Pre- and post-natal growth and protein turnover in the lung of the rat

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The growth of the rat lung was studied at six ages, from 18 days of fetal life to old age (i.e. 105 weeks). Most of the increase in lung size appeared to involve cellular hyperplasia rather than hypertrophy, the DNA content of the lung increasing 96-fold from one extreme of life to the other. Pulmonary rates of protein turnover were high and were, age for age, consistently greater than the rates in the whole body. The age-related decline in the rate of lung growth corresponded to a marked decrease in the fractional rate of protein synthesis, i.e. from 93 to 33% per day during fetal and neonatal life. This in turn correlated with a 58% fall in the ribosomal capacity. From weaning onwards, synthesis rates remained between 30 and 40% per day. In contrast, the degradation of lung proteins was unchanged, at 28–38% per day throughout both fetal and post-natal life.

INTRODUCTION

During fetal life, the lung grows from a small dense structure with high vascular resistance, to become a compliant tissue of large surface area essential for gaseous exchange at birth. Subsequent post-natal growth ensures a further increase in the surface area (Thurlbeck, 1975). However, the lungs are more than an inert tissue functioning simply as a vehicle for gaseous exchange. They are in fact a metabolically active organ for other substrates, such as lipids and carbohydrates (Maniscalco *et al.*, 1982; Jones & Rolph, 1985). Surprisingly little attention has been devoted to studying the growth of the whole lung over the entire life span of an animal. Other investigators have measured protein turnover in the lung in order to explain changes in lung size, either during normal growth or under various experimental conditions (Rannels *et al.*, 1979; Watkins & Rannels, 1980; Chiang *et al.*, 1979; Garlick *et al.*, 1980; Laurent, 1982). However, these studies have all been confined to single ages, mainly using adult animals, and only the last two studies have measured protein synthesis *in vivo*.

The present study is essentially different from all previous investigations in that it deals with the developmental growth of the lungs over most of the life span of the rat, i.e. from the fetus through to senescence. The changing growth patterns are explained by the age-related changes in protein turnover (measured *in vivo*). This study on the lung also represents part of a much larger investigation involving the developmental changes in the whole rat and several of its major tissues (Goldspink & Kelly, 1984; Lewis *et al.*, 1984; Goldspink *et al.*, 1984). Since the techniques were standardized and rigorously applied throughout, it is now possible to compare meaningfully the developmental changes in the lung alongside other body tissues.

METHODS

Male albino rats of the CD strain were used from our own breeding colony, stock having originally been derived from Charles River (U.K.) Ltd. (Manston, Kent,

U.K.). All animals were housed in small groups in a room controlled for temperature (22 °C), and having a daily photoperiod of 12 h light between 06:00 and 18:00 h. Each animal had free access to water and was fed *ad libitum* on a modified laboratory cubed diet (Diet 41B; Bruce & Parkes, 1949) containing a minimum of 21% (w/w) protein. Pregnant female rats (approx. 270 g) from the same colony were used for studies on fetal lungs. To minimize variations in the number and weights of the fetuses during such pregnancies, only females undergoing their second pregnancies were used. The presence of sperm in vaginal smears taken 12 h after a mating period was taken to signify the commencement (i.e. day 0) of pregnancy. Fetal lungs were subsequently removed at 18 or 20 days of gestation. All experimental procedures were, however, standardized and performed between 09:00 and 12:00 h to minimize possible diurnal variations.

Protein synthesis was measured *in vivo* after an intravenous injection of a large dose of phenylalanine to flood the precursor pool(s) (Garlick *et al.*, 1980). This injection was administered to each rat via a lateral tail vein and contained 150 µmol of the amino acid, including 65 µCi of L-[4-³H]phenylalanine (sp. radioactivity 24 Ci/mmol; from The Radiochemical Centre, Amersham, Bucks., U.K.), in 1 ml of 0.9% NaCl per 100 g body wt. Pregnant rats were treated similarly. In preliminary studies rats (approx. 200 g males, or 20-day-pregnant females) were initially decapitated 2.5, 5, 10, 20 or 30 min after injection, and blood was collected over the following 10 s. The time-related changes in the specific radioactivity of phenylalanine in the tissues pool(s) relative to the plasma and covalently bound in protein were measured to enable us to determine a suitable time for measuring protein synthesis. This was found to be 10 min after the injection (see below).

In all of these studies the thoracic cavity of the injected animal was rapidly opened immediately after decapitation and the entire animal was completely immersed in ice-cold water. The chilled lungs were immediately removed and frozen in liquid N₂. From each pregnant rat

three fetuses were removed at random from the chilled uterus, and their thoracic cavities in turn were rapidly opened. The lungs were removed while remaining immersed beneath the ice-cold water. Strict emphasis was placed throughout on the rapid chilling and dissecting out of the tissues to ensure the stoppage of any further metabolism beyond the point of killing.

Frozen lungs were then reduced to a powder by pulverizing the tissue between chilled metal plates. Samples were immediately homogenized in ice-cold 0.3 M-HClO₄ with a ground-glass homogenizer. The specific radioactivities of the phenylalanine in both the 'flooded' tissue pool(s) and covalently bound in protein were measured in these homogenates, as described by Garlick *et al.* (1980). This involved the prior hydrolysis of the washed protein pellets in 6 M-HCl at 100 °C for 24 h and the conversion of phenylalanine into β -phenethylamine (Garlick *et al.*, 1980). All measurements of radioactivity were made in an LKB scintillation counter (efficiency for ³H of 25%) in a Triton X-100/xylene-based scintillant with the use of an external standard. The fractional rate of synthesis (i.e. k_s , the percentage of the protein mass synthesized per day) was calculated from

$$k_s = \frac{S_B}{S_A t} \times 100$$

where S_A and S_B are the specific radioactivities of phenylalanine in the free tissue pool (i.e. intracellular and extracellular) and protein respectively and t is the time in days.

Protein was measured in the same tissue preparations by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, Poole, Dorset, U.K.) as standard. RNA and DNA were also extracted and measured as previously described (Goldberg & Goldspink, 1975). Growth rates (k_g) were measured as the net accumulation of protein in the lung between two time intervals close to, or spanning, the point at which synthesis rates were measured. The exact time intervals varied, being 1 day (i.e. between days 19 and 20 of gestation) for fetal tissues where growth was rapid, 5 days at 3 and 8 weeks, and 7 days at 44 and 105 weeks of age. That is, the time intervals necessarily lengthened as growth slowed. Growth rates (k_g) were then expressed as the percentage change in the protein content per day. Since growth arises from an imbalance in the rates of synthesis (k_s) and degradation, protein breakdown (k_b) was calculated by subtraction, from the two measured parameters, i.e. $k_b = k_s - k_g$.

RESULTS AND DISCUSSION

Various parameters of growth were initially measured in relation to the whole lung at, and between, the extremities of life in this strain of rat (Table 1). From the 18-day fetus to senescence the wet tissue weight increased 120-fold, whereas the protein content increased 174-fold (Table 1). The disproportionately larger increase in lung protein reflects a progressive increase in the protein composition within the enlarging tissue mass, i.e. from 6.9 to 22.8% (Table 1). The lung's contribution to the protein mass of the whole animal, however, progressively decreased, from 5.6% at 18 days of fetal life to less than 1.0% in old age.

The overall age-related increase in lung RNA (53-fold)

was less than that of DNA (96-fold). This was particularly the case during fetal and neonatal life, as evidenced by the fall in the RNA/DNA ratio (Table 1). This developmental feature distinguishes the lung from most other body tissues, e.g. the liver, kidney, heart, skeletal muscle, oesophagus, the large and small intestine, and the animal as a whole. These other tissues possess RNA/DNA ratios which are greater than unity and usually increase with increasing animal age (Waterlow *et al.*, 1978; Goldspink & Kelly, 1984; Lewis *et al.*, 1984; Goldspink *et al.*, 1984). Furthermore, in contrast with many other tissues, both nucleic acids in the lungs appear to be accumulated at a similar, and now slower, rate than the increase in tissue mass, hence the constancy of the values when expressed per g of lung (Table 1).

The age-related enlargement of the lungs appears to be mainly a consequence of increased nuclear and cellular proliferation (i.e. DNA; Table 1) rather than cellular hypertrophy (i.e. protein/DNA; Table 2). This conclusion is based on only a doubling of the latter value, compared with the 96-fold increase in the former between fetal life and senescence. At the moment it is not possible to say whether the hyperplasia represents a proportionate, or disproportionate, increase in the cell types present (see below; Thurlbeck, 1975). Despite the continuous increase in the protein mass of the lungs with increasing age, the fractional rate of growth fell dramatically, from 25% to 0.1% per day between late fetal life and old age (Table 2). In order to explain this change in the growth rates, protein turnover was measured *in vivo* in the lungs at each age. The method of Garlick *et al.* (1980) has been shown to be suitable for measuring average rates of protein synthesis in several fetal, as well as adult, tissues (Goldspink & Kelly, 1984; Lewis *et al.*, 1984). Nonetheless preliminary investigations were undertaken to ascertain a suitable time period over which protein synthesis could be measured in the lungs, during both pre- and post-natal life. Certain criteria need to be satisfied if accurate rates of protein synthesis are to be obtained (Waterlow *et al.*, 1978). These include an accurate knowledge of the specific radioactivity of the tracer amino acid both free in the precursor pool (S_A) and covalently bound in lung proteins (S_B). Hence the time-related changes in both S_A and S_B were monitored in both fetal (Fig. 1*b*) and adult (Fig. 1*a*) lungs for 30 min after an intravenous injection of [³H]phenylalanine.

In the 200 g adult animals, S_A values for the lung had already reached 80% of the specific radioactivity in the plasma at 2.5 min (Fig. 1*a*), demonstrating rapid equilibration between the plasma and tissue. Thereafter, although the plasma values declined with time, S_A values in the lungs remained constant over 30 min. Over the same period S_B increased linearly. In the pregnant mothers the specific radioactivities in the fetal plasma and lung had equilibrated with the maternal plasma at the earliest time studies (i.e. 2.5 min; Fig. 1*b*), indicating very rapid transfer across the placenta. Thereafter, values for fetal blood and lung (S_A) remained constant, but S_B increased linearly (Fig. 1*b*).

It was clear from these preliminary studies that accurate rates of protein synthesis could indeed be measured over 10 min and in both fetal and adult lungs, as shown for other body tissues (Garlick *et al.*, 1980; Goldspink & Kelly, 1984; Lewis *et al.*, 1984). The total amount of protein synthesized per day in the lungs (i.e.

Table 1. Pre- and post-natal changes in the protein and nucleic acid contents of the lung

Frozen lungs were pulverized between chilled metal plates. The powdered tissues were then allowed to thaw in ice-cold 0.3 M-HClO₄ (50 vol., v/w) and homogenized in a ground-glass homogenizer. Total protein (collagenous and non-collagenous) was measured in these homogenates by the method of Lowry *et al.* (1951), and RNA and DNA were extracted and assayed as described by Goldberg & Goldspink (1975). The values at each age are means $\pm$ S.E.M. derived from a minimum of 15 fetuses or five animals *post partum*. Values in parentheses give the percentage contributed by the lung towards the whole-body protein and nucleic acid contents, as reported by Goldspink & Kelly (1984).

Age	Protein content (mg)	Protein/wet wt. (%)	Total RNA P (μ g)	RNA P/wet wt. (μ g/g)	Total DNA P (μ g)	DNA P/wet wt. (μ g/g)	RNA P/DNA P
Foetal (days)							
18	4.6 $\pm$ 0.2 (5.6)	7.6 $\pm$ 0.1	33 $\pm$ 1.5 (5.5)	562 $\pm$ 13	22.1 $\pm$ 1.3 (4.7)	376 $\pm$ 27	1.52 $\pm$ 0.08
20	9.2 $\pm$ 0.5 (3.9)	6.9 $\pm$ 0.3	64 $\pm$ 2.1 (4.8)	476 $\pm$ 8.8	62.7 $\pm$ 2.3 (6.4)	466 $\pm$ 11	1.01 $\pm$ 0.03
Post partum (weeks)							
3	90 $\pm$ 6.2 (1.6)	11.8 $\pm$ 0.9	285 $\pm$ 24 (2.5)	374 $\pm$ 36	449 $\pm$ 36 (7.0)	556 $\pm$ 33	0.64 $\pm$ 0.03
8	243 $\pm$ 14 (0.61)	14.4 $\pm$ 0.4	524 $\pm$ 45 (1.3)	334 $\pm$ 14	682 $\pm$ 71 (4.0)	456 $\pm$ 18	0.73 $\pm$ 0.04
44	795 $\pm$ 74 (0.66)	22.8 $\pm$ 1.9	1240 $\pm$ 171 (1.8)	341 $\pm$ 25	1701 $\pm$ 159 (5.3)	489 $\pm$ 34	0.70 $\pm$ 0.06
105	799 $\pm$ 76 (1.1)	11.0 $\pm$ 0.5	1747 $\pm$ 302 (2.6)	293 $\pm$ 22	2131 $\pm$ 307 (7.1)	329 $\pm$ 39	0.77 $\pm$ 0.04

Table 2. Changes in protein turnover in the lung during pre- and post-natal growth

The values presented here are means $\pm$ S.E.M. of measurements made on the same lung tissues as in Table 1. Protein synthesis was measured *in vivo* in the lungs 10 min after an intravenous injection of 150 μ mol of phenylalanine/100 g body wt., via pregnant mothers in the case of fetal tissues. The fractional synthesis rates were calculated at each age after measurement of the specific radioactivities of both the free and protein-bound phenethylamine in the lungs (Garlick *et al.*, 1980). The total amount of protein synthesized per day represents the product of the fractional rate and the protein mass (Table 1). The percentage contributions of the lung towards whole-body synthesis are presented in parentheses. Lung growth rates were determined as the daily percentage change in the protein mass, as measured over appropriate time intervals close to the measurement points for protein synthesis (see the Methods section). Protein breakdown was subsequently calculated by subtracting the measured rates of growth from synthesis.

Age	Protein/ DNA P (mg/ μ g)	Fractional rate of synthesis (%/day)	Total protein synthesized		RNA P/ protein (mg/g)	Growth rate (%/day)	Calculated rate of breakdown (%/day)
			(mg/day)	(mg/day per mg of RNA P)			
Foetal (days)							
18	213 $\pm$ 14	92.6 $\pm$ 6.4	3.9 $\pm$ 0.3 (9.1)	118 $\pm$ 5.9	7.4 $\pm$ 0.2	–	–
20	149 $\pm$ 7.7	54.3 $\pm$ 3.9	5.0 $\pm$ 0.3 (5.5)	79 $\pm$ 4.2	7.2 $\pm$ 0.3	25.3	29.0
Post partum (weeks)							
3	205 $\pm$ 12	32.7 $\pm$ 0.9	29.4 $\pm$ 2.1 (1.7)	104 $\pm$ 3	3.2 $\pm$ 0.1	5.2	27.5
8	391 $\pm$ 11	38.1 $\pm$ 1.9	95.1 $\pm$ 11 (2.0)	176 $\pm$ 11	2.6 $\pm$ 0.2	7.1	31.0
44	467 $\pm$ 21	41.1 $\pm$ 5.6	413 $\pm$ 96 (4.3)	285 $\pm$ 42	1.4 $\pm$ 0.1	2.9	38.2
105	353 $\pm$ 41	28.2 $\pm$ 2.6	227 $\pm$ 26 (2.6)	119 $\pm$ 5	2.7 $\pm$ 0.3	0.1	28.1

fractional rate $\times$ protein mass) was found to increase with animal age (Table 2). This was mainly due to the progressive enlargement of the tissue and hence its increasing protein mass (Table 1). In contrast, the fractional rate of protein synthesis actually decreased

during fetal and neonatal life, to remain fairly static at 30–40% per day from weaning onwards (Table 2). The dramatic decline in the synthetic rate between fetal life and weaning was more closely associated with a fall in the ribosomal capacity (i.e. RNA/protein) than in the

Fig. 1. Time-related changes in the specific radioactivity of phenylalanine in plasma and lung of fetal and adult rats

(a) Male rats (200 g) were killed at selected times between 2.5 and 30 min after receiving an intravenous injection containing [^3H]phenylalanine. The specific radioactivities of both free (i.e. S_A ; $\circ$, $\square$) and protein-bound (i.e. S_B ; $\bullet$, $\blacksquare$) phenylalanine were determined at each time in the plasma ($\square$, $\blacksquare$) and lung ($\circ$, $\bullet$), as described by Garlick *et al.* (1980). Each value is the mean $\pm$ S.E.M. for at least five individual tissues. (b) Here, 20-day-pregnant rats (270 ± 10 g) were injected in an identical manner as for the animals above. The specific radioactivity of the free amino acid was then measured in both maternal ($\square$) and fetal ($\blacksquare$) plasma. The free ($\circ$) and protein-bound ($\bullet$) specific radioactivities were also determined in the fetal lungs. Values are presented as means $\pm$ S.E.M. Maternal and fetal plasmas were taken from four injected mothers and 16 fetuses respectively. Nine individual lungs were analysed, with three fetuses taken at random from each of three injected mothers.

ribosomal activity (i.e. protein synthesis/RNA) within the lung tissue (Table 2). In contrast with these changes in synthesis, the rate of protein breakdown (calculated) appeared to remain at 28–38% throughout the age range studied. Owing to various technical difficulties in measuring protein breakdown *in vivo* (Waterlow *et al.*, 1978), indirect rates of degradation have been determined in this study. This approach is believed to be as good as, if not better than, any other method currently available (Garlick *et al.*, 1979). However, the accuracy of these calculated rates in turn depends on the accuracy of the two measured parameters from which they are derived, i.e. the rates of growth and protein synthesis. As such,

any errors could be compounded, hence these indirect rates of protein degradation are presented as indicators of the likely developmental changes. This being so, the developmental slowing of growth in the lungs appears to be primarily due to the decline in the synthesis rate, with little or no change in the rate of degradation (Table 2).

These developmental changes in protein turnover in the lung are similar to those in the liver, although the absolute rates and ribosomal capacity in the lungs are, age for age, lower than those in the liver (Goldspink & Kelly, 1984). In contrast, several other tissues, e.g. kidney, heart, skeletal muscle, and the animal as a whole, have been shown to exhibit continuous developmental decreases in their protein-synthetic and degradative rates throughout both pre- and post-natal life (Waterlow *et al.*, 1978; Goldspink & Kelly, 1984; Lewis *et al.*, 1984). Clearly differentiation and the growth patterns in individual body tissues are controlled in different ways, in accord with variations in their functional roles and the age-related expressions of those functions (Jones & Rolph, 1985). These rapid rates of protein turnover in the lung are higher than those measured in the whole animal (Goldspink & Kelly, 1984). This is consistent with the appreciation of the lung as a metabolically active tissue, as well as an organ designed for gaseous exchange (Fishman & Pietra, 1974; Jones & Rolph, 1985). The rates of protein synthesis described here also compare favourably with the few previously reported values on the lung, where such comparisons can be meaningfully made. Using the same method, tracer amino acid and timing for measuring protein synthesis *in vivo*, Garlick *et al.* (1980) reported a fractional rate of 33% per day for the lung of 130 g (approx. 5 weeks old) Wistar rats. In addition, Laurent (1982), using the same basic method, but with a longer post-injection time (3 h) and [^3H]proline instead of phenylalanine, reported a rate of 35% per day in the lungs of young (approx. 115 days) rabbits. Hence, where the same, or very similar, experimental procedures have been employed, good agreement exists. Less satisfactory, however, are comparisons made with the synthesis rates of perfused lung preparations from adult rats. Such values *in vitro* vary appreciably, ranging from 14% per day (Rannels *et al.*, 1979; Watkins & Rannels, 1980) to 72% per day (Chiang *et al.*, 1979). Numerous experimental differences could, however, account for the variance in synthetic rates when measured *in vivo* and *in vitro*.

It should be emphasized that the rates of protein turnover that are reported here are only average rates for the whole lung. They do not therefore identify possible changes in the size or proportions of the different cell types, or individual intracellular and extracellular proteins within the heterogeneous tissue. For example, at least five major cell types may be present. These include pneumocytes I (9%) and II (15%), interstitial (37%) and endothelial (33%) cells and pulmonary macrophages (6%), which may comprise relative volumes ranging from 15 to 26% of the parenchymal mass in human lungs (Barry *et al.*, 1979). From the very limited information available, it appears that protein turnover in these different cell types may vary appreciably. For example, the fractional rate of synthesis in isolated pulmonary macrophages has been reported (Hammer & Rannels, 1981; Oliver *et al.*, 1984) to be 46–86% per day, i.e. probably more than twice the average rate determined in the rat lungs *in vivo* (Table 2; Garlick *et al.*, 1980;

Laurent, 1982) and 6 times those measured in some perfused lung preparations (Rannels *et al.*, 1979; Watkins & Rannels, 1980). Hence, although this particular cell type is in the minority (6%) (Barry *et al.*, 1979), an appreciable change in its population within the lung could have a significant bearing on the average rate of protein synthesis. However, considerable caution should be exercised in extrapolating the limited data currently available to us. For example, it is possible that alveolar macrophages are activated when isolated and cultured *in vitro*. In a single study undertaken by Oliver *et al.* (1984), the same cell type was reported to possess a protein-synthesis rate of only 9.3% per day, when measured *in vivo*. Hence it is possible that macrophages in the normal lung are relatively inert and in a quiescent state. Furthermore, the present study does not attempt to distinguish between the synthesis of the different protein types, e.g. collagenous and non-collagenous. Other workers (Laurent, 1982) have found collagen to represent only 6.7% of the total protein mass and a small proportion (2–3%) of the protein synthesized in the whole lung. Hence it seems probable that the synthesis rates described here (Table 2) largely reflect developmental changes in non-collagenous proteins.

One further note of caution should be attached to some of the values derived from the lungs of the very old animals (i.e. 105 weeks). In some of these particular lungs, localized areas of infection were noted. Care was therefore taken to avoid the inclusion of any areas of infection within the samples subsequently taken for analysis. As such, most values in Tables 1 and 2 (i.e. those expressed per wet wt., the various ratios and fractional rates) are unaffected by this potential complication. However, the total protein, RNA and DNA contents (i.e. columns 1, 3 and 5; Table 1) are likely to be over-estimated by the presence of these denser, infected, regions within the whole lung. The larger standard errors (15–20%) associated with these particular mean values can also be attributed to this complication at 105 weeks of age.

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