Bradykinin Modulation of Tumor Vasculature: II. Activation of Nitric Oxide and Phospholipase A₂/Prostaglandin Signaling Pathways Synergetically Modifies Vascular Physiology and Morphology to Enhance Delivery of Chemotherapeutic Agents to Tumors

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ABSTRACT

Intravenous infusions of the bradykinin agonist Cereport (l-Bradimil, formerly RMP-7) enhance delivery of concomitantly administered hydrophilic chemotherapeutic agents to solid tumors. The enhanced delivery produces greater in vivo efficacy of chemotherapeutic agents, manifested as suppressed tumor growth and increased survival in tumor-bearing rats. Here we elucidate the mechanisms of action involved with this unique phenomenon, at both the physical and biochemical levels. At the physical level we demonstrate that Cereport modifies the tumor vasculature in several important ways, including transient 1) reductions in interstitial fluid pressure within the tumor, 2) increases in pore size of the vasculature, and 3) increases in total vascular surface area. All three of these changes modify tumor-specific characteristics of the vasculature known to impede drug delivery to the tumor interstitium. Biochemically, we demonstrate that the activation of both of bradykinin"s major signaling pathways, the nitric oxide and phospholipase A₂/prostaglandin E₂, are necessary events. Although pharmacologically blocking either pathway greatly reduced the effects of Cereport, stimulation of either pathway alone did not enhance delivery. However, simultaneous stimulation of both pathways (without exogenous bradykinin B₂ receptor stimulation) produced a nearly 2-fold increase in delivery of carboplatin to the tumor. Thus, stimulation of endogenous bradykinin B₂ receptors induces at least two parallel biochemical cascades that act synergistically to uniquely modify the tumor vasculature in ways that increase delivery and efficacy of chemotherapeutic agents.

It is generally recognized that treatment of solid, peripheral tumors is impeded because the abnormal nature of the tumor vasculature limits delivery of chemotherapeutic agents to the tumor interstitium (Jain, 1990, 1991; Less et al., 1991). Recent studies in rat tumor models demonstrate that stimulation of endogenous B₂ bradykinin receptors with the selective agonist Cereport leads to enhanced delivery of chemotherapeutic agents into solid peripheral tumors (Emerich et al., 2001). This effect is relatively selective for tumor tissue, in that little or no effect is achieved in healthy tissue or organs. Moreover, the enhanced delivery is manifested as suppression of tumor growth and increased survival in tumor-bearing rats. Interestingly, because the plasma levels of Cereport required to achieve this effect are relatively low (i.e., well below the Kᵢ established for Cereport at the B₂ receptor (Bartus et al., 1996b, 2000), this phenomenon may reflect a natural role of bradykinin in mediating vascular flow of blood and nutrients to solid tumors.

Given the novelty of this phenomenon, as well as its potential application for improving the treatment of cancer patients, the present paper attempts to further increase our understanding of the underlying physical and biochemical mechanisms of action. A series of studies was performed to characterize the physical changes of the tumor vasculature induced by Cereport and to identify those that may be responsible for the enhanced delivery. Interstitial fluid pressure within the tumor, tumor blood flow and systemic blood pressure were independently measured during Cereport infusion. Separate measurements of the physical properties of the tumor vasculature included areas of hypoperfusion

ABBREVIATIONS: NO, nitric oxide; NECA, 5′-N-ethylcarboxamidoadenosine; IAP, 4-iodo-N-methyl-[¹⁴C]antipyrine; IFP, interstitial fluid pressure; L-NAME, L-N-nitro-L-arginine methyl ester hydrochloride; SNAP, S-nitroso-N-acetylpenicillamine; PGE, prostaglandin E; PDE-V, phosphodiesterase V; PL/PG, phospholipase A₂/prostaglandin E₂.
within the tumor, intravascular pore size and surface area of the tumor vessels. Finally, a parallel series of pharmacological studies was performed to define the roles played by bradykinin’s two major signaling pathways.

Bradykinin uses a G-coupled receptor capable of inducing a variety of different effector responses, including activation of phospholipases, channels, and protein kinases (Burch et al., 1993). Two major pathways have been shown to be activated by B<sub>2</sub> receptor stimulation in nearly all tissues (Burch et al., 1993) and they are the focus of our studies here. The first involves the release of arachidonic acid by activation of phospholipase A<sub>2</sub>. Arachidonic acid, in turn, is metabolized into a variety of eicosanoids, including prostaglandin E<sub>2</sub> through the action of cyclooxygenase. In this article, we pharmacologically stimulate one component of this pathway by administering exogenous prostaglandin E<sub>2</sub> and suppress the pathway by inhibiting cyclooxygenase activity.

The second major pathway involves the activation of phospholipase C and phosphatidylinositols to release diacylglycerol and inositol phosphates (Burch et al., 1993). Inositol phosphates have been shown to be instrumental in the release of calcium from the endoplasmic reticulum (Fisher, 1995). The elevation in cytosolic calcium has, in turn, been linked to numerous calcium-dependent events, including protein kinase C and calmodulin-dependent protein kinases (Fisher, 1995). Recently, an important signaling role of nitric oxide (NO) has been linked to the bradykinin-induced calcium fluxes (McGehee et al., 1992). NO, in turn, stimulates cGMP activity as an important modulating event (Murad, 1994). We focused our attention on the NO-cGMP component of this pathway, stimulating it by administering NO donors as well as inhibiting the degradation of cGMP. Furthermore, we dampened the effects of this pathway by inhibiting nitricoxide synthetase activity.

Collectively, the studies we report reveal a complex chain of events that occurs following B<sub>2</sub> receptor stimulation within solid tumor vessels. This involves at least two parallel signaling pathways and multiple, transient physiological and morphological changes within the tumor that can be linked to the increased delivery of chemotherapeutic agents following Cereport infusion.

### Materials and Methods

**Animals.** Male Fischer rats (N = 569; 170–220 g; Taconic Farms, Germantown, NY) were housed in polypropylene cages with free access to food and water. The vivarium was maintained on a 12-h light/dark cycle with a room temperature of 22°C and relative humidity levels of 50 ± 5%. All studies were approved in advance by Alkermes’ Institutional Animal Care and Use Committee and were conducted in a manner that met or exceeded National Institutes of Health guidelines.

**Cell Maintenance and Implantation.** A rat ascites mammary adenocarcinoma cell line (MATB-III; ATCC CRL-1666) was used in the following studies. Cells were grown and maintained as previously described (Emerich et al., 2001). Before implantation, cells were harvested and suspended in serum-free media containing 1.2% methyl cellulose at a density of 5 × 10<sup>6</sup> cells/mL and 200 µl of the suspension (10<sup>6</sup> cells) was injected subcutaneously into the rear flank, using a 22-gauge needle. All tumors were palpitated and measured daily until they reached a size of 1 cm<sup>2</sup> (7–10 days), at which time the animals were used in dosing studies.

**Systemic Blood Pressure.** Two separate and mutually corroborating studies examined the relationship between alterations in blood pressure and the enhanced delivery of [14C]carboplatin produced by Cereport. In the first studies, continuous on-line measures of mean arterial blood pressure were recorded from an intrafemoral cannula over the course of the Cereport (0.1, 0.2, 0.5, or 1.0 µg/kg/min) infusion using a MacLab 8 physiology recording system (ADInstruments, Milford, MA). In the second series of studies, the adenosine agonist 5′-N-ethylcarboxamidoadenosine (NECA) (Research Biochemicals International, Natick, MA) was infused i.v. to decrease blood pressure in a manner that mimicked both the magnitude and time course produced by Cereport. In these experiments, animals received a 15-min i.v. infusion of [14C]carboplatin (mol. wt. = 371, specific activity = 144 µCi/mg; Amersham, Arlington Heights, IL) followed by a 10-min i.v. infusion of either saline (N = 8) or Cereport (2 µg/kg) (N = 6). Parallel groups of animals received [14C]carboplatin infusions together with a 10-min i.v. infusion of NECA at a dose of either 0.2 µg/kg/min (N = 8) or 2.0 (N = 8) µg/kg/min.

**Regional Blood Flow and Autoradiography.** To determine whether significant changes in regional blood flow occur in peripheral tumors following Cereport administration, 4-iodo-N-methyl-[14C]antipyrine (IAP) autoradiography was used. IAP (25 µCi; Amersham) was administered 30 s before the completion of the Cereport infusion (0.15 µg/kg/min) using a progressively increasing infusion rate (0.8 to 3.3 ml/min over 30 s). Timed arterial blood samples were collected from a catheterized femoral artery directly into heparinized 60-µl glass capillary tubes (Ciba Corning Diagnostics Ltd., Suffolk, England) every 5 s over the 30-s tracer infusion period for subsequent analysis of whole-blood radioactivity levels. At the end of the IAP infusion, the tumor was rapidly removed and frozen in 2-methylbutane at −30°C. Blood samples were analyzed using scintillation counts.

Tumors were sectioned at 20-µm intervals and thaw-mounted onto microscope slides. Using standard autoradiographical techniques, the slides were apposed to radiosensitive film (Kodak Biomax MR-1) with [14C] calibration standards (0.002–3.58 µCi/g; American Radiolabeled Chemicals, St. Louis, MO) for 2 days and developed. The slides were removed and stained with H&E to verify tumor demarcation. Quantitative analysis of the regional radioactivity within the tumor sections was performed using an image analysis system (MCID; Imaging Research Corp., St. Catharines, Ontario, Canada). Individual H&E-stained sections from the center of the tumor were digitized and the same section on the autoradiograph was then precisely overlaid and digitized. Using the stained section to locate the tumor boundary, the total radioactivity within it was measured. Autoradiographical image optical densities were converted to radioactivity level/tumor tissue (nCi/g) using a standard curve from [14C] standards.

Total tissue radioactivity was converted into regional blood flow (mL/100 g/min) using the tissue equilibration method of Sakurada et al. (1978). The operational equation for tissue equilibration blood flow is as follows:

\[
A(T) = F \int_0^T C_s(t) e^{-\lambda T} dt \text{ ml}/100 \text{ g/min}
\]  

where \(A(T)\) is the amount of the tracer per unit weight of tissue at the end of experiment, \(\lambda\) is the estimated equilibrium tissue-blood partition coefficient, \(F\) is the actual rate of blood flow per unit weight of tissue, \(\lambda C_s(t)\) is the concentration of tracer in arterial blood perfusing a tissue at time \(t\). To solve for \(F\), the equation (Patlak et al., 1984) is simplified to the following:

\[
F = \frac{A(T)}{\int_0^T C_s(t) dt}
\]  

**Effect of Cereport on Perfusion of Tumor Blood Vessels.** A double fluorescent dye technique was used to determine whether...
Cereport altered the pattern of perfusion within the tumor vasculature. Animals received an i.v. bolus injection of the fluorescent dye Hoechst 33342 (30 mg/kg; Molecular Probes, Eugene, OR). Five minutes later, the same animals received a 10-min i.v. infusion of saline or Cereport (0.15 µg/kg/min) together with a bolus injection of a second, different fluorescent dye (DiOC₆, 1 mg/kg; Molecular Probes), 2 min into the Cereport infusion.

At the end of the Cereport infusion, the tumors were removed and sectioned (20 µm) on a cryostat. Labeled vessels were counted in three individual sections (comprising the beginning, quarter, and middle of the tumor) in sequential 500-µm² fields beginning at one edge of the tumor and continuing across the entire tumor to the other edge. The average number of blood vessels per field labeled with Hoechst 33342 (pre-Cereport treatment) was compared with those labeled with DiOC₆, (post-Cereport treatment) under a fluorescence microscope (Hoechst 33342: excitation = 376, emission = 418; DiOC₆: excitation = 490, emission = 520).

**Effect of Cereport on Interstitial Fluid Pressure.** Interstitial fluid pressure (IFP) of subcutaneous MATB-III tumors was measured using the wick-in-needle technique (Scholander et al., 1968). A recording needle was constructed by removing the hub of a 23-gauge × 1-inch needle and drilling a 0.8-mm hole 4 mm from the needle tip. Five 2.5-cm lengths of 6-0 nylon monofilament suture were inserted into the needle (extending 0.5 mm beyond the needle tip) to permit transmission of tumor IFP through the individual channels formed by the suture to the pressure transducer. The recording needle was connected to an ultralow compliance pressure transducer (TXC-310; Micro-Med, Lexington, KY) using 10 cm of PE50 tubing filled with heparinized saline (50 U/ml). Interstitial fluid pressure (mm Hg) was continuously measured six times per second using a Digi-Med Low Pressure analyser and DMSI-200 software (Micro-Med) and the results were averaged across each minute.

For on-line IFP measures, animals were placed in the supine position and a 2-cm incision was made immediately lateral to the tumor. The recording needle was inserted into the center of the tumor and left in place until a 10-min stable baseline was achieved. Saline or Cereport (0.1 or 1.0 µg/kg/min) was infused i.v. for 5 to 10 min and IFP was recorded throughout the drug administration and for an additional 5 min.

**Effect of Cereport on Transvascular Pore Size.** Specific-size fluorescent polymer microspheres (Duke Scientific Corporation, Palo Alto, CA) were infused i.v. as pairs of different diameter microspheres (0.025 and 0.05 µm or 0.1 and 0.2 µm) containing different fluorescent dyes (red or green) just before an i.v. infusion of Cereport (0.15 µg/kg/min) or vehicle. Animals were sacrificed 2 or 24 h later (to allow the microspheres to clear from the circulation, ensuring that any remaining microspheres within the tumor had extravasated from the vasculature into the interstitial space). The tumor tissue was frozen and sectioned with a cryostat at 20-µm intervals. Using a florescence microscope, the two different-sized microspheres within a single section representing the center of the tumor were independently visualized using fluorescein isothiocyanate and rhodamine filters. Beginning at the edge of the tumor, the section was scanned sequentially across the entire tumor. The first 12 fields (200 µm² each) containing microspheres were digitized using a color charge-coupled device camera interfaced with an image analysis system (Image Pro-Plus; Media Cybernetics, Silver Spring, MD).

A semiquantitative ranking of the intensity of the fluorescence for each microsphere size was performed by six independent raters blinded to (i.e., unaware of) which treatment group each animal belonged. Fluorescence within individual tumors was ranked ordered from the least (ranking of 1) to the most intense (maximum ranking equal to that number of animals within each microsphere size group) independent of whether an animal received Cereport or vehicle. Data are expressed as the mean overall ranking for saline and Cereport-treated animals, independently for each microsphere size. By statistically analyzing the vehicle versus Cereport scores, it was possible to determine the extent to which Cereport facilitated extravasation of the microspheres from the vasculature to the tumor interstitium for each microsphere size (although it was not possible to compare the relative effects between microsphere sizes).

**Immunocytochemistry.** MATB-III tumors were removed and quickly frozen in chilled isopentane (−30°C). Tumors were then sectioned on a cryostat (20 µm), thaw-mounted onto microscope slides, and processed for the selective visualization of tumor blood vessels using CD-31 immunohistochemistry (Scholander et al., 2000) as follows: 1) slides washed 6 × 10 min in dilution media (Triton X-100 and Tris buffer) followed by 0.1 M sodium periodate for 1 h; 2) slides washed 6 × 10 min in dilution media followed by 0.1 M sodium 3 × 10 min in dilution media, followed by normal horse serum and bovine serum albumin for 1 h; 3) slides incubated for 48 h (24 h at 22°C and 24 h at 4°C) in the primary monoclonal antibody to CD-31 (1:300; Chemicon, Tempecula, CA); 4) slides washed 6 × 10 min in dilution media followed by a 1-h incubation in the appropriate biotinylated secondary IgG antibody (1:200; Vector, Burlingame, CA); 5) slides washed 6 × 10 min in dilution media, rinse slides; 6) slides incubated with “Elite” avidin-biotin complex (1:1000; Vector) for 1.25 h; 7) slides rinsed 3 × 10 min in imidazole/acetate buffer; 8) slides incubated in a solution containing 3,3’-diaminobenzidine and nickel ammonium sulfate dissolved in imidazole/acetate buffer with hydrogen peroxide for 8 min; and 9) reaction terminated by rinsing 3 × 10 min in imidazole/acetate buffer. Sections were stored in phosphate-buff ered saline and mounted, dehydrated in alcohol, and cover slipped. Control sections were processed in an identical manner except the primary antibody solvent was substituted for the primary antibody.

**Effect of Cereport on Tumor Blood Vessel Size.** Cereport (0.15 µg/kg/min) or saline was infused i.v. into tumor-bearing animals. At 2, 5, or 8 min into the infusion, the animals were sacrificed, the peripheral tumors were rapidly removed. A 1- to 2-mm-thick slice from the center of tumor was removed, weighed, and placed into a scintillation vial and the amount of radioactivity (dis/µg) was computed using scintillation counts.

**Pharmacological Interactions between Bradykinin and Nitric Oxide and Prostaglandins.** A series of studies examined the relationship between stimulation of bradykinin B₂ receptors, alterations in the endogenous NO activity, and delivery of [14C]carboplatin to peripheral tumors. In the first experiment, rats received a
15-min i.v. infusion of [14C]carboplatin followed by a 10-min i.v. infusion of either saline or Cereport (0.1 μg/kg/min). L-NAME was administered as a 30-min infusion (0.2 μg/kg/min) beginning 15 min before the initiation of the carboplatin infusion and continuing until the end of the Cereport infusion. Tumors were removed at the end of the infusion and processed for scintillation as described above.

A second experiment investigated the effects of inhibiting phospholipase A2/prostaglandin E2 signaling using the cyclooxygenase inhibitor indomethacin (Sigma, St. Louis, MO) on [14C]carboplatin delivery to tumors. Indomethacin was initially administered as an i.v. bolus (1 or 2 mg/kg) and then as a 25-min i.v. infusion of (0.4 or 0.8 mg/kg/min) that ran concurrent with the [14C]carboplatin and Cereport (0.15 μg/kg/min) dosing.

A third experiment determined whether infusions of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (Research Biochemicals International) could mimic the ability of Cereport to enhance delivery of [14C]carboplatin to peripheral tumors. Animals received a 10-min i.v. infusion of SNAP, at a concentration of either 0.005, 0.01, or 0.05 mg/kg/min, immediately following a 15-min infusion of [14C]carboplatin.

The final experiment directly compared the effects of administering SNAP and prostaglandin E2 (PGE2, Research Biochemicals International), either alone or in combination, on delivery of [14C]carboplatin to tumors. Animals were divided into groups that received either the saline vehicle, SNAP alone (0.01 or 0.025 mg/min), PGE2 alone (1.0 mg/kg/min), or SNAP (0.01 or 0.025 mg/min) combined with PGE2 (1.0 mg/kg/min). SNAP and PGE2 were delivered i.v. for 10 min immediately following a 15-min i.v. infusion of [14C]carboplatin. The doses used in the combination studies were based on two considerations. First, the doses of the individual compounds represent the maximally tolerated doses. The dose of SNAP was chosen based on studies (under Results) that demonstrated that doses higher than 0.025 μg/kg/min produced significant hypotension (under Results). Additional pilot studies demonstrated that increasing the dose of PGE2 beyond 1.0 mg/kg/min produced significant hypotension. Second, pilot studies were conducted to ensure that combining PGE2 and SNAP did not produce unexpected or untoward vasoactive consequences. These studies confirmed that the doses used were the maximally tolerated doses when used both alone and in combination with each other.

Modulation of Cereport’s Effects with PDE-V Inhibition. Rats bearing MATB-III tumors were used to determine whether prolonging the action of the NO-mediated second messenger cGMP could modulate the ability of Cereport to enhance delivery of [14C]carboplatin to the tumor. Animals received i.v. infusions of either saline or Cereport (0.05, 0.2, or 0.5 μg/kg/min) alone or together with the PDE-V inhibitor zaprinast (20 mg/kg, Research Biochemicals International). Zaprinast is a selective, competitive inhibitor of phosphodiesterase V, which enhances the elevations of cGMP by inhibiting its hydrolysis by that enzyme (Thompson, 1991). This dose of zaprinast was determined to be the maximally tolerated dose based on pilot studies demonstrating that significant hypotension occurred with higher doses. All zaprinast infusions were given i.v., to ensure distribution to the tumor vasculature, and continuously with the 15-min [14C]carboplatin infusion and the 10-min Cereport infusion.

Statistics. The effects of Cereport on blood pressure and IPF were compared using a repeated measures ANOVA. The ranked data in the transvascular pore size studies were compared using a Mann-Whitney nonparametric analysis. The effects of Cereport on transvascular pore size, perfusion of blood vessels, and blood flow were also compared using a one-way ANOVA. Finally, all the effects of pharmacologically modifying delivery of [14C]carboplatin to tumors were evaluated in rats using a one-way ANOVA (JMP; SAS Institute, Inc., Cary, NC).

Results

Systemic Blood Pressure. Cereport produced a transient, dose-related decrease in blood pressure (Fig. 1). The hypotensive effects were maximal within 1 min and in all cases recovered to control levels within 3 min of beginning the Cereport infusion. The dose-response curve revealed that the hypotension observed with Cereport was not correlated with the shape of Cereport’s dose-response curve for enhancing drug delivery to peripheral tumors. For example, although 0.1 and 0.2 μg/kg/min Cereport produced significant and equivalent effects on delivery of [14C]carboplatin (Emerich et al., 2001), the effects on blood pressure differed, for 0.1 μg/kg/min was without reliable effects, whereas 0.2 μg/kg/min produced a reliable mean decrease of about 10% from baseline. Moreover, although the 0.2-μg/kg/min dose significantly enhanced [14C]carboplatin delivery, its effects on blood pressure were equivalent to 0.5 μg/kg/min, which produced much less effect on delivery into tumor (Emerich et al., 2001). Finally, the greatest effect on blood pressure was seen with a Cereport dose of 1.0 μg/kg/min, and this dose is beyond the active range of the inverted U dose response for enhanced drug delivery (determined in initial pilot dose range test; data not shown).

The lack of a simple relationship between hypotension and enhanced drug delivery with Cereport was further supported by studies using the adenosine agonist NECA to produce equivalent hypotension to Cereport. Intravenous administration of NECA (0.1 and 1.0 μg/kg/min) completely bracketed the range of hypotensive effects induced by therapeutic doses of Cereport (data not shown). However, although Cereport (0.2 μg/kg/min) again increased delivery of [14C]carboplatin into tumor, no increase in delivery was observed with either
dose of NECA (196% increase with Cereport, \( p < 0.05 \); 11–20% increase with NECA, \( p > 0.10 \)).

**Regional Blood Flow and Autoradiography.** Using iodinated antipyrine, in conjunction with quantitative autoradiography, a significant decrease in blood flow to the tumor was observed following Cereport infusions (Fig. 2). This effect began within the 1st min of the infusion and persisted throughout the duration of a 15-min Cereport infusion (decreased 86%, \( p < 0.001 \)). Thus, although profound changes in blood flow were observed, it seems unlikely that these paradoxical decreases can directly account the increased drug delivery observed.

**Effect of Cereport on Perfusion of Tumor Blood Vessels.** The ability of bradykinin B\( _2 \) receptor stimulation to alter areas of hypoperfusion within the tumor was evaluated by injecting different fluorescent dyes before and following Cereport administration. This enabled direct comparisons to be made between the patency of vessels within the vascular bed of the tumor under Cereport versus vehicle conditions. Although a trend for decreased areas of perfusion was observed under saline conditions (decreased 24% relative to pretreatment), this effect did not reach statistical significance (\( p > 0.1 \)). However, perfusion to subregions of the tumor was significantly decreased (66% relative to pretreatment, \( p < 0.001 \)) during the Cereport infusion (Fig. 3). Thus, these studies did not support the hypothesis that stimulation of B\( _2 \) receptors enhances drug delivery to tumors by improving blood flow to regions of the tumor that are normally hypoperfused.

**Effect of Cereport on Interstitial Fluid Pressure.** Cereport produced a progressive decline in IFP in the tumor that began within 2 to 3 min of its initiation and continued throughout the infusion (Fig. 4). The reduced IFP was most robust within the most effective Cereport dose range (e.g., 0.10 \( \mu \)g/kg/min, \( p < 0.01 \)), and was only marginal at the high end of the inverted U dose response, where Cereport’s uptake effects are greatly diminished (e.g., 1.0 \( \mu \)g/kg/min, \( p > 0.1 \)).

**Effect of Cereport on Transvascular Pore Size.** The ability of Cereport to increase the pore size of the vasculature supplying solid peripheral tumors was evaluated by comparing the extravasation of different-sized fluorescent microspheres from the tumor vasculature to the tumor interstitium. As shown in Table 1, semiquantitative analysis revealed that the extravasation of two consecutive-sized microspheres (0.05 and 0.1 \( \mu \)m) was significantly increased (\( p < 0.05 \)) by Cereport infusions. In contrast, no effect of Cereport
TABLE 1

Effect of Cereport on transvascular pore size

Data are presented as the mean overall ranking for saline- and Cereport-treated animals, independently for each microsphere size (0.025–0.2 μm). Fluorescence from individual tumors was rank ordered under blinded conditions from the least (ranking of 1) to the most intense (maximum ranking equal to that number of animals within each microsphere size group), independent of whether an animal received Cereport or vehicle. Data in the top of the table depict extravasation 2 h following saline or Cereport, while the bottom depicts extravasation 24 h following saline or Cereport. Note that in both cases, Cereport facilitated extravasation of two consecutive sized microspheres (0.05 and 0.1 μm) relative to saline-infused animals.

<table>
<thead>
<tr>
<th>Bead Size (μm)</th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h post-Cereport/saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline Mean ± S.E.M.</td>
<td>4.50 ± 1.24</td>
<td>2.93 ± 1.6</td>
<td>2.50 ± 1.17</td>
<td>4.50 ± 1.52</td>
</tr>
<tr>
<td>Median</td>
<td>4.25</td>
<td>2.75</td>
<td>2.5</td>
<td>4.17</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cereport (1.5 μg/kg) Mean ± S.E.M.</td>
<td>4.50 ± 1.22</td>
<td>6.06 ± 0.87*</td>
<td>6.50 ± 0.51*</td>
<td>4.50 ± 0.39</td>
</tr>
<tr>
<td>Median</td>
<td>5.125</td>
<td>6.375</td>
<td>6.585</td>
<td>4.17</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>24 h post-Cereport/saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline Mean ± S.E.M.</td>
<td>5.54 ± 1.14</td>
<td>4.89 ± 1.03</td>
<td>5.86 ± 0.77</td>
<td>4.63 ± 1.23</td>
</tr>
<tr>
<td>Median</td>
<td>5.58</td>
<td>5.75</td>
<td>5.5</td>
<td>4.59</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Cereport (1.5 μg/kg) Mean ± S.E.M.</td>
<td>3.46 ± 1.1</td>
<td>9.46 ± 1.42*</td>
<td>8.33 ± 0.78*</td>
<td>4.38 ± 1.22</td>
</tr>
<tr>
<td>Median</td>
<td>3.33</td>
<td>10.63</td>
<td>8.25</td>
<td>4.09</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. saline at the same microspheres size. Note also that this analysis does not permit comparisons of the relative effects between microsphere sizes.

was seen at either the smallest (0.025 μm) or largest (0.2 μm) diameter microspheres (p values 0.1). Collectively, these data indicate that one of the consequences of Cereport infusions is to increase the transvascular pore size of the blood vessels supplying solid peripheral tumors, thereby increasing the opportunity for diffusion of compounds into the tumor tissue.

Effect of Cereport on Tumor Blood Vessel Size. Using the endothelial cell-specific marker CD-31 to visualize individual blood vessels within the tumor, the circumference of vessels within the tumor was measured following saline and Cereport infusions (Figs. 5 and 6). As shown in Fig. 6A, Cereport infusions significantly increased the diameter and circumference of vessel size. This effect was manifest within 2 min (p < 0.001) (the earliest time point examined), peaked at about 5 min (p < 0.01), and persisted throughout the 8-min (p < 0.05) infusion. Thus, another likely mechanism by which Cereport enhances delivery to peripheral tumors is a transient increase in the surface area of the tumor vessels at the precise time the vessels contain high concentrations of drug within their luminal space.

Infusions of L-NAME prevented the increase in tumor vessel size produced by Cereport (Fig. 6B). Examination of blood vessel size 5 min into the i.v. infusions of Cereport/L-NAME (the time point at which Cereport alone maximally increased vessel size) revealed that the increases in vessel circumference were completely blocked by L-NAME (p < 0.0001 versus Cereport only). Together with the previous observation that L-NAME completely blocks the ability of Cereport to enhance delivery of [14C]carboplatin (see below) to the tumor, these data provide further support for a fundamental role in NO signaling as a mediator of Cereport’s effects in peripheral tumors.

Pharmacological Interactions between Bradykinin, Nitric Oxide, and Prostaglandins. As shown in Fig. 7, top, i.v. administration of the bradykinin B2 agonist Cereport significantly enhanced the delivery of [14C]carboplatin to solid peripheral tumors. Using a dose previously shown to optimally enhance delivery (Emerich et al., 2001), levels of [14C]carboplatin were increased 140% (p < 0.01). To gain insight into the biochemical pathways involved with the increased drug delivery to peripheral tumors produced by Cereport infusions, a series of pharmacological studies was performed. An initial study revealed that the enhanced delivery produced by Cereport was completely suppressed by infusing the NO synthetase inhibitor L-NAME (Fig. 7, top). A second study replicated the enhanced delivery of [14C]carboplatin produced by Cereport, demonstrating a 164% increase relative to vehicle (p < 0.05). Administration of the cyclooxygenase inhibitor indomethacin produced a significant, but incomplete dose-related suppression of Cereport’s effects (Fig. 7, bottom). Cereport plus indomethacin, at a dose of 0.4 μg/kg/min, increased [14C]carboplatin levels in tumor by

Fig. 5. Representative sections of tumor immunostained for the endothelial cell marker CD-31 to visualize blood vessels. A, low-power photomicrograph of a dense torturous network of vessels. Solid arrows in A and B depict cross-sections of blood vessels to illustrate examples used for quantitative determinations of vessel diameter. B and C, higher power photomicrographs of selected immunopositive vessels depicting cross-sectional areas. Staining was eliminated in sections processed in an identical manner except that the primary antibody was deleted. Bar, 50 μm for A and 20 μm for B and C.
100% (64% less than the maximal effect obtained with Cereport alone). Increasing the dose of indomethacin (0.8 mg/kg/min) further dampened the effects of Cereport, with the delivery of [14C]carboplatin to the tumor being increased by only 59% (p < 0.05) relative to vehicle-infused animals. Administration of indomethacin alone, at either dose, did not affect [14C]carboplatin levels in tumor (data not shown).

The next experiments infused the NO donor SNAP (to increase NO activity) to determine whether the effects of Cereport could be mimicked by pharmacological activation of endogenous NO. Intravenous infusions of SNAP alone produced subtle (p > 0.05) trends toward increased drug delivery across a wide range of SNAP doses, including the maximum tolerated dose (0.05 µg/kg/min), which increased [14C]carboplatin levels in tumor by 52% relative to saline (Fig. 8). Parallel studies demonstrated that i.v. infusions of PGE2 alone failed to alter the delivery of [14C]carboplatin to the tumor (Fig. 9). However, when SNAP and PGE2 were coadministered, the two significantly increased levels of [14C]carboplatin in the tumor. Although combining a low
dose of SNAP (0.01 μg/kg/min) with PGE₂ (1.0 mg/kg/min) did not alter [¹⁴C]carboplatin levels, a higher dose of SNAP (0.025 μg/kg/min) combined with PGE₂ produced a 75% increase in delivery to the tumor (p < 0.05, Fig. 9).

**Modulation of Cereport’s Effects with PDE-V Inhibition.** Consistent with the above-described studies, i.e. Cereport significantly (p < 0.01) enhanced delivery of [¹⁴C]carboplatin to MATB-III tumors. As before, this effect was maximal with 0.2 μg/kg/min Cereport with lesser but still significant (p < 0.05) effects at 0.05 and 0.5 μg/kg/min Cereport. Zaprinast (selective PDE-V inhibitor) given alone (data not shown) did not alter delivery of carboplatin to the tumor. Moreover, administration of zaprinast (20 mg/kg) did not alter the ability of Cereport to enhance delivery of [¹⁴C]carboplatin at any dose tested (Fig. 10).

**Discussion**

The experiments described in this manuscript explored the physical and biochemical mechanisms that may be responsible for the novel finding that increased delivery of chemotherapeutic agents to solid peripheral tumors can be achieved by stimulating B₂, bradykinin receptors with the selective agonist Cereport. These experiments used a MATB-III cell line derived from F-344 rats that forms solid peripheral tumors with an infiltrating vasculature that bears considerable homology to the vasculature in human solid tumors (see Emerich et al. (2001) for a detailed discussion).

Consistent with the general vasoactive properties established for both bradykinin (Wahl et al., 1983; Unterberg et al., 1984; Yong et al., 1992) and Cereport (Elliott et al., 1996a,b), a number of vascular responses occurred during Cereport infusion. Three of these seem sufficiently linked to the increased drug delivery to the tumor to suggest important roles in the physical mechanism of action. First, a steady decrease in IFP occurred during the infusion of an effective concentration of Cereport (but not during infusion of a higher, ineffective concentration). Second, the diameter and surface area of the tumor vessels was markedly increased during the Cereport infusion. Importantly, just as inhibiting NO activity (with L-NAME) completely blocked Cereport’s ability to enhance delivery to tumors, it also completely blocked Cereport’s effect on tumor vessel size. Both the decrease in IFP and the increase in vessel surface area were empirically linked to the increased delivery afforded by Cereport, providing at least partial explanations for how Cereport achieves the enhanced delivery. Although the vasculature feeding many solid tumors is known to be leaky (Jain, 1987), increased fluid pressure within the tumor interstitium contributes to the inability of drugs to transit from the lumen of the tumor vessel to the interstitial fluid of the tumor (most likely by reducing convection currents in that direction) (Jain, 1990, 1991). By reducing IFP, Cereport shifts the pressure gradient between the tumor vasculature and tumor interstitium to favor enhanced flow from the lumen into the tumor. By simultaneously increasing vessel surface area, Cereport provides an even greater opportunity for drugs within the vascular lumen to flow to the tumor interstitium. Finally, evidence for increased pore size was also observed, which would further facilitate flow of agents from the vascular lumen to the tumor interstitium (Jain, 1989; Monsky et al., 1999).

Several other physiological effects of Cereport were ob-
served but were not linked to the increased drug delivery to the tumors. For example, the well known hypotensive effects of Cereport and bradykinin were observed (Wahl et al., 1983; Unterberg et al., 1984; Yong et al., 1992; Elliott et al., 1996a,b), but could not have produced the enhanced delivery described here. First, the timing of the hypotensive effects were not linked to the enhanced delivery, because studies manipulating the timing of Cereport and carboplatin demonstrate that peak hypotension occurred at the worst time point for delivery (Emerich et al., 2001). Second, the dose-response curve for hypotension did not correlate with the respective shape of the dose-response curve for enhancing delivery. Finally, dropping blood pressure in the same temporal pattern shape of the dose-response curve for enhancing delivery. Together, these mutually corroborating data suggest that hypotension is neither necessary nor sufficient for Cereport’s ability to enhance drug delivery to tumors. This pathway may participate in the phenomenon. Nonetheless, these data do suggest important roles for the PL/PG pathway as well. The fundamentally important role of these biochemical pathways was further highlighted by additional pharmacological studies. Stimulating NO activity with SNAP produced modest, but nonsignificant trends toward enhanced delivery, whereas administration of exogenous PGE produced no measurable effects on drug delivery. However, simultaneous stimulation of both pathways produced a significant, nearly 2-fold increase of carboplatin levels in the tumor, clearly supporting an important role of both of these bradykinin-linked pathways, working in concert.

The fact that simultaneous, pharmacological stimulation of these two pathways did not achieve the same 3-fold effect achieved by Cereport stimulation of the B2 receptor might be explained in a least three different (but not mutually exclusive) ways: 1) the dose combination and temporal parameters of SNAP and PGE required to maximize the response may not have been optimized, 2) flooding the vascular lumen with NO donors and PGE may never be able to produce as great a response as that which occurs naturally following receptor stimulation and subsequent natural activation of these two signaling pathways, and 3) another, still-undefined signaling pathway may participate in the phenomenon. Nonetheless, the data reported here clearly establish that both pathways are necessary and that neither is sufficient by itself to achieve the effects reported here.

As briefly discussed (Emerich et al., 2001) the increased delivery to peripheral tumors achieved with Cereport and that previously reported for improved drug delivery to brain tumors differ in several phenomenological ways, including the dose level, the shape of the dose-response function, and to administer the chemotherapeutic agent before initiating the Cereport infusion. That is, the concentration of the chemotherapeutic agent within the tumor vessel must be elevated prior to the onset of the physiological changes induced by Cereport (Emerich et al., 2001). Otherwise, the decreased perfusion of the tumor caused by Cereport would limit, rather than enhance, delivery. For this reason, as long as chemotherapeutic drug concentrations within the lumen of the tumor vessels are sufficiently high before the Cereport infusion, the decrease in blood flow to the tumor does not obviate the benefits induced by the reduction in IFP, increase in vessel surface area and increase in pore size.

The mechanistic studies demonstrated that Cereport achieves increased drug delivery by activating NO and phospholipase A2/prostaglandin E2 (PL/PG) pathways as essential, synergistically acting intracellular signaling events. Although each of these pathways is known to be stimulated by bradykinin (Burch et al., 1993), the fact that both had to be activated to achieve the enhanced delivery to tumors reported here was not expected. It is well established that both NO and PL/PG are common signaling pathways used by bradykinin receptor systems (Burch et al., 1993). Our pharmacological manipulations demonstrated that inhibiting NO synthetase completely blocked Cereport’s ability to enhance drug delivery to the tumor, indicating that this pathway is necessary for the phenomenon reported here. Similarly, blocking PL/PG activity substantially reduced Cereport’s effects (from >150% increase to approximately 50%), although the emergence of confounding hypotensive side effects limited our ability to fully test the involvement of this pathway in the tumor delivery phenomenon. Nonetheless, these data do suggest important roles for the PL/PG pathway as well.

**Fig. 10.** Effect of i.v. zaprinast, a selective PDE-V competitive inhibitor, on Cereport’s ability to enhance delivery of [14C]carboplatin to peripheral MATB-III tumors. Animals received infusions of saline or zaprinast concurrent with a range of Cereport doses. Note the nonmonotonic dose-response curve with maximal effects at 0.2 µg/kg/min Cereport and lesser effects with 0.05 and 0.5 µg/kg/min. Zaprinast alone (data not shown) did not impact delivery of [14C]carboplatin to the tumors. Zaprinast (20 mg/kg) combined with Cereport did not affect delivery of [14C]carboplatin, relative to Cereport alone, at any dose treated. Group sizes were vehicle (N = 21), 0.05 Cereport (N = 7), 0.05 Cereport plus zaprinast (N = 8), 0.2 Cereport (N = 10), 0.2 Cereport plus zaprinast (N = 8), 0.5 Cereport (N = 9), and 0.5 Cereport plus zaprinast (N = 8). Data are presented as mean ± S.E.M. nanocuries per gram of tissue. *p < 0.05 versus saline, **p < 0.01 versus saline.
the optimal dosing protocol required to achieve the desired effect. A number of apparent mechanistic differences are also apparent, for, in contrast to the findings presented here, no evidence of reduced cerebral blood flow or changes in intracranial tumor pressure has been seen in studies with brain tumors following Cereport infusion. Moreover, although a disengagement of the tight junctions comprising the blood-brain tumor barrier has been implicated as playing a major role in Cereport's effects on brain tumors (Sanovich et al., 1995), an analogous effect on vascular pore size (in the present studies) plays a less exclusive role. Finally, although both the increased delivery to brain tumors and solid, peripheral tumors involves activation of bradykinin B₂ receptors and NO as an intracellular second messenger, even here an interesting difference was revealed. In prior studies with brain tumors, enhancing the duration of the NO-linked second messenger cGMP (via the PDE-V inhibitor zaprinast) potentiated the effects of both bradykinin (Sugita and Black, 1998) and Cereport (Dean et al., 1999). However, in the present studies, zaprinast neither enhanced the effects of an optimal dose nor a range of suboptimal doses of Cereport. This suggests that, in contrast to brain tumors, the NO-induction of cGMP in solid peripheral tumors that occurs following B₂ stimulation may achieve near-optimal levels and therefore does not benefit from a pharmacologically aided extension of second messenger duration. Thus, although a number of similarities are shared between Cereport's effects on enhancing delivery to brain tumors and peripheral, solid tumors, several interesting differences exist as well.

In summary, the data presented offer new and detailed information regarding the novel finding that stimulation of bradykinin B₂ receptors can increase delivery of chemotherapeutics to solid, peripheral tumors. This phenomenon appears to require the participation of two parallel bradykinin signaling pathways working in tandem. Additionally, among the many hemodynamic changes observed, a decrease in interstitial fluid pressure, an increase in vascular surface area, and an increase in vascular pore size all appear to contribute to the end effect. On the other hand, significant decreases in systemic blood pressure, tumor blood flow, and increases in areas of hyoperfusion within the tumor are unlikely participants in the phenomenon. Further studies might next determine why the bradykinin-mediated responses are more robust and consistent in tumor vasculature (e.g., higher density of receptors, more efficient signaling pathways, etc.) relative to healthy, nontumor vasculature.

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