Increased Brain Tumor Microvessel Permeability after Intracarotid Bradykinin Infusion Is Mediated by Nitric Oxide

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ABSTRACT

Nitric oxide (NO), a free radical gas implicated in a wide variety of biological reactions, is a novel signaling molecule that may regulate vasodilation, cerebral blood flow, and vascular permeability. This study was performed to determine whether NO mediates the selective increase in brain tumor microvessel permeability after intracarotid infusion of bradykinin in the RG2 rat glioma model. Intracarotid infusion of bradykinin selectively increased the transport of radiolabeled α-aminoisobutyric acid and dextran into brain tumors. Transport into normal brain was not increased. The administration of an NO synthase inhibitor, NG-nitro-l-arginine methyl ester, significantly inhibited the increased transport into tumors for both tracers. The inhibitory effect of NG-nitro-l-arginine methyl ester on the response to bradykinin was reversed by l-arginine. The expression of two NO synthase (NOS) isoforms in cultured RG2 glioma cell lines and intracerebral RG2 glioma was examined by immunohistochemistry and Western blot analysis. High levels of expression of neuronal NOS were detected in cultured and intracerebral RG2 cells but not in normal brain tissue, except in rare neuronal cells. The endothelial form of NOS was also expressed in cultured RG2 cells, but not as strongly as neuronal NOS expression. In intracerebral RG2 gliomas, expression of endothelial NOS in the tumor was detected at higher levels than in normal brain. These findings indicate that RG2 rat gliomas express high levels of NOS, which regulate the production of NO, compared with normal brain. We suggest that the selective permeability increase in brain tumor microvessels after bradykinin infusion is mediated by NO. Furthermore, the absence of high levels of NOS in normal brain may account for the attenuated permeability response to bradykinin in normal brain microvessels.

INTRODUCTION

The blood-brain barrier restricts entry of nonlipid soluble molecules that are not recognized by specific transport carriers into the brain (1). Brain tumor microvessels also have a BTB, which limits delivery of nonlipid soluble molecules to tumor tissue (2–4). One approach to improve therapy of brain tumors has focused on methods that increase delivery of anti-tumor compounds to tumor tissue (5). Leukotrienes and bradykinin were shown to selectively increase transport of compounds to brain tumors and abnormal brain (6–8). Intracarotid infusion of bradykinin was reported to selectively increase transport of tracers, ranging from Mf 103 to 70,000, into tumors. The permeability of normal brain microvessels was unaffected by intracarotid infusion of bradykinin. The magnitude of increased transport by bradykinin appears to relate to tracer size, because transport of smaller tracers (Mf 100–300) were increased by 2–4-fold, whereas transport of larger tracers (Mf 70,000) were increased 10–12-fold (7). Recently, it was reported that intracarotid infusion of the bradykinin analogue RMP-7 selectively increases permeability in RG2 gliomas. Intracarotid infusion of RMP-7 selectively increased transport of carboplatin to brain tumors and increased survival in rats with experimental gliomas (9). Clinical studies in the United States and Europe are investigating whether RMP-7 will increase delivery of carboplatin to tumor tissue and increase survival in patients with recurrent gliomas. However, the mechanism of bradykinin-selective action on tumor capillaries is not understood.

NO is a prominent vascular and neuronal messenger molecule first identified as the chemical responsible for endothelium-derived relaxing factor activity (10, 11). Studies have suggested a role for NO in regulation of tumor blood flow (12), edema, and vascular permeability (13–16). NOS is a cell type-specific enzyme that catalyzes the synthesis of NO. The cells in which NO synthesis was first demonstrated were the vascular endothelium, the neurons (eNOS and nNOS, respectively), and activated macrophages (17–19). In endothelial cells and neurons, constitutive NOS is activated by agonists that increase intracellular Ca2+ concentrations and enhance calmodulin binding.

We hypothesized that NO mediated the effect of bradykinin on brain tumor microvessel permeability, and that the effect of bradykinin on tumor microvessels, but not normal brain microvessels, was the result of higher expression of NOS in tumors. In this study, the effect of L-NAME, a NOS inhibitor, on blood-tumor barrier permeability enhanced by bradykinin was studied using the RG2 rat glioma model. Also investigated was the expression of NOS in rat glioma cell lines, implanted intracerebral tumors, and normal brain tissue.

MATERIALS AND METHODS

Female Wistar rats, weighing 150–200 g each, were used for this study.

Tumor Inoculation

RG2 is a cell line derived from a rat glioma induced by ethylnitrosourea (20, 21). The RG2 glioma cells were kept frozen until use, then thawed and maintained in a monolayer culture in Ham’s F12 medium with 10% calf serum. The rats were anesthetized with i.p. ketamine (50 mg/kg). Glial tumors were implanted into the right hemisphere by intracerebral injections of 1 × 106 RG2 glioma cells in 5 μl Ham’s F12 medium (1.2% methylcellulose) by a Hamilton syringe. The coordinates used were 5 mm lateral, 2 mm anterior to the bregma, and 4.5 mm deep to the dural surface.

Animal Preparation

Seven or 8 days after tumor implantation, the rats were anesthetized with i.p. urethane (1 g/kg), and a polyethylene catheter was inserted retrograde through the external carotid artery to the common carotid artery bifurcation ipsilateral to the tumor. The external carotid artery and pterygopalatine artery were then ligated. Both femoral veins were cannulated for administration of drugs or tracers. One femoral artery was cannulated to monitor cardiac blood pressure, and the other femoral artery was cannulated to withdraw arterial blood. Body temperature was maintained at 37°C, and arterial blood gases, blood pressure, and hematocrit were monitored. Animals with abnormal physiological parameters were eliminated from this study. In a preliminary study to determine the dose of L-NAME (Sigma Chemical Co., St. Louis, MO), we infused different doses (100, 200, and 300 μg/kg/min for 30 min) of L-NAME i.v., and physiological parameters were monitored. Serious systemic hypertension occurred at the dose of 300 μg/kg/min, and the respiratory condition became unstable. Therefore, we chose the dose of 200 μg/kg/min for 30 min for L-NAME.
**Study 1: Effect of Bradykinin on BTB Permeability.** The tumor-implanted rats were divided into two tracer groups, AIB or dextran. Fifteen minutes after the start of the i.v. vehicle 30-mm load, bradykinin (Sigma) or saline (control) was infused into the right carotid artery at a rate of 53.3 μl/min (10 μg/kg/min) for 15 min. In regional permeability studies, 5 min after the start of the intracarotid infusion, 100 μCi/kg [14C]dextan (M, 103; DuPont New England Nuclear, Boston, MA) or [14C]dextran (M, 70,000; DuPont New England Nuclear) were injected as an i.v. bolus. A peristaltic withdrawal pump was used to withdraw femoral arterial blood at a constant rate of 0.083 ml/min immediately after the injection of the tracer for the determination of serum radioactivity. Fifteen minutes after the start of intracarotid infusions, the animals were killed by decapitation, and the brains were rapidly frozen and frozen.

**Study 2: Effect of L-NAME on BTB Permeability Enhanced by Bradykinin.** L-NAME or saline was administered continuously into the venous cannula at a rate of 27.0 μl/min for 30 min. The dose of L-NAME was 200 μg/kg/min (6–mg/kg total dose). Fifteen minutes after the start of the L-NAME infusion, intracarotid infusion of bradykinin (or saline) was initiated at the same rate as in study 1. Injection of tracer, withdrawing of arterial blood, and other protocols were in the same manner as described in study 1.

**Study 3: Effect of L-NAME during Inflow of L-Arginine on BTB Permeability Enhanced by Bradykinin.** Continuous i.v. infusion of L-arginine (Sigma) into the femoral vein was started at the same time as the start of the infusion of L-NAME through the other femoral vein. The rate of infusion of L-arginine was 3.33 mg/kg/min for 30 min (100–mg/kg total dose). The remaining protocol was performed in the same manner as described in study 2.

**Blood Volume Studies**

In blood volume studies, 5 and 14 min after the start of the intracarotid infusion with L-NAME or saline, 100 μCi/kg [14C]dextran were injected as an i.v. bolus to make the circulation time of dextran 10 and 1 min, respectively. In this study, the infusion of saline, bradykinin, and L-NAME was the same as in study 2, but the time of tracer injection and duration of arterial blood withdrawal were different from those in study 2. After the circulation of dextran, the brains were removed immediately and frozen. At least five rats were used in each part of circulation time of any treatment group.

**Autoradiography**

The frozen brains were mounted onto pedestals with M-1 embedding matrix (Lipshaw, Pittsburgh, PA), and 20-μm coronal sections were cut with a cryotome. The sections were thaw mounted onto coverslips, and autoradiograms were generated by coexposing the sections on Kodak XAR-5 film with tissue-calibrated 14C standards (Amersham, Arlington Heights, IL) for 2 weeks. The sequential section was stained with hematoxylin for the correlation of areas of histologically verified tumor areas with autoradiograms. The regional radioactivity was measured in the tumor. BST (areas within a 2-mm distance from the border of the tumor), right cortex (ipsilateral cortex), right basal ganglia, and left basal ganglia. Quantitative analysis of the regional radioactivity was performed using a computer (Power Macintosh 7100) and Image 1.55 software (NIH).

**K0 Value and Blood Volume Calculation**

For quantitative autoradiographic examination, the regional permeability in the brain and tumor tissues was expressed by the unidirectional transfer constant \( K_0 \) value (μg/min). The initial rate for blood-to-brain transfer was calculated using the following equation (22, 23):

\[
K_0 = \frac{Cbr - V_0 Cbl}{\int_0^T Cpl \times dt}
\]

\( Cbr \) is the brain or tumor concentration of the tracer at the end of the experiment (dpm/g); \( Cbl \) is the blood concentration of the tracer at the end of experiment (dpm/g); \( T \) is the duration of the experiment (min); and \( Cpl \) is the arterial plasma concentration (dpm/ml). \( V_0 \) is the regional cerebral blood volume in the tissue (μl/g).

To determine the \( V_0 \) values, we performed the blood volume studies by graphic method using [14C]dextran as described previously (7, 24). In brief, plotting \( \int_0^T Cpl \times dt / Cpl(T) \) as X values and volume of distribution \( [V_d = Cbr / Cpl(T)] \) as Y values, \( V_0 \) was obtained as the Y-intercept of the regression line. For all tracers, statistical comparison of regional \( K_0 \) values or blood volume between same regions with or without bradykinin, L-NAME, or L-arginine were determined using the Mann-Whitney U test and ANOVA. \( P < 0.05 \) was considered significant.

**Immunohistochemical Analysis of the RG2 Cell Line and Intracerebral RG2 Gliomas**

The RG2 cell line was trypsized and resuspended at 5 x 106 cells/ml and grown on chamber slides for immunocytochemistry, which was done 48 h after seeding. Before immunostaining, the cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Next, they were incubated with 2% hydrogen peroxide in 60% methanol for 30 min, to quench endogenous peroxidases, and 0.1% Triton X-100 in PBS for 20 min, to permeabilize the membranes. After washing with PBS, the cells were incubated sequentially with 3% normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min, 1:50 or 1:100 dilution of monoclonal eNOS or nNOS antibody (Transduction Laboratories, Louisville, KY) for 1 h, and biotinylated horse antimouse immunoglobulin (Vector Laboratories) for 30 min. Mouse IgG was used at identical concentrations as a control primary antibody. Biotinylated conjugates were detected with avidin-biotin peroxidase complex (Vector Laboratories) for 30 min at room temperature. After washing three times with PBS, the peroxidase sites were visualized with aminoethylcarbazol for 20 min. The slides were then washed in distilled water and counterstained with Gill’s hematoxylin for 1 min. The cells were mounted in glycerin and examined for the presence of positively stained cells.

Tumor inoculations of intracerebral RG2 gliomas were performed using the same methods as in the permeability studies. On the seventh day after tumor inoculation, rats were sacrificed, and brains were removed and paraffin embedded. Brain sections (6 μm) were deparaffinized in xylene and hydrated through graded alcohols to PBS. These sections were stained in a similar manner as described for the RG2 cell line.

**Western Blot Analysis**

The RG2 cells seeded on a 10-cm-diameter dish were scraped with buffer A (50 mM Tris-HCI (pH 7.4), 1 mM EDTA, 10 mg/liter antipain, 10 mg/liter leupeptin, 10 mg/liter soybean trypsin inhibitor, 10 mg/liter pepstatin, 10 mg/liter chymostatin, and 100 mg/liter phenylmethylsulfonyl fluoride). Protein homogenate from enucleated intracerebral RG2 gliomas or normal rat brain was prepared by rapidly homogenizing in 10 volume buffer A. Samples were centrifuged at 20,000 X g for 15 min, and the protein concentration of soluble materials was measured by the bicinchoninic acid method. Samples were diluted to equal concentrations and were partially purified using 2′,5′-ADP agarose chromatography as described previously (25). Control protein lysates for nNOS and eNOS were provided by Transduction Laboratories. Samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and probed with anti-eNOS antibody or anti-nNOS antibody (1:500 dilution). After incubating with primary antibody, membranes were next incubated with biotinylated horse antimouse immunoglobulin. Biotinylated conjugates were detected with avidin-biotin peroxidase complex for 30 min at room temperature. After washing, the peroxidase sites were visualized with diaminobenzidine for 2 min.

**RESULTS**

**Effects of Bradykinin, L-NAME, or L-Arginine on Physiological Parameters**

Physiological parameters, which included systemic blood pressure, arterial blood gas, and hemoglobin concentration, were not significantly changed by intracarotid bradykinin infusion at a rate of 10 μg/kg/min during the experiments. The physiological parameters were not significantly changed in any experimental conditions except

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for L-NAME infusion. i.v. infusion of L-NAME significantly increased mean arterial blood pressure in a dose-dependent manner. Mean arterial blood pressures were increased 33.7 ± 8.4% by i.v. infusion of L-NAME (200 μg/kg/min, 30 min). L-NAME did not change blood gas parameters and hemoglobin concentration. i.v. infusion of L-arginine (3.33 mg/kg/min, 30 min) did not affect the mean arterial blood pressure significantly.

Blood Volume in Tumor and Normal Brain

The blood volume (μL/g) for quantitative examination of permeability was calculated by a graphic method. The blood volumes in tumors in the saline, bradykinin, and L-NAME-only groups were 12.6, 14.4, and 14.1, respectively. The blood volumes in normal brain in the saline, bradykinin, and L-NAME groups were 3.6, 3.3, and 3.8, respectively. The tumor or normal brain blood volumes were not significantly different among treatment groups.

Regional Permeability

Study 1. The K₁ values were calculated using the method described in previous studies (7, 22). The transfer constant K₁ in tumors to [¹⁴C]dextran (M, 70,000) after bradykinin infusion (n = 7) was 9-fold higher than in controls (n = 6; mean ± SD. 15.86 ± 8.70 versus 1.85 ± 1.02; P < 0.01; Fig. 1a). The transport in tumors to [¹⁴C]AIB (M, 103) after infusion of bradykinin (n = 6) was also increased compared with controls (n = 6; 27.41 ± 8.32 versus 9.15 ± 3.07; P < 0.005; Fig. 1b). Brain tissue within 2 mm of the tumor was evaluated, because this BST may be affected by tumor compression and vasoactive agents released directly by the tumors. The K₁ for dextran and AIB was not significantly increased in BST after bradykinin infusion, and the infusion did not increase transport in any normal brain regions of either tracer tested (Fig. 1, a and b).

Study 2. The combined administration of L-NAME and bradykinin significantly decreased the K₁ value in tumors compared with the bradykinin infusion group (4.31 ± 2.45 versus 15.86 ± 8.70; P < 0.005; Fig. 1a). L-NAME treatment also decreased K₁ values in tumors to AIB, which initially were increased by intracarotid infusion of bradykinin (20.22 ± 6.12 versus 27.41 ± 8.32; P < 0.05; Fig. 1b). In all normal brain regions, the K₁ values of both tracers showed no significant differences between the bradykinin with L-NAME treatment group and the bradykinin-alone treatment group. The K₁ values in tumors in the saline infusion group with an L-NAME load were not significantly different from the K₁ values in the saline infusion groups with a saline load in study 1 (dextran, 1.63 ± 1.30; n = 6; AIB, 8.79 ± 3.91; n = 6).

Study 3. With the L-arginine continuous infusion, the K₁ in the tumors of the group treated with bradykinin and L-NAME was significantly higher than in those without L-arginine infusion (dextran, 11.23 ± 6.24 versus 4.31 ± 2.45; P < 0.05; AIB, 28.85 ± 3.64 versus 20.22 ± 6.12; P < 0.005; Fig. 1, a and b). There were no significant changes in K₁ between the two groups treated with bradykinin alone and with bradykinin, L-NAME, and L-arginine.

Immunohistochemical Analysis of RG2 Cell Line and Intracerebral RG2 Gliomas

Immunohistochemistry, using the monoclonal anti-nNOS, showed intense staining in cultured rat RG2 glioma cells and in sections of implanted intracerebral RG2 tumors. Immunoreactivity of this isoform was detected in the nuclei and cytoplasm of these tumor cells (Fig. 2, a and c). Positive staining of nNOS in normal brain tissue was detected only in neurons. Normal glial cells were not stained by the anti-nNOS antibody. On immunohistochemical analysis, the expression of nNOS in RG2 glial tumors was stronger than in normal brain tissue (Table 1). Weak positive immunoreactivity was present using the monoclonal anti-eNOS antibody in the cultured RG2 cell line (Fig. 2b). eNOS expression was detected in tumor capillaries and tumor cells in intracerebral tumors (Fig. 2d). However, in normal brain regions, eNOS expression was detected only in rare endothelial cells.

Western Blot Analysis

Immunoreactive proteins of the expected size (M, 155,000) for nNOS were stained in both the normal cerebral hemisphere and intracerebral tumor lysates. The intensity of the bands in the normal cerebral tissue and RG2 tumors were similar using the anti-nNOS antibody. In the lysates of normal cerebellum, intense immunoreactivity, believed to be from neuronal cells, was detected (Fig. 3). The anti-eNOS antibody produced bands of the appropriate size (M, 140,000) in intracerebral RG2 tumors and normal cerebral tissue. The band observed in RG2 tumors was more intense than that in normal tissue (Fig. 3).
**Table 1** NOS expression by immunohistochemistry

<table>
<thead>
<tr>
<th>Tissue</th>
<th>nNOS</th>
<th>eNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured RG2 cell line</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>Intracerebral RG2 tumors</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>Normal cerebral tissue</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Normal cerebellum</td>
<td>3+</td>
<td>1+</td>
</tr>
</tbody>
</table>

* +, weakly positive; 2+, moderately positive; and 3+, strongly positive.

**DISCUSSION**

This study, as in previous studies, demonstrates that intracarotid infusion of bradykinin significantly increased uptake of radiolabeled tracers to tumors but not to normal brain. In this study, the selective increases in permeability in tumor microvessels were significantly inhibited by the NOS inhibitor L-NAME. L-NAME, an L-arginine analogue, inhibits the action of endogenous L-arginine, which is required in the NO synthase reaction (26–28). To confirm the inhibitory effect of L-NAME on permeability, we infused L-arginine to saturate endogenous L-arginine. Continuous i.v. infusion of L-arginine reversed the inhibitory effect of L-NAME. These results suggest that NO and NOS mediate the effects of increase permeability by bradykinin on tumor microvessels. It was demonstrated that bradykinin caused a transient increase in cytosolic Ca\(^{2+}\) through the binding to bradykinin B2 receptors in brain endothelial cells (29, 30). Reiser (31) showed that NO synthesis was activated by elevation of Ca\(^{2+}\), and as a result, the level of cGMP was increased in neuronal cells through Ca\(^{2+}\) and calmodulin-dependent activation of NOS. In addition, it is known that NO mediates glutamate stimulation of cGMP formation in the cerebellum via N-methyl-D-aspartate receptors (32, 33), and cGMP could alter the permeability of brain microvessels (34). Antagonist of the bradykinin B2 receptor inhibited the effect of bradykinin on tumor permeability.4

In this study, NOS expression was also demonstrated in rat RG2 glial tumors by immunohistochemistry and Western blot analysis. Total concentrations of nNOS were not significantly different between implanted tumors and normal cerebral tissue by Western blot analysis. However, the expression of nNOS was more evident in intracerebral RG2 tumors and the cultured RG2 cell line using immunohistochemistry, compared with normal cerebral and cerebellum tissue, in which the expression of this protein was limited only to neuronal cells. The reason for the discrepancy in nNOS expression observed between immunohistochemistry and Western blot analysis may be due to the higher nNOS expression in neuronal

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4 Unpublished data.
cells. Western blot analysis of eNOS also revealed weak expression in normal brain. By both Western blot analysis and immunohistochemistry, higher levels of eNOS were observed in RG2 tumors compared with normal brain. It is known that high levels of NOS expression have been detected in a variety of malignant brain tumors (35). NO may be responsible for the increased blood flow to tumors expressing high levels of NOS (36). NOS inhibitors can decrease local edema formation in experimental tumors (15), and NO production by endothelial cells can change the permeability in microvessels. A NOS inhibitor decreased blood-brain barrier transport in the focal ischemic area of the brain without causing significant changes in the nonischemic tissue (37). Thus, NO produced by tumor cells may have a role in the regulation of blood flow and vascular permeability in brain tumors.

Our data suggested that NO mediates the effect of increased permeability in brain tumor microvessels by bradykinin. It is suggested that higher levels of NOS expression in brain tumors account for the selective bradykinin increase in permeability in brain tumor microvessels compared with normal brain. Microvessels in human brain tumors and the rat gliomas are different biologically from normal brain capillaries (38, 39). Bradykinin did not induce cGMP production in pure cultures of normal brain capillary endothelial cells (40, 41). However, NO produced by neuroblastoma cells and by aortic endothelial cells in response to bradykinin increased cGMP levels in brain capillary endothelial cells, and these effects were inhibited by NOS inhibitors (41). We hypothesized two possible mechanisms for the effect of bradykinin on brain tumor capillaries. First, bradykinin may pass through the BTB and bind to B2 receptors on the tumor surface, activating NOS and initiating NO production in tumor cells. As a result, this NO production causes the increased permeability in tumor capillaries. Second, higher levels of NOS in tumor capillaries may be activated by bradykinin, which binds to B2 receptors on tumor endothelial cells directly. NO produced in the endothelium can then increase tumor microvessel permeability. Further studies are planned to explore these hypotheses in the laboratory.

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