Cultured Lung Cells: Effects of Isotopic Dilution and Some Undefined Stimulation on the Incorporation of Radiolabeled Palmitate and Choline into Phosphatidyl Choline (Lecithin)

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Using radiolabels, we studied the effect of certain experimental conditions on the incorporation of choline and palmitate into phosphatidyl choline (lecithin) of cloned type II rat lung cells. When the label was changed from methyl-^{3}H to 1,2-^{14}C the incorporation of choline was reduced to 1/3; in contrast, when the label was moved from 1-^{14}C to 9,10-^{3}H, the incorporation of free palmitate was more than doubled. Removal of choline from the culture medium caused trebling of choline incorporation and appreciable decrease in palmitate uptake, indicating respectively an expected effect of increased choline label concentration in the absence of carrier, and a marked dependence of palmitate on choline incorporation. Removal of fetal calf serum produced more than 2/3 decrease in palmitate incorporation (instead of an expected increase because of palmitate isotope concentration), whereas choline uptake was not affected, meaning respectively that either serum hormones or serum lipids, or both simultaneously, are important for palmitate but not for choline incorporation. This is only the beginning of a host of studies required to clarify the role of fetal calf serum constituents onto lecithin biosynthesis in cultured lung cells and finally gain a full appreciation of the biosynthetic pathways of phosphatidyl choline ("surfactant") in type II cells of alveolar epithelium.

The importance attributed to dipalmitoyl phosphatidyl choline (DPPC) in pulmonary surfactant and alveolar stability has prompted a number of studies on the biosynthetic mechanisms leading to DPPC formation in lung type II cells [1]. In a previous study with a cell line (A 549) derived from human lung adenocarcinoma and resembling type II epithelial cell, we established that the incorporation of palmitate was several fold greater than that of choline [2]. Such results are consistent with the belief and evidence that the prevalent mechanism of biosynthesis of DPPC in alveolar type II cell is one of acylation and transacylation [1, 3].

However, in the attempt to evaluate the quantitative data and elucidate biosynthetic pathways, one becomes overwhelmed by questions related to the complexity of lipid, protein and hormone composition of the culture media (the fetal calf serum especially) and to their roles in metabolism, including their interaction with the cell membrane and their transport into the cell. In brief, one would like to know at what stage in DPPC biosynthesis do the fatty acids and phospholipid constituents of the culture media play their role and which. Although a systematic study of these questions is desirable,

certain variables are still imponderable, especially in regard to the chemical composition of the fetal calf serum; it becomes a prohibitive task if in one single study one wishes to take into account dose curves, cell growth phases, and the like. Probably because of the difficulties attending these complexities, best studies of the kind have been restricted to a one dose effect [4]. The following experiments will be used to identify certain phenomena and formulate the pertinent questions.

For obvious reasons we focused our attention onto palmitic acid and choline. They are precursors in two major mechanisms of DPPC biosynthesis, the acyl and the CDP-phosphoryl choline transferases [1, 3], and both precursors are present in the culture media [2]. In fetal calf serum, palmitic acid is found free as well as in the lecithin fraction [2] in concentrations that bear some similarity to those found in serum of adult mammals [5]. In the present communication we introduce the study of the effects of three parameters: (a) position of the isotope label in the precursor's molecule; (b) withdrawal of choline; and (c) withdrawal of fetal calf serum.

Materials and Methods

Unlike in a previous study [2], we utilized type II epithelial cells from adult rat lung, which were ob-

tained by a modification of the cloning method described by Douglas and Kaighn [6]. Because of difficulties encountered in keeping the A 549 and the cloned rabbit lung type II cells in monolayer culture, irrespective of whether we used Petri dishes or Falcon flasks, we resorted to rat lung cells, which seemed much more resistant and grew much more uniformly. In any event, it should be appreciated that, after a certain number of transfers, the cells in monolayer cultures lose the morphologic characteristic of the original type II cell, especially in terms of the inclusion bodies; these become less numerous, while the cells themselves either are unable to survive and multiply (as with A 549 and cloned rabbit type II cells) or possibly transform in some kind of fibroblasts, as it could be said of the cloned rat type II cells. What intrinsic character of type II cells is left in such cultures is not exactly known. The monolayer culture is, however, one of the accepted models systems for the study of metabolic processes in lung cell physiology [4].

The cells were grown in Petri dishes of 250 cm^2 area, using 20 ml Dulbecco's modified Eagle's medium at $37\,^\circ\text{C}$ in 5% CO_2 and 15% air. Among other constituents, the medium contained 15% fetal calf serum, $100\,\mu\text{g/ml}$ streptomycin and 100 units/ml penicillin. The cells were transferred weekly at $1:4$ split. At the beginning of the 7th day of growth, when the cells had reached confluency, the precursors were supplied in $10\,\mu\text{l}$ 0.15 M NaCl each to the flask containing 20 ml of medium. After five hours incubation, the cells were collected from the monolayer by scraping with a rubber policeman.

When cell counting was required, the medium was removed, the monolayer was washed $3\times$ with 20 ml phosphate buffer saline (0.15 M; pH 7.3) each time, then were added 10 ml of aqueous medium containing 500 mg trypsin and 200 mg EDTA per liter of Ca^{2+} and Mg^{2+} free Hank's balanced medium (Grand Island's #610-5300). After five minutes incubation at $37\,^\circ\text{C}$, the cells became lifted off, whereby they were easily dispersed and counted in the usual way.

For the study of the effect of choline and fetal calf serum withdrawals, at the beginning of the seventh day, the regular medium was removed, and after the cell monolayer was washed $3\times$, about 20 seconds with 20 ml of the desired medium each time at $37\,^\circ\text{C}$, the monolayer was covered with 20 ml of the new medium, and then immediately

the radioactive precursor solutions were added in 5 to $10\,\mu\text{l}$ aliquots.

After gentle centrifugation at $400\times g$ for ten minutes with two washings in 0.15 M NaCl, the cell pellet was finally dispersed in four volumes of 0.15 M NaCl; and aliquots were extracted twice with four volumes chloroform-methanol $2:1$. The organic phase was concentrated and the lipids therein were separated on precoated thin layer chromatography plates by methods already described [2]. The lipid spots were scraped, and the radioactivity was measured by liquid scintillation spectrometry. All the washings and pertinent fractions in the process of lipid extraction were also examined, to determine losses during washing and amounts of lipid secretion. The methods for protein, phosphorus and DNA determinations have been described elsewhere [2].

The labeled precursors [1-^{14}C]palmitic acid (sp. act. 55 mCi/mmol), [9,10-^{3}H]palmitic acid (sp. act. 190 mCi/mmol), [methyl-^{3}H]choline HCl (sp. act. 4.4 Ci/mmol), and [1,2-^{14}C]choline·HCl (sp. act. 6.3 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts. The palmitic acid sample, after removal of the organic solvent (hexane), was dispersed with rabbit serum albumin as described by Smith *et al.* [7].

The fatty acid distribution in fetal calf serum, in both the free fatty acid fraction and the phosphatidyl choline (lecithin) fraction were determined by gas liquid chromatography according to standard procedures; the composition was presented in a previous report [2]. As pointed out on that occasion, the serum's free fatty acid fraction contained only members between C_{12} and C_{18}, the most abundant of which was palmitic (41%); in contrast, the phosphatidyl choline fraction contained 31% fatty acids with chain length greater than C_{18}, and palmitic acid was the single most abundant one with 27% of the whole mixture. Unfortunately, the fatty acid composition of the other lipids (such as phosphatidyl ethanolamine and cholesteryl esters) in fetal calf serum was not determined; that notwithstanding, the above information must be taken into account when one wishes to extricate the role of the medium's lipids in pathways involving acylations and transacylations [1, 3] as well as in other pathways.

Results and Discussion

The data, averages of at least two separate experiments, are expressed in both nmol label/100 μg

DNA and % incorporation, keeping into account the quantities of label put in the culture flask at start. The variations in counting and DNA determinations did not exceed ±10%; in line with another study [4], the quantities of labels measured in the lecithin collected as "secretion" in the culture medium [4] and in the various washings amounted also to much less than 10% of those found in the pellet. Both errors were, therefore, negligible as compared to the effects reported below as biosynthesis.

Influence of label's position

Since decarboxylation of fatty acids is a known biological phenomenon [8] and so is also transmethylation from the quaternary ammonium group of choline [1, 8], it occurred to us that palmitate with label in carbon one [2] and choline with label in the methyl group [2] could provide incomplete information, if the access of unlabeled carriers to various pools is qualitatively and quantitatively not uniform and different from that of the medium's analogs, and the labels end up in molecules other than phosphatidyl choline. Also, in view of a metabolism [1, 3] in which the medium's lecithin can be degraded to provide fatty acid, choline, and glyceryl phosphoryl choline, diversity of pools is a strong possibility.

To begin to deal with this intriguing problem, we sought palmitate with label in the middle of the chain, and choline with label in carbons one and two. Since these two precursors ultimately had to be found in the same phosphatidyl choline molecule, and a two-channel [³H/¹⁴C] scintillation spectrometer was used, the double change from [1-¹⁴C] to [9,10-³H]palmitate and from [methyl-³H]choline to [1,2-¹⁴C]choline became convenient and necessary.

When the label was changed from the carboxyl group [1-¹⁴C] to carbons 9 and 10, the % incorporation of palmitate was more than doubled. A speculation comes to mind. If decarboxylation of palmitate takes place, less [1-¹⁴C]label should be found in the product; thereby, the tracer technique will not reflect with fidelity the true incorporation of unlabeled palmitate, if two different pools are available, one decarboxylating and the other not, and labeled and unlabeled precursors are not equally distributed in the two pools; the unequal distribution of precursors could occur for two reasons: first, the pools have different both site and metabolic origin; secondly,

whereas we introduce palmitate, this may not reach the pools of metabolic palmitate deriving from hydrolysis of the medium's phosphatidyl choline, phosphatidyl ethanolamine, cholesteryl esters and some other phospholipids present in the serum. Future studies must consider labels in all those molecules.

Rather interesting was the fact that when the label in choline was changed from ³H in the methyl groups to ¹⁴C in carbons one and two, the % choline incorporation decreased to 1/3. In spite of the large differences in specific activities in the precursors used (see Methods), these results cannot be due to specific activity, for the calculation of % label incorporation excludes such effects. A reason for our seeking a label other than in the methyl group of choline was our suspicion that the labeled methyl group could be lost into uninteresting pools. That would have in part accounted for the magnitude of the palmitate/choline incorporation ratio observed in previous studies [2]. The data in Table I confirm that suspicion and suggest an additional implication of the methyl transfer. Namely, when the methyl was labeled, some of it may have indeed reached the choline of the cell lecithin by transfer onto the ethanolamine [1], whereby the final product showed more label (0.69%). In contrast, the ethanol moiety of choline participated less (0.23%) through the CDP-phosphoryl choline transferase. To a first approximation the observed 0.69% incorporation of [methyl-³H]choline would be then the sum of two contributions, the methyl transferase and the CDP-phosphoryl choline transferase mechanisms. Further studies are required to confirm and separate these contributions; other possibilities cannot be excluded in the maze of reactions connecting transacylation

Table I. Effect of label's position on the incorporation of [1-¹⁴C]palmitate, [9,10-³H]palmitate, [methyl-³H]choline and [1,2-¹⁴C]choline in the lecithin of cultured type II cells from rat lung.

| | Incorporation | |
	$\dfrac{\text{nmol}}{100\ \mu\text{g DNA}}$	%
[¹⁴C]palmitate	0.245	5.30
[³H]choline	0.008	0.69
palmitate/choline	30.6	7.8
[³H]palmitate	1.385	13.65
[¹⁴C]choline	0.099	0.23
palmitate/choline	14.0	59.4

and total synthesis. One should bear in mind that the free choline present in the culture medium and that deriving from the medium's lecithins do not necessarily follow the same pools. Finally, it may have to be investigated if the label itself is subject to phenomena of steric specificity.

Isotopic dilution and serum hormones

In this study we used [9,10-³H]palmitate and [1,2-¹⁴C]choline precursors. The ordinary growth medium had 15% fetal calf serum. Since the latter contains appreciable amounts of palmitate, 27% in the lecithin fraction and 41% in the free fatty acid fraction (see ref. [2], and Methods section above), two questions come to mind: one is isotopic dilution of choline and palmitate, the other is the possible involvement of choline and palmitate and choline-laden and palmitate-laden phosphatidyl choline in different routes of transport across the cell membrane and then in the various pathways of DPPC biosynthesis [1 – 3]. The first question can be answered readily, the second implies an extended investigation.

When a choline-free medium was used (Table II) the increase in choline label incorporation from 0.23% to 0.69% reflected the effect of radio-isotope enrichment. The decrease in palmitate incorporation from 14% to 9% could be related to choline deficiency, whereby less of a glyceryl phosphoryl choline backbone would be available for an optimal utilization of the palmitate via either transmethylation or

CDP-phosphoryl choline transferase. This would indicate some interdependence among the two pathways and possibly also among the various choline and palmitate pools.

The effect of the serum-free medium was quite revealing. When 5% albumin was used in place of the fetal calf serum (FCS), the incorporation of choline was obviously not affected, for the choline had not been omitted from the culture medium. This indicates that, under the given experimental conditions, choline incorporation is independent of palmitate, whereas (above) palmitate incorporation is strongly dependent on free choline. However, the marked fall in palmitate incorporation from 13.65% to 3.80% in the absence of FCS is contrary to an increase to be expected from an isotope concentration effect because of the absence of nonlabeled precursors (free and phospholipid palmitate) in the FCS-free medium [2]. Such a loss in activity could suggest either of two things or both. First, the lecithins and palmitate in the medium are indispensable mediators of the acyl transfer at the basis of DPPC biosynthesis; also, since in the absence of fetal calf serum the incorporation of choline was not affected, in this particular direction choline and palmitate pools may be unrelated, and choline incorporation under these specific conditions is not controlled by serum hormones. Secondly, the fetal calf serum contains hormones (estrogens) that activate the enzymes of the acyl transferase pathway and not those of the CDP-phosphoryl choline transferase pathway. Both hypotheses are equally valid. However, one must still ascertain if and how the effect of the fetal calf serum constituents is limited to these enzymes or it extends to others and also to transport of label and carrier across the cell membrane.

The hormone specificity is consistent with a recent observation, whereby serum estrogen exhibited a marked activity in stimulating DPPC biosynthesis by fetal lung [9] and should, therefore, be considered as the natural stimulant of fetal lung maturation, in preference to the widely used glucocorticosteroids (cortisol and betamethasone), whose untoward effects have also been reported [10 – 13]. Although the marked negative effect of fetal calf serum withdrawal on palmitate incorporation into cell phosphatidyl choline would appear to conflict with the marked enrichment in palmitate observed by Smith in the A 549 cells upon serum withdrawal

Table II. Effect of isotope dilution by exogenous choline and fetal calf serum (FCS) on incorporation of [9,10-³H]palmitate and [1,2-¹⁴C]choline in phosphatidyl choline of cultured type II cells from rat lung. Notice the remarkable inhibitory effect of the serum-free medium on palmitate incorporation.

Control	Incorporation	
	nmol / 100 μg DNA	%
[³H]palmitate	1.385	13.65
[¹⁴C]choline	0.099	0.23
palmitate/choline	14.0	59.4
No choline		
[³H]palmitate	0.930	9.10
[¹⁴C]choline	0.300	0.69
palmitate/choline	3.1	13.2
No FCS		
[³H]palmitate	0.390	3.80
[¹⁴C]choline	0.088	0.22
palmitate/choline	4.4	17.3

[4], the two studies cannot be compared on several grounds. For instance, (a) the cell lines and species were different, [6] the conditions of withdrawal were different, being in our case only a few minutes, in the other case [4] amounting to 48 hour starvation before the experiment; (c) we report % palmitate incorporation, the others [4] report % palmitate in the total fatty acid composition of phosphatidyl choline.

Biological significance

At present it is not possible to provide significant interpretations of biosynthetic data of lung type II cells in culture, before one obtains a total picture of the lipid metabolism. Such a picture will be possible when we will have information concerning the role of the culture medium constituents. Particular attention must be paid to the effects of the fetal calf serum constituents with regard not only to metabolic processes but also to the transport processes across the plasma membrane preceding and following the

biosynthetic result that we measured as incorporation. Extensive experimentation is still required, using possibly inhibitors and activators in order to separate mechanisms or pathways and precursors' pools. Also, although the state of the art in the isolation and maintenance of viable type II cells is such [14] that metabolic studies may appear to have dubious relevance to type II cells *in vivo*, and viable type II cells are not yet available that are capable to afford the numerous transfers required for a complete study, the cell monolayer still remains an indispensable model. Whether the cells used are comparable to type II or they are transformed, the information is not only valid but also fundamental for us to begin to dissect in the complexity of the structural and metabolic phenomena of lung cells in particular and the living cell in general.

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