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Studies on the transport of thyrotropin-releasing hormone (TRH) analogues in Caco-2 cell monolayers

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Abstract

The transport mechanisms of thyrotropin-releasing hormone (TRH) and its pharmacologically active analogues ((3-methyl-His^2)TRH (MeTRH), taltirelin, montirelin, azetirelin) across Caco-2 cell monolayers were characterized. The results of kinetic analysis showed a linear relationship between the concentration (over the range 0.5–10 mM) and apical-to-basolateral transport rate of these agents. The permeability coefficients (P_{app}) of these agents were not substantially different from each other, and their P_{app} ratios of the basolateral-to-apical over the apical-to-basolateral transport were close to one (0.73–1.23). The cellular transport of [^{3}H]MeTRH at low concentrations (3–15 nM) showed a linear relationship between the concentration and transport rate. The transport of [^{3}H]MeTRH in Caco-2 cell monolayers was neither affected by TRH nor TRH analogues, and there was little difference in P_{app} values between [^{3}H]MeTRH and [^{14}C]mannitol. The cell-per-medium ratio of [^{3}H]MeTRH in the cellular uptake experiment was similar to the value of [^{14}C]mannitol. A large excess of TRH and MeTRH did not significantly influence cell-per-medium ratios of [^{3}H]MeTRH in Caco-2 cell monolayers. The k'_{IAM} value, which represents lipophilicity, was decreased in the following order: montirelin > taltirelin > TRH > azetirelin, and the values varied from 0.234 to 1.028. These results indicate that a paracellular passive diffusion may be the major route for the transport of TRH and its analogues in Caco-2 cell monolayers.

Introduction

Thyrotropin-releasing hormone (TRH) has been used clinically for the treatment of spinocerebellar degeneration (Mori et al 1999) and disturbance of consciousness (Manaka et al 1977). However, TRH has some disadvantages, such as its short duration of action and lack of pharmacological effects following oral administration. To overcome these problems, a number of novel TRH analogues that are resistant to enzymatic degradation have been synthesized. Thus, some stable TRH analogues, compared with TRH, have been shown to exert a more potent pharmacological effect in both animals and man (Horita 1998). Our previous studies have revealed that the novel pharmacologically active TRH analogues, JTP-2942, taltirelin and montirelin, significantly bind to the brain TRH receptors in-vivo (Urayama et al 1999, 2001).

There are several studies focused on the transport of TRH itself across biological membranes (Gan et al 1993; Thwaites et al 1993). The intestinal epithelium is a barrier for oral absorption of hydrophilic drugs, because these compounds cannot easily permeate the lipid bilayer of the cell membrane and the tight junctions restrict their passage through intercellular space. It has been reported that the bioavailability of TRH in rats, dogs and man is usually low (< 5%) and dose-dependent (Yokohama et al 1984). The Caco-2 cell line, well-differentiated human intestinal cells derived from a colorectal carcinoma, has been used as a potential in-vitro model for the absorption and metabolism studies of therapeutic agents (Fisher et al 1999; Chu et al 2001). Recent studies on drug transport in the Caco-2 cell line have demonstrated the presence of carrier-mediated transport systems for various drugs as well as natural substrates (Tamai et al 1995, 1997). It has been shown that a peptide transport system is also expressed in the Caco-2 cell line (Dantzig & Bergin 1990; Inui et al 1992) and it has

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broad substrate specificity for di- and tri-peptides with K_m values of millimolar order (Walter & Kissel 1994; Moore et al 2000). Gan et al (1993) and Thwaites et al (1993) postulated a passive and paracellular transport of TRH through Caco-2 cell monolayers, but Walter & Kissel (1994) observed an active transport component. Thus, the transport pathway of TRH across biological membranes is controversial. Furthermore, the transport mechanisms of pharmacologically active TRH analogues across biological membrane have not been examined. Thus, this study was undertaken to investigate the mechanism of cellular transport of several pharmacologically active TRH analogues (MeTRH, taltirelin, montirelin and azetirelin) in Caco-2 cell monolayers. Our data revealed that paracellular passive diffusion might be the major pathway for the transport of these agents across Caco-2 cell monolayers.

Materials and Methods

Chemicals

[^{3}H]-(3-methyl-His^2)TRH (MeTRH; 98 Ci mmol^{-1}) and [^{14}C]mannitol (58.1 mCi mmol^{-1}) were purchased from DuPont-NEN Research Products Co. Ltd (Boston, MA). Taltirelin, montirelin and azetirelin were donated by Tanabe Seiyaku Co. Ltd (Osaka, Japan), Nippon Shinyaku Co. Ltd (Kyoto, Japan) and Yamanouchi Pharmaceutical Co. Ltd (Tokyo, Japan), respectively. TRH and MeTRH were obtained from Peptide Institute Inc. (Osaka, Japan) and Sigma Chemical Co. Ltd (St Louis, MO), respectively. Each agent was dissolved in distilled water and diluted in Hanks' balanced salt solution (HBSS, pH 7.4) for experiments. All solutions were prepared freshly each day. All other chemicals were obtained from commercial sources.

Caco-2 cell culture model

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and were cultured at 37 °C in Dulbecco's modified Eagle medium, containing 10% FBS and 1% non-essential amino acids, in an atmosphere of 5% CO_2 and 90% relative humidity according to Tsuji et al (1994). The cells used in this study were between passage 35 and 50. Cells grown in 75-cm^2 T-flasks (Costar, Cambridge, MA) were supplied in 25 mL of culture medium. Cells were passed every 3–4 days at a split ratio of 3–5 and confluence was reached within 4–5 days. For transport and cellular uptake studies, cells were seeded on either 1.0-cm^2 or 4.7-cm^2 polycarbonate membranes of Transwells (0.4 μm pore size, 1 cm^2 × 12 wells; Corning, Bedford, MA) or well dishes (Nunclon multidishes, 4.5 cm^2 × 6 wells; Nalge Nunc Int. Co., Rochester, NY), respectively. Media were changed every 2–3 days after seeding. The transport study was performed on separate sets of Caco-2 cell monolayers 21 days after seeding onto transwells. The integrity of each batch of cells was tested by measuring the flux of [^{14}C]mannitol in representative cell monolayers. Apical-to-basolateral side flux for this paracellular marker was approximately 1% h^{-1}.

Cellular transport study

The transepithelial transport of TRH and its analogues across Caco-2 cell monolayers was determined in duplicate. Cell monolayers were rinsed twice with prewarmed HBSS (pH 7.4) and pre-incubated for 30 min at 37 °C. Each agent was dissolved in HBSS at a concentration of 0.5–10 mM for unlabelled compounds, 3–15 nM for [^{3}H]MeTRH, and 4.1 kBq mL^{-1} for [^{14}C]mannitol. The solution of these agents was applied to the donor compartment, and HBSS without agents was placed in the receiver compartment. Transport experiments were performed by measuring the apical-to-basolateral and basolateral-to-apical flux of TRH and its analogues. The volumes added were 500 μL (apical side) and 1500 μL (basolateral side), respectively. From the receiver compartment, samples were removed up to 120 min. The transport rates of TRH and its analogues were determined by measuring the amount of these agents present in the receiver compartment at various time points. The transported amount of unlabelled and labelled compounds was determined by HPLC or liquid scintillation counter (Aloka, LSC-400), respectively.

Cellular uptake study

The uptake of [^{3}H]MeTRH in Caco-2 cells was measured in the absence or presence of TRH and MeTRH at a concentration of 1 mM. Cell monolayers were pre-incubated for 30 min at 37 °C in HBSS. The uptake study was initiated by adding 1.0 mL of HBSS containing [^{3}H]MeTRH (15 nM) and [^{14}C]mannitol (4.1 kBq mL^{-1}). After an incubation period of 1–5 min, cell monolayers were washed 3 times with PBS(−) and solubilized in 1 mL of 1 M NaOH for 40 min at 60 °C. The cellular uptake of [^{3}H]MeTRH compared with [^{14}C]mannitol was determined from the radioactivity measured by liquid scintillation counter. The protein assay was carried out with BCA Protein Assay Reagent (Pierce, Rockford, IL).

HPLC analysis and determination of partition coefficient

The concentration of TRH and TRH analogues was measured by the HPLC procedure (Werner et al 1997). The instrumentation consisted of a pump (880-PU; Nippon Bunko Co., Ltd) and a UV detector (UVIDEC-100-V, Nippon Bunko, Co. Ltd). All substances were separated on a reverse-phase column (STR-ODSII, 4.6ϕ × 250 mm; Shinwa Chemical Industries Ltd) using a mobile phase of 0.1% trifluoroacetic acid, pH 2.0 (A) and acetonitrile (B). The mobile phase consisted of 97.5% A and 2.5% B for TRH and MeTRH, 94.0% A and 6.0% B for taltirelin and 92.5% A and 7.5% B for montirelin.

The lipophilicity of TRH analogues was determined using an immobilized artificial membrane (IAM) column

(IAM.PC.MG, 4.6$\phi \times 150$ mm; Regis Technologies Inc., Morton Grove, IL) and hydrophilic mobile phase (10 mM sodium phosphate, pH 7.4) according to Sudoh et al (1998). The chromatographic partition coefficient, k'_{IAM}, which is proportional to the octanol/water partition coefficient, was determined. The k'_{IAM} values of each solute were calculated from the equation:

$$k'_{IAM} = (t_R - t_0)/t_0 \quad (1)$$

where t_R is the retention time of the compound of interest and t_0 corresponds to the void volume of the column. The solution of each TRH analogue was injected on the column (flow rate 1.0 mL min^{-1}), and solutes were detected with a UV detector ($\lambda = 215$ nm). The detection limit was 0.5 μM for each agent.

Data analysis

Apparent permeability coefficients (P_{app}) were calculated from the receiver compartment concentrations and following relationship:

$$P_{app} = (dC/dt \times V)/(C_0 \times A) \quad (2)$$

where V is the volume of receiver compartment, A is the membrane surface area (1 cm^2), C_0 is the initial donor concentration of solute and dC/dt is the slope of the cumulative receiver concentration versus time. Statistical analysis of data was performed by analysis of variance followed by Dunnett's test with the Prism 3.0 program (GraphPad Inc., San Diego, CA).

Results

Cellular transport study

The transport of TRH and its analogues across Caco-2 cell monolayers was investigated. The concentration dependency of transport rates of TRH, MeTRH, taltirelin, montirelin and azetirelin is shown in Figure 1. There was a linear relationship between concentration (over the range 0.5–10 mM) and apical-to-basolateral transport rates of these agents. When TRH and its analogues were applied to the donor compartment, concentration-dependent linear fluxes to the receiver compartment were observed, independent of whether the analogue was initially applied to the apical or basolateral compartment. As shown in Table 1, the permeability coefficients (P_{app}) of these agents did not differ substantially, and their P_{app} ratios of the basolateral-to-apical over the apical-to-basolateral transport were close to one (0.72–1.23).

The apical-to-basolateral transport of low concentrations (3–15 nM) of [^{3}H]MeTRH across Caco-2 cell monolayers was measured, and there was a linear relationship between concentration and transport rate. The apical-to-basolateral transport of [^{3}H]MeTRH across Caco-2 cell monolayers was affected by neither TRH nor TRH analogues (Figure 2), and there was little difference in P_{app} values between [^{3}H]MeTRH and [^{14}C]mannitol ($1.40 \pm 0.14 \times 10^{-6}$ cm s^{-1} and $1.23 \pm 0.18 \times 10^{-6}$ cm s^{-1}, respectively).

Also, when TRH and its analogues were incubated with Caco-2 cell monolayers, little metabolic degradation products and little disintegration of transported [^{3}H]MeTRH was detected by HPLC method (data not shown).

Cellular uptake study

The time dependency of [^{3}H]MeTRH uptake in Caco-2 cells was examined. The cell-per-medium ratio of [^{3}H]MeTRH reached equilibrium at 3–5 min after an initial application of [^{3}H]MeTRH (15 nM) to the medium (Figure 3). In addition, the influence of TRH and MeTRH on the cellular uptake of [^{3}H]MeTRH was examined. The cell-per-medium ratio of [^{3}H]MeTRH at 1 min after incubation was similar to the value of [^{14}C]mannitol, and their values were 0.19 ± 0.03 and 0.18 ± 0.04 μL (mg protein)$^{-1}$, respectively (Table 2). Also, TRH and MeTRH (1 mM) had little significant inhibition of the cell-per-medium ratio of [^{3}H]MeTRH.

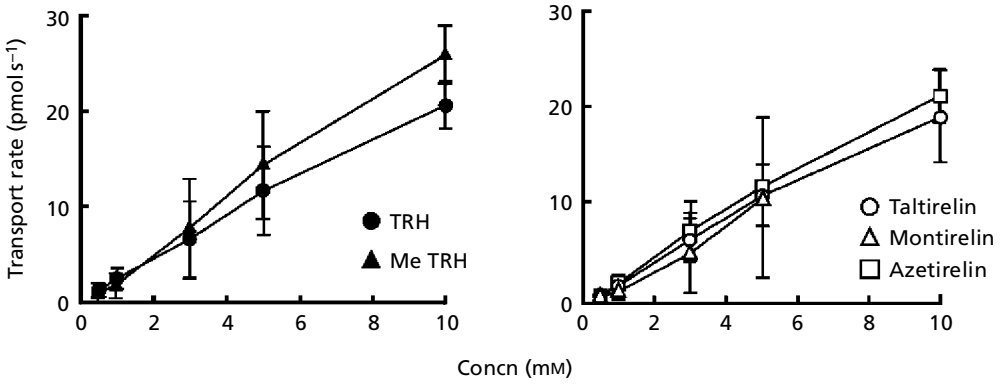

Figure 1 Concentration dependency of transport of TRH and its analogues across Caco-2 cell monolayers. The monolayers were incubated with TRH, MeTRH, taltirelin, montirelin and azetirelin for 60 min at 37 °C. Each agent was added to the apical side at a concentration of 0.5–10 mM. Each point represents the mean ± s.d. of 3–5 determinations.

Table 1 Permeability of TRH and its analogues across Caco-2 cell monolayers in comparison with their physicochemical properties.

	MW	k'_{IAM}	P_{app} (cm s^{-1}) $\times 10^{-6}$		Ratio (BL → AP/AP → BL)
			AP → BL	BL → AP	
TRH	362.39	0.338 ± 0.000	2.05 ± 0.44	1.51 ± 0.37	0.73
MeTRH	376.40	ND	1.84 ± 1.30	2.07 ± 0.07	1.13
Taltirelin	477.47	0.391 ± 0.000	1.83 ± 1.01	1.55 ± 0.17	0.85
Montirelin	480.54	1.028 ± 0.000	1.32 ± 0.84	1.62 ± 0.28	1.23
Azetirelin	350.37	0.234 ± 0.000	2.32 ± 0.76	1.67 ± 0.20	0.72

Values are mean ± s.d. of 3 or 4 determinations. ND, not determined.

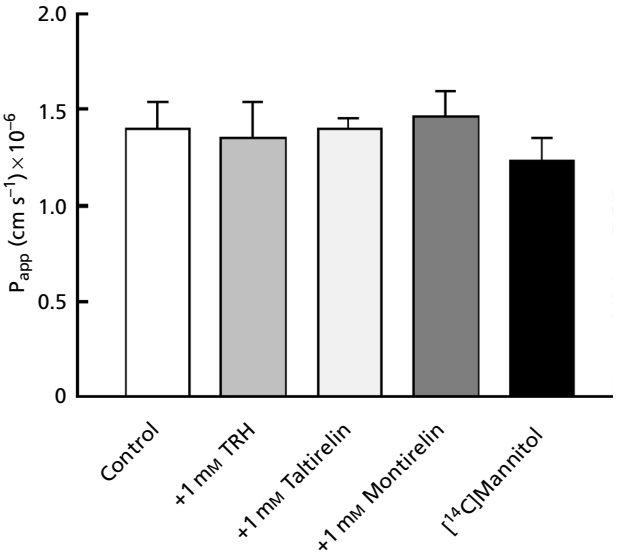

Figure 2 Influence of TRH and its analogues on the permeability coefficient (P_{app}) of [^{3}H]MeTRH in Caco-2 cell monolayers. The monolayers were incubated with [^{3}H]MeTRH (15 nM) added to the apical side in the absence or presence of TRH and its analogues, for 60 min at 37 °C. Also, the permeability of [^{14}C]mannitol (4.1 kBq mL^{-1}) was determined. Each column represents the mean ± s.d. of 3–5 determinations.

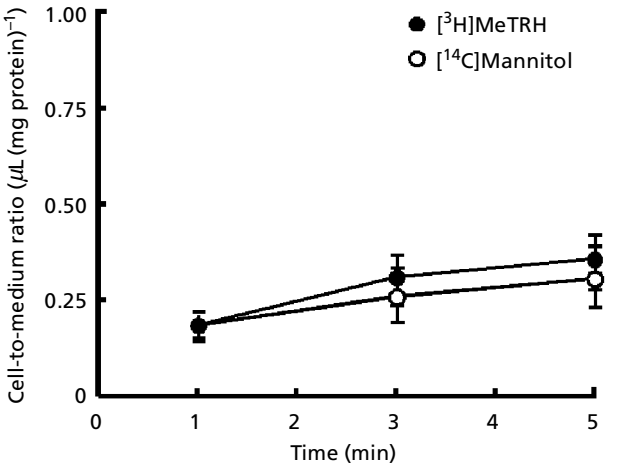

Figure 3 Time course of cell-per-medium ratios for [^{3}H]MeTRH and [^{14}C]mannitol in Caco-2 cell monolayers. The monolayers were incubated with [^{3}H]MeTRH (15 nM) and [^{14}C]mannitol (4.1 kBq mL^{-1}) at 37 °C for the designated times. Each point represents the mean ± s.d. of 6 determinations.

Physicochemical characteristics

The ability of TRH and its analogues to interact with biological membrane was assessed by IAM chromatography. The k'_{IAM} values of each agent were decreased in the following order: montirelin > taltirelin > TRH > azetirelin, and the values varied from 0.234 to 1.028 (Table 1).

Discussion

The purpose of this study was to characterize systematically the transport of TRH and its analogues across Caco-2 cell monolayers because peroral administration of peptide drugs and analogues could offer significant advantages over parenteral application, especially in the long-term treatment of chronic diseases such as spinocerebellar degeneration. The P_{app} values of TRH, MeTRH, taltirelin, montirelin and azetirelin did not substantially differ from each other, and their P_{app} ratios of the basolateral-to-apical

Table 2 Influence of unlabelled MeTRH and TRH on initial uptake of [^{3}H]MeTRH in Caco-2 cell monolayers.

	Concn	Cell-per-medium ratio (μL (mg protein)$^{-1}$)	
		[^{3}H]MeTRH	[^{14}C]mannitol
Control		0.19 ± 0.03	0.18 ± 0.04
+ MeTRH	1 mM	0.22 ± 0.08	0.25 ± 0.09
+ TRH	1 mM	0.19 ± 0.05	0.22 ± 0.05

The monolayers were incubated with [^{3}H]MeTRH (15 nM) and [^{14}C]mannitol (4.1 kBq mL^{-1}) for 1 min at 37 °C in the absence or presence of MeTRH and TRH (1 mM). Values are the mean ± s.d. of 5 or 6 determinations.

over the apical-to-basolateral transport were close to one. The results of kinetic analysis showed a linear relationship between the concentration and transport rate of TRH and its analogues. Even at low concentrations of [^{3}H]MeTRH, there was linear relationship between the concentration and transport rate. Furthermore, the transport of [^{3}H]MeTRH was not affected by TRH, taltirelin, montirelin and azetirelin, and there was little difference in the P_{app} values between [^{3}H]MeTRH and [^{14}C]mannitol. Thus, these data indicate that TRH and its analogues may be transported across Caco-2 cell monolayers by paracellular passive diffusion. Our results confirm previous observations, made by Gan et al (1993), of a passive diffusion of TRH across Caco-2 cell monolayers. Furthermore, our study has first noted that the paracellular passive diffusion process could be extended to pharmacologically potent analogues of TRH.

The cellular uptake of [^{3}H]MeTRH was also examined. The cell-per-medium ratio of [^{3}H]MeTRH was similar to the ratio of [^{14}C]mannitol, a paracellular marker. Furthermore, the cell-per-medium ratio of [^{3}H]MeTRH was not affected by large excess of TRH or MeTRH. Thus, the cellular uptake of [^{3}H]MeTRH has been shown to be negligible in Caco-2 cell monolayers.

It is known that the enzymatic deamination of TRH is catalysed by a cytosolic prolyl-endopeptidase, cleaving the C-terminal prolyl-amide (Wilk 1983; Cummins & O'Connor 1998). In the HPLC analysis of receiver-compartment samples in the transport study, TRH and its analogues remained intact. Thus, TRH and its analogues have been shown to be stable during the course of transport experiment across Caco-2 cell monolayers, supporting the paracellular passive diffusion of these agents. Such a transport mechanism may be advantageous in terms of the avoidance of intracellular enzymatic degradation.

The lipophilicity of each agent, determined by IAM chromatography, was decreased in the order montirelin > taltirelin > TRH > azetirelin. The k'_{IAM} values of these agents varied from 0.234 to 1.028 (Table 1), suggesting high hydrophilicity. It is well known that the transported amount of drug correlates inversely with its molecular weight, and is proportional to its lipophilicity. In this study, the molecular weight, lipophilicity and P_{app} values of TRH, MeTRH, taltirelin and montirelin varied only slightly, and neither molecular weight nor lipophilicity of TRH and its analogues was correlated with permeability across Caco-2 cell monolayers. Thus, it seems unlikely that the transport activity of these agents may depend on the difference in their lipophilicity and size.

This study has shown that TRH, MeTRH, taltirelin, montirelin and azetirelin may be predominantly transported via paracellular route in Caco-2 cell monolayers. The permeability of TRH analogues was similar to that of TRH. Our previous studies have revealed that novel pharmacologically active TRH analogues, JTP-2942, taltirelin and montirelin, bind significantly to the brain TRH receptors in-vivo (Urayama et al 1999, 2001). Furthermore, it has been shown that novel TRH analogues have fairly potent and sustained CNS effects compared with TRH (Itoh et al 1994; Kinoshita et al 1996). Thus, such potent pharmacological effects of TRH analogues may be ascribed more largely to their enzymatic stability in blood or high affinity for brain receptors rather than to the difference in membrane permeability.

Conclusions

The transport mechanisms of TRH and its pharmacologically active analogues (MeTRH, taltirelin, montirelin, azetirelin) across Caco-2 cell monolayers were characterized. There was a linear relationship between the concentration and apical-to-basolateral transport rate of these agents. The permeability coefficient (P_{app}) ratios of basolateral-to-apical over apical-to-basolateral transport of these agents were close to one. Also, the cellular transport of [^{3}H]MeTRH at low concentrations showed a linear relationship between the concentration and transport rate. The transport of [^{3}H]MeTRH in Caco-2 cell monolayers was affected by neither TRH nor TRH analogues, and there was little difference in P_{app} values between [^{3}H]MeTRH and [^{14}C]mannitol. The cell-per-medium ratio of [^{3}H]MeTRH in the cellular uptake experiment was similar to the value of [^{14}C]mannitol. A large excess of TRH and MeTRH did not significantly influence the cell-per-medium ratios of [^{3}H]MeTRH in Caco-2 cell monolayers. The k'_{IAM} values of TRH, taltirelin, montirelin and azetirelin varied from 0.234 to 1.028, suggesting high hydrophilicity. These results indicate that a paracellular passive diffusion may be the major route for the transport of TRH and its analogues in Caco-2 cell monolayers.

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