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Tripeptide transport in rat lung

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Abstract

Transport of L-alanyl-D-phenylalanyl-L-alanine was investigated with an in situ vascular perfusion preparation of rat lung and brush border membrane vesicles prepared from type II pneumocytes. In the perfused lung 1 mM tripeptide was transported intact from the alveolar lumen to the vascular perfusate at a mean rate of 25.1 ± 1.29 (3) nmol/min per g dry weight. D-Phenylalanine also appeared in the vascular perfusate at a rate of 21.9 ± 1.74 (3) nmol/min per g dry weight indicating that 47% of the absorbed tripeptide was split during passage across the epithelial layer. No dipeptide could be detected in the vascular effluent during perfusions with tripeptide. Rapid L-alanyl-D-phenylalanyl-L-alanine uptake occurred with fresh apical membrane vesicles prepared from type II pneumocytes and this was abolished by treatment with 0.1% triton. The related tripeptide, D-alanyl-L-phenylalanyl-D-alanine, was taken up significantly more slowly by the vesicles. D-phenylalanyl-L-alanine and D-phenylalanyl-D-alanine, were also studied with the vascularly perfused preparation; the mixed dipeptide appeared in the vascular perfusate significantly faster than L-alanyl-D-phenylalanyl-L-alanine whereas D-phenylalanyl-D-alanine appeared more slowly and was not hydrolysed.

Key words: Tripeptide; Lung; Membrane transport; (Rat)

1. Introduction

The pulmonary epithelium is embryologically derived from the primitive foregut and can transport sugars and amino acids [1,2]. Previously, we have reported [3] that dipeptides of L-phenylalanine and L-alanine are absorbed intact from the alveolar lumen (air space), but are split within the epithelial layer, releasing the constituent amino acids into the vascular perfusate. When mixed D/L-dipeptides with an N-terminal D-amino acid were placed in the alveolar lumen only about half of the absorbed peptide was hydrolysed and the remainder was transferred intact to the vascular perfusate [3]. The dipeptide D-phenylalanyl-D-alanine, which was not split by the perfused lung preparation, appeared in the vascular perfusate at a rate which was significantly slower than that of the mixed dipeptides. The experiments reported here provide the first evidence of tripeptide transport in the lung and the first indication that a peptide with an L-amino acid at the N-terminal end can cross the pulmonary epithelium without being hydrolysed.

The uptake of amino acids and peptides from the alveolar lumen of the lung may be physiologically significant for removal of the products of surfactant breakdown, for helping to regulate the thickness of the alveolar lining fluid layer and for removing potential nutrients which could support bacterial growth.

2. Materials and methods

Materials

All chemicals were of analytical grade. The reagents used for peptide synthesis were purchased from the Sigma, Dorset, UK. Sodium pentobarbitone was purchased from May and Baker, Dagenham, UK.

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Animals

Male Wistar rats (250 g) were purchased from Harlan Olac, Oxfordshire, UK and fed ad libitum on rat and mouse diet (Bantin & Kingham, Humberside, UK) until they reached a weight of 280 g.

Peptide synthesis

The tripeptide, L-alanyl-D-phenylalanyl-L-alanine, was synthesised by standard techniques [4] using two stages; first D-phenylalanyl-L-alanine was formed by condensation of *N-tert*-butyloxycarbonyl-D-phenylalanine with carboxybenzyl-L-alanine; after removal of the *N*-block from the dipeptide it was converted to the L-alanyl-D-phenylalanyl-L-alanine by condensation with *N*-benzyloxycarbonyl-L-alanine. The same technique was used to synthesize D-alanyl-L-phenylalanyl-D-alanine using the appropriate starting materials. The dipeptides, D-phenylalanyl-L-alanine and D-phenylalanyl-D-alanine were synthesised by condensation of *N-tert*-butyloxycarbonyl-D-phenylalanine with either carboxybenzyl-L-alanine or carboxybenzyl-D-alanine, respectively. The products were subjected to a series of acid and alkali washes to eliminate starting materials and byproducts before removal of the protecting groups. Finally, the samples were freeze-dried and the purity checked by mass spectrophotometry, NMR and HPLC.

Perfusion of rat lung

To establish the in situ vascular lung perfusion rats were anaesthetised by intraperitoneal injection of 20 mg of sodium pentobarbitone, the rat was placed on his back and the thorax opened to expose the lungs. The trachea was cannulated and the alveolar lumen of both lungs was rinsed with luminal perfusate (consisting of bicarbonate Krebs-Ringer at 37°C, gassed with 95% O_2-5% CO_2 and containing 1 mM peptide) and then a weighed amount (approx. 5 ml) of the luminal perfusate was instilled into the alveolar lumen. Although a larger volume can be instilled into the lumen of rat lungs, 5 ml was chosen because it gave reproducible perfusions without the risk of obstructing the vascular channels [5]. The vascular bed was perfused for 20 min through the pulmonary artery in single pass at 3 ml/min with bicarbonate Krebs-Ringer at 37°C which had previously been gassed with 95% O_2-5% CO_2 and containing 5% bovine serum albumin, 5 mM D-glucose, streptomycin and heparin. Samples of the vascular effluent were collected at 1 min intervals. At the end of each perfusion the luminal perfusate was recovered from the lungs in order to measure the volume and solute concentration. The lungs were then dissected out and dried to constant weight.

Membrane vesicle experiments

Vesicles were prepared from the lungs of two rats by the method of Shaw et al. [6], resuspended in 298 mM mannitol, 0.1 mM $MgSO_4$, 2 mM Tris-HCl at pH 7.4 and incubated in a medium containing 125 mM NaCl, 30 mM mannitol, 0.1 mM $MgSO_4$, 20 mM Tris-HCl at pH 7.4 plus 4 mM tripeptide. Each incubation was started by mixing equal volumes of vesicle suspension and incubation medium; uptake was terminated by rapid filtration through 0.65 μm Millipore filters, and the vesicles were lysed with 3% perchloric acid and neutralised with 1 M KOH.

Analysis of samples by HPLC

Samples from the vascular effluent and the alveolar lumen of the perfused lung preparations were deproteinised with 6% perchloric acid and centrifuged at $1800 \times g$ for 2 min; an aliquot of the supernatant was neutralized with 0.6 M KOH, rapidly frozen in liquid nitrogen, thawed, and recentrifuged at $1800 \times g$. The deproteinized, neutralized samples from the lung perfusions and the vesicle experiments were analysed for peptide and free phenylalanine by isocratic HPLC on a 5 μm ODS c18 column using a Kontron 400 automated HPLC system (Kontron Instruments, Watford, UK). The mobile phase was 20% methanol/80% 21 mM KH_2PO_4 (pH 5) and the elution profile was determined by measuring the absorbance of the eluant at 210 nm.

Expression of results

Rates of transport across the pulmonary epithelium in the perfused lung were calculated by linear regression from cumulative plots of solute appearance in the vascular effluent and are reported as rates per g dry weight ±standard error of the mean (S.E.) with the number of perfusions in brackets. The significance of differences between the rates of appearance were determined by covariance analysis.

3. Results

Tripeptide structure

Fig. 1 shows the structure of the tripeptide L-alanyl-D-phenylalanyl-L-alanine which we have found to be transported across the epithelial lining of the rat lung.

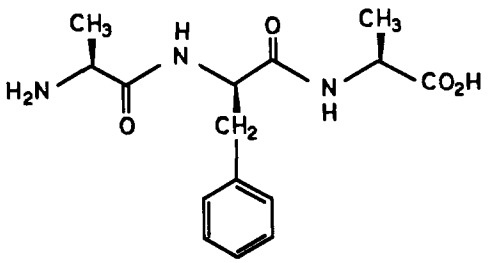

Fig. 1. Structure of the tripeptide, L-alanyl-D-phenylalanyl-L-alanine, which was used to study transport in rat lung. The tripeptide was synthesised in two stages from *N-tert*-butyloxycarbonyl-D-phenylalanine, carboxybenzyl-L-alanine and *N*-benzyloxycarbonyl-L-alanine.

Table 1
Rates of vascular appearance of phenylalanine in perfused rat lungs with amino acids or peptides present in the alveolar lumen

Luminal transport substrate	Concentration (mM)	Rate of appearance (nmol/min per g dry wt.
L-Phenylalanine	1	1560 ± 120
D-Phenylalanine	1	620 ± 30
D-Phenylalanyl-L-alanine	1	49 ± 4
L-Alanyl-D-phenylalanyl-L-alanine	1	22 ± 2
D-Phenylalanyl-D-alanine	1	0

This structure was verified by mass spectrometry and the sample was shown to be more than 95% pure by NMR and HPLC.

Transport of phenylalanine in the perfused rat lung

The viability of our perfused lung preparation was assessed by the stability of the vascular flow rate, the capacity of the preparation for amino acid transport and by the extent to which it was capable of discriminating between D-amino acids and L-amino acids. We have carried out sets of lung perfusion experiments with 1 mM free L-phenylalanine in the lumen which show (Table 1) that the L-amino acid was transported by the perfused rat lung at a rate of 1.56 ± 0.12 (3) μmol/min per g dry weight whereas the transport rate for D-phenylalanine was only 40% of that for the L-amino acid. At the conclusion of the perfusions with L-phenylalanine more than 92% of the amino acid had been absorbed from the alveolar lumen and the rate of uptake declined sharply due to depletion of the transport substrate in the alveolar lumen.

Peptide transport in the perfused rat lung

When the lungs were perfused for 20 min with 1 mM L-alanyl-D-phenylalanyl-L-alanine the luminal tripeptide concentration did not decrease significantly, although it was only possible to recover 90% of the initial fluid volume. Fig. 2 shows that after the initial 3 min the cumulative appearance of tripeptide in the vascular effluent (heavy line) was approximately linear giving a mean rate of 25.1 ± 1.29 (3) nmol/min per g dry weight for the three perfusions (see Table 2). The tripeptide could be detected in the first sample of vascular perfusate and it rose to a maximum concentration of 2.8 μM in the vascular effluent. In addition to tripeptide we also found D-phenylalanine in the vascular perfusate indicating that some of the tripeptide absorbed from the lumen was hydrolysed. The mean rate of vascular appearance of phenylalanine in the perfusions with the L-alanyl-D-phenylalanyl-L-alanine in the lumen is shown in Table 1 and indicates that the quantity of tripeptide appearing in the vascular effluent was approximately half that taken up from the

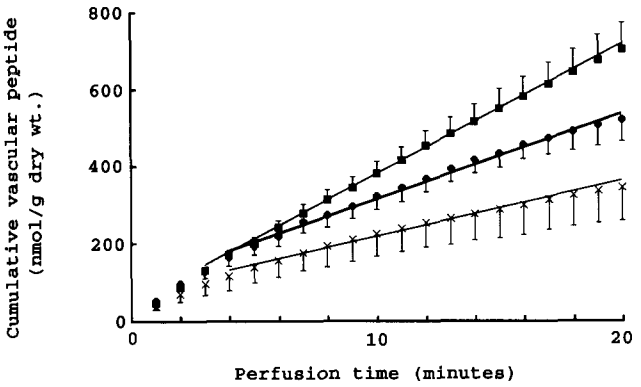

Fig. 2. Time-course of the vascular appearance of a tripeptide and two dipeptides in three groups of in situ perfusions of rat lung. The points show the mean cumulative appearance of L-alanyl-D-phenylalanyl-L-alanine (●), D-phenylalanyl-L-alanine (■) and D-phenylalanyl-D-alanine (X) in the vascular effluent of rat lungs in which the lumen was perfused with either the tripeptide or one of the dipeptides at a concentration of 1 mM. The values are nmol/g dry weight ± S.E. ($n = 3$). The lines are calculated regressions from the individual data points over the time range indicated by each line.

alveolar lumen of the lung. Table 2 shows that when the concentration of L-alanyl-D-phenylalanyl-L-alanine in the alveolar lumen was increased to 2 mM the rate of vascular dipeptide appearance was doubled, although there was no significant increase in the rate of D-phenylalanine appearance in the vascular effluent. There was no evidence for D-phenylalanine or other hydrolysis products of the tripeptide in the lumen at the end of the perfusion.

The results of two groups of 3 perfusions with 1 mM dipeptides (D-phenylalanyl-L-alanine and D-phenylalanyl-D-alanine) are also shown in Fig. 2 for comparison with the L-alanyl-D-phenylalanyl-L-alanine. Both dipeptides cross the pulmonary epithelium intact and the cumulative rates of appearance of the two dipeptides, which are shown in Table 2, differed significantly from the cumulative rate for the tripeptide ($P < 0.01$). It should be pointed out that the perfused lung handled the two D-phenylalanine dipeptides quite differently. In the case of the mixed dipeptide D-phenylalanine appeared in the vascular effluent at approximately the same rate as the dipeptide (see Table 1)

Table 2
Rates of vascular appearance of peptides in perfused rat lungs with peptides present in the alveolar lumen

Luminal peptide	Concentration (mM)	Rate of appearance (nmol/min per g dry wt.)
D-Phenylalanyl-L-alanine	1	34.6 ± 1.4
L-Alanyl-D-phenylalanyl-L-alanine	1	25.1 ± 1.3
L-Alanyl-D-phenylalanyl-L-alanine	2	56.9 ± 1.7
D-Phenylalanyl-D-alanine	1	15.6 ± 2.0

indicating that the dipeptide appearing in the vascular effluent was only half of that absorbed from the lumen. However, the dipeptide composed entirely of D-amino acids was not split in the lung preparation, so that the rate at which this peptide was taken up from the alveolar lumen was about 20% of that for the mixed dipeptide.

The time-course of cumulative vascular peptide appearance in Fig. 2 indicates that for both the tripeptide and for D-phenylalanyl-D-alanine the rapid initial rate of appearance was followed by a steady rate for a period of 12–14 min, and at the end of the perfusion period the rate declined somewhat. In Table 2 we choose to report the rates over the 4–18 min period rather than the initial rates because we considered that they most closely approximated the steady state rates of vascular appearance. The fact that D-phenylalanyl-D-alanine is not split in the perfused lung may indicate that this peptide is crossing the epithelial layer by a paracellular route or a leak, and if the rate of appearance of this dipeptide is subtracted from that of the tripeptide the difference is linear throughout the 20 min perfusion period at a rate of 8.8 ± 1.0 (3) nmol/min per g dry weight.

Tripeptide uptake by membrane vesicles

We have investigated the cellular basis of this transport with membrane vesicles prepared from type II pneumocytes [7]. Fig. 3 shows the time-course of the uptake of 2 mM L-alanyl-D-phenylalanyl-L-alanine by fresh vesicles as well as that by vesicles lysed with 0.1% triton. Each point represents the mean value ±S.E. from four vesicle experiments. The fact that no tripeptide was associated with the lysed vesicles is an indication that there was no appreciable binding of the tripeptide to the brush border membranes. The uptake of L-alanyl-D-phenylalanyl-L-alanine was also shown to be into an osmotically active space.

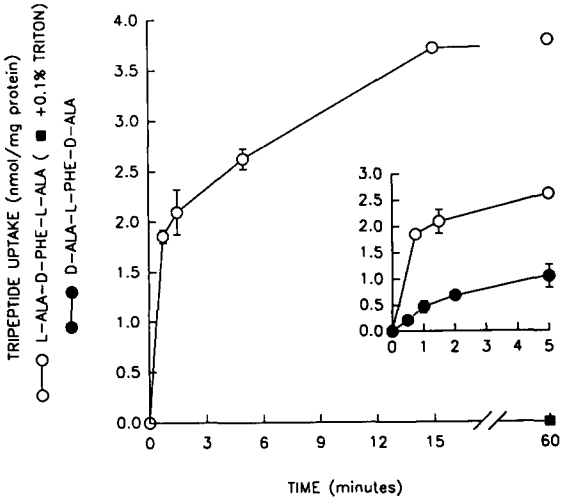

Fig. 3. Time-course of uptake of 2 mM L-alanyl-D-phenylalanyl-L-alanine (○) and of 2 mM D-alanyl-L-phenylalanyl-D-alanine (●) into isolated membrane vesicles prepared from type II pneumocytes. The values are nmol of tripeptide/mg protein ±S.E. ($n = 4$); (■) vesicles incubated with 2 mM L-alanyl-D-phenylalanyl-L-alanine plus 0.1% Triton. The vesicles were used at a concentration of 2.2 mg protein/ml. The vesicle volume at equilibrium was 1.92 μl/mg protein.

The insert plot in Fig. 3 compares the initial rates of uptake of L-alanyl-D-phenylalanyl-L-alanine with that of the reverse tripeptide, D-alanyl-L-phenylalanyl-D-alanine. As can be seen the initial rate of uptake of L-alanyl-D-phenylalanyl-L-alanine is very much more rapid than that of D-alanyl-L-phenylalanyl-D-alanine, and the half-time of filling of the vesicles is approximately 1 and 3 min, respectively.

4. Discussion

The results reported in this paper show that L-alanyl-D-phenylalanyl-L-alanine (Fig. 1) is transported intact from the alveolar lumen (air space) to the vascular effluent of the in situ vascular perfusion preparation of rat lung. The cumulative plots in Fig. 2 show that the tripeptide appeared in the vascular perfusate at a steady rate for most of the perfusion period although the rate for the first 3 min was approximately 80% faster. This was also true for the mixed dipeptide, D-phenylalanyl-L-alanine, and for D-phenylalanyl-D-alanine. It is possible that the decline in rate after the first few minutes could result from the lungs being incompletely filled.

In view of the relatively slow rate at which the tripeptide was transported it is tempting to conclude that transfer of the tripeptide across the epithelial layer was due to a leak, or possibly to paracellular transport. However, such a conclusion is not supported by the results we have obtained with the two dipeptides (D-phenylalanyl-L-alanine and D-phenylalanyl-D-alanine). A comparison of the rates of transport of the three peptides in Fig. 2 shows that L-alanyl-D-phenylalanyl-L-alanine appears significantly faster than D-phenylalanyl-D-alanine ($P < 0.01$) and the difference between these two rates is linear throughout the perfusion period. Furthermore, since approximately half of the tripeptide absorbed from the lumen was hydrolysed during passage across the epithelium, the rate of tripeptide uptake from the lumen is three times that of D-phenylalanyl-D-alanine, The fact that no hydrolysis products were found in the lumen, makes it is reasonable to conclude that the L-alanyl-D-phenylalanyl-L-alanine must have entered the epithelial cells before any hydrolysis occurred.

The conclusions we have drawn from our experiments with the the in situ vascular perfusion preparation are reinforced by the results we have obtained with the brush border membrane vesicles from type II

pneumocytes, which clearly show rapid uptake of L-alanyl-D-phenylalanyl-L-alanine. Uptake of the reverse tripeptide, D-alanyl-L-phenylalanyl-D-alanine, by the vesicles occurs significantly more slowly which suggests that there is a specific peptide transporter in the vesicle membrane. Preliminary studies with the reverse tripeptide in the in situ vascular perfusion preparation indicate that it is also transported across the epithelial layer of the rat lung (unpublished observations).

Although the cellular basis of peptide transport in the lung is not yet clear, it seems likely to involve type II pneumocytes [7]. The doubling of the rate of appearance of L-alanyl-D-phenylalanyl-L-alanine in the vascular effluent when the luminal substrate concentration is raised from 1 to 2 mM (Table 2) is consistent with an estimated K_m for L-alanyl-D-phenylalanyl-L-alanine in lung brush-border membrane vesicles of 3.9 mM. The V_{max} for the transport of this tripeptide by the membrane vesicles was 1.3 nmol/min per mg protein which is consistent with the hypothesis that the type II cells are the site of transepithelial tripeptide transfer. (1 mg of apical membrane vesicle protein may be estimated to be derived from approximately 10^8 type II cells; with $1.5 \cdot 10^{10}$ such cells in a adult rat [8], the observed rate of transfer at 2 mM tripeptide (15 nmol/min per rat by the in situ vascularly perfused lung is less than the measured rate of uptake into the brush-border membrane vesicles when expressed in the same units (80 nmol/min per rat even if the higher initial rates of appearance are used.)

Previous studies have indicated that the epithelial lining of the small intestine is capable of transporting hydrolysis-resistant tripeptides [9–11], but this report provides the first evidence that a tripeptide can be transported intact across the epithelial barrier of the lung. It is also of interest to note that in preliminary studies with in vitro loops of rat small intestine we have found that L-alanyl-D-phenylalanyl-L-alanine is absorbed from the lumen of the intestine; the tripeptide accumulates within the mucosa and is slowly released into the serosal effluent together with the dipeptide D-phenylalanyl-L-alanine (unpublished observations). The fact we find no evidence for intestinal transport of the reverse tripeptide, D-alanyl-L-phenylalanyl-D-alanine, suggests that there is a clear difference between the specificity of tripeptide transport across the epithelial lining of the lung and that of the small intestine.

5. Acknowledgement

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