Solute Absorption from the Airways of the Isolated Rat Lung. IV. Mechanisms of Absorption of Fluorophore-Labeled Poly-α,β-[*N*(2-Hydroxyethyl)-DL-Aspartamide]

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The pulmonary absorption kinetics of a single molecular weight distribution (MWD) of fluorophore-labeled poly-α,β-[*N*(2-hydroxyethyl)-DL-aspartamide] (F-PHEA), a hydrophilic and biocompatible synthetic polypeptide, were studied in the isolated, perfused rat lung (iprl) as functions of administered polymer concentration, dose, vehicle, and presence and absence of fluorophore. The MWD was characterized before and after absorption by measurement of weight- and number-averaged molecular weights (M_w and M_n, respectively) using high-performance gel-permeation chromatography. Values for M_w and M_n were 8.6 and 5.3 kD before, and 6.7 and 4.7 kD after, absorption into the perfusate; there was no significant metabolism and the MWD of the absorbed polymer was independent of both dose and sampling time over a 3-hr period. F-PHEA failed to show any evidence of aggregation in solution or changes in dose distribution within the airways as functions of increasing polymer concentration and dose. A concentration ranging study indicated the presence of a saturable, carrier-mediated transport process for F-PHEA with a maximum absorption rate, V_{max}, of approximately 180 μg or 0.027 μmol/hr. Coadministration of fluorophore-free PHEA was capable of depressing the absorption of F-PHEA. The transport process for F-PHEA appeared to have a molecular weight limit of about 7 kD for this hydrophilic polymer.

KEY WORDS: aerosols; isolated perfused rat lung; polypeptides; pulmonary absorption; carrier-mediated transport; molecular weight; epithelial tight junctions; endocytosis.

INTRODUCTION

Our last paper in this series (1) described the pulmonary absorption of fluorophore-labeled poly-α,β-[*N*(2-hydroxyethyl)-DL-aspartamide] (F-PHEA) as a function of molecular weight. We have described the structure, synthesis, and characterization of this peptidase-resistant, synthetic polypeptide in detail previously (1–3). It was chosen as a model to explore the effects of molecular variation upon residence kinetics and absorption from the airways, in the absence of significant metabolism (2). The smallest of the F-PHEAs studied previously had a molecular weight distribution (MWD) characterized by weight- and number-averaged molecular weights of M_w = 4.7 kD and M_n = 3.3 kD, respectively, and was absorbed in a dose-dependent fashion (1). Also, unlike larger polymers, it was absorbed in the same MWD in which it was administered [larger-MWD polymers may have smaller MWDs after pulmonary absorption, indicating size selectivity in the transfer process (1)]. In this paper, to assess the importance of the fluorophore label in the transfer process, we describe the absorption of F-PHEA from different doses, and different vehicles, following administration alone and in admixture with the unlabeled polymer, PHEA. Here a polydisperse MWD was chosen (M_w = 8.6 kD, M_n = 5.3 kD), which we anticipated would lead to the preferentially rapid absorption of smaller molecules from the distribution. By comparing the MWDs of absorbed F-PHEA as functions of time and administered dose, we hoped to gain further insight into the mechanisms of F-PHEA transfer from airways to perfusate.

MATERIALS AND METHODS

Preparation of PHEA and F-PHEA. Preparation of F-PHEA has been described in detail previously (1). PHEA was prepared in a similar MWD. Briefly, a single batch of polysuccinimide was prepared and reacted with either 2-aminoethylcarbonyl-6-aminofluorescein followed by excess ethanolamine or excess ethanolamine alone in order to obtain the ring-opened F-PHEA or PHEA (Fig. 1). Polymers were lyophilized and characterized in terms of MWD and fluorophore content as described previously (1).

Polymer Absorption from the Airways of the Isolated Perfused Rat Lung (iprl). We have described the administration of polymers to the airways of the iprl in previous papers (1–3). Identical procedures were used here. Briefly, polymer solutions (Table I) were administered in nominal 0.1-mL volumes as coarse aqueous sprays directly to the airways through an endotracheal cannula. Sprays were propelled by a metered volume of fluorocarbon propellant (4). Administered dose (dose delivered to the airways) was determined by subtracting the polymer recovered from nonbiological equipment components from the product of polymer concentration and known volume originally placed in the dosing cartridge. Samples were withdrawn from the circulating perfusate (a 15-mL/min flow rate through the pulmonary vasculature was employed for 180 min in each experiment) at different times for quantitative assay of F-PHEA and its MWD by gel-permeation chromatography (GPC) as described previously (2,5). A series of experiments was performed in which the dosing solutions were varied (Table I). Each solution was administered to at least four separate rat lung preparations. Values for the number of replicates, *n*, are reported.

Polymer Formulation Effects. Table I summarizes the content of the various dosing solutions. F-PHEA absorption kinetics were studied as functions of dose and concentration (both were varied simultaneously, with dosing volume held constant; solutions A, B, C, and D), polymer vehicle (solutions A and H, the latter containing 0.9% NaCl), and PHEA/F-PHEA ratio (all solutions except H). The airway distribu-

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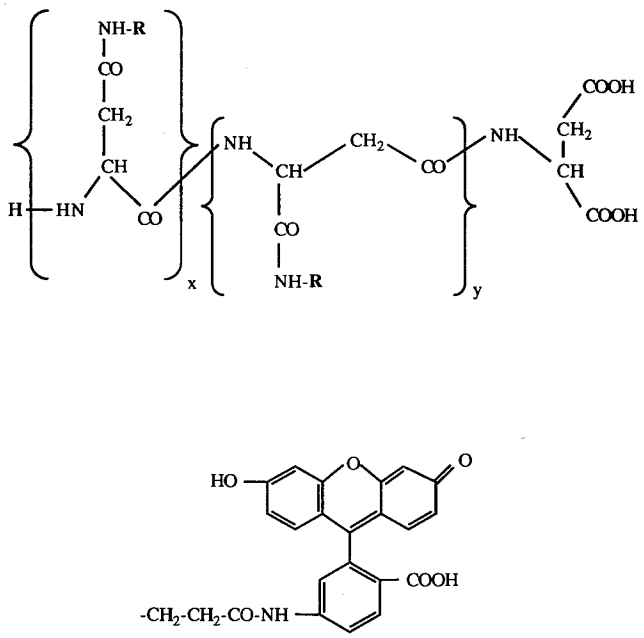

Fig. 1. Polyaspartamide (top) and fluorophore (bottom) structures. F-PHEA was 99.7 mol% hydroxyethylated (R = $-CH_2CH_2OH$) with 0.3 mol% fluorophore (R = ethylcarbonyl-6-aminofluorescein). PHEA was 100% hydroxyethylated.

Table I. Composition of Dosing Solutions and Administered Doses in Each Group of Experiments

Solution	Solvent	Polymer concentration (mg/mL) F-PHEA	PHEA	Total	Mean administered dose F-PHEA ± SD (mg)[a]
A	Water	50	—	50	3.198 ± 0.490[b]
B	Water	25	—	25	1.774 ± 0.127
C	Water	10	—	10	0.810 ± 0.074
D	Water	2	—	2	0.155 ± 0.013[c]
E	Water	2	48	50	0.133 ± 0.007
F	Water	10	40	50	0.757 ± 0.037
G	Water	2	8	10	0.159 ± 0.006
H	0.9% NaCl	50	—	50	3.869 ± 0.488

[a] The administered dose was determined in each experiment from the difference between the mass loaded into the dosing apparatus and that remaining after administration. It was defined as milligrams of F-PHEA reaching the airways.

[b] An administered dose of 3.060 ± 0.240 mg was achieved when the distribution of this solution was studied immediately after administration to separate lung preparations.

[c] An administered dose of 0.136 ± 0.010 mg was achieved when the distribution of this solution was studied immediately after administration to separate lung preparations.

tion of F-PHEA following dosing of the concentration extremes was assessed independently to ensure that changes in concentration did not affect polymer distribution in the airways. In that particular study, preparation of the iprl was followed by administration of solutions A and D. Perfusion was halted immediately after administration. The rat lung was removed from the artificial thorax. Trachea and major bronchi were separated from each lobe. Trachea and major bronchi and individual lobes were homogenized in separate 10-mL volumes of 0.1 M, pH 7.4, sodium phosphate buffer at room temperature using a BioHomogenizer (Fisher Scientific, Fairlawn, NJ). Fluorescent assays were performed on the clear supernatant of centrifuged tissue suspensions to determine the relative distribution of the F-PHEA between lung lobes and the upper airways (4). Also, to exclude the possibility that concentration-dependent polymer aggregation might influence F-PHEA absorption kinetics, a series of experiments was performed at 37°C with solutions A, B, C, and D as donor solutions in a rotating diffusion cell spinning at 120 rpm (6). F-PHEA concentrations were determined (30-min intervals over 4 hr) in a 350-mL receiving compartment containing phosphate-buffered saline (0.05 M phosphate, 0.15 M NaCl) at pH 7.4. An F-PHEA-permeable dialysis membrane (Spectra/Por; MW cutoff, 50 kD; Fisher Scientific, Raleigh, NC) was used to separate the donor and receiver solutions. Sink conditions were maintained and the surface area available for diffusive transfer was 3.14 cm^2 in all cases. Amount of F-PHEA-vs-time profiles in the receiver compartment were rectilinear throughout the 4-hr sampling period. The slopes of these curves (polymer flux as μg/hr) were determined by linear regression analysis.

RESULTS AND DISCUSSION

The single batch of F-PHEA which was synthesized for this study was polydispersed with respect to molecular weight and had values for M_w and M_n of 8.6 and 5.3 kD, respectively. PHEA was synthesized from an identical batch of polysuccinimide with a known MWD in the same way as F-PHEA and differed only with respect to the absence of the fluorophore. The stability of F-PHEA and PHEA has been studied and reported previously (1). Both were stable for the period of this investigation.

Polymer Absorption from the Airways of the iprl. The pulmonary absorption of this batch of F-PHEA has been reported previously at a high dose level [approximately 2–4 mg (2)] and found to occur as an altered MWD. The weight- and number-averaged molecular weights of absorbed F-PHEA in the perfusate 120 min after dosing were 6.7 ± 0.5 and 4.7 ± 0.6 kD, respectively, where the standard deviations were due to interanimal variation (n = 8). In the present experiments, much smaller doses were administered in some cases (D, Table I), resulting in significantly enhanced absorption in a 3-hr period (Fig. 2). Even though we anticipated larger MWDs in these cases (due to a greater percentage of the dose being absorbed), MWDs determined in the perfusate differed insignificantly from the values reported above (2); at sampling times ≥30 min, GPC chromatograms and, thus, MWDs appeared to be independent of both administered dose and sampling time. We had suspected previously (2) that the small molecular weight fraction of the absorbed polymer slowly decreased with increasing sampling time. Thus, we reanalyzed GPC data to determine mean peak elution volumes (V_e) and mean elution volumes at one-tenth peak height (0.1 V_e); the latter from the tail of the chromatogram to emphasize the low molecular weight fraction. However, when we compared values for V_e and 0.1 V_e across doses and sampling times (30 through 180 min) using analysis of variance, we were unable to detect any signifi-

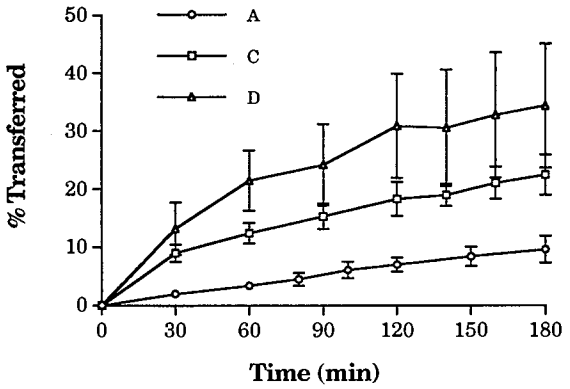

Fig. 2. Percentage administered dose of F-PHEA transferred from airways to circulating perfusate versus time. Error bars are standard deviations. F-PHEA was administered alone in aqueous solution (n = 4) in all cases as solutions A, C, and D (Table I).

cant differences. At sampling times ≤30 min, V_e was again unchanged, although there was insufficient polymer in the perfusate to permit MWDs to be determined from the chromatograms. For this polymer, we conclude that there is no significant MWD change over time in the perfusate in the 180-min duration of these experiments.

Polymer Formulation Effects. The F-PHEA absorption-versus-time profiles at different dose levels are shown in Fig. 2. These formulations were modified by changing solution concentrations to vary the dose. Percentage absorbed increased with decreasing dose and dosing solution concentration (Table I). The profiles tended toward rectilinearity when dose was increased, indicating the existence of a capacity limited process for F-PHEA transport. An absorption rate of approximately 3 μg/min occurred throughout the absorption of solution A. A similar rate was also seen for the first 30-min absorption from solutions B and C (Fig. 3). The effect of changing vehicle tonicity was determined at a single nominal dose and administered F-PHEA concentration (solutions A and H; Table I). There was no significant difference

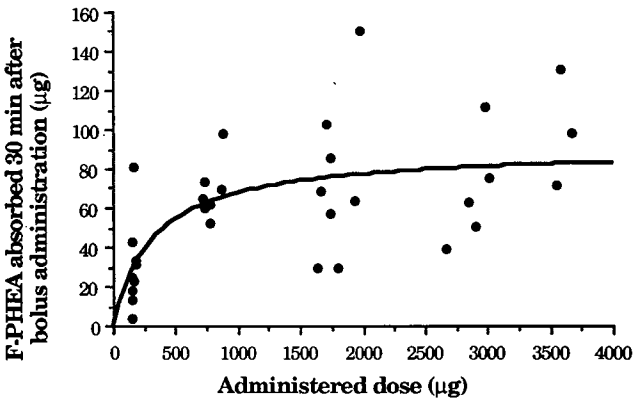

Fig. 3. F-PHEA transfer between t = 0 and t = 30 min versus administered dose (μg). F-PHEA was administered alone in aqueous solution as dosing solutions A, B, C, and D (Table I). Additional experiments were performed (beyond those reported as time profiles in Fig. 2) sampling only at time = 30 min, in order to increase the number of data points on this curve. The solid curve is the best fit according to Eq. (1), with V_{max} = 90.1 ± 10.9 μg/30 min and K_m = 331 ± 163 μg, respectively.

in the absorption of F-PHEA presented at 50 mg/mL in either 0.9% NaCl or water [percentage of administered dose absorbed at 2 hr, 6.5 ± 2.3 (n = 4) and 7.0 ± 1.2 (n = 4), respectively]. All these results (Fig. 2) are consistent with our previous report (1) of dose dependence for a much smaller MWD of F-PHEA. In that case (M_w and M_n = 4.7 and 3.3 kD, respectively) there was no preferential absorption of the smallest molecules from the distribution, presumably because all molecules had sizes smaller than the approximate 7-kD "cutoff" for rapid absorption.

An experiment was performed to ensure that polymer distribution in the airways was not a function of dose. We reasoned that different polymer concentrations (Table I) may have penetrated the airways of the iprl to different degrees and produced the dose dependence shown in Fig. 2 as an artifact of depth of penetration. Two extreme dose levels were tested in which F-PHEA was administered as different concentrations in solution (solutions A and D in Table I; six rats per group). The percentage of the administered dose (±SD) reaching the lung lobes for each dosing solution was 84.6 ± 7.4 and 82.9 ± 3.5 for solutions A and D (Table I), respectively. There was no significant difference in deep lung penetration. The results imply that approximately 84% of an administered dose could potentially be absorbed in each case [the bronchial circulation supplying the upper airways is unperfused and incomplete in this preparation; absorption is believed to occur primarily across the alveolar epithelium (7)].

The dose dependence shown in Fig.2 could also have been produced by aggregation of the polymer in solution, at high administered concentrations (reducing the effective driving force for diffusion and the thermodynamic activity of F-PHEA in solution at high doses). For this reason, diffusion kinetics were determined under identical conditions through a 50-kD cutoff dialysis membrane from dosing solutions A, B, C, and D (Table I). Diffusion rates were determined by linear regression from curves for amount of F-PHEA in the receiver solution vs time in 4-hr experiments at 37°C. Rates were 82.2, 39.4, 18.1, and 3.34 μg/hr for solutions A, B, C, and D, respectively. Values for the quotient, (diffusion rate)/(donor solution starting concentration) were 1.64, 1.58, 1.81, and 1.67 μL/hr, respectively. The apparent proportionality between diffusion rate and donor concentration indicated the absence of concentration-dependent polymer aggregation in these solutions.

In an attempt to describe the capacity limitation of the data in Fig. 2 mathematically, the amount of F-PHEA absorbed 30 min after bolus administration to the airways was plotted for each iprl preparation vs administered dose, D, according to Eq. (1), the Michaelis–Menten equation:

$$\text{Amount absorbed in 30 min} = \frac{V_{max} \times D}{K_m + D} \qquad (1)$$

where V_{max} is the maximum amount transferable in 30 min and K_m is the value for D at which 50% of V_{max} is achieved. Despite the scatter, the solid curve in Fig. 3 shows the case where Eq. (1) was forced through the data using NONLIN (8), with best estimates of K_m = 331 ± 163 μg and V_{max} = 90.1 ± 10.9 μg F-PHEA (per 30 min), respectively (values were independent of the assigned initial parameter values for

K_m and V_{max} in NONLIN). The profile assumes the existence of an active transport process which remains to be convincingly proven. Even so, a value for V_{max} of approximately 90 μg/30 min in the rat lung is large for a molecule of this size, especially considering that a typical aerosol dose to the human lung from a metered-dose inhaler is often only 10 to 50 μg. Similar absorption rates should be sufficient for effective peptide and small polypeptide delivery to the systemic circulation provided that significant metabolism can be avoided (9).

Carrier-mediated transport has been reported previously in the rat lung for the antiallergic chromone, disodium cromoglycate, and the dye, phenol red (10,11). Maximum rates of absorption were 0.18 μmol/hr (92 μg/hr) and 0.0034 μmol/hr (1.2 μg/hr), respectively. Transport was inhibited by other organic anions, suggesting the existence of some relatively nonspecific anion transport process in the rat lung. At physiological pH, F-PHEA carries a net negative charge and it was possible that the dose-dependent transport (Fig. 2) was due in part to the bound fluorophore (Fig. 1), which is dianionic (12). The molecule also carries one positive charge due to the protonated amine and two additional negative charges through its unprotonated succinate moiety. The pK_a's of these groups are such that at physiologic pH they are all almost totally ionized.

Figure 4 shows the effect of the administered F-PHEA concentration and the PHEA/F-PHEA ratio (average number of unlabeled PHEA molecules per labeled F-PHEA molecule) upon absorption at 2 hr. The dose and concentration dependence of F-PHEA's absorption kinetics was not due to the presence of the fluorophore. Increasing the concentration of F-PHEA alone, from 2 to 50 mg/mL decreased the average 2-hr absorption to 7% of the administered dose. However, the addition of fluorophore-free PHEA, so that the total polymer concentration increased (2, 10, and 50 mg/mL) with F-PHEA held constant at 2 mg/mL, also produced significant reductions in absorption: in this case, to 14%. By varying the F-PHEA concentration (10 mg/mL) and diluting with PHEA simultaneously (solution F; total polymer concentration, 50 mg/mL; PHEA/F-PHEA ratio, 25.5), it was possible to reduce F-PHEA's absorption to 6.8%, a value which differed insignificantly from that for solution A. The values for the PHEA/F-PHEA ratio in Fig. 4 result from the concentration of 0.3 mol% F (Fig. 1) in F-PHEA with respect to the hydroxyethyl aspartamide monomer, HEA (0.3 mol F/100 HEA U). On average, approximately 1 molecule in 6 of F-PHEA bore a fluorophore label. More precisely, the PHEA/F-PHEA ratio for the fluorophore-labeled polymer used in this study was 5.1. Five- and 25-fold dilutions with PHEA led to ratios of 25.5 and 127.5, respectively (Fig. 4).

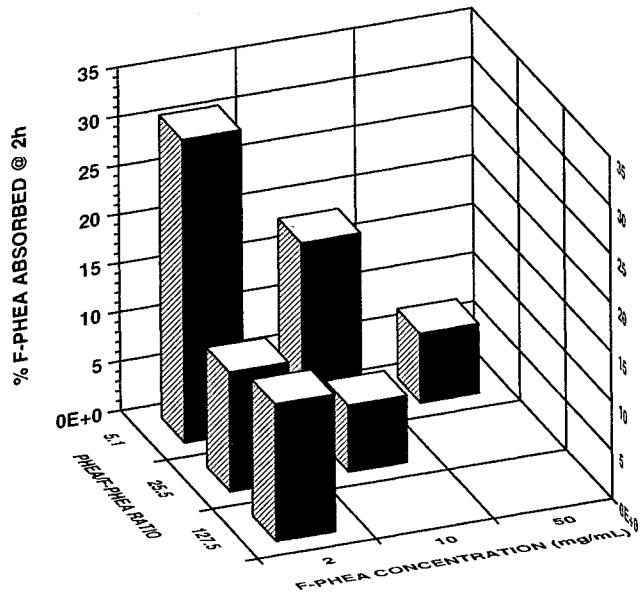

Fig. 4. Average (n = 4) percentages F-PHEA transferred to the perfusate 2 hr after administration as functions of the PHEA/F-PHEA ratio and the concentration of F-PHEA in the dose. For reasons of clarity, the diagram is not to scale on the x or z axis. Individual results for solutions A, C, D, E, F, and G (Table I) were 7.0 ± 1.2, 18.3 ± 2.9, 30.9 ± 9.0, 14.0 ± 3.3, 6.8 ± 1.6, and 12.2 ± 2.3%, respectively.

Because PHEA has no analytical marker, we have no results for its absorption through the lung. Thus, it is impossible to say at this stage whether PHEA acts as a direct competitor for a carrier-mediated F-PHEA transport process. If this were the case, the results in Fig. 4 imply that F-PHEA has a higher affinity for the carrier than PHEA. Unfortunately, these statements are simplistic and presume the importance of the carrier process. At high airway concentrations, known carrier processes for cromoglycate and phenol red become saturated. Under these conditions, absorption then proceeds according to first-order kinetics (10,11) due to the predominance of passive diffusion at high airway concentrations. This "multiple-mechanism" type of transfer is equally possible for F-PHEA. However, F-PHEA's diffusion should be slower than that of cromolyn or phenol red on the basis of its increased molecular weight, and it appears that a transport process which is saturable may be responsible for accelerating its pulmonary absorption. Even though the precise spatial location of the transport process is currently unknown, F-PHEA's pulmonary absorption may proceed by a combination of endocytosis and diffusion through interepithelial cell tight junctions, as suggested by Wangensteen (13) for inulin and dextran passage through the tracheal epithelium. Both mechanisms may display molecular selectivity on the basis of size, and endocytosis is known to be energy consuming (1,13).

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