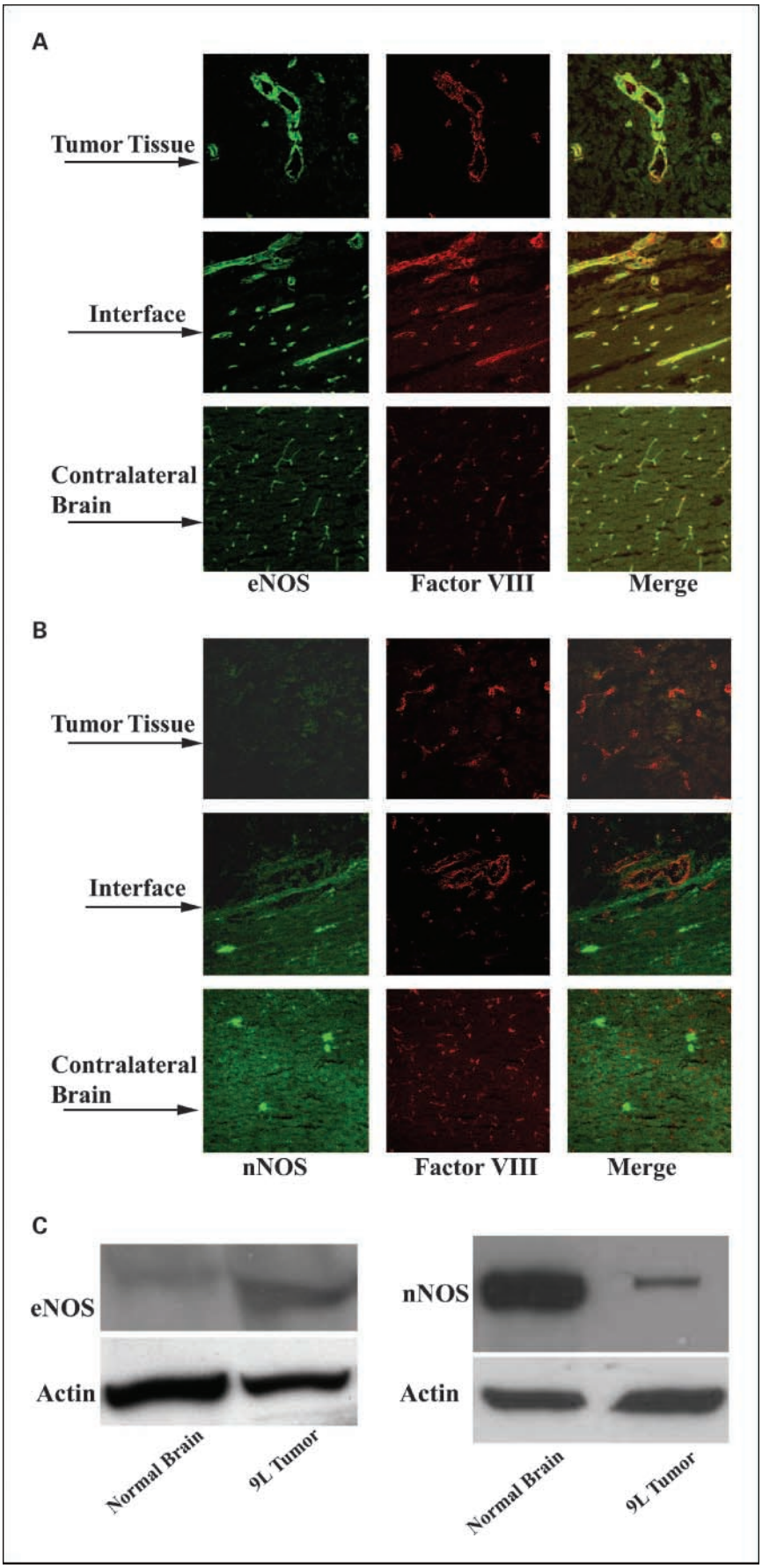

Fig. 3. Expression of eNOS and nNOS in tumor tissue, the tissue surrounding the tumor (interface), and contralateral normal brain from 9L gliosarcoma-bearing rats. *A,* immunohistochemistry image of eNOS, factor VIII, and their merge. *B,* immunochemistry image of nNOS, factor VIII, and their merge. *C,* Western analysis of eNOS and nNOS. Factor VIII is a marker for microvessel endothelial cells. Immunohistochemistry and/or Western blot analysis for eNOS, nNOS, factor VIII, and actin were done as described in Materials and Methods. *A* and *B,* original magnification, ×20. Actin was used as an internal standard for quantization of eNOS and nNOS.

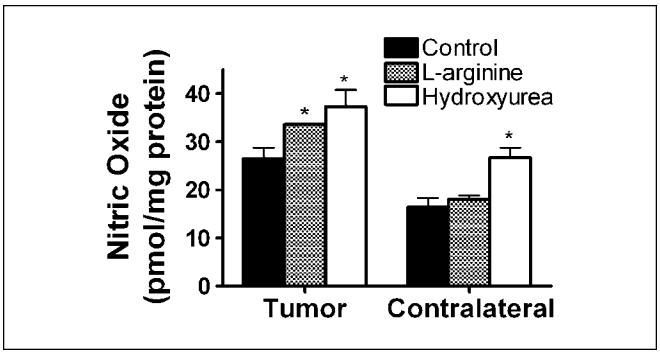

Fig. 4. NO (nitrite and nitrate) levels in brain tissues with and without oral administration of hydroxyurea or L-arginine. Nitrite and nitrate were detected by a fluorometric assay as described in Materials and Methods. Brain tumors were removed before and 60 min after oral administration of L-arginine (200 mg/kg) or hydroxyurea (80 mg/kg). *Columns,* mean; *bars,* SE ($n = 3$ in each group). *, $P < 0.05$, significantly different from saline control group. Each group of tumor is significantly higher that the corresponding group of contralateral brain tissue, which is not shown in the figure.

Discussion

The present study investigated the effect of NO donors, L-arginine and hydroxyurea, on tumor permeability in a rat brain tumor model. We found that oral administration of L-arginine or hydroxyurea significantly increased BTB permeability, indicated by an increased uptake of a radiolabeled tracer to the brain tumor but not to normal brain. Consistently, higher levels of eNOS were detected in the blood vessels of implanted intracerebral 9L tumor when compared with normal brain. Both hydroxyurea and L-arginine enhanced NO levels in tumor tissues. L-Citrulline and cGMP was found to increase in tumor tissue of the rats treated with L-arginine. The data suggest that NO and cGMP may mediate the effect of increasing permeability by NO donors on tumor microvessels. Taken together, the findings constitute a rationale for oral administration of NO donors as a strategy to enhance the delivery of chemotherapeutic compounds to brain tumors.

Many activities of supplemental L-arginine are explained by its role as the precursor to NO (19), and hydroxyurea can be metabolized to NO *in vivo* (25, 26). NO plays very important roles in the cardiovascular system, immune system, and nervous system (19). It has bee assumed that the NO formed from L-arginine and hydroxyurea diffuses to nearby target cells, in which it stimulates the soluble guanylate cyclase to enhance synthesis of cGMP (19). Increased cGMP levels have been related to enhancement of BTB permeability (34). Therefore, NO donors would be expected to augment the physiologic action of cGMP and increase BTB permeability. In this study, oral administration of L-arginine and hydroxyurea selectively increased the permeability of brain tumor to [^{14}C]sucrose, a tracer we used for K_i measurement that is similar in molecular weight, water solubility, and ability to cross the BTB as many of the chemotherapeutics currently used to treat human tumors (35). Neither L-arginine nor hydroxyurea increased the permeability for tracers in brain surrounding tumor and normal brain in our animal model, thereby reducing possible toxicity of chemotherapeutic drugs to normal brain tissue. These data are consistent with our previous observations that agents such as bradykinin (9), K_{Ca}, and K_{ATP} channel agonists (11, 36) selectively increase permeability in brain tumor capillaries or injured brain capillaries, whereas normal brain capillaries seem to resist the effect by vasoactive compounds (9). However, it should be noted that increasing drug delivery to the tumors infiltrated into normal brain is critical to successful chemotherapy for malignant brain tumors as shown by Levin (37). The tumor model and experimental methods in this study did not allow us to directly test whether the NO donors increase the permeability of the infiltrated tumors. Further studies are required to address this.

Both L-arginine and hydroxyurea increased tumor permeability and NO production in this study. However, the mechanisms could be somewhat different. Whereas L-arginine is the main source for the generation of NO via NOS (19),

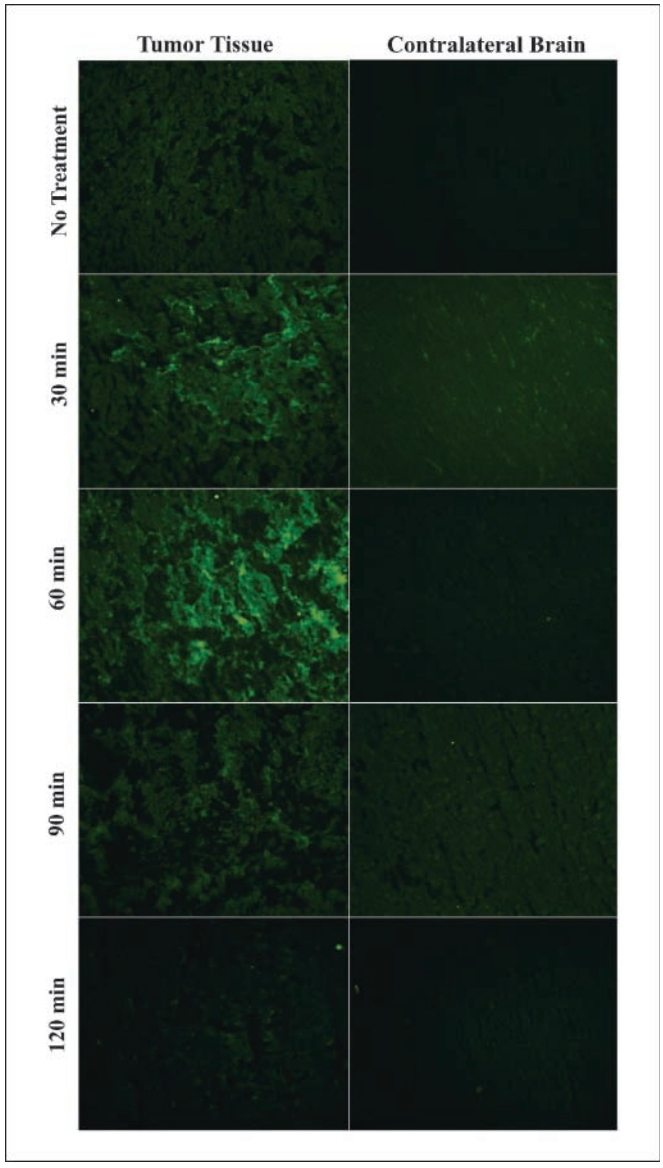

Fig. 5. L-Citrulline levels in brain tissues with and without oral L-arginine treatment. L-Citrulline was detected by immunohistochemistry as described in Materials and Methods. Brain tumors were removed before and 30, 60, 90, and 120 min after oral administration of L-arginine (200 mg/kg). Fluorescent microscopy was done using anti-L-citrulline primary and FITC-conjugated secondary antibodies. Original magnification, ×20. *Columns,* mean; *bars,* SE.

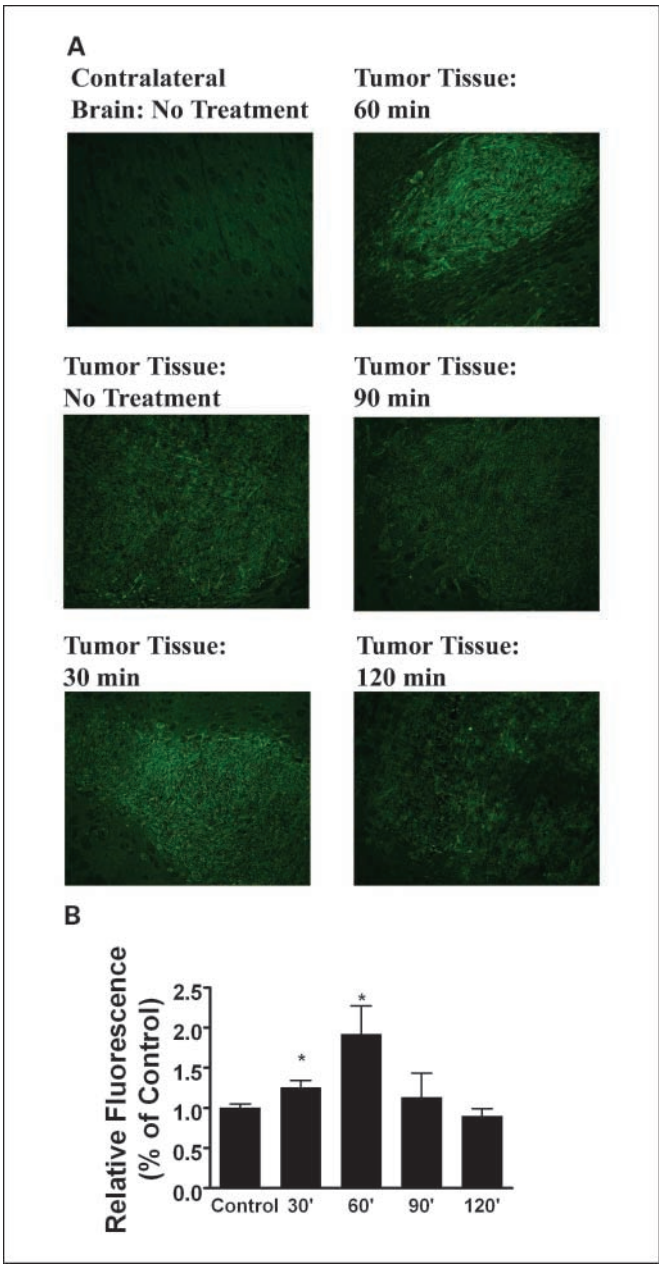

Fig. 6. cGMP levels in brain tissues with and without oral L-arginine treatment. cGMP levels were determined by immunohistochemistry as described in Materials and Methods. *A,* images representing the effect of L-arginine on cGMP levels in 9L tumor. Brain tumors were removed before and 30, 60, 90, and 120 min after oral administration of L-arginine (200 mg/kg). Fluorescent microscopy was done using anti-cGMP primary and FITC-conjugated secondary antibodies. Original magnification, ×20. *B,* Semiquantification of immunofluorescent intensity of cGMP staining. As described in Materials and Methods, the immunofluoresence intensity of cGMP staining was measured at four randomly chosen areas of tumor using Zeiss AxionVersion software. *Columns,* mean; *bars,* SE. *, $P < 0.05$, significantly different from saline control group.

hydroxyurea can be oxidized by heme group to produce NO via a series of intermediate reactions (25, 26, 38). Hydroxyurea also has been found to induce NO production in an eNOS-dependent manner (38). The maximal effect of hydroxyurea on BTB permeability was comparable with that of L-arginine (1.8-fold versus 2.0-fold over control; $P > 0.05$; Fig. 1). However, there is a trend that hydroxyurea is more effective than L-arginine in increasing K_i and NO production, which may be related to their differences in chemical and pharmacologic characteristics. Interestingly, we found only a single dose (80 mg/kg) of hydroxyurea to be effective in increasing BTB permeability and high doses of both hydroxyurea (160 and 320 mg/kg) and L-arginine (600 mg/kg) were ineffective. The reason for the observations remains unclear. It may be related to certain feedback mechanisms that deplete NO downstream messenger molecules. It should be noted that our results are consistent with a report using bradykinin and its analogue, RMP-7, to increase the permeability of solid tumors (39). In that report, dose-response curve for RMP-7 displayed an inverted U shape with less permeability increase by high doses. Both bradykinin and NO are critical molecules involved in a pathway regulating BTB permeability that we have proposed previously (11).

We have shown previously that both bradykinin and RMP-7 increase tumor permeability in rat brain tumor models and humans (7–9, 21, 40). However, increased transport across the BTB mediated by bradykinin and RMP-7 is transient (~15 minutes) and dependent on bradykinin-2 receptor expression in individual brain tumors (40). Moreover, the dose-limiting side effect of hypotension from both compounds has discouraged their clinical use (12). In the present study, oral administration of L-arginine or hydroxyurea resulted in a comparable but much longer duration of increased tumor permeability in contrast to bradykinin. This extended period of BTB opening may facilitate a greater accumulation of antitumor therapeutic agents in malignant brain tumors when administration is optimized with the pharmacokinetics of the drugs. In addition, both L-arginine and hydroxyurea, at the optimal doses of increasing BTB permeability, did not cause any significant blood pressure changes. Although further preclinical studies (e.g., survival studies and dynamic monitoring tumor progression) are critical and ongoing, the present study has shown great advantages of NO donors over other vasoactive compounds such as bradykinin and RMP-7 while translating preclinical findings to clinical settings.

Less nNOS expression was detected in the implanted tumors when compared with that in normal cerebral tissue, suggesting that nNOS is not important in mediating the increase of BTB permeability by hydroxyurea and L-arginine. The reason for the difference may be that the expression of nNOS is limited to neuronal cells (21). In contrast, higher levels of eNOS were observed in the tumors compared with normal brain. This is consistent with previous studies that high levels of eNOS expression have been detected in rat (21) and human malignant brain tumors (41). The high levels of eNOS in brain tumors may be one of the reasons, if not all, why L-arginine and hydroxyurea enhanced BTB permeability.

Higher levels of NO were detected in tumor tissues collected from 9L tumor rats treated with hydroxyurea and L-arginine than those from untreated controls. Moreover, higher levels of L-citrulline, a byproduct of NO formatted from L-arginine (31–33), were detected in 9L tumors of rats after L-arginine treatment compared with that of untreated rats. NO has been reported to account for the increased blood flow to tumors expressing high levels of eNOS (27), and NOS inhibitor decreased blood-brain barrier transport in the focal ischemic area of the brain without causing significant changes in the nonischemic tissue (42). In this study, NO

produced in tumor endothelial cells in response to NO donors may be an underlying mechanism for the increased BTB permeability.

Oral administration of L-arginine also increased cGMP levels in the tumor tissue compared with that in untreated-rats. This is in accordance with the reports that L-arginine (19) and hydroxyurea (27) enhance cGMP levels in the target cells or tissue. Previously, we found that zaprinast, an inhibitor of phophodiesterase 5 that breaks down cGMP, can enhance bradykinin-induced BTB permeability increase (34). Very recently, we also observed that other phophodiesterase 5 inhibitors such as vardenafil and sildenafil significantly increased BTB permeability.[1] Interestingly, increased permeability induced by L-arginine, hydroxyurea, or phophodiesterase 5 inhibitors was abolished by the selective K_{Ca} channel antagonist, iberiotoxin (Fig. 1C).[1] Taken together, current data are well fitted to a pathway regulating BTB permeability, which have been proposed previously (11). In brief, NO donors may selectively increase NO levels in brain tumor tissue, resulting in cGMP increase. The increased levels of cGMP in tumors may activate cGMP-dependent protein kinase, which subsequently stimulates K_{Ca} channels (43) that are highly expressed in tumor microvessels. The activation of K_{Ca} channels leads to increased vesicular transport of drug from blood to brain tumor tissue (11).

In conclusion, this study used oral NO donors to pharmacologically modulate BTB permeability in a malignant brain tumor model. Oral administration of either L-arginine or hydroxyurea selectively increased brain tumor permeability, which appears to be mediated by elevated cGMP levels in tumor tissue. Currently, both L-arginine and hydroxyurea are approved for patients. Our findings have significant implications in improving drug delivery to brain tumors in patients.

[1] Unpublished data.

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