

## Riboflavin-Enhanced Transport of Serum Albumin Across the Distal Pulmonary Epithelium

O. Douglas Wangenstein,<sup>1,4</sup> Marilyn M. Bartlett,<sup>2</sup> Jonathan K. James,<sup>1</sup> Zhen-fan Yang,<sup>3</sup> and Philip S. Low<sup>3</sup>

Received April 11, 1996; accepted October 6, 1996

**Purpose.** Conjugation of bovine serum albumin (BSA) with riboflavin (BSA-riboflavin) increases its uptake into cultured epithelial cells. Our purpose was to determine whether transport of BSA-riboflavin across the intact distal pulmonary epithelium is also increased, and whether transcytosis plays a role.

**Methods.** In anesthetized rats, we instilled <sup>3</sup>H-BSA-riboflavin or <sup>3</sup>H-BSA into the trachea and measured their appearance in blood. In isolated, perfused rat lungs we measured the distal pulmonary epithelium permeability-surface area product (PS) for FITC-BSA or FITC-BSA-riboflavin.

**Results.** In intact rats we found 2.1 times more <sup>3</sup>H-BSA-riboflavin than <sup>3</sup>H-BSA appeared in blood 60 min after intratracheal instillation of the protein. In isolated, perfused rat lungs we found that BSA-riboflavin had double the PS of BSA (2.63 vs.  $1.46 \times 10^{-5}$  cm<sup>3</sup>/sec). The addition of transcytosis inhibitors monensin or nocodazole (both  $3 \times 10^{-5}$  M) reduced the BSA-riboflavin PS to that of BSA and had no effect on the PS of unconjugated BSA. Simultaneous measurements of <sup>3</sup>H-sucrose PS showed no differences in paracellular transport among any of the experimental groups.

**Conclusions.** Conjugation with riboflavin increases the flux of BSA across the distal pulmonary epithelium. The increased transport appears to be due to transcytosis, which apparently does not play a significant role in the movement of unconjugated BSA across the distal pulmonary epithelium.

**KEY WORDS:** transcytosis; paracellular transport; permeability; sucrose; pulmonary drug delivery.

### INTRODUCTION

Uptake across the distal pulmonary epithelium is a potential means for the delivery of protein and peptide pharmaceuticals to the systemic circulation. A major problem encountered is that this epithelium is a barrier with very low permeability to water-soluble solutes, especially those with a large molecular weight. Hydrophilic proteins can potentially cross healthy pulmonary epithelia by diffusion through the junctions between cells (paracellular transport) or transport through epithelial cells by vesicular activity (transcytosis) (1). The latter may be either nonspecific or it may involve specific protein receptors. Attempts have been made to increase the normally slow protein movement across pulmonary epithelia by the use of substances

or strategies which either (a) open the tight junctions between cells and thereby increase paracellular transport or (b) increase transcytosis. This report describes enhancement of serum albumin transport across the distal pulmonary epithelium by exploitation of an intrinsic transcytosis pathway.

Transcytosis involves the uptake of material into a cell by endocytosis, movement of the vesicles across the cell, and discharge of vesicle contents into the extracellular space by exocytosis. A general strategy that has been used to increase the cellular uptake of proteins by endocytosis is to alter the protein in a way that increases its binding to the cell surface (2). One method to accomplish this is to conjugate the protein to a vitamin, such as folic acid (3) or riboflavin (4), for which there are specific receptors on some cell surfaces. In both of these cases increased cellular uptake of protein has been observed (3,4). However, since receptor-mediated endocytosis is just the first step in the overall transcytosis process, those results only suggest the possibility that vitamin conjugation will increase protein transport across an epithelial barrier.

In anesthetized rats, we instilled either bovine serum albumin (BSA) or BSA conjugated with riboflavin (BSA-riboflavin) into the lungs via the trachea and compared their rates of appearance in the blood. We also used an isolated, perfused rat lung preparation to measure the distal pulmonary epithelial permeabilities of BSA and BSA-riboflavin in the presence and absence of two known inhibitors of transcytosis, monensin and nocodazole (5). Our data show that conjugation of BSA with riboflavin increases its pulmonary uptake and suggest that the enhanced uptake is entirely due to transcytosis.

### METHODS

#### Preparation of FITC-Labelled, Riboflavin-Conjugated BSA

BSA was first conjugated to riboflavin and then labelled with fluorescein isothiocyanate (FITC) as described by Holladay, *et al.* (4). Dry riboflavin was dissolved in pyridine, treated with thionyl chloride, and heated at 65°C for 16 hrs. Dimethylformamide was added to solubilize all components and an aliquot of the resulting solution was added to BSA dissolved in PBS (125 mM NaCl, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH = 8.0), and the reaction was allowed to proceed in the dark for 3 hrs at 23°C. The solution was then centrifuged to remove particulates and the product was separated from the starting materials by use of a Sephadex G-25 desalting column equilibrated with PBS. The BSA-riboflavin thus prepared had 5–7 riboflavin molecules per BSA molecule.

Labelling with FITC was accomplished by incubating BSA or BSA-riboflavin dissolved in PBS with a 20-fold molar excess of FITC for 3–4 hrs in the dark at 23°C. The labelled proteins were separated from unreacted FITC with Sephadex G-25 desalting columns equilibrated in PBS. The eluted fractions were collected, sterile filtered, and stored in the dark at 4°C. The final products had 5–7 FITC molecules per BSA molecule.

Prior to their use in isolated lung experiments, solutions of FITC-BSA or FITC-BSA-riboflavin in PBS were maintained under constant dialysis against PBS at 4°C, pH = 7.40 (Dia-Cell™, InstruMed, Inc., 6–8 kD cutoff membrane). This was

<sup>1</sup> Department of Physiology, University of Minnesota, Minneapolis, Minnesota 55455.

<sup>2</sup> Life Sciences Sector Laboratory, 3M, St. Paul, Minnesota 55144.

<sup>3</sup> Department of Chemistry, Purdue University, West Lafayette, Indiana 47907.

<sup>4</sup> To whom correspondence should be addressed.

done to remove any fluorescein that might have hydrolyzed from the protein surface.

### In Vivo Experiments

Rats weighing 300–350 g were anesthetized with sodium pentobarbital (7 mg/kg, i.p.) and a polyethylene tube (PE 240) was tied into the trachea below the larynx. Throughout the experiment the animals breathed spontaneously. A polyethylene tube (PE 50) attached to a syringe was used to instill into the trachea approximately 200  $\mu$ l of PBS (pH = 7.40) containing 2  $\mu$ Ci of  $^3$ H-BSA or  $^3$ H-BSA-riboflavin. The amount instilled was measured to within 1%. Initial experiments with Evans Blue-dyed instillate showed that a substantial, but unquantitated, volume of the instillate reached the alveoli. After 60 min the animal was sacrificed and duplicate blood samples were taken for measurements of hematocrit and plasma  $^3$ H activity. It was assumed that  $^3$ H in the plasma was associated with the same proteins as in the instillate. From those data, and knowing the total activity of  $^3$ H-labelled protein instilled into the lungs, the fraction of instilled protein that appeared in the plasma in 60 min was calculated. For this calculation it was assumed that the blood volume was 7% of body weight (6).

### Measurement of Distal Pulmonary Epithelium Permeability-Surface Area (PS)

#### Isolated, Perfused Rat Lung Preparation

The PS of the distal pulmonary epithelium was measured using an isolated, perfused lung technique previously described (7). Male Sprague-Dawley rats weighing 300–350 g were anesthetized, a polyethylene tube (PE 240) was placed into the trachea just below the larynx, the chest was opened, and heparin sodium (50U in 0.5 ml) was injected into the beating right ventricle. A polyethylene catheter (PE 190) connected to the perfusion system was inserted into the pulmonary artery and the ventricles and atria were removed to allow unobstructed flow from the pulmonary veins. The lungs were removed, suspended from a force transducer, and loosely wrapped with plastic to prevent cooling and evaporation from the pleural surface. After a brief initial period of perfusion, 30 ml of perfusate was recirculated at a flow of 8.0 ml/min. Preparation weight and pulmonary artery pressure were monitored throughout; the pulmonary artery pressure was always 8–10 cm H<sub>2</sub>O and varied less than  $\pm 0.5$  cm H<sub>2</sub>O. Experiments lasted about 80 min.

The perfusate was a modified Ringer-Tyrode solution that has been used in this laboratory for several years. It contained (in mM) 137 NaCl, 2.68 KCl, 1.25 MgSO<sub>4</sub>, 1.82 CaCl<sub>2</sub>, 5.55 glucose, and 12.0 HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), as well as 1% dextran 70 and 0.5% BSA. The osmolarity was 280–285 mosmol/liter, the pH was 7.4 and the experiments were done at 37° C.

#### Measurement of Distal Pulmonary Epithelium PS

We determined the permeability-surface area product (PS) by filling the air spaces with a test solution that contained  $^3$ H-sucrose and FITC-labelled BSA (conjugated or unconjugated) and measuring the rate at which those solutes appeared in the recirculating perfusate. Sucrose was included as an independent

monitor of changes in paracellular transport between the different experimental groups.

Isolated lungs were perfused for 5–15 min to assure stable weight and perfusion pressure. After this, 5.0 ml of test solution were instilled into the airspaces, slowly removed and reinstalled twice more to assure uniform filling, and a portion was withdrawn so that 4.0 ml (approximately 40% of total lung capacity) remained in the lungs. Finally, a small volume of air (0.1 ml) was pushed into the trachea to move the solution out of the airways, and the trachea was tied. (In earlier studies we found that this added gas causes an immeasurable (<0.5 cm H<sub>2</sub>O) increase in alveolar pressure and has no effect on fluid or solute transport.) The test solution consisted of perfusate to which the two test solutes were added: 0.2  $\mu$ Ci/ml  $^3$ H-sucrose (specific activity = 14.4 Ci/mmol) and 0.3–0.4 mg/ml FITC-BSA or FITC-BSA-riboflavin. The alveolar albumin concentration in the filled lungs was about 5.4 mg/ml, which is 10–25% of the albumin concentration that has been reported in the epithelial lining fluid of normal lungs.

Perfusate samples (1.0 ml) were taken at 15', 30', 40', 50', 55' and 60' after fluid instillation. Airspace fluid samples were obtained from the initial instillate and from fluid recovered after the 60 min sample. All samples were assayed for FITC with an SLM-Aminco fluorimeter (model SPF-500C) and for  $^3$ H with a Packard Tri-Carb scintillation counter (model 4530), with EcoLite+ (ICN Biomedicals, Inc.) as the scintillation fluid. The FITC concentration was expressed in arbitrary units/ml and  $^3$ H-sucrose concentration as cpm/ml.

The PS for each test solute was calculated for every perfusate sample as follows:

$$PS = (\text{solute flux}) / (C_{af} - C_{perf}) \quad (1)$$

where  $C_{af}$  and  $C_{perf}$  are test solute concentrations in airspace fluid and perfusate, respectively, and (solute flux) is the rate of solute appearance in the recirculating perfusate during the time since the previous sample. The latter was calculated from the measured perfusate concentrations and the perfusate volume, corrected for the samples previously removed. Airspace solute concentrations were recalculated for each perfusate sample, based upon airspace solute loss and the change in instilled fluid volume (assumed equal to the change in preparation weight) since the last sample. PS values were stable after 30–40 min. and a single PS value for each solute was calculated as the average of the 50', 55' and 60' values.

The presence of free FITC in perfusate and airspace fluid samples was determined with a Centrifree™ filtration system (Amicon, Inc). Free FITC was not detectable in the instilled test solution. In the final airspace fluid sample, however, free FITC accounted for 1–4% of the total FITC detected, and in the final perfusate sample about 35% of the total FITC was unbound. The reason for FITC separating from the riboflavin-conjugated or unconjugated BSA after instillation into the lungs is not clear. PS values for BSA and BSA-riboflavin were calculated using the concentrations of intact FITC-BSA or FITC-BSA-riboflavin in the perfusate samples.

#### Experimental Groups, Statistics

Six experimental groups were studied. FITC-BSA and  $^3$ H-sucrose were the test solutes in three of the groups, while FITC-BSA-riboflavin and  $^3$ H-sucrose were the test solutes in the other

three. In each of these sets of three, one group had only the test solutes in the instilled test solution and the other two had either  $3.5 \times 10^{-5}$  M monensin or  $3.5 \times 10^{-5}$  M nocodazole added to inhibit transcytosis (5). Stock solutions ( $10^{-2}$  M) of either monensin in ethanol or nocodazole in dimethyl sulfoxide were prepared fresh each day and aliquots were added to the test solution before it was instilled into the alveoli. Preliminary experiments confirmed that the presence of the vehicle alone had no effect on PS (data not shown).

Results are presented as means  $\pm$  SEM. A one-way analysis of variance, followed by a Sheffé test for multiple comparisons was used to compare data among the six groups.  $P < .05$  was considered significant.

## RESULTS

### In Vivo Measurements

A total of four animals were studied in each group. The results, shown in Table 1, indicate that following intratracheal instillation, 2.1 times more BSA-riboflavin moves into the blood in 60 min than does unconjugated BSA. This suggests that the conjugation of BSA with riboflavin has enhanced BSA transport across the distal pulmonary epithelium.

### PS Measurements

Data were obtained from 29 isolated rat lung preparations. All lungs continuously lost weight following instillation of the test solution. The average weight loss over the 60 min experimental period was  $0.730 \pm .033$  gm and there were no significant differences among the groups. The steady loss of airspace fluid has been observed previously in isolated, fluid-filled lung preparations (7,8,9). It is undoubtedly a consequence of active  $\text{Na}^+$  transport out of the fluid by epithelial cells (10).

PS data determined in the six experimental groups are presented in Table 2. The mean PS of BSA-riboflavin was approximately twice that of unconjugated BSA. Furthermore, the addition of either nocodazole or monensin brought the BSA-riboflavin PS values back to the unconjugated BSA level. Neither inhibitor affected the PS of unconjugated BSA. There was no significant difference in the sucrose PS values between any of the groups, which indicates that the paracellular transport pathway was the same in all cases.

## DISCUSSION

The purpose of this study was to determine whether the transport of BSA across the distal pulmonary epithelium could be increased by its conjugation with riboflavin, a water-soluble vitamin for which there are receptor sites on most cells. Holla-

**Table II.** Permeability  $\times$  Surface Area (PS) Values for Isolated, Perfused Rat Lungs

	n	PS [ $\text{cm}^3/\text{sec} \times 10^{-5}$ ] $\pm$ sem	
		BSA	Sucrose
BSA	7	$1.46 \pm .28$	$34.0 \pm 6.7$
+ nocodazole	2	$1.60 \pm .09$	$56.3 \pm 16.3$
+ monensin	4	$1.11 \pm .36$	$47.9 \pm 12.4$
BSA-riboflavin	6	$2.63 \pm .32^a$	$38.6 \pm 7.6$
+ nocodazole	5	$0.98 \pm .14$	$29.3 \pm 7.1$
+ monensin	5	$0.94 \pm .17$	$34.0 \pm 6.6$

Note: Data are means  $\pm$  SEM.

<sup>a</sup> Significantly different from all other PS(BSA) values,  $p < .005$ .

PS(BSA) values for the five groups other than BSA-riboflavin were not significantly different from each other. PS(sucrose) values for all groups were not significantly different.

day, et al. (4) have shown that BSA-riboflavin is taken up by cultured cells, including human lung cell lines, whereas the uptake of unconjugated BSA is undetectable. Although the mechanism for that enhanced uptake is not completely clear, a form of receptor-mediated endocytosis seems a reasonable explanation. Their data suggest the possibility that riboflavin conjugation could increase BSA transport across pulmonary epithelia, but an increased uptake *into* cells does not necessarily lead to increased transport *through* cells. However, our *in vivo* and *in vitro* data show that conjugation with riboflavin does, indeed, increase BSA transport through distal pulmonary epithelial cells, and suggest that this increase is due to vesicular transport.

In the measurement of PS with the isolated lung technique one cannot separately determine permeability and surface area. Thus, a possible explanation for our results is that the permeability of BSA-riboflavin and BSA were the same, but the surface area for exchange was doubled in the BSA-riboflavin experiments. The best evidence against this is that the PS for sucrose, which also depends upon surface area, was the same in the BSA and BSA-riboflavin groups. Furthermore, there is no apparent reason why the addition of a trace amount of BSA-riboflavin to instilled fluid would increase surface area of the pulmonary epithelium, so we believe this explanation to be very unlikely.

It has been suggested that filling isolated, perfused lungs with liquid alters the transport properties of the distal pulmonary epithelium, but there is no functional evidence to support this. By electron microscopy the epithelium appears essentially the same as in air-filled lungs, except for a possible increase in the number of vesicles in Type I alveolar epithelial cells, which might be an artifact of the fixation process. Even if filling lungs with fluid did increase vesicle numbers, that does not mean that transcytosis would also be increased. In our study, if instilling test solution into the lungs had increased transcytosis this should have affected both BSA and BSA-riboflavin, and been inhibited by monensin or nocodazole. The fact that those inhibitors did not alter the PS for BSA suggests that additional fluid in the airspaces did not increase transcytosis.

There are two reasons why we believe transcytosis is the most likely explanation for enhanced BSA-riboflavin permeability. The first is the fact that monensin and nocoda-

**Table I.** In Vivo Uptake of BSA and BSA-Riboflavin

Protein	Fraction of Instilled Appearing in Plasma in 60 min ( $\pm$ sem)
BSA	$0.0180 \pm .0030$
BSA-riboflavin	$0.0383 \pm .0042^a$

Note:  $n = 4$  for each group.

<sup>a</sup>  $p = .020$  compared to BSA.

zole reduce the permeability of BSA-riboflavin to that of unconjugated BSA (Table 2). Monensin inhibits endocytosis by preventing acidification of intracellular vesicles and thereby interfering with the recycling of membrane receptors and phospholipids. Nocodazole inhibits transcytosis by disrupting intracellular microtubules, which are needed for vesicles to move from one side of a cell to another. Hastings, *et al.* (5) used both ultrastructural and immunohistochemical studies to confirm the effectiveness of these two compounds in alveolar epithelial cells. They also showed that, at  $3 \times 10^{-5}$  M, neither compound alters alveolar epithelial cell functions related to gas exchange or fluid reabsorption. The fact that our PS sucrose values were not altered in the presence of either compound also indicates that they did not significantly affect alveolar epithelial cell function. The second reason is that the PS for sucrose was unchanged when BSA-riboflavin values were increased. If the increased BSA-riboflavin permeability had been due to an "opening" of tight junctions or leak pathways, there would also have been a significant increase in sucrose permeability. An increase in transcytosis that doubles BSA-riboflavin permeability, however, would not affect the measured sucrose permeability, because the paracellular sucrose flux is so large compared to the flux of BSA-riboflavin by transcytosis (Table 2).

The mechanism by which unconjugated BSA (or native albumin) crosses the distal pulmonary epithelium is not completely clear. There is substantial evidence that a large part of the small BSA flux observed in healthy lungs is by passive diffusion through paracellular or other "leak" pathways. Whether transcytosis plays a role is controversial. Recently Hastings, *et al.* (5) found that both monensin and nocodazole inhibited endocytosis by alveolar epithelial cells, but neither affected the rate of removal of albumin from the alveolar lumen. They concluded that processes other than transcytosis are involved in the clearance of albumin from the alveolar space. Our data support that conclusion.

In conclusion, with a combination of in vivo and in vitro experiments we have shown that the transport of BSA across the distal pulmonary epithelium is increased when the protein is conjugated with riboflavin. Studies with inhibitors suggest that the increased flux is due to transcytosis. The observed increase in BSA transport was not large, however, and it is thus not clear whether riboflavin conjugation will be useful in enhancing the uptake of a protein that has importance as a pharmaceutical agent. Our results nevertheless suggest that it is possible to improve the systemic delivery of an inhaled protein by increasing transcytosis across the distal pulmonary epithelium.

#### ACKNOWLEDGMENTS

Supported in part by grants to O. D. W. and P. S. L. from 3M Pharmaceuticals.

#### REFERENCES

1. P. B. Breitfeld, J. E. Casanova, N. E. Simister, S. A. Ross, W. C. McKinnon, and K. E. Mostov. *Am. J. Respir. Cell Mol. Biol.* 1:257-262 (1989).
2. W.-C. Shen, J. Wan, and H. Ekrami. *Adv. Drug Del. Rev.* 8:93-113 (1992).
3. C. P. Leamon and P. S. Low. *Proc. Nat. Acad. Sci. USA* 88:5572-5576 (1986).
4. S. R. Holladay, Z.-F. Yang, C. P. Leamon, R. J. Lee, M. Jayamani, T. Mason, and P. S. Low. (*Submitted for publication*)
5. R. H. Hastings, J. R. Wright, K. H. Albertine, R. Ciriales, and M. A. Matthay. *Am. J. Physiol.* 266:L544-L552 (1994).
6. P. L. Altman and D. S. Dittmer. *Respiration and Circulation*, FASEB, Bethesda, 1971.
7. R. M. Palazzo, O. D. Wangenstein, and D. E. Niewoehner. *J. Appl. Physiol.* 73:1881-1887 (1992).
8. G. Basset, C. Crone, and G. Saumon. *J. Physiol. Lond.* 384:311-324 (1987).
9. B. E. Goodman, K. J. Kim, and E. D. Crandall. *J. Appl. Physiol.* 62:2460-2466 (1987).
10. G. Saumon and G. Basset. *J. Appl. Physiol.* 74:1-15 (1993).
11. H. G. Folkesson, M. A. Matthay, B. R. Weström, K. J. Kim, B. W. Karlsson and R. H. Hastings. *J. Appl. Physiol.* 80:1431-1445 (1996).