JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 599–606

Received November 20, 2008
Accepted February 10, 2009
DOI 10.1211/jpp/61.05.0008
ISSN 0022-3573

Research Paper

Specific aminopeptidases of excised human nasal epithelium and primary culture: a comparison of functional characteristics and gene transcripts expression

Remigius U. Agu[a], Dominic U. Obimah[b], Wendy J. Lyzenga[a], Mark Jorissen[c], Emad Massoud[d] and Norbert Verbeke[b]

[a]Biopharmaceutics and Drug Delivery Laboratory, College of Pharmacy, [b]Experimental Otorhinolaryngology, Department for Human Genetics, Campus GHB, [c]Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven, Leuven, Belgium, [d]Department of Surgery, Dalhousie University, Halifax, Canada

Abstract

Objectives To investigate whether growing human nasal epithelium as primary cultures alters aminopeptidase B (APB), aminopeptidase N (APN) and dipeptidyldipeptidase (DPPIV) metabolic characteristics, and mRNA gene transcript expression.

Methods The formation of 7-amino-methyl coumarin from specific substrates for APN (L-alanine-4-methyl-coumaryl-7-amide), APB (L-arginine-4-methyl-coumaryl-7-amide) and DPPIV (glycyl-L-proline-4-methyl-coumaryl-7-amide) was used to estimate the K_M, V_{max} and the effect of aminopeptidases inhibitors on the enzymes. Polymerase chain reaction was used to investigate gene expression.

Key findings Results of this study showed that: (1) both the excised tissues and primary cultures of human nasal epithelium expressed APN, APB and DPPIV activity; (2) the K_M of APB, APN and DPPIV was not significantly different in cell and tissue homogenates; (3) except for APN, the V_{max} was not significantly different in the two metabolism models; (4) there was no statistically significant difference in the behaviours of APB, APN and DPPIV in response to inhibition by puromycin and bestatin in the two models; (5) the mRNA transcripts that encode APB, APN and DPPIV were expressed in both cell culture and tissue homogenate.

Conclusions Based on the results of this study, it may be concluded that nasal primary culture system is suitable for investigating peptide and protein metabolism and enzymatic stability in human nasal epithelium. Except for APN, the tissue culture conditions did not significantly alter the functional and molecular expression of the aminopeptidases.

Keywords 7-amino-methylcoumarin; aminopeptidases; drug metabolism; nasal cell culture; nasal tissue homogenates; protease inhibitors

Introduction

Biologically active peptides and proteins derived from recombinant DNA technology have been identified as drugs of the future.[1] However, there are many problems with systemic delivery of these drugs.[2] Almost all peptide and protein drugs are administered by injection. As this method is invasive, various alternative routes have been investigated, including buccal,[3,4] vaginal,[5,6] rectal[7] and pulmonary[8] routes.

Nasal drug delivery is a very attractive approach for drugs that are active in low doses and show low or no oral bioavailability.[9] The nasal route circumvents first-pass metabolism associated with oral delivery. It is easily accessible and also suitable for self-medication. The nasal route has a relatively large surface area, porous endothelial basement membrane and a highly vascularised epithelial layer.[10] Fast absorption and rapid onset of action are desirable therapeutic advantages. Indeed, the rate of absorption, peak plasma concentrations and therapeutic response compare well with intravenous administration of some drugs.[11] Despite the advantages of the nasal route for drug administration, there are significant barriers to nasal drug absorption, especially for proteins and peptides. The biopharmaceutical characteristics of proteins and peptides make their delivery a challenge. Such challenges stem from their high molecular weight, short plasma half-life,

Correspondence: Remigius U. Agu, Biopharmaceutics and Drug Delivery Laboratory, College of Pharmacy, Dalhousie University, 5968 College Street Halifax, NS B3H 3J5, Canada.
E-mail: remigius.agu@dal.ca

susceptibility for breakdown in both physical and biological environments, and the tendency to undergo self-association, immunogenicity and lack of precise dosing.[12] Despite these challenges, some proteins and peptides, including salmon calcitonin (e.g. Miacalcin), desmopressin (e.g. DDAVP) and nafarelin (e.g. Synarel), have made it on to the market.[13] In order to successfully develop other peptides for nasal administration, the impact of metabolic and penetration barriers must be recognised.[14] Enzymatic barriers comprise exopeptidases and endopeptidases that cleave peptides and proteins at N- and C-terminals and at internal peptide bonds, respectively. The exopeptidases include aminopeptidase, dipeptidyldipeptidase, diaminopeptidase, dipetidylcarboxypeptidase, serine carboxypeptidase, metalocarboxypeptidase and cysteine carboxypeptidase. Aminopeptidases are one of the major enzymes that metabolise peptides and proteins. They cleave the N-terminal amino acids of dipeptides and tripeptides. The expression of these enzymes has been reported in various organs, including the intestine, liver, kidney, spleen, muscle and the nasal epithelium.[15,16] Similarly, dipeptidyldipeptidases (DPPIV) specifically recognise and cleave N-terminal dipeptides. The dipeptidyldipeptidases are classified as DPP I to IV. Each type has different specific activity, physicochemical characteristics and cellular localisation. DPPIV, known to be expressed in plasma membranes, contributes towards the degradation of peptide drugs.[17]

Aminopeptidases result in presystemic metabolism of intranasally administered peptides. One of the best approaches for investigating the effect of drug metabolism on systemic bioavailability is the use of tissue culture models. In the area of nasal drug delivery, information on cell culture models for drug evaluation is limited. The aim of this study was to compare the functional, mRNA expression and activity of specific aminopeptidases of excised human nasal mucosa and cells in primary culture. We also aimed to determine whether tissue culture environment significantly affects aminopeptidase expression and activity. The results of this study may be useful as a reference for cross-validation of yet-to-be developed nasal epithelial cell lines with respect to aminopeptidase metabolic characteristics. Unlike our previous studies that demonstrated the degradation of enkephalins by the nasal aminopeptidases,[16] this study further explored the relative contributions of specific aminopeptidases, aminopeptidase N (APN), aminopeptidase B (APB) and DPPIV, in peptide degradation in human nasal epithelium. Furthermore, this study showed for the first time that the genes that encode APN, APB and DPPIV *ex vivo*[18] are expressed in nasal primary cultures.

Materials and Methods

Chemicals

Enzyme substrates L-arginine-7-amino-methyl-coumarin (Arg-MCA), L-alanine-4-methyl-coumaryl-7-amide (Ala-MCA), glycyl-L-proline 4-methyl-coumaryl-7-amide (Gly-MCA) and 7-amino-methyl-coumarin were obtained from ICN Biomedicals (Aurora, OH, USA). Puromycin hydrochloride, bestatin hydrochloride, protease type XIV (pronase) and bovine serum albumin were obtained from Sigma (St Louis, MO, USA). Phenol red, phosphate-buffered saline, Hanks' balanced salt, Trizol, M-MVL reverse transcriptase, cDNA buffer, and dNTP were supplied by Invitrogen (Carlsbad, CA, USA). MgCl, Taq PCR buffer and Taq polymerase were provided by Fermentas (Burlington, ON, USA). Agarose powder and oligo dT primers were from Fisher Scientific (Ottawa, ON, Canada) and Promega (Madison WI, USA), respectively. All other chemicals and reagents used during the study were of analytical grade and were used without further purification.

Cell culture

The cell culture method used for the study has been described in detail elsewhere.[19] Human nasal epithelial tissues (without secondary ultrastructural abnormalities) were obtained from 12 different adult patients that underwent endoscopic transnasal skull laser surgery. Smokers and patients with chronic inflammatory respiratory diseases were excluded from the study. Samples from the same patients were compared in homogenate and cell culture experiments. The use of human biopsies was approved by the QEII Regional Hospital Research Ethics Board (REB CDHA-RS/2006-352). The tissues were transported in DMEM–F12 1/1 (Life Laboratories, Paisley, UK) culture medium supplemented with streptomycin 100 μg/ml and penicillin 100 IU/ml (Boehringer, Mannheim, Germany) and used for cell culture. Tissues collected for tissue homogenate studies were immediately frozen in liquid nitrogen after excision to preserve the enzymes. The medium for the monolayer culture was Ultroser G 2.0% (Biosepra, Cergy, France) in DMEM–F12 1/1 supplemented with 10 ng/ml cholera toxin, streptomycin 100 μg/ml and penicillin 100 IU/ml.

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 before cell washing with DMEM–F12 1/1. The washing solution was removed after centrifugation at 70*g* for 5 min on each occasion. The resulting suspension of cells was filtered through a 70-μm pore size polycarbonate filter (Pall, Portsmouth, UK) and pre-plated 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 at a density of 5.0×10^5 cells/well. The cells were incubated at 37°C in a 95% O_2 and 5% CO_2 environment using DMEM–F12 supplemented with Ultroser G 2%. The medium was changed every other day. The cells were used for experiments after becoming confluent (7–14 days).

Preparation of nasal homogenates

The ultrastructurally normal human nasal epithelial tissues and cultured cells were homogenised in Hanks' balanced salt buffer with a tissue homogeniser (Kinematica GmbH, Lucerne, Switzerland) at 2500 rev/min. The homogenised cells were centrifuged at 10 000*g* for 10 min at 4°C to remove cellular and nuclear debris. The supernatant was used

for protein assay and for the determination of aminopeptidase activity. The supernatant was kept on ice and used within 4 h of preparation.

Optimisation of incubation time and protein content

Homogenate samples (5–100 μg) were pre-incubated at 37°C for 2 min. Subsequently, 0.03125–1 mM of substrate was added to initiate metabolism. The mixture was incubated for 5–60 min at 37°C. After the incubation period, metabolism was terminated using a stop buffer (0.12 M monochloroacetic acid and 0.13 M sodium acetate, pH 4.3). Then, 7-amino-methylcoumarin formed from the substrates was measured using a Perkin Elmer LS-55 Luminescence spectrofluorimeter (Perkin Elmer Instruments, MA, USA; excitation and emission wavelengths of 380 and 440 nm, respectively). Cell or tissue lysates over a concentration range of 5–20 μg/100 μl was adequate to produce a measurable reaction. Within this protein range, the rate of 4-methyl coumaryl-7-amide formation was linear. Based on these studies, the ideal protein concentration and incubation time were optimised to be 15 μg/100 μl and 10 min, respectively.

Assay for aminopeptidase activity

The method described by Quan *et al.*[17] was used to determine the aminopeptidase activity of the tissue and cell culture homogenates. Three aminopeptidase substrates (Arg-MCA, Ala-MCA and Gly-MCA) were used for APB, APN and DPPIV, respectively. The homogenate samples (5–50 μg protein in 950 μl PBS) were pre-incubated at 37°C for 2 min. Metabolism was initiated by the addition of 50 μl (0.125 mM) of the substrate and incubated for 10 min at 37°C. The reaction was terminated by adding 2 ml of stop buffer and 7-amino-methyl coumarin was measured as described above. Aminopeptidase assays were also conducted for each substrate in the presence of aminopeptidase inhibitors (bestatin and puromycin). The inhibitors were used over a concentration range of 0.01–1.1 mM. A 100-μl volume of the inhibitors in buffer was added to the homogenate samples and they were incubated for 15 min at 37°C. Following the incubation, 50 μl of the substrate solution was added and incubated for another 20 min. The reaction was subsequently terminated using the stop buffer, and the fluorescent activity of 7-amino-methyl-coumarin was determined as previously described.

RNA isolation

Total RNA was extracted from human nasal tissues and epithelial cells using Trizol according to the manufacturer's instructions. In brief, cells were lysed with Trizol at 1 ml per 10 cm^2. Subsequently, 200 μl of chloroform was added per 1 ml of Trizol and vortexed. Three phases were separated by centrifuging for 15 min at 12 000*g* at 4°C. Only the colourless upper aqueous RNA phase was removed and vortexed with isopropanol to precipitate the RNA. Samples were incubated at room temperature for 10 min before centrifugation for 10 min at 12 000*g* at 4°C. The resulting RNA pellet was washed twice with ice cold 75% ethanol and then re-suspended in double-distilled water. Concentration and purity of RNA was measured using GeneQuant Pro. All samples had A_{260}/A_{280} absorbance readings greater than 1.6, confirming high RNA purity.

cDNA synthesis

Isolated RNA (2 μg) was transcribed into cDNA by M-MVL reverse transcriptase using 5 mM dNTP, 1 μl of oligo dT primers and 2 μl of cDNA buffer.

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in a solution containing 1.5 μl of 50 μM MgCl, 2.5 μl Taq PCR buffer, 1 μl of 5 mM dNTP, 1 μl of 10 mM forward and reverse primers, 2 μl of cDNA, 0.5 units of Taq polymerase and water up to 25 μl. Gene sequences for primer design were obtained from the National Center for Biotechnology Information's GenBank. Primers were designed using OligoPerfect Designer from Invitrogen. APN (ANPEP) forward and reverse primer sequences were as follows: forward 5′-CCTCAATGTGACGGGCTATT-3′ (corresponding to bases 2161–2180), reverse 5′-TCATTGAC-CAGTGTGGCATT-3′ (corresponding to bases 2763–2744). The PCR product was 603 bp. APB (RNPEP) forward and reverse primer sequences were as follows: forward 5′-GAGCCCGTGAGCTTCTACAC-3′ (corresponding to bases 345–364), reverse 5′-GTGGCATGAAGAGCAAGTCA-3′ (corresponding to bases 906–877). The PCR product was 562 bp. DPPIV forward and reverse primer sequences were as follows: forward 5′-CAAATTGAAGCAGCCAGACA-3′ (corresponding to bases 2377–2396), reverse 5′-TGAAGTGGCTCATGTGGGTA-3′ (corresponding to bases 2836–2817). The PCR product was 460 bp. The solution experienced an initial denaturation at 94°C for 5 min and then 35 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min. A final extension was applied for 10 min at 72°C. A negative control was done using RNA and APN primers only. Amplified DNA was electrophoresed on a 1% agarose gel containing ethidium bromide.

Statistical analysis

Unless stated otherwise, each experiment was carried out three times and the data expressed as mean ± SD. Michaelis–Menten kinetic constants were calculated using non-linear regression curve-fitting using Graphpad Prism 5.0 (Graphpad Software Inc., CA, USA). Percentage inhibition due to bestatin and puromycin were computed by comparing the specific activity of the tissue and cell culture homogenate enzymes in the presence of the inhibitors with the specific activity of the enzymes in the absence of the inhibitors. Kruskal–Wallis analysis followed by Dunn's test was used to compare differences between the tissue and cell culture aminopeptidase kinetic parameters and the effect of protease inhibitors on the enzyme activity. Significant P values were set at $P < 0.05$.

Results

Figure 1 shows the formation of 7-amino-methylcoumarin from Ala-MCA, the substrate for APN in nasal tissue homogenates and primary culture. The formation of 7-amino-

methylcoumarin was concentration dependent. At a concentration of 0.5 mM Ala-MCA, more metabolite was formed in tissue homogenates compared with the cultured cells (approx. 3-fold). At the lowest concentration (0.0625 mM), approximately 1 μM of 7-amino-methylcoumarin was formed, whereas less than 0.5 μM was formed in cell culture homogenates. Similarly, at the highest concentration of the substrate (0.5 mM), more 7-amino-methylcoumarin was formed in the tissue homogenates. Overall, the results indicated more metabolic activity of APN in the tissue samples compared with cultured cells. Despite this quantitative difference, the formation of 7-amino-methylcoumarin in both experimental models showed that APN activity is preserved in primary culture.

The metabolic cleavage of Arg-MCA to 7-amino-methylcoumarin was used to compare the APB activity of tissue and primary culture homogenates. The results of the metabolic degradation of the substrate in both models are summarised in Figure 2. Whereas the breakdown of Arg-MCA to 7-amino-methylcoumarin was rapid in the nasal tissue homogenates (saturation was reached within 15 min), the degradation of the compound in the tissue culture homogenates occurred gradually over a 1-h period. As observed for APN, the formation of 7-amino-methylcoumarin was concentration dependent. At a much lower concentration of the substrate (0.007812 nM), approximately 1 μM of 7-amino-methylcoumarin was formed in both models. A comparable result was also obtained with higher substrate concentrations.

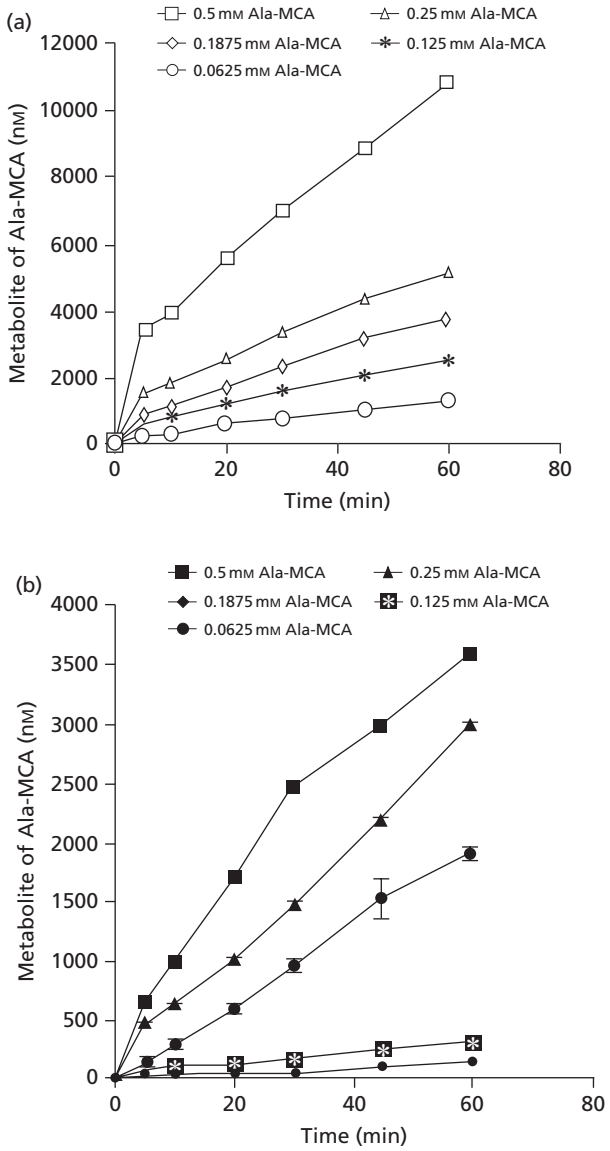

Figure 1 Formation of 7-amino-methyl-coumarin from L-alanine-4-methyl-coumaryl-7-amide. Formation of 7-amino-methyl-coumarin was determined in excised tissue (a) and primary culture (b) homogenates of human nasal epithelium. Each point represents the mean ± SD, $n = 3$.

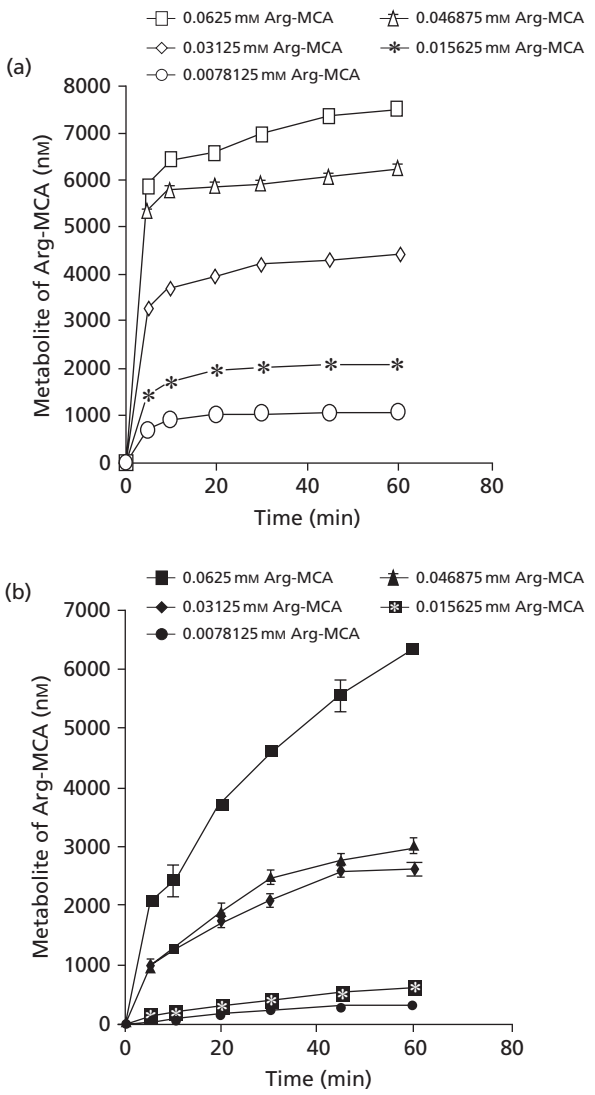

Figure 2 Formation of 7-amino-methyl-coumarin from L-arginine-4-methyl-coumaryl-7-amide. Formation of 7-amino-methyl-coumarin was determined in excised tissue (a) and primary culture (b) homogenates of human nasal epithelium. Each point represents the mean ± SD, $n = 3$.

Following the exposure of the cells to Gly-MCA, the metabolic substrate for DPPIV, the formation of 7-amino-methylcoumarin was comparable in both the tissue and nasal culture homogenates. As with the substrates for APN and APB, the release of the fluorescent metabolite from Gly-MCA was linear at the lowest concentrations of the substrates (0.02–0.06 mM). In line with the substrates for APN and APB, both the lowest (0.02 mM) and highest (0.1 mM) concentrations of the DPPIV substrate yielded comparable quantities of 7-amino-methylcoumarin in 1 h (Figure 3).

Table 1 summarises the kinetic parameters (K_M and V_{max}) for the formation of 7-amino-methylcoumarin by the tissue and cell culture homogenates following incubation with APN, APB and DPPIV specific substrates. The K_M (mM) for 7-amino-methylcoumarin formation was 2.9 ± 0.2, 0.04 ± 0.01 and 0.2 ± 0.04 (excised tissue homogenates) and 0.11 ± 0.01, 0.17 ± 0.02 and 0.74 ± 0.3 (cell culture homogenates) for APN, APB and DPPIV, respectively. Similarly, the V_{max} (nmol/mg protein per min) for Ala-MCA, Arg-MCA and Gly-MCA conversion to 7-amino-methylcoumarin were 41.7 ± 9.2, 10.1 ± 0.9, 3.4 ± 0.2 (excised tissue homogenates) and 1.3 ± 0.2, 1.62 ± 0.4 and 2.1 ± 0.3 (cell culture homogenates), respectively. Dunn's analysis showed no statistically significant difference between the K_M of the aminopeptidases (APB, APN and DPPIV) in tissue versus cell culture homogenates ($P > 0.05$). Similar results were observed for APB and DPPIV V_{max} ($P > 0.05$). However, the APN V_{max} for the tissue homogenates was significantly higher ($P < 0.05$) compared with cell homogenates. The observed metabolism of Ala-MCA, Arg-MCA and Gly-MCA in both the tissue and cell culture homogenates showed that the functional expression of APN, APB and DPPIV in the epithelium *in vivo* was preserved. The decreased V_{max} noted for APN and APB of cultured nasal cells relative to the tissue homogenates has been observed in aminopeptidases of cultured retinal pigment epithelium.[20] This may explain the more rapid breakdown of Arg-MCA that was observed in tissue homogenates relative to cultured cells.

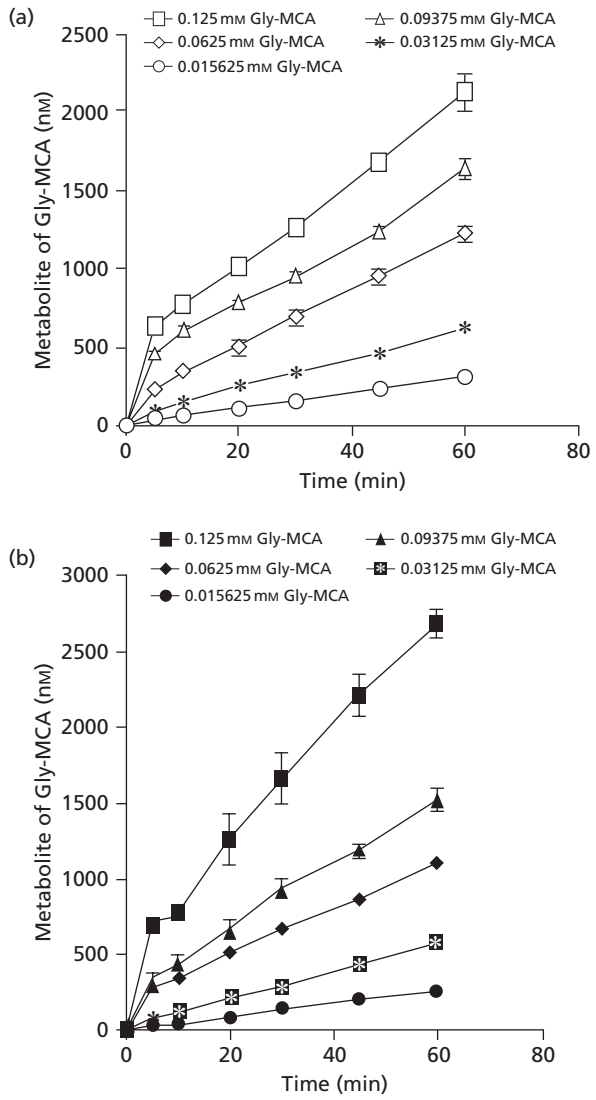

Figure 3 Formation of 7-amino-methyl-coumarin formation from glycyl-L-proline-4-methyl-coumaryl-7-amide. Formation of 7-amino-methyl-coumarin was determined in excised tissue (a) and primary culture (b) homogenates of human nasal epithelium. Each point represents the mean ± SD, $n = 3$.

In vivo, the predominant aminopeptidase in human nasal epithelial and submucosal gland cells is membrane-bound puromycin-resistant aminopeptidase.[18] It was therefore important to subject the aminopeptidases of the nasal tissue and primary culture homogenates to the inhibitory effect of specific aminopeptidase inhibitors (puromycin and bestatin). Table 2 shows concentration-dependent inhibition of APN in the nasal tissue and primary culture homogenates by bestatin and puromycin. Similar results were also obtained for APB. As for DPPIV, the enzyme showed remarkable resistance to the inhibitory effect of bestatin and puromycin in both the tissue and cell culture homogenates. Bestatin showed higher inhibition than puromycin at equimolar concentrations. Whereas 0.2 mM bestatin resulted in more than 90% inhibition of APN and APB in the tissue and cell culture homogenates, it resulted in only 30–60% inhibition of DPPIV. The inhibitors significantly reduced specific aminopeptidase activity in both tissue and cell culture homogenates ($P = 0.006$, Kruskal–Wallis test). However, Dunn's post-test analysis showed that APB, APN and DPPIV were inhibited in a similar manner in both tissue and cell culture homogenates ($P > 0.05$).

Although the various functional studies highlighted some characteristics of specific aminopeptidases in human nasal and tissues homogenates, PCR was used to compare the expression of gene transcripts that encode APN, APB and DPPIV. The PCR results are summarised in Figure 4. No gene product was seen in the reaction with negative control. Reactions with the RNA isolated from nasal cultures yielded the predicted PCR product size for all three genes studied. For APN (ANPEP) an expected 600-bp product was seen. A product of about 550 bp was seen for APB (RNPEP) and a product just under 500 bp was amplified using the DPPIV gene specific primers. Reactions with the RNA from nasal

Table 1 Michaelis–Menten parameters of tissue and monolayer culture homogenates of human nasal epithelium for aminopeptidase N, B and dipeptidyldipeptidase IV

	Substrate	Enzyme	$K_m \pm SD$ (mM)	$V_{max} \pm SD$ (nmol/mg protein per min)
Excised tissue	Ala-MCA	Aminopeptidase N	2.9 ± 0.9	41.7 ± 9.2*
	Arg-MCA	Aminopeptidase B	0.04 ± 0.01	10.1 ± 0.9
	Gly-MCA	Dipeptidyldipeptidase IV	0.2 ± 0.04	3.4 ± 0.2
Monolayer culture	Ala-MCA	Aminopeptidase N	0.11 ± 0.01	1.34 ± 0.2
	Arg-MCA	Aminopeptidase B	0.17 ± 0.02	1.62 ± 0.4
	Gly-MCA	Dipeptidyldipeptidase IV	0.74 ± 0.3	2.1 ± 0.3

Michaelis–Menten parameters (K_m and V_{max}) of tissue and monolayer culture homogenates of human nasal epithelium for aminopeptidase N, B and dipeptidyldipeptidase IV. Each value represents the mean ± SD of three experiments. *$P < 0.05$, aminopeptidase N V_{max} for the tissue homogenate was significantly higher compared with the cell homogenate.

tissue homogenates also showed the predicted PCR products; however, other PCR products of unexpected sizes were also observed (Figure 4).

Discussion

Most of the currently available data on presystemic metabolic clearance of proteins and peptides in the human nasal mucosa were based on studies with excised tissue from animal species.[21] Species and anatomical differences, tissue viability and sometimes difficulties encountered in extrapolating some animal tissue based study results to humans are important concerns when using animal models.[22] Human nasal epithelium cultured on permeable supports is a viable alternative for overcoming some of the problems associated with excised tissue models for studying nasal drug absorption and metabolism. Although cell lines remain the most popular models for investigating drug absorption and metabolism via the oral and pulmonary routes, this is not the case for the nasal route, as there are currently no acceptable nasal epithelial cell lines for drug delivery studies. In order to increase our understanding of the suitability and potential application of the human nasal epithelium in primary culture for investigating protein and peptide enzymatic stability following nasal administration, we investigated the functional expression and metabolic characteristics of different aminopeptidases in human nasal primary culture. We also compared the results of the cultured cells to that of homogenised tissues to find out to what extent growing the cells possibly alters the aminopeptidase activity.

Using leucine-enkephalin and alanine-nitroanilide, Ohkubo *et al.*[18] showed that freshly harvested human nasal epithelium expressed aminopeptidases and DPPIV. These enzymes are typically involved in the presystemic metabolism of most nasally administered proteins and peptides. Therefore in this study, APN, APB and DPPIV were investigated using their specific substrates. Based on this approach, non-fluorescent amides (Ala-MCA, Arg-MCA, Gly-MCA) were cleaved by the enzymes to yield their respective amino acids and a fluorescent metabolite (7-amino-methylcoumarin).

Table 2 Effect of aminopeptidase inhibitors on the metabolic activity of aminopeptidases and dipeptidyldipeptidases

	Inhibitor	Concn (mM)	Inhibition (%)		
			Aminopeptidase N	Aminopeptidase B	Dipeptidyldipeptidase IV
Excised tissue	Puromycin	0.05	29.1 ± 1.1	34.4 ± 0.5	7.5 ± 1.3
		0.2	34.8 ± 0.6	91.0 ± 0.7	16.8 ± 4.5
		0.5	ND	ND	24.9 ± 2.2
		1.0	ND	ND	24.9 ± 1.6
	Bestatin	0.05	78.9 ± 0.9	92.0 ± 0.7	8.7 ± 3.2
		0.2	97.9 ± 9.6	95.6 ± 0.1	32.2 ± 1.4
Monolayer culture	Puromycin	0.05	59.3 ± 4.9	19.0 ± 0.6	21.4 ± 5.9
		0.2	82.0 ± 2.1	40.0 ± 1.0	31.5 ± 0.9
		0.5	ND	ND	36.8 ± 0.5
		1.0	ND	ND	37.6 ± 9.8
	Bestatin	0.05	94.7 ± 1.4	95.3 ± 0.2	11.9 ± 8.7
		0.2	98.9 ± 0.2	97.7 ± 0.1	61.2 ± 7.2

Effect of puromycin and bestatin on the metabolic activity of aminopeptidase N, aminopeptidase B and dipeptidyldipeptidases of the human nasal tissue and primary culture homogenates. Each value represents the mean ± SD of three experiments. ND, not determined as significant inhibition was recorded before the concentration. The inhibitors significantly reduced specific aminopeptidase activity in tissue and cell culture homogenates ($P = 0.006$, Kruskal–Wallis test, KW = 16.3). Dunn's post-test analysis indicated that aminopeptidase B, N and dipeptidyldipeptidase IV were inhibited in a similar manner in both tissue and cell culture homogenates ($P > 0.05$).

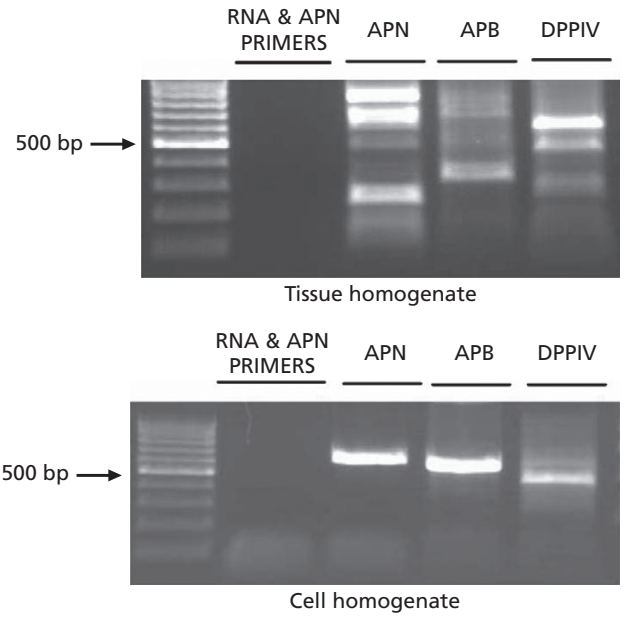

Figure 4 Polymerase chain reaction results. Expression of mRNA transcripts for aminopeptidase B (APB), aminopeptidase N (APN) and dipeptidyldipeptidase IV (DPPIV) in human nasal tissue and cell homogenates.

The observed significant inhibition of the aminopeptidases in the tissue and cell culture homogenates by puromycin and bestatin confirmed the presence of these enzymes in the nasal tissues, as well as preservation of their metabolic characteristics when cultured as monolayers. The relative resistance of DPPIV to puromycin and bestatin in both nasal homogenates and cultured cells implied that the cleavage of N-terminal dipeptides in the mucosa by DPPIV may require unreasonably high concentrations of the inhibitors to significantly inhibit the enzyme *in vivo*. Puromycin resistant aminopeptidase is an integral membrane protein and its cDNA for the rat kidney has been cloned, giving insights to its amino acid sequence.[23,24] It is yet to be determined whether the amino acid sequence of the puromycin-resistant DPPIV is similar to the rat kidney sequence.

Although tissue and cell culture homogenates were used in this study, it important to highlight the fact that the rate and extent of peptide and protein metabolism during delivery across intact cells may not be the same as in homogenised enzymes. Therefore, the aminopeptidase kinetic parameters such as K_m and V_{max} may vary significantly. Disruption of cell membranes by homogenisation does not take into account subcellular enzyme organisation relative to intracellular transport. Moreover, homogenisation liberates enzymes from their physiological compartments making both membrane-bound and cytosolic enzymes available for substrate metabolism. Differences in both patterns and extent of metabolite formation have been observed in homogenate versus intact cells.[25–27] It is also important to mention that full enzyme preservation may not be maintained during homogenisation. Therefore, loss of enzyme activity may also be possible. It is therefore important to consider experimental variables when interpreting the results of metabolism studies. Moreover, the kinetics and specificity of proteases *in vivo* are likely to be different from those *in vitro* because of differences in substrate concentration and harsh treatments often used for enzyme isolation.[28]

PCR was used to compare the expression of gene transcripts that encode APN, APB and DPPIV. Reactions with the RNA isolated from nasal cultures yielded the predicted PCR product size for all three genes studied. Reactions with the RNA from nasal tissue homogenates also showed the predicted PCR products and other PCR products of unexpected sizes. The unpredicted PCR products are most likely to be due to RNA fragments. The RNA from smaller pieces of tissue was probably semi-degraded by the time the RNA was extracted. The time lapse between tissue harvesting from patients and incubation in Trizol (~1 h) might have led to certain RNA degradation and subsequent annealing of the RNAs, which led to the unpredicted bands in the tissue homogenates. Furthermore, trauma during tissue excision may cause complimentary sequences in sRNA to bind to mRNA, thus creating double-stranded RNA molecules that are substrates for certain classes of RNAses.[29] This might explain the source of the unexpected bands as the tissue RNAs become very unstable immediately after harvest.

Conclusions

Based on the results of the study, it seems reasonable to conclude that the observed cleavages of Ala-MCA, Arg-MCA and Gly-MCA in both nasal tissue and primary culture homogenates were due to APB, APN and DPPIV. The following key observations were made: (1) the K_M of APB, APN and DPPIV was not significantly different in cell and tissue homogenates; (2) except for APN, the V_{max} was not significantly different in the two metabolism models; (3) there was no statistically significant difference in the behaviours of APB, APN and DPPIV in response to inhibition by puromycin and bestatin in the two models; (4) the mRNA transcripts that encode APB, APN and DPPIV were expressed in both cell culture and tissue homogenate. Based on these observations, it may be concluded that the nasal primary cell culture system is suitable for investigating peptide and protein metabolism and enzymatic stability in the human nasal epithelium. Except for APN, it may also be concluded that the tissue culture conditions did not significantly alter the kinetic and molecular expression of the aminopeptidases.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was partially supported by start-up funding granted to R. U. Agu by the Dalhousie University Pharmacy Endowment Fund.

References

1. Tirucherai GS *et al*. Novel approaches to nasal delivery of peptides and proteins. *STP Pharma Sci* 2002; 12: 3–12.
2. Scheuch G, Siekmeier R. Novel approaches to enhance pulmonary delivery of proteins and peptides. *J Physiol Pharmacol* 2007; 58(Suppl 5): 615–625.
3. Morley P. Delivery of parathyroid hormone for the treatment of osteoporosis. *Expert Opin Drug Deliv* 2005; 2: 993–1002.
4. Sudhakar Y *et al*. Buccal bioadhesive drug delivery – a promising option for orally less efficient drugs. *J Control Release* 2006; 114: 15–40.
5. Justin-Temu M *et al*. Intravaginal gels as drug delivery systems. *J Womens Health (Larchmt)* 2004; 13: 834–844.
6. Steidler L. Delivery of therapeutic proteins to the mucosa using genetically modified microflora. *Expert Opin Drug Deliv* 2005; 2: 737–746.
7. Goto T *et al*. Novel mucosal insulin delivery systems based on fusogenic liposomes. *Pharm Res* 2006; 23: 384–391.
8. Arnolds S, Heise T. Inhaled insulin. *Best Pract Res Clin Endocrinol Metab* 2007; 21: 555–571.
9. Merkus FW *et al*. Cyclodextrin in nasal drug delivery. *Adv Drug Deliv Rev* 1991; 36: 41–57.
10. Ugwoke MI *et al*. Nasal mucoadhesive drug delivery: background, applications, trends and future perspectives. *Adv Drug Deliv Rev* 2005; 57: 1640–1665.
11. Borland M *et al*. D. A randomized controlled trial comparing intranasal fentanyl to intravenous morphine for managing acute pain in children in the emergency department. *Ann Emerg Med* 2007; 49: 335–340.
12. Shoyele SA, Slowey A. Prospects of formulating proteins/peptides as aerosols for pulmonary drug delivery. *Int J Pharm* 2006; 314: 1–8.
13. Costantino HR *et al*. Intranasal delivery: physicochemical and therapeutic aspects. *Int J Pharm* 2007; 337: 1–24.
14. Lee VH, Yamamoto A. Penetration and enzymatic barriers to peptides and protein absorption. *Adv Drug Del Rev* 1990; 4: 171–211.
15. Torp N *et al*. Lactase-phlorizin hydrolase and aminopeptidase N are differentially regulated in the small intestine of the pig. *Biochem J* 1993; 295: 177–182.
16. Agu RU *et al*. Metabolism and absorption enhancement of methionine enkephalin in human nasal epithelium. *Peptides* 2004; 25: 563–569.
17. Quan YS *et al*. Transport kinetics of leucine enkephalin across Caco-2 monolayers: quantitative analysis for contribution of enzymatic and transport barrier. *Life Sci* 1999; 64: 1243–1252.
18. Ohkubo K *et al*. Aminopeptidase activity in human nasal mucosa. *J Allergy Clin Immunol* 1998; 102: 741–750.
19. Agu RU *et al*. In-vitro nasal drug delivery studies: comparison of derivatised, fibrillar and polymerised collagen matrix-based human nasal primary culture systems for nasal drug delivery studies. *J Pharm Pharmacol* 2001; 53: 1447–1456.
20. Fröhlich E, Klessen C. Regional differences and post-mortem stability of enzymatic activities in the retinal pigment epithelium. *Graefes Arch Clin Exp Ophthalmol* 2003; 241: 385–393.
21. Agu RU, Ugwoke MI. *In situ* and *ex vivo* nasal models for preclinical drug development studies. In: Ehrhardt C, Kim KJ, eds. *Drug Absorption Studies - In situ and In silico Tools for Drug Absorption Studies*. New York: Springer Verlag, 2008: 112–129.
22. Morimoto K *et al*. Effects of proteolytic enzyme inhibitors on the nasal absorption of vasopressin and an analogue. *Pharm Res* 1991; 8: 1175–1179.
23. Knight M, Klee WA. The relationship between enkephalin degradation and opiate receptor occupancy. *J Biol Chem* 1978; 253: 3843–3847.
24. Tobler AR *et al*. Cloning of the human puromycin-sensitive aminopeptidase and evidence for expression in neurons. *J Neurochem* 1997; 68: 889–897.
25. Malfroy B *et al*. Molecular cloning and amino acid sequence of rat kidney aminopeptidase M: a member of a super family of zinc-metallohydrolases. *Biochem Biophys Res Commun* 1989; 161: 236–241.
26. Choi HK *et al*. Transdermal delivery of bioactive peptides: the effect of n-decylmethyl sulfoxide, pH, and inhibitors on enkephalin metabolism and transport. *Pharm Res* 1990; 7: 1099–1106.
27. Ademola JI *et al*. Metabolism of propranolol during percutaneous absorption in human skin. *J Pharm Sci* 1993; 82: 767–770.
28. Lee VHL *et al.* Enzymatic barriers to peptide and protein delivery. In: Lee VHL, ed. *Peptide and Protein Drug Delivery*. New York: Marcel Dekker Inc., 1991: 303–358.
29. Jaronczyk K *et al*. Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? *Biochem J* 2005; 387: 561–571.