Cultured Lung Cells: Interplay Effects of Beta-Mimetics, Prostaglandins and Corticosteroids in the Biosynthesis of Dipalmitoyl Lecithin

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Z. Naturforsch. 34c, 101—105 (1979) ; received October 6, 1978

Ethanol Metabolism, Beta-Stimulants, Lung Type II Cells, Pulmonary Surfactant, Cortisol

Cultured lung cells were used to study interplays of substances affecting incorporation of labeled precursors [1-14C]palmitate and [methyl-3H]choline into phosphatidyl choline. Ethanol stimulated markedly biosynthesis of dipalmitoyl phosphatidyl choline in cloned rabbit lung cells; the stimulating action of ethanol was reduced very much by cortisol and less by ritodrine. In the presence of 0.1 μM isoproterenol, two prostaglandins, E2 and F2α, caused marked depressions in the incorporation of both precursors by cell line A549 derived from human lung adenocarcinoma. One concluded that among the agents studied, ethanol and cortisol are potent antagonists, and so were also the prostaglandins and isoproterenol.

Our interest in beta-stimulators, glucocorticosteroids and prostaglandins stems from work on pulmonary surfactant [1] and fetal lung maturation [2]. In the latter, these three groups of compounds appear to be physiologically, clinically and pharmacologically related. The prenatal process of lung maturation has been fairly well characterized, biochemically [3] and morphologically [4]; it consists of accelerated biosynthesis of dipalmitoyl phosphatidyl choline (DPPC) in type II epithelial lung cells. This phospholipid (of the phosphatidyl choline or lecithin class) is then stored in the osmiophilic inclusion bodies, whose contents are ultimately released from the type II cells into the alveolar lining layer [5], and is an essential constituent of the air-blood barrier of the lung.

The probable interplay of these various drugs and hormones in the perinatal period of the infants prompts several questions with regard to the physiology of the lung in child and mother. Since lecithin biosynthesis and its incorporation in the inclusion bodies was demonstrated by autoradiographic electron microscopy also in the adult lung [20], the introduction (either natural, experimental or clinical) of relatively large doses of beta-stimulant, corticosteroid and prostaglandin in the vascular circulation of the pregnant woman in late gestation prompts interest in the effects of these agents in the adult lung. The latter thus becomes a convenient model, also more accessible, to study the role of substances of known chemical structure in processes of transport, mechanics, metabolism and morphology across the air-blood barrier of the lung.

Because of the complexities attending the study of biological processes in vivo, we have restricted our early investigations to simpler systems and thus...
focused our attention onto the effects of these agents on the lipid biosynthesis of type II epithelial lung cells in culture. We used adult lung cells because they are readily available in our laboratories [19, 21].

In this communication we present the results of experiments concerning the influence of selected mixtures of ethanol, cortisol, prostaglandins and beta-stimulants on the incorporation of labelled precursors, $[^3]$H choline and $[^14]$C palmitate, into phosphatidyl choline (PC, lecithin) in type II cells of adult lung in culture.

**Materials and Methods**

**Cells**

We used cloned type II cells from rabbit lung, and line A549 cells from human lung carcinoma resembling type II epithelial cells. The rabbit lung cells were obtained in our laboratory by a modification of the cloning method described by Douglas and Kaighn [22]. Cell line A549 was kindly provided by Drs. A. Szakal and G. Todaro [23]. The cells were grown in monolayers in Falcon flasks using Dulbecco's modified Eagle's medium containing 100 µg/ml streptomycin and 100 units/ml penicillin at 37 °C, in 5% CO$_2$ and 15% air. The cells were transferred weekly at 1 : 4 split.

**Chemicals and Procedures**

The labelled precursors [methyl-$^3$H]choline·HCl and [1-$^14$C] palmitic acid were purchased from New England Nuclear, Boston, Massachusetts; the specific activities were 4.4 Ci/mmol and 55 mCi/mmol respectively. The precursors, prepared by a procedure that was already described [13, 19], were supplied in 10 µl aliquots each to 10 ml of culture medium at the beginning of the 8th day of growth. After five hours, cells and media were harvested and separated, and aliquots of both the cell pellet and the supernatant were processed as follows. The lipids were extracted, separated and identified by their migration on thin layer chromatography (TLC) plates, and incorporation of the radioactive precursors was determined by liquid scintillation counting of the lipid spots. Cell counting and DNA analysis, performed in the usual way [19], showed that the deviation in the number of cells per flask was not greater than 15%. Most of the experimental procedures with regard to culture, harvesting, lipid extraction and identity, and radioactivity measurements were already described [19].

The administration of cortisol in aqueous medium with serum albumin [13] to the cell culture presented some difficulty because of improper dispersion of cortisol. We thus found it convenient to dilute the ethanolic solution of cortisol with 0.15 M NaCl and then add 10 µl of the dilute solution to the culture medium; the final concentration of ethanol was 80 µM.

Although choline·HCl is water soluble, much of it was found with the lipid in the chloroform-methanol extract of the cell aliquot as it was indicated by the usually large $^3$H-choline counts in two TLC spots, a large one near the origin (choline) and the other (smaller) overlapping with both the phosphatidyl ethanolamine and phosphatidyl choline spots. In order to remove this serious interference, we washed the chloroform phase of the lipid extract (4 ml) 2 × with 2 ml 0.15 M NaCl at a time. In this operation, most of the free palmitate and the other lipids remained in the chloroform phase; further washing caused large lipid losses. Using TLC plates precoated with silica gel G (Analtech, Inc., Newark, Delaware) and the appropriate lipid standards, equal aliquots of the lipid mixtures were developed in chloroform-methanol-conc. NH$_4$OH — water 70 : 30 : 1 : 4 and hexane-diethyl ether-acetic acid 80 : 20 : 1 to resolve the phospholipids and the neutral lipids respectively [19, 24].

**Results**

In most of the experiments, we use the crude data, cpm, since the purpose of this communication is only to compare the effects of various drugs and hormones under given experimental conditions. We focus our attention mostly onto the radio-activity found in the cell pellet lecithin, to which we refer as biosynthesis. Although interesting effects were seen in the lecithin secretion in the supernatant, the secretion was so small (between 1% and 10% of the total incorporation) that for the sake of clarity and for the objective of this paper we choose to ignore it.

The average incorporation of $[^3]$H choline and $[^14]$C palmitate from four separate experiments was expressed in cpm per 1 ml aliquots of cell dispersion in 0.15 M NaCl; the standard deviation was not greater than 10%. The higher cpm values from $^3$H as compared to $^14$C are due to the greater specific
activity of [3H]choline; when the latter and isotope dilution in the culture medium were taken into account, the incorporation of palmitate was much greater than that of choline [19]. This information is important for a correct reading of the following Tables.

**General lipid pattern.** In Table I is a typical view of incorporation of both [3H]choline and [14C]palmitate in the various lipids of the pelleted cells (Line A 549, "control" in experiment below). Most of the choline was found in lecithin (PC) \( \Rightarrow \) lysolecithin (LL) + sphingomyelin (Sph); the few [3H]counts found in the phosphatidyl ethanolamine (PE) spot could be due either to contamination from the heavy PC spot which followed PE, to some choline in salt linkage with palmitate, to free palmitate itself, and/or to some other palmitate or choline adduct. For undefined reasons, some of the free palmitate migrated with the phospholipid in the TLC system used. Whatever the reason for this spurious choline activity, for all practical purposes those few counts may be ignored.

Unlike choline, which was found mostly in PC, the radioactivity of [14C]palmitate was distributed as follows: PC \( \Rightarrow \) FFA > TG > PE > Sph > LL > CE (see Legend Table I). The large amount of free fatty acid (FFA) is probably the unreacted 14C-palmitate that entered or had already remetabolized from higher products in the cells; as expected, it partitioned into the organic phase during the lipid extraction. Similar qualitative lipid patterns were observed with various cell cultures. Since our prevailing interest is phosphatidyl choline, at present we will provide data with this lipid only.

**Cloned rabbit lung cells**

In this experiment (Table II) we remarked the effect of ethanol on lecithin biosynthesis. Ethanol was used for two reasons, first to dissolve the water insoluble cortisol, secondly to see the influence of ethanol on the incorporation of DPPC precursors, since ethanol is known to participate in lipid biosynthesis [25]. Ethanol, 80 \( \mu M \), caused a marked increase in the incorporation of both choline (3.5 \( \times \)) and palmitate (5.5 \( \times \)) with respect to the control.

In the presence of 80 \( \mu M \) ethanol, cortisol, 5.5 \( \mu M \), depressed to about 1/5 the incorporation of choline and to less than 1/2 the incorporation of palmitate with respect to the ethanol alone. Since cortisol, by itself, stimulated lecithin biosynthesis by rabbit fetal [13] and adult [27] lung cells in culture as well as by fetal lungs in vivo [10], it is interesting how, in these experiments, ethanol and cortisol were antagonistic; also interesting is the precursor and pathway selectivity. For instance, in the presence of ethanol, cortisol depressed to values below the control (13,555 cpm) the incorporation of choline (8,961 cpm) but still above the control (1,492 cpm) the incorporation of palmitate (3,015 cpm). This selectivity in inhibition of ethanol activity by cortisol suggests two possible phenomena. First, the two precursors (choline and palmitate) may be derived from separate pools over the two different biosynthetic pathways (the CDP-phosphoryl choline and the acyl transferases respectively); secondly, the agonists and antagonists have dose dependent responses.

Ritodrine, 5.5 \( \mu M \), in the presence of 80 \( \mu M \) ethanol, reduced only by 40\% the incorporation of choline and by 30\% the incorporation of palmitate with respect to the ethanol alone. Thus, the depressing action of ritodrine on the stimulating action of ethanol was modest as compared to that of cortisol.

**Line A 549 cells from human lung carcinoma**

In this experiment, we used two beta-stimulants (isoproterenol and ritodrine) and two prostaglandins (E and F2, \( \alpha, \beta \)), but no ethanol (Table III). Isoproterenol, 0.1 \( \mu M \) did not affect appreciably the incorporation of either choline or palmitate.

### Table I. Distribution of radioactivity in various lipids of cultured cells from human lung carcinoma (Line A 549)

<table>
<thead>
<tr>
<th>cpm</th>
<th>LL and Sph</th>
<th>PC</th>
<th>PE</th>
<th>FFA</th>
<th>TG</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H</td>
<td>1,395</td>
<td>15,188</td>
<td>122</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14C</td>
<td>1,532</td>
<td>9,961</td>
<td>2,133</td>
<td>5,205</td>
<td>3,046</td>
<td>373</td>
</tr>
</tbody>
</table>

### Table II. Influence of ethanol on the incorporation of [3H]choline and [14C]palmitate in lecithin by cloned rabbit lung type II cells in culture.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Ritodrine</th>
<th>Cortisol</th>
<th>Ethanol</th>
<th>[3H] cpm</th>
<th>[14C] cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>13,555</td>
<td>1,492</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
<td>80</td>
<td>46,044</td>
<td>7,541</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>5.5</td>
<td>80</td>
<td>9,861</td>
<td>3,015</td>
</tr>
<tr>
<td>4</td>
<td>5.5</td>
<td>—</td>
<td>80</td>
<td>28,146</td>
<td>5,636</td>
</tr>
</tbody>
</table>
### Table III. Effects of isoproterenol (Isop), ritodrine (Rit) and prostaglandins, $E_2$ and $F_{2a}$, on the incorporation of $[^3H]$choline and $[^14C]$palmitate in lecithin by cultured cells, Line A 549 from human lung carcinoma.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Isop $[^\mu M]$</th>
<th>Rit $[^\mu M]$</th>
<th>PGE$_2$ $[^\mu M]$</th>
<th>PGF$_{2a}$ $[^\mu M]$</th>
<th>$[^3H]$</th>
<th>$[^14C]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>15,188</td>
<td>9,961</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>13,707</td>
<td>8,508</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>1.0</td>
<td>$-$</td>
<td>$-$</td>
<td>9,055</td>
<td>5,864</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>$-$</td>
<td>1.0</td>
<td>$-$</td>
<td>5,360</td>
<td>5,012</td>
</tr>
<tr>
<td>5</td>
<td>$-$</td>
<td>1.0</td>
<td>$-$</td>
<td>$-$</td>
<td>20,330</td>
<td>8,705</td>
</tr>
<tr>
<td>6</td>
<td>$-$</td>
<td>1.0</td>
<td>$-$</td>
<td>$-$</td>
<td>11,195</td>
<td>6,210</td>
</tr>
</tbody>
</table>

However, in the presence of 0.1 $\mu M$ isoproterenol, PGF$_{2a}$ (1 $\mu M$) was more effective than PGE$_2$ in depressing the incorporation of both choline and palmitate. This is interesting, for in separate studies with the same cells in the absence of isoproterenol, PGE$_2$ (more than PGF$_{2a}$) stimulated markedly the incorporation of both precursors [19]. Thus, isoproterenol and these prostaglandins are antagonistic.

Ritodrine, 1 $\mu M$, stimulated the incorporation of choline but affected little the uptake of palmitate. A higher concentration, 10 $\mu M$, ritodrine depressed to 1/2 the incorporation of choline and to a lesser extent the incorporation of palmitate as compared to 1 $\mu M$ ritodrine. This is suggestive of dose-dependent effects.

### Discussion

In general, ethanol, isoproterenol, ritodrine, prostaglandins and cortisol influenced differently the incorporation of choline and palmitate into lecithin. It is clear that the various drugs and hormones act selectively on different biosynthetic pathways, as it was predicted by previous experiments [19]. This observation could be exploited to tailor chemical structure to biological function and to investigate the pharmacologic interplay of beta-stimulants, corticosteroids and prostaglandins in adult as well as in perinatal lung. The importance of the interplays seen in the foregoing data is confirmed by recent work of Smith [27], regarding the effect of beta-adrenergic and cholinergic agents on the biosynthesis and secretion of lecithins in monolayer cultures of the same A 549 cell line.

Interestingly, ethanol and isoproterenol are smooth muscle relaxants [18, 26], yet ethanol stimulated whereas isoproterenol barely affected the incorporation of choline and palmitate into phos-phetidyl choline. Ethanol and cortisol, which separately promote biosynthesis of phosphatidyl choline (Table I and Ref. 13), together are so antagonistic that their mixture abolished the stimulating action of ethanol and depressed below the control level the incorporation of choline. Future studies must (a) consider the effect of inhibitors in order to define the unique mechanisms of the various agents and (b) take into account the kinetics of precursor incorporation in order to identify the various possible precursor pools and the logistics of enzymic sequences in both the cell ultrastructure and the pathways of dipalmitoyl phosphatidyl choline biosynthesis. The remarkable effect of ethanol may offer a lead to such mechanisms, for it probably implies the action of alcohol and acetaldehyde dehydrogenases [25] and formation of Ac-SCoA pools, which then serve as sources of $\text{SCoA}$ in the transacylating reactions [25, 28] that lead to the biosynthesis of this unique lung phospholipid. Other effects of ethanol cannot be excluded.

**Biological significance**

A note of caution is appropriate. Cells that have undergone many transfers are bound to lose the physiologic and morphologic characteristics of the original cells, and one rightfully questions the relevance of studies carried out with cells that no longer represent the *in vivo* cell. The ultrastructural pictures of the cloned rabbit and the A 549 human lung cells after 6 transfers showed progressive losses of the inclusion bodies and degeneration of the cells (unpublished); this property was reflected in poor growth as apparent from visual and light microscopic inspection of the monolayer culture as well as from large deviations in cell counts. Such cultures were not used, and the studies with those cell lines had to be abandoned. More successful was in contrast the experience with the cloned rat lung cells, which appear to be much more resistant after innumerable transfers. Although we do not claim that type II cells in monolayers remain such, or indeed they may resemble prosperous fibroblasts, one has to accept the fact that a model of some kind is necessary to carry out a host of compelling experiments, while awaiting felicitous results from attempts, thus far unsuccessful [29], to preserve type II cells in culture.

This work was supported by NIH Grant, and contract NOI-HR5-2952, both from the Heart, Lung and Blood Institute.


