Insulin Enhancement of Opioid Peptide Transport across the Blood-Brain Barrier and Assessment of Analgesic Effect

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ABSTRACT

Insulin crosses the blood-brain barrier (BBB) via receptor-mediated transcytosis and has been suggested to augment uptake of peripheral substances across the BBB. The δ-opioid receptor-selective peptide d-penicillamine^{2,5} (DPDPE), a Met-enkephalin analog, produces analgesia via a central nervous system-derived effect. In vitro (K_{cell}, \mu M \cdot min^{-1} \cdot mg^{-1}) and in situ (K_{trans}, \mu M \cdot min^{-1} \cdot g^{-1}) analyses of DPDPE transport (K_{cell} = 0.56 ± 0.15; K_{trans} = 0.28 ± 0.03) revealed significant (P < .01) increases in DPDPE uptake by the BBB with 10 \mu M insulin (K_{cell} = 1.61 ± 0.26; K_{trans} = 0.48 ± 0.04). In vitro cellular uptake was significantly increased (P < .05) at 1 \mu M insulin, whereas no significant uptake was observed with CTAP (a somatostatin opioid peptide analog) or sucrose (a paracellular diffusionary marker). No significant change in uptake was seen with DPDPE, CTAP, or sucrose in the presence of holo-transferrin (0–100 \mu M), indicating that the effect of insulin on DPDPE was not a generalized effect of receptor endocytosis. Insulin did not affect P-glycoprotein efflux, a mechanism that has shown affinity for DPDPE. Similar uptake of DPDPE into the brain (64% increase) was seen with the in situ brain perfusion model. Analgesic assessment revealed a significant decline in DPDPE (i.v.)-induced analgesia with increasing concentrations of insulin (i.v., i.c.v., s.c.) in a dose-dependent manner. Thus, insulin significantly increases DPDPE uptake across the BBB by a specific mechanism. The analgesic effect seen with DPDPE and insulin coadministration was shown to decrease, indicating that insulin reduces the analgesic effect within the central nervous system rather than at the BBB.

Reduced efficacy of opioids in various pain states is a common complication of diabetes. It has been shown that hypoinsulinemic states reduce the analgesic capacity of opioids. The role of diabetes in decreased opioid efficacy is supported by a number of studies (Simon and Dewey, 1981; Suh et al., 1996; Courteix et al., 1998). In contrast to streptozotocin-induced diabetic models, insulin-independent models have shown to induce hyperinsulinemic states (Coleman and Hummel, 1967). The analgesic potency of opioids has been shown to decrease in spontaneously hyperinsulinemic diabetic mice (Kamei et al., 1998). Because hyperinsulinemia (as well as hypoinsulinemia) contributes to the reduction of pain-relieving effects from opioids, diabetic patients requiring opioid treatment for pain may require greater concentrations of analgesic to counterbalance changes in their insulin levels.

The mechanisms by which diabetes alters opioid potency are unclear. Courteix et al. (1998) showed no differences in brain receptor binding between normal and diabetic rats at either the \mu-opioid or \delta-opioid receptors using DAMGO and DPDPE (d-penicillamine^{2,5}), respectively. Injected (i.c.v.) insulin (3 \mu M) was shown to reduce antinociception (i.e., analgesic effect) of i.c.v.-injected DAMGO (\mu-opioid-specific) (Kamei et al., 1998). The mechanism of insulin’s effect on opioid-induced analgesia is unknown, and the degree by which the blood-brain barrier (BBB) participates in this reduced effect has not been elucidated. Previous studies have shown that insulin injected into the brain often exhibits effects opposite to insulin injected peripherally (Banks et al., 1997b), such as the lowering of serum insulin, while increasing serum glucose levels (Ajaya and Haranath, 1982; Florant et al., 1991). These effects are mediated directly by the BBB.

The BBB acts as a selective partition between the peripheral circulation and central nervous system, thereby limiting the passage of blood-borne substances into the CNS. However, essential substances required for proper functioning and development of the CNS, such as insulin, transferrin, glucose, and some amino acids, are transported across the BBB by carrier-mediated mechanisms. Insulin gains access to the brain via receptor-mediated transport (King and Johnson, 1985; Banks et al., 1997a) and regulates CNS activity by having neurotrophic action (Baskin et al., 1988), directing brain metabolism (Henneberg and Hoyer, 1994), and aug-

ABBREVIATIONS: DAMGO, [\alpha-Ala^{2},N-Me-Phe^{5},Gly^{\beta}-ol]-enkephalin; DPDPE, d-penicillamine^{2,5}; H-Tyr-d-Pen-Gly-Phe-d-Pen-OH; BBB, blood-brain barrier; CTAP, d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH_{2}; CNS, central nervous system; CSF, cerebrospinal fluid; BBMEC, bovine brain microvessel endothelial cell; % MPE, percentage maximal possible effect; AUC, area under the curve; P-gp, P-glycoprotein.
menting autonomic outflow (Muntzel et al., 1994). Additionally, insulin enhances the blood plasma concentration of opioids (Owens and Smith, 1987) and is augmented in concentration by opioids (Pfeiffer and Herz, 1984). Insulin, shown to regulate various CNS functions (Palovcik et al., 1984; Boyd et al., 1985), has the potential for a reciprocal effect on CNS-acting analgesics (Akunne and Soliman, 1987).

The first aim of the present study was to characterize the effect of insulin on the delivery of the cyclic Met-enkephalin analog DPDPE across the BBB using in vitro and in situ methodologies. CTAP (d-Phe-Cys-Tyr-b-Trp-Arg-Thr-Pen-Thr-NH₂), a somatostatin opioid analog known to gain entry into the brain solely via diffusion (Abbruscato et al., 1997), and sucrose (a paracellular diffusionary marker) were used for comparison. DPDPE, a δ-opioid receptor-selective peptide, has been shown previously to enter the CNS by way of a saturable uptake system (Thomas et al., 1997). DPDPE has become the prototypical δ-opioid receptor agonist and has served as a valuable tool for the characterization of the δ-opioid receptor (Knapp et al., 1991). Additionally, DPDPE shows promise as a therapeutic analgesic without the adverse side effects associated with morphine and other μ-selective opioid drugs (Heyman et al., 1986). Analgesia, via δ-opioid receptors, is understood to be a centrally mediated event; therefore, only those δ-selective opioids that can cross the BBB intact will achieve biological effect. Saturable uptake of DPDPE is most likely through the BBB, with the blood-CSF barrier playing a minor role in brain uptake (Williams et al., 1996). In an initial examination of the potential mechanism by which DPDPE enters the CNS, Thomas et al. (1997) revealed that in the presence of insulin, DPDPE entry into the CNS was significantly increased. Our investigation analyzed this effect in greater detail. The second aspect of our investigation was to assess changes in analgesic effect by administering a peripheral (i.v.) concentration of DPDPE with coadministration of insulin (i.v., i.c.v., and s.c.) using a Pierce (Rockford, IL) BCA-protein kit with analysis on a Beckman UV spectrometer (model 25). Rcell% is the percentage ratio of labeled compound taken up by the cell relative to the concentration of the labeled compound in the buffer. Unidirectional rate constants were determined by multiple time point analysis and normalized for protein content (Egleton et al., 1998):

\[ K_{\text{cell}} = \frac{\text{uptake into cell (t)/conc. in buffer (t)}}{x} \]

where uptake into cell is the disintegrations per minute (dpm) of radioactivity per milligram of protein at time (t), concentration in buffer is the dpm · ml⁻¹ of buffer, and \( x \) is the time point assessed.

**In Situ Brain Perfusion Analysis.** Female adult Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 250 to 300 g were housed under standard 12-h light/12-h dark conditions and provided food ad libitum. Approval of study protocols was given by the Institutional Animal Care and Use Committee at the University of Arizona. Rats were anesthetized with a 1·ml⁻¹ kg⁻¹ i.m. injection of a cocktail containing ketamine (3.1 mg · ml⁻¹), xylazine (78.3 mg · ml⁻¹), and acepromazine (0.6 mg · ml⁻¹) and then heparinized (10,000 U · kg⁻¹). Both common carotid arteries were exposed and cannulated with silicone tubing connected to a perfusion circuit. The perfusate consisted of a protein-containing mammalian Ringer’s solution containing 117 mM NaCl, 4.7 mM KCl, 0.8 mM MgSO₄, 24.8 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, and 10 mM D-glucose, plus 3.9% dextran (mol. wt. = 70,000) and bovine serum albumin type V (10 g · l⁻¹). The addition of Evans blue dye (0.055 g · l⁻¹) albumin to the Ringer’s solution provided a control for BBB integrity. Receptor saturable concentrations of insulin (10 μM) or transferrin (1 μM) were added to perfused Ringer’s solution. The perfusate was aerated with 95% O₂, 5% CO₂ and warmed to 37°C. The right jugular vein was sectioned upon initiation of the perfusion to serve as a reference. The desired perfusion pressure and flow rate were achieved (85–95 mm Hg; 1 ml · min⁻¹), the contralateral carotid artery was cannulated and perfused in the same manner as described above, and the left jugular vein was then sectioned. Radiolabeled compounds were infused (1.5 ml · min⁻¹) into the inflow of the perfusate using a slow-drive syringe pump (model 22; Harvard Apparatus, South Natick, MA). After a set perfusion time of 20 min, a cisterna magna CSF sample (~50 μl) was taken using a glass cannula. The animal was decapitated, and the brain was removed. Choroid plexi were excised, and the brain was sectioned and homogenized. The perfusate containing the radiolabeled compounds was collected from each respective carotid cannula at the termination of the perfusion to serve as a reference.

Brain tissue (~500 mg wet weight), along with the CSF and 100-μl perfusate samples, was prepared for liquid scintillation counting. Samples were treated in a uniform manner, with 1 ml of tissue solubilizer (TS-2; Research Products, Mount Pleasant, IL) added to each respective sample. After a 2-day solubilization, 100 μl of 30% glacial acetic acid was added to the samples to eliminate chemiluminescence. Four milliliters of Budget Solve Liquid Scintillation Cocktail (Research Products) was added, and samples were measured for radioactivity using a model LS 5000 TD counter (Beckman Instruments, Fullerton, CA) model LS 5000 TD counter.

**Materials and Methods**

**Radioisotopes/Chemicals.** [H]-labeled DPDPE (mol. wt. = 649.8) and CTAP (mol. wt. = 1107.0) were obtained from Multiple Peptide Systems (San Diego, CA). [U-14C]Sucrose (mol. wt. = 342.3) was obtained from ICN Pharmaceuticals Inc. (Irvine, CA). Radiolabeled compound purity and stability were confirmed by the supplier using HPLC analysis; all radiolabeled compounds were used within 5 weeks of the assay date. Insulin (bovine), holo-transferrin (iron saturated), and all other chemicals, unless noted otherwise, were purchased from Sigma (St. Louis, MO).

**In Vitro Bovine Brain Microvascular Endothelial Cell (BBMEC) Uptake Analysis.** BBMECs were isolated from the gray matter of cerebral cortices as detailed previously and characterized (Audus and Borchardt, 1987). BBMECs were grown to confluence on 24-well plates precoated with rat-tail collagen and fibronectin. At confluence, confirmed microscopically 10 to 12 days after seeding, the growth medium was removed, and the cells were preincubated for 30 min in an assay buffer containing 122 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.4 mM K₂HPO₄, 1.4 mM CaCl₂, 10 mM d-glucose, and 10 mM HEPES. The cells were then incubated for 20 min with each respective radiolabeled compound, on a shaker table at 37°C, with insulin, transferrin, or cyclosporin A. [¹⁴C]Sucrose was incubated under the same conditions and time points to serve as control. Analysis of DPDPE uptake in the presence of receptor saturable insulin (10 μM) and holo-transferrin (1 μM) was measured over multiple time points. After the appropriate times, the radioactive buffer was removed and the cells washed three times with ice-cold assay buffer. Then, 1 ml of 1% Triton X-100 was placed into each well and shaken for 30 min. A 200-μl portion of the Triton X-100 was prepared for radioactive counting using a Beckman Instruments (Fullerton, CA) model LS 5000 TD counter. The other portion of the sample was assayed for protein concentration using a Pierce (Rockford, IL) BCA-protein kit with analysis on a Beckman UV spectrometer (model 25). Rcell % is the percentage ratio of labeled compound taken up by the cell relative to the concentration of the labeled compound in the buffer. Unidirectional rate constants were determined by multiple time point analysis and normalized for protein content (Egleton et al., 1998):

\[ K_{\text{cell}} = \frac{\text{uptake into cell (t)/conc. in buffer (t)}}{x} \]

where uptake into cell is the disintegrations per minute (dpm) of radioactivity per milligram of protein at time (t), concentration in buffer is the dpm · ml⁻¹ of buffer, and \( x \) is the time point assessed.
<p>Capillary Depletion. Measurement of the vascular component to total brain uptake was performed using capillary depletion (Triguero et al., 1990). After a 20-min in situ perfusion, the brain was removed, and the choroid plexi were excised. The brain tissue (500 mg) was homogenized (Polytron homogenizer; Brinkman Instruments, Westbury, NY) in 1.5 ml of capillary depletion buffer [10 mM 4-(2-hydroxyethyl)-piperaxineethanesulfonic acid, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM d-glucose (pH 7.4)] kept on ice. Ice-cold 26% dextran (2 ml; mol. wt. = 70,000) was added, and homogenization was repeated. Aliquots of homogenate were centrifuged at 5400 g for 15 min in a Microfuge (Beckman Instruments). The capillary-depleted supernatant was separated from the vascular pellet. All of the homogenization procedures were performed within 2 min of sacrificing the animal. The homogenate, supernatant, and pellet were taken for radioactive counting.</p>

**Expression of In Situ and Capillary Depletion Data.** The amount of <sup>3</sup>H]DPDDE, <sup>3</sup>H]CTAP, and <sup>14</sup>C]sucrose in the whole brain, CSF, homogenate, supernatant, and pellet was expressed as the percentage ratio of tissue (<i>C<sub>Tissue</sub></i>) disintegrations per minute per gram<sup>−1</sup> or disintegrations per minute per milliliter<sup>−1</sup> to plasma activities (<i>C<sub>Plasma</sub></i>, disintegrations per minute per milliliter<sup>−1</sup>) and expressed as a percentage ratio of brain tissue uptake (<i>R<sub>Br</sub></i> %):</p>

\[
R_{Br} \% = \frac{C_{Tissue}/C_{Plasma}}{100}
\]

The unidirectional transfer constant in vivo (<i>K<sub>in</sub></i>, μl·min<sup>−1</sup>·g<sup>−1</sup>) was determined by single time point calculation at 20 min (Zlokovic et al., 1986).

\[
K_{in} = \frac{C_{Plasma}}{C_{perf}}t
\]

The blood-brain unidirectional transfer constants were corrected for vascular space by subtraction of vascular space marker <sup>14</sup>C]sucrose <i>R<sub>Br</sub></i> % from the <sup>3</sup>H]DPDPE <i>R<sub>Br</sub></i> % values.

**Analgesia Analysis.** A radiant-heat, tail-flick analgesia meter (model-33; IITC Scientific Products, Woodland Hills, CA), was used to assess pain sensitivity after the administration of the test compounds. The analgesia meter was set to produce a baseline latency of 2 to 3 s, with a cutoff time of 15 s. Male ICR mice (20–25 g) (Harlan Sprague-Dawley) were administered 30 or 120 mU of insulin injected i.v., 30, 120, or 240 mU injected s.c., or 3.0 or 12 mU injected i.c.v. followed by a single i.v. dose (tail vein) of DPDPE (6.2 nM) dissolved in sterile saline. Measurements were taken at 15, 30, 45, 60, 75, and 90 min. The mice (n = 4–5) were placed into restraint holders, and their tails were properly placed under the radiant heat beam. The beam was turned on and then automatically shut off upon flicking of the tail. Analyses were stopped at any given time point at which the maximal possible analgesic effect fell within 5% of the baseline. DPDPE was used to establish the proper dosage level and served as the control. Morphine (20 μg · kg<sup>−1</sup> i.v.) analgesia measurements were performed as an additional control and with i.v.-injected insulin (30 mU).

Nociceptive sensitivity was determined by converting the recorded analgesic tail-flick times to a percentage maximal possible effect (% MPE):

\[
\% \text{MPE} = \frac{\text{recorded flick time} - \text{baseline}}{\text{maximum time (15 s) - baseline}}
\]

**Data Analysis.** For all experiments, the data are presented as mean ± S.E.M. values. The slopes (<i>K<sub>cell</sub></i>) of curves were determined by least-squares linear regression analysis, with slopes compared by ANOVA. Linear regressions were carried out using the Pharmacological Calculation System statistical analysis program (Tallarida and Murry, 1987). All other analyses used ANOVA comparison, followed by Newman-Keuls statistical analysis when applicable.

**Results**

**BBMVEUptake.** In vitro uptake analysis of <sup>3</sup>H]DPDPE in the presence of various concentrations of insulin (Table 1) showed a significant increase in percentage ratio of cellular uptake (<i>R<sub>cell</sub></i> %) in a dose-dependent manner at concentrations greater than 0.1 μM (<i>n</i> = 6). <sup>14</sup>C]Sucrose exhibited no increase in cellular uptake when coadministered with insulin at concentrations of 10 and 100 μM. <sup>3</sup>H]CTAP also showed no increase in cellular uptake with coadministration of insulin at concentrations of 1, 10, and 100 μM. Analysis of radiolabeled compounds in the presence of various concentrations of transferrin (Fig. 1) showed no differences in <i>R<sub>cell</sub></i> % compared with control. BBMVE uptake of <sup>3</sup>H]DPDPE with 1.6 μM cyclosporin A showed no significance in BBMVE uptake of <sup>3</sup>H]DPDPE, whereas 10 μM insulin showed significant (<i>P</i> < .05) increase in uptake. BBMVE uptake of <sup>3</sup>H]DPDPE with 1.6 μM cyclosporin A and 10 μM insulin showed a significant (<i>P</i> < .01) increase in cellular uptake (Fig. 2). Table 2 shows <i>K<sub>cell</sub></i> (μl·min<sup>−1</sup>·mg<sup>−1</sup>) uptake values from multiple time point analysis of DPDPE in the presence of insulin (10 μM) and holo-transferrin (1.0 μM).

**In Situ Brain Perfusion and Capillary Depletion.** The effects of <sup>3</sup>H]DPDPE, <sup>3</sup>H]CTAP, and <sup>14</sup>C]sucrose in the presence of insulin (10 μM) and transferrin (1 μM) were assessed after a 20-min in situ perfusion (Fig. 3) (n = 5–6). Permeability of radiolabeled compounds across the intact BBB is expressed as a ratio of brain uptake (<i>R<sub>Br</sub></i> %). <sup>3</sup>H]DPDPE exhibited a significant (<i>P</i> < .01) increase in brain uptake (64%) in the presence of insulin. No significant difference in brain uptake was observed with <sup>3</sup>H]DPDPE in the presence of transferrin. No significant change in uptake was seen with <sup>3</sup>H]CTAP or <sup>14</sup>C]sucrose in the presence of either insulin or transferrin. Capillary depletion analysis of <sup>3</sup>H]DPDPE showed no difference in concentration associated with the vascular component (i.e., pellet) after a 20-min in situ brain perfusion in the presence of either insulin or transferrin compared with control. The <i>K<sub>in</sub></i> values (<i>μl·min<sup>−1</sup>·g<sup>−1</sup></i>) for <sup>3</sup>H]DPDPE in the presence of insulin or holo-transferrin (Table 2) showed similar trends seen with cellular uptake analysis <i>K<sub>cell</sub></i> (μl·min<sup>−1</sup>·mg<sup>−1</sup>).

**Analgesia.** The i.v. administration of DPDPE (6.2 nM) with each respective route/concentration of insulin (n = 4–5)

**Table 1.** Uptake of DPDPE in the presence of various concentrations of insulin, expressed as a percentage ratio of cellular uptake (<i>R<sub>cell</sub></i> %)

<table>
<thead>
<tr>
<th>Insulin Concentration (μM)</th>
<th>&lt;i&gt;R&lt;sub&gt;cell&lt;/sub&gt;&lt;/i&gt; %</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>6.30 ± 0.53</td>
<td>N.S.</td>
</tr>
<tr>
<td>0.01</td>
<td>6.13 ± 0.46</td>
<td>N.S.</td>
</tr>
<tr>
<td>0.1</td>
<td>6.39 ± 0.76</td>
<td>N.S.</td>
</tr>
<tr>
<td>1.0</td>
<td>7.71 ± 0.35</td>
<td>&lt;i&gt;P&lt;/i&gt; &lt; .05</td>
</tr>
<tr>
<td>5.0</td>
<td>8.34 ± 0.48</td>
<td>&lt;i&gt;P&lt;/i&gt; &lt; .01</td>
</tr>
<tr>
<td>10.0</td>
<td>8.35 ± 0.38</td>
<td>&lt;i&gt;P&lt;/i&gt; &lt; .01</td>
</tr>
<tr>
<td>50.0</td>
<td>19.6 ± 1.84</td>
<td>&lt;i&gt;P&lt;/i&gt; &lt; .001</td>
</tr>
<tr>
<td>100</td>
<td>25.0 ± 1.10</td>
<td>&lt;i&gt;P&lt;/i&gt; &lt; .001</td>
</tr>
</tbody>
</table>

N.S., nonsignificant.
Significance of DPDPE in the presence of insulin (10 μM) for DPDPE in the presence of insulin (10 μM) or cyclosporin A (1.6 μM) or in combination, compared with DPDPE alone, determined by ANOVA, followed by Newman-Keuls analysis, denoted by **P < .01; *P < .05.

Table 2

<table>
<thead>
<tr>
<th>K&lt;sub&gt;in&lt;/sub&gt; (μl·min&lt;sup&gt;-1&lt;/sup&gt;·mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;out&lt;/sub&gt; (μl·min&lt;sup&gt;-1&lt;/sup&gt;·g&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPDPE</td>
<td>0.56 ± 0.15</td>
</tr>
<tr>
<td>DPDPE and insulin</td>
<td>1.61 ± 0.25</td>
</tr>
<tr>
<td>DPDPE and holo-transferrin</td>
<td>0.08 ± 0.03</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M.

was evaluated (Fig. 4). Significant (P < .01) decreases in analgesic effect of DPDPE was seen with i.v. injections (tail vein) of insulin at both 30 and 120 μU in a dose-dependent manner (Fig. 4a, 4 days). Injection (s.c.) of insulin at 30 μU increased (P < .05) the analgesic area under the curve (AUC) of DPDPE, whereas 120 μU was not different from control, and 240 μU showed a decreased (P < .01) analgesic effect (Fig. 4b, 4 days). Significant (P < .05) decreases in analgesic effect of DPDPE were seen with i.v. injection of insulin at 12 μU (Fig. 4c, 4 days). Morphine (20 μg · kg<sup>-1</sup> i.v.), run as control, showed significant (P < .01) reduction in analgesic effect when coadministered with 30 μU of insulin (i.v.).

**Discussion**

Assessment of [³H]DPDPE uptake into BBMECs with co-administration of insulin showed that insulin significantly enhanced DPDPE cellular transport in a dose-dependent manner at concentrations greater than 0.1 μM. [³H]CTAP, which enters the brain solely via diffusionary mechanisms, and [¹⁴C]sucrose were not affected by coadministration of insulin. This indicates that insulin enhanced DPDPE uptake via the saturable, rather than the nonsaturable, pathway of DPDPE. Insulin binding to its receptor may induce cellular changes that augment DPDPE surface receptor concentration, production, and/or transport. Insulin has been suggested to augment cellular lipid synthesis and membrane profile, which may result in changes of membrane fluidity (Schilsky et al., 1981). However, with insulin modification of membrane profile, the uptake of CTAP and sucrose would also be expected to change. Because this does not appear to take place here, it suggests that the carrier mechanism for DPDPE or some other component of the DPDPE binding domain is altered.

BBMEC uptake analyses of DPDPE, CTAP, and sucrose were also assessed with the coadministration of holo-transferrin. In a manner similar to that of insulin, transferrin is transported at the BBB via receptor-mediated endocytosis (Roberts et al., 1993). The analyses revealed no change in R<sub>cell</sub>% uptake of DPDPE, CTAP, or sucrose with saturable and supersaturable concentrations of holo-transferrin. This suggests that insulin enhancement of DPDPE uptake may be via a specific action of insulin rather than via a nonspecific endocytotic process. This was confirmed in situ, where increased DPDPE uptake into the brain was significantly increased with insulin, whereas holo-transferrin had no significant effect. The R<sub>SH</sub>% of DPDPE/insulin coadministration was 64% greater than that of DPDPE control. The capillary depletion data revealed no significant differences among control, insulin coadministration, and holo-transferrin coadministration in DPDPE, CTAP, or sucrose. This indicates that the DPDPE/insulin increase in uptake into the brain is a...
transcytotic increase, and therefore DPDPE is not merely accumulating within the capillary endothelial cell.

The unidirectional transfer constants calculated from the in situ \( K_{\text{in}} \) (\( \mu l \cdot \text{min}^{-1} \cdot g^{-1} \)) brain perfusion analysis of DPDPE in the presence of insulin and holo-transferrin showed an excellent correlation with the in vitro \( K_{\text{cell}} \) (\( \mu l \cdot \text{min}^{-1} \cdot mg^{-1} \)) BBMEC uptake analysis. The in situ \( K_{\text{in}} \) values exhibited smaller increases in permeability compared with the \( K_{\text{cell}} \) values of the in vitro model. The rank order of permeability is preserved across both models, providing validation of the respective models. To understand the differences between the models one should realize that \( K_{\text{cell}} \) is solely representative of in vitro apical membrane permeability, with a greater degree of error associated with extracellular surface binding, by volume, as compared to the in situ perfusion analysis.

Another factor of importance associated with DPDPE is the recent finding of P-glycoprotein (P-gp) affinity (Chen and Pollack, 1999). The P-gp efflux mechanism is present throughout the capillary endothelium of the BBB (Lum and Gosland, 1995). Although predominately associated with cancer chemotherapeutics, opioids such as morphine (Lerent et al., 1999) have been shown to interact with P-gp. Because earlier studies show that morphine has reduced analgesic effect in the presence of insulin (Ginawi, 1992), we examined, in vitro, the P-gp effects on DPDPE and DPDPE/insulin transport. The \( R_{\text{cell}} \) % uptakes of DPDPE with coadministered insulin and insulin/cyclosporin A showed a significant increase over control. However, there was no significant difference between DPDPE coadministered with insulin and DPDPE coadministered with insulin/cyclosporin A, indicating that the effects of insulin on DPDPE transport are independent of P-gp. These data correlate with Banks et al. (1997a) showing insulin uptake across the BBB was P-gp-independent.

Studies have shown that insulin can enhance the passage of chemicals across the BBB, such as azidodeoxythymidine (Ayre et al., 1989) and DPDPE (Thomas et al., 1997). The variation in structure of these drugs supports the theory that insulin potentiation occurs via a nonspecific mechanism. However, insulin has not been shown to enhance the permeability of other drugs or compounds when coadministered, as demonstrated in this investigation. Significant saturation of insulin binding to its receptor at the BBB may induce a change in the lipid membrane profile, as suggested by Schilsky et al. (1981), thereby inducing or freeing up delivery mechanisms at the cell membrane. Additionally, the endocytotic mechanism of insulin may be colocalized with certain peptide receptors (i.e., DPDPE) resulting in a cointernalization, with an increase in receptor recycling at saturable concentrations of insulin resulting in increasing DPDPE uptake. To date, the large neutral amino acid carrier mechanism, absorptive endocytosis, and the carrier by which Leu-enkephalin crosses the BBB have been examined and ruled
out as potential mechanisms by which DPDPE might enter the BBB (Thomas et al., 1997).

Because DPDPE enters the CNS at an increased rate with insulin receptor-saturable concentrations, the question remains as to the actual in vivo effect insulin has upon DPDPE-induced analgesia. This question prompted the analgesic assessment of DPDPE in the presence of various concentrations and routes of insulin administration. In contrast to BBB transport analysis, coadministration of insulin induced a general decrease in analgesic effect of i.v.-administered DPDPE in a dose-dependent manner. This coincides with similar studies of peptide analogues in the presence of insulin (Kamei et al., 1998; Ohsawa et al., 1999). A point of significance between BBB assessment and analgesic outcome may be in the concentration of insulin administered, as well as in the route. Because of limitations of insulin dosing, concentrations equivalent to those used in assessing BBB augmentation could not be used. However, no significant change in DPDPE blood-brain barrier permeability was observed at any concentration less than 1 μM. Thus, the decrease in analgesia most likely does not result from decreased uptake at the BBB. This is in agreement with the i.c.v. insulin analgesia data, in which insulin administration circumvents the BBB. It is presumed that insulin increases the transport of DPDPE across the BBB in conjunction with increased transport of insulin itself. The increased insulin concentration in the brain may result in a decreased analgesic effect. Insulin-induced alterations in brain opiate receptors, interactions at the binding site, or adaptation of second messenger systems may account for this effect. In an investigation of the i.c.v. insulin-induced decreased analgesic effect of DAMGO (μ-opioid receptor), Ohsawa et al. (1999) reported that insulin activation of protein kinase C and subsequent activation of various cytoplasmic substrates. Because opioid receptors contain consensus protein kinase sites (Miotto et al., 1995), it is possible that phosphorylation of the δ-opioid receptor by protein kinases desensitizes opioid receptor function. The administration of insulin s.c. most accurately models the actual dose (relative to weight) and route a patient with diabetes may use, and exhibits a similar AUC trend to that of i.v. and i.c.v. administered doses of insulin, with exception of the 30 μL dose. As expected, there is a rightward shift in the s.c. time-response curve, resulting from the time required for insulin to gain entry to the systemic circulation. The 30 μL dose of insulin s.c. resulted in a significant (P < .05) increase in the AUC compared with control, although the % MPE of this dose did not reach that of control. This may be a result of the time insulin takes to enter the blood stream or potentially an effect on BBB influx of DPDPE without sufficient insulin entering the brain to induce an antianalgesic effect. A caveat with regard to our assessment of analgesic results is the potential for increased peripheral distribution of DPDPE, when coadministered with insulin, which may also result in a decreased analgesic effect. Yet, with the increased BBB transport of DPDPE in the presence of insulin shown in the in vitro and in situ analyses, as well as the decreased analgesic effect exhibited by DPDPE when insulin was administrated i.c.v., we believe that the observed effect is not attributable to an increased peripheral uptake of DPDPE.

In conclusion, the present study has shown that insulin significantly increases the uptake of DPDPE across the BBB in a dose-dependent manner by a potentially specific mechanism. Insulin did not enhance the uptake of CTAP or surce at concentrations up to 100 μM in vitro and 10 μM in situ. This potentiation of DPDPE is not related to its affinity for the P-gp efflux system or a generalized effect of receptor-mediated endocytosis. Yet, the end analgesic effect seen with DPDPE and insulin coadministration was shown to decrease, indicating that insulin's effect occurs within the CNS rather than at the BBB. The implications of these results are far reaching. The effects of insulin therapy may inhibit the pain-relieving effect of opioid analgesics. Additionally, diabetics have been shown to have an increased insulin transport at the BBB (Banks et al., 1997c) and may be more susceptible to insulin's antianalgesic effect. Likewise, newborns with an underdeveloped BBB and potentially enhanced insulin-BBB binding capacity (Frank et al., 1985) may likewise suffer from a decreased opioid analgesic effect. It remains unclear as to the exact mechanism by which insulin inhibits analgesic function of opioids, and therefore further examination is required.

References


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