Isolation of Animal Cell Mutants Defective in Long-chain Fatty Aldehyde Dehydrogenase*

SENSITIVITY TO FATTY ALDEHYDES AND SCHIFF’S BASE MODIFICATION OF PHOSPHOLIPIDS: IMPLICATIONS FOR SJÖGREN-LARSSON SYNDROME*

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(Received for publication, March 21, 1997, and in revised form, June 25, 1997)

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Using trinitium suicide, we have isolated a variant of the Chinese hamster ovary cell line, CHO-K1, that is deficient in long-chain fatty alcohol:NAD⁺ oxidoreductase (FAO; EC 1.1.1.192). Specifically, it was the fatty aldehyde dehydrogenase component that was affected. The enzymatic deficiency found in this mutant strain, designated FAA.K1A, was similar to that displayed by fibroblasts from patients with Sjögren-Larsson syndrome (SLS), an inheritable neurocutaneous disorder. Complementation analyses suggested that the deficiency in fatty alcohol oxidation in the FAA.K1A cells and the SLS fibroblasts is a result of lesions in homologous genes. The FAA.K1A cells were unable to convert long chain fatty aldehydes to the corresponding fatty acids. This resulted in a hypersensitivity of the FAA.K1A cells to the cytotoxic effects of long chain fatty aldehydes. The difference between the mutant and wild-type cells was most obvious when using fatty aldehydes between 14 and 20 carbons, with the greatest difference between wild-type and mutant cells found when using octadecanal. Fibroblasts from a patient with SLS also displayed the hypersensitivity phenotype when compared with FAldDH⁺ human fibroblasts. In both CHO and human FAldDH⁻ cell lines, addition of long chain fatty aldehydes to the medium caused a dramatic increase in aldehyde-modified phosphatidylethanolamine, presumably through Schiff’s base addition to the primary amine of the ethanolamine head group. When 25 μM hexadecanal was added to the growth medium, approximately 10% of the phosphatidylethanolamine was found in the fatty aldehyde-modified form in FAA.K1A, although this was not observed in wild-type cells. Modified phosphatidylethanolamine could be detected in FAldDH⁻ cells even when exogenous fatty aldehydes were not added to the medium. We propose a possible role for fatty aldehydes, or other aldehydic species, in mediating some of the symptoms associated with Sjögren-Larsson syndrome.

Sjögren-Larsson syndrome (SLS),¹ first described in Sweden in 1957, is an autosomal recessive inherited human genetic disorder characterized by mental retardation, spastic di- or tetraplegia, and chronic ichthyosis (scaling of the skin) (1). The world-wide incidence of SLS is low; as of 1993, there had been about 200 diagnosed cases (2), but the incidence in Northern Sweden has been calculated to be as high as 1 in 12,000 (3). While the clinical diagnosis of SLS is based upon three obligate symptoms described above, other symptoms have been reported to be associated with this syndrome. Pigmental degeneration (3, 4), seizures (5), dental anomalies, speech impediments, and short stature (6, 7) have been noted, although not universally.

A biochemical abnormality associated with SLS was first reported in 1988 by Rizzo et al. (8) who showed that the levels of the long chain fatty alcohols (>14 carbons in length) were elevated in the serum as well as in fibroblasts obtained from SLS patients. This was shown to be a result of the decreased ability of these cells to oxidize long chain fatty alcohols to the corresponding fatty acids, due to a decrease in long-chain fatty alcohol:NAD⁺ oxidoreductase (FAO) activity (8, 9). FAO activity is comprised of two separable components; an alcohol dehydrogenase and an aldehyde dehydrogenase (10). It is the aldehyde dehydrogenase (FAldDH) that is deficient in SLS cells (11). The cDNA for human FAldDH has been isolated and used to demonstrate that patients with SLS have abnormalities within the structural coding sequence (12). These findings are good indicators that this enzymatic deficiency is the primary lesion involved in SLS.

Although the biochemical defect has been identified, the relationship between FAldDH deficiency and the symptoms observed in the patients has not been defined. As tools to study SLS, we have isolated mutants in a rodent cell line, CHO-K1, that share the same biochemical lesion as SLS fibroblasts. The animal cells were used since they are immortal, grow rapidly, and a number of experiments can be performed more rapidly than with human fibroblasts. Phenotypic changes found in the CHO mutants can then possibly be extended to the human condition.

We have previously reported the isolation of a CHO mutant cell line, FAA.1, that also displayed a deficiency in FAldDH activity (13). However, the FAA.1 variant was a double mutant, in that it was isolated from a CHO mutant cell line, ZR-82 (14), that was deficient in ether lipid biosynthesis (EL⁻) and perox-

¹ The abbreviations used are: SLS, Sjögren-Larsson syndrome; PBS, phosphate-buffered saline; FAO, fatty alcohol:NAD⁺ oxidoreductase; CHO, Chinese hamster ovary; EL, ether lipid; FAldDH, fatty aldehyde dehydrogenase; HAT, hypoxanthine/aminopterin/thymidine; Bicine, \( N,N\)-bis[2-hydroxyethyl]glycine.

This paper is available on line at http://www.jbc.org
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isome function. While FAA.1 is phenotypically similar to SLS fibroblasts with respect to the FAldDH deficiency, the EL phenotype and loss of functional peroxisomes complicates analyses concerning the relationship between the FAldDH deficiency and changes in cellular functions. For example, EL cells can accumulate significant amounts of long chain fatty alcohols (13, 15), a phenotype that has also been reported in FAldDH cells (8).

In this study, we used a selection procedure, similar to that used previously (13), to isolate an FAldDH− mutant cell line, FAA.K1A, from a wild-type (EL+) background. We report that animal cells defective in FAldDH activity (FAldDH−) were less able to metabolize long chain fatty aldehydes and were hypersensitive to the cytotoxic effects of these compounds. Importantly, this hypersensitivity phenotype could be extended to fibroblasts from an SLS patient. We also present evidence that Schiff’s base formations between long chain fatty aldehydes and biologically important amines are enhanced in FAldDH− cells. These findings may help to provide a possible connection between the biochemical defect and the symptoms associated with SLS.

EXPERIMENTAL PROCEDURES

Materials—[9,10-3H]Hexadecanoic acid (28 Ci/mmol; 1 Ci = 37 Gq), methylethyl-Hexadecanoic acid (248 Gq/mmol), and ENHANCE spray were purchased from NEN Life Science Products. [9,10-3H]Hexadecanol was synthesized from [9,10-3H]hexadecanoic acid by the method of Davis and Hajra (16). [1-14C]Hexadecanal (12.5 mCi/mmol) was purchased from Sigma. [1-14C]Hexadecanal was synthesized from [1-14C]hexadecanol, and other aldehydes were synthesized from the corresponding fatty alcohols (purchased from Sigma) by the method of Corey and Suggs (17). Once synthesized, aldehydes were quantitated spectrophotometrically after derivatization with p-nitrophenyldihydrazine (18). Decanal, hexanal, and NaCNBH₃ were purchased from Aldrich. All other biochemicals were purchased from Sigma unless otherwise noted in the text. N-Alkyl-phosphatidylethanolamines was synthesized using the method of Borch et al. (19) using [1-13C]Hexadecanal and egg yolk phosphatidylethanolamine.

Cell Lines and Culture Conditions—CHO-K1 was obtained from the American Type Culture Collection (Rockville, MD). Normal human fibroblasts (D.P.) and an unrelated SLS fibroblast (E.C.) cell line were generously donated by Dr. William B. Rizzo, Medical College of Virginia. The E.C. cells have been shown to be FAldDH− by Dr. Rizzo. All cell lines were maintained in Ham’s F12 nutrient mixture (Whittaker Bioproducts, Walkersville, MD), supplemented with 10% fetal bovine serum (Whittaker Bioproducts), 1 mM glutamine, 100 units/ml penicillin G, and 75 units/ml streptomycin, unless otherwise indicated. This growth medium is referred to as “F12c” throughout the text. Cells were routinely grown at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Tritium Suicide Selection—CHO-K1 cells (10⁶) were mutagenized using ethyl methanesulfonate (14), and the mutagenized population was allowed to grow for several generations to allow establishment of the phenotypes prior to selection. Mutagenized CHO-K1 cells were plated into several 75-cm² tissue culture flasks at 2 × 10⁶ cells/flask and incubated in 10 ml of F12c medium overnight at 33 °C. The following day, growth medium containing [9,10-3H]hexadecanol was added to achieve a final concentration of 20 μM hexadecanol at 2 μCi/ml. The cells were incubated for 3 h at 37 °C; the labeled medium was removed, and the cells were washed once with fresh F12c and then incubated for 1 h in unlabeled F12c at 37 °C. The medium was removed, and the cells were harvested with trypsin, pelleted by centrifugation at 600 × g, and then resuspended in F12c containing 10% (w/v) glycerol. This cell suspension was placed in vials (5 × 10⁶ cells/vial) and frozen in liquid N₂ (21). The cells were thawed periodically, and viable cells were allowed to grow and form colonies (7–10 days). When less than 100 colonies appeared from a vial (i.e., less than 0.02% survival), the surviving population was harvested and used for the generation of clonal isolates.

Complementation Analyses—When fusing CHO strain with another CHO strain, in each fusion pair, one cell line bearing the secondary mutations, resistance to ouabain, and a deficiency in hypoxanthine-guanine phosphoribosyltransferase/ouabain-sensitive phenotypes (20). The cell lines were plated together (2 × 10⁶ cells of each strain) in one well of a 24-well tissue culture plate and incubated overnight in F12c at 33 °C. The growth medium was removed, and the cells were washed twice with sterile PBS and then resuspended in F12c medium (5 × 10⁶ cells/ml) and triturated for 1 min. The suspension was mixed with 50% (w/v) polyethylene glycol (average molecular weight = 3400). The polyethylene glycol-containing medium was removed, and the cells were washed 7 times with PBS and incubated overnight in 1 ml of F12c. The medium was removed, and the cells were harvested with trypsin and plated into 100-mm tissue culture plates in 15 ml of F12c containing hypoxanthine aminopterin (5 × 10⁻⁵ M), and thymidine (1.5 × 10⁻⁵ M) (HAT medium; Ref. 21) containing 1 mM ouabain (22). After 2 weeks of selection in HAT medium (with medium changes every 3 to 5 days) the surviving hybrids were used for biochemical analyses.

When fusing CHO cells with human fibroblasts, CHO cells bearing the secondary mutations were fused to human fibroblasts. Due to the low frequency of successful CHO/human fusions, greater numbers of cells were used. Cells were plated together in 100-mm tissue culture dishes (3 × 10⁶ human/L × 10⁶ CHO) in 10 ml of F12c and incubated at 33 °C overnight. The medium was removed, and the cells were washed twice with sterile PBS and incubated for 3 min with F12, 50% PEG. The PEG-containing medium was removed, and the cells were washed 7 times with 7 ml of PBS. The medium was replaced with 15 ml of HAT selection medium containing 50 μM ouabain and 2 mM CaCl₂ (HOC medium). Twice during the selection period, the cells were harvested with trypsin, pelleted by centrifugation, and resuspended in 100-mm tissue culture dishes. This was followed, the next day, with a medium change to remove dead cells. The selection medium was changed every 3 to 5 days, and the surviving cells were evaluated for the ability to convert fatty alcohols to fatty acids after 4 weeks of selection.

Fatty Alcohol Oxidase and Fatty Aldehyde Dehydrogenase Activities in Whole Cell Homogenates—Cells were grown to near confluence in 100-mm diameter tissue culture dishes in F12c at 37 °C. For each dish, medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped into 3 ml of PBS. The cells were pelleted by centrifugation at 600 × g for 7 min, washed once with 5 ml of PBS, and resuspended in 1 ml of homogenization buffer (25 mM Tris-HCl, pH 8.0, 0.25 mM sucrose). The cell suspension was homogenized with 15 up and down strokes of a motorized glass-Teflon homogenizer and frozen at −70 °C. Protein content of each homogenate was determined by the method of Lowry et al. (23). Frozen whole cell homogenates were thawed on ice and sonicated for 30 s using a sonic water bath (Branson Ultrasonics Corporation, Danbury, CT; model 2210) prior to assay.

FAO activity was assayed by measuring the NAD+−dependent conversion of [9,10-3H]hexadecanol to the fatty acid essentially as described (13). The reaction mixture consisted of 50 mM Bicine, pH 8.5, 0.25 mg/ml fatty acid-free bovine serum albumin (2.5 mM NAD+, 5 μM [9,10-3H]hexadecanol (5 × 10⁶ cpm), and 25–50 μg of cellular protein in a final volume of 200 μl. Samples containing the complete reaction mixture (except the cell homogenate, replaced with the appropriate volume of homogenization buffer), were used as blanks. The reactions were initiated by the addition of NAD+ following a 5-min preincubation at 37 °C. After 20 min at 37 °C, in a shaking water bath, the reactions were terminated by the addition of 2 ml of 2% acetic acid in methanol. Following the addition of 100 μg of carrier lipid (total lipid from mouse liver), 2 ml of chloroform, and 1.5 ml of PEG were added to form a two-phase system (24). After vortexing, the mixture was centrifuged for 5 min at 600 × g to separate the phases, and the lower (organic) phase was recovered. The upper phase was washed once with 2 ml of chloroform and the two lower phases were combined. Lipids were separated by TLC on silica gel 60 (Merck) using hexane:ethyl ether:acetone (70:30:1) as the solvent system. After drying, the thin layer plates were sprayed with ENHANCE and exposed to Kodak X-AR film at −70 °C. Fatty alcohol, fatty acid, and fatty aldehyde were located using the x-ray film pattern and identified based on co-migration with authentic standards. The bands of interest were scraped into scintillation vials containing 1 ml of methanol and were counted using liquid scintillation spectrometry after adding 8 ml of Ecoscint A (Ecoscint A; National Diagnostics, Atlanta, GA) as scintillation fluid.

FAKDH activity was determined identically to FAO activity with the exception that 75 μM [1-14C]hexadecanal was used as substrate. All assays were performed under conditions in which the rate of fatty acid formation was linear.

Conversion of Fatty Alcohol and Fatty Aldehyde to Fatty Acid by Intact Cells—To measure fatty alcohol conversion, cells were plated into sterile glass scintillation vials, at cell densities that would yield 10–60

² William B. Rizzo, Dept. of Pediatrics and Human Genetics, Medical College of Virginia, Richmond, VA, personal communication.
µg of total cell protein, in 1 ml of F12c and allowed to attach overnight at 37 °C. Medium was removed, replaced with 0.8 ml of medium containing 5 µg [9,10-3H]hexadecanol (3.8 × 10^6 cpm/vial), and cells were incubated for 3 h at 37 °C. Following this, 3 ml of chloroform: methanol (2:1), 900 µg of carrier lipid (total lipid extract from bovine heart), and 50 µl of 1 M HCl were added to form a single-phase Bligh and Dyer system (24). This single phase mixture was transferred to a test tube, and a two-phase system was formed by the addition 1 ml of chloroform and 1 ml of PBS followed by vortexing. After separation of the phases by centrifugation, the lower (organic) phase was recovered.

The upper phase was washed once with 2 ml of chloroform, and the lower phases were combined. The lipid extracts were taken up in 3 ml of a solution of urea:ethanol (1:3). After drying, DNA was solubilized by adding 0.3 ml of 0.5% (w/v) Coomassie Blue in methanol:H2O:acetic acid (45:45:10) followed by sonication for 5 min in a sonicating bath immediately prior to addition to the cells in all cases.

Detection of Schiff’s Base-modified Phosphatidylethanolamine—Cells (10^5) were plated, in 1 ml of F12c, into sterile glass scintillation vials and allowed to attach overnight at 33 °C. The following day, 0.5 ml of F12c containing 1.5 µCi of [1-14C]hexadecanal was added, and the cells were incubated for 3 days at 37 °C. The labeled medium was removed and replaced with 1 ml of F12c containing 20 µg [9,10-3H]hexadecanol (5 × 10^6 cpm/ml). The cells were incubated at 37 °C after which the medium was removed, and the cells were washed once with 0.4 ml of PBS, and the medium and wash were combined in a 1.5 ml microcentrifuge tube. The medium was centrifuged at 12,000 × g for 1 min to pellet out any residual cells, and 0.8 ml of the supernatant was used for liquid extraction (24) as described above, under neutral conditions (no HCl added). After evaporation of the solvent, fatty aldehyde was isolated on silica gel 60 using a triple development system (25). This involved successive developments of the TLC plate in benzene, hexane, and hexane:diethyl ether-acetic acid (70:20:1) with drying between runs. The thin layer plates were sprayed then with EN3HANCE and exposed to Kodak X-AR film at −70 °C. Radioactive fatty aldehyde was identified based on co-migration with authentic standard. The aldehyde band was scraped into a scintillation vial containing 1 ml of methanol and was counted using liquid scintillation spectrometry after adding 8 ml of scintillation fluid.

Fatty Aldehyde Release into the Medium—Cells were plated into sterile glass scintillation vials (10^5 cells/vial) and allowed to attach overnight at 37 °C. The next day, medium was removed and replaced with 1 ml of F12c containing 2 µg [9,10-3H]hexadecanol (5 × 10^6 cpm/ml). The cells were incubated at 37 °C after which the medium was removed, and the cells were washed once with 0.4 ml of PBS, and the medium and wash were combined in a 1.5 ml microcentrifuge tube. The medium was centrifuged at 12,000 × g for 1 min to pellet out any residual cells, and 0.8 ml of the supernatant was used for liquid extraction (24) as described above, under neutral conditions (no HCl added).

Cytotoxicity Assays—Cytotoxicity was determined either using a u.v. clonogenic assay or by measuring the incorporation of [methyl-3H]thymidine into DNA (26) following aldehyde treatment. For experiments using the clonogenic assay, cells were plated in 24-well tissue culture plates at 4 × 10^4 cells/well in 0.4 ml of growth medium, F12c, and allowed to attach overnight at 37 °C. The following day, medium was removed and replaced with 0.4 ml of F12c containing the test compound at the indicated concentration, and the cells were incubated at 37 °C. After 8 h, a 0.2-ml bolus of F12c containing the same concentration of test compound was added to the well, and the cells were incubated for an additional 20 h at 37 °C. The aldehyde-containing F12c medium was removed and replaced with 1 ml of F12c containing no additions, and the cells were incubated for an additional 5–7 days at 37 °C to allow the surviving cells to grow. The colonies resulting from the surviving cells were visualized by staining with Coomassie Blue. For staining, the medium was removed, the cells were washed once with 2 ml of PBS, and 0.5 ml of 0.5% (w/v) Coomassie Blue in methanol:H2O:acetic acid (45:45:10) was added for 30 min. This was removed, and the cells were washed twice with 2 ml in methanol:H2O:acetic acid (45:45:10) to remove residual stain.

For [methyl-3H]thymidine labeling, cells were labeled after 3 days of growth following aldehyde treatment. Medium was removed and replaced with 0.4 ml of F12c containing [methyl-3H]thymidine at 2.0 µCi/ml, and the cells were incubated for 2 h at 37 °C. The labeling medium was then removed, and 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) were added to cell monolayers were washed 5 times with 1 ml of 10% trichloroacetic acid (ice-cold) and twice with 1.5 ml of ice-cold ethyl ether:ethanol (1:3). After drying, DNA was solubilized by adding 0.3 ml of 0.5 N NaOH and incubating for 2 h at 37 °C. Aliquots were counted by liquid scintillation spectrometry after neutralization with HCl.

Aldehydes were added to the medium and sonicated for 5 min in a sonicating bath immediately prior to addition to the cells in all cases.

Detection of Schiff’s Base-modified Phosphatidylethanolamine—Cells (10^5) were plated, in 1 ml of F12c, into sterile glass scintillation vials and allowed to attach overnight at 33 °C. The following day, 0.5 ml of F12c containing 1.5 µCi of [1-14C]ethanolamine was added, and the cells were incubated for 3 days at 37 °C. The labeled medium was removed and replaced with 1 ml of F12c containing 10 µg [9,10-3H]hexadecanol. The cells were worked up immediately, or, alternatively, 1 ml of F12c containing the indicated addition (usually fatty aldehyde) was added to the labeled cells, which were then incubated for an additional 3 h at 37 °C prior to workup.

To work up the samples, medium was removed, and the cells were washed once with PBS. One-half of the vials for any given treatment were treated with PBS alone (no NaCNBH3). Following this, the cells could be easily detached from the glass surface with gentle agitation, after which the cell suspension was transferred to a 15-ml conical centrifuge tube. Residual cells were recovered with 5 ml of PBS, and this wash was added to the original cell suspension. The cells were pelleted by centrifugation for 7 min at 600 × g. The supernatant was removed, and the cells were washed 3 times using 10 ml of PBS, and the final cell pellet was resuspended in 1 ml of PBS. For each sample, 0.1 ml of the cell suspension was used for protein determination, and 0.8 ml was added to a glass tube containing 2 ml of methanol, 1 ml of chloroform, and 300 µg of carrier lipid (total mouse liver lipid extract) to form a single-phase Bligh and Dyer system (24). Treatment of cells with NaCNBH3, resulted in the same recovery of cellular protein when compared with cells that had been treated with PBS alone. The lipids were extracted as described above for the FAO assays, under neutral conditions. Organic solvent was removed, and the lipids were resuspended in chloroform and separated on silica gel 60 (Merck) using chloroform: methanol (90:10; v:v). The TLC plates were exposed to x-ray film at −80 °C following treatment with EN3HANCE spray. Bands of interest were scraped into scintillation vials and quantitated by liquid scintillation spectrometry.

RESULTS

Tritium Suicide Selection of FAO-deficient Mutants—Our intent was to isolate cells that were unable to convert long chain fatty alcohols (e.g. hexadecanol) to the corresponding fatty acid due to a loss of long-chain fatty alcohol oxidase (FAO) activity. Tritium suicide was used as the selection method. Cells that have accumulated a tritiated compound will be damaged by the radioactive decay of the tritium while frozen in liquid nitrogen. If the damage is extensive enough, the cells will not survive when thawed. The severity of the damage is dependent on the amount of tritium accumulated and the amount of time frozen. Cells that have accumulated less tritium will survive longer freezing periods. The majority of [9,10-3H]hexadecanol taken up by a cell is incorporated, as the alcohol, into ether lipids or is converted to the corresponding fatty acid and incorporated into complex lipids (28). Mutant cells that cannot form ether lipids (EL−) or that cannot convert [9,10-3H]hexadecanol to hexadecanolic acid (FAO−) incorporate less tritium than wild-type cells (13) and survive longer freezing times after incubation with [9,10-3H]hexadecanol.

A mutagenized population of CHO-K1 cells were allowed to take up [9,10-3H]hexadecanol over a period of 3 h. This was followed by a 1-h incubation in growth medium that contained no hexadecanol, to allow any uncomplexed fatty alcohol to diffuse out of the cells. Under these conditions, the cells accumulated an average of 0.75 dpm/cell. The labeled cells were then frozen in cryogenic medium in a series of vials. At 1 week intervals, a vial was thawed, and the surviving cells were allowed to grow out and form colonies. After 14 weeks of frozen storage, only 25–50 colonies were generated from a vial of approximately 5 × 10^6 labeled cells. This surviving population was harvested, and eight cell lines were cloned by limiting dilution.

Of eight isolates generated from this population, all but one displayed a defect in long-chain fatty alcohol oxidase (FAO) activity when measured using whole cell homogenates (Fig. 1). The homogenates from these FAO− strains (lanes 2 and 4–9)
were unable to convert hexadecanol to the fatty acid; instead, fatty aldehyde accumulated. This labeling pattern was observed in the previously described (13) FAO− strain, FAA.1 (lane 10). Homogenates from one of the isolates, FAA.K1B (lane 3), showed a labeling pattern that was similar to wild-type cells (lane 1). This mutant was further characterized and shown to have a defect in acyl/alkyl-DHAP reductase (the third step in ether lipid biosynthesis) as reported in the accompanying paper (29). We chose FAA.K1A (lane 2), an FAO− strain, for further analysis in this study.

Quantitation of FAO activity in the CHO cells (Table I) showed that FAA.K1A homogenates were severely deficient in the ability to convert fatty alcohol to fatty acid, with a reduction to 2% of wild-type cells. FAO activity is catalyzed by two polypeptides, a fatty alcohol dehydrogenase (which forms the aldehyde) and a fatty aldehyde dehydrogenase (FAldDH, which completes the oxidation to fatty acid). The accumulation of the aldehyde on the autoradiogram (Fig. 1) suggested that the latter component of the FAO system, FAldDH, was defective in FAA.K1A. Measurement of FAldDH activity in whole cell homogenates of FAA.K1A confirmed this (Table I). The mutants displayed 10% of the activity found in CHO-K1.

We also examined the FAO system in human fibroblasts. In general, whole cell homogenates from human fibroblasts displayed higher levels of FAO activity (Table I). The specific activity of normal human cells was three times that observed in the wild-type CHO cells (Table I). Fibroblasts from a patient diagnosed with SLS2 displayed much lower FAO activity than the normal human fibroblasts although the decrease was less severe (22% of normal fibroblasts). The increase in FAO activity of normal human cells was three times that observed in CHO-K1. Mutants and FAldDH activity in whole cell homogenates of FAA.K1A and the SLS fibroblasts are the result of mutations in FAA.K1A, and the SLS fibroblasts are the result of mutations in FAA.K1A.

**Table I**

| Cell line | FAO activity in cell homogenates | FAldDH activity in cell homogenates | Fatty acid formed from fatty aldehyde by intact cells |
|-----------|----------------------------------|-------------------------------------|---------------------------------------------------|
| CHO-K1    | 17.0 ± 3.0                      | 1,311 ± 97                          | 1,186                                              |
| FAA.K1A   | 0.3 ± 0.5                       | 136 ± 50                            | 157                                               |
| Normal human fibroblasts | 51.6 ± 9.5 | 11,934 ± 195 | 3,949 |
| SLS fibroblasts | 11.6 ± 0.8 | 3,027 ± 197 | 2,079 |

*Whole cell homogenates were prepared, and assays were performed as described under "Experimental Procedures." Each value represents the average ± S.D. of three determinations.

**Table II**

| Cell lines | Fatty acid formed by intact cells |
|------------|----------------------------------|
| K1         | 13.9 ± 1.1                       |
| K1A        | 3.4 ± 0.4                        |
| NH fibroblasts | 24.3 ± 4.9                      |
| SLS fibroblasts | 5.2 ± 2.2                      |
| Hybridsa   | CHO × CHO                        |
| K1 × K1    | 16.2 ± 1.4                       |
| K1 × K1A   | 10.7 ± 1.1                       |
| Hybridsa   | CHO × fibroblast                |
| K1 × SLS   | 15.7 ± 0.5                       |
| K1A × SLS  | 1.8 ± 0.6                        |
| K1A × NH   | 15.4 ± 2.3                       |

*All values represent the average ± S.D. of three determinations.

The abbreviations used are: K1, CHO-K1; K1A, FAA.K1A; NH, normal human fibroblasts; SLS, fibroblast from patient with SJögren-Larsson syndrome; ND, not determined.

**Figure 1**

Fatty alcohol oxidation by tritium suicide survivors. Homogenates (25 μg of cellular protein) from clonal isolates obtained from the surviving population were incubated in the presence of [9,10-3H]hexadecanol and NAD+ (13) for 20 min at 37 °C. The lipids were extracted from the assay mix under acidic conditions and separated using silica gel 60 (Merck) and n-hexane:ethyl ether:acetic acid (70:30:1) as the solvent system. The thin layer plate was sprayed with

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  <li>aldehyde</li>
  <li>acid</li>
  <li>alcohol</li>
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in homologous genes. Alternatively, these results could suggest that the FAldDH in one species was unable to interact and function in coordination with the fatty alcohol dehydrogenase of the other species.

**Hypersensitivity of Mutant CHO Cells to Long Chain Fatty Aldehydes**—The FAldDH− cells were less able to oxidize long chain fatty aldehydes. These reactive molecules can chemically interact with a variety of macromolecules, including proteins and phospholipids. Should fatty aldehydes accumulate within the cell, cell functions could be disrupted. We hypothesized that the FAldDH− cells, less able to metabolize these compounds, should be hypersensitive to the toxic effects of exogenous long chain fatty aldehydes. Fig. 2A shows the cytotoxic effects of octadecanal (18:0 fatty aldehyde) on CHO-K1 and FAA-K1A. In this assay, the cells were treated twice, over a period of 28 h, with these compounds. The second round of additions enhanced the cytotoxicity. The FAldDH− strain, FAA.K1A, was dramatically more sensitive to octadecanal than the wild-type cells. At 60 μM, the lowest dose tested in this experiment, there was little survival in the FAA.K1A population, whereas the CHO-K1 cells were unaffected. We obtained identical results using other FAldDH− CHO strains. The SLS fibroblasts were also more sensitive to lower concentrations of fatty aldehyde than the normal fibroblasts (Fig. 2B). In general, the normal and SLS fibroblasts were more resistant to octadecanal than their CHO counterparts. This may have been the result of higher levels of FAldDH activity and the greater capacity to metabolize fatty aldehyde (Table I).

A variety of fatty aldehydes were tested using wild-type and mutant CHO cells (Fig. 3). The major difference between aldehydes was the chain length. The FAA.K1A cells were more sensitive to almost all of the aldehydes we examined. Higher levels of aldehyde were required when using compounds with aliphatic chains of less than 12 carbons or greater than 18 carbons. Aldehydes of 6 and 26 carbons were not toxic under the conditions used in this study. Hypersensitivity of FAA.K1A was observed only with the aldehyde. Under no conditions did we observe a difference in the sensitivity of the two cell lines to either fatty alcohols or fatty acids (not shown).

**Aldehyde-modified Phosphatidylethanolamine in FAldDH−**

3 We have also examined the sensitivity of a FAldDH− strain, FAA.1, which had been isolated from an ether lipid-deficient background (13), to octadecanal and found this variant strain to display an identical hypersensitivity phenotype (unpublished data). FAA.1 and FAA.K1A are in the same complementation group (unpublished data).
We quantitated the amount of ethanolamine label found as N-alkyl-phosphatidylethanolamine in CHO cells under different conditions (Fig. 5). Under normal growth conditions (F12c) the level of NaCNBH₃-dependent labeling of the modified lipid was increased in FAA.K1A cells severalfold over the CHO-K1 cells. Under these conditions the FAldDH-cells contained 6 times as much label in this lipid than wild-type cells, comprising 2–3% of the total ethanolamine-labeled lipid. In wild-type cells, the addition of 25 μM hexadecanal to the medium for 3 h prior to extraction of the lipids caused only a slight increase in label found in the modified lipid, although there was a dramatic increase in the mutants. Approximately 10% of the total ethanolamine label was now found in this lipid in the FAA.K1A cells. Concurrently, 6–10% of the label was lost from phosphatidylethanolamine (not shown). The addition of decanal, an aldehyde that should be oxidized by other dehydrogenase activities (30), resulted in no increase in the labeling of the new lipid when compared with the untreated cells. Increasing the level of serum in the medium from 10 to 50% had no effect on the labeling pattern, showing that components within the serum do not stimulate the modification of phosphatidylethanolamine.

Similar results were observed when using human cells. Fibroblasts from an SLS patient also displayed more NaCNBH₃-dependent labeling of the modified lipid (2.0% of the lipid-associated label) compared with normal fibroblasts (0.7%) following an additional 3-h incubation in medium containing 25 μM hexadecanal (160 Ald), 25 μM decanal (10:0 Ald), or 50% fetal calf-serum (50% serum). The data are presented as the percent of label found in the N-alkyl-phosphatidylethanolamine band following NaCNBH₃ treatment, lipid extraction, and development on TLC as described in Fig. 4. This workup was performed immediately following ethanolamine labeling (F12c) or following an additional 3-h incubation in medium containing 25 μM hexadecanal (16:0 Ald), 25 μM decanal (10:0 Ald), or 50% fetal calf-serum (50% serum). The data are presented as the percent of label found in the N-alkyl-phosphatidylethanolamine band following NaCNBH₃ treatment. Cell samples that were not treated with NaCNBH₃ were used as controls and contained little radioactivity. The increase in N-alkylphosphatidylethanolamine-associated label found in the hexadecanal-treated cells was accompanied by a loss of label in phosphatidylethanolamine. All values represent the average ± S.D. of three samples. Open bars, CHO-K1; cross-hatched bars, FAA.K1A.

**DISCUSSION**

The biochemical basis for Sjögren-Larsson syndrome has been identified as a defect in long-chain fatty aldehyde dehydrogenase (9), but the relationship between the loss of this activity and the symptoms associated with this disease have not been defined. We isolated a FAldDHvariant of the CHO.K1 cell line, FAA.K1A, and have demonstrated that the loss of this activity results in a hypersensitivity to the cytotoxic effects of long-chain fatty aldehydes. This is a phenotype that was consistent with the loss of FAldDH in other CHO variants. Importantly, these observations could be extended to the human fibroblasts. The SLS fibroblasts, which demonstrated the same enzymatic and, possibly, the same genetic lesion, also demonstrated a marked hypersensitivity to fatty aldehyde. The fact that it required higher levels of fatty aldehydes to kill the human fibroblasts than their CHO counterparts may have been due to the higher levels of FAldDH activity. Still the difference in sensitivity between the normal and the SLS fibroblasts was striking.

We also present evidence for the interaction of fatty aldehydes with ethanolamine phospholipids, through Schiff's base formation, in the FAldDHcells (both FAA.K1A and SLS fibroblasts) under normal growth conditions. The levels of modified ethanolamine phospholipids could be dramatically increased by the addition of long chain fatty aldehydes to the growth medium. Although the increased toxicity of fatty aldehydes toward the FAldDH CHO cells correlated with the increased formation of aldehyde-modified phosphatidylethanolamine.
amine, we could not determine if fatty aldehydes’ interactions with ethanolamine phospholipids were responsible for the cytotoxicity of the fatty aldehydes. Certainly, such modification to a significant percentage of a major phospholipid class could result in lethal changes in membrane structure and function. However, a major contributor to fatty aldehydes’ cytotoxic effects may well be their ability to modify other macromolecules, such as other lipids, proteins, and DNA.

Due to earlier findings that long chain fatty alcohols accumulate in the serum of homozygous SLS patients (8), attention has focused on the possibility that these molecules accumulate in tissues and membranes, disrupting cellular functions. Certainly, this may contribute to the symptoms presented in these patients. Fatty alcohols have been shown to have physiological effects; short and medium chain fatty alcohols have a fluidizing effect on biological membranes and do display anesthetic properties (31). A long chain fatty alcohol, docosanol, has been shown to be neurotrophic (32). Aldehydes can also have significant effects on functions of the cell and the organism as a whole. Chronic, abnormal protein/aldehyde interactions have been implicated in complications associated with diabetes (33), alcoholism (34), and aging (35). Fatty aldehydes may also interfere in biological processes that involve other aldehydes. For example, Schiff’s base interactions are important in the formation of collagen fibers (36,37) and in the light-activated signal transduction mediated by rhodopsin (38). Long chain fatty aldehydes may impair the formation of retinoic acid from dietary β-carotene, a process that involves a retinal intermediate (39).

No data have yet been presented regarding accumulation of long chain fatty aldehyde or fatty aldehyde-modified macromolecules in the tissues or plasma of patients with SLS. In our studies, using the human fibroblasts, we did not observe release of fatty aldehydes into the medium by SLS fibroblasts. We observed this only in the FAldDH-CHO cells that displayed a more stringent enzymatic deficiency (Fig. 7). Consistent with these data, analysis of plasma from SLS patients has failed to reveal measurable levels of long chain fatty aldehydes. The evidence of aldehyde-modified lipid in SLS cells does suggest that tissues in patients with SLS may be exposed to chronic, low levels of fatty aldehyde or other aldehydic species, which have been generated within those tissues. Cell functions could be affected in more subtle but important ways under these circumstances, and this may play a significant role in the symptoms associated with this disorder.

In light of the data presented here, the role of fatty aldehydes in Sjögren-Larsson syndrome should be considered. It may be worthwhile examining patients’ tissues for evidence of aldehyde modifications and developing or using drugs that block the fatty alcohol dehydrogenase(s) to reduce the rate of formation and accumulation of the fatty aldehydes.

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J. Biol. Chem. 1997, 272:23532-23539.
doi: 10.1074/jbc.272.38.23532

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