Sjögren-Larsson Syndrome

Impaired Fatty Alcohol Oxidation in Cultured Fibroblasts due to Deficient Fatty Alcohol:Nicotinamide Adenine Dinucleotide Oxidoreductase Activity

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Abstract

Lipid metabolism was studied in cultured skin fibroblasts from patients with the inherited disorder, Sjögren-Larsson syndrome (SLS). Intact SLS fibroblasts incubated in the presence of [1-^{14}C]palmitate accumulated more radioactive hexadecanol than did normal cells, whereas incorporation of radioactivity into other cellular lipids was unaltered. The hexadecanol content of SLS fibroblasts was abnormally elevated. Hexadecanol accumulation was not due to increased fatty alcohol synthesis nor its deficient utilization for glycerol ether synthesis. The half-life of intracellular hexadecanol loaded into SLS fibroblasts was increased (70 min) compared with normal (15 min), and intact SLS fibroblasts showed impaired oxidation of [^{14}C]-hexadecanol to fatty acid. Fatty alcohol:NAD$^+$ oxidoreductase, the enzyme catalyzing this reaction, was deficient in SLS fibroblasts. Mean total activity in SLS fibroblasts (n = 5) was 13% of that in normal fibroblasts, and palmitoyl CoA–inhibitable activity was 1% of normal. Fibroblasts from two obligate SLS heterozygotes had enzyme activities intermediate between that in normal fibroblasts and individuals with SLS. These results suggest that the primary defect in SLS is deficiency of fatty alcohol:NAD$^+$ oxidoreductase. SLS represents the first inherited disorder in man associated with an isolated abnormality in fatty alcohol metabolism.

Introduction

Sjögren-Larsson syndrome (SLS)[1] is an autosomal recessive disorder characterized by congenital ichthyosis, mental retardation, and spasticity (1). Most patients have been reported from Sweden, where the prevalence of SLS in certain regions is as high as 1 in 12,000 (2), although the disease occurs worldwide at a much lower frequency.

The primary biochemical defect in SLS is unknown. Altered lipid metabolism has been demonstrated in other inherited forms of ichthyosis, including X-linked ichthyosis (steroid sulfatase deficiency) (3), neutral lipid storage disease (4, 5), multiple sulfatase deficiency (6), and Refsum disease (7). We now report that cultured skin fibroblasts from SLS patients accumulate fatty alcohol due to deficient activity of the enzyme, fatty alcohol:NAD$^+$ oxidoreductase, which catalyzes the oxidation of fatty alcohol to fatty acid.

Received for publication 31 March 1987 and in revised form 17 August 1987.

1. *Abbreviation used in this paper:* SLS, Sjögren-Larsson syndrome.

J. Clin. Invest.

0021-9738/88/03/0738/07 $2.00
Volume 81, March 1988, 738–744

Methods

Cells. Human cultured skin fibroblasts were derived from normal subjects by skin punch biopsy after obtaining informed consent. Fibroblast cultures from two Swedish SLS patients (T.J. and E.N.) were originally obtained by Dr. S. Jagell and Dr. G. Holmgren, University Hospital, Umeå, Sweden, and kindly provided by Dr. Joel Avigan, National Institutes of Health, Bethesda, MD, and cells from two SLS siblings (An.B. and Am.B.) and their parents (T.B. and B.B.) were generously provided by Dr. Susan Black, Genetics and IVF Institute, Fairfax, VA. Dr. Enrique Chaves-Carballo, Eastern Virginia Medical School, Norfolk, VA, provided a skin biopsy from an unrelated black SLS patient (C.B.). Fibroblasts from patients with X-linked ichthyosis (GM3034), multiple sulfatase deficiency (GM3245), Zellweger syndrome (GM4340 and GM6231), and Refsum disease (GM3896) were obtained from the Human Mutant Genetic Cell Repository, Camden, NJ. Cells were routinely grown at 37°C in an atmosphere of 5% CO_2, 95% air in Dulbecco's MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All experiments were performed on cells at passage 4 through 12. Lipid-free fetal bovine serum was produced by solvent extraction and extensive dialysis (8).

Chemicals. [1-^{14}C]Palmitate (58 mCi/mmol) was obtained from ICN Radiochemicals, Irvine, CA, and [9,10-^{3}H]palmitate (30 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Both radioactive fatty acids were purified by thin-layer chromatography on silica gel G plates (Whatman, Inc., Clifton, NJ) using hexane/diethyl ether/acetic acid (90:10:1) before use. Solvents were either reagent grade or HPLC grade from J.T. Baker Chemical Co., Phillipsburg, NJ. [1-^{14}C]Hexadecanol was synthesized from [1-^{14}C]palmitate and purified by thin-layer chromatography as described (9). High performance thin-layer chromatography plates and reverse phase plates were obtained from Whatman, Inc. All other chemicals were from Sigma Chemical Co., St. Louis, MO.

Fatty alcohol metabolism in intact fibroblasts. To screen for incorporation of radioactive palmitate into cellular lipids, fibroblasts were grown to confluency in 100-mm diam culture dishes and medium was changed to fresh MEM containing 10% fetal bovine serum one day before the study. [1-^{14}C]Palmitic acid (1.5 μCi/ml)/bound to fatty acid–free bovine serum albumin at a fatty acid/albumin ratio of 1:1 was added, and cells were incubated for varying periods of time at 37°C. Radioactive medium was removed and monolayers were washed three times with ice-cold PBS. Cells were collected by trypsinization, pelleted by centrifugation at 3,000 g, washed with PBS, and extracted overnight with 3 ml chloroform/methanol (1:1). The solubilized lipids were submitted to a Folch extraction (10), dried under a stream of nitrogen, and separated by high performance thin-layer chromatography using the solvent system for total lipid separation described by Yao and Rastetter (11). Radioactive lipids were visualized by autoradiography after spraying the plate with Enhance (New England Nuclear).

In experiments to screen for incorporation of radioactive palmitate into nonsaponifiable lipids, cells were placed in MEM containing 10%

lipid-free fetal bovine serum and pulsed with [1-^{14}C]palmitate as above. After extraction of cellular lipids with chloroform/methanol and drying under nitrogen, the dried lipids were resuspended in 2 ml of 0.3 N NaOH in 95% ethanol and saponified by incubation at 80°C for 1 h. Nonsaponifiable lipids were extracted into 3 ml of hexane after addition of 2 ml of water, and separated by high performance thin-layer chromatography using the development protocol for nonpolar lipids described by Yao and Rastetter (11). Radioactive lipids were visualized by autoradiography.

To measure fatty alcohol synthesis, the growth media of fibroblasts in culture dishes were changed to MEM containing 10% lipid-free fetal bovine serum one day before study. [9,10-^{3}H]Palmitic acid bound to bovine serum albumin was added to a final concentration of 2 μCi/ml. In some dishes, hexadecanol in ethanol was added to a final concentration of 40 μM just before radioactive palmitate addition; final ethanol concentration was 0.1% in the medium. Cells were incubated at 37°C for 20 min. Dishes were removed and placed on ice, radioactive medium was aspirated, monolayers were washed three times with ice-cold PBS, and cells were scraped into 3 ml of ice-cold methanol. Lipids were extracted with chloroform/methanol (1:1) and nonsaponifiable lipids were isolated as described above. After addition of carrier hexadecanol (25 μg), fatty alcohol was separated from cholesterol and contaminating fatty acid by development on silica gel G plates using benzene/ethyl acetate/diethyl ether/acetic acid (80:9.5:10:0.2). The fatty alcohol was visualized under ultraviolet (UV) light after staining the plate with rhodamine G, and scraped into scintillation vials for quantitation of radioactivity. A replicate dish of fibroblasts was harvested for protein determination (12). Under these conditions, radioactive cellular hexadecanol increased linearly with time for at least 20 min.

To measure hexadecanol oxidation to fatty acid in intact fibroblasts, [1-^{14}C]hexadecanol in ethanol was added at a final concentration of 1.7 μM (0.06 μCi/ml) to cells in MEM containing 10% lipid-free serum. After incubation at 37°C for various times, culture dishes were removed and placed on ice. Medium was aspirated, monolayers were washed three times with ice-cold PBS, and cells were collected by scraping into 3 ml of ice-cold methanol. Cellular lipids were extracted overnight with chloroform/methanol (1:1), and nonsaponifiable lipids were prepared and removed by extraction into hexane as described above. 1 ml of 1 M HCl was added to the remaining ethanolic phase, and saponifiable lipids (fatty acids) were extracted twice into 3 ml hexane. The hexane extracts were combined, back extracted with water, and transferred into scintillation vials. After evaporation of the hexane under a stream of air, radioactive fatty acids were quantitated by liquid scintillation spectroscopy. Fibroblasts in parallel dishes were used for protein determination (12).

The half-life of radioactive hexadecanol loaded into fibroblasts was measured by pulsing cell monolayers in MEM containing 10% lipid-free serum with 0.3 μM [1-^{14}C]hexadecanol for 20 min. Radioactive medium was removed, monolayers were washed (× 3) with PBS, and nonradioactive medium was replaced. After various periods of time at 37°C, cell monolayers were washed with ice-cold PBS, scraped into ice-cold methanol, and radioactive hexadecanol was isolated from nonsaponifiable lipids by thin-layer chromatography and quantitated as described above.

Glycerol ether synthesis in intact fibroblasts. Incorporation of radioactive hexadecanol into the ether linkage of phosphatidylethanolamine of fibroblasts was measured by incubating confluent fibroblasts in MEM containing 10% lipid-free serum with 7 μM [1-^{14}C]-hexadecanol for 2 h. Cells were harvested, washed, and lipids extracted with chloroform/methanol (1:1). Incorporation of radioactive hexadecanol into the ether linkage of phosphatidylethanolamine was determined by measuring radioactive acid-labile fatty aldehyde released after separation of phospholipids by two-dimensional thin-layer chromatography (13).

Palmitate oxidation in intact fibroblasts. Confluent fibroblasts were collected by trypsinization and washed twice with PBS. Cells (100–250 μg protein) were incubated in glass scintillation vials capped with rubber stoppers (Kontes Co., Vineland, NJ) containing 1 ml of Krebs-Ringers phosphate buffer, pH 7.4, and 30 μM [1-^{14}C]palmitate bound to fatty acid-free bovine serum albumin. Cells were incubated for 2 h at 37°C with shaking, and reactions were terminated by addition of 0.1 ml of 60% perchloric acid. Aliquots (0.5 ml) were incubated on ice for 1 h, and centrifuged at 12,000 *g* for 5 min. The amount of acid-soluble radioactivity was determined by scintillation spectroscopy. Radioactivity present in control incubations lacking cells, typically 5% of that present in incubations containing cells, was subtracted from that present in each incubation to determine actual palmitate oxidation.

Enzyme assays. Confluent fibroblasts from one 75-cm^2 culture flask were collected by trypsinization and washed three times with PBS. The cell pellet was homogenized in a glass teflon motor-driven homogenizer in 1 ml of 25 mM Tris-HCl, pH 8.0, 0.25 M sucrose.

Fatty alcohol:NAD$^+$ oxidoreductase was assayed by modification of the method described by Lee (14). Activity was measured in a total volume of 0.2 ml containing 50 mM glycine-NaOH buffer, pH 9.8, 2.5 mM NAD$^+$, 0.5 mg/ml fatty acid–free bovine serum albumin, 12 μM [1-^{14}C]hexadecanol (220,000 cpm added in 3 μl ethanol), and 10–25 μg of cell homogenate protein; control incubations lacked homogenate. After 20 min at 37°C, reactions were stopped by addition of 2 ml of methanol containing 2% acetic acid. 1 ml water and 1 ml chloroform was added to each reaction tube. Tubes were vortexed for 1 min and centrifuged for 5 min at 3,000 *g* to facilitate phase separation. The upper phase together with the interphase was carefully removed and the lower chloroform phase was dried under nitrogen. 15 μg of carrier palmitate and hexadecanol were added. The dried lipids were dissolved in chloroform/methanol (1:1) and spotted on silica gel G plates. Palmitate was separated from hexadecanol by development in benzene/ethyl acetate/diethyl ether/acetic acid (80:9.5:10:0.2) or hexane/diethyl ether/acetic acid (60:40:1). The fatty acid spot was visualized under UV light after staining the plate with rhodamine G. The areas of silica gel containing palmitate were scraped into scintillation vials for measurement of radioactivity. Radioactivity present in control incubations was typically 100–200 cpm, or 5–10% of the radioactivity measured in complete incubations containing homogenate from normal fibroblasts. This background radioactivity was subtracted from that measured in incubations with homogenate to determine actual enzyme activity. Protein concentration of cell homogenates was determined according to Lowry et al. (12). Total fatty alcohol:NAD$^+$ oxidoreductase activity was expressed as picomoles per minute per milligram protein. Enzyme activity was also measured in the presence of 80 μM palmitoyl CoA, which is an inhibitor of fatty alcohol:NAD$^+$ oxidoreductase (9). Palmitoyl CoA-inhibitable activity was determined by subtracting enzyme activity measured in the presence of palmitoyl CoA from total fatty alcohol:NAD$^+$ oxidoreductase activity (without palmitoyl CoA).

Palmitoyl CoA synthetase activity was assayed by minor modification of the method of Krisans et al. (15) in a total volume of 0.2 ml containing 50 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol, 0.1 mM CoA, 0.1% Triton X-100, and 0.2 mM [1-^{14}C]palmitate (dissolved in 0.5% Triton X-100). The reaction was initiated by addition of fibroblast homogenate (10–30 μg protein) and allowed to proceed for 10 min at 37°C. Control reactions lacked fibroblast homogenate. The reactions were terminated by addition of 3.25 ml of methanol/chloroform/hexane (140:125:100) and 1.0 ml of 0.1 M sodium acetate, pH 4.0. Reaction tubes were vigorously agitated on a vortex stirrer for 1 min and centrifuged at 3,000 *g* for 5 min to facilitate phase separation. The upper aqueous phase containing palmitoyl CoA was removed for determination of radioactivity.

Fibroblast hexadecanol content. For measurement of fibroblast hexadecanol content, confluent cells were incubated overnight in MEM containing lipid-free serum. To some culture dishes, palmitate bound to fatty acid–free bovine serum albumin was added at a final concentration of 50 μM for 2 h. Media were quickly removed and the monolayers were washed three times with ice-cold PBS. Cells were harvested by scraping into ice-cold methanol, and lipids were extracted overnight with chloroform/methanol (1:1). 2 μg of pentadecanol was

added as internal standard. Cells were centrifuged at 3,000 *g* and the solvent was removed. The pellet was assayed for protein (12). The chloroform/methanol was dried under a stream of nitrogen. The dried lipids were resuspended in 1 ml of ethanolic NaOH (0.3 N NaOH in 95% ethanol), capped, and incubated at 80°C for 2 h. After cooling to room temperature, 2 ml of water was added and nonsaponifiable lipids were extracted twice into 3 ml of hexane. The combined hexane extracts were dried under a stream of nitrogen, and acetate derivatives of the fatty alcohols were made by reaction overnight at room temperature with 0.5 ml acetate anhydride and 1 ml pyridine. After addition of water, acetate derivatives were extracted into petroleum ether and purified by thin-layer chromatography on silica gel G plates by development in hexane/diethyl ether (95:5). Alcohol acetates were visualized under UV light after spraying the plate with rhodamine G. The appropriate area of silica gel was collected by scraping. Alcohol acetates were eluted with hexane/benzene (3:2) and dried under nitrogen.

Alcohol acetates were analyzed by gas-liquid chromatography on a gas chromatograph equipped with a flame ionization detector (5880A; Hewlett-Packard Co., Palo Alto, CA). An SP2100 methyl silicone glass capillary column (60 m × 0.75 mm, internal diameter; Supelco, Inc., Bellefonte, PA) was used to separate alcohol acetates. The temperature of the injection port was 250°C and of the detector 320°C. Direct on-column injection was performed. The initial column temperature was 100°C; after 3 min, the column temperature was raised at a rate of 10°C per minute to a final temperature of 300°C. A series of alcohol acetate standards were used to identify peaks according to retention time.

Results

Abnormal hexadecanol metabolism in intact SLS fibroblasts. Cultured skin fibroblasts from a normal subject and a patient with SLS were incubated for various times in medium containing 10% fetal bovine serum and [1-^{14}C]palmitate. Incorporation of radioactivity into cellular lipids was examined after extraction and separation of lipids by high performance thin layer chromatography (Fig. 1 *A*). SLS fibroblasts accumulated more radioactive material in the region corresponding to that of fatty alcohol than did normal cells. No significant differences were seen in the incorporation of radioactive palmitate into other neutral lipids or phospholipids. When cells were incubated in lipid-free medium containing radioactive palmitate, and the nonsaponifiable lipids were separated by thin-layer chromatography using a different solvent system, SLS fibroblasts accumulated an increased quantity of radioactive material that co-migrated with hexadecanol (Fig. 1 *B*). The identity of this material as fatty alcohol was established by (*a*) co-migration of radioactivity with hexadecanol on silica gel G plates in two additional solvent systems (hexane/ether/acetic acid, 60:40:1; and benzene/ether/ethyl acetate/acetic acid, 80:10:9.5:0.2); (*b*) formation of an acetate derivative and co-migration on thin-layer chromatography with a standard acetate derivative of hexadecanol using a solvent system consisting of hexane/ether (95:5); and (*c*) greater than 90% of this derivatized radioactive material co-migrated with standard hexadecanol acetate on reverse-phase thin-layer chromatography plates composed of octadecylsilane (C18) using methanol as a solvent.

Cultured human fibroblasts synthesize and oxidize fatty alcohol (9). To measure the rate of palmitate reduction to fatty alcohol in normal and SLS fibroblasts, cells were incubated in lipid-free medium with [^{3}H]palmitate and accumulation of cellular radioactive hexadecanol was determined. Since fatty alcohol is rapidly oxidized to fatty acid in fibroblasts under

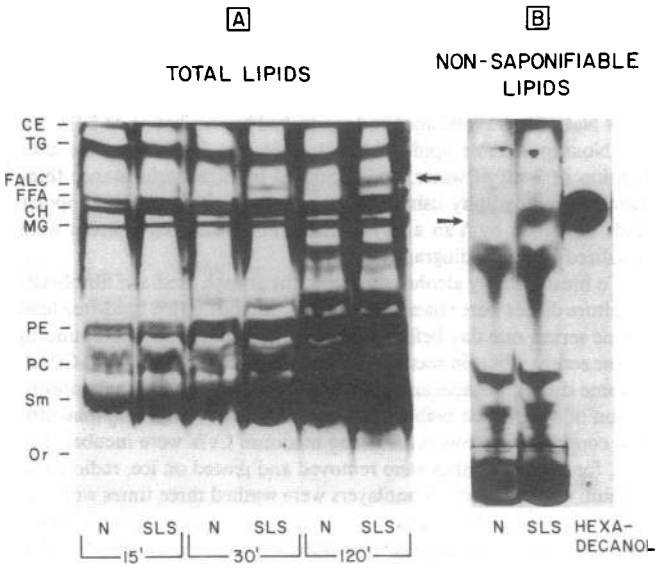

Figure 1. Incorporation of radioactive palmitate into fibroblast lipids of normal (N) and SLS cells. (*A*) Separation of total radioactive lipids. Confluent fibroblasts were incubated with 1.5 μC/ml [1-^{14}C]-palmitate in MEM containing 10% fetal bovine serum for the indicated times. Cells were harvested, washed, and total lipids extracted as described in Methods. Total lipids were separated on high performance thin-layer chromatography plates using the solvent system described by Yao and Rastetter (11). Radioactive lipids were visualized by autoradiography. The arrow indicates accumulation of radioactive fatty alcohol in SLS cells (An.B.) compared with normal. CE, Cholesterol ester; TG, triglyceride; FALC, fatty alcohol; CH, cholesterol; MG, monoacylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sm, sphingomyelin; and Or, origin of migration. (*B*) Separation of radioactive nonsaponifiable lipids. Confluent fibroblasts were incubated for 2 h with 1.5 μC/ml [1-^{14}C]palmitate in MEM containing 10% lipid-free fetal bovine serum. Cells were harvested, washed, and total lipids extracted as above. Lipids were saponified as described in Methods and nonsaponifiable lipids were separated on high performance thin-layer chromatography plates using the solvent system for nonpolar lipids described by Yao and Rastetter (11). Radioactive lipids were visualized by autoradiography. Authentic [^{14}C]hexadecanol was run as a standard in lane *3*. The arrow indicates accumulation of radioactive fatty alcohol in SLS cells (An.B.) compared with normal.

these conditions, incubations were also performed in the presence of nonradioactive hexadecanol, which "traps" the newly synthesized radioactive hexadecanol and prevents its metabolism. As shown in Table I, SLS fibroblasts accumulated about threefold more radioactive hexadecanol than normal cells when no exogenous unlabeled hexadecanol was present. However, in the presence of unlabeled exogenous hexadecanol, which permits a more valid measure of hexadecanol synthesis, the mean rate of radioactive hexadecanol accumulation in SLS fibroblasts was increased by only 40% compared with normal cells, and one SLS cell line (T.J.) was clearly normal. These results are consistent with an expanded fatty alcohol pool in SLS fibroblasts under conditions where no exogenous hexadecanol is present in the medium, and suggest that an increased rate of hexadecanol synthesis is not the basis for radioactive hexadecanol accumulation in SLS cells.

Measurement of cellular hexadecanol content confirmed the accumulation of hexadecanol in SLS fibroblasts (Table II). The mean hexadecanol content of SLS fibroblasts was in-

Table I. Accumulation of Radioactive Hexadecanol by Intact Cultured Fibroblasts Exposed to 2 μC/ml [^{3}H]Palmitate (3×10^4 cpm/pmol)

Cells	[^{3}H]Hexadecanol accumulated	
	−Exogenous hexadecanol	+Exogenous hexadecanol
	pmol/mg protein	
Normal (n = 5)	0.152±0.040	0.969±0.363
SLS		
T.J.	0.357	0.740
An.B.	0.614	1.828
Am.B.	0.587	1.517
Mean±SD	0.485±0.182	1.361±0.560

Incubations were performed in lipid-free medium with or without exogenous unlabeled hexadecanol (40 μM), which "traps" the radioactive hexadecanol and prevents its metabolism. Cells were incubated for 20 min at 37°C, during which time radioactive hexadecanol accumulation increased in a linear fashion (9). Data are the mean of two experiments for each cell line tested. Number in parentheses indicates the number of different cell lines tested.

creased approximately twofold above normal when cells were incubated in the absence of exogenous palmitate. When fibroblasts were incubated with palmitate, SLS cells accumulated only 39% more hexadecanol than normal cells. Notably, the hexadecanol content of SLS fibroblasts incubated in the absence of exogenous fatty acids was similar to that seen in normal cells incubated with palmitate. Normal fibroblasts incubated with palmitate showed a 115% increase in hexadecanol content compared with cells incubated in the absence of this fatty acid, whereas the mean hexadecanol content of SLS fibroblasts increased only 51% in the presence of palmitate. These results suggest that the cellular hexadecanol pool of SLS fibroblasts may not be regulated in a normal manner by exogenous palmitate.

The half-life of the intracellular fatty alcohol pool in normal and SLS fibroblasts was determined by loading cells with [^{14}C]hexadecanol and measuring the decrease in radioactivity over time (Fig. 2 *A*). Radioactive hexadecanol was rapidly lost from both normal and SLS cells during the first 5 min. Thereafter, radioactive hexadecanol was lost from normal fibroblasts with a half-life of 15 min, whereas the half-life was increased to 70 min in SLS fibroblasts. In these experiments, the initial amount of radioactive hexadecanol loaded into the fibroblasts was ~ 2.5-fold higher in the SLS cells than the normal cells. Normal fibroblasts loaded with fivefold more radioactive hexadecanol than the experiments summarized in Fig. 2 *A* showed no significant increase in half-life (data not shown), suggesting that the increased hexadecanol half-life in SLS fibroblasts was not simply due to an increased amount of cellular radioactive hexadecanol or an expanded intracellular fatty alcohol pool.

Table II. Hexadecanol Content of Fibroblasts Incubated in Lipid-free Medium (−Palmitate) or Medium Supplemented with 50 μM Palmitate for 2 h (+Palmitate)

Cells	Hexadecanol content	
	−Palmitate	+Palmitate
	pmol/mg protein	
Normal (n = 4)	144±49	310±68
SLS (n = 3)	284±44	431±109

Data are expressed as the mean±SD. Numbers in parentheses indicate the number of different cell lines tested.

Fatty alcohol is a substrate for glycerol ether biosynthesis. To determine whether the increased hexadecanol half-life in SLS fibroblasts was due to its decreased utilization for glycerol ether biosynthesis, incorporation of [^{14}C]hexadecanol into the ether linkage of plasmalogen phosphatidylethanolamine was measured in intact fibroblasts. Normal fibroblasts incorporated 104±67 pmol [^{14}C]hexadecanol/h per mg protein (range, 35 to 161, n = 5 cell lines), and fibroblasts from two SLS patients (T.J. and E.N.) had normal [^{14}C]hexadecanol incorporation rates (157 and 132 pmol/h per mg protein, respectively).

The major fate of hexadecanol in cultured human fibroblasts is oxidation to fatty acid (9). [^{14}C]Hexadecanol oxidation to fatty acid was impaired in intact SLS fibroblasts compared with normal cells (Fig. 2 *B*). After incubation for 20 min with labeled substrate, SLS fibroblasts had oxidized only 12–32% of the mean amount of hexadecanol oxidized by normal cells. In contrast, oxidation of [1-^{14}C]palmitate to acid-soluble material was not decreased in SLS fibroblasts; intact SLS fibroblasts (T.J.) oxidized palmitate at 5,730 pmol/h per mg protein, and control fibroblasts oxidized 5,060±450 pmol/h per mg protein (n = 3).

Deficient fatty alcohol:NAD$^+$ oxidoreductase activity in SLS fibroblasts. Studies on intact SLS fibroblasts pointed to a defect in hexadecanol oxidation to fatty acid, a reaction catalyzed by fatty alcohol:NAD$^+$ oxidoreductase. In normal fibroblast homogenates, activity of fatty alcohol:NAD$^+$ oxidoreductase was dependent on the amount of protein added to the assay up to 25 μg. The amount of radioactive palmitate produced from [^{14}C]hexadecanol increased linearly over time for at least 20 min. The enzyme showed an apparent K_m of 0.8 μM for hexadecanol and 42 μM for NAD$^+$.

Activity of fatty alcohol:NAD$^+$ oxidoreductase was deficient in SLS fibroblast homogenates compared with normal (Table III). Mean total fatty alcohol:NAD$^+$ oxidoreductase activity in fibroblasts from five SLS patients (two from Sweden and three from the United States) was decreased to 13% of normal. Fatty alcohol:NAD$^+$ oxidoreductase activity in normal fibroblasts is inhibited ~ 50% in the presence of 80 μM palmitoyl CoA. This palmitoyl CoA-inhibitable activity, expressed as the difference between total activity minus activity measured in the presence of palmitoyl CoA, was 32.4±8.3 pmol/min per mg protein in normal cells. In contrast to normal cells, residual fatty alcohol:NAD$^+$ oxidoreductase activity in SLS fibroblasts showed little or no inhibition by palmitoyl CoA. Mean palmitoyl CoA-inhibitable activity in SLS fibroblasts was decreased to 1% of normal. Activity of a control enzyme, palmitoyl CoA synthetase, was normal in SLS cells. Fatty alcohol:NAD$^+$ oxidoreductase activity was normal in fibroblasts from patients with several other inherited forms of ichthyosis associated with altered lipid metabolism or other neurological disorders. In fibroblasts from two obligate SLS heterozygotes, total fatty alcohol:NAD$^+$ oxidoreductase activity was 46 and 48% of mean normal activity (Table III).

Mixing experiments failed to show evidence of a diffusible inhibitor of enzyme activity in SLS cell homogenates (Table IV).

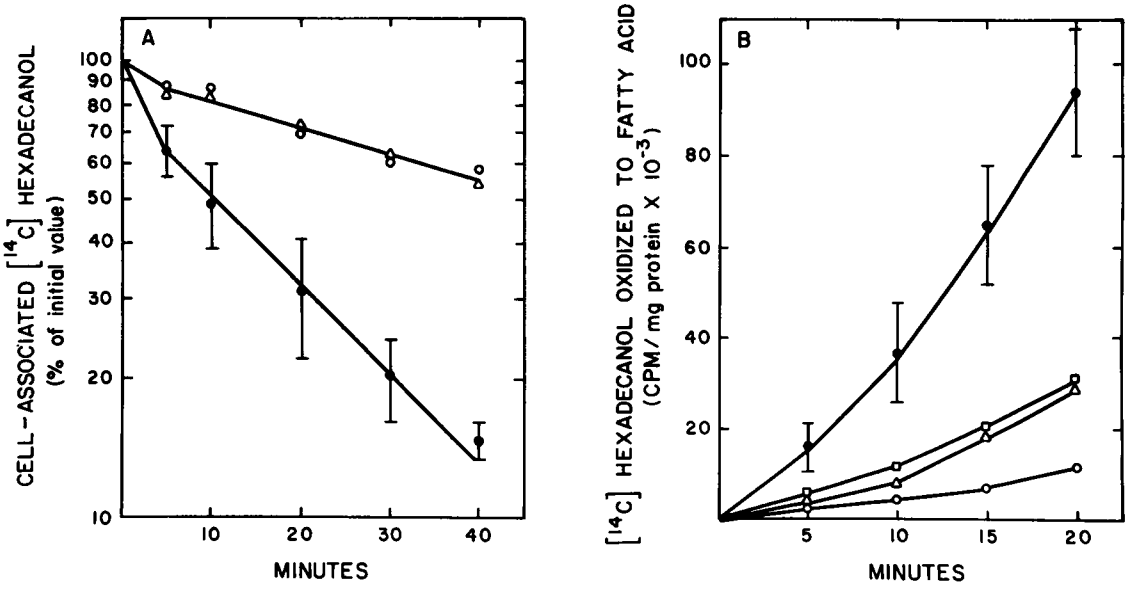

Figure 2. Hexadecanol metabolism by intact fibroblasts from normal subjects and patients with SLS. (*A*) Determination of the half-life of radioactive hexadecanol loaded into fibroblasts. Confluent fibroblasts in MEM containing 10% lipid-free fetal bovine serum were exposed to 0.3 μM [1-^{14}C]hexadecanol for 20 min. The radioactive medium was removed, monolayers were washed with PBS, placed in fresh medium, and incubated at 37°C for the indicated times. Cell monolayers were washed and harvested. Lipids were extracted with chloroform/methanol, saponified, and nonsaponifiable lipids were isolated. Radioactive hexadecanol was separated by thin-layer chromatography and quantitated. Data are expressed as the percent of cell-associated radioactive hexadecanol remaining at each time compared with that initially loaded into the fibroblasts at time zero (100%). Closed symbols represent the mean±SD for four normal cell lines. Open symbols represent the mean of duplicate dishes for SLS cells (△, T.J.; ○, An.B.). The amount of hexadecanol loaded into normal cells at time zero was 122±61 pmol/mg protein, whereas the mean amount loaded into SLS cells was 302 pmol/mg protein. (*B*) Oxidation of [1-^{14}C]hexadecanol by intact fibroblasts. Confluent fibroblasts were incubated in MEM containing 10% lipid-free fetal bovine serum and 1.7 μM radioactive hexadecanol (90 cpm/pmol). At the indicated times, cell monolayers were washed, harvested, and extracted with chloroform/methanol. Incorporation of radioactivity into fatty acids was determined as described in Methods. Closed circles represent the mean±SD for five normal cell lines. Open symbols represent the mean of duplicate dishes for SLS fibroblasts (△, T.J.; ○, An.B.; □, Am.B.). Data shown are the result of three separate experiments.

Discussion

No consistent biochemical abnormality has been identified in SLS (16, 17). A defect in lipid metabolism has been suspected based on clinical improvement seen in three patients placed on dietary fat restriction with supplementation of medium chain fatty acids (18, 19) and the histological finding of lipid accumulation in the brain of an SLS patient (20). A recent report showed that SLS patients had abnormal serum polyunsaturated fatty acids characterized as deficient delta 6-desaturation products of linoleic acid (21), but cultured SLS fibroblasts had normal fatty acid desaturase activity and fatty acid composition (22).

We were initially led to investigate fatty alcohol metabolism in SLS because of the importance of this pathway in supplying substrate for the biosynthesis of wax esters and glycerol ether lipids, which are particularly prominent in the skin and nervous system, respectively. Our studies indicate that SLS fibroblasts have impaired fatty alcohol oxidation due to deficient fatty alcohol:NAD$^+$ oxidoreductase activity. No abnormality was seen in the incorporation of radioactive palmitate into other cellular lipids, in palmitate oxidation, incorporation of hexadecanol into the ether linkage of phosphatidylethanolamine, or in palmitoyl CoA synthetase activity, suggesting that the deficiency of fatty alcohol:NAD$^+$ oxidoreductase is a specific metabolic defect. Fatty alcohol:NAD$^+$ oxidoreductase activity was normal in fibroblasts from patients with several other inherited forms of ichthyosis or neurological disease. Mean total activity of this enzyme in SLS cells was 13% of normal, while mean palmitoyl CoA-inhibitable activity was decreased to 1% of normal. It is possible that total fatty alcohol:NAD$^+$ oxidoreductase activity is due to multiple enzymes, only one of which is inhibited by palmitoyl CoA and is deficient in SLS, or the defect may alter an enzyme binding site for palmitoyl CoA to a greater extent than the catalytic site. Fatty alcohol:NAD$^+$ oxidoreductase has not been purified and little is known about its properties. In rodent liver, enzyme activity is almost entirely particulate (14).

The finding that intact SLS fibroblasts oxidized some hexadecanol to fatty acid suggests that fatty alcohol:NAD$^+$ oxidoreductase activity is not completely deficient within the intact cell or that alternate pathways may exist for fatty alcohol oxidation, perhaps through a fatty aldehyde intermediate.

We have recently found deficiency of fatty alcohol:NAD$^+$ oxidoreductase in leukocytes from SLS patients (Rizzo, W. B., A. L. Dammann, D. Craft, S. Black, A. Tilton Henderson, D. Africk, and E. Chaves-Carballo, manuscript in preparation). It seems likely that accumulation of fatty alcohol or its metabolites may be important in the pathogenesis of this disease. The in vivo effects of fatty alcohol accumulation in the skin or nervous system are unknown. In steroid sulfatase deficiency (X-linked ichthyosis) and in multiple sulfatase deficiency, skin changes are associated with accumulation of cholesterol sulfate (23, 24). The ichthyosis and neurological abnormalities in

Table III. Enzyme Activities Involved in Fatty Alcohol Metabolism in Normal, SLS, and Related Fibroblasts

Cells	Fatty alcohol:NAD+ oxidoreductase*		
	Total activity	Palmitoyl CoA-inhibitable activity	Palmitoyl CoA synthetase*
Normal (12)			
Mean±SD	59.5±12.7	32.1±7.4	6,240±1,840
Range	36.6–79.6	19.6–47.1	3,450–9,290
SLS			
T.J.	13.1	1.4	7,200
E.N.	4.3	0	4,960
An.B.	6.5	0	6,790
Am.B.	7.1	0	6,580
C.B.	6.9	0.1	6,330
Mean±SD	7.6±3.3	0.3±0.6	6,372±851
Obligate SLS heterozygotes			
T.B.	27.5	20.1	9,550
B.B.	28.7	16.0	6,940
Non-SLS abnormal controls			
X-linked Ichthyosis (1)	43.5	30.0	3,060
Multiple sulfatase deficiency (2)	37.7	24.1	4,180
Refsum disease (1)	51.9	32.3	3,630
Hypohidrotic ectodermal dysplasia (1)	51.9	23.5	6,300
Zellweger syndrome (2)	61.6	41.1	5,270
Adrenoleukodystrophy (3)	50.4±11.4	31.4±12.2	4,270±280
Pompe's disease (1)	74.5	28.9	11,250
Leber optic atrophy (1)	79.0	39.5	8,790

* Enzyme activity is expressed as picomoles per minute per milligram protein. Each cell line was assayed on two to four separate occasions; numbers in parentheses indicate the number of different cell lines tested.

Refsum disease may be the result of accumulation of phytanic acid due to defective alpha-oxidation (7, 25). Patients with ichthyosis due to neutral lipid storage disease accumulate triglyceride in skin and other tissues, including cultured fibroblasts (26, 27). Some other inherited forms of ichthyosis have been associated with epidermal or scale lipid abnormalities (28, 29), although no enzymatic defects have been identified; it is uncertain if the lipid changes have been primary or secondary to the basic genetic defect.

Table IV. Activity of Fatty Alcohol:NAD+ Oxidoreductase in Mixtures of Normal and SLS Fibroblast Homogenates

Normal homogenate added	SLS homogenate added	Fatty alcohol:NAD+ oxidoreductase activity observed (% of normal activity)*
	%	
100	0	100
75	25	73
50	50	42
25	75	25
0	100	0

* Enzyme activity represents total activity measured. The residual activity present in the assay containing 100% SLS homogenate was normalized to zero, and appropriate proportions of this activity were subtracted from the measured enzyme activity in each mixture to determine the observed activity.

In mammals, fatty alcohol synthesis occurs mainly by reduction of fatty acyl CoA substrates (30) (Fig. 3). Fatty alcohol may be utilized for biosynthesis of wax esters and glycerol ether lipids. Cultured human fibroblasts apparently do not synthesize wax esters, and incorporation of fatty alcohol into glycerol ether lipids (plasmalogens) is a minor pathway for hexadecanol metabolism (9). The finding that hexadecanol is simultaneously synthesized by cultured fibroblasts and oxidized to fatty acid has led to the hypothesis of a fatty alcohol cycle operating in these cells rather than a reversible interconversion of fatty acid and fatty alcohol (9). Our studies on SLS fibroblasts lend further support to this hypothesis. Since SLS cells accumulate radioactive hexadecanol during incubation with radioactive palmitate and are deficient in fatty alcohol:NAD+ oxidoreductase, it is unlikely that a significant proportion of fatty alcohol synthesis occurs by simple reversal of this reaction. However, it is still possible that hexadecanol is synthesized from palmitate or oxidized to palmitate to some extent through a free fatty aldehyde intermediate.

In normal cultured fibroblasts, fatty alcohol concentrations may be regulated in part by availability of acyl CoA as substrate for its biosynthesis and by activity of fatty alcohol:NAD+ oxidoreductase. Fatty alcohol oxidation is inhibited in intact fibroblasts by addition of exogenous palmitate to the medium, and fatty alcohol:NAD+ oxidoreductase activity is inhibited by palmitoyl CoA (9). The intracellular hexadecanol content of SLS fibroblasts was abnormally increased when cells were incubated in the absence of exogenous fatty acids in the growth medium, conditions which should normally allow maximal activity of fatty alcohol:NAD+ oxidoreductase. Furthermore, hexadecanol content of SLS fibroblasts seemed to show a blunted increase after addition of palmitate to the medium compared with normal cells. This correlates with the observation that the residual activity of fatty alcohol:NAD+ oxidoreductase in SLS cells was not subject to inhibition by palmitoyl CoA. If this phenomenon occurs in vivo, SLS patients may

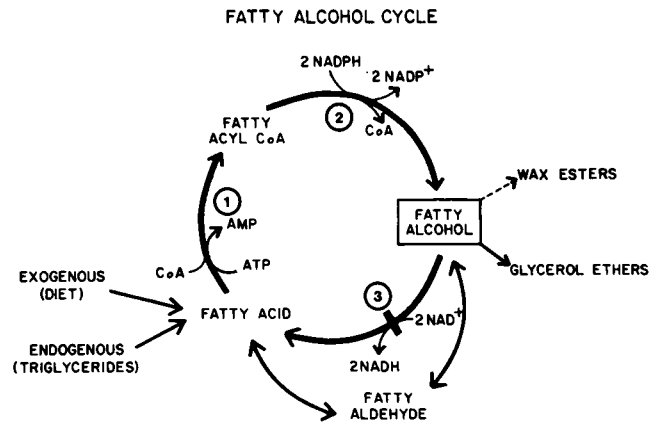

Figure 3. Proposed pathway of fatty alcohol metabolism in man: the "fatty alcohol cycle." Reaction 1 is catalyzed by acyl (palmitoyl) CoA synthetase; reaction 2 is catalyzed by acyl CoA reductase; and reaction 3 is catalyzed by fatty alcohol:NAD+ oxidoreductase. The bar across reaction 3 indicates the enzyme deficiency in SLS fibroblasts. The dashed arrow from fatty alcohol to wax esters indicates a reaction that does not occur in cultured human fibroblasts, although it presumably occurs in other human tissues.

exhibit both accumulation of fatty alcohol (or its metabolites) due to deficient fatty alcohol oxidation and deranged regulation of cellular fatty alcohol concentrations. This may be therapeutically important. Some SLS patients have responded favorably to a low fat diet supplemented with medium chain triglycerides (18, 19), which would be expected to decrease long-chain fatty alcohol synthesis and accumulation.

Our studies on cultured SLS fibroblasts suggest that deficiency of fatty alcohol:NAD^+ oxidoreductase is the primary enzymatic defect in this disease. The presence of intermediate levels of enzyme activity in obligate SLS heterozygotes is consistent with this hypothesis, and raises the possibility of heterozygote detection. Further investigations of fatty alcohol metabolism in SLS patients may provide insight into the etiology and pathogenesis of this inherited disorder, as well as highlight a neglected area of metabolism in man.

Acknowledgments

We thank Dr. Joel Avigan, Dr. Enrique Chaves-Carballo, and Dr. Susan Black for kindly providing SLS fibroblasts.

This research was supported by National Institutes of Health grant AM33914 and by Basil O'Connor Starter Research Grant 5-476 from the March of Dimes.

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