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Transport and metabolism of opioid peptides across BeWo cells, an in vitro model of the placental barrier

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Abstract

In keeping with the advance of biotechnology, cell culture becomes an important tool for investigating the transport and the metabolism phenomena. A cell line of human origin, the BeWo choriocarcinoma cell line, was used for the study of the transport and metabolism of opioid peptides across the in vitro model of the placental barrier. Opioid peptides, both naturally occurring and their synthetic analogs, are of interest to be developed as potent analgesics and were included in this study. The apparent permeability coefficients (P_e)s of the peptides containing 4–11 amino acid or analog residues were in the range of $0.23–14.6 \times 10^{-5}$ cm/s. The (P_e)s of these peptides were comparable to those of sucrose or dextrans, hydrophilic markers. The (P_e)s of low molecular weight (MW) peptides was not dependent on their MW or molecular size, whereas an inversely linear correlation between (P_e)s and molecular size was observed with the larger peptides. Molecular sieving of the BeWo monolayer restricted the transport of the peptides with MW $\geq$ 1033 Da or molecular size $\geq$ 6.6 Å. Membrane partitioning ability and charge of the peptides were also investigated and found to be the minor factors regulating the extent of peptide permeation. Contrasting to the transport of Tyr-[D-pen-Gly-Phe-D-Pen] (DPDPE) peptide analog across the blood–brain barrier, the transport of DPDPE across the BeWo monolayers were not indicated to be via carrier-mediated transport. The major transport pathway of the opioid peptides across the BeWo monolayers was found to be via paracellular route. In metabolism studies, aminopeptidase was found to be a major enzyme type responsible for the degradation of naturally occurring peptides but not for the synthetic analogs. The finding obtained from the present study reveals the applicability of the BeWo cell line as an in vitro model for investigating placental transport and metabolism of opioid peptides.

Keywords: Placenta; Permeability; Metabolism; BeWo; Opioid peptides

1. Introduction

Since the discovery of multiple opioid receptors (Lord et al., 1977) and endogenous opioid peptides (Hughes et al., 1975), several attempts have

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been made to synthesize safe and effective agonists to selective opioid receptors. The clinically used μ-opioid receptor agonists such as morphine and meperidine are associated with side effects, which range from constipation to respiratory depression and dependence liability (Galligan et al., 1984). In addition, the above-mentioned μ-receptor agonists have the ability to rapidly cross the placental barrier, thus posing risks to the developing fetus.

The developing fetus is not known to express the δ-opioid receptor (Rapaka and Porreca, 1991). Therefore, selective δ-opioid receptor analogs can be developed without the risk of any adverse effects to the fetus. The endogenous δ-opioid receptor-specific enkephalins (leu- and met-enkephalins) have been shown to have significant analgesic properties (Hughes et al., 1975). However, due to their high susceptibility to enzymatic degradation in vivo, attempts are being made to synthesize highly selective, enzymatically stable analogs of enkephalins which can be clinically used in the treatment of pain. Mosberg et al. (1983) and Schiller et al. (1992) reported the synthesis of several synthetic analogs of leu-enkephalin with high selectivity towards the δ-opioid receptor. In addition, some of these peptides have shown good in vivo stability and appear to be promising candidates for pain treatment (Greene et al., 1996). The blood–brain barrier (BBB) transport of one synthetic analog of leu-enkephalin, Tyr-[D-pen-Gly-Phe-D-Pen] (DPDPE), was found to be carrier-mediated (Williams et al., 1996). However, no information currently exists on the placental transport and metabolism of this peptide.

The primary function of the placenta is to transport nutrients and excretory products to and from the fetus. Although passive diffusion is the principal transport mechanism across the placenta (Mirkin, 1975), several carrier-mediated transport mechanisms (e.g. for glucose, fatty acids and amino acids) have been identified (Lemons and Schreine, 1983). To study the mechanistic aspects of drug transport across the placental barrier, several in vitro models were developed for a simple and rapid method of studying. Several cell lines (e.g. JAR, JEG and BeWo) have been evaluated for their potential applications in the study of the mechanisms of trans-trophoblast transport of drugs (Ugele and Simon, 1999). The BeWo cell line is a choriocarcinoma cell line developed from a malignant gestational choriocarcinoma of the fetal placenta and was characterized in 1968 (Patillo and Gey, 1968). This cell line has been shown to exhibit morphological and biochemical characteristics, similar to the human trophoblasts. BeWo cells have been found to have transporters for glucose, amino acids (Eaton and Soorna, 1996), serotonin (Prasad et al., 1996), fatty acids (Knipp et al., 2000), multidrug resistance (Utoguchi et al., 2000), and monocarboxylic acids. Each of these transporters may also function as a drug transporter (Utoguchi et al., 1999; Utoguchi and Audus, 2000). In addition, the BeWo cell line is stable, easy to maintain by passage and forms confluent monolayers on collagen-coated polycarbonate filters in a relatively short period of time (4–5 days), making it an attractive in vitro model to study trans-trophoblast transport, uptake and metabolism of drugs (Liu et al., 1997).

Peptide transport across the placenta has not been studied extensively. Of the few reported studies, the primary mechanism of peptide transport across the placenta appears to be via paracellular diffusion (Malek et al., 1996). However, a low affinity transporter for dipeptides has been shown to exist (Meredith and Laynes, 1996). The objectives of the present work were to investigate the trans-trophoblast transport of a series of opioid peptides and to determine the effect of physicochemical properties (molecular size, molecular weight, charge and membrane partitioning ability) on their transport across the trophoblast barrier. In addition, the susceptibility of the peptides to enzymatic degradation and the mechanisms of transport of one synthetic analog (DPDPE) across the trophoblast monolayers were also investigated.

2. Materials and methods

2.1. Materials

Translucent polycarbonate filters (12 mm diameter, 0.4 μM pore size) were obtained from Fisher Scientific. ^{3}H-DPDPE and ^{3}H-DAMGO

were obtained from DuPont-NEN Research (Boston, MA). Amastatin ([(2S, 3R)-3-amino-2-hydroxy-5-methylhexanoyl]-Val-Val-Asp) was obtained from ICN (Cosa Mesta, CA). Leu-enkephalin (LE) was purchased from Sigma (St. Louis, MO). All other peptides [Tyr-D-Arg-Phe-D-Leu-NH_2 (DADLE), Tyr-Tic-Phe-Phe (TIPP), Tyr-Ticψ[CH_2–NH]-Phe-Phe (TIPP-ψ), dynorphin A (1–11), dynorphin A (2–11), dynorphin A (1–8), Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO), Tyr-D-Arg-Phe-Lys-NH_2 (DALDA), Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET), and Tyr-[D-Pen-Gly-Phe-D-Pen] (DPDPE)] were obtained from the National Institute on Drug Abuse (NIDA). Dulbecco's modified Eagles medium (DMEM), Hank's balanced salt solutions (HBSS) and trypsin–EDTA solutions were purchased from Sigma. Fetal bovine serum, heat activated (FBS/HI), was from JRH Biosciences (Lenexa, KS). Penicillin–streptomycin as a mixture was obtained from GIB Co. (Gaithersburg, MD). Rat-tail collagen was prepared in our laboratory. For performing the sensitive determination of peptides [LE, dynorphin A (1–11), dynorphin A (2–11), and dynorphin A (1–8)], 1*H*-naphtho(2,3-*d*)imidazole-6,7-dicarboxaldehyde (IMNDA), a fluorogenic derivatizing reagent, was synthesized in our laboratory. Sodium cyanide (NaCN) was purchased from Aldrich (St. Louis, MO). All other chemicals were of the highest, commercially available grade.

2.2. BeWo cell culture

The BeWo clone (b30) was obtained from Dr Alan Schwartz (Washington University, St. Louis, MO). The cells were cultured and harvested as previously described (Liu et al., 1997). For transport studies, the cells were passed onto polycarbonate membranes coated with rat-tail collagen. At a seeding density of 100 000 cells/cm^2, the cells formed monolayers in 4–5 days. The medium was changed every other day and all cells used for these studies were from passages 35–90.

2.3. Trans-monolayer permeability

Trans-monolayer permeability studies were carried out using a Side-Bi-Side™ diffusion apparatus (Crown Glass Company). The donor and receiver chambers were each filled with 3.0 ml of phosphate buffer saline solution fortified with 0.1 mM ascorbic acid (PBSA, pH 7.4). The cells faced the donor chamber as the apical (maternal) side and the membranes faced the receiver chamber as the basolateral (fetal) side. The temperature during the studies was maintained at 37 °C using thermostated water jacket. The contents of the two chambers were stirred continuously at 600 rpm with magnetic stir bars to prevent the formation of an aqueous boundary layer. Prior to the studies, the monolayer cultures were equilibrated for 30 min. Experiments were initiated by adding either 5 nM (^{3}H-DPDPE and ^{3}H-DAMGO) or 100 μM of the other peptides (dissolved in PBSA) to the donor chamber. A 100 or 176 μl sample was obtained from the receiver chamber at different time points over a 60 (^{3}H-DPDPE and ^{3}H-DAMGO) or 120 min (all other peptides) period with replacement of the buffer after each sample withdrawal. Samples were analyzed by liquid scintillation spectrometry (^{3}H-DPDPE and ^{3}H-DAMGO) using a Beckman scintillation counter (LS 6800) or by High-Performance Liquid Chromatography (HPLC). In addition, transport experiments were conducted with ^{14}C-sucrose, a marker for paracellular transport. Polycarbonate membranes treated with rat-tail collagen but without cell monolayers were used as a control. The apparent permeability coefficients were estimated using the following equation:

$$P\ (\mathrm{cm/s}) = X/(A^{*}t^{*}C_{\mathrm{d}})$$

where P is the apparent permeability coefficient, X is the amount of substance (mol) in the receiver chamber at time t (s), A is the diffusion area (0.636 cm^2) and C_d is the concentration of the substance in the donor chamber (mol/cm^3). The permeability coefficients were expressed as the mean ± SD from three to six different monolayers.

The apparent permeability coefficients for the BeWo monolayers, P_e, were calculated from the following relationship:

$$1/P_{\mathrm{t}} = 1/P_{\mathrm{c}} + 1/P_{\mathrm{e}}$$

where P_t is the apparent permeability coefficients for the collagen-coated polycarbonate membranes

in the presence of BeWo cell monolayers and P_c is the apparent permeability coefficients for collagen-coated polycarbonate membranes alone (Adson et al., 1994).

In order to determine the mechanism of DPDPE transport across the BeWo monolayers, transport experiments ($n = 4–6$) of 5 nM ^{3}H-DPDPE were carried out in the presence of excess (300 μM) unlabeled DPDPE. In addition, to ensure that DPDPE was not being metabolized by the enzymes of the BeWo cells during its transport, separate transport studies of ^{3}H-DPDPE were conducted in the presence of an aminopeptidase inhibitor, amastatin (50 μM). Apparent permeability coefficients for DPDPE were calculated in the presence and absence of amastatin.

2.4. Peptide analysis

Analyses of four peptides (DALDA, DADLE, DPDPE and DSLET) were conducted using a reversed-phase HPLC (RP-HPLC) with UV detection. Aliquots of samples (30 μl in triplicate) were injected on a 4.5×250 mm ODS (C18) column (Waters, Milford, MA) by an autosampler (Shimadzu SIL-10 AD). The column was controlled at the temperature of 37 °C. The mobile phase was consisted of a mixture of 0.1 M sodium phosphate buffer, pH 3.0 (containing 0.1% heptanesulfonic acid) and acetonitrile (75:25 v/v), at a flow rate of 1.5 ml/min. The eluting peptides were detected at a wavelength of 210 nm using a Shimadzu UV detector (SPD 6A).

For some peptides (LE and dynorphins), the permeants in the receiver chamber were not detected at low levels with the above analytical method. Thus, the detection sensitivity was enhanced by using a precolumn derivatization combined with an RP-HPLC. The peptides were treated with 1*H*-napthol(2,3-*d*)imidazole-6,7-dicarboxaldehyde–sodium cyanide (IMNDA–NaCN) to form fluorescent products. The derivatization reaction was optimized in order to yield the highest levels of peptide derivatives. The optimal fluorescent product was obtained when the volume of sample was utilized at 176 μl. Subsequently, the aliquot of sample was automatically derivatized with 12 μl of 5 mM NaCN and 12 μl of 5 mM IMNDA derivatizing reagent using the derivatized program of an autosampler. Under these conditions, the concentrations of peptides in nanomolar levels could be detected within 10 min. Therefore, the 176 μl sample volume was utilized with the considerations of the maximum derivatized product with appropriate reaction time and reactant concentrations, volume of total sample and instrumentation requirement. Subsequently, an aliquot of 50 μl of reaction mixture was injected onto the HPLC column. A Spherisorb® ODS (5 μM, 80 Å, 250×4.6 mm) column (Alltech, Deerfield, IL) was used for the determination of LE- and dynorphin-IMNDA(CN) derivatives. Gradient elution with solution A [10% acetonitrile (ACN) in 0.05% trifluoroacetic acid (TFA) and 20 mM 1-heptane sulfonic acid (HSA)] and solution B [10% of 0.05% TFA and 20 mM 1-heptane sulfonic acid (HSA) in ACN] was performed with a linear gradient of 37–77% of solvent B within 20 min. For TIPP and TIPP-ψ determinations, an in-house packed Hypersil® C18 (5 μM, 80 Å, 150×4.6 mm) was used in the study. Isocratic elution with 28% ACN in 26 mM TFA was employed to elude the native form of TIPP and TIPP-ψ. All separations were performed at an ambient temperature at the flow-rate of 1.0 ml/min. Fluorescence intensity was monitored either at $\lambda_{ex} = 467$ nm and $\lambda_{em} = 510$ nm for the IMNDA derivatives or at $\lambda_{ex} = 208$ nm and $\lambda_{em} = 305$ nm for the native TIPP and TIPP-ψ peptides.

2.5. Stability of opioid peptides upon exposure to the BeWo monolayers

The BeWo cells were seeded at 100 000 cells/cm^2 into the 12-well tissue culture plates. Collagen-coated polycarbonate membranes were not required in this study. Cell maintenance was performed as described earlier; however, care was taken due to an easier detachment of the cell membranes from the plastic surface than the surface of the collagen-coated polycarbonate filters.

The cell monolayers were washed three times with 37 °C PBSA solution to eliminate other primary amines such as amino acids in cell culture media or the other peptides that cells might have released to the medium. Subsequently, the tissue culture plates were incubated in a temperature-controlled shaking water-bath (60 rpm, 37 °C) for 10 min. Afterwards, 1 ml of a peptide solution (25 μM) was added to each well including control wells, the wells without the presence of BeWo cell monolayers. Immediately after the addition of the peptide solution, peptide aliquots were withdrawn for the initial concentration determination. At 5, 15, 30, and 60 min, 176 μl aliquots were pipetted out from the subsequent wells. The samples were collected in polypropylene vials and were then frozen immediately until the analysis.

2.6. Membrane partitioning ability

In this study, the relative membrane partitioning ability of the opioid peptides was determined from the ability of the peptides to interact with the immobilized artificial membrane (IAM). An IAM chromatography column (4.6 mm i.d. × 100 mm, 5 μm, 300 Å, Regis Technologies, Inc., Morton Grove, IL) with 5–10% acetonitrile (ACN) in 10 mM phosphate buffer as a mobile phase was used in determining the ability of the peptides to interact with phosphatidylcholine (PC) analog membranes. The PC analog membranes are the artificial membranes that mimic the lipid environment found in the cell membrane (Pidgeon et al., 1995). A 20 μl aliquot of each peptide solution (100 μM) was injected onto the column (flow rate 1.0 ml/min), and the eluate was monitored by the UV (210 nm) detector (Shimadzu, Columbia, MD). The IAM capacity factors (k'_{IAM}) of the opioid peptides were calculated from the following equation:

$$k'_{IAM} = \frac{t_R - t_0}{t_0}$$

where t_R is the retention time of a peptide and t_0 is the column void volume. Permeability coefficients were plotted against the k'_{IAM} of the opioid peptides in order to evaluate the relationship between the relative membrane partitioning ability of the peptides and ability to cross the BeWo monolayers.

2.7. Molecular size

^{1}H-NMR spectroscopy was utilized to determine the diffusion coefficients of the opioid peptides and analogs. An opioid peptide in a concentration of 1 mM was dissolved in 10% D_2O and 90% H_2O. A 500 MHz NMR spectrometer (Bruker AM-500) interfaced to an auxiliary PC-driven 15 A gradient pulse generator specifically designed for diffusion studies (Digital Specialties, Chapel Hill, NC) was employed in this study. The NMR spectra were acquired using the pulsed field gradient bipolar longitudinal eddy current delay pulse sequence. During the experiments, the diffusion delay time was held constant at 200 ms and the gradient pulse duration at 2 ms. The gradient pulses was sequentially increased (i.e. 0.9, 1.4, 1.8, 2.6, 3.4, 4.2, 5.0 and 5.6 ms) until one order of magnitude decreasing of a peak height of interest was observed. The experimental gradient strength was calculated from a coil constant of 5.15 G/cm/A based on the known diffusion coefficient of β-cyclodextrin at 25 °C, 3.23×10^{-6} cm^2/s. By linear regression analysis, the diffusion coefficients at 25 °C were obtained from the slope of the semilogarithm plots of the peak height versus the square of the pulse gradient. Applying the Stokes–Einstein equation, molecular radii (radius of gyration) of the peptides were calculated from their diffusion coefficients determined from the NMR experiments:

$$D = kT/6\pi\eta r \quad \text{(Stokes–Einstein equation)}$$

where D is the diffusion coefficient (m^2/s), k is Boltzmann's constant, T is the absolute temperature (K), r is the radius of a spherical particle and using viscosity, $\eta = 0.9183$ cP calculated from the standard D and η of water at 25 °C and D of 10% D_2O and 90% H_2O at 25 °C.

3. Results and discussion

3.1. Molecular weight and size of peptides versus permeability coefficients

Although very few studies have been conducted, peptide transport across the placenta appears to be primarily via passive diffusion (Malek et al., 1996). The placenta, unlike the gastrointestinal or the BBB, is a 'leaky' barrier and allows for a relatively free exchange of substances. The apparent permeability coefficients of most of the peptides studied (Table 1) were in the same range of the permeability coefficients of hydrophilic markers such as ^{14}C-sucrose, fluorescein and fluorescein isothiocyanate conjugated dextrans (FITCDs) (Liu et al., 1997). Interestingly, limited permeation was observed with large molecular weight peptides, e.g. dynorphin A series (1–11, 2–11 and 1–8) (Table 1, Fig. 1). Dynorphin A (1–11) (MW 1363) was the least permeable across the BeWo monolayers. Compared with the set of high molecular weight peptides (MW $\geq$ 1033 Da), the permeability coefficients of small peptides (MW 513–645 Da) were higher and not correlated to their molecular weights.

Concerning various conformations of peptides in an aqueous solution, an investigation of the relationship between peptide permeability and their size may give more meaningful comparison than the correlation of their molecular weights. Fig. 2 shows the relationship between the apparent permeability coefficients and the molecular radii of the peptides determined from their diffusion coefficients using NMR spectroscopy technique. Similar to the relationship between the permeability coefficients and the molecular weights, an inverse linear correlation was observed only for the peptides with MW range of 1033 Da or higher and radius range of 6.6 Å and larger (Fig. 2). For smaller peptides, poor relationships were found when either molecular weights or molecular size were employed, indicating that other factors such as lipophilicity, hydrogen bonding or peptide charge may influence the permeation properties of the peptides. A good linear correlation was observed between molecular weights and molecular radii of the peptides ($R^2 = 0.94$), suggesting that molecular weight could be used interchangeably with molecular size as the parameter for the prediction of peptide permeability across the BeWo monolayers. As the peptides included in our study are relatively hydrophilic and their P_e(s) are comparable to the hydrophilic markers, passive diffusion through water-filled transmembrane channels or pores which is a primary transport mechanism of most hydrophilic substances was suggested to be a major transport mechanism for these peptides. In general, water-

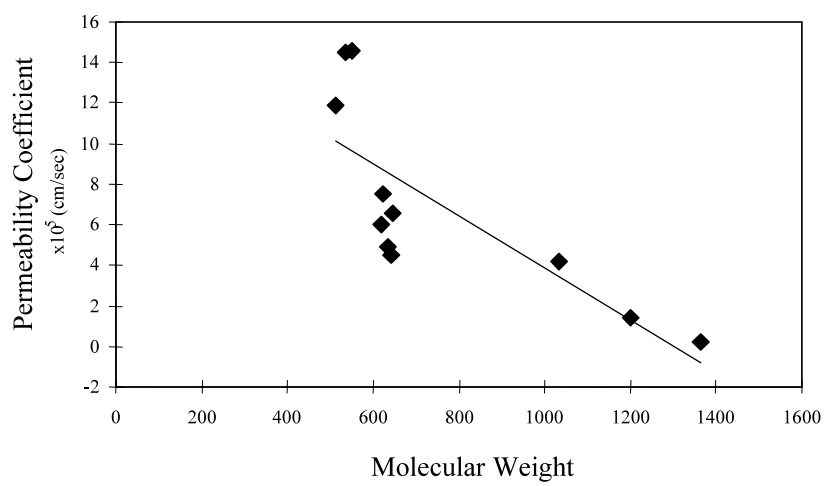

Fig. 1. Plot showing the relationship between the molecular weights of model peptides and apparent permeability coefficients for permeation across BeWo cell monolayers.

Table 1
Physicochemical properties and apparent permeability coefficients (P_e) of opioid peptides

Sample	Amino acid composition	MW	Permeability coefficient $\times 10^5$ (cm/s)	Radius (Å)	Charge at pH 7.4	Specific experiment
Sucrose		321	8.34 ± 1.32 [a]	–	–	–
DAMGO	Tyr-Ala-Gly-N-Me-Phe-Gly-ol	513	11.90 ± 2.90 [a]	4.64	1	–
Leu-Enkephalin	Try-Gly-Gly-Phe-Leu	537	9.57 ± 4.63	4.85	0	Stability
DALDA	Tyr-D-Arg-Phe-Lys-NH_2	552	14.6 ± 2.50 [a]	5.41	2	–
TIPP-ψ	Tyr-Ticψ[CH_2NH]-Phe-Phe	618	5.99 ± 0.62	4.85	1	Stability
DADLE	Tyr-D-Ala-Gly-Phe-D-Leu	624	7.49 ± 1.44	5.10	0	Stability
TIPP	Tyr-Tic-Phe-Phe	634	4.93 ± 0.60	4.84	0	Stability
DSLET	Tyr-D-Ser-Gly-Phe-Leu-Thr	641	4.53 ± 2.89 [a]	5.63	0	–
DPDPE	Tyr-[D-Pen-Gly-Phe-D-Pen](cyclic)	645	6.45 ± 0.97 [a]	5.18	0	Carrier mediated
Dynorphin A (1–8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Lle	1033	4.19 ± 2.40	6.66	2	–
Dynorphin A (2–11)	Gly-Gly-Phe-Leu-Arg-Arg-Lle-Arg-Pro-Lys	1200	1.40 ± 0.34	7.43	4	–
Dynorphin A (1–11)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Lleu-Arg-Pro-Lys	1363	0.23 ± 0.04	7.82	4	Stability

The (P_e)s were expressed as the mean $\pm$ SD from three to six different monolayers.
[a] Data was previously reported by Chandorkar et al. (1999).

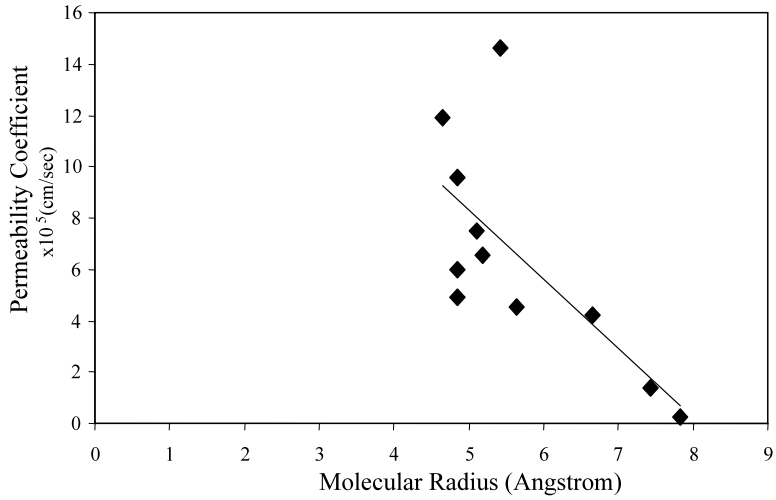

Fig. 2. Plot showing the relationship between the molecular radii of model peptides and the apparent permeability coefficients for permeation across BeWo cell monolayers.

soluble drugs with molecular weights below 800 Da (Morriss et al., 1994) and perhaps closer to 1000 Da (Willis et al., 1986; Malek et al., 1996) appear to passively diffuse across the human placenta. Similarly, the peptides included in our study whose molecular weights are higher than 1033, such as dynorphin A (1–11), (2–11) and (1–8), encountered some permeability difficulties due to a sieving property of the membrane. These observations implied that the BeWo cell monolayers described by Liu et al. (1997) are likely to have similar permeability properties, such as pore size and porosity, to the human placental membrane.

3.2. Membrane partitioning ability versus permeability coefficients

Solute-membrane partition coefficients, k'_{IAM}, is another parameter that was investigated. Immobilized artificial membrane (IAM) chromatography was a selected method to observe the relative membrane partitioning ability of the peptides. In general, an organic modifier is usually required in a mobile phase for compounds, which strongly interact, with the IAM stationary phase. Additional isocratic elutions at various percentages of organic modifier are normally required to extrapolate to obtain theoretical capacity factors at 0% organic modifier ($k'_{IAM,w}$). However, the retention time and elution order of the compounds did not change dramatically as the organic modifier was incorporated in a small fraction (5–10%) (Bidlingmeyer et al., 1979; Barbato et al., 1997). Valko et al. (2000) reported the use of organic modifier for the IAM interaction determination of 48 structurally unrelated compounds. One finding was a good correlation ($r = 0.98$) between $k'_{IAM,w}$ and $k'_{IAM,20}$ obtained from the mobile phase with 0 and 20% acetonitrile. Consequently, the mobile phase with a low percentage of acetonitrile could be used interchangeably with the 0% acetonitrile mobile phase in order to obtain the relative capacity factors for the correlation determination purpose. Fig. 3a showed the plot between the capacity factor (k'_{IAM}, at pH 7.4) and the permeability coefficients of the opioid peptides. Dynorphin A (1–8) has the longest retention time (~ 25 min) followed by dynorphin (2–11), TIPP-ψ, and dynorphin (1–11), respectively. However, broad peaks were observed from only the basic peptides, such as dynorphin A(s), which possessed multiple positive charges at physiological pH. This phenomenon was clearly explained by Avdeef et al. (1998), in which an electrostatic interaction occurred between the charged side chains and the phosphate head groups of phosphatidylcholine

analogs. Similarly the prolonged retention time and thus increased k'_{IAM} value of acids and bases was primarily due to the electrostatic interaction of the drug with the column silanol groups, although the column was filled with end-capped packing material (Ottiger and Wunderli-Allenspach, 1999). Therefore, the k'_{IAM} is highly dependent on the non-specific binding and the ionization states of drugs in the physiological pH range.

The peptides with multiple positive charges [i.e. dynorphin A(s)] strongly interacted with the stationary phase. Valko et al. (2000) studied the retention behavior of 48 compounds at various pH and found charged basic compounds strongly interacted with the IAM column. The interaction of basic compounds to the IAM stationary phase at pH 2 was also shown to be less than that observed from pH 7.4. When lipophilicity was considered, the results from IAM chromatography could be significantly different from the results determined by other lipophilicity scales. Ottiger and Wunderli-Allenspach (1999) reported the pH-dependent partition behavior of model acidic and basic compounds. A gradual decrease of the partition coefficients (K_{IAM}) was observed for the protonated bases with decreasing pH values and for the deprotonated acids with increasing pH. To suppress the ionic interaction between solute and stationary phase, a well-known ion-

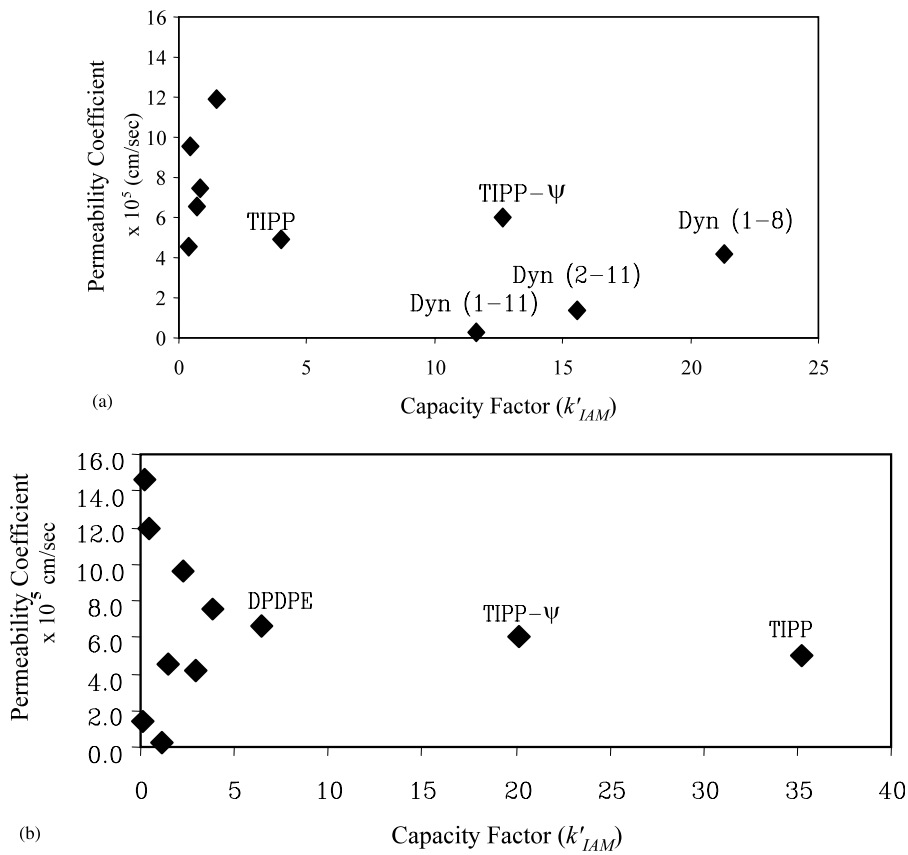

Fig. 3. (a) Plot showing the relationship between the capacity factors (k_{IAM}) at pH 7.4 and the permeability coefficients for opioid peptide permeation across BeWo cell monolayers. Chromatographic conditions: IAM (4.6 mm × 100 mm, 300 Å) column with 10% ACN in 10 mM sodium phosphate buffer, pH 7.4, UV detection at 210 nm. (b) Plot showing the relationship between the capacity factors (k_{IAM}) at pH 3.0 and the permeability coefficients for opioid peptide permeation across BeWo cell monolayers. Chromatographic conditions: IAM (4.6 mm × 100 mm, 300 Å) column with 5% ACN in 10 mM sodium phosphate buffer, pH 3.0 and 2 mM 1-heptane sulfonic acid, UV detection at 210 nm.

pairing agent, 1-heptane sulfonic acid (2 mM), was included in an acidic mobile phase (10 mM sodium phosphate buffer, pH 3.0). In this mobile phase, the solute-IAM stationary phase interaction was primarily from hydrophobic interaction. As shown in Fig. 3(b), TIPP and TIPP-ψ possessed long retention times (45.3 and 26.5 min), suggesting the lipophilic character of these compounds, whereas the dynorphins' retention times were in the range of 1.4–4.9 min. However, this mobile phase and the values obtained need to be validated by other experiments for lipophilicity measurement. In this study, the IAM capacity factors were not correlated to the permeability coefficients of the peptides across the BeWo monolayers. Other factors, such as molecular weight and molecular size, were found to play more important role in the permeability. Nevertheless, IAM chromatography can be used to predict the extent and types of interaction between solutes and cell membranes. Pidgeon et al. (1995) suggested that the IAM chromatography is unsuitable for the prediction of the small peptides or compounds which permeate through water-filled channels, since the transport via this pathway is independent of the lipophilicity or cell membrane partitioning ability of these compounds.

Due to the hydrophilic nature of these peptides, it is unlikely for them to be transported via a trans-cellular mechanism. However, additional experiments were carried out to determine whether a carrier-mediated mechanism exists in the trans-trophoblast transport of the opioid peptides. Williams et al. (1996) reported the presence of a saturable transport system involved in the BBB transport of DPDPE. Therefore, studies were initiated to determine the trans-trophoblast transport of DPDPE using the in vitro cell culture model. Although DPDPE has been shown to be stable against enzymatic degradation, the transport of ^{3}H-DPDPE was studied in the presence of amastatin (50 μM). As seen from the data (Fig. 4), the permeability coefficients of ^{3}H-DPDPE were not influenced by the presence of an excess amount (300 μM) of unlabelled DPDPE or amastatin [($P < 0.05$) from ^{3}H-DPDPE alone]. These studies show that DPDPE is resistant to enzymatic hydrolysis by the placenta and its primary mechanism of transport across the placenta is via passive diffusion.

Although a number of observations had been reported about the transport systems of nutrients and electrolytes across the placenta (Morriss et al., 1994), very limited information is available on peptide transport. To date, one uptake study reported on the presence of very low affinity dipeptide transporter(s) found in brush-border membrane vesicles (BBMV) prepared from human full-term placenta (Meredith and Laynes, 1996). However, these peptide transporters were not responsible for peptide uptake into human placental BBMV. A simple diffusion mechanism was also suggested to be a transport pathway of oxytocin from the study using in vitro dually perfused isolated cotyledons from term human placenta (Malek et al., 1996) and also for the placental transport of cyanocobalamin (Vit B12) across the human and guinea pig placenta (Willis et al., 1986), while the other types of transport mechanisms remain unclear. For all the peptides included in this study, less than 10% of the total amount of the peptides were observed in the receiver chambers at the chosen sampling intervals. Unlike the other hydrophilic peptides, the permeation of rather lipophilic peptides, TIPP, was observed with the lag-time of 30 min. Contrasting to TIPP-ψ, saturation of the permeation was observed after 120 min in TIPP transport study (Audus et al., 1998). TIPP also had the longest k'_{IAM} in the conditions that electrostatic interactions were suppressed. From mass balance consideration, the recovery of most peptides was approximately 100%; except for TIPP. About 18% of the TIPP was lost from the initial amount. Since TIPP is an enzymatically stable peptide, the loss should not result from the metabolic instability. These observations suggested that a partial amount of TIPP could remain adhered to the membrane and/or in the cells, implying that the trans-cellular transport could be another pathway for relatively hydrophobic peptides, such as TIPP in this case. Surprisingly, TIPP-ψ and DPDPE, which are also relatively hydrophobic, did not show the same transport behavior as the TIPP peptide. The difference in their transport behavior

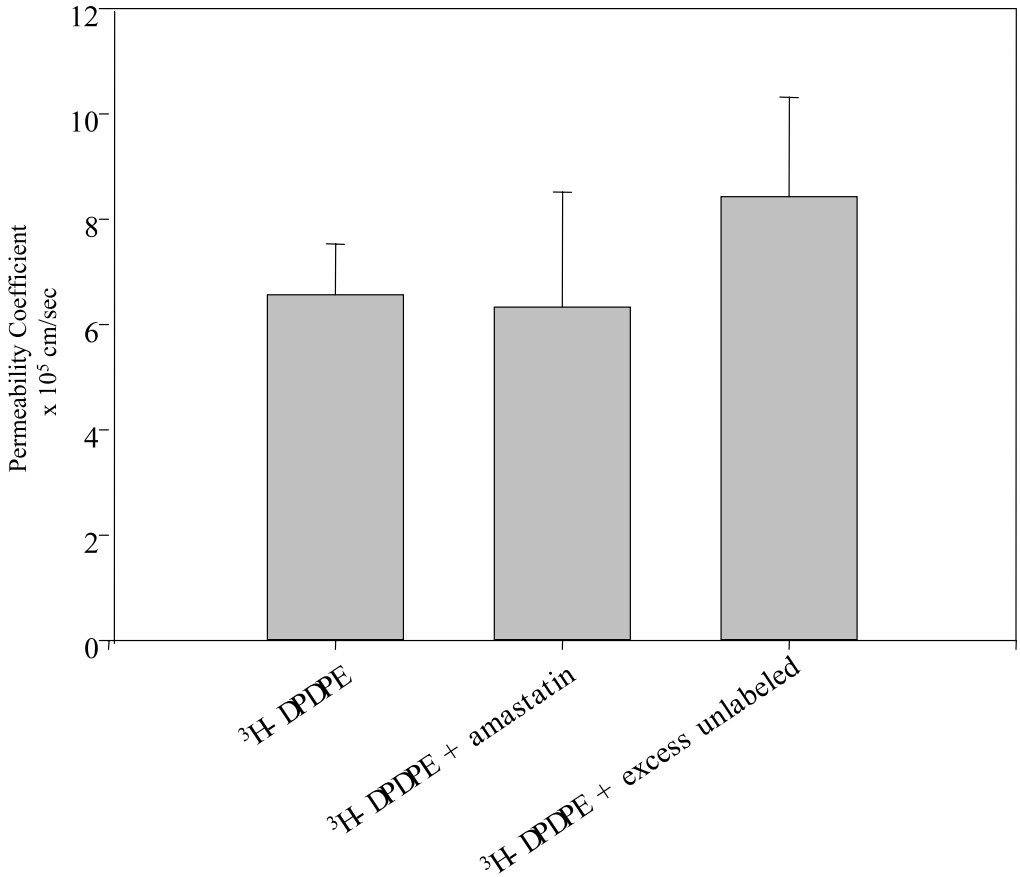

Fig. 4. Apparent permeability coefficients for ^{3}H-DPDPE permeation across BeWo cell monolayers in the presence of 50 mM amastatin or 300 μM unlabeled DPDPE at 37 °C.

could be partly due to the difference in the other types of interactions, such as hydrogen-bonding between the peptides and cell membrane. However, the transport behavior of TIPP, TIPP-ψ and DPDPE should be investigated further.

Berhe et al. (1987) reported the importance of charge effects on the permeability of positive or negative horseradish peroxidase across guinea pig placenta. Positively charged protein was found to permeate across the placenta better than the negatively charged protein. However, no relationship between the charges and the permeability coefficients for the peptides was observed in this study (Fig. 5). Therefore, the changes of maternal and fetal plasma pH would not be expected to affect the permeability of these peptides.

3.3. Stability of opioid peptides upon exposure to BeWo cell monolayers

Aminopeptidases, carboxypeptidases, endopeptidases and dipeptidases were previously reported to be the enzymes responsible for degrading peptides in the human placenta. Widely distributed in different parts of the placental cells, the transports of both naturally occurring and synthetic peptides are largely inhibited by peptidases (Shimamori et al., 1988; Kenagy et al., 1998). In this study, the naturally occurring opioid peptides, leu-enkephalin (LE), dynorphin A (1–11) and the synthetic peptides, DADLE, TIPP and TIPP-ψ, were incubated with the BeWo cell monolayers at 37 °C. The surface area of a 12-well plate used in

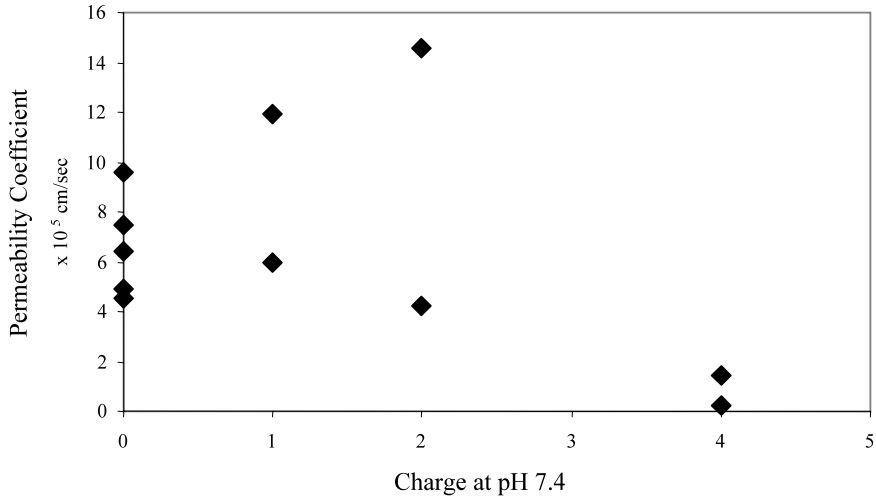

Fig. 5. Plot showing the relationship between the charge of peptides at pH 7.4 and the permeability coefficients.

the metabolism study was larger than the surface area of Side-Bi-Side™ diffusion unit, and the degradation of the naturally occurring peptides became significant. In the case of LE, although more than one site of the peptide bonds of LE is vulnerable to the enzymatic degradation, the major metabolite observed on chromatograms was identified to be des-Tyr-leu-enkephalin. Rapid degradation was also observed for the dynorphin A (1–11), another naturally occurring opioid peptide (Fig. 6). The half-life of the intact dynorphin A (1–11) was found to be about 15 min. The presence of aminopeptidases in the BeWo cells was also confirmed in this study. The dynorphin A (1–11) was rapidly converted to several small fragments including des-Tyr peptide [dynorphin A (2–11)], which was the major identifiable metabolite. The dynorphin A (2–11) was subsequently metabolized to other unidentified fragments. Contrasting to the observation in plasma, dynorphin A (1–13) were 80% metabolized by carboxypeptidases (Muller and Hochhaus, 1995), yielding dynorphin A (1–12). In this study, dynorphin A (1–10) was found to be of insignificant amounts on the exposure of dynorphin A (1–11) to the BeWo cells, suggesting that aminopeptidases are the major enzymes in this cell type. The peptidase activity in the BeWo cells was reported to be comparable to the activity in the placental cells from the primary cultures of the human cytotrophoblasts. However, the presence of gestation time, environment, smoking habits, drugs, metal and hormonal factors were shown to be important to the activity of the peptidases (Shimamori et al., 1988; Kenagy et al., 1998). In contrast to the naturally occurring peptide counterparts [LE and dynorphin A (1–11)], the synthetic peptides (DADLE, TIPP and TIPP-ψ) were more stable upon incubation with the BeWo cells as we have shown in a previous report (Chandorkar et al., 1999). Therefore, the incorporation of unnatural

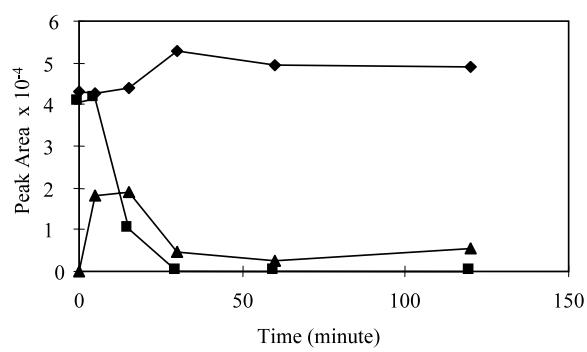

Fig. 6. Stability of dynorphin A (1–11) upon the incubation with the BeWo cell monolayers, at 37 °C, data points are the means averaged from $n = 3$. –◆– control without cells; –■– samples with cells; –▲– identified degradant [dynorphin A (2–11)].

amino acid residues into the peptide structures gave the peptides more resistance to placental enzyme degradation. However, awareness should be made to the chemical modification of the peptide structure, since the ability of the peptides to bind with opioid receptors is highly dependent on the peptide stereochemistry (Schiller et al., 1992). The introduction of D-amino acid residue(s) may compromise or change the binding selectivity to the receptors. In addition, the presence of varieties of peptidases that can cleave at different sites of peptides increases the challenges of employing the chemical modification approach for the enhancement of peptide stability against enzymatic degradations.

4. Conclusions

The BeWo cells served as both metabolic and physical barriers to the permeation of the peptides. Restricted permeation due to a sieving property of the membrane was observed with large peptides, which have their molecular weights ≥ 1033 or molecular radii ≥ 6.6 Å. The membrane partitioning ability and charge character of peptides appeared to be minor factors influencing the peptide permeation. Therefore, the passage through the paracellular route was suggested to be a primary pathway for the transport of hydrophilic peptides. However, the presence of aminopeptidase in BeWo cells largely inhibited the distribution of the peptides across the BeWo placental barrier. This study has confirmed the stability of structurally modified peptidomimetics, in which peptides containing D-amino acid or unnatural residues were found to resist enzymatic degradation upon incubation with the BeWo cells. Unlike the other types of in vitro blood–brain or gastrointestinal barriers, the BeWo placental membrane is a leaky barrier, which allows the easy passage of substances, especially small hydrophilic compounds. Finally, information from this study can provide a basis for eventual future designs of peptide molecules with minimal penetration through the placental barrier and minimal side effects for obstetric analgesic applications while maintaining analgesic activity in the mother.

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