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RAPID REGULATION OF P-GLYCOPROTEIN AT THE
BLOOD-BRAIN BARRIER BY ENDOTHELIN-1

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Abbreviations:

[N-ε(4-nitrobenzofurazan-7-yl)-D-Lys⁸]-cyclosporine A, NBDL-CSA; p-glycoprotein, p-gp;
endothelin-1, ET-1; Dulbecco's phosphate buffered saline, DPBS; sodium nitroprusside, SNP;
NO synthase, NOS

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ABSTRACT

The ATP-driven xenobiotic transporter, p-glycoprotein, is a critical element of the blood-brain barrier. To study regulation of p-glycoprotein function, we measured specific transport (PSC833-sensitive) of a fluorescent cyclosporine A derivative (NBDL-CSA) into the lumens of isolated rat brain capillaries using confocal microscopy and quantitative image analysis. Luminal NBDL-CSA accumulation was rapidly and reversibly reduced in a concentration-dependent manner by 0.1-100 nM endothelin-1 (ET-1). In this concentration range, ET-1 did not affect junctional permeability. The ET_B receptor agonist, sarafotoxin 6c, also reduced transport. Effects of ET-1 and sarafotoxin 6c were blocked by an ET_B receptor antagonist; an ET_A receptor antagonist was without effect. Consistent with this, immunostaining and Western blotting showed expression of the ET_B receptor in brain capillary membranes. NBDL-CSA transport was also reduced by sodium nitroprusside (SNP), a NO donor, and by phorbol ester, a protein kinase C (PKC) activator. Inhibition of NO synthase (NOS) or PKC abolished the ET-1 effects. Thus, ET-1, acting through an ET_B receptor, NOS and PKC, rapidly and reversibly reduced transport mediated by p-glycoprotein at the blood-brain barrier.

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INTRODUCTION

The structural basis of the blood-brain barrier is the non-fenestrated, brain capillary endothelium. Functionally, this barrier tissue is defined by low passive permeability and the expression of selective, plasma membrane-bound transporters. These combine to both nourish the central nervous system (CNS) and protect it from potentially toxic chemicals. One critical selective component of the blood-brain barrier is p-glycoprotein, an ATP-driven, drug efflux pump (Schinkel et al., 1994). This multispecific, primary active transporter, located at the luminal membrane of the brain capillary endothelium, is considered to be an important “gatekeeper” of the blood-brain barrier (Schinkel, 1999). As such, it both protects the CNS from neurotoxicants, but also limits brain penetration of a large number of drugs used to treat CNS disorders (Schinkel et al., 1996). In this regard, reduced p-glycoprotein function, whether the result of pump inhibition, genetic manipulation or pathological change, substantially increases brain levels of administered drugs, including, chemotherapeutics, HIV protease inhibitors, anticonvulsants, antipsychotics and glucocorticoids (Fellner et al., 2002; Goralski et al., 2003; Schinkel et al., 1996)

Clearly, for CNS therapy it would be advantageous to be able to modulate p-glycoprotein function over the short-term, while still retaining its protection over the longer-term. One strategy that might accomplish this would be to transiently decrease specific efflux to the blood through rapid regulation. Unfortunately, we currently know little about the regulation of p-glycoprotein at the blood-brain barrier and what we do know concerns mechanisms that work over hours to days rather than minutes (Bauer et al., 2004; Demeuse et al., 2004; Nwaozuzu et al., 2003). In other barrier and excretory tissues, regulatory mechanisms have been described that signal rapid changes in p-glycoprotein function. For example, in hepatocytes, endocytic insertion

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and retrieval of p-glycoprotein from the canalicular membrane occurs in response to cyclic AMP and taurocholate (which cause insertion) and to cholestasis (which causes retrieval; (Kipp and Arias, 2002). In addition, in renal proximal tubule, endothelin-1 (ET-1), signaling through an ET_B receptor, nitric oxide synthase (NOS) and protein kinase C (PKC), rapidly reduces transport mediated by p-glycoprotein and the multidrug resistance-associated transporter isoform 2 (Mrp2; (Masereeuw et al., 2000; Terlouw et al., 2001)). The molecular mechanism by which ET signaling reduces transport is unclear (Miller, 2002a).

Here we provide the first evidence for hormonal signaling causing rapid and reversible changes in blood-brain barrier p-glycoprotein-mediated transport. We found that exposing isolated rat brain capillaries to subnanomolar to nanomolar concentrations of ET-1 rapidly decreased p-glycoprotein-mediated, luminal accumulation of a fluorescent cyclosporin A (NBDL-CSA) derivative. Transport was rapidly restored when ET-1 was removed. As in renal proximal tubule (above), in brain capillaries, ET-1 signaled through an ET_B receptor, NOS and PKC.

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MATERIALS and METHODS

Chemicals

Endothelin-1, RES-701-1, JKC-301, N^G-monomethyl-L-arginine (L-NMMA), phorbol-12-myristate-13-acetate (PMA) and bis-indolylmaleimide I (BIM) were purchased from Calbiochem-Novabiochem (La Jolla, CA). MK 571 was purchased from Cayman Chemical (Ann Arbor, MI). NBDL-CSA was custom-synthesized by R. Wenger (Basel, CH) (Schramm et al., 1995), PSC833 was a kind gift from Novartis (Basel, CH) and 1-*O*-pentylglycerol was a kind gift from Dr. Bernhard Erdlenbruch (Children's Clinic, University of Göttingen, Germany). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Isolation of rat brain capillaries

Sprague-Dawley rats (male retired breeders, Taconic, Germantown, NY) weighing approximately 500-750 g were used. Animals were acclimated to the housing facility (1 rat/cage, 12-h light/dark cycle) for at least 5 days and fed NIH-31 rodent diet (Zeigler Brothers, Gardners, PA) and water ad libidum. Animal housing was in accordance with institutional guidelines and the NIH Guide for the Use and Care of Laboratory Animals. Isolation of rat brain capillaries was performed as described previously (Miller et al., 2000). Briefly, rats were euthanized by CO₂ inhalation and decapitated, brains were taken immediately and kept at 4°C in DPBS buffer (2.7 mM KCl, 1.46 mM KH₂PO₄, 136.9 mM NaCl, 8.1 mM Na₂HPO₄) supplemented with 5 mM D-glucose and 1 mM Na-pyruvate, pH 7.4. Rat brains were dissected and homogenized in the same volume of buffer. After addition of Ficoll[®] (final concentration 15%, Sigma, St. Louis, MO), the homogenate was centrifuged at 5800 g for 20 min at 4°C, the pellet was resuspended in DPBS containing 1% BSA and then passed over a glass bead column. Capillaries adhering to the glass

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beads were collected by gentle agitation in DPBS with 1% BSA. Capillaries were washed three times in BSA-free DPBS buffer and then used immediately for experiments.

Transport and efflux experiments

Freshly isolated capillaries were transferred to glass cover slips, preincubated for 30 min with the effectors and then incubated for 1 hour at room temperature with a fluorescent-labeled cyclosporine A derivative (NBDL-CSA). In some experiments, capillaries were first loaded with NBDL-CSA to steady state, and then, modulators were added. For each treatment, confocal fluorescence images of 7-15 capillaries were acquired and luminal fluorescence intensity was measured using Scion Image software as described previously (Miller et al., 2000). For efflux experiments, brain capillaries were transferred to glass cover slips and loaded with 2 μ M Texas Red (sulforhodamine 101 free acid) for 1 h at room temperature. After washing with DPBS, efflux of Texas Red from the lumens of control and treated brain capillaries was followed using confocal microscopy. Confocal images of capillaries were analyzed as described above. First order efflux rate constants were calculated using nonlinear regression (GraphPad Prism[®] 4 Software, San Diego, CA).

Western blot analysis

Brain capillaries were homogenized and lysed in mammalian tissue lysis buffer (Sigma, St. Louis, MO) containing protease inhibitor cocktail (Roche, Mannheim, FRG). After 1 hour on ice and occasional vortexing, samples were centrifuged at 10,000g for 30 min. Denucleated supernatants were centrifuged at 100,000g for 90 min. Pellets were resuspended in DPBS containing protease inhibitor cocktail and protein concentrations were determined. Western blots

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were performed using the Invitrogen NuPage™ Bis-Tris electrophoresis system and conducted according to the manufacturer's protocol. Briefly, protein samples were electrophoresed on 4-12% Bis-Tris NuPage™ gradient gels, subsequently blotted on Invitrolon PVDF membranes (Invitrogen, Carlsbad, CA) and blocked with SuperBlock® buffer (Pierce, Rockford, IL) for 8 hours. Then, membranes were incubated overnight with a 1:200 dilution (1.5 µg/ml) of polyclonal rabbit anti-ET_B primary antibody (Alomone Labs, Jerusalem, Israel). Membranes were washed and incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated ImmunoPure® secondary IgG (1:10,000; Pierce, Rockford, IL). Membranes were again washed and ET_B was detected using SuperSignal® West Pico Chemoluminescent Substrate (Pierce, Rockford, IL). Protein bands were visualized with a BioRad Gel Doc 2000™ gel documentation system (BioRad, Hercules, CA).

Immunohistochemistry

Freshly isolated rat brain capillaries adhering to glass cover slips were fixed for 15 minutes with 3% paraformaldehyde/0.2% glutaraldehyde at room temperature. After washing with DPBS, capillaries were permeabilized for 30 minutes with 0.1% (v/v) Triton X-100 in DPBS, and subsequently washed with DPBS, now containing 1% BSA for blocking. Then, capillaries were incubated for 1 hour at 37°C with a 1:100 dilution (3 µg/ml) of polyclonal rabbit anti-ET_B antibody (Alomone Labs, Jerusalem, Israel). After washing (DPBS, 1% BSA), capillaries were incubated with anti-rabbit Alexa Fluor® 488-conjugated secondary IgG (1:1,000; Molecular Probes, Eugene, OR) for 1 hour at 37°C. Negative controls were incubated with secondary antibody only, nuclei were counterstained with 5 µg/ml propidium iodide for 15 min. Immunostained ET_B was visualized using confocal microscopy.

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RESULTS

P-glycoprotein-mediated transport in isolated brain capillaries

We have developed a simple, but powerful method to assess p-glycoprotein function in intact brain capillaries. It is based on measuring the accumulation of a fluorescent cyclosporin A derivative (NBDL-CSA) in capillary lumens using confocal microscopy and image analysis (Miller et al., 2000). In previous studies, with isolated brain capillaries from rats, pigs and fish, we could demonstrate specific and metabolism-dependent bath to lumen transport of a number of p-glycoprotein substrates including, NBDL-CSA (Fellner et al., 2002; Miller, 2003; Miller et al., 2002; Schramm et al., 1995). We are now able to maintain isolated rat brain capillaries in a simple physiological saline for up to 8 h without loss of metabolism-driven, concentrative transport (Bauer et al., 2004).

Figure 1A shows a representative rat brain capillary after 60 min in 2 μ M NBDL-CSA. Fluorescence intensity is low in the bath, moderate in the endothelium and highest in the capillary lumen. In this capillary, luminal fluorescence exceeds bath fluorescence by a factor of at least ten. PSC833, a specific inhibitor of p-glycoprotein, blocked luminal NBDL-CSA accumulation (Fig. 1B). In agreement with previous studies (Bauer et al., 2004; Fellner et al., 2002), luminal accumulation was also inhibited to the same extent by the metabolic inhibitor NaCN. NBDL-CSA accumulation was not reduced by the multidrug resistance-associated protein (Mrp) inhibitor leukotriene C₄ (not shown). Note that recent studies indicate that brain capillary endothelial cells express another multispecific, drug efflux pump at the luminal plasma membrane, breast cancer resistance protein (BCRP, Eisenblatter and Galla, 2002; Cooray et al., 2002). Although BCRP and p-glycoprotein have substrates in common, PSC833 is at best a low affinity inhibitor of BCRP (Chen et al., 2000) and it does not affect the blood to brain transport of

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BCRP substrates (Cisternino et al, 2004). Although we cannot exclude participation of as yet unidentified transporters, concentrative, specific and energy-dependent transport of NBDL-CSA in these brain capillaries appears to be mediated by p-glycoprotein- .

Figure 1C shows the time course of 2 μ M NBDL-CSA accumulation in the lumens of isolated rat brain capillaries. Control capillaries exhibited an initial increase of luminal NBDL-CSA and reached steady state levels after about 30 min. Exposing capillaries to 5 μ M PSC833 decreased luminal NBDL-CSA accumulation significantly. Other experiments (not shown) demonstrated that this concentration of PSC833 elicited maximal inhibition of transport. Note that roughly 40% of steady state luminal NBDL-CSA is not affected by PSC833 or NaCN. This component of accumulation likely represents simple diffusion and binding of the CSA derivative to luminal contents and cell surfaces.

To better define the time course of PSC833 action, capillaries were loaded for 60 min (steady state) with NBDL-CSA and PSC833 was then added. Upon addition of the transport inhibitor, luminal fluorescence declined rapidly to less than 50% of control levels (Fig. 1D). Removing PSC833 from the medium completely restored luminal fluorescence to control values. Note that luminal fluorescence in control capillaries remained constant over the entire time course of the experiment (nearly 3 h), further evidence for extended capillary viability and retained transport function.

Endothelin Effects on Transport

Exposing capillaries to subnanomolar to nanomolar concentrations of ET-1 reduced steady state luminal NBDL-CSA in a concentration-dependent manner (Fig. 2A). Maximal ET-1 effects were comparable to those seen with 5 μ M PSC833. As with PSC833, adding ET-1 to

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capillaries incubated to steady state with NBDL-CSA rapidly decreased luminal fluorescence. This effect was entirely reversed when hormone was removed from the medium (Fig. 2B).

The decrease in luminal NBDL-CSA accumulation caused by ET-1 could have resulted from reduced p-glycoprotein function or from opening of tight junctions and leakage of pumped dye out of the lumen. Indeed, mannitol, used to osmotically open tight junctions at the blood-brain barrier (Kroll and Neuwelt, 1998), rapidly reduced steady state, luminal NBDL-CSA fluorescence to about 50% of control levels, an effect comparable to that seen with PSC833 and ET-1 (Fig. 3A). After removal of mannitol, luminal fluorescence returned to control levels. Thus, mannitol showed the same pattern of effects as ET-1.

To experimentally distinguish between opening of tight junctions and reduced pumping, we developed a kinetic assay based on efflux of a preloaded fluorescent test compound from the lumens of capillaries. The compound used was Texas Red (sulforhodamine 101 free acid), a rhodamine-based, fluorescent organic anion. This organic anion is a substrate for the multidrug resistance-associated protein isoform 2 (Mrp2), another transporter located in the luminal membrane of the rat brain capillary endothelium (Miller et al., 2000). Consistent with this, steady state luminal accumulation of Texas Red in rat brain capillaries was not affected by the p-glycoprotein inhibitor, PSC833, but was reduced by the Mrp2 inhibitor LTC₄ (Fig. 3B). Importantly, Texas Red accumulation was not affected by 100 nM ET-1, a concentration that is 100 times higher than that causing maximal reduction in NBDL-CSA accumulation in capillary lumens, nor by SNP concentrations up to 1 μ M (Fig. 3B). These results indicate that mediated, concentrative, transport of an organic anion into brain capillary lumens was neither reduced by ET-1 nor by NO. They imply that ET-1 does not increase junctional permeability.

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To test the latter point directly, capillaries were loaded to steady state with Texas Red and then washed with dye-free medium. Luminal fluorescence was followed for 60 min after washing using the confocal microscope. Figure 3C shows the results of a representative experiment. In control capillaries, loss of dye from the lumen was slow, but significant. Adding the osmotic agents, mannitol or sucrose (100 mM), to the medium immediately increased Texas Red efflux from the capillary lumens, as did 1-*O*-pentylglycerol, a chemical agent that opens the blood-brain barrier by a non-osmotic mechanism (Erdlenbruch et al., 2003). In contrast to mannitol, sucrose and 1-*O*-pentylglycerol, 100 nM ET-1 did not alter Texas Red efflux (Fig. 3D).

We modeled Texas Red efflux as a first order exponential and calculated rate constants using non-linear regression. For 42 control capillaries, the rate constant averaged $0.053 \pm 0.004 \text{ min}^{-1}$. The mean rate constant for capillaries exposed to 100 mM mannitol was $0.192 \pm 0.009 \text{ min}^{-1}$ ($n = 42$). Thus, mannitol increased the efflux rate for Texas Red about four-fold ($P < 0.01$). In parallel experiments, the mean efflux rate constant for capillaries exposed to 100 nM ET-1 was $0.053 \pm 0.003 \text{ min}^{-1}$ ($n = 21$), a value that is not significantly different from controls. We take this result to mean that ET-1 reduced luminal NBDL-CSA accumulation by decreasing p-glycoprotein-mediated transport rather than by opening tight junctions.

ET-1 Signaling

Endothelins signal through two receptors, ET_A and ET_B. Both are known to be expressed in the brain and in the brain vasculature (Kedzierski and Yanagisawa, 2001; Levin, 1995). We used ET receptor-specific antagonists and an ET_B receptor agonist to determine which receptor signaled the decrease in p-glycoprotein-mediated transport in rat brain capillaries. As shown in

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Fig. 4A, RES-701-1, an ET_B antagonist, blocked the action of ET-1. JKC-301, an ET_A antagonist was without effect (Fig. 4B). Consistent with these results, the ET_B-receptor-specific agonist, sarafotoxin 6c, significantly reduced in a concentration-dependent manner luminal NBDL-CSA accumulation (Fig. 4C). The decrease in luminal NBDL-CSA fluorescence caused by sarafotoxin 6c was blocked by RES-701-1 (Fig. 4D), but not by JKC-301 (Fig. 4E). Thus, ET-1 acted through an ET_B-receptor. Consistent with these results, immunostaining localized the ET_B-receptor to the luminal (apical) as well as abluminal (basolateral) membranes of brain capillaries (Fig. 5A). Western blot analysis confirmed the immunostaining findings, showing a strong band for the ET_B-receptor in capillary lysates and in capillary membranes (Fig. 5B).

Further experiments implicated activation of NOS and PKC as downstream events in ET-1 signaling. Exposing capillaries to the NO donor, sodium nitroprusside (SNP), caused a concentration-dependent decrease of luminal NBDL-CSA fluorescence (Fig. 6A). With 1-100 nM SNP, fluorescence was reduced to about 60-70% of control levels ($P < 0.001$). As one might expect N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS, did not reverse the SNP effect (Fig. 6B). However, blocking NOS with L-NMMA did abolish the effect of ET-1 (Fig. 6C), indicating that activation of NOS and NO generation follows ET-1 binding to its receptor.

Phorbol-12-myristate-13-acetate (PMA), which activates PKC, also reduced luminal NBDL-CSA accumulation in a concentration-dependent manner (Fig. 7A). This effect was abolished by the PKC selective inhibitor, bis-indolylmaleimide (BIM, Fig. 7B). Although PMA can affect targets other than PKC, the low concentration causing significant effects (1 nM) and the protective effect of BIM suggest that PMA reduces NBDL-CSA transport in brain capillaries by acting through PKC. Importantly, BIM also blocked the decrease in luminal NBDL-CSA fluorescence caused by SNP (Fig. 7C) and by ET-1 (Fig. 7D), indicating that PKC signaling

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followed ET-1 binding and NOS activation. Thus, ET-1, acting through an ET_B receptor, activated in turn NOS and PKC, and this chain of events rapidly decreased p-glycoprotein-mediated transport.

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DISCUSSION

Because of its wide specificity limits, ability to drive concentrative transport and placement on the luminal side of the brain capillary endothelium, p-glycoprotein is a critical selective component of the blood-brain barrier. By limiting penetration of foreign chemicals into the brain, p-glycoprotein protects the CNS. However, because it poorly distinguishes between neurotoxicants and therapeutic drugs, p-glycoprotein is a primary obstacle to therapy of CNS disorders and thus a potential target for maneuvers designed to increase drug penetration into the brain. In this regard, little is known about physiological mechanisms that modulate p-glycoprotein function at the blood-brain barrier.

The present experiments are focused on mechanisms that regulate p-glycoprotein transport function over the short-term (minutes). Using intact, isolated rat brain capillaries, we found that subnanomolar to nanomolar concentrations of the polypeptide hormone, ET-1, rapidly and reversibly decreased p-glycoprotein-mediated transport function. With 1-100 nM ET-1, steady state NBDL-CSA accumulation in capillary lumens was reduced to the same level as found with 5 μ M PSC833, which maximally inhibits p-glycoprotein-mediated transport in the capillaries. Decreased luminal accumulation of NBDL-CSA caused by ET-1 was not accompanied by increased capillary permeability, e.g., due to opening of tight junctions, as measured using a dye efflux assay.

ET-1 release has been shown to occur in many CNS disorders, including, cerebral vasospasm/subarachnoid hemorrhage, stroke, HIV encephalitis/AIDS dementia complex, multiple sclerosis and other inflammatory diseases of the brain (Brooks et al., 1998; Didier et al., 2002; Kedzierski and Yanagisawa, 2001; Levin, 1995; Narushima et al., 1999; Nie and Olsson, 1996). The literature contains conflicting reports as to the effects of ET-1 on brain capillary

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permeability. Thus, injection of ET into the brain has been reported to increase permeability (Narushima et al., 2003) or to have no significant effect (Hughes et al., 2003). In these in vivo studies, permeability measurements were made after many hours to days of ET-1 treatment. In the present experiments, we only examined ET-1 effects over 1-2 h. Thus, it is possible that ET-1 does increase brain capillary permeability over longer time periods than those used here and that decreased p-glycoprotein transport function with no change in capillary permeability is an early effect of ET-1 exposure. Additional experiments will be needed to address this issue.

Note that transport of Texas Red by rat brain capillaries was not affected by 100 nM ET-1, a concentration that was two orders of magnitude higher than that which caused maximal reduction in NBDL-CSA transport. We previously showed that Texas Red transport into the lumens of these capillaries is mediated by a member of the Mrp family of transporters, most likely Mrp2 (Miller et al., 2000). The present findings indicate that, unlike renal proximal tubule (Masereeuw et al., 2000), ET-1 signaling does not affect Mrp2 function in brain capillaries.

The present experiments provide an outline of the mechanism by which ET-1 signals a change in p-glycoprotein function. Transport was reduced by ET-1 activation of ET_B receptors and subsequent activation of two intracellular enzymes, NOS and PKC. ET-1 actions were mimicked by the ET_B receptor agonist, sarafotoxin 6c; both ET-1 and sarafotoxin 6c effects were blocked by an ET_B receptor antagonist but not by an ET_A receptor antagonist. ET_B receptor was immunolocalized to both the luminal and abluminal surfaces of the capillary endothelium. Thus, in vivo hormone released into the blood or produced in the brain could affect transport. With the present in vitro model, ET-1 exposure was from the brain side of the capillaries.

SNP, which generates NO, also reduced p-glycoprotein-mediated transport, presumably mimicking the effects of NOS activation. Inhibition of PKC blocked the effects of ET-1 and of

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SNP, while inhibition of NOS blocked the effects of ET-1 but not of PKC activation. The minimal signaling pathway that fits the data is linear, going from the ET_B receptor to NOS to PKC. This is the same signaling pathway by which ET-1 reduces transport on p-glycoprotein and Mrp2 in renal proximal tubule (Masereeuw et al., 2000; Miller, 2002a; Miller et al., 1998; Notenboom et al., 2002; Terlouw et al., 2001). In proximal tubule, recent studies have implicated guanylyl cyclase, cyclicGMP and PKG in signaling between NO and PKC (Notenboom et al., 2004). There are likely to be additional intermediate signals in brain capillaries that have not yet been identified.

At present it is not clear how PKC modifies p-glycoprotein function. Given the rapid time course of action seen with isolated brain capillaries, three types of mechanism seem possible: 1) PKC directly phosphorylates the transporter, changing turnover number or substrate affinity; 2) PKC phosphorylates an accessory protein that modifies p-glycoprotein activity; 3) PKC influences p-glycoprotein trafficking, e.g., stimulating retrieval from the plasma membrane into a vesicular compartment, as has been demonstrated for p-glycoprotein and other ABC transporters in hepatocytes (Kipp and Arias, 2002). At present, we are not able to distinguish among the possibilities.

Finally, we show here for the first time that p-glycoprotein function at the blood-brain barrier can be rapidly reduced and that this reduction in transport function is completely reversible. This finding raises the possibility that activation of signaling could provide a narrow window in time during which normally impermeant drugs that are p-glycoprotein substrates, e.g., many chemotherapeutics, would selectively enter the brain and gain access to sites of action within the CNS. Because of vascular effects, it would not be practical to administer ET-1 itself. However, as we learn more about downstream signals that alter p-glycoprotein function in brain

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capillaries, it may be possible to identify a molecular target that can be pharmacologically manipulated to transiently reduce p-glycoprotein-mediated drug efflux with minimal side effects.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. NBDL-CSA transport in isolated rat brain capillaries. (A) Representative confocal image of a brain capillary after 60 min incubation with 2 μ M NBDL-CSA; note the high luminal accumulation of the fluorescent drug derivative. (B) Capillary after 60 min incubation in medium with 2 μ M NBDL-CSA plus 5 μ M PSC833; luminal accumulation is greatly reduced. (C) Time course of 2 μ M NBDL-CSA accumulation in the lumens of isolated rat brain capillaries; data are shown for control capillaries and those exposed to 5 μ M PSC833. (D) Effect of PSC833 on steady state luminal NBDL-CSA accumulation. Capillaries were loaded to steady state (60 min) in medium with 2 μ M NBDL-CSA. Then (time zero on graph), 5 μ M PSC833 was added to the medium; 45 min later, PSC833 was removed. Each point represents the mean value for 7-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SE bars. Units are arbitrary fluorescence units (scale 0-255). The scale bars in (A) and (B) indicate 5 μ m.

Figure 2. Effects of ET-1 on NBDL-CSA transport in isolated capillaries. (A) Concentration-dependent decrease in steady state luminal NBDL-CSA accumulation caused by ET-1. (B) Effect of ET-1 on steady state luminal NBDL-CSA accumulation. Capillaries were loaded to steady state (60 min) in medium with 2 μ M NBDL-CSA. Then (time zero on graph), 10 nM ET-1 was added to the medium; 90 min later, ET-1 was removed. Each point represents the mean value for 7-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SE bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly smaller than control, $P < 0.001$.

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Figure 3. ET-1 actions on transport do not involve increased junctional permeability. (A) Effect of increased medium osmolality on steady state luminal NBDL-CSA accumulation. Capillaries were loaded to steady state (60 min) in medium with 2 μ M NBDL-CSA. Then (time zero on graph), 100 mM mannitol was added to the medium; 60 min later, mannitol was removed. (B) Effect of transport inhibitors, ET-1 and SNP on steady state (60 min) luminal accumulation of Texas Red. Data from 2 experiments are shown. (C) Osmotic agents and 1-O-pentylglycerol increase efflux of Texas Red. (D) ET-1 does not affect Texas Red efflux. Each point represents the mean value for 7-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SE bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly smaller than control, $P < 0.001$.

Figure 4. ET-1 acting through an ET_B receptor reduces luminal NBDL-CSA accumulation. (A) Effect of an ET_B receptor antagonist. (B) Lack of effect of an ET_A receptor antagonist. (C) The ET_B receptor antagonist, sarafotoxin 6c reduces luminal NBDL-CSA accumulation. (D) Sarafotoxin effects are blocked by an ET_B receptor antagonist. (E) Sarafotoxin effects are not blocked by an ET_A receptor antagonist.. Each point represents the mean value for 7-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SE bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly smaller than control, $P < 0.001$.

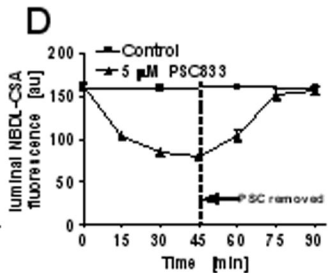
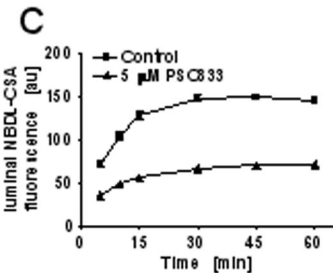
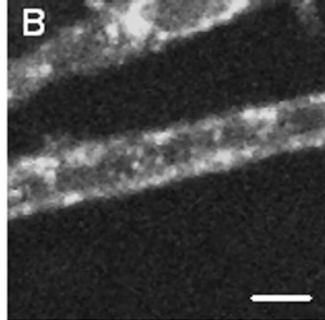
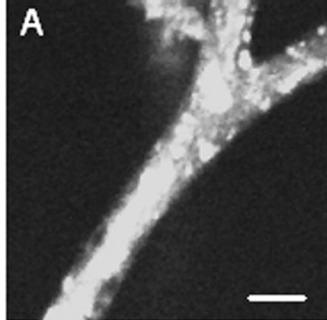
Figure 5. Expression of ET_B receptor in rat brain capillaries. (A) Representative confocal image of capillary immunostained for the ET_B -receptor (green) and counterstained for nuclei with propidium iodide (red); the scale bar indicates 10 μ m. The insert shows at higher magnification

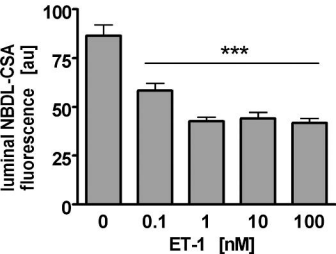
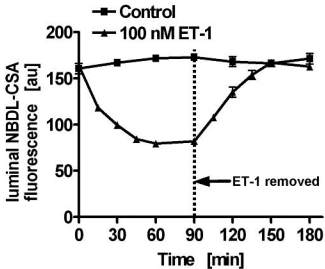
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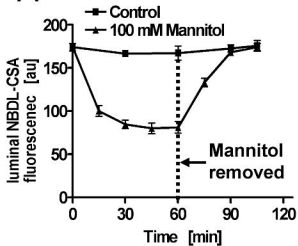
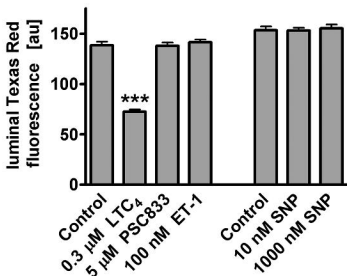
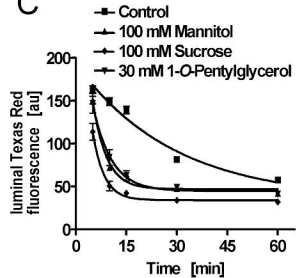
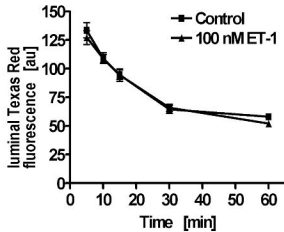
ET_B-receptor immunostaining on the apical and basolateral membrane. Left: ET_B-receptor immunostaining, right: negative control (top left: red channel with propidium iodide staining of nuclei, top right: green channel, bottom left: transmitted light, bottom right: overlay of green, red and transmitted light channels; the scale bar indicates 10 μm). (B) Western blot analysis of ET_B-receptor expression in capillary lysates and isolated capillary membranes.

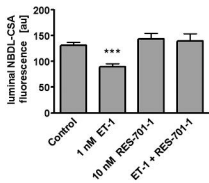
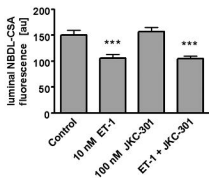
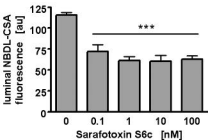
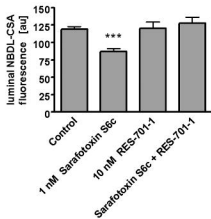
Figure 6. Evidence for involvement of NOS in ET-1 signaling. (A) Concentration dependence of sodium nitroprusside (SNP)-induced reduction in steady state (60 min) luminal accumulation of NBDL-CSA. (B) Lack of effect of the NOS inhibitor, L-NMMA, on SNP action. (C) Blockade of the ET-1-induced decrease in luminal NBDL-CSA fluorescence by L-NMMA. Each point represents the mean value for 7-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SE bars. Units are arbitrary fluorescence units (scale 0-255).
Statistical comparisons: *** significantly smaller than control, $P < 0.001$.

Figure 7. Evidence for involvement of protein kinase C (PKC) in ET-1 signaling. (A) Concentration dependence of phorbol ester (PMA)-induced reduction in steady state (60 min) luminal accumulation of NBDL-CSA. (B) Blockade of the PMA effect by the PKC-selective inhibitor, BIM. (C) Blockade of the sodium nitroprusside effect by the PKC-selective inhibitor, BIM. (D) Blockade of the ET-1 effect by the PKC-selective inhibitor, BIM. Each point represents the mean value for 7-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SE bars. Units are arbitrary fluorescence units (scale 0-255).
Statistical comparisons: ** significantly smaller than control, $P < 0.01$; *** significantly smaller than control, $P < 0.001$.



A**B**

A**B****C****D**

A**B****C****D****E**