Neutral lipid storage disease: a genetic disorder with abnormalities in the regulation of phospholipid metabolism

R. Ariel Igal and Rosalind A. Coleman[1]

Departments of Nutrition and Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Abstract Neutral lipid storage disease (NLSD) is an autosomal recessive disorder characterized by the presence of numerous lipid droplets in virtually all tissues examined. The increased cellular triacylglycerol content results from defective recycling of triacylglycerol-derived diacylglycerol to phospholipids (Igal, R. A. and R. A. Coleman. 1996. *J. Biol. Chem.* **271:** 16644–16651). In order to determine whether de novo glycerolipid synthesis is also altered in NLSD, we compared the ability of normal human skin fibroblasts and fibroblasts from a patient with NLSD to incorporate phospholipid precursors into cell lipids. NLSD cells had increased rates of incorporation of [^{14}C]oleic acid and [^{3}H]glycerol into triacylglycerol and all phospholipid species except phosphatidylethanolamine. However, the cell content of each phospholipid species was similar in control and NLSD cells, indicating a higher turnover rate in NLSD cells for phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and sphingomyelin. Labeling with [^{14}C]choline and [^{14}C]ethanolamine confirmed the increase in the rate of phosphatidylcholine synthesis and the decreased rate of phosphatidylethanolamine synthesis through their respective CDP pathways. The activities of the major regulatory enzymes of triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine biosynthesis were similar in control and NLSD cells. Taken as a whole, this study provides strong evidence for an underlying regulatory defect in NLSD that alters the rates of synthesis and degradation of the major cellular phospholipids.**—Igal, R. A., and R. A. Coleman.** Neutral lipid storage disease: a genetic disorder with abnormalities in the regulation of phospholipid metabolism. *J. Lipid Res.* 1998. **39:** 31–43.

Supplementary key words triacsin C • neutral lipid storage disease • phospholipid synthesis • lipid droplets • glycerolipids

Neutral lipid storage disease (NLSD), also known as Dorfman-Chanarin syndrome, is an autosomal recessive disorder characterized by a large accumulation of triacylglycerol droplets in most tissues examined, including liver, muscle, intestinal mucosa, neutrophils, and skin fibroblasts (1). Although the triacylglycerol droplets, which are not membrane-bound, had been thought to result from a defect in triacylglycerol lipolysis (2), our studies with triacsin C, a competitive inhibitor of acyl-CoA synthetase, demonstrated that triacylglycerol (TAG) accumulates in cells because of a defect in the recycling of TAG-derived diacylglycerol as a substrate for phospholipid synthesis (3).

Phospholipids are essential structural components of intracellular membranes and the plasma membrane and form a precursor pool for the generation of lipid second messengers such as diacylglycerol, the eicosanoids, phosphatidic acid, and platelet activating factor. As such, it is critical to understand how cells regulate the synthesis of each of the individual phospholipids as well as the fatty acid species esterified to these phospholipids. A genetic defect like NLSD provides a cellular model of disturbed complex lipid metabolism that can shed light on the normal regulation of phospholipid synthesis. Because it was unclear whether the NLSD defect lies in recycling triacylglycerol-derived diacylglycerol to one specific phospholipid or to all phospholipids, and whether the defect is manifested in both recycling and de novo synthetic pathways, we examined the de novo synthesis of the major phospholipid species in NLSD and control fibroblasts from labeled oleate, glycerol, choline, and ethanolamine.

Abbreviations: BSA, bovine serum albumin; CT, CTP:phosphocholine cytidylyltransferase; DCPT, diacylglycerol cholinephosphotransferase; DEPT, diacylglycerol ethanolaminephosphotransferase; DGAT, diacylglycerol acyltransferase; EMEM, Eagle's minimal essential medium plus 1% nonessential amino acids; ET, CTP:phosphoethanolamine cytidylyltransferase; FBS, fetal bovine serum; NLSD, neutral lipid storage disease; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol; TCL, thin-layer chromatography.

[1]To whom correspondence should be addressed.

[2]DAG concentrations were virtually identical in NLSD and control cells (J-H. Kim, K. A. daCosta, and R. A. Coleman, unpublished data).

This report describes in NLSD fibroblasts: *1*) a higher rate of incorporation of radiolabeled oleic acid and glycerol into TAG and all major phospholipid species except PE; *2*) a higher turnover rate of PC, PI, PS, and sphingomyelin; *3*) stimulated PC synthesis and decreased PE synthesis through their respective CDP-pathways; and *4*) normal catalytic activities of the likely regulatory enzymes of triacylglycerol, PC, and PE biosynthesis. Taken as a whole, this study provides strong evidence for an underlying regulatory defect in NLSD that alters the rates of synthesis and degradation of the major cellular phospholipids.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]oleic acid and [2-^{3}H]glycerol, CDP-[^{14}C]choline, and CDP-[^{14}C]ethanolamine were from Amersham Life Sciences Co. [^{14}C-methyl]choline, [1,2-^{14}C]ethanolamine hydrochloride, phospho-[^{14}C]choline, and phospho-[^{3}H]ethanolamine were from American Radiolabel Company. [^{3}H]palmitic acid was from New England Nuclear. Tissue culture media and supplies were purchased from Gibco BRL. TLC Plates were from Whatman. Choline, ethanolamine, and sodium oleate were from Sigma. Lipid standards and *sn*-1,2-dioleoylglycerol were from Serdary. Bovine serum albumin (essentially fatty acid-free) was from ICN.

Cell culture

Normal human skin fibroblasts (ATCC Collection, cell line CCD) and fibroblasts from a child with NLSD (1) were routinely grown in Eagle's Minimum Essential Medium with Earle salts plus 1% nonessential amino acids (E-MEM) supplemented with heat-inactivated FBS (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 at 37°C.

Oleic acid and glycerol labeling

Normal and NLSD cells were cultured in 60-mm dishes until near confluence. Fibroblasts were incubated for 24 h either with [^{14}C]oleic acid (0.25 μCi/dish) or [^{3}H]glycerol (4 μCi/dish) in the presence of 100 μM Na oleate dissolved in 10% FBS, EMEM, 1% BSA. At the end of the labeling period, the radioactive media were discarded and residual label was removed by washing the monolayer three times with a solution of 0.1% BSA in PBS at 37°C. Cells were scraped from the dishes in two additions of 1 ml CH_3OH and 1 ml water. Total lipid from both cell lines was extracted (4).

Metabolism of [^{14}C]choline or [^{14}C]ethanolamine by NLSD and control fibroblasts

Near-confluent NLSD and control fibroblasts in 100-mm dishes were incubated with 2 μCi/dish of [^{14}C]choline or [^{14}C]ethanolamine in either 10% FBS (which adds about 60 μM choline as PC), EMEM (which contains 7 μM choline and no ethanolamine), or in RPMI 1640 (which contains no choline or ethanolamine) in the absence or presence of 0.5 mM Na oleate in 1% BSA. After a 2-h incubation, the labeling media were aspirated and cells were washed three times with 0.1% BSA in PBS, and scraped from the dishes in two additions of 3 ml CH_3OH and then 1.5 ml water. Finally, 3 ml of $CHCl_3$ was added to the combined solvents and total lipids were extracted (4). The aqueous phases were saved for analysis of water-soluble metabolites.

Pulse-chase labeling of fibroblasts with [^{14}C]choline or [^{14}C]ethanolamine

In pulse-chase experiments, control and NLSD fibroblasts grown in 100-mm dishes were pulsed for 24 h with either [^{14}C]choline or [^{14}C]ethanolamine (2 μCi/dish) in 10% FBS, EMEM supplemented with 50 μM choline or ethanolamine. The prelabeled cell monolayers were washed three times with 0.1% BSA in 37°C PBS in order to eliminate residual label. Fibroblasts were then chased in 10% FBS, EMEM. Aliquots of the chase media were counted in order to quantify the radioactive metabolites released. Total lipids were extracted as above (4), and the aqueous phases were saved for analysis.

TLC analysis of lipids and aqueous metabolites

Neutral and polar lipid species were separated on silica gel 0.25-mm 150A LK50 plates using a one-dimensional double-development procedure. The chromatoplate was first run in chloroform–methanol–30% ammonium hydroxide 65:25:4 (v/v/v) to 8 cm from the top. After evaporating residual solvents with a N_2 stream, the plate was rerun in heptane–isopropylether–glacial acetic acid 60:40:4 (v/v/v) to the top of the plate. Pure lipid standards were run in parallel. To separate PI and PS, which were not resolved by this solvent system, the spot corresponding to PI and PS was scraped and extracted from the silica gel with $CHCl_3$–CH_3OH 1:1 (v/v). Lipids were concentrated and respotted on a new TLC plate. PI was separated from PS by chromatography with $CHCl_3$–CH_3OH–CH_3COOH–H_2O 85:15:10:3 (v/v/v/v). For cells labeled with [^{14}C]ethanolamine, [^{14}C]diacyl-PE and [^{14}C]plasmalogen-PE were separated by spotting one-half of the total lipid sample on a silica gel plate and exposing the plate to concentrated HCl

vapor for 20 min to hydrolyze vinyl ether bonds while preserving ester bonds. The remainder of the sample was then spotted on a separate lane. When the plate was run in $CHCl_3$–CH_3OH–30% NH_4OH 65:25:4 (v/v/v) the former alkenyl, acyl-PE moved to a spot similar to that of lyso-PE. To separate aqueous metabolites of choline and ethanolamine, the aqueous phases were dried and dissolved in H_2O, spotted on silica gel plates, and chromatographed with 0.6% NaCl–CH_3OH–30% NH_4OH 50:50:5 (v/v/v). Carrier standards were added to the samples before the chromatography. All ^{14}C-labeled lipids and water-phase metabolites were detected and quantified using a Bioscan Image 200 System. ^{3}H-labeled lipids were visualized with iodine vapor, scraped into vials, and counted.

Cell phospholipid content

Near confluent control and NLSD fibroblasts, grown in 100-mm dishes, were scraped in two additions of 3 ml CH_3OH, then 1.5 ml H_2O. Total lipids were extracted and the phospholipids were separated as described above. Areas corresponding to each phospholipid were scraped into glass tubes and lipids were extracted with ether–$CHCl_3$–CH_3OH 3:1:2 (v/v/v). Phosphate was measured by the method of Bartlett (5).

Preparation of subcellular fractions and enzyme assays

Near-confluent control and NLSD cells, grown in 150-mm dishes, were washed twice with ice-cold PBS (or 0.9% NaCl when samples were required for phosphatidate phosphohydrolase assay) and scraped into 5 ml of the same solution (twice). The cells were pelleted by centrifugation and the pellet was resuspended in 2 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose. The fibroblasts were homogenized with 30–40 strokes of a motor-driven Teflon-glass homogenizer and centrifuged at 100,000 *g* for 1 h. The supernatant was used as a cytosolic fraction and the pellet (total particulate preparation) was resuspended in the same buffer. Both fractions were stored in aliquots at −80°C until used. Protein was measured using serum bovine albumin as the standard (6). CTP:phosphoethanolamine cytidylyltransferase was measured in the cytosolic fraction with 10 to 20 μg protein, 100 μM [^{3}H]phosphorylethanolamine and 40 μM CTP (7). CTP:phosphocholine cytidylyltransferase was assayed using 10 to 30 μg of either cytosolic or total particulate protein and 4 mM [^{14}C]phosphocholine, 5 mM CTP, 50 μM PC, and 50 μM oleic acid (8). Diacylglycerol cholinephosphotransferase and diacylglycerol ethanolaminephosphotransferase were assayed using 25 to 100 μg of total particulate protein with 100 μM CDP-[^{14}C]choline (10 μCi/μmol) or 100 μM CDP-[^{14}C]ethanolamine, respectively, and 100 μM *sn*-1,2-dioleoylglycerol in acetone (9). Acyl-CoA synthetase was assayed using 10 to 30 μg of total particulate protein, 50 μM [^{14}C]oleate or [^{3}H]palmitate, 10 mM ATP, and 0.2 mM CoA (10). Acyl-CoA hydrolase was measured using 50 μM [^{3}H]palmitoyl-CoA and 5 to 15 μg of total particulate protein (11). Diacylglycerol acyltransferase activity was determined using 5 to 25 μg of total particulate protein, 200 μM *sn*-1,2-dioleoylglycerol in acetone, and 30 μM [^{3}H]palmitoyl-CoA (12). Choline kinase was measured using 10–30 μg cytosolic protein, 10 mM ATP, and 1 mM [^{14}C-methyl]choline (13). Assays were proportional to the amount of protein used.

Other methods

DNA content was determined fluorometrically, using calf thymus DNA as the standard (14). For each point, DNA was measured in three separate dishes. [^{3}H]palmitoyl-CoA was synthesized enzymatically (15).

RESULTS

NLSD cells incorporate more oleate into neutral lipids and all phospholipids except PE

NLSD cells incorporated more [^{14}C]oleic acid into total cellular lipids than did control cells (**Fig. 1A**). The increase was observed as early as 3 and 6 h (23 and 54% more, respectively) and was 78% more than control cells at 24 h. The enhanced formation of oleate-labeled TAG by NLSD fibroblasts was also observed as early as 3 h (24% more), increasing to 72% at 6 h and 2.5-fold at 24 h. Incorporation of [^{14}C]oleate into the non-esterified fatty acid fraction was similar in control and NLSD fibroblasts (data not shown).

The initial incorporation of [^{14}C]oleate into total polar lipids was increased in NLSD cells at early time points (23% at 3 h and 37% at 6 h), but became similar by 24 h (Fig. 1B). Analysis of the individual phospholipid species showed that [^{14}C]oleate incorporation into PC increased 27% by 6 h, but was equal in NLSD and control cells by 24 h. In contrast to other phospholipids that showed increased labeling at both early and late time points, incorporation of [^{14}C]oleate into PE by NLSD cells was equivalent to control cells at early time points and 20% less at 24 h (Fig. 1C). Unlike the small changes observed with [^{14}C]oleate incorporation into PC and PE, NLSD fibroblasts incorporated 50% more [^{14}C]oleate into PI/PS at all time points. Higher values were also observed at 3 (110%) and 6 h (82%), and persisted at 24 h (30%). Analysis of the radiolabeled PI/PS fractions from several experiments showed that 70–90% of the label was in PI and that PS was a minor constituent (10–30%) (data not shown). Increased la-

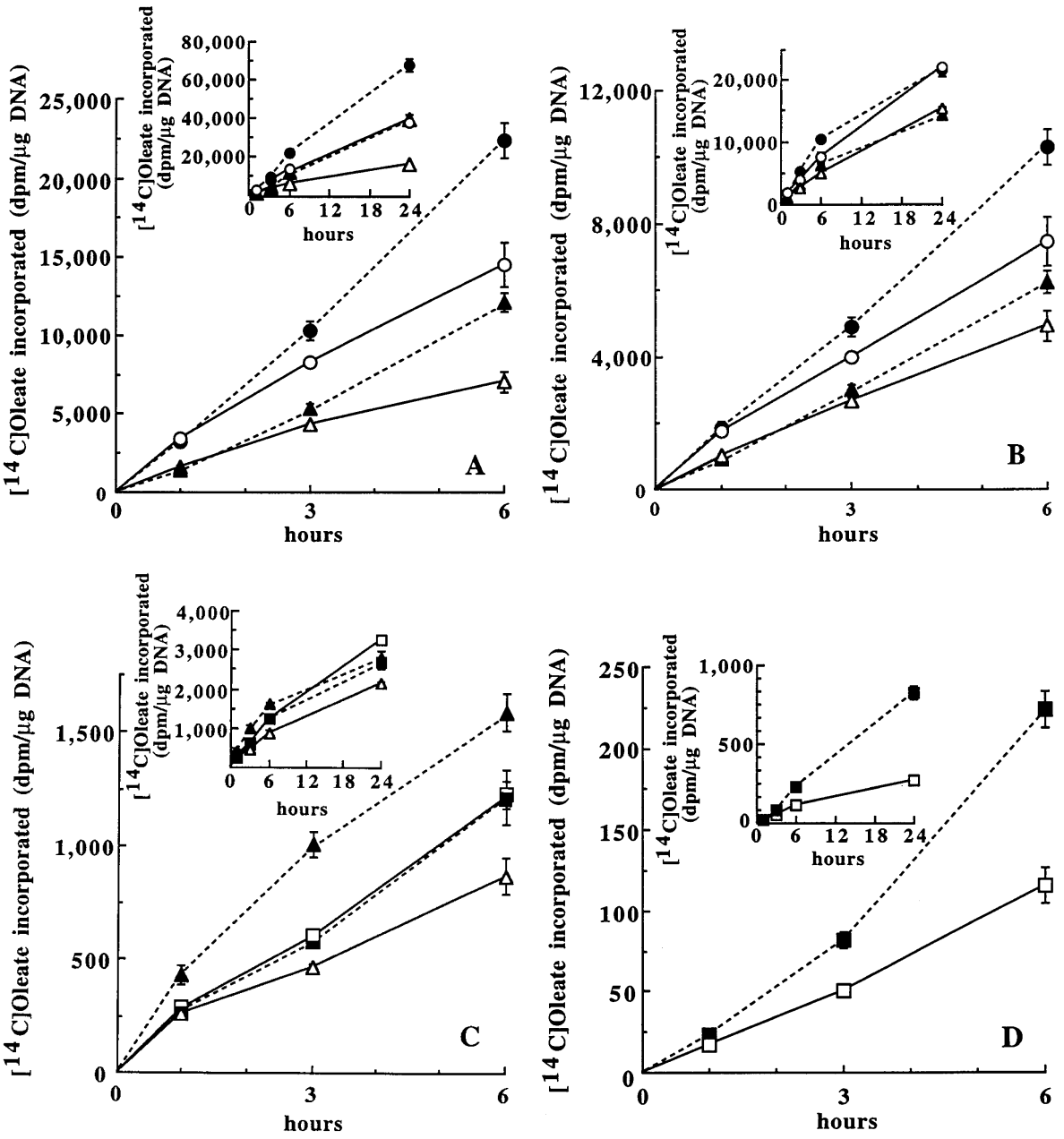

Fig. 1. Incorporation of [^{14}C]oleic acid into total lipids, TAG, and PL in control and NLSD fibroblasts. Cells were incubated with 100 μM [^{14}C]oleic acid in 1% BSA, 10% FBS, EMEM. Lipids were extracted and chromatographed as described in Experimental Procedures. Data points represent the means ± SD of an experiment performed in triplicate. Some error bars are smaller than the symbols. A)Total lipids (circles) and TAG (triangles); B) total phospholipid (circles) and PC (triangles); C) PE (squares) and PI/PS (triangles); D: sphingomyelin (squares). Inset: 1 to 24 h values. Labels: Control (open symbols, solid lines), NLSD (closed symbols, dashed lines). The data are representative of three experiments, each performed in triplicate.

beling was observed in both PI and PS (2- to 8-fold) in NLSD cells compared to control cells. NLSD cells also incorporated more [^{14}C]oleate into sphingomyelin throughout the entire time period (Fig. 1D). By 24 h, NLSD cells contained more than twice as much labeled sphingomyelin as did control cells.

NLSD cells incorporate more glycerol into neutral lipid and all phospholipids except PE

Incorporation of labeled fatty acid into a phospholipid can occur via de novo synthesis from glycerol-3-P, from reacylation of the analogous lysophospholipid, or from

recycling of diacylglycerol derived from triacylglycerol. In contrast, incorporation of labeled glycerol represents de novo synthesis alone and can help distinguish among these possibilities. Incorporation of [^{3}H] glycerol confirmed the results observed with [^{14}C]oleate, indicating that the differences observed with oleate represented, at least in part, differences in de novo synthesis. NLSD cells incorporated more [^{3}H]glycerol into total lipids at 3 h (46%) and 24 h (93%) compared to control cells (**Fig. 2A**). As with [^{14}C]oleic acid, 50% of the total [^{3}H]glycerol incorporated was present in TAG in both cell lines after 1 h of incubation, but in NLSD cells the percentage of glycerol incorporated into TAG continued to increase. [^{3}H]TAG was 44% and 67% higher in NLSD cells at 3 and 6 h, respectively, and by 24 h, [^{3}H]TAG was 3-fold higher in NLSD cells than in controls.

Similar to the [^{14}C]oleate incorporation studies, the de novo synthesis of total phospholipids from [^{3}H]glycerol (Fig. 2B) was 48% higher in NLSD cells at 3 h, but decreased to 16% by 6 h, and was similar in the two cell lines by 24 h (Fig. 2, inset). Analysis of individual phospholipid species showed that, similar to the oleate studies, NLSD cells incorporated 39 and 18% more label into PC at 3 h and 6 h, respectively but that this difference did not persist at 24 h. NLSD cells incorporated twice as much [^{3}H]glycerol into PI/PS at 3 h compared to control cells (Fig. 2C). The difference declined to 50% at 6 h, and by 24 h, incorporation was equivalent in the two cell lines. Normalization of label incorporation suggests an increased rate of PC, PI, and PS turnover in NLSD cells. In contrast, [^{3}H]glycerol incorporation into PE was 25% lower in NLSD cells at 6 h and 30% lower at 24 h, com-

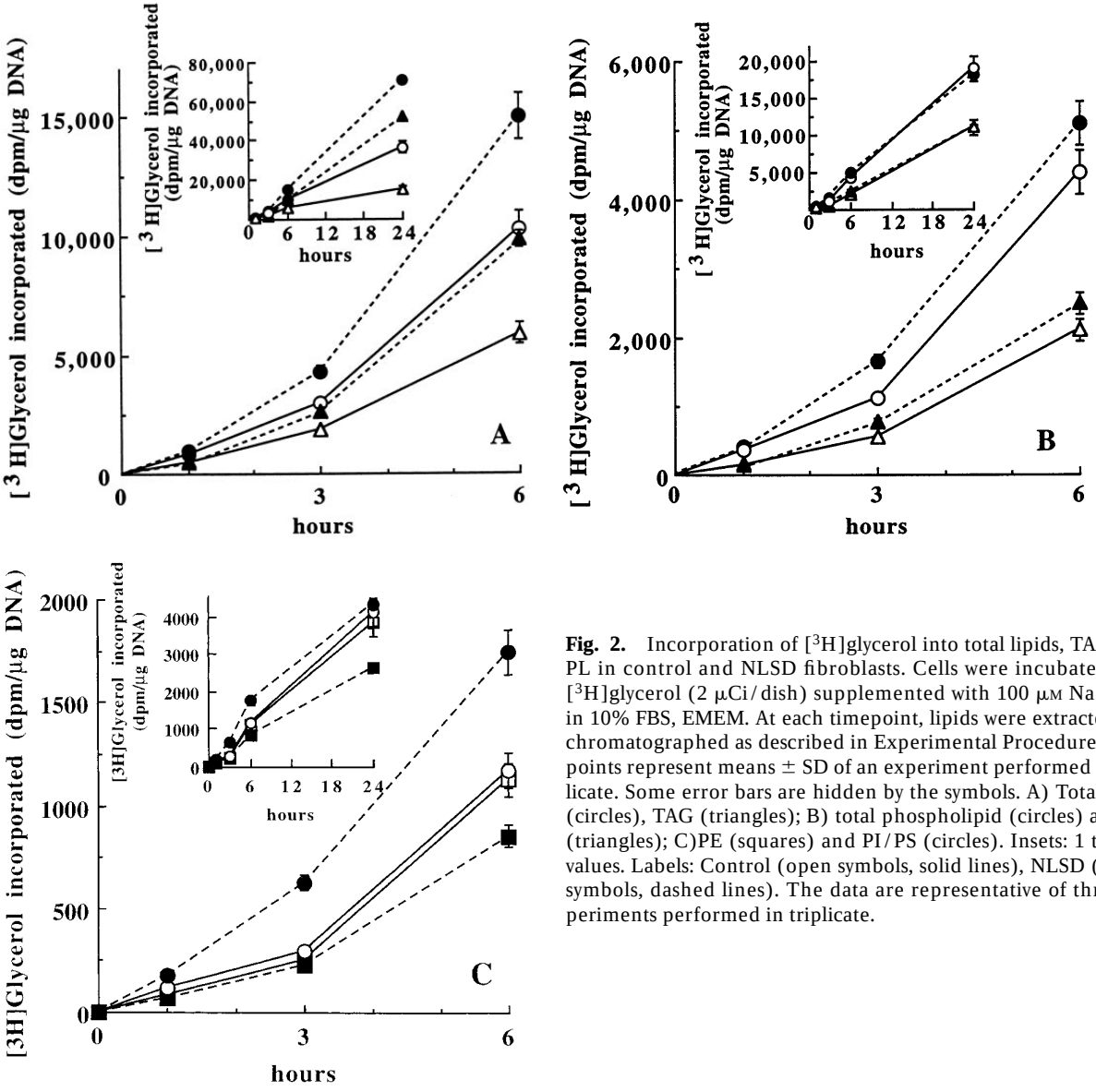

Fig. 2. Incorporation of [^{3}H]glycerol into total lipids, TAG, and PL in control and NLSD fibroblasts. Cells were incubated with [^{3}H]glycerol (2 μCi/dish) supplemented with 100 μM Na oleate in 10% FBS, EMEM. At each timepoint, lipids were extracted and chromatographed as described in Experimental Procedures. Data points represent means ± SD of an experiment performed in triplicate. Some error bars are hidden by the symbols. A) Total lipids (circles), TAG (triangles); B) total phospholipid (circles) and PC (triangles); C)PE (squares) and PI/PS (circles). Insets: 1 to 24 h values. Labels: Control (open symbols, solid lines), NLSD (closed symbols, dashed lines). The data are representative of three experiments performed in triplicate.

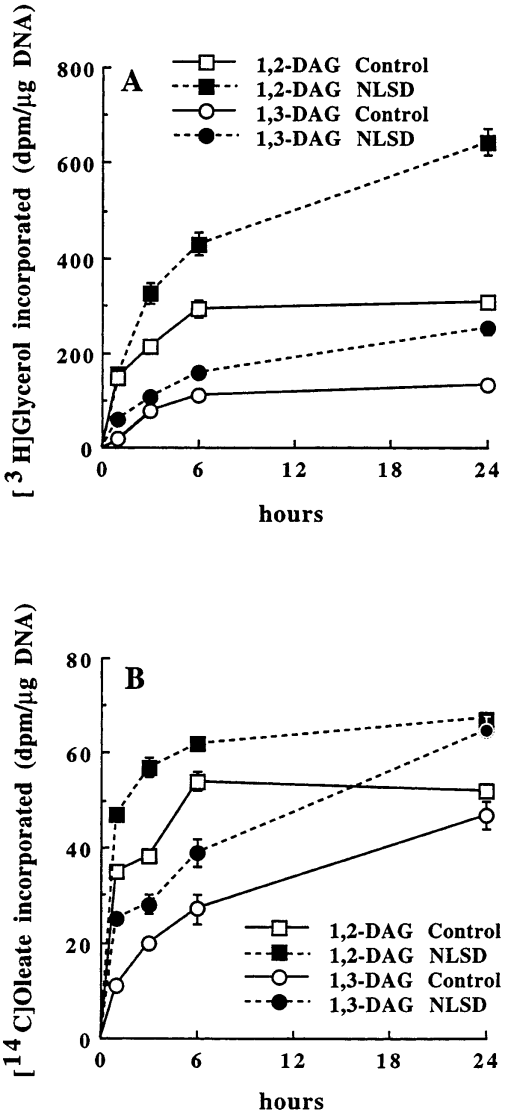

Fig. 3. Incorporation of [^{3}H]glycerol and [^{14}C]oleate into diacylglycerol. Cells were incubated with both 100 μM [^{14}C]oleic acid in 1% BSA, 10% FBS, EMEM and [^{3}H]glycerol (2 μCi/dish). At each timepoint, lipids were extracted and chromatographed in heptane–isopropyl ether–glacial acetic acid 60:40:4 (v/v) with *sn*-1,2- and 1,3-DAG as carrier and standards. Data points represent means ± SD of an experiment performed in triplicate. Some error bars are hidden by the symbols.

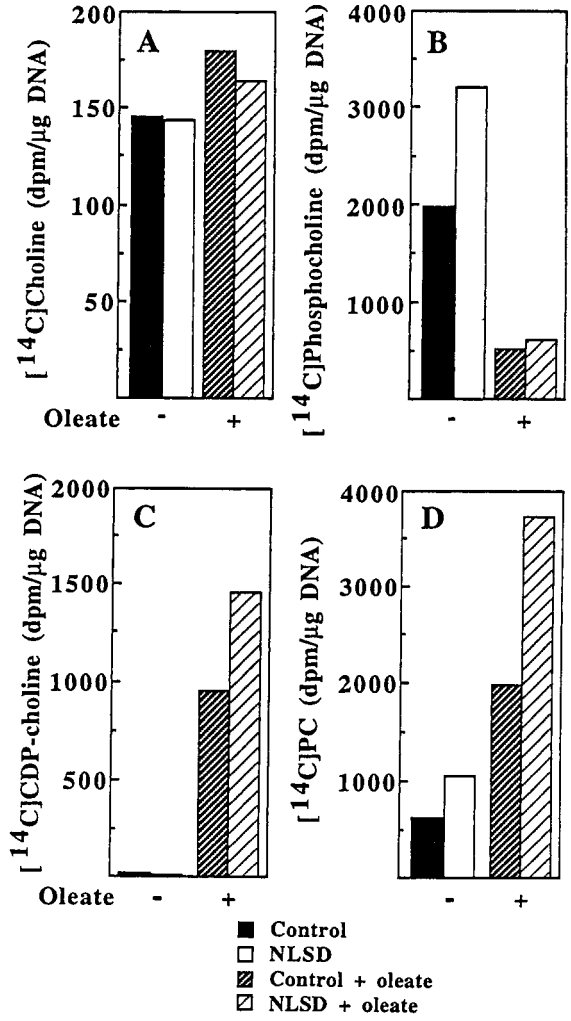

Fig. 4. Incorporation of [^{14}C]choline into aqueous- and lipid-soluble metabolites in the absence and presence of 0.5 mM oleic acid. Control and NLSD fibroblasts were incubated with 2 μCi/dish [methyl-^{14}C]choline for 2 h in the presence or absence of 0.5 mM Na oleate in 1% BSA, 10% FBS, EMEM. Labeled water-soluble metabolites and total lipids were extracted and separated by TLC as described in Experimental Procedures. Each bar expresses the average of measurements from two 100-mm dishes. Panels: A) [^{14}C]choline; B) [^{14}C]phosphocholine; C) [^{14}C]CDP-choline; and D) [^{14}C]PC. The basal data are representative of five experiments, each performed in duplicate. The data with oleate are from a single experiment. Variation between the duplicates was less than 5%.

pared to the control cells (Fig. 2C). This decrease had been observed with oleate-labeling only at 24 h (Fig. 1C).

Incorporation of [^{14}C]oleate and [^{3}H]glycerol into diacylglycerol

A double label experiment was used to determine whether there might be differences in the synthesis of the intermediate diacylglycerol (DAG) from acylation and de novo pathways. Although very little DAG is present in fibroblasts, NLSD cells incorporated more [^{14}C]oleate into 1,2- and 1,3-DAG at each time point (**Fig. 3A**). Similarly, the [^{3}H]glycerol labeling of 1,2- and 1,3-DAG by NLSD cells was 50% or even higher than in control at any single time point (Fig. 3B). The amounts of labeled DAG in NLSD cells do not appear to plateau like those in control cells at 6 h of incubation; however, these two fractions represented only a small percentage of the total lipid labeling.

Increased PC and decreased PE formation by the CDP-choline and CDP-ethanolamine pathways

Because the [^{3}H]glycerol studies, examining de novo synthesis, suggested that NLSD cells incorporate more fatty acid and glycerol into PC, PI/PS, and sphingomyelin and less into PE than do control cells, we examined the incorporation of [^{14}C]choline and [^{14}C]ethanolamine into cellular lipids. When near-confluent cells were incubated with [^{14}C]choline for 2 h, NLSD cells incorporated 75% more label into lipid than did control cells (**Fig. 4D**). Ninety-eight percent of this [^{14}C]choline-labeled lipid was PC (data not shown). Very little free cellular choline was present (Fig. 4A), but total incorporation of [^{14}C]choline into aqueous plus lipid-soluble products was 60% higher in NLSD cells. As has been reported for other cultured cell lines (16–18), most of the label present in water-soluble metabolites was phosphocholine, and NLSD cells accumulated 62% more phosphocholine than did the control fibroblasts (Fig. 4B). [^{14}C]CDP-choline, which represented less than 1% of the aqueous metabolites, was 46% lower in NLSD cells than in controls (Fig. 4C).

Fatty acids cause CTP:phosphocholine cytidylyltransferase (CT), the rate-limiting enzyme of PC synthesis, to translocate from the cytosol to membranes where it is activated (19). Because NLSD cells incorporated more oleate, glycerol, and choline into PC than did control cells, we studied the effect of oleate on PC formation in order to determine whether PC synthesis was regulated normally by fatty acid. When 0.5 mM sodium oleate was added into the labeling media, incorporation of [^{14}C]choline into PC increased about 3.3-fold in both control and NLSD cells (Fig. 4D). In addition, the presence of oleic acid in the incubation media stimulated by 30% the total incorporation of choline into lipid plus aqueous metabolites in both cell lines (data not shown). Oleate did not alter the low amount of free [^{14}C]choline (Fig. 4A) in the cells, but the amount of [^{14}C]phosphocholine decreased 80% in both cell lines (Fig. 4B), and [^{14}C]CDP-choline formation increased 50-fold in control cells and 100-fold in NLSD cells (Fig. 4C). These results are consistent with normal fatty acid-induced activation of CT, thereby enhancing conversion of phosphocholine to CDP-choline (17, 20). Thus, in addition to the constitutive increase of PC synthesis in NLSD cells, it appears that CT activity can be additionally stimulated.

Ethanolamine glycerolipids represent 20–30% of the total phospholipid present in most mammalian cells, including CCD fibroblasts (human) and fibroblasts from our NLSD patient (**Table 1**). Synthesis of diacyl-PE can occur via three different routes: DCPT condensation of CDP-ethanolamine and diacylglycerol, decarboxylation of PS, or Ca^{2+}-dependent base exchange with free ethanolamine. When sufficient ethanolamine is available, the major route of PE synthesis in mamma-

TABLE 1. Quantification of phospholipid species in NLSD and control cells

	Control		NLSD	
Phospholipid	μg phospholipid/mg protein	%	μg phospholipid/mg protein	%
PI/PS	19.0 ± 2.9	10.8	11.9 ± 0.9	8.0
PE	54.8 ± 3.3	31.1	48.1 ± 3.1	32.2
Sphingomyelin	4.3 ± 0.4	2.4	<0.53	<0.4
PC	98.4 ± 5.8	55.8	89.0 ± 6.3	59.5

Lipids from five 100-mm dishes of NLSD cells and five of control cells at near confluence were extracted, separated by TLC, and analyzed by phosphorous content as described in Experimental Procedures. Results given as mean ± SD.

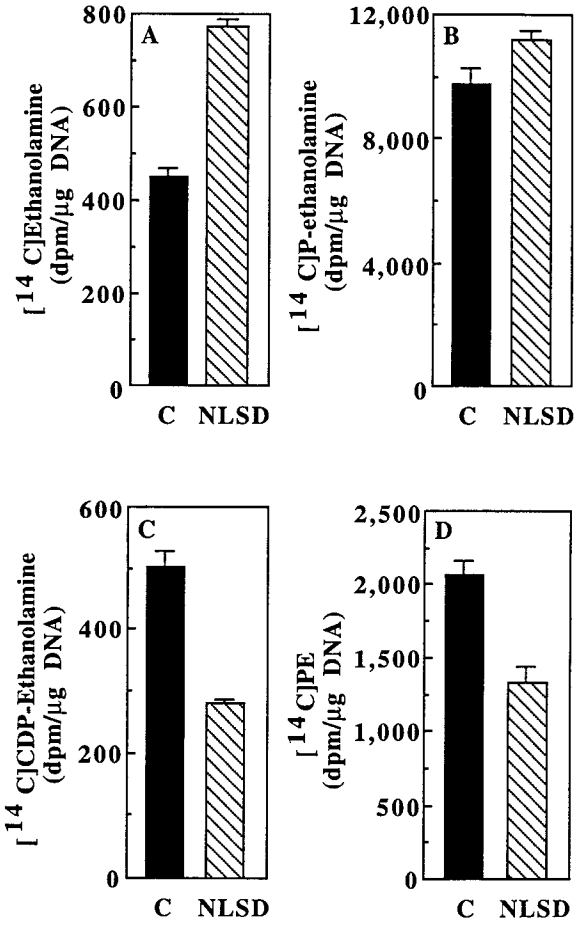

Fig. 5. Incorporation of [^{14}C]ethanolamine into water-soluble metabolites and PE. Control and NLSD fibroblasts were incubated with 2 μCi/dish of [^{14}C]ethanolamine for 2 h in 10% FBS, EMEM. Aqueous and $CHCl_3$ phases were extracted and chromatographed as described in Experimental Procedures. Each bar represents the means ± SD for three 100-mm dishes. Panels: A) [^{14}C]ethanolamine; B) [^{14}C]phosphoethanolamine; C) [^{14}C]CDP-ethanolamine; and D) [^{14}C]PE. The data are representative of five experiments.

lian cells occurs via the CDP-ethanolamine pathway (7). Because NLSD cells incorporated less [^{3}H]glycerol into PE than did the control cells (Figs. 2 and 5), we examined the CDP-ethanolamine pathway of PE formation. More than 95% of the total labeled lipid was PE in both control and NLSD fibroblasts incubated with [^{14}C]ethanolamine for 2 h (data not shown), but NLSD cells incorporated 35% less [^{14}C]ethanolamine into PE than did control cells (**Fig. 5D**). Although the total labeling of water-soluble metabolites was 15% higher in NLSD cells, the distribution of the three main water-soluble metabolites differed in the two cell lines. Free [^{14}C]ethanolamine and [^{14}C]phosphorylethanolamine were 42% and 15% higher in NLSD cells, respectively, than in control cells (Fig. 5A, B), whereas CDP-[^{14}C]ethanolamine was 45% lower (Fig. 5C).

A decrease in CDP-[^{14}C]ethanolamine and [^{14}C]PE synthesis could indicate a partial block in the conversion of phosphoethanolamine to CDP-ethanolamine by the ethanolamine cytidylyltransferase (ET) and explain the decreased rate of synthesis of PE in NLSD fibroblasts. In order to test the hypothesis that the reason for decreased incorporation of [^{14}C]ethanolamine into PE lay in the production of CDP-ethanolamine rather than in the diacylglycerol substrate, we took advantage of differences in the synthetic pathways of alkyl, acyl- and diacyl-PE. Synthesis of alkyl, acyl-PE (plasmalogen-PE), occurs via a peroxisomal pathway that begins with the addition of a long-chain fatty alcohol at the *sn*-1 position of dihydroxyacetone-P (**Fig. 6**) (21). The *sn*-2 position of alkyl-DHAP is then reduced and acylated and the phosphate is hydrolyzed to form an *sn*-1-alkyl,2-acyl-diacylglycerol analog. Microsomal DEPT then catalyzes the addition of phosphoethanolamine from CDP-ethanolamine. Finally, the alkyl-1-enyl moiety is formed by a specific 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine Δ^1-desaturase. Because alkyl, acyl-PE cannot be synthesized directly from either diacylglycerol or diacyl-PE, decreased incorporation of [^{14}C]ethanolamine into both PE and plasmalogen-PE would indicate a defect in the production of CDP-ethanolamine, the common co-substrate, rather than a defect involving the amount of diacylglycerol available for diacyl-PE synthesis. In order to determine whether [^{14}C]ethanolamine incorporation into both plasmalogen-PE and diacyl-PE decreased similarly in NLSD cells, we determined the

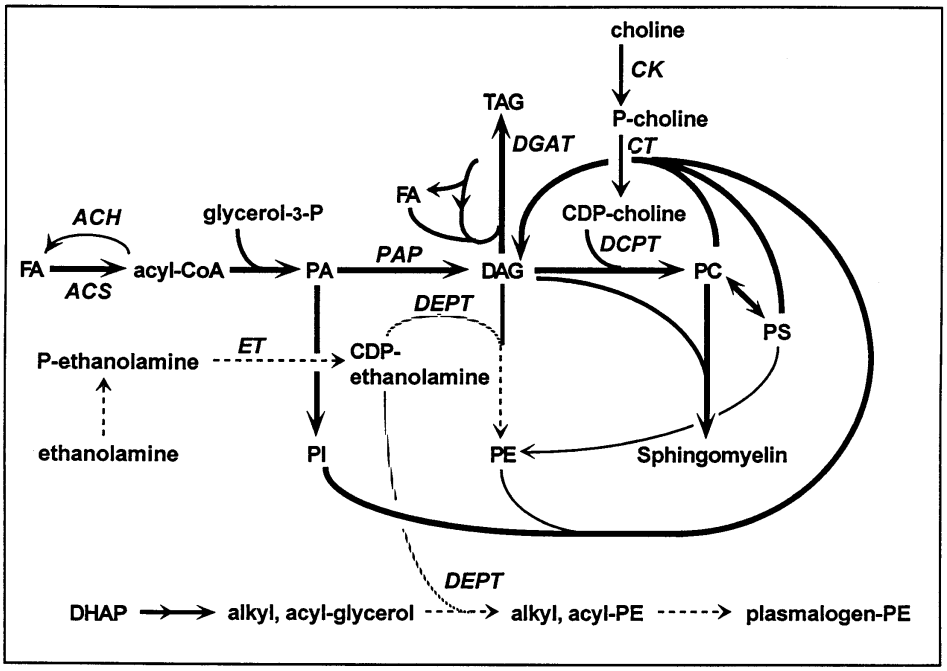

Fig. 6. Abnormal glycerolipid metabolism in NLSD. Synthesis and degradation of all phospholipids except PE is increased. The decreases in PE and plasmalogen-PE arise from a lack of availability of CDP-ethanolamine, not DAG. TAG lipolysis is normal but the DAG released is primarily used for TAG resynthesis rather than for synthesis of phospholipids. ACS, acyl-CoA synthetase; ACH, acyl-CoA hydrolase; CK, choline kinase; CT, CTP:choline cytidylyltransferase; DGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone-P; DCPT, diacylglycerol cholinephosphotransferase; DEPT, diacylglycerol ethanolaminephosphotransferase; ET, CTP:ethanolamine cytidylyltransferase; FA, fatty acid; PA, phosphatidic acid; PAP, phosphatidate phosphohydrolase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol.

amount of plasmalogen produced during a 2-h incubation with [^{14}C]ethanolamine. [^{14}C]plasmalogens formed 22% of total ethanolamine phospholipid in both NLSD and control cells, and NLSD cells showed a similar (75%) decrease in [^{14}C]ethanolamine incorporation into both PE fractions. These data indicate that the decreased PE synthesis in NLSD cells results from decreased production of CDP-ethanolamine and that the pathway from glycerol-3-P is affected only indirectly (Fig. 6).

To determine the effect of a choline- and ethanolamine-deficient media on PC and PE synthesis, we labeled cells with [^{14}C]choline or [^{14}C]ethanolamine in RPMI 1640 media which contains no choline or ethanolamine. As was observed when cells were labeled in 10% FBS, EMEM, both NLSD and control cells labeled for 2 h with [^{14}C]choline produced only PC (98% of total radiolabeled lipids). However, when cells were incubated with [^{3}H]ethanolamine in choline- and ethanolamine-deficient media, the labeled lipids were 90% PE, 6–7% PC, and about 2% sphingomyelin (data not shown). In the choline- and ethanolamine-deficient media, as in the sufficient media, [^{14}C]choline incorporation into PC in NLSD cells increased 25%, whereas [^{14}C]ethanolamine incorporation into PE formation was 75% lower than in the control cells. Thus, insufficient choline and ethanolamine appears to further modify phospholipid synthesis in NLSD cells by decreasing both the enhanced PC formation as well as the already decreased synthesis of PE.

Glycerolipid synthetic enzyme activities are similar in NLSD and control cells

Because NLSD cells show increased incorporation of oleate and glycerol into TAG and PC and decreased incorporation into PE, we measured the specific activities of several relevant enzymes in the TAG, PC, and PE pathways (**Table 2**). The two cell lines had similar acyl-CoA synthetase and DGAT specific activities, in agreement with a previous report (22). The activity of acyl-CoA hydrolase from NLSD cells was also similar to that of the control cells. DCPT and DEPT specific activities were similar in particulate fractions from NLSD and control fibroblasts, as were the rate-limiting enzymes in PC and PE formation, CTP:choline cytidylyltransferase and CTP:ethanolamine cytidylyltransferase, respectively. Choline kinase activity in NLSD cells was also similar to that of controls.

Metabolism of [^{14}C]choline in NLSD and control cells during a chase

Because the rate of PC synthesis might be modified by the pool size of the water-soluble precursors of the CDP-choline pathway, we labeled cells for 24 h with 50 μM [methyl-^{14}C]choline. As observed in 2 h incubations, the incorporation of [^{14}C]choline into total phospholipid was 2-fold higher in NLSD cells than in controls (**Fig. 7**). After 24 h of labeling, more than 90% of the choline-labeled phospholipid was PC; the remainder was sphingomyelin. During the first 6 h of a chase with unlabeled media, the incorporation of [^{14}C]choline into PC increased in both cell lines. However during most of the chase, the PC fraction in both cell lines plateaued, while sphingomyelin labeling continued to increase (Fig. 7, inset), consistent with increased PC synthesis and its precursor–product relationship with sphingomyelin.

After the 24-h labeling period, NLSD cells contained almost twice as much [^{14}C]choline water-soluble metabolites as did control cells (**Fig. 8A**). Most of the label was released into the media during the chase period. Phosphocholine was the major intracellular metabolite (>95%) labeled with choline. A comparatively small

TABLE 2. Specific activities of enzymes of complex lipid metabolism

Enzyme	Control	NLSD
	nmol/mg protein/min	
Diacylglycerol phosphoethanolamine transferase (DEPT)	0.18	0.16
Diacylglycerol phosphocholine transferase (DCPT)	0.31	0.31
CTP:phosphocholine cytidylyltransferase (CT)		
Cytosolic	2.87	3.54
Microsomal	1.20	1.40
CTP:phosphoethanolamine cytidylyltransferase (ET)	0.52	0.52
Choline kinase	1.03	1.15
Acyl-CoA hydrolase	16.4	15.1
Acyl-CoA synthetase		
Substrate C18:1	0.91	0.82
Substrate C16:0	0.31	0.35
Phosphatidate phosphohydrolase (microsomal)	1.13	1.2
Diacylglycerol acyltransferase (DGAT)	0.45	0.52

Cytosolic or total particulate protein from four to six 150-mm dishes was prepared and assayed for enzyme activities as described in Experimental Procedures. Activities were assayed at three different protein concentrations and individual results varied by less than 15%.

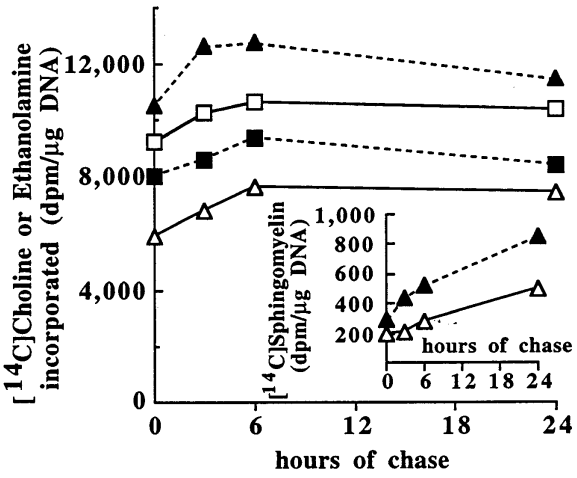

Fig. 7. Incorporation of [^{14}C]choline into PC and sphingomyelin, and of [^{14}C]ethanolamine into PE during a 24-h chase. Control and NLSD fibroblasts were incubated for 24 h with 50 μM [^{14}C]choline or 50 μM [^{14}C]ethanolamine in 10% FBS, EMEM. Residual label was removed and the cells were chased for up to 24 h in 10% FBS, EMEM. Lipids were extracted and chromatographed as described in Experimental Procedures. Labels: PC (triangles), PE (squares) and sphingomyelin (triangles-inset) from control (open symbols-solid lines) and NLSD (closed symbols-dashed lines). The figure shows determinations from duplicate 100-mm dishes.

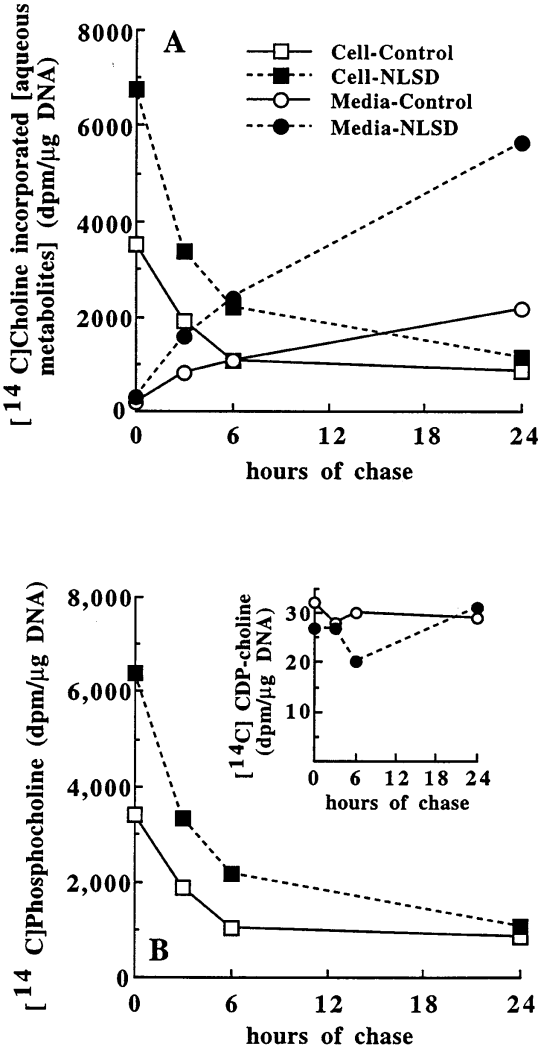

Fig. 8. Recycling of phosphocholine pools after a 24-h incubation with [^{14}C]choline. Control and NLSD cells were treated as described in Fig. 7. Metabolites were separated as described in Experimental Procedures. A) [^{14}C]choline incorporated into cell (squares) and media (circles) total aqueous metabolites; (B) [^{14}C]phosphocholine (squares) and [^{14}C]CDP-choline (circles) (inset) in control (open symbols-solid lines) and NLSD (closed symbols-dashed lines) cells. The figure shows determinations from duplicate 100-mm dishes.

amount of CDP-choline was present (Fig. 8B). During the chase, there was a massive loss of intracellular [^{14}C]phosphocholine, the only water-soluble compound present. During the first 6 h of chase, a significant amount of phosphocholine (about 50% of the released metabolites) was used for PC synthesis in both cell lines (Fig. 7). However, the cellular concentration of phosphocholine in NLSD cells was 2-fold greater than in control cells, except at 24 h when it was 30% greater. A very small amount of free [^{14}C]choline was present in cells at the end of the labeling period. Although this [^{14}C]choline pool was 3.7-fold higher in the NLSD cells (85 vs. 317 dpm / μg DNA), it was almost undetectable in both cell lines by the third hour of the chase. The very low amount of labeled CDP-choline present did not change during the chase (Fig. 8B, inset), and no differences in [^{14}C]CDP-choline were observed in the two cell lines.

Metabolism of [^{14}C]ethanolamine in NLSD and control cells during a chase

In order to examine the metabolism of PE during a similar chase, we labeled the intracellular ethanolamine pool for 24 h. As had been previously observed during 2-h incubations (Fig. 5), after a 24-h incubation with 50 μM [^{14}C]ethanolamine, incorporation into [^{14}C]PE was about 25% lower in the NLSD cells than in control cells (Fig. 5). Incorporation into PE, the only labeled lipid formed, continued to increase for 6 h during the chase in both cell lines and then to plateau.

The total amount of water-soluble metabolites labeled with ethanolamine was only 10% increased in NLSD cells at the end of the labeling period. As with the choline metabolites, most of the [^{14}C]ethanolamine label was released into the media as phosphoethanolamine during the chase (**Fig. 9A**). No difference was observed between NLSD and control fibroblasts. Phosphoethanolamine formed more than >90% of the

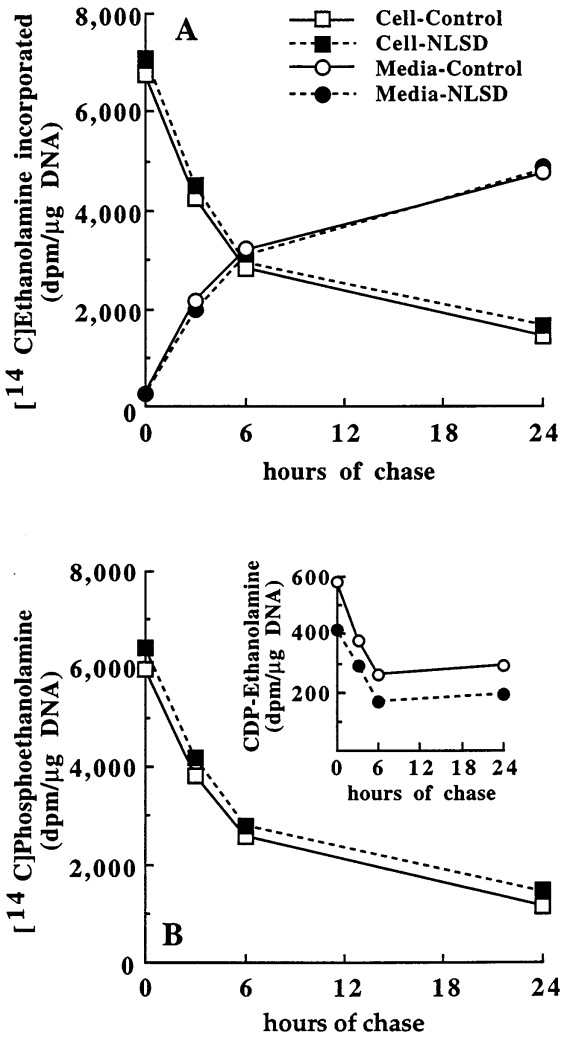

Fig. 9. Recycling of phosphoethanolamine pools after a 24-h incubation with [^{14}C]ethanolamine. Normal and NLSD fibroblasts were labeled for 24 h with 50 μM [^{14}C]ethanolamine in 10% FBS, EMEM. Residual label was removed and the cells were chased with 10% FBS, EMEM. Metabolites were separated as described in Experimental Procedures. A) [^{14}C]ethanolamine incorporated into cell (squares) and media (circles) aqueous- and lipid-soluble metabolites; (B) [^{14}C]phosphoethanolamine and [^{14}C]CDP-ethanolamine (inset) in control (open symbols-solid lines) and NLSD (closed symbols-dashed lines) cells. The figure shows determinations from duplicate 100-mm dishes.

aqueous cell metabolites as well (Fig. 9B). During the chase the amount of [^{14}C]phosphoethanolamine was always slightly higher in NLSD cells compared to control cells. Free [^{14}C]ethanolamine could be detected in cells only at the start of the chase, and was 20% higher in the NLSD line (data not shown).

Cellular [^{14}C]CDP-ethanolamine decreased 50% during the first 6 h of the chase and then plateaued. At every time point in the chase, NLSD cells contained 30–35% less [^{14}C]CDP-ethanolamine than did the control cells (Fig. 9B, inset). This finding, together with the presence of between 10 and 30% more [^{14}C]phosphoethanolamine in NLSD cells at each time point (Fig. 7B), is consistent with the interpretation that the lower PE synthesis by NLSD cells before and during the chase results from decreased CDP-ethanolamine formation. As had occurred with PC formation, about 30% of the [^{14}C]phosphoethanolamine initially present was incorporated into PE during the first 6 h of the chase. At every time point, however, the incorporation of [^{14}C]ethanolamine into PE remained lower in NLSD fibroblasts than in controls (Fig. 5).

DISCUSSION

PC synthesis and turnover

Studies with labeled oleate, glycerol, and choline all indicate that the rate of de novo PC synthesis is increased in NLSD cells, but that there is no net PC accumulation (Table 1). These data are consistent with an increased rate of phospholipid turnover in NLSD cells. Although NLSD cells from our patient and others are able to maintain a normal long-term precursor incorporation into phospholipid (2) and normal phospholipid composition (22), the secondary result of the increased turnover rates appears to be that TAG stores are not readily recycled to phospholipid.

The presence of higher amounts of [^{14}C]choline and [^{14}C]phosphocholine, as well as the low and steady CDP-choline labeling in non-oleate-treated NLSD cells, compared to controls, suggest that a high flux of water-soluble choline metabolites is a primary cause for the increased rate of PC synthesis. Despite this higher basal rate of PC synthesis in NLSD cells, the rate of incorporation of [^{14}C]choline into PC in both control and NLSD cells could be further increased when added fatty acid was present in the media, suggesting that CT, the rate-limiting step of PC synthesis, was not fully activated. Further, the increase in PC synthesis by NLSD cells cannot be explained by an increased specific activity of CT because its specific activity in both cytosol and membrane fractions was equivalent to that of control cells. The comparable CT specific activities in NLSD and control fibroblasts also rule out an underlying cellular increase of DAG or fatty acid concentration.[2] The constitutively high rate of PC synthesis in NLSD cells might result from the presence of increased phosphocholine derived from rapid PC turnover (Figs. 5, 8). In any case, the over-synthesis and increased turnover of PC is likely to be a secondary problem, related indirectly to the underlying NLSD defect.

Increased PC turnover is a feature of several other cell lines

In *ras*-activated C3H10T $^{1}/_{2}$ cells (16) and oocytes (23), and in NIH 3T3 cells (24) the rate of PC turnover is increased and PC content is decreased compared to non-transfected cells. The increased phosphocholine levels observed in these cells appear to result from an increase in choline kinase (16, 24). Although CT specific activity is decreased in C3H10T $^{1}/_{2}$ cells, calculations indicated that the rate of PC synthesis is increased and that label arises from both added label in the media and reuse of [^{3}H]choline-containing glycerolipid (16). In *ras*-transfected HaCaT human keratinocytes, elevated PC formation from [^{14}C]choline is accompanied by increases of choline uptake into cells and CT activity (without increased cellular diacylglycerol levels), but choline kinase activity is unchanged (18). In HeLa cells after stimulation with EGF or insulin, more labeled choline is incorporated into PC, as a result of increases in choline kinase, glycerol-3-P acyltransferase, and an expanded phosphocholine pool (25). Thus, it appears that increases in PC synthesis can be produced via several different mechanisms. Of interest is the finding that the PE content of *ras*-activated cells is unchanged (16) though both phosphocholine and phosphoethanolamine are markedly elevated (16, 23). PE and TAG synthesis from labeled precursors was not measured in any of these cell models.

Even though NLSD cells normalized the amount of [^{14}C]oleate and [^{3}H]glycerol incorporated into PC by 24 h (Figs. 2A, 4A), a 24-h incubation with [^{14}C]choline continued to show a 2-fold increase in PC labeling compared to control cells (Fig. 7). The similar increase in [^{14}C]choline-labeled sphingomyelin in NLSD cells (Fig. 7, inset) may have resulted from the increased rate of PC synthesis, as the primary route for sphingomyelin synthesis occurs via the transfer of phosphocholine from PC to ceramide (26, 27). Increased [^{14}C]oleate-labeled sphingomyelin formation was also observed (Fig. 1D), and remained 16% higher in NLSD cells during a 24-h chase.

These studies clearly indicate that NLSD fibroblasts have an increased rate of TAG and PC synthesis, rapid PC turnover, and a decreased rate of PE synthesis (Fig. 6). Despite these metabolic alterations, NLSD cells are still able to maintain a normal content of each phospholipid species. Agents that increase PC synthesis, like oleic acid (17), *ras*-oncogene (16), diacylglycerol (28), or the over-expression of CT in cultured cells (29), also increase the rate of PC degradation. Thus, in several experimental models in which PC synthesis is stimulated, the cellular concentration of PC remains virtually unchanged, indicating, as in NLSD cells, a compensatory increase in degradation.

PE synthesis and turnover

The decreased rate of PE synthesis via the CDP-ethanolamine pathway cannot be explained by a decrease in the availability of ethanolamine. If the cell PE pool had been low, the ^{14}C-label would have been diluted into a smaller endogenous pool in the NLSD cells compared to control cells, and one would have observed increased, rather than decreased, labeling with [^{14}C]ethanolamine. Further, adding 100 μM ethanolamine to NLSD cells did not alter TAG or phospholipid turnover (R. A. Igal and R. A. Coleman, unpublished data). Thus, no evidence exists in NLSD cells for a primary block in sphingolipid degradation, the primary cellular source of ethanolamine (30). The observation that NLSD fibroblast PE content is normal might be explained by either a decreased rate of PE turnover or by the increased rate of PS synthesis (Figs. 2B and 4B), which could provide an alternative source of PE by decarboxylation. In baby hamster kidney cells, decarboxylation of PS is a major source of PE (31).

Like the present study, the report by Williams et al. (22) on NLSD fibroblasts from two members of a family unrelated to our patient showed that the rate of incorporation of both [^{14}C]acetate and [^{14}C]oleic acid into complex lipids was higher in NLSD cells. However, unlike the present study, which shows a decrease in the rate of de novo PE synthesis, Williams et al. (22) reported an increase in oleate-labeled PE. These differences might result if the two NLSD families had different, but related genetic defects. A more likely reason is that Williams et al. (22) used only tracer quantities of [^{14}C]oleate. Under these conditions label is incorporated primarily into phospholipid, whereas when high concentrations of fatty acid are present as in the present study, label is incorporated into both polar and neutral lipids.

The present data narrow the range of possible defects in NLSD. The underlying problem is not limited to the recycling of TAG-derived DAG to phospholipid synthesis, but, instead, is the result of profound alterations in glycerolipid metabolism that include an increase in the rate of PC turnover. The increased synthesis of PC could underlie the increase in sphingomyelin synthesis directly and the increase in PS synthesis by base exchange. One cannot, however, relate the increased rate of PI synthesis directly to changes in PC metabolism as PI and PC are not synthesized via a common intermediate (Fig. 6). Changes in PC turnover also do not explain the decreased rate of PE synthesis. Not only are cellular DAG concentrations normal, but the equivalent decreases in plasmalogen-PE and diacyl-PE point to a problem in CDP-ethanolamine availability. In NLSD cells it appears that there is abnormal regulation of the major phospholipids via a common or

linked signaling pathway and that a primary signaling defect may increase the activities of phospholipases specific for PC and PI. The cardinal NLSD increase in cellular TAG appears to be a secondary manifestation of profound alterations in phospholipid metabolism.

This work was supported by grant HD 19068 from the National Institutes of Health and by the Lineberger Cancer Center Core Support Grant CA 16086. We are grateful to Dr. Patricia A. Gallagher for her help with the GC analyses.

Manuscript received 29 July 1997 and in revised form 25 September 1997.

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