Lack of Lecithin: Retinol Acyltransferase Activity in Chick Lungs

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Summary Our previous study revealed that no retinyl esters were detectable in chick and hen lungs, suggesting that the retinol esterification system may be absent in these tissues. This possibility encouraged us to investigate whether chick lungs exhibit the activity of a retinol esterifying enzyme, i.e., lecithin: retinol acyltransferase (LRAT). The LRAT activity was assayed with dilauroyl phosphatidylcholine and either complex of retinol-cellular retinol-binding protein, type two or retinol-cellular retinol-binding protein in microsomal preparations of lung, duodenum and liver of 7-day-old chicks. Relatively high levels of LRAT activity were present in the duodenum and the liver of chicks as well as in the rat lung. However, the chick lung exhibited no LRAT activity. The lungs of both rat and chick showed similar and low levels of acyl-CoA: retinol acyltransferase (ARAT) activity, but only rat lung, but not chick lung, contained a detectable amount of retinyl esters. Thus, the retinyl ester storage in the lung seems to depend on the presence of LRAT activity in the lung, but it is independent of the presence of ARAT activity in the lung. The absence of LRAT activity and retinyl esters in the chick lung suggests that the retinol in the chick lung may not be provided from retinyl ester storage, and the retinol transferred directly from serum should be utilized to generate retinoic acid.

Key Words lung, lecithin: retinol acyltransferase, retinol esterification, chick

Many studies have suggested that vitamin A is a necessary factor to differentiate and to maintain proper lung epithelial cells (1, 2). Squamous metaplasia of the lungs has been reported in children suffering from vitamin A deficiency (3, 4). It is conceivable that lungs in perinatal stages of humans and animals may utilize a large amount of retinol to produce retinoic acid, which is essential for differentiation of

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Abbreviations: LRAT, lecithin: retinol acyltransferase; ARAT, acyl-CoA: retinol acyltransferase; CRBP, cellular retinol-binding protein; CRBP(II), cellular retinol-binding protein, type two; NRC, National Research Council.

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epithelial cells. Therefore during the developmental period of lung growth and differentiation, the storage of lung retinyl esters (mainly retinyl palmitate) may be inadequate because of hydrolysis to retinol. Indeed, the rat lungs stored a large amount of retinyl palmitate in the fetal stages, but the lung was depleted of retinyl esters during perinatal development (5, 6).

The esterification of retinol with long chain fatty acids, mainly palmitic acid, occurs in the small intestine, liver and other epithelial tissues. Recent works have shown that retinol esterification involves cellular retinol-binding protein (CRBP) or cellular retinol-binding protein, type two (CRBP(II)) (7). These proteins possibly form the ligand complex that serves as a substrate for microsomal lecithin: retinol acyltransferase (LRAT) (8); retinol-CRBP(II) would be used in the small intestine and retinol-CRBP would be the substrate in other tissues (9, 10). The other enzyme which catalyzes the esterification of free retinol has been known as acyl-CoA: retinol acyltransferase (ARAT) (11). However, recent studies suggested that LRAT is the physiologic retinol esterifying enzyme (7).

We previously found that no retinyl esters were detectable in chick lungs at any developmental stages examined, nor even in the adult hen. In contrast with this, the rat lung has plenty of retinyl esters (12).

Accordingly, questions arose as to whether there might be a lack of retinol esterification enzyme, the LRAT activity in the lungs of chicks. The present study demonstrates that the chick lungs are devoid of LRAT activity, which depletes stored retinyl esters in the lung of this species.

MATERIALS AND METHODS

Animals. Fertile eggs of white Leghorn breed were obtained from a hatchery (Ohata Hatchery Co., Yaizu, Japan), and hatched in our laboratory. The hatched chicks were given free access to a commercial chick starter ration (Broiler-Diet, Shizuoka Agriculture Inc., Shizuoka) 1 day after hatching. The 7-day-old chicks were used for the study. Wistar rats weighing about 150g (Japan SLC, Hamamatsu) were used as control animals for a comparative study on the retinol esterification enzyme (LRAT). The rats were provided ad libitum with a standard chow diet (MF; Oriental Yeast Co., Japan). The chicks and rats received free access to deionized water.

The chicks were dissected and the tissue samples of lung, duodenum and liver were collected. The duodenum was flushed with ice-cold 0.9% NaCl and then mucosa was scraped using a microscope glass slide. The rats were dissected and the lungs were collected. These tissue samples were quickly frozen in liquid nitrogen and stored at $-80\degree C$.

Preparation of tissue samples. The tissue samples of chicks or rats were homogenized in 2 volumes (v/w) of ice-cold potassium phosphate buffer (pH 7.2) using a Teflon-glass homogenizer. After centrifugation of the homogenates at 10,000 $\times g$ for 15 min ($4\degree C$), the resulting supernatant was saved and the pellet was
re-homogenized in 1 volume (v/w) of the same buffer, followed by recentrifugation at 10,000 × g for 15 min. The supernatants of the above two centrifugations were combined and centrifuged at 105,000 × g for 1 h (4°C). The microsomal pellets were suspended in 1 volume of 0.2 M potassium phosphate buffer containing 1 mM dithiothreitol and quickly frozen in liquid nitrogen and stored at −80°C. The protein content of the microsome preparation was determined by the method of Lowry et al. (13).

Assays of LRAT and ARAT activity. Microsomes were assayed for LRAT and ARAT activity using protein concentrations and incubation times that have been validated for initial rate determinations.

LRAT activity in the microsome samples was determined as described previously (14) according to the procedure reported (8) using retinol bound to CRBP (II) or CRBP as substrate.

CRBP(II) was purified from the small intestine of the chicken (15) and rat (16), respectively, as described previously. CRBP was purified from the livers of the chicken (15) and rat (17), respectively, as described previously. Complex of retinol bound to CRBP(II) or CRBP was prepared as described previously (14, 18). Briefly, 1.5 mg of the purified CRBP(II) or the purified CRBP was incubated with 1 μmol of retinol (about 10-fold mol) for 5 min at 4°C and unbound retinol was removed with a Sephadex G-25 column (PD-10, Pharmacia P-L Biochemicals) equilibrated with 0.2 M potassium phosphate buffer (pH 7.2).

The CRBP(II)-retinol or CRBP-retinol (3 μM retinol each), respectively, was incubated with 80 μM dilauroyl phosphatidylcholine (lecithin-laurate) and the microsomes (60 μg protein) in 0.2 M potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol and 2.4 mg/ml bovine serum albumin (final volume, 250 μl) for 15 min or 30 min at 37°C. The reaction was terminated by the addition of 1 ml of cold ethanol, and retinyl laurate was extracted from the reaction mixtures into 4 ml of hexane containing butylated hydroxytoluene (BHT, 200 μg/ml). A portion (3 ml) of the upper hexane phase was evaporated to dryness under nitrogen gas, and the residue was dissolved in 50 μl of methanol for high-performance liquid chromatography (HPLC) analysis. The HPLC analysis was performed using a Shimadzu LC-6A system fitted with a μBondapak C18 column (10 nm particle, 3.9 mm × 25 cm; Waters Associates Inc., Milford, MA, USA), with 100% methanol as the mobile phase at a flow rate of 2 ml/min. Absorbance of retinyl laurate in the eluates was determined using a spectrophotometer (Shimadzu SPD-6AV) with a wavelength of 330 nm. An external standard of the purified retinyl palmitate was used for quantification of eluted retinyl laurate peak.

The ARAT activity was assayed according to the procedure described previously (10). The reaction was initiated by incubating the microsome samples (60–240 μg protein) and 80 μM lauroyl-CoA and 3 μM solvent (dimethyl sulfoxide)-dispersed retinol in 0.2 M potassium phosphate buffer (pH 7.2) containing 20 mM bovine serum albumin and 5 mM dithiothreitol for 20 min at 37°C (total volume, 250 μl). The extraction of the reaction mixture and HPLC analysis of the extracts
were conducted by the same procedure described above for the LRAT assay.

RESULTS AND DISCUSSION

The esterification of retinol-CRBP(II) or retinol-CRBP was examined with dilauroyl phosphatidylcholine (lecithin-laurate) as an exogenous acyl donor. The acyl donor involved in the intestinal esterification of retinol-CRBP(II) by LRAT has been suggested to be phosphatidylcholine (8). Thus, we used dilauroyl phosphatidylcholine for the examination of esterification by LRAT in the lung samples, because its acyl moieties are different from the retinyl esters produced with endogenous acyl donor and the retinyl laurate from the exogenous acyl donor was clearly distinct from the ester products of the endogenous donor.

When the chick lung microsomal preparation was incubated with the lecithin laurate and either retinol-chicken CRBP(II) or retinol-chicken CRBP as substrates, retinyl laurate was not detected in the hexane extracts of the reaction mixtures by the HPLC analysis (Fig. 1, panels A, B). Furthermore, the HPLC analysis showed no other retinyl esters which were endogenously present in the extracts. In contrast, the rat lung microsomal preparations esterified the retinol provided with both retinol-rat CRBP(II) and retinol-rat CRBP, and produced retinyl laurate, which was eluted at a retention time of about 5.6 min on the HPLC (Fig. 1, panels C, D). The peaks eluted after the retinyl laurate peak indicate retinyl esters produced from the endogenous acyl donors in rat lung microsomes, including retinyl palmitate which was eluted at around 9 min of the retention time (Fig. 1, panels C, D).

Also evident in each chromatogram of Fig. 2 was the retinyl laurate produced from the exogenous acyl donor with the microsome preparation of chick small intestine (duodenum, panel A) and liver (panel B). In this study, we used retinol-chicken CRBP(II) as the substrate for the esterifications in the small intestine and liver. Although we did not examine the LRAT activity with retinol-chicken CRBP, we consider that the retinol esterification catalyzed by LRAT would be possible with the retinol-CRBP. Other studies suggest that the liver LRAT is involved in the esterification of retinol-CRBP rather than retinol-CRBP(II) and the intestinal LRAT is involved in the esterification of retinol-CRBP(II) rather than retinol-CRBP (7).

The LRAT activities in chick lung, small intestine and liver, and rat lung are summarized in Table 1. The 7-day-old chicks showed LRAT activity of 16.7 pmol/min/mg protein in the duodenum and 10.8 pmol/min/mg protein in the liver. Although LRAT activity was not detected in the 7-day-old chick lungs, the rat lungs exhibited LRAT activities of 16.4 pmol/min/mg protein with the substrate of retinol-rat CRBP(II) and 11.3 pmol/min/mg protein with the substrate of retinol-rat CRBP.

The results in this study indicate that LRAT activity is absent in the chick lungs. This evidence is consistent with the previous findings (12) that chicken lungs...
Fig. 1. Comparison of chick lung and rat lung for esterification of retinol-CRBP (II) or retinol-CRBP in their microsomes. Lung microsomes (60 µg microsomal protein) prepared from 7-day-old chick or rat weighing about 150g were incubated with 80 µM dilauryl phosphatidylcholine and 3 µM either retinol-CRBP(II) (panels, A and C) or retinol-CRBP (panels, B and D) for 30 min at 37°C. The final volume was 250 µl. Reaction products were extracted into hexane and analyzed by HPLC. R-la, retinyl laurate. The retinol-protein complexes for the chick lung samples were prepared with the purified chicken CRBP(II) or CRBP. The retinol-protein complexes for rat lung samples were prepared with the purified rat CRBP(II) or CRBP.

had no retinyl palmitate and no other retinyl esters, but had a considerable amount of retinol. As shown in Table 1, the rat lungs contained approximately 5 µg of retinyl palmitate per gram of tissue, but the chick lungs contained no detectable amount of retinyl palmitate. The previous study demonstrated that the adult hen (about 60 days of age) did not show any retinyl esters in the HPLC analysis (12).
Fig. 2. Demonstration of retinyl laurate produced in microsomes of chick duodenum and liver. The microsomes (60 μg microsomal protein) prepared from duodenum or liver of 7-day-old chick were incubated with 80 μM dilauroyl phosphatidylcholine and 3 μM retinol-chicken CRBP(II) for 30 min at 37°C. The final volume was 250 μl. Reaction products were extracted into hexane and analyzed by HPLC. R-la, retinyl laurate.

Table 1. Activities of lecithin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase (ARAT) in microsomes of chick lung, duodenum and liver and rat lung, and retinyl palmitate contents in chick and rat lungs.

| Enzyme source       | Activity (pmol/mg protein/min) | Retinyl palmitate (μg/g tissue) |
|---------------------|-------------------------------|---------------------------------|
|                     | LRAT                          | ARAT                           |                                |
|                     | Retinol-CRBP(II) | Retinol-CRBP | Retinol | Retinol-CRBP(II) | Retinol-CRBP | Retinol | Retinol-CRBP(II) | Retinol-CRBP | Retinol |
| Chick intestinal duodenum | 16.7±2.4 | — | — | 48.3±2.5 |
| Chick liver | 10.8±0.7 | — | — | — |
| Chick lung | ND | ND | 1.2±0.2 | ND |
| Rat lung | 16.4±2.4 | 11.3±2.5 | 1.8±0.3 | 5.2±0.7 |

Data represent the M±SE of three or four determinations. The values for the lung, duodenum and liver of chick represent the means of four individual determinations of 7-day-old chicks. The value for the rat lung represents the mean of three individual determinations of rats weighing about 150 g. Retinol-CRBP(II): retinol bound to CRBP(II) as a substrate, and the retinol-CRBP(II) complex was prepared with chicken CRBP(II) for chick samples and with rat CRBP(II) for rat samples. Retinol-CRBP: retinol bound to CRBP as a substrate, and the retinol-CRBP complex was prepared with chicken CRBP for the chick samples and with rat CRBP for the rat samples.

ND, not detectable; —, not determined; *, data from the previous report (12).

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Accordingly, we concluded that chick and hen lungs did not have LRAT and retinol esterification possibly did not occur.

The ARAT activity in chick and rat lungs is shown in Table 1. The chick and rat lungs exhibited trace ARAT activity, showing 1.2 pmol/min/mg protein and 1.8 pmol/min/mg protein, respectively. These levels of ARAT activity were about 10% of the LRAT activity in rat lungs. Such low ARAT activity in the rat lungs is unlikely attributable to the retinyl palmitate content in the lungs, as the trace ARAT activity in the chick lungs did not reflect the absence of retinyl palmitate (Table 1). Therefore, in this study, it was clear that there was a linkage between the presence of LRAT activity in tissue and the tissue retinyl ester content, but not between the existence of ARAT activity and the tissue retinyl ester content.

Recently, LRAT has been shown to be a major enzyme for retinol esterification (10). The ARAT has been considered to be involved in the esterification of free retinol when animals receive a large amount of vitamin A (7). However, we recently found that ARAT activity in the intestine of rats fed the diet containing 1,000 times the NRC recommended requirement (4.2 μmol/kg, I9) of vitamin A had not increased, but the LRAT activity in the same tissue had increased (20).

We previously observed that developing chicks accumulated only retinol, instead of the ester forms, in embryonic and posthatch lungs. The change in serum retinol concentration in the developmental stages of embryonic and posthatch chicks paralleled the changes in lung retinol content. The retinol levels in serum and lung were very high at 5 days before hatching and then the levels decreased to the lowest level around hatching, followed by a remarkable increase in both the levels of serum and lung retinol within the first 7 days after hatching. However, this developmental pattern of serum retinol concentration was different from those of retinol and retinyl palmitate contents in the liver (12). Therefore, the developmental change in the lung retinol level greatly reflected the changes in the serum retinol level.

The ontogeny of vitamin A storage in rat lungs of the fetus and neonate was examined (6). Only a trace amount of retinol was detected in the rat lung throughout the perinatal period (about 0.12 μg/g), but the concentration of retinyl esters (mainly retinyl palmitate) changed in developing rat lungs. It increased rapidly from the low level in fetal lungs on gestation day 14 to a peak on gestation day 18, and its concentration steadily declined thereafter. The postnatal levels from birth to 14 days averaged around 1.2 μg/g of tissue. Therefore, the rat lung is capable of storing a substantial amount of retinyl esters, because of the presence of LRAT activity in lung tissue.

It is clear that chicken lungs can not store much retinol (about 0.2 μg/g at 7 days of age) (12) and any of its esters (Table 1). Therefore, the lungs may use the retinol absorbed directly from serum in order to generate retinoic acid needed for cellular differentiation and lung growth. The lack of LRAT and retinyl esters would partly explain the fact that chicks are more easily prone to vitamin A deficiency compared to rats (21).
It is well known that rat liver vitamin A is stored in the stellate cells. The rat lung is also capable of storing vitamin A. The rat lung alveolar epithelium has been reported to be composed of type I and type II cells (22). The rat lung type II cells contain a substantial amount of retinyl esters (mainly retinyl palmitate) compared with the type I cells (23). This type II cell in rat lung tissue has been postulated to be a vitamin A-storing cell, which might be comparable to stellate cells in the liver. Further studies are required to elucidate whether such a specific cell type for vitamin A storage is absent in the chicken lung.

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