Active Transport of Fentanyl by the Blood-Brain Barrier

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ABSTRACT

Previous studies have shown that uptake of the lipophilic opioid, fentanyl, by pulmonary endothelial cells occurs by both passive diffusion and carrier-mediated processes. To evaluate if the latter mechanism also exists in brain endothelium, transport of [3H]fentanyl was examined in primary cultured bovine brain microvessel endothelial cell (BBMEC) monolayers. Uptake of fentanyl appears to occur via a carrier-mediated process as uptake of [3H]fentanyl by BBMECs was significantly inhibited in a dose-dependent manner by unlabeled fentanyl. Fentanyl uptake was also significantly inhibited by either 4°C or sodium azide/2-deoxyglucose, suggesting that carrier-mediated uptake of fentanyl was an active process. Fentanyl was also tested to determine whether it might be a substrate of the endogenous blood-brain barrier efflux transport system, P-glycoprotein (P-gp). Release of [3H]fentanyl or rhodamine 123, a known substrate of P-gp, previously loaded in the BBMECs was studied in the presence or absence of either fentanyl or verapamil, a known competitive inhibitor of P-gp. Both fentanyl (10 μM) and verapamil (100 μM) decreased release of rhodamine 123 from BBMECs, indicating that fentanyl is a substrate of P-gp in the BBMECs. This was further supported by the observation that uptake of [3H]fentanyl was significantly increased in Mg2+-free medium, a condition known to reduce P-gp activity. However, release of [3H]fentanyl was significantly increased when incubated with either unlabeled fentanyl or verapamil. These results suggest that the active P-gp-mediated extrusion of fentanyl in these cells is overshadowed by an active inward transport process, mediated by an as yet unidentified transporter. In addition, verapamil was shown to be a substrate of both P-gp and the fentanyl uptake transporter.

Drug distribution to tissue affects pharmacokinetics, and thus, observed pharmacodynamics. For example, drug uptake by pulmonary tissue, if extensive, markedly reduces peak systemic arterial drug concentrations in the moments after rapid i.v. drug administration (Roerig et al., 1994). Thus, for drugs with rapid onsets of action, such as i.v. anesthetics, pulmonary drug uptake could act to reduce peak drug effect. We recently have demonstrated that the marked pulmonary uptake of fentanyl (a lipophilic synthetic μ-opiate agonist and prototypic high pulmonary uptake drug) is generated by the pulmonary endothelium and results from both first order passive diffusive and higher capacity saturable processes, suggesting transporter mediation (Waters et al., 1999).

It has been assumed that entry of lipophilic xenobiotics into tissues occurs by passive diffusion with the equilibrium between plasma and tissue drug concentrations determined by physicochemical properties such as octanol-water partitioning and protein binding (Ishizaki et al., 1997; Wood, 1997). More recently, transport proteins such as the ATP-binding cassette (ABC) transporter superfamily [e.g., P-glycoprotein (P-gp)] have been found to be extensively distributed in many endothelia (Thiebaut et al., 1987) and act to create and maintain tissue/plasma partition gradients of lipophilic xenobiotics that would not be produced by simple passive processes alone.

Although the increased pulmonary tissue/plasma partitioning of fentanyl may affect observed drug effects after rapid i.v. administration, the presence of a similar uptake phenomenon at the blood-brain barrier (BBB) would have even more immediate pharmacodynamic implications. Firstly, if fentanyl concentration at its site(s) of action is controlled by an endothelial transporter, not passive diffusion, then intra- and interindividual potency variability may not be solely dependent on μ receptor differences, but on variable plasma/brain partitioning. Secondly, if fentanyl transport is inward at the brain endothelium, then such a mechanism may be exploitable for reducing fentanyl effects.
or enhancing the central nervous system effects of other drugs.

Here we report the kinetics of fentanyl transport in a model of the BBB to determine to what extent fentanyl uptake into bovine brain microvascular endothelial cells (BBMECs) is saturable, energy-dependent, and inhibitable.

**Experimental Procedures**

**Materials.** Dispase and collagenase/dispase were obtained from Boehringer Mannheim (Indianapolis, IN). Type I rat tail collagen and endothelial cell growth supplements (ECGS) were purchased from Collaborative Biomedical (Bedford, MA). Cell culture medium was obtained from Gibco (Grand Island, NY). [3H]fentanyl (8.89 Ci/mmol) was obtained from Research Diagnostics, Inc. (Flanders, NJ). 3H-antipyrine (7.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Rhodamine 123 (R123), fentanyl citrate, platelet poor horse serum, sodium azide, 2-deoxyglucose, dimethyl sulfoxide, fibronectin, and verapamil hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents, unless specifically stated otherwise, were purchased from Sigma Chemical Co.

**BBMEC Isolation and Culturing.** BBMEC were isolated from the cerebral gray matter of bovine brain as described previously (Audus et al., 1996; Ng and Schallenkemp, 1996). Briefly, brain grey matter was collected and minced to 1- to 2-mm cubes with razor blades before undergoing a 2.5-h dispase digestion (4 ml 12.5% dispase solution/50 g of gray matter). The microvessels were then separated from the cell debris by centrifugation in 13% dextan. The isolated microvessels were further incubated on a per gram basis with 3 ml of collagenase/dispase (at 1 mg/ml or 0.3 U collagenase and 4.12 U dispase/ml) for 4 h at 37°C. At the conclusion of this incubation, the microvessels were subjected to Percoll gradient centrifugation for final separation of microvessels from pericytes, cell debris, and other contaminating cells. The purified microvessels were stored frozen at −180°C in freezing medium (36% minimum essential medium, 36% F-12 medium, 18% platelet poor horse serum, 10% dimethyl sulfoxide, 50 U/ml penicillin, 50 μg/ml streptomycin, and 125 μg/ml heparin) until used.

For cellular accumulation and release studies, purified BBMECs were seeded at a density of 50,000 cells/cm² onto collagen-coated and fibronectin-treated 48-well cell culture plates in BBMEC plating medium (45% minimum essential medium, 45% F-12 medium, 10% platelet poor horse serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 50 μg/ml polymixin B and 25 μg/ml ECGS). The cells were grown in a 37°C incubator with 5% CO₂ and 95% humidity. The plating medium was replaced with BBMEC culture medium (plating medium less polymixin B and ECGS) on the third day after plating and every other day thereafter. BBMECs were allowed to grow to confluence before cellular accumulation and release studies. Typically, formation of monolayers took about 6 to 8 days.

**[3H]Fentanyl Cellular Accumulation Studies.** After the establishment of a confluent BBMEC monolayer was confirmed (by phase contrast microscopy examination), cellular accumulation of [3H]fentanyl was measured. Briefly, confluent cell cultures were washed twice with serum-free BBMEC culture medium. Subsequently, the BBMEC monolayers were incubated with 0.25 ml serum-free BBMEC culture medium containing either 50 nM [3H]fentanyl (0.447 μCi) or 4 μM R123 for 60 min at 37°C. After incubation was complete, the culture media was aspirated gently, and cells were washed three times with 1 ml of ice-cold serum-free BBMEC culture medium to remove any extracellular [3H]fentanyl or R123. After the washing was complete, the cells were restored to either fresh serum-free BBMEC culture medium or serum-free BBMEC culture medium containing no additional drugs or the P-gp inhibitor verapamil (100 μM) or nonlabeled fentanyl (10 μM) at 37°C. At the indicated time intervals for fentanyl or at 60 min for R123 after starting the secondary postwash incubation, the culture medium was removed and the cells were washed three times with 1.0 ml of ice-cold PBS. The cells were solubilized in 1 ml 0.2 N NaOH and 500 μl and 25 μl aliquots of the cell lysate solution were removed for measurement of [3H]fentanyl or R123 and protein, respectively. The intracellular concentration of R123 was determined quantitatively by fluorescence spectrophotometry as described previously (Fontaine et al., 1996). Briefly, sample cell lysate solutions were diluted to 1 ml with 0.5 ml 0.2 N HCl. Sample fluorescence was then measured using a Shimadzu RF5000 Fluorescence Spectrophotometer (excitation wavelength 505 nm; emission wavelength 534 nm; Shimadzu Scientific Instruments Inc., Columbia, MD). The concentration of R123 in each sample was determined from the fluorescence measurements by the construction of a R123 standard curve and standardized by the protein content of each sample.

**Effect of Magnesium on Intracellular Accumulation of [3H]Fentanyl.** Confluent cell cultures were washed twice with serum-free Earl's balanced salt solution (EBSS; 108 mM NaCl, 26 mM NaHCO₃, 10 mM KCl, 1.8 mM CaCl₂, 1 mM NaH₂PO₄, 5.5 mM glucose). Subsequently, the BBMEC monolayers were preincubated with either serum-free EBSS or serum-free magnesium-plus EBSS (EBSS plus 5 mM MgSO₄) for 30 min at 37°C. At the end of this preincubation, the culture media was aspirated gently. The cells were then restored to either serum-free EBSS or serum-free magnesium-plus EBSS containing 50 nM [3H]fentanyl (0.447 μCi). After incubation with the medium containing [3H]fentanyl, the culture medium was removed and the cells were washed three times with 1.0 ml of ice-cold PBS. The BBMEC were then solubilized in 1 ml 0.2 N NaOH and aliquots of the cell lysate solution were removed for measurement of [3H]fentanyl and protein as described above.

**Kinetic Analysis.** To evaluate whether cellular accumulation of fentanyl is governed by processes which are first order passive, Michaelis-Menten (active), or both, a model developed previously for analysis of fentanyl uptake by bovine pulmonary artery endothelial cells (BPAECs) was used (Waters et al., 1999). In this model, the constant defining the equilibrium between the supernatant and the endothelial cells, KEQ, is represented by the sum of two terms:

\[ K_{EQ} = H + \frac{R_{MAX}}{(k_1/k_2) + C_s} \]  

\[ (1) \]

where H is the first order term, H, representing the diffusional partition coefficient and a saturable term in which R_{MAX} is the total transport
capacity and $k_r/k_s$ is the $K_M$ or the supernatant drug concentration $C_s$ which leads to 50% occupancy (inhibition) of transporters.

Cell-associated [$^3$H]fentanyl data, after being normalized for their protein content, were all fit simultaneously to eq. 1 using a constant weight and TableCurve2D (SPSS, Chicago, IL). Simpler models in which the transport component was linearly related to protein content, were all fit simultaneously to eq. 1 using a constant and saturable transporter rate constants). Note that in this experiment the transporter was assumed to be at either the $R_{\text{MAX}}$ state (no added fentanyl) or at a fully inhibited state ($10 \mu M$ fentanyl added), thus reducing the model to two rate constants. In addition, a simpler model in which there only is only a bidirectional diffusional rate constant (i.e., there are no differences in the [$^3$H]fentanyl concentrations at the various times for the 0 $\mu M$ versus the $10 \mu M$ fentanyl conditions) was tested. All data were fit simultaneously using reciprocal weighting and the compartmental module of SAAM II (SAAM Institute, Seattle, WA). Model selection was based on the Akaike information criterion (Ludden et al., 1994).

Statistical Analysis. All other data were compared to control with a one-way ANOVA. If statistically significant differences were detected, post hoc analysis consisted of a Tukey test. $P$ was set to $p < .05$. The statistical analyses were performed with SigmaStat (SPSS, Inc., Chicago, IL).

**Results**

**Inhibition of Fentanyl Endothelial Uptake and Release.** [$^3$H]fentanyl uptake into BBMECs was found to occur by both a first order (passive) and saturable (carrier-mediated) processes. The evidence for this is in Fig. 1, which shows that $K_{R_{\text{EQ}}}$ is significantly higher at low doses of unlabeled fentanyl and lower at high doses. The line in Fig. 1 is the best fit of eq. 1 to our data; a sigmoidal relationship that was well predicted by our model (three parameters, adjusted $r^2 = 0.662$). From this fit we determined $H$ to be 0.040 ml/µg protein, $R_{\text{MAX}}$ to be 0.11 pmol/µg protein and $K_M$ to be 3.19 µM. This fit was significantly better than a linear concentration-dependent relationship (two parameters, adjusted $r^2 = 0.352$) or one that assumed fentanyl uptake occurred only by a simple diffusion mechanism (one parameter, i.e., a constant $K_{R_{\text{EQ}}}$, adjusted $r^2 < 0.001$).

To corroborate these findings, a study was done to determine the rates of release of [$^3$H]fentanyl from cells preloaded with [$^3$H]fentanyl under control conditions, and one was done in which the supernatant contained 10 µM cold fentanyl (EC$_{75}$ for saturating the inward transport mechanism). Figure 2 shows that [$^3$H]fentanyl was released from BBMECs at a faster rate when 10 µM cold fentanyl was present, consistent with its inhibition of a transporter directed inwardly. The ratio of the rate constants for passive diffusion (unsaturable) to that for the transporter (saturable) was 0.38, which is identical with the ratio of $H$ to $R_{\text{MAX}}$ from the uptake model (eq. 1) of 0.38. A simpler model was also fit to the data. In this model there was no inhibitable rate constant directed inward (i.e., all data in Fig. 2 is fit to a single line). The full model in which there is an additional parameter for the inward transporter (under control conditions) was supported by the Akaike information criterion. The percent coefficients of variation for the diffusive rate constant and the transporter rate constant were 7.0 and 8.5%, respectively.

**Energy Depletion and Fentanyl Endothelial Uptake.** Pretreatment of the BBMECs with the metabolic inhibitors sodium azide and 2-deoxyglucose reduced [$^3$H]fentanyl uptake into BBMECs to that of diffusion alone (Fig. 3) as predicted by the saturation model (eq. 1 and Fig. 1). These data indicate that the saturable component of the uptake process for fentanyl is energy-dependent. In contrast, pretreatment of the BBMECs with the same metabolic inhibitors had no effect on the uptake of antipyrine, a prototypic lipophilic diffusion tracer (data not shown). Fentanyl uptake was similarly reduced when the experiments were conducted at $4^\circ C$, confirming the energy dependence of this process.

**P-gp Inhibition.** Because fentanyl is very lipophilic (Stanski and Hug, 1982) and metabolized by CYP3A4 (Labroo et al., 1997), it is a candidate substrate for the endogenous BBB efflux system, P-gp (Zhang et al., 1998), which

![Fig. 1](image1.png) **Fig. 1.** The ratio of cell-associated [$^3$H]fentanyl concentrations (nmol/mg protein) to supernatant fentanyl concentrations (nmol/ml) versus supernatant fentanyl concentrations (nmol/ml, micromolar). Observed values are solid circles and the line represents the best fit of these data to eq. 1. Note the sigmoid shape of this line with the upper plateau delineating the combined effects of diffusional equilibrium (H) and active transport, the lower plateau representing H alone, and the $K_M$ at the midpoint of the vertical portion.

![Fig. 2](image2.png) **Fig. 2.** Results of the pulse-trace experiment showing the labeled cellular fentanyl concentrations versus time for the BBMECs preloaded with [$^3$H]fentanyl. At time $t = 0$ the culture medium containing [$^3$H]fentanyl was removed and after washing, replaced with either culture medium (○, $n = 3$ at each point) or culture medium plus 10 µM unlabeled fentanyl (∆, $n = 3$ at each point). The lines represent the best fit of these data to the respective models (see Materials and Methods).
functions to actively pump out lipophilic drugs that enter the brain endothelial cells (Schinkel et al., 1996). To determine whether fentanyl may also be a substrate for P-gp, pulse-trace experiments were performed to study the effects of both unlabeled fentanyl and the competitive P-gp inhibitor verapamil on the intracellular release (trace-step) of [3H]fentanyl or the P-gp substrate R123 (Fontaine et al., 1996; Rose et al., 1998). If fentanyl is also a competitive inhibitor of P-gp, both fentanyl and verapamil should decrease the egress of R123 and [3H]fentanyl from BBMECs. Figure 4 shows that both verapamil (100 μM) and fentanyl (10 μM) significantly decreased the egress of R123 from BBMECs. However, the reverse was true for [3H]fentanyl for which both verapamil (100 μM) and unlabeled fentanyl (10 μM) significantly increased the egress of [3H]fentanyl from BBMECs (Fig. 5). These inhibition findings suggest that verapamil is a substrate for the inwardly directed fentanyl transporter (in addition to its being a substrate for P-gp) and that fentanyl is a substrate for the outwardly directed P-gp (in addition to the evidence for an inward transporter shown in Figs. 1 and 2). To determine whether P-gp alone was transporting bidirectionally (i.e., fentanyl in and R123 out) we performed an [3H]fentanyl uptake study in which the incubation media did or did not contain Mg2+, an essential ion for P-gp ATPase function (Awasthi et al., 1994). In the Mg2+-depleted condition there was more fentanyl uptake by BBMECs (Fig. 6), consistent with the loss of outward transport of fentanyl by P-gp.

Discussion

The distribution of lipophilic drugs to tissue has traditionally been attributed to the processes of tissue blood flow and passive diffusion with the ultimate partitioning of drug between blood and tissue being dependent on the physicochemical properties of drug as it relates to the composition of adjacent media (Ishizaki et al., 1997). For instance, drugs with high octanol/water partition ratios tend to have high tissue/blood partition ratios, especially in tissues such as adipose, which have high lipid content (Barton et al., 1997). This simplistic view of drug distribution has been challenged recently by the discovery of transporters for lipophilic drugs at the blood/tissue interface (Wood, 1997). There is evidence for both inward and outward vectors of transport relative to the microvascular lumen. The ABC transporter P-gp has been found to be extensively distributed in many endothelia (Thiebaut et al., 1987; Schinkel et al., 1995), where it transports lipophilic xenobiotics away from tissue back into the microvascular lumen. At the BBB, P-gp actively pumps a variety of lipophilic drugs away from brain tissue, thus reducing the tissue/blood partition ratio that would exist by passive diffusion alone (Schinkel et al., 1996).

In contrast to the outward vector produced by P-gp, the processes mediating the extensive first pass pulmonary uptake of the lipophilic basic amine fentanyl (Roerig et al., 1987) include a saturable mechanism, suggesting a trans-
porter directed toward lung tissue, thus increasing the tissue/blood partition ratio (Waters et al., 1999). Because fentanyl is an opioid with full \( \mu \)-agonist properties, a lung-directed drug transporter has pharmacokinetic implications after rapid i.v. administration (i.e., the initial arterial fentanyl concentrations delivered to the brain depend on the extent of pulmonary uptake). However, a tissue-directed transporter at fentanyl’s central nervous system site of action would affect its pharmacodynamics (i.e., onset time and plasma-apparent EC\(_{50}\)).

This report demonstrates that BBMECs, like BPAECs, possess a high-capacity fentanyl uptake process that is saturable (\( K_M = 3.19 \mu M \)). The results in Fig. 1 indicate that at the low clinical concentrations of fentanyl (2–10 nM; Shafer and Varvel, 1991), the saturable mechanism increased the uptake of fentanyl into brain endothelium by more than 2.6-fold over that produced by simple diffusion. These results are similar to findings in BPAECs where the saturable process (\( K_M = 2.8 \mu M \)) increased endothelial uptake 3.8-fold over that by simple diffusion alone (Waters et al., 1999). In addition, BBM ECT uptake of fentanyl was reduced by ATP depletion and 4°C (Fig. 3) to a similar degree, confirming that the saturable uptake process is energy-dependent and suggesting that an active transport process is involved.

In uptake studies, whether in vivo or in vitro, cellular equilibration with the supernatant (or blood) is assumed to occur very quickly (Audi et al., 1995). In in vitro studies, equilibrium between cells and the supernatant is assumed to be complete within seconds. Indeed, in pilot studies we found that fentanyl uptake into endothelial cells was indistinguishable at incubation times between 5 and 120 min. Therefore, in uptake experiments, partition coefficients rather than transfer rate constants are estimated. In pulse-trace studies, changes in cellular drug content can be measured over time because of the large dimensions and capacity of the supernatant relative to the cellular monolayer, allowing estimation of transfer rate constants. In theory, the ratio of the diffusional equilibrium constant to the total transporter capacity from an uptake equilibrium model ought to mirror the ratio of the diffusional rate constant to the transporter rate constant of the pulse-trace kinetic model. Indeed, we found these ratios to be 0.38 in both instances, confirming that the inward transporter capacity is more than 2.6-fold greater than diffusional transport as well as confirming that direct comparisons between results from uptake and pulse-trace paradigms can be made.

Previous findings suggest that fentanyl may be a substrate of the outwardly-directed P-gp in BBMECs. First, fentanyl is a lipophilic drug and a substrate of CYP3A4 (Labroo et al., 1997), characteristics shared by other P-gp substrates (Zhang et al., 1998). Secondly, mice treated with the P-gp inhibitor PCS 833 (a cyclosporin A analog) and cyclosporin A are more sensitive to the sedative (Mayer et al., 1997) and analgesic (Cirella et al., 1987) effects of fentanyl than untreated animals, respectively. For these reasons, we performed pulse-trace studies of \(^3\)Hfentanyl and R123, a known substrate of P-gp in brain endothelium (Rose et al., 1998), using both fentanyl (10 \( \mu M \)) and the known P-gp substrate/competitive inhibitor verapamil (100 \( \mu M \); Maia et al., 1998) as potential competitive inhibitors. As reported above, 10 \( \mu M \) fentanyl greatly enhanced the egress of fentanyl from BBMECs, consistent with saturation (competitive inhibition) of an inwardly-directed transporter (Fig. 5). Verapamil (100 \( \mu M \)) was an equally effective inhibitor of this process. These results suggest that verapamil is a substrate of the fentanyl uptake transporter and, because verapamil is a classic P-gp substrate/competitive inhibitor, fentanyl uptake may be mediated by P-gp. In contrast to these results, verapamil greatly reduced the egress of R123 from BBMECs (Fig. 4), confirming the findings of others that verapamil and R123 are substrates of the outwardly directed P-gp. Fentanyl was equally effective to verapamil at reducing R123 transport by P-gp, suggesting that fentanyl too is a substrate of this outwardly directed transporter. This finding is consistent with the increased sensitivity to fentanyl of mice treated with PCS 833 and patients treated with cyclosporin A, but is inconsistent with the current findings of a net inward transport of this drug at the BBB under control conditions.

To clarify this issue we inhibited P-gp by means other than competitive inhibition, i.e., removal of Mg\({ }^{2+}\) from the supernatant. Under these conditions, fentanyl uptake was increased, suggesting that fentanyl is indeed actively transported out of BBMECs by P-gp and consistent with both the current R123 results and increased fentanyl sensitivity in subjects treated with P-gp inhibitors other than verapamil. The other major implication of these findings is that there is an active transporter for fentanyl in addition to P-gp— one that transports fentanyl and verapamil inward and has a higher capacity than P-gp at the BBB. The lower transporter contribution to the inward transport of fentanyl in BBMECs in this study (2.6 times greater than diffusion alone) compared with the previous results in BPAECs (3.8 times greater than diffusion alone) may owe to the fact that P-gp content is higher in the BBB, thus negating a portion of the inward transport in BBMECs. It is not entirely surprising that verapamil is a substrate, like fentanyl, of a transporter directed inward across the endothelium. Both drugs are lipophilic basic amines, a fact that may underlie a shared substrate specificity, and both are classic examples of drugs demonstrating high pulmonary uptake (Roerig et al., 1987, 1989).

Currently, works are being undertaken to identify and characterize this transporter. It can be expected that this information should greatly enhance our understanding of the contribution of the BBB to the pharmacokinetics and pharmacodynamics of fentanyl. It is further anticipated that this transporter may be exploitable for either reducing fentanyl effects or enhancing the central nervous system effects of other drugs.

In conclusion, these results demonstrate that the major factor governing the uptake of fentanyl into BBMECs is active carrier-mediated transport, not passive diffusion. The results also indicate that fentanyl is a substrate of P-gp in BBMECs, but the active P-gp-mediated extrusion of fentanyl in these cells is overshadowed by an active inward transport process, mediated by an as yet unidentified transporter. In addition, verapamil was shown to be a substrate of both P-gp and the fentanyl uptake transporter.

### References


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