# Role of phosphatidylethanolamine methylation in the synthesis of phosphatidylcholine by hepatocytes isolated from choline-deficient rats

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## 1. INTRODUCTION

In recent years the transmethylases involved in phosphatidylcholine (PC) synthesis [1] have been the object of a number of studies [2–5]. These attempted to elucidate the mechanism of their action as well as their role in several biological processes. However, the importance of transmethylation in the synthesis of the PC moiety of lipoproteins have not yet been established. Previous studies that used labelled *S*-adenosyl-L-methionine (SAM) as a precursor for PC synthesis via the transmethylase pathway, showed that the latter is enhanced in choline-deficient animals [6–8]. In contrast, measurements of the transformation of phosphatidylethanolamine (PE) to PC suggested that phospholipid methylation falls in choline deficiency [9,10]. This decrease could reflect the existence of a limited SAM pool in hepatocytes of choline-deficient animals. In fact, SAM was shown to be a limiting factor for the methylation of phospholipids by rat liver microsomes [5]. Thus, the cellular pool of SAM could be considered as a limiting factor for the synthesis of the PC moiety of lipoproteins in choline-deficiency. As an initial test for this hypothesis we studied the effect of SAM on lipoprotein secretion by hepatocytes isolated in vitro from choline-supplemented (CS) and choline-deficient (CD) rats. The results indicate that the transmethylase pathway may be substituted for the CDP-choline pathway [11] to supply the PC moiety for lipoproteins when a high cellular SAM pool is maintained by addition of the methyl donor to the incubation medium.

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## 2. MATERIALS AND METHODS

Male Wistar rats (70–100 g) were fasted 18 h and then fed a CS or a CD diet ad libitum for 3 days [12]. Hepatocyte isolation was performed by the method in [13] at noon. Cells were centrifuged 10 min at 400 × *g* and washed once in a medium containing 60 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM $Na_2HPO_4$, 5 mM glucose and 50 mM Hepes buffer (pH 7.4). They were finally suspended in a modified HAM F-12 medium (Laboratoires Eurobio, Paris) containing 0.4% defatted bovine serum albumin (Sigma Chem. Co., St. Louis, MO, USA), 0.1% penicillin G and 20 mM Hepes buffer (pH 7.4), and free of choline, methionine, folic acid and sodium bicarbonate. The hepatocytes were 90–95% viable as tested by the trypan blue exclusion. Incubations were done in 25 ml Erlenmeyer flasks, at 37°C. To each flask were added sequentially: 0.5 ml of modified HAM F-12 medium containing, when indicated, SAM and/or *N,N*-diethylethanolamine; 1 ml of cell suspension ($3 \times 10^6$) and 0.05 ml of labelled precursors. SAM was added as a disulphate–di-*p*-toluene–sulfonate conjugate [14], (Bioresearch Co., Liscate, Milano, Italy). This conjugate is stable for several hours at neutral pH. Cell triacylglycerol (TG) was labelled by [1-$^{14}$C]-palmitate (0.01 μCi/μmol per sample, New England Nuclear, Dreieich, West Germany). Stock so-

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lutions of [1-$^{14}$C]palmitate-albumin (fraction V from bovine serum, defatted, Sigma Chem. Co.) and [$^{12}$C]palmitate were made as in [15]. Cell phospholipid was labelled with [$^{3}$H]ethanolamine (0.15 $\mu$Ci/2 × $10^{-3}$ $\mu$mol per sample, The Radiochemical Centre, Amersham, England). The reaction was stopped by addition of 6 ml of cold saline followed by centrifugation at 400 × $g$ per 10 min. The sediments were washed once in saline. The incubation flasks were washed with saline and then freeze–thawed once in order to detach the cells adhering to the bottom. These cells were quantitatively collected and added to the washed sediments. Lipoproteins were precipitated from supernatants as in [16]. Lipids were extracted from both sediments and supernatants and purified as described [17,18]. They were used for radioactivity measurements in a liquid scintillation counter.

For the acrylamide gel electrophoresis experiments, the lipoproteins secreted by hepatocytes incubated in the presence of labelled palmitate, were precipitated, lyophylized and then dissolved in 3 × $10^{3}$ mM Tris–HCl buffer (pH 8.3). Electrophoresis was performed at room temperature using 7% slab gels and 20 mM glycine–Tris buffer (pH 8.3). Coomassie blue and Sudan black staining were made by the methods in [19] and [20], respectively. The labelled bands were identified by fluorography [21]. Rat serum lipoproteins, isolated by ultracentrifugation [22], were used as standards for electrophoresis.

Triacylglycerol was determined as in [23] and phospholipid as previously published [17].

## 3. RESULTS

Table 1 shows that the TG content of intact liver of CD rats was 3.5 times higher than that of control livers. This ratio was similar, averaging 3.05, for the hepatocytes isolated from CS and CD rats. These results indicate that a loss of TG due to cell rupture during isolation from CD rats can be excluded.

Figure 1 illustrates the kinetics of TG synthesis and secretion in isolated hepatocytes. It shows that the cells from CS rats did actively secrete TG. The secretion rate rose progressively from 15 to 180 min of incubation. This phenomenon was associated to an increase in palmitate uptake by cellular TG, whose rate, however, decreased as the secretion rate increased. Conversely, a low TG release coupled to an active palmitate incorporation into cellular TG was observed in hepatocytes from CS rats. This uptake was higher in CD than in CS rats because of the larger TG content in the liver of the former animals. However, the specific activity of hepatocyte TG, at 120 min of incubation, was 55% lower in experimental than in control hepatocytes

Table 1

Triacylglycerol content of intact liver and isolated hepatocytes of control and choline-deficient rats

| Diet | Liver (mg triacylglycerol/g) | Hepatocytes (mg triacylglycerol/$10^{6}$ cells) |
|---|---|---|
| CS | (7) 17.19 ± 2.98 | (2) 0.28 |
| CD | (9) 60.13 ± 9.49 | (8) 0.84 ± 0.22 |

Mean values ± SD; in parenthesis the number of experiments

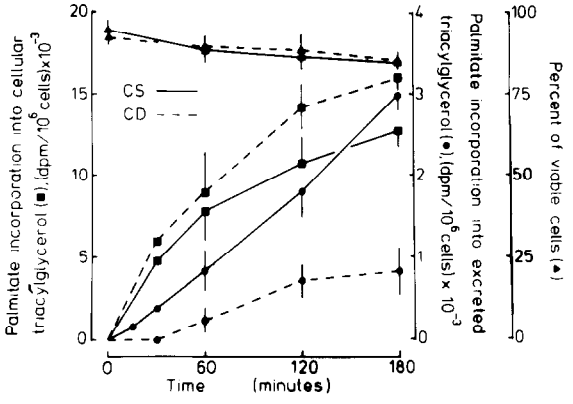


Fig. 1. Time-course of the synthesis and secretion of triacylglycerol in hepatocytes isolated from rats fed a CS or a CD diet. Hepatocytes (3 × $10^{6}$) were preincubated 10 min at 37°C in 'methyl-group-deficient' HAM F-12. The reaction was started by the addition of labelled palmitate. TGs were extracted and purified by TLC from hepatocytes or lipoproteins as in [17] and [18], and used to measure the radioactivity. Points with vertical bars are means of at least 4 triplicate experiments ± SD; other points are means of 2 experiments.

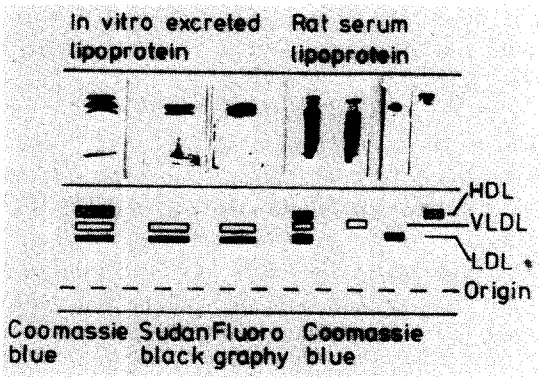


Fig.2. Acrylamide gel electrophoresis of lipoproteins secreted by isolated hepatocytes. Hepatocytes were incubated 180 min at 37°C in the conditions described in the legend of fig. 1. At the end of the incubations the lipoproteins were precipitated and used for the gel electrophoresis as described in the Materials and Methods section.

(10 200 *vs* 26 000 dpm/mg of TG). At 180 min of incubation this figure was 89% (11 510 *vs* 30 333 dpm/mg of TG). TG secretion, whether expressed as incorporation of palmitate per $10^6$ cells (fig.1) or as specific activity (not shown), was significantly reduced in hepatocytes from CD rats.

Gel electrophoresis of the lipoproteins secreted by control hepatocytes (fig.2) indicated that the labelled palmitate was contained mostly in VLDL. Some radioactive material migrated with LDL band. A band reacting with Coomassie blue and migrating with HDL was also visible, but it did not contain lipids reacting with Sudan black, nor did it incorporate the labelled precursor. No investigations were made to further elucidate the nature of this band. The electrophoresis of lipoproteins secreted by hepatocytes from CD rats, in the presence of SAM, gave the same results (not shown).

The effects of SAM in TG synthesis and secretion are shown in table 2. The addition of SAM to the hepatocytes from CS rats did not modify TG secretion and synthesis. In contrast, SAM addition to the hepatocytes from CD rats resulted in a large rise of lipoprotein secretion back to the control values, parallel to a fall of palmitate incorporation into cellular TG. Consequently, the total values (secretion plus synthesis) did not change. The effects of SAM on the experimental hepatocytes were suppressed by *N,N*-diethylethanolamine, a strong inhibitor of the transmethylase pathway [24]. The addition of the inhibitor alone had no effect on the hepatocytes from CD rats (not shown).

The effects of SAM on the synthesis of the membrane PC as well as the PC moiety of lipoproteins is shown in table 3. The synthesis was measured using labelled ethanolamine as a precursor for the transmethylase pathway. The fraction of labelled ethanolamine found in membrane and lipoprotein phospholipids, after 120 min of incubation, was low for the hepatocytes of CS and CD rats. This could depend in part on radioisotope dilution by endogenous PE, since this phospholipid is present in high amounts in liver cells [25] and its content is further increased by ethanolamine addition to hepatocytes suspensions [26]. The incorporation of ethanolamine into membrane PE was 3.4 and 4.8 times higher than that into PC for CS and CD rats, respectively. This probably indicates that PE synthesis was not followed by that of relevant amounts of PC. SAM addition to control hepatocytes did not alter ethanolamine uptake by lipoprotein and membrane phospholipids (not

Table 2

Effect of *S*-adenosyl-L-methionine and *N,N*-diethylethanolamine on the synthesis and secretion of triacylglycerol in the hepatocytes isolated from rats fed a choline-supplemented or a choline-deficient diet

| Conditions | Labelled triacylglycerol | | Total |
|---|---|---|---|
| | Secretion | Synthesis | |
| | (dpm per $10^6$ cells) | | |
| CS | 1984 ± 172 | 10 759 ± 2767 | 12 743 |
| CS ± SAM | 1900 | 10 852 | 12 752 |
| CD | 741 ± 143 | 14 228 ± 2743 | 14 969 |
| CD ± SAM | 1886 ± 164 | 11 465 ± 2948 | 13 351 |
| CD ± SAM ± DE | 593 | 10 997 | 11 590 |

Conditions are the same as described in the legend of fig.1. Incubation time, 120 min. SAM and *N,N*-diethylethanolamine (DE) were added to the incubation medium up to a final concentration of 3 and 2 mM, respectively. Data are means ± SD of at least 3 triplicate experiments or means of 2 experiments

Table 3

Incorporation of radioactivity of [$^3$H]ethanolamine into phosphatidylethanolamine and phosphatidylcholine of lipoprotein and hepatocyte membranes

| Conditions | Phospholipid | Percent of intracellular labelled precursor | |
|---|---|---|---|
| | | Lipoprotein | Membranes |
| CS | PE | 1.34 ± 0.04 | 8.47 ± 0.80 |
| | PC | 0.77 ± 0.05 | 2.49 ± 0.22 |
| CD | PE | 2.05 ± 0.01 | 11.80 ± 1.61 |
| | PC | 0.29 ± 0.01 | 2.45 ± 0.11 |
| CD + SAM | PE | 4.70 ± 0.33 | 29.70 ± 2.50 |
| | PC | 9.61 ± 2.29 | 18.20 ± 7.40 |

Hepatocytes were incubated 120 min as described in the legend of fig.1 except that [$^3$H]ethanolamine was added to the medium in place of labelled palmitate. 3 mM SAM was present when indicated. In order to isolate membranes the hepatocytes were subjected to a mild sonic oscillation and then centrifuged 70 min at 165 000 × *g*. Phospholipids were purified by TLC as in [17] and used for radioactivity measurements. Intracellular labelled precursor was calculated as the sum of the total cell radioactivity plus the radioactivity found in the secreted lipoproteins. Data are means ± SD of 3 triplicate experiments

shown). In contrast, when SAM was added to experimental hepatocytes the ethanolamine incorporation into PC increased 33 times for lipoproteins and 7.4 times for hepatocyte membranes. In the case of PE, the precursor incorporation increased 2.3–2.5 times for both lipoproteins and membranes. The data expressed as specific activity (not included in the table) show that ethanolamine incorporated into secreted PC was increased by SAM addition to CD rat hepatocytes from 0.180 to 0.650 dpm/nmol of phospholipid. Specific activity rose in membranes from 0.33 to 16.37 dpm/nmol of PC.

## 4. DISCUSSION

The present results indicate that hepatocytes isolated either from CS or CD rats do actively synthesize and release TG during the incubation in vitro. TG secretion by control hepatocytes was higher and proceeded faster than that observed in experimental hepatocytes. In contrast, TG synthesis proceeded at a higher rate and in larger amounts in experimental than in control hepatocytes. Thus, as previously observed in experiments in vivo [27], a significantly higher fraction of labelled precursor was present in the secreted TG of CS rats than of CD animals. A larger fraction was present in liver TG of CD rats than in those of CS animals.

The hepatocytes isolated from CD rats were suspended in a medium lacking choline and methyl donors. In these conditions, while hepatocyte PC is low, PE is high [28]. This should lead to a fall in the phospholipid available for the assembly of lipoproteins and for the synthesis of membranes involved in lipoprotein secretion [29,30]. On the other hand an increase of hepatocyte PE activates phospholipid *N*-methylation [26]. Our data show that TG release by hepatocytes isolated from CD rats is high only if SAM is added to the incubation medium. Moreover, SAM significantly enhances the fraction of labelled ethanolamine incorporated into PC used for both membrane synthesis and lipoprotein assembly in hepatocytes from CD rats. The stepwise methylation of PE is catalyzed by at least two methyltransferases [2,5]. The first methyltransferase transforms PE into phosphatidylmonomethylethanolamine. It has a low $K_m$ for SAM and it is inhibited by low amounts of *S*-adenosyl-L-homocysteine. It has been suggested that this enzyme is rate limiting for the pathway [1–3]. SAM was shown to freely enter isolated hepatocytes [31]. Consequently, when intact liver cells are incubated in the presence of saturating SAM concentrations, the first methyltransferase should be protected from *S*-adenosyl-L-homocysteine inhibition and the second methyltransferase should function close to its maximum rate [2,5]. Therefore the overall pathway should be activated. Interestingly, SAM addition to control hepatocytes failed to enhance lipoprotein secretion and ethanolamine uptake. Thus, transmethylation may be stimulated only when the CDP-choline pathway is inhibited by choline deficiency. This observation strengthens recent evidence that the contributions by each pathway are reciprocally compensatory [32].

In conclusion, the transmethylase pathway supplies sufficient PC for the synthesis of membranes involved in lipoprotein secretion and for lipoprotein assembly in the hepatocytes isolated from CD

rats and incubated in the presence of externally added SAM. Thus, TG accumulation during choline deficiency probably occurs because the lack of choline for the CDP-choline pathway is coupled to a decrease of the transmethylase pathway due to a limited methionine supply.

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