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Characterization of human nasal primary culture systems to investigate peptide metabolism

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Abstract

The objectives of this study were to validate and compare the suitability of different primary cell culture systems as models to investigate peptide enzymatic stability following nasal administration. The degradation kinetics of a model peptide, leucine enkephalin (Tyr–Gly–Gly–Phe–Leu, Leu–Enk), was determined in four nasal cell culture systems: immersion, air–liquid interface, sequential monolayer-suspension, floating collagen. The influence of enzyme inhibitors (bestatin, puromycin) and Leu–Enk metabolite analogs (Tyr–Gly, Phe–Leu, Tyr–Gly–Gly, Gly–Phe–Leu) on the Leu–Enk degradation profile was also investigated. The disappearance of Leu–Enk in all the cell culture systems followed first order kinetics. The specific activity in the cell culture systems followed the rank: sequential monolayer-suspension (32.60 $\mu M\ min^{-1}\ mg^{-1}$) > air–liquid interface (15.19 $\mu M\ min^{-1}\ mg^{-1}$) > immersion (11.49 $\mu M\ min^{-1}\ mg^{-1}$) > floating collagen (4.57 $\mu M\ min^{-1}\ mg^{-1}$). At equimolar concentration, bestatin had a higher inhibitory effect than puromycin. The rate of hydrolysis of Leu–Enk was reduced significantly by co-incubation with Leu–Enk metabolite analogs. This study showed that immersion, sequential monolayer-suspension and air–liquid interface culture systems may be potentially suitable for further studies on peptide enzymatic stability following nasal administration.

Keywords: Nasal cell culture; Peptides; Leucine enkephalin; Metabolism; Protease inhibitors

1. Introduction

Several experimental models have been used to investigate the limitations posed by nasal proteases to the absorption of nasally administered proteins and peptides, and strategies to overcome them. The most commonly used approaches include rat in situ perfusion model, nasal

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mucosal washing and extract, nasal mucosal homogenate, in vivo animal models (rat, sheep, dog, rabbit), human nasal washing, turbinate and cultured epithelium (Kashi and Lee, 1986; Maitani et al., 1986; Faraj et al., 1990a; Gizurarson and Bechgaard, 1991; Sayani et al., 1993; Irwin et al., 1994; Hussain et al., 1995; Pietrowsky et al., 1996; Kissel and Werner, 1998).

As a result of experimental, methodological and ethical limitations associated with the use of human species, animal models are frequently used for metabolism studies. However, they have several disadvantages that justify the preference of in vitro nasal models based on human nasal washing, tissues or cultured cells. These disadvantages include species differences, differences in anatomy of the animal nasal cavity in comparison to the human nose and high variability when large numbers of animals are used (Kissel and Werner, 1998). The use of intact cultured human nasal epithelium may help to overcome some of the problems mentioned above. In general, cell culture models have many advantages over conventional experimental models (e.g. in situ perfusion and in vivo surgical models). Such advantages include (a) possibility to elucidate the transport mechanism of drug molecules and the pathway(s) of drug degradation, (b) rapid evaluation of strategies for promoting drug permeation and minimizing drug metabolism, (c) possibility to mimic the real human conditions and (d) avoidance of expensive and sometimes controversial animal studies (Audus et al., 1990). Nevertheless, the level of epithelial cell differentiation may affect the suitability of in vitro nasal cell culture models for drug metabolism studies.

Cell culture studies have shown that cell culture techniques and conditions for growing human nasal epithelium (e.g. immersion culture, air–liquid interface culture, growing the cells on collagen, media supplement, etc.) may affect the morphological and functional features of the cells and the expression of differentiated phenotype (Jorissen et al., 1989; Yamaya et al., 1992; Blank et al., 1995). These attributes may also affect the expression of drug metabolizing enzymes and the suitability of cultured human nasal epithelium as models to study the metabolism of proteins and peptides in vitro.

The aim of this study was to compare and validate human nasal epithelium cultured under different conditions using different methods as models to investigate peptide metabolism. The four cell culture systems investigated include immersion, air–liquid interface, floating collagen and sequential monolayer-suspension culture systems.

2. Materials and methods

2.1. Chemicals

Leucine enkephalin (Leu–Enk) and puromycin hydrochloride were purchased from Fluka (Buchs, Germany). Bestatin hydrochloride was obtained from Sigma (St. Louis, MO, USA). Professor P. Augustijns (Farmacotechnologie en Biofarmacie, KU Leuven, Belgium) kindly provided Des–Tyr–Leu–Enk acetate, Tyr–Gly, Phe–Leu, Tyr–Gly–Gly and Gly–Phe–Leu. All other chemicals and reagents used for sample analysis were of analytical grade.

2.2. Cell culture media and other components

Human nasal epithelial tissues (without secondary ultrastructural abnormalities) were obtained during elective surgeries from patients. The tissues were transported in DMEM-F12 1/1 (Life Laboratories, Paisley, UK) culture medium supplemented with streptomycin 100 μg ml^{-1} and penicillin 100 IU ml^{-1} (Boehringer, Mannheim, Germany) and used for cell culture. Other cell culture components used include physiological saline (Braun, Melsungen, Germany) containing antibiotics (streptomycin 100 μg ml^{-1} and penicillin 100 IU ml^{-1}), pronase (Sigma, Missouri, USA), 0.2% collagen (extracted from rat tail), NH_4OH (UCB, Leuven, Belgium), phenol red (GIBCO, Paisley, UK) and collagenase type IV (Worthington, New Jersey, USA). Phosphate buffered saline and Hanks' balanced salt (HBBS) were obtained from Life Technologies (Paisley, UK). Transwell® polycarbonate inserts were supplied by Corning (NY, USA). The medium for the monolayer culture was Ultroser G 2.0% (Life Laboratories,

Paisley, UK) in DMEM-F12 1/1 supplemented with 10 ng ml^{-1} choleratoxin, streptomycin 100 μg ml^{-1} and penicillin 100 IU ml^{-1}. The medium for the suspension culture was NU-serum 10.0% (Life Laboratories, Paisley, UK) in DMEM-F12 1/1 supplemented with streptomycin 100 μg ml^{-1} and penicillin 100 IU ml^{-1}.

2.3. Cell culture

The cell culture protocol used for this study was based on the selection, modification and combination of optimal conditions of the cell culture methods described for differentiated human nasal and tracheal epithelia (Jorissen et al., 1989; Yamaya et al., 1992; Blank et al., 1995).

The human nasal epithelial tissues obtained during surgery were washed three times with physiological saline solution supplemented with antibiotics. The cells were dissociated enzymatically for a period of 16–24 h at 4 °C using 0.1% pronase. The pronase was deactivated with 10% NU-serum prior to cell washing with DMEM-F12 1/1. The washing solution was removed after centrifugation at $70 \times g$ for 5 min on each occasion. The resulting suspension of cells were filtered through a 70 μm pore size polycarbonate filter (Pall, Portsmouth, UK) and preplated on plastic for 1 h at 37 °C in a 95% O_2 and 5% CO_2 environment to reduce fibroblast contamination. Subsequently, the cells were counted with a Coulter multisizer (Northwell, UK) and seeded on different culture dishes at a density of 5.0×10^5–1.0×10^6 cell per cm^2 depending on the cell culture method. The cells were incubated at 37 °C in a 95% O_2 and 5% CO_2 environment.

For immersion culture, cells were seeded in six-well cell culture plates coated with 1 mm thick rat-tail collagen gel (0.2%). The wells had previously been filled with 2 ml monolayer culture medium. After cell plating, an additional 1 ml of this medium was added to each well. The medium was changed the day after seeding and subsequently three times a week.

Regarding the air–liquid interface culture, the cells (150–250 μl suspension in monolayer culture medium) were seeded on Transwell® polycarbonate inserts coated with 1 mm thick collagen gel. The medium was also changed a day after cell plating. For subsequent replacement of the monolayer medium, the medium (400 μl) was added only to the receiver compartment. Therefore, the apical side of the cells was exposed to 95% O_2 and 5% CO_2 environment while the basolateral side was immersed in the culture medium. The cells were maintained in this condition until when they were used for experiments. For sequential monolayer-suspension, the cells were cultured as described by Jorissen et al. (1989).

Cells for floating collagen culture were seeded in six-well cell culture plates coated with 0.2% collagen and containing 3–4 ml monolayer culture medium. The medium was changed the day after cell plating and subsequently three times a week. After 1 week (within this period the cells were confluent), the thin layers of collagen with cells grown on them were carefully lifted using a flame-bent glass Pasteur pipette. The cells were maintained in the 'lifted' form with 10.0% NU-serum for 1 week. Within the first 2 days of lifting the collagen, the cells were kept on a gyratory rotor to avoid re-attachment to the plastic surface. The culture medium was added in such a manner that the floating collagen was completely immersed in the culture medium. The medium was changed every 2 days until when they were used for experiments.

2.4. Metabolism studies

2.4.1. Degradation of Leu–Enk in different cell culture systems

Cells grown using the immersion and air–liquid interface culture methods were used for metabolism studies on day 7 following establishment of the culture. For the sequential monolayer-suspension and floating collagen culture systems, the cells were used for metabolism studies after 3 weeks and 1 week of releasing the cells as suspension and floating the collagen substratum, respectively.

On the day of the experiment, the cells were washed three times with HBSS (supplemented with HEPES buffer [4-(2-hydroxyethyl)-1 piperazineethane sulfonic acid], glucose 15 mM, pH 7.4). For air–liquid interface-based culture, the

filters (with cell layers) were carefully cut out from the inserts and placed in test-tubes. For suspension cells, the cells were separated from the culture medium by centrifugation ($70 \times g$, 5min) and the experiments were carried out in 1.5 ml eppendorfs. For immersion and floating collagen cultures, the experiments were conducted in multi-well culture dishes used for growing cells.

Prior to the metabolism studies, each batch of cells was washed three times using HBSS. After washing and 15 min pre-incubation period at 37 °C, the HBSS was removed and the cells were incubated with 100 μM Leu–Enk (1 ml for immersion and floating collagen and 0.5 ml for air–liquid interface and sequential monolayer-suspension cultures). Samples (50 μl) were taken at pre-determined intervals (0, 10, 20, 30, 40, 60, 90, 120 min) and were immediately diluted with 100 μl citric acid (0.1 M) to prevent further hydrolysis. Samples were stored at −40 °C (for not more than 1 week) until analyzed by HPLC. The stability of Leu–Enk was maintained upon storage.

Except for the sequential monolayer-suspension culture, control experiments were conducted in which collagen gel-coated plates were incubated with Leu–Enk. For studies involving the use of protease inhibitors (bestatin and puromycin) and Leu–Enk metabolite analogs (Tyr–Gly, Phe–Leu, Tyr–Gly–Gly, Gly–Phe–Leu), similar procedure as described above was used. However, after washing the cells Leu–Enk was co-incubated with the test compounds and samples were collected at set time intervals. The protein content of all cell batches used was assayed using the method described by Bradford (1976).

2.4.2. Sample analysis

High performance liquid chromatography (HPLC) was used to analyze Leu–Enk and its metabolites. The system consisted of HPLC pump 420 (Tegimenta, Rotkreuz, Switzerland), injector model U6K and Lamda-Max model 480 UV detector (Waters Associates, Milford, MA, USA), and PC Integration pack, version 1.00 (Softron, Gräfelfing, Germany). Reversed-phase isocratic separation was achieved using Hypersil BDS C-18 (5 μm), 250 × 4.6 mm column (Thermo Hypersil, Cheshire, UK). A protective guard column packed with silica Nova-Pack C-18 (4 μm) (Waters Associates) was used to protect the HPLC column. The mobile phase used for the assay was a 23:77 (v/v) mixture of acetonitrile and 0.05 M phosphate buffer (pH 2.5). The mobile phase was filtered through a nylon membrane filter (0.45 μm) and degassed before use by ultrasonication for 10 min. The flow rate and detection wavelength were set at 1.3 ml min^{-1} and 210 nm, respectively. Retention times were Leu–Enk, 8.1 min; Des–Tyr–Leu–Enk, 5.9 min.

2.5. Data presentation and statistical analysis

Specific activity of the cells and degradation rate constants for Leu–Enk metabolism were calculated from the remaining concentrations of Leu–Enk at stipulated time intervals and their natural logarithms, respectively, using a least square fit. Half-lives of Leu–Enk degradation were calculated using the degradation rate constant.

Unless stated otherwise, each experiment was carried out in triplicate and the data were expressed as mean ± S.D. Where necessary, statistical analysis was conducted using Bonferroni's one-way analysis of variance (ANOVA) to compare differences between the various groups. The level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Metabolism of Leu–Enk in different cell culture systems

Leucine enkephalin possesses interesting characteristics that make it a useful substrate to investigate transmucosal permeation and metabolism of peptides. First, this compounds is rapidly inactivated by peptidases, but still chemically stable up to 3 h over the time course of incubation at 37 °C (Kashi and Lee, 1986). Second, leucine enkephalin may be degraded by many enzyme systems including aminopeptidase, enkephalinase and carboxypeptidase (Schwartz et al., 1981). Based on above-mentioned characteristics, this compound was selected to characterize the

aminopeptidase activity of human nasal primary cultures.

This study showed that the four cell culture systems investigated degraded Leu–Enk (100 μM) to Des–Tyr–Leu–Enk (Fig. 1(a) and (b)). Control experiments in which collagen gels alone were incubated with Leu–Enk showed that the Leu–Enk remaining after 2 h incubation was not significantly different from 100 μM and no Des–Tyr–Leu–Enk was detected. This confirmed that the disappearance of Leu–Enk was due to enzymatic degradation. Except for cells cultured

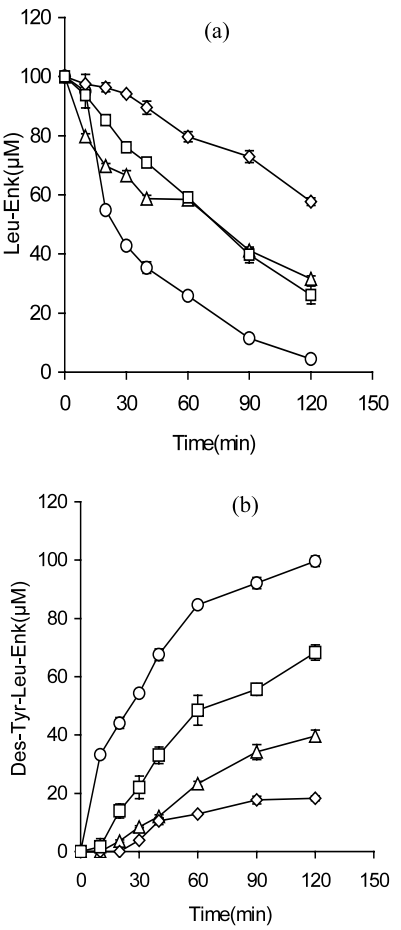

Fig. 1. Disappearance of Leu–Enk (a) and appearance of Des–Tyr–Leu–Enk (b) in human nasal cell culture systems. Each point represents the mean ± S.D., $n = 3$ experiments. ○, Immersion; □, floating collagen; △, sequential monolayer-suspension; ◇, air–liquid interface.

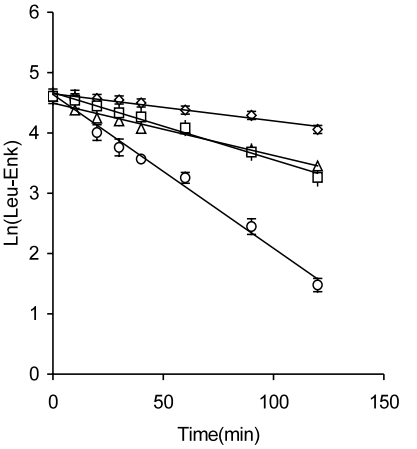

Fig. 2. First-order degradation profiles of Leu–Enk in human nasal cell culture systems. Each point represents the mean ± S.D., $n = 3$ experiments. ○, immersion; □, floating collagen; △, sequential monolayer-suspension; ◇, air–liquid interface.

using the air–liquid interface method in which less than 0.5% Phe–Leu was seen, no other degradation products of Leu–Enk such as Tyr–Gly, Tyr–Gly–Gly, Gly–Phe–Leu could be detected under our chromatographic conditions. This observation suggests that aminopeptidases are predominantly responsible for the degradation of Leu–Enk to its major metabolite (Des–Tyr–Leu–Enk) in primary cultures of human nasal epithelium. A similar metabolic pattern was reported in albino rabbits (Kashi and Lee, 1986). Also this study showed that Leu–Enk or Des–Tyr– Leu–Enk could be further degraded into smaller peptides (e.g. Phe–Leu) by cultured human nasal epithelium.

3.2. Kinetics of Leu–Enk degradation in human nasal primary cultures

The degradation of Leu–Enk was proportional to the concentration of Leu–Enk remaining with time. Therefore, a plot of Ln (Leu–Enk) versus time resulted in a straight line with slope equal to $-k$, $r \geq 0.99$ (Fig. 2). Considering the fact that after 2 h incubation period, the Leu–Enk concentration in the sequential monolayer-suspension, floating collagen and immersion culture systems was reduced by more than 70%, the degradation

of Leu–Enk in the culture systems was considered to follow first order kinetics.

Generally, the rate of Leu–Enk hydrolysis is proportionally dependent upon aminopeptidase activity. In a particular cell culture system, it was directly proportional to the amount of cell protein content. This implies that steeper disappearance slope of Leu–Enk in a particular cell culture model does not necessarily mean greater metabolic capacity. Therefore, it is more precise to refer the kinetic parameters to the protein content of each batch of cells used for the study (Mentlein and Lucius, 1997; Table 1).

The specific activity for Leu–Enk degradation in the sequential monolayer-suspension culture system (32.6×10^{-1} μM min^{-1} mg^{-1}) was two to seven times higher than in other cell culture systems. The specific activity for the degradation of Leu–Enk was lower in floating collagen culture in comparison with other cell culture systems. The rank order of specific activities for Leu–Enk degradation was: sequential monolayer-suspension > air–liquid interface > immersion > floating collagen.

A comparison of the degradation rate constants obtained in this study with literature data (Table 2) shows that the rate constants for the immersion (2.34×10^{-2} min^{-1}) and air–liquid interface (2.23×10^{-2} min^{-1}) cultures agreed with results obtained using rat model (Hussain et al., 1985). On the other hand, the rate constants for Leu–Enk degradation in sequential monolayer-suspension (0.95×10^{-2} min^{-1}) and floating collagen (1.05×10^{-2} min^{-1}) culture systems though smaller than values reported in literature (Table 2) did not correlate with their specific activities. This may be explained by differences in cell protein content in the various cell culture systems. Based on differences in experimental models, concentrations of Leucine enkephalin investigated, data treatment and species differences, direct comparison of kinetic data for peptides in various nasal models is rather difficult. Nevertheless, the results of this study compared favorably well with literature data (Tables 1 and 2).

The degradation rate constants of Leu–Enk observed in this study (intact cultured nasal epithelium) was found to be smaller than in rabbit nasal homogenate. This may be attributed to the fact that nasal homogenates contain both membrane-bound and cytosolic aminopeptidases (Kashi and Lee, 1986).

3.3. Influence of protease inhibitors

In order to confirm the expression of proteases in the cultured human nasal epithelium, the effects of bestatin and puromycin on the degradation of Leu–Enk were investigated. These inhibitors were found to concentration dependently protect Leu–Enk from the hydrolytic effects of the proteases (Table 3).

The various concentrations of bestatin and puromycin (0.02, 0.1 and 1 mM) investigated caused significant reduction in Leu–Enk degradation ($P < 0.05$). Bestatin showed a stronger inhibitory effect than puromycin at equimolar concentrations. This may be explained by the fact that while bestatin inhibits leucine aminopeptidase, aminopeptidase B and N (Suda et al., 1976),

Table 1
Kinetic parameters of Leu–Enk degradation in human nasal primary cultures

Kinetic parameters	Cell culture models			
	Immersion	Air–liquid interface	Sequential-monolayer suspension	Floating collagen
Specific activity [$\times 10$ (μM min^{-1} mg^{-1})]	11.49 ± 0.10	15.19 ± 3.10	32.60 ± 5.58	4.57 ± 0.19
Degradation rate constants [$\times 10^2$ (min^{-1})]	2.34 ± 0.29	2.23 ± 0.18	0.95 ± 0.09	1.05 ± 0.34
Half life ($t_{1/2}$) (min)	29.6 ± 3.8	31.1 ± 2.7	72.9 ± 7.5	66.0 ± 8.4

Table 2
Kinetic parameters of Leu–Enk degradation obtained in different nasal models

Species	Experimental models	Substrate	Degradation rate constant	Order of degradation	Half-life ($t_{1/2}$) (min)	References
Human	Nasal washing	Leu–Enk (120 μg ml^{-1})	NP	1	40	Hussain et al., 1990; Werner and Kissel, 1995
	Cell culture	Leu–Enk [115 ng ml^{-1} ($\approx$0.0002 mM)]	0.24 nmol min^{-1}	0	NP	
Rabbit	Mucosa extract	Leu–Enk [50 μg ml^{-1} ($\approx$0.09 mM)]	75.7×10^{-4} min^{-1}	1	NP	Sayani et al., 1993
	Homogenate	Leu–Enk (2.5 mM)	3.45×10^{-2} min^{-1}	1	20.1	Kashi and Lee, 1986
Rat	In situ	Leu–Enk (1 mM)	0.55 (μg ml^{-1} min^{-1})	0	NP	Hussain et al., 1992
		Leu–Enk [60 μg ml^{-1} ($\approx$0.1 mM)]	2.25×10^{-2} min^{-1}	1	NP	Hussain et al., 1985
		Leu–Enk [60 μg ml^{-1} ($\approx$0.1 mM)]	NP	NP	45	Hussain et al., 1989
		Leu–Enk [60 μg ml^{-1} ($\approx$0.1 mM)]	NP	NP	60	Faraj et al., 1990b
Sheep	Homogenate	Leu–Enk [120 μg ml^{-1} ($\approx$0.2 mM)]	1.72×10^{-2} min^{-1}	1	40	
	Nasal washing	Leu–Enk [120 μg ml^{-1} ($\approx$0.2 mM)]	5.81×10^{-2} min^{-1}	1	12	Irwin et al., 1994

NP, not provided; Leu–Enk, leucine–enkephalin; Met–Enk, methionine–enkephalin.

Table 3
Inhibition of Leu-Enk degradation by protease inhibitors

Cell culture systems	Change in specific activity relative to control[a](%)					
	Bestatin			Puromycin		
	0.02 mM	0.1 mM	1 mM	0.02 mM	0.1 mM	1 mM
Suspension	26.7 ± 3.8	100	100	12.5 ± 6.9	77.4 ± 4.5	100
Air–liquid interface	82.6 ± 2.2	100	100	64.5 ± 2.5	74.6 ± 3.4	90.5 ± 6.5
Immersion	65.0 ± 9.4	72.8 ± 2.7	100	25.3 ± 10.6	45.8 ± 1.9	100
Floating collagen	100	100	100	100	100	100

[a] Control = Leu–Enk alone. 0.02, 0.1, 1 mM bestatin and puromycin caused significant decrease in specific activity in all the cell culture systems ($P < 0.05$).

puromycin inhibits only aminopeptidase B and N (McDonald et al., 1964). This observation strongly suggests the existence of different aminopetidases in the cultured human nasal epithelium.

3.4. Influence of Leu–Enk metabolite analogs

Literature information shows that co-incubation of peptides with metabolically competing peptide results in reduced metabolism and increased enzymatic stability of the peptides under investigation (Hussain et al., 1985). The aminopeptidases of the cultured cells was further characterized by investigating the effect of Leu–Enk metabolite analogs (Tyr–Gly, Tyr–Gly–Gly, Gly–Phe–Leu, Phe–Leu) on Leu–Enk metabolism (Table 4).

From Table 4, the rate of hydrolysis of Leu–Enk was reduced significantly ($P < 0.05$) when co-incubated with Leu–Enk metabolite analogs (ratio 1:1).

Among the Leu–Enk metabolite analogs, Gly–Phe–Leu had the greatest effect on Leu–Enk stabilization against aminopeptidases in human nasal primary cell culture systems. When compared with the other cell cultures, the enzyme activity of the immersion culture was particularly susceptible to inhibition by Leu–Enk metabolite analogs. While, the enzyme activity of the air–liquid interface culture was more resistant to inhibition. The variation in the extent of metabolism inhibition by the various metabolite analogs may be due to the ratio of Leu–Enk and Leu–Enk metabolite analogs used (1:1). Presumably, if the concentration of Leu–Enk metabolite analogs is increased (e.g. 20-fold molar excess), a more pronounced inhibition could be observed (Hussain et al., 1985). This study suggests the possibility of using cultured human nasal epithelial cells to study peptide enzymatic stabilization by co-administration with pharmacologically inactive peptidase substrates. This has already been demonstrated for Leu–Enk using the rat in situ model (Hussain et al., 1985).

4. Conclusions

Although valuable insights into the mechanisms of nasal drug metabolism and absorption can be obtained using animal models, their predictive power allowing an extrapolation to nasal drug administration in humans remains a controversial issue. The use of human-based model is a viable alternative in solving this problem. The aminopetidase activity of the various culture systems, their inhibition by protease inhibitors and peptides metabolite analogs reasonably agreed with literature data. Thus, this study showed that human nasal epithelium cultured as sequential monolayer-suspension, air–liquid interface and immersion cultures are potentially useful for studying enzymatic stability of peptides in vitro. Due to better epithelial cell differentiation and the possibility to keep the cells for a longer period of

Table 4
Influence of Leu–Enk metabolite analogs on Leu–Enk kinetic parameters

Cell culture systems	Experimental conditions	Specific activity ($\times 10^{-1}$ μM per mg protein $min^{-1} \pm$ S.D.)	Change in specific activity relative to control (%)
Suspension	Leu–Enk alone (control)	32.6 ± 0.56	100 ± 1.7
	Leu–Enk + Tyr–Gly–Gly	13.5 ± 0.40	41.4 ± 1.2*
	Leu–Enk + Gly–Phe–Leu	8.7 ± 0.16	26.7 ± 0.5*
	Leu–Enk + Phe–Leu	13.2 ± 0.37	40.5 ± 1.1*
	Leu–Enk + Tyr–Gly	13.5 ± 0.28	41.4 ± 0.9*
Air–liquid interface	Leu–Enk alone (control)	15.2 ± 0.31	100 ± 2.0
	Leu–Enk + Tyr–Gly–Gly	13.6 ± 0.42	89.5 ± 2.8*
	Leu–Enk + Gly–Phe–Leu	11.0 ± 0.33	72.4 ± 2.2*
	Leu–Enk + Phe–Leu	12.8 ± 0.27	84.2 ± 1.8*
	Leu–Enk + Tyr–Gly	12.6 ± 0.02	82.9 ± 0.1*
Immersion	Leu–Enk alone (control)	11.5 ± 0.01	100 ± 0.1
	Leu–Enk + Tyr–Gly–Gly	1.7 ± 0.05	14.8 ± 0.4*
	Leu–Enk + Gly–Phe–Leu	1.1 ± 0.02	9.6 ± 0.2*
	Leu–Enk + Phe–Leu	1.5 ± 0.02	13.0 ± 0.2*
	Leu–Enk + Tyr–Gly	1.5 ± 0.01	13.0 ± 0.1*
Floating collagen	Leu–Enk alone (control)	4.6 ± 0.02	100 ± 0.4
	Leu–Enk + Tyr–Gly–Gly	2.3 ± 0.03	50.0 ± 0.7*
	Leu–Enk + Gly–Phe–Leu	2.8 ± 0.07	60.9 ± 1.5*
	Leu–Enk + Phe–Leu	3.4 ± 0.06	73.9 ± 1.3*
	Leu–Enk + Tyr–Gly	4.0 ± 0.06	86.9 ± 1.3*

*, Significantly different from control (Leu–Enk alone) ($P < 0.05$).

time, the sequential monolayer-suspension culture may be preferred. Nevertheless, cost (in terms of cell processing time and finance) is an important consideration when using this model for metabolism studies. Due to low aminopeptidase activity, the use of floating collagen culture to study nasal metabolism of peptides is not advisable.

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