Defective activity of acyl-CoA:cholesterol *O*-acyltransferase in Niemann–Pick Type C and Type D fibroblasts

David M. BYERS,* Stella R. RASTOGI, Harold W. COOK, Frederick B. St. C. PALMER and Matthew W. SPENCE

Departments of Pediatrics and Biochemistry and the Atlantic Research Centre for Mental Retardation, Dalhousie University, 5849 University Avenue, Halifax, Nova Scotia, Canada B3H 4H7

The activity of acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) was measured in fibroblast homogenates from Niemann–Pick Type C (NPC) and Type D (NPD) patients to determine whether these cells exhibit similar defects in the regulation of cholesterol esterification. ACAT activity in normal cells cultured in the absence of serum lipoproteins responded rapidly (within 6 h) to the addition of serum and reached peak levels at 12–24 h, whereas little stimulation of activity in NPC cells was observed. In contrast, ACAT activity in NPD fibroblasts (cell lines from four different patients) began to increase between 6 and 12 h after serum addition, reaching levels up to 50 % of normal values at 24 h. ACAT activity in NPC and NPD cell extracts could not be stimulated by preincubation with normal cell homogenates, nor was complementation between NPC and NPD homogenates observed. Addition of 25-hydroxycholesterol to fibroblasts cultured in delipidated serum increased ACAT activity for all three cell types, although stimulation in NPD cells was less than that observed in NPC cells. ACAT activity of deoxycholate-solubilized homogenates reconstituted into phosphatidylcholine vesicles was independent of the presence of serum lipoproteins during culture and dependent on cholesterol present in the vesicles for all cell types. However, ACAT activities of mutant fibroblasts in vesicles plus cholesterol were significantly (about 40 %) lower than control levels. These results suggest that the metabolic lesions in NPC and NPD cells are biochemically distinct and that both may involve factors in addition to the availability of cholesterol substrate for the ACAT enzyme.

INTRODUCTION

The human fibroblast has been an important model for the study of intracellular cholesterol metabolism and regulation in peripheral cells [1,2]. The normal response of fibroblasts cultured in lipoprotein-deficient (LPD) serum to the addition of low-density lipoprotein (LDL) to the medium is three-fold: (1) a decrease in the synthesis of LDL receptors and their expression on the cell surface, (2) regulation of 3-hydroxy-3-methylglutaryl-CoA reductase activity resulting in decreased cholesterol synthesis *de novo* and (3) an increase in the activity of acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) leading to the storage of excess cholesterol as cholesteryl esters [1,3]. Mutations affecting intracellular cholesterol metabolism, such as defective LDL receptor activity in familial hypercholesterolaemia [1], have been invaluable in the elucidation of these pathways.

The Niemann–Pick group of diseases is characterized by variable accumulation of cholesterol in addition to the sphingomyelin storage considered typical of the condition [4]. In one group of patients, called Type I [4], the primary defect appears to be a deficiency of the lysosomal hydrolase, sphingomyelinase. In a second group of patients, called Type II, sphingomyelinase activity may be normal. The latter group includes the panethnic Niemann–Pick disease Type C (NPC), and the

Acadian Niemann–Pick Type D (NPD) [4]. Recently, Pentchev and his coworkers [5,6] demonstrated an abnormality in intracellular cholesterol esterification in fibroblasts from patients with NPC. Subsequent studies have suggested the defect to be more general in intracellular cholesterol metabolism since all three regulatory pathways are affected in NPC cells [7,8] and mutant cells do not exhibit abnormal responses to non-LDL regulators such as 25-hydroxycholesterol and mevalonate [8]. Moreover, NPC cells show a delayed expression of normal responses following addition of LDL, indicating a partial block in the intracellular delivery of cholesterol to regulatory sites [7,8]. This conclusion is supported by the abnormal storage of LDL-derived cholesterol in lysosomes from NPC cells [9]. A similar defect in NPD has been suggested, based on studies of patients whose maternal ancestry was Acadian [10].

The present investigation was initiated to establish a system for the study of intracellular cholesterol metabolic regulation in Niemann–Pick cells *in vitro*, and to determine whether the biochemical phenotype of cultured fibroblasts from NPC and NPD patients is distinct. The results indicate that although both cell types possess abnormal ACAT activity, each type exhibits characteristic and distinct features with respect to this activity. The present observations also provide evidence that the

Abbreviations used: ACAT, acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26); FBS, fetal bovine serum; LPD-FBS, lipoprotein-deficient FBS; NPC, Niemann–Pick Type C; NPD, Niemann–Pick Type D; LDL, low-density lipoprotein; MEM, minimum essential medium.

* To whom correspondence should be addressed, at the Atlantic Research Centre for Mental Retardation.

primary defect in Niemann–Pick Type II cells affects ACAT in a complex manner, involving both regulatory and substrate effects.

EXPERIMENTAL PROCEDURES

Materials

[1-¹⁴C]Oleoyl-CoA (57.4 Ci/mol) was purchased from NEN-Dupont (Lachine, Quebec, Canada). T.l.c. plates (Redi-Plate Silica G) were obtained from Fisher Scientific (Dartmouth, Nova Scotia, Canada). Sodium deoxycholate, cholestyramine resin, bovine serum albumin, dithiothreitol, egg yolk phosphatidylcholine, cholesterol, 25-hydroxycholesterol and lipid standards for t.l.c. were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Organic solvents were h.p.l.c. grade, while all other chemicals were reagent grade or better.

Delipidated serum was prepared from fetal bovine serum (FBS) from Flow Laboratories (Mississauga, ON, U.S.A.) by overnight incubation with 3% (w/v) acetone-washed Cab-O-Sil (Packard Instruments, Downers Grove, IL, U.S.A.) essentially as described by Liscum & Faust [8]. Human serum, obtained fresh from local donors, was delipidated by preparative ultracentrifugation [11].

Cell culture

Normal and NPD fibroblasts were obtained from skin biopsy of four healthy individuals and from four local patients diagnosed with Niemann–Pick disease Type D, respectively. The paternal and maternal ancestry of all Type D subjects can be traced to the Acadian population of southwestern Nova Scotia [4]. Two NPC fibroblast cell lines (GM0110 and GM3123) were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.); these particular cell lines have previously been shown to be defective in cholesterol esterification [5]. Another NPC cell line was a gift from Dr. J. Callahan, Hospital for Sick Children, Toronto, Canada. Fibroblasts were maintained at 37 °C in an atmosphere of 5% CO₂ and were used between the 7th and 13th passage.

In most experiments, cells were seeded in 4 ml of minimal essential medium (MEM; Gibco, Grand Island, NY, U.S.A.) containing 10% (v/v) LPD-FBS in 60 mm dishes at a density of $(1.6\text{--}2) \times 10^5$ cells per dish. Following a culture period of 3 days, this medium was replaced with 2 ml of MEM containing 10% LPD-FBS or 10% FBS. After further incubation (typically 12 h), fibroblasts were harvested as outlined below and used for preparation of cell homogenates and assay for ACAT within 2 h.

Cell harvest and lysis

All operations were carried out at 0 °C. Fibroblasts were harvested by scraping in phosphate-buffered saline; cells from three identical dishes were pooled to obtain a cell pellet by centrifugation (2000 g, 10 min). Cell pellets were transferred to a manual ground-glass homogenizer and disrupted by 30 strokes in a total volume of 150 µl of 10 mM-potassium phosphate (pH 7.4)/1 mM-dithiothreitol/1 mM-EDTA. The resulting homogenate was used for duplicate or triplicate enzyme assay after removal of aliquots for protein determination [12].

Regular ACAT assay

ACAT activity in cell homogenates was measured by the incorporation of [1-¹⁴C]oleoyl-CoA into cholesteryl [¹⁴C]oleate as monitored by t.l.c. and liquid scintillation spectrometry [13]. Assays (total volume 100 µl) were carried out by preincubation of cell homogenates (typically 50–100 µg of protein in 50 µl) with 40 µl of 10 mM-potassium-phosphate (pH 7.4)/0.1% bovine serum albumin for 10 min at 37 °C; the reaction was initiated by the addition of 10 µl of [¹⁴C]oleoyl-CoA substrate (final concentration in assay was 15–20 µM; 18.8 d.p.m./pmol). After a 10 min incubation at 37 °C, the reaction was terminated by adding 375 µl of methanol/chloroform (2:1, v/v) containing 10 µg of neutral lipid standards and total lipid was extracted by the method of Bligh & Dyer [14]. The entire lipid extract from a single sample was redissolved in chloroform/methanol (2:1, v/v) and separated on t.l.c. plates (20 cm × 20 cm) developed in light petroleum (boiling range 30–60 °C)/diethyl ether/acetic acid (90:10:1, by vol.). The position and recovery of carrier and radiolabelled lipid were determined by spraying with Rhodamine and by scanning with a Bioscan System 200 imaging detector (5% counting efficiency for ¹⁴C); lipids were identified by comparison with known standards. The cholesteryl [¹⁴C]oleate band was recovered by scraping and was counted in Beckman HP scintillation cocktail. Preliminary experiments indicated that cholesteryl ester formation was linear with both time and protein concentration under the above conditions for all three cell types.

Vesicle ACAT assay

The measurement of ACAT activity in phospholipid/cholesterol vesicles was carried out essentially as described by Cadigan *et al.* [15]. Vesicles containing 10 mg of phosphatidylcholine/ml with or without 1.5 mg of cholesterol/ml were prepared in 50 mM-Tris/HCl/5 mM-EDTA at pH 7.8 (buffer A) by treatment with cholestyramine resin [16]. Cell homogenates prepared as described above were solubilized by addition of 0.25 vol. of 10% (w/v) deoxycholate/4% (w/v) phosphatidylcholine in buffer A for 30 min at 0 °C. Solubilized cell homogenate (15 µl) was then preincubated with 225 µl of vesicles at 0 °C (10 min) and the assay was initiated by adding 60 µl of [¹⁴C]oleoyl-CoA (final assay concentration 15 µM, 18.8 d.p.m./pmol)/0.25% bovine serum albumin in buffer A. After incubation for 10 min at 37 °C the reaction was terminated with 1.125 ml of methanol/chloroform (2:1, v/v); lipid extraction, isolation and quantification of cholesteryl [¹⁴C]oleate were performed as described above for the regular assay.

For both the regular and vesicle ACAT assays, values for a single cell line were the means of duplicate or triplicate assays performed using a single homogenate prepared from three pooled dishes. An unpaired Student's *t* test was used to compare means derived from *n* independent analyses or different cell lines.

RESULTS

NPC and NPD fibroblasts exhibit distinct temporal stimulation of ACAT activity by serum

It has been well documented that human fibroblasts cultured in the absence of serum lipoproteins respond to

Fig. 1. Time course of stimulation of ACAT activity by FBS in normal and mutant fibroblasts

Normal (○), NPC (▲) and NPD (■) fibroblasts cultured in MEM + 10 % LPD-FBS were switched to MEM + 20 % FBS at zero time (except for the 0 h controls which were harvested directly). Cells were harvested at the times indicated and ACAT activity was measured by the regular assay as described in the text. Each point represents the mean $\pm$ S.D. of n independent samples (each assayed in duplicate), where n corresponds to four different normal cell lines, two different NPC cell lines and four different NPD cell lines.

Table 1. Lipid synthesis in extracts of normal and Niemann–Pick fibroblasts

Subconfluent fibroblasts were incubated for 72 h in MEM containing 10 % LPD-FBS followed by 12 h in medium containing 10 % LPD-FBS or 20 % FBS as indicated. Cells were harvested, lysed, and incorporation of [14 C]oleoyl-CoA into different lipids during a 10 min incubation was determined as outlined in the text. The means and S.D. were obtained from n independent samples (duplicate determinations for each) using four different normal cell lines (3–9 samples per cell line), three different NPC cell lines (5–7 samples per cell line) and four different NPD cell lines (2–6 samples per cell line). *Values are significantly different from each other ($P < 0.005$) using an unpaired Student's t test. For all other comparable sets of data, P was > 0.05 .

Cell type	[14 C]Oleoyl-CoA incorporation (pmol/min per mg)			
	Phospho- lipid	Fatty acid	Tri- acyl- gly- cerol	Cholesteryl ester
Normal ($n = 22$)				
LPD-FBS	590 $\pm$ 220	680 $\pm$ 370	83 $\pm$ 32	7.0 $\pm$ 4.3
FBS	650 $\pm$ 260	530 $\pm$ 280	69 $\pm$ 30	57 $\pm$ 27*
NPC ($n = 19$)				
LPD-FBS	540 $\pm$ 270	620 $\pm$ 410	68 $\pm$ 32	1.7 $\pm$ 1.3
FBS	510 $\pm$ 240	480 $\pm$ 310	70 $\pm$ 39	5.7 $\pm$ 4.4*
NPD ($n = 14$)				
LPD-FBS	600 $\pm$ 200	490 $\pm$ 240	72 $\pm$ 26	1.7 $\pm$ 1.6
FBS	550 $\pm$ 190	470 $\pm$ 320	82 $\pm$ 34	15.8 $\pm$ 8.6*

the addition of exogenous LDL or whole serum containing LDL by increasing (up-regulating) the activity of the microsomal enzyme ACAT [1,3]. The stimulation of ACAT activity in homogenates from normal, NPC and NPD cells harvested at different times after addition of FBS was measured by the incorporation of [14 C]oleoyl-CoA into cholesteryl [14 C]oleate; under these assay conditions the cholesterol substrate is endogenous (i.e. derived from fibroblast cellular membranes).

The results shown in Fig. 1, obtained using four different normal cell lines, four NPC cell lines and two previously characterized NPC cell lines (GM0110 and GM3123 [5]), reveal a distinct temporal pattern of ACAT stimulation by serum for each cell type. Normal fibroblasts responded rapidly (within 6 h) to FBS, with maximum ACAT values being attained at 12–24 h. In contrast, both NPC and NPD fibroblasts exhibited no stimulation of ACAT in the first 6 h in the presence of FBS. Unlike NPC cells, however, NPD cells showed a delayed response at 12 and 24 h, with ACAT activity approaching about 50 % of that of normal cells. ACAT activities of all three cell types were similar at 48 h. In the set of experiments shown in Fig. 1, ACAT activity in NPD fibroblasts was significantly ($P < 0.01$) lower than in normal cells between 6 and 24 h and clearly greater than in NPC cells at 12 h and 24 h.

Differences in ACAT activity among the three cell types did not appear to be due to general difference in the ability to utilize [14 C]oleoyl-CoA as a lipid precursor. Table 1, obtained from several experiments in which LPD-FBS or FBS was present 12 h prior to harvest, reveals that the labelling of phospholipid, free fatty acid or triacylglycerol was similar in the three cell types; moreover, no substantial effect of serum on this labelling was noted. In the majority of experiments with all cell lines tested, ACAT activity in NPD cells was observed to be greater than in NPC cells at 12 h; in certain experiments (also included in Table 1), activity in NPD cells did not diverge from that in NPC cells until after 12 h. Nevertheless, the data in Fig. 1 and Table 1 clearly indicate that ACAT activity in NPD cells, but not NPC cells, begins to respond in the period 6–12 h after the addition of FBS.

Distinct patterns of ACAT activity among the cell types did not appear to be due to differing affinities of the cells for serum lipoproteins, as maximal ACAT stimulation was achieved at similar levels of serum present; i.e. at 10 % FBS (Fig. 2). Experiments conducted using human serum at 12 or 24 h revealed no qualitative differences in the relative stimulation of ACAT activity from that observed using FBS (results not shown). On the basis of the above observations, FBS at a final concentration of 10 or 20 % was used in the present study.

Effect of mixing homogenates from normal and mutant cells prior to ACAT assay

To determine whether impaired ACAT activity in NPC and NPD cells was due to the presence of an inhibitor or the absence of an activator, homogenates from different cell types were co-incubated for 1 h at 37 °C prior to the addition of [14 C]oleoyl-CoA to start the ACAT assay; the combined results of two such experiments are shown in Fig. 3. Mixing normal and NPC (or NPD) homogenates yielded ACAT activities that were intermediate between those for the individual

Fig. 2. Effect of serum concentration during culture on ACAT activity of normal and mutant fibroblasts

Normal (○), NPC (▲) and NPD (■) fibroblasts cultured in MEM + 10% LPD-FBS were switched to MEM containing FBS at the concentrations indicated. After 12 h, cells were harvested and ACAT activity was measured using the regular assay. Each point represents the mean of duplicate assays from a homogenate obtained from a single cell line.

cell lines, indicating the absence of cholesterol equilibration or of any soluble factor(s) that might stimulate or inhibit ACAT activity between different extracts. Moreover, no restoration of activity was observed upon mixing of NPC and NPD homogenates, suggesting that complementation cannot occur under these conditions.

Previous studies by Sokol *et al.* [9] have indicated that the ability of NPC fibroblast homogenates to catalyse cholesterol esterification is dependent upon the conditions used for cell lysis and homogenization. In particular, it was demonstrated that NPC fibroblasts subjected to repeated freeze-thawing and vigorous homogenization exhibited stimulation of ACAT activity by serum similar to that seen with normal cells, while gentle cell lysis resulted in ACAT deficiency (presumably by failure to disrupt a latent lysosomal pool of LDL-derived cholesterol). While differences in the lysis and assay conditions preclude direct comparison with the above observations, we have found that using frozen (−20 °C) instead of fresh NPC or NPD cells does not significantly influence ACAT activity. Moreover, no substantial effect on the ACAT activity from normal, NPC or NPD fibroblasts was noted when a 1 h instead of 10 min preincubation at 37 °C was performed, nor when unesterified cholesterol (2 µg/ml) was present in the assay (results not shown). These results indicate that membrane-bound cholesterol pools do not freely exchange under typical assay conditions *in vitro*, in agreement with the results shown in Fig. 3.

Effects of 25-hydroxycholesterol on ACAT activity

It has been reported that the apparent regulatory defect in NPC fibroblasts which results in low ACAT activity is specific for LDL-derived cholesterol, since cholesterol esterification in intact cells can be partially [7] or completely [8] stimulated to normal levels by the polar derivative 25-hydroxycholesterol. As shown in Fig. 4, the addition of 25-hydroxycholesterol during culture in the presence of LPD-FBS also produced an increase

Fig. 3. Effect of mixing normal and mutant fibroblast homogenates on ACAT activity *in vitro*

Cells grown in MEM + 10% LPD-FBS (3 days) followed by MEM + 20% FBS (12 h) were harvested and lysed as outlined in the text. Aliquots (40 µl) of each homogenate were used either directly or after preincubation for 10 min at 37 °C with homogenate from another cell type (20 µl each). Each value represents the mean ± s.d. of four determinations from two separate experiments using different cell lines in each case. N, normal fibroblasts, C, NPC; D, NPD.

in the resultant ACAT activity of both normal and Niemann–Pick fibroblast homogenates. In the case of NPC cells, this stimulation was substantially greater than that produced by FBS. In contrast to the effects of serum on ACAT activity, 25-hydroxycholesterol appeared to produce a greater stimulation of ACAT in NPC than in NPD cells (Fig. 4). This was also observed with cells cultured for different time intervals in the presence of serum LDL (Table 2). Although the nature of the data shown in Fig. 4 and Table 2 precludes statistical comparison of values due to the different categories, they provide several independent paired observations indicating that NPC and NPD cells respond differently to 25-hydroxycholesterol than to serum LDL, further supporting our conclusions that these cell types are biochemically distinct.

ACAT activity in phospholipid/cholesterol vesicles

Experiments such as that shown in Fig. 4 suggest that impaired ACAT activity in Niemann–Pick cells is at least in part due to a proximal effect on cholesterol substrate concentration in the microsome rather than a molecular defect in ACAT itself. To address this question directly, we have taken advantage of a recently described assay system in which detergent-solubilized homogenates are reconstituted into phosphatidylcholine vesicles containing a specific amount of cholesterol [15]. Under these conditions, ACAT activity is completely dependent upon added vesicle cholesterol rather than on endogenous sterol levels [15,17]. Indeed, when normal and mutant cells grown in FBS were reconstituted into vesicles without cholesterol, ACAT activity was very low (< 5 pmol/min per mg, results not shown).

In a representative experiment shown in Table 3, the ACAT activities of normal, NPC and NPD cells cultured in either FBS or LPD-FBS were compared using both the regular and vesicle (plus cholesterol) assays. For all

Fig. 4. Effect of addition of 25-hydroxycholesterol during culture on ACAT activity of normal and mutant fibroblasts

Normal (○), NPC (▲) and NPD (■) cells grown on MEM+10% LPD-FBS were switched to the same medium containing the indicated concentrations of 25-hydroxycholesterol for a period of 12 h before cell harvest, lysis and ACAT assay. Each point represents the mean of duplicate determinations from a homogenate obtained from a single cell line.

three cell types, the effect of serum LDL on ACAT activity was abolished in the presence of vesicle cholesterol. With normal cells, ACAT activity in vesicles was comparable with regular ACAT activity in homogenates from FBS-stimulated cells. In homogenates from NPC cells, vesicle ACAT activities were significantly higher than values measured using the regular assay, while vesicle ACAT activities in NPD cells were only slightly higher (Table 3). In any case, ACAT activity of NPC and NPD homogenates reconstituted into phosphatidylcholine/cholesterol vesicles appeared to be significantly below normal values: data pooled from several experiments (using at least two cell lines for each type) yielded ACAT values of 62 ± 7 pmol/min per mg (normal, $n = 7$ independent samples), 40 ± 12 pmol/min per mg (NPC, $n = 7$ samples), and 36 ± 11 pmol/min per mg (NPD, $n = 6$ samples). This corresponds to $P < 0.005$ using an unpaired Student's *t* test. This difference did not appear to arise from any preference of ACAT in mutant cells for different levels of cholesterol, since all three cell types exhibited maximal ACAT activity in vesicles con-

Table 2. Effect of 25-hydroxycholesterol on ACAT activity of normal and Niemann–Pick fibroblasts cultured in the presence of FBS

Subconfluent fibroblasts were incubated for 72 h in MEM containing 10% LPD-FBS and switched to medium containing 10% FBS+10 μg of 25-hydroxycholesterol (25-HC)/ml for the times indicated prior to cell harvest. ACAT assay was performed as described in the text. Each value represents the average of triplicate ACAT assays obtained using a single cell line. Similar data were obtained at 24 h in an additional experiment (results not shown) using different cell lines for each type.

Cell type	Period of incubation...	ACAT activity (pmol/min per mg)		
		6 h	12 h	24 h
Normal:				
FBS alone		34	62	60
+ 25-HC		48	51	56
NPC:				
FBS alone		7	9	10
+ 25-HC		40	37	56
NPD:				
FBS alone		12	9	16
+ 25-HC		17	16	32

taining 1.5% cholesterol (results not shown). Although the reasons for cell-specific differences in ACAT activity are not known at this time, the above data indicate that decreased cholesterol substrate availability is only partly responsible for the observed ACAT deficiency in Niemann–Pick Type II cells.

DISCUSSION

Despite the importance of ACAT in the regulation of intracellular cholesterol metabolism [3], this enzyme has never been purified to homogeneity nor is its amino acid sequence known. ACAT activity, situated in the RNA-rich region of the rough endoplasmic reticulum [18], is subject to a complex array of regulatory influences, including the local substrate concentration of cholesterol [17,19], regulatory effects of sterols [20,21] and protein factors [22], membrane fluidity [23], and covalent modification by phosphorylation [24–26]. These signals

Table 3. Comparison of ACAT activities in normal and mutant cell homogenates by regular assay or following reconstitution into phosphatidylcholine/cholesterol vesicles

Fibroblasts grown in MEM plus 10% LPD-FBS were switched to fresh medium containing either 10% LPD-FBS (–) or 10% FBS (+) for 12 h. Cells were harvested and homogenates were assayed for ACAT activity either directly using the regular assay (Reg) or after deoxycholate solubilization and reconstitution into vesicles containing 10% phosphatidylcholine and 1.5 mg of cholesterol/ml (Ves). The means $\pm$ s.d. of four determinations from two separate experiments using the same cell lines are shown.

Cell type	Assay...	ACAT activity (pmol/min per mg)			
		Reg(–)	Reg(+)	Ves(–)	Ves(+)
Normal		15.0 ± 2.0	66.6 ± 3.2	62.5 ± 2.8	58.8 ± 9.6
NPC		2.8 ± 0.4	14.1 ± 4.6	30.0 ± 3.2	35.7 ± 1.8
NPD		3.4 ± 0.9	23.8 ± 3.3	30.4 ± 11.0	33.9 ± 12.7

appear to be generated in turn by the cholesterol status within cellular membranes, resulting from the uptake of LDL and non-LDL cholesterol, and by other events such as the degradation of sphingomyelin at the cell surface [27].

It is not known which of the above factors might be affected in Niemann-Pick Type II fibroblasts. The responses of cholesterol esterification and other cholesterol regulatory systems, as well as the lysosomal storage of cholesterol, have indicated that the molecular defect in NPC cells is located upstream of ACAT in the LDL-mediated pathway [7,8]. A defect in intracellular processing of LDL-derived cholesterol could affect ACAT activity by reducing cholesterol transport to the enzyme itself (substrate, allosteric and fluidity effects) or by failure to generate the appropriate second messenger(s) involved in regulation of enzyme activity [8].

The present study *in vitro* was initiated to address the regulation of cholesterol esterification under conditions in which the environment of the enzyme can be more carefully controlled. The rapid increase and eventual decline of ACAT activity that we have observed for normal fibroblasts after addition of serum containing LDL is consistent with the known effects of LDL on ACAT stimulation and receptor turnover in intact cells [1-3]. Gavigan & Knight [19] also observed a transient LDL-stimulated increase in ACAT activity of fibroblast microsomal preparations and further demonstrated that this response is due to an increase in local cholesterol substrate concentration. We have adapted a vesicle assay originally developed for Chinese hamster ovary cells [17] and have demonstrated that endogenous cholesterol concentration is an important determinant of ACAT activity in both normal and NP fibroblasts.

On the other hand, our data provide evidence that impaired ACAT activity in NPC and NPD cells is not completely corrected by increasing local substrate availability. This further limitation on ACAT activity in mutant cells could involve incomplete activation of the enzyme by a covalent mechanism (i.e. phosphorylation), or by non-covalent factors which survive detergent solubilization and reconstitution, or it could reflect depressed levels of ACAT protein in these cells. The previous finding that normal levels of ACAT activity can be observed in NPC homogenates prepared under certain lysis conditions [7,9] is particularly interesting in this regard. In addition to using a different lysis protocol from that employed here, these workers have used an assay in which [³H]oleic acid, ATP and CoA are incubated with homogenates for a period of 2 h at 37 °C. Since the specific activities obtained were much lower (< 5 pmol/min per mg of protein [9]) than those we have observed using [¹⁴C]oleoyl-CoA in a 10 min assay, it is possible that a different rate-determining step was being measured in their assay. In any case, we suggest that careful attention to the differences in these protocols might be exploited in further localization of the molecular defect in Niemann-Pick cells.

The present experiments clearly indicate that the responses of ACAT activity to culture manipulations are different in NPC and NPD fibroblasts. In particular, NPD cells exhibited a stronger, more rapid response to LDL than did NPC cells, while the latter showed greater stimulation by a non-lipoprotein effector, 25-hydroxycholesterol. In the only previous report of

cholesterol esterification in NPD cells, Butler *et al.* [10] found no significant difference in cholesterol esterification rates or in ACAT activity *in vitro* between NPC and NPD fibroblasts do not accumulate excess total cholesterol. Further studies will be necessary to determine whether biochemical and clinical differences between NPC and NPD are due to mutations in different genes or to a single mutation differing in severity and thus in effects on the complex regulatory network of intracellular cholesterol metabolism.

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