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Sjögren-Larsson syndrome: A biochemical rationale for using aldehyde-reactive therapeutic agents

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ABSTRACT

Sjögren-Larsson syndrome (SLS) is a neurocutaneous disease caused by mutations in *ALDH3A2* that result in deficient fatty aldehyde dehydrogenase (FALDH) activity and impaired fatty aldehyde and fatty alcohol oxidation. The pathogenesis of SLS is thought to involve accumulation of long-chain fatty aldehydes and alcohols and/or metabolically-related ether glycerolipids. Fatty aldehydes are particularly toxic molecules that can covalently react with proteins and certain amino-containing lipids such as phosphatidylethanolamine (PE), generating an unusual aldehyde adduct, N-alkyl-PE (NAPE). Using Faldh-deficient Chinese hamster ovary cells (FAA-K1A) as a cellular model for SLS, we investigated the ability of an aldehyde trapping agent, ADX-102 [2-(3-amino-6-chloro-quinolin-2-yl)-propan-2-ol], to mitigate the harmful effects of fatty aldehydes. FAA-K1A cells were protected from octadecanal (C18:0-al) induced cytotoxicity and apoptosis by ADX-102. Metabolism of C18:0-al to fatty alcohol (octadecanol) was also inhibited by ADX-102. FAA-K1A cells accumulated 5-fold more NAPE with C16- and C18-linked N-alkyl chains compared to wild-type cells, but NAPE levels decreased to normal after growth for 4 days with 50 μM ADX-102. Our results suggest that small aldehyde-reactive molecules, such as ADX-102, should be explored as novel therapeutic agents for SLS by preventing aldehyde adduct formation with critical cellular targets and inhibiting fatty aldehyde metabolism to fatty alcohol.

1. Introduction

Sjögren-Larsson syndrome (SLS) is a rare inborn error of fatty aldehyde oxidation caused by mutations in the *ALDH3A2* gene, which result in deficient activity of fatty aldehyde dehydrogenase (FALDH) [1]. Patients with SLS have characteristic clinical features of ichthyosis, spastic diplegia, intellectual disability, seizures, and a distinctive retinopathy. The ichthyosis is usually present at birth and neurologic symptoms appear later in the first or second year of life. These symptoms are thought to arise from accumulation of long-chain fatty aldehydes and related lipids, such as fatty alcohols, which are catabolized through FALDH-dependent pathways [2].

Aliphatic aldehydes are derived enzymatically from several diverse biochemical pathways and non-enzymatically during lipid peroxidation [2,3]. FALDH is important for elimination of long-chain aldehydes by catalyzing their oxidation to fatty acids (Fig. 1). This enzyme also participates in the sequential oxidation of fatty alcohols to fatty acids as a component of the fatty alcohol:NAD^+ oxidoreductase complex. Thus, FALDH deficiency in SLS impairs oxidation of both fatty aldehydes and alcohols. In cultured SLS keratinocytes [4], skin [5] and brain [6], excess fatty alcohols build up and are diverted into synthesis of ether glycerolipids and/or wax esters, which also accumulate and likely contribute to tissue dysfunction. In contrast, free fatty aldehydes have not been found to accumulate appreciably, although evidence for their metabolism to fatty alcohols has been demonstrated in cultured cells [7,8].

Unlike fatty alcohols and their metabolic products, the reactive carbonyl group of fatty aldehydes has led to the suspicion that these lipids are particularly toxic and intimately involved in the pathogenesis of SLS [2]. Aldehydes are highly reactive molecules that can form covalent adducts with proteins, DNA and certain amino containing lipids such as phosphatidylethanolamine (PE) [9]. Recent studies indicate that certain fatty aldehydes that are normally oxidized by FALDH, such as 2-

Abbreviations: ADX-102, [2-(3-amino-6-chloro-quinolin-2-yl)-propan-2-ol]; C16:0-al, hexadecanal; C16:1-al, 2-hexadecenal; C18:0-al, octadecanal; CHO, Chinese hamster ovary; FALDH, fatty aldehyde dehydrogenase; NAE, N-alkyl-ethanolamine; NAPE, N-alkyl-phosphatidylethanolamine; N-16:0-PE, N-hexadecanyl-phosphatidylethanolamine; N-18:0-PE, N-octadecanyl-phosphatidylethanolamine; PE, phosphatidylethanolamine; SLS, Sjögren-Larsson syndrome..

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hexadecenal (C16:1-al) and hexadecanal (C16:0-al), can covalently react with a wide range of proteins [10] and certain small molecules including amino acids and glutathione [11]. The spectrum of potential molecular targets for aldehyde adduct formation is wide and could impact a variety of metabolic pathways, both lipid and non-lipid in nature.

Currently, there is no effective therapy for SLS. Therapeutic strategies to mitigate aldehyde toxicity in other conditions have generally focused on inducing alternative enzyme pathways to eliminate aldehydes [12], using antioxidants to prevent their formation from lipid peroxidation [13] and exploring carbonyl-reactive chemicals to inactivate or "trap" aldehydes [14]. Recently, an aldehyde trapping agent, ADX-102 (Aldeyra Therapeutics), was developed for treating eye diseases associated with inflammation and short-chain aldehyde production. Its use as a long-chain aldehyde blocking agent in SLS has not been demonstrated. Here, we report that ADX-102 has efficacy in preventing formation of covalent long-chain aldehyde adducts with PE in cultured FALDH-deficient cells, protecting the cells from aldehyde cytotoxicity, and blocking fatty aldehyde conversion to fatty alcohol. These results provide a biochemical rationale for investigating aldehyde blocking agents in the treatment of SLS.

2. Materials and methods

2.1. Chemicals and materials

L-α-Phosphatidylethanolamine (PE) from egg yolk was obtained from Sigma Chemical Company (St. Louis, MO). ADX-102 [2-(3-amino-6-chloro-quinolin-2-yl)-propan-2-ol] was a gift from Aldeyra Therapeutics (Lexington, MA). [9,10-^{3}H]-Stearic acid (60 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and used to synthesize radioactive octadecanol by chemical reduction with LiAlH as described [15]. Solvents were HPLC-grade from Fisher Scientific Company (Hampton, NH). All other chemicals were from Sigma Chemical Company. Dulbecco's MEM/F12 cell media were obtained from GE Healthcare Life Sciences, HyClone Laboratories (Logan, UT). The Cell Counting Kit-8 was obtained from Sigma Chemical Company. Fatty aldehydes were synthesized from their corresponding alcohols using dipyridine chromic anhydride [16], purified by TLC in a solvent system consisting of petroleum ether/diethyl ether (90/10), and stored in hexane at −20 °C under a nitrogen atmosphere. Channelized silica gel G thin-layer chromatography plates were obtained from Silicycle (Quebec, Canada).

2.2. Cells

Wild-type Chinese hamster ovary cells (CHO-K1) and Faldh-deficient cells (FAA-K1A) [17] were kindly provided by Dr. R. Zoeller, Boston University. CHO cells were grown in a 5% CO2 atmosphere at 37 °C in DMEM/F12 media supplemented with 10% fetal bovine serum, penicillin and streptomycin.

2.3. Chemical synthesis of NAPE

To synthesize NAPE, 7.46 mg phosphatidylethanolamine and 4.8 mg C16:0-al (or other fatty aldehydes) were dissolved in a 1:2 M ratio in 400 μl of chloroform/methanol (1/1) and allowed to react at room temperature under a nitrogen atmosphere for 2 h [18]. To reduce Schiff base adducts, 100 μl of freshly made stock $NaBH_3CN$ (125 mM) in ethanol was added and the reaction was placed at 4 °C for 30 min. After warming to room temperature, 2 ml of chloroform/methanol (1/1) and 0.75 ml water were added and the mixture was shaken in a vortex shaker. The tube was centrifuged at 3000 xg for 5 min to hasten phase separation. The upper aqueous phase was discarded. The lower organic phase containing NAPE, PE and reduced C16:0-al (now hexadecanol) was dried under nitrogen, dissolved in chloroform and spotted on a channelized silica gel G thin-layer chromatography plate using most of the lanes. PE was spotted on an adjacent lane as standard. The plate was developed in chloroform/methanol (90/10). After drying the TLC plate, the lane containing PE and one lane containing the NAPE reaction product were visualized by spraying with rhodamine G and examining under UV light. A NAPE spot at Rf 0.4-0.5 and a prominent PE spot at Rf 0.05 were seen. The silica regions containing NAPE were collected by scraping and NAPE was eluted with 2 ml of chloroform/methanol (1/1), dried under nitrogen and stored in chloroform at 4 °C.

To quantitate the purified NAPE, an aliquot was dried under nitrogen and dissolved in 2 ml of 1 N methanolic-NaOH in an 8 ml Teflon-lined screw-capped glass tube. The NAPE was hydrolyzed in a 150° oven for 1 h to generate N-alkylethanolamine (NAE). After cooling to room temperature, 2 ml of water was added and NAE was extracted twice with

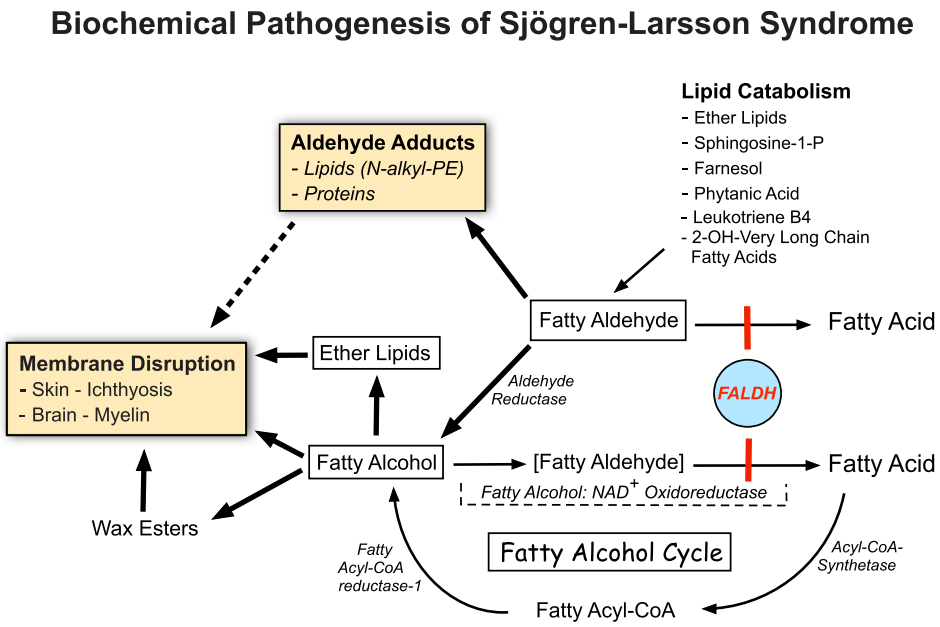

Fig. 1. Biochemical pathway for fatty aldehydes and fatty alcohols in SLS. Note that FALDH deficiency blocks oxidation of both fatty aldehydes and alcohols. Dashed arrow indicates hypothesized contribution of aldehyde adducts to membrane disruption.

2 ml of hexane. The overall yield of NAPE was typically 20-30% of the initial PE, determined by quantitation of NAE by GC-MS.

2.4. Chemical synthesis N-alkylethanolamine (NAE)

NAEs were synthesized by a modification of the method for NAPE using ethanolamine in place of PE. A 2:1 M ratio of fatty aldehyde to ethanolamine was incubated in 200 μl methanol for 2 h in a shaking water bath at 37 °C. The Schiff base adduct was reduced by adding $NaBH_3CN$ in methanol to a final concentration of 25 mM and incubating at 4° for 30 min. NAE was extracted according to Folch et al. [19] and the organic phase was recovered and dried under nitrogen. NAE was purified by TLC in a solvent system consisting of chloroform/methanol/acetic acid (90/10/1). NAE migrated with Rf 0.18 and was visualized by spraying the plate with water. After drying, NAE was recovered by extracting the silica with chloroform/methanol (1/1) and stored at 4 °C in chloroform under a nitrogen atmosphere. The yield was approximately 20% of the added fatty aldehyde. N-16:0-ADX-102 was synthesized in a similar manner.

2.5. GC-MS analysis of NAE

The trimethylsilyl derivatives of NAE were prepared by reaction with 50 μl N,O-Bis(trimethysilyl)trifluoroacetamide/trimethylchlorosilane at 70 °C for 1 h. Derivatized NAEs were analyzed on an Agilent 6890 gas chromatograph equipped with a 5973 Mass Selective Detector using a HP-5MS (30 m × 0.25 mm ID) column with helium carrier gas at a column flow rate of 1 ml/min. The samples were injected in the splitless mode. The inlet temperature was 250 °C. The Initial oven temp was 100 °C for 2 min, ramp temp 6 °C/min to 324 °C, and final time 2 min. The mass detector was operated in electron impact mode with MS source at 230 °C. Single ion monitoring was used to identify and quantitate NAEs according to their characteristic fragment ions (Table 1). NAEs were quantitated using C22:0-NAE (*m/z* 338) as internal standard or, in experiments with liver microsomes and intact cells, NAE content was expressed as a ratio to cellular cholesterol (m/z 458).

2.6. Measurement of NAPE in cultured cells

Cultured CHO cells were grown in 75-cm flasks and used at 70-80% confluence. Stock solutions of fatty aldehydes were dissolved in ethanol and added to warm cell media at a final ethanol concentration of <0.2%, whereas ADX-102 was dissolved in DMSO. In experiments involving ADX-102, cells were initially preincubated for 2 h with media containing ADX-102 prior to adding media containing fatty aldehydes and subsequently incubated for 24 h at 37 °C. Cell culture media were removed and cell monolayers were washed once with phosphate-buffered saline (PBS). Aldehyde adducts were reduced by incubation in 10 ml PBS containing freshly-made 25 mM $NaBH_3CN$ for 1 h at 37 °C. Cells were subsequently collected by scraping, placed in 8 ml screw-capped tubes and pelleted by centrifugation at 3000 xg for 5 min. Cell pellets were washed twice with PBS and lipids extracted immediately or stored frozen prior to lipid extraction. Cell pellets were resuspended in 250 μl HPLC-grade water and sonicated for 10 s using a microtip probe at 50% maximum power. An aliquot (50 μl) was removed for protein measurement. To the remaining cell sonicate was added 2.5 ml chloroform: methanol (1:1). Tubes were tightly capped with a Teflon-lined screw cap, mixed by vortexing for 1 min and lipids extracted at room temperature for 2 h. To each tube was added 1.25 mL chloroform and 0.8 mL water. Tubes were vortexed for 1 min and centrifuged to help separate the aqueous and organic phases. The upper aqueous phase was discarded and the lower organic phase was dried under a stream of nitrogen in a 40 °C water bath. After drying, lipids were hydrolyzed by addition of 2 ml of 0.3 M NaOH in 95% ethanol in a 120 °C oven for 1 h. After cooling to room temperature, 2 ml water was added and the NAEs (and cholesterol) were extracted into 2 ml hexane, back extracted with 2 ml water and dried under a nitrogen stream. NAEs were quantitated by GC-MS using SIM and expressed as a ratio to cholesterol (Section 2.5).

Table 1
Major ions used to identify and measure N-alkyl-ethanolamine (NAE).

Silylated NAE	m/z ions[a]
N-15:0-EtOTMS	146, 240, 328, 343
N-16:0-EtOTMS	146, 254, 342, 357
N-17:0-EtOTMS	146, 254, 268, 356, 371
N-18:0-EtOTMS	146, 282, 370, 385
N-22:0-EtOTMS	146, 338, 410, 426, 498

[a] Underlined ions were used for quantitation of NAEs. Cholesterol TMS was quantitated using m/z 458.

2.7. NAPE formation in mouse liver microsomes

Microsomes were prepared from liver of BALB/C mice by differential centrifugation. All steps were performed at 4 °C. Liver was homogenized in 3 volumes of 50 mM potassium phosphate, pH 7.4, 250 mM sucrose, 1 mM Na_2EDTA at 4 °C. The homogenate was centrifuged at 500 xg for 10 min and the supernatant was removed and centrifuged at 19,000 xg for 30 min. The supernatant was subsequently centrifuged at 100,000 xg for 30 min. The microsome-enriched pellet was resuspended in homogenization buffer and stored at -70 °C. PE content was measured by HPTLC as described [4].

Microsome incubations were performed in 200 μl of 50 mM potassium phosphate, pH 7.4, 250 mM sucrose, 1 mM Na_2EDTA, 50 μl microsomes (containing 0.365 mg protein and 6.8 μg PE), and varying amounts of C16:0-al added in 2 μl ethanol. In ADX-102 inhibition experiments, the ADX-102 was added at varying concentrations in 2 μl ethanol. Reactions proceeded at 37 °C for 2 h and PE adducts were subsequently stabilized by addition of 100 μl of 50 mM $NaBH_3CN$ and incubation at 4 °C for 30 min. The lipids were extracted according to Folch et al. [19] and NAPE was hydrolyzed with ethanolic-NaOH as described (Section 2.6) to produce NAEs for analysis. The C16:0-NAE was quantitated by GC-MS and expressed as a ratio to cholesterol (Section 2.5).

2.8. Fatty aldehyde cytotoxicity experiments

Cells were seeded into black-walled 96-well plates at a density of 20,000 cells/well and grown overnight. C18:0-al in ethanol was added to growth media at a final ethanol concentration of 0.2%. In experiments testing the effects of ADX-102, cells were preincubated for 2 h in media containing ADX-102 (added from a DMSO stock solution) prior to replacing with media containing C18:0-al and/or ADX-102. Control cells lacking aldehydes or ADX-102 had an equal amount of ethanol/DMSO. Cells were grown for 24 h. Cell viability was determined using the Cell Counting Kit-8, which measures the reduction of WST-8 to WST-8 formazan. WST-8 was added to the cell growth media and absorbance at 450 nm was measured after 4 h using a microplate reader. All incubations were done in quadruplicate and the absorbance of media from aldehyde-treated cells was compared to that of untreated control cells lacking C18:0-al exposure to calculate cell viability. Results were confirmed by trypan blue staining.

2.9. Caspase 3/7 activity

Cells were grown in 6-well dishes. For ADX-102 treated cells, cells were pretreated with media containing 300 μM ADX-102 for 2 h before media was switched to that containing 300 μM C18:0-al and/or ADX-102. After 3 h, the cell media were removed, cell monolayers were washed with PBS and collected by scraping and centrifugation. Cell pellets were resuspended in 25 mM Tris-HCl, pH 8.0 and homogenized in a motor–driven glass-Teflon homogenizer. Caspase 3/7 activity in the

cell homogenates was measured using the Caspase-3-DEVD-R110 Assay Kit from GeneCopoeia, Inc. according to the manufacturer's instructions. Data were expressed as % of mean caspase 3/7 activity in untreated cells.

2.10. FALDH enzyme activity

FALDH activity was measured in CHO-K1 and FAA-K1A cells using C18:0-al substrate as described [20]. Cell and tissue protein was measured according to Lowry et al. [21].

2.11. Measurement of cellular fatty alcohol content

Cellular fatty alcohols were extracted as previously described [22] and measured as pentafluorobenozyl-derivatives using negative ionization-chemical ionization GC-MS with pentadecanol as internal standard [23].

2.12. Fatty alcohol metabolism

Octadecanol biosynthesis was measured by incubating FAA-K1A cells in 6-well dishes containing 2 ml complete F12 media with 2 μCi ^{3}H-stearic acid ±50 μM ADX-102 for 120 min at 37 °C. Media also contained 50 μM non-radioactive octadecanol to metabolically trap ^{3}H-octadecanol synthesized by the cells [15]. Media were subsequently removed, cell monolayers were washed with PBS and collected by scraping twice into 2 ml methanol. Radioactive octadecanol was purified by TLC and quantitated as described [15]. Octadecanol synthesis was expressed as cpm/min/mg cell protein. ^{3}H-Octadecanol oxidation to fatty acid was measured in FAA-K1A cells in 6-well dishes with 2 ml media containing 400,000 cpm ^{3}H-octadecanol. After 3 h, cell monolayers were washed with PBS, collected by scraping into 2 ml methanol and lipids were extracted with chloroform/methanol. After Folch washing, radioactive stearic acid was purified by TLC and quantitated as described [15]. Octadecanol oxidation was expressed as cpm/h/mg cell protein.

2.13. Statistical analysis

Data were plotted with Prism 9.0 software (GraphPad Software, LLC) and analyzed using unpaired Students *t*-test with significance determined at $p < 0.05$.

3. Results

Free fatty aldehydes may not be reliably detected in cells due to their reactive nature and typically low concentrations. As a surrogate measure of fatty aldehydes, we therefore investigated the formation of aldehyde adducts with PE by monitoring the unique N-alkyl-ethanolamine (NAE) hydrolysis product of NAPE [24] (Fig. 2). To identify NAEs, authentic standards were synthesized and their major fragmentation ions were identified by GC-MS (Table 1). NAEs were measured by single ion monitoring using C22:0-NAE as internal standard, or by expressing NAEs as a ratio with cellular cholesterol.

3.1. Chemical synthesis of NAPE and inhibition by ADX-102

To establish that C16:0-al was capable of forming an adduct with PE, we initially investigated this reaction in an organic solution. In methanol, the reaction of C16:0-al and PE resulted in formation of a Schiff base product, which was converted to the more stable N-C16:0-al-PE (N-16:0-PE) lipid after chemical reduction with $NaBH_3CN$ (Fig. 3A). N-16:0-PE was not detected unless the C═N Schiff base bond was stabilized to C-N by chemical reduction. When ADX-102 was added to the reaction in varying amounts, N-16:0-PE formation was prevented in a concentration dependent manner with essentially complete inhibition at ADX-102/PE

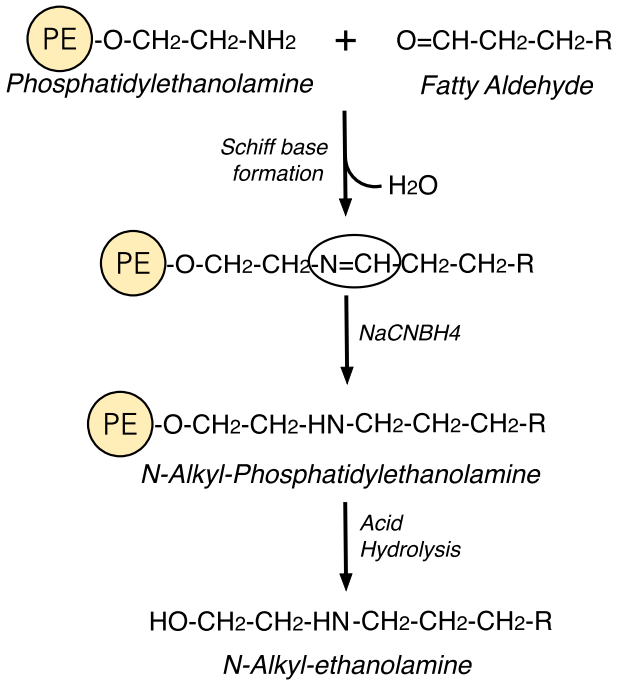

Fig. 2. NAPE formation from phosphatidylethanolamine (PE) and fatty aldehyde, and its chemical hydrolysis to the unique lipid N-alkyl-ethanolamine (NAE) analyzed. The Schiff base bond is circled.

molar ratios greater than 4. This decrease in N-16:0-PE was accompanied by a commensurate increase in formation of N-16:0-al-ADX-102 product (Fig. 3A).

3.2. ADX-102 inhibits NAPE formation in microsomal membranes

To determine whether a similar reaction occurs under aqueous conditions with biological membranes containing PE, we reacted C16:0-al with mouse liver microsomes and found a concentration-dependent formation of N-16:0-PE (Fig. 3B). The addition of ADX-102 to the reaction blocked N-16:0-PE formation (Fig. 3C).

3.3. ADX-102 prevents C18:0-al cytotoxicity

We next investigated whether ADX-102 could inhibit fatty aldehyde effects in intact cells by using a Faldh-deficient Chinese hamster ovary cell line (FAA-K1A) as a model for SLS [17]. Faldh activity in the FAA-K1A cells (<100 pmol/min/mg protein) was profoundly reduced compared to wild-type CHO-K1 cells (2125 ± 497 pmol/min/mg protein; $n = 3$), and similar to the enzyme deficiency seen in SLS [4]. The FAA-K1A cells are reported to be more sensitive to aldehyde cytotoxicity than wild-type cells [18]. We therefore investigated the ability of ADX-102 to protect the cells from C18:0-al. As shown in Fig. 4A, C18:0-al at a concentration of 200-300 μM was more cytotoxic to the FAA-K1A cells than the CHO-K1 cells, demonstrating the role of Faldh in detoxification of this fatty aldehyde. A shorter aldehyde, octanal, was not cytotoxic at the highest concentration tested (300 μM), indicating greater susceptibility to longer chain aldehydes (data not shown). When mutant cells were treated with 300 μM C18:0-al, cell cytotoxicity was largely prevented by co-incubation with ADX-102 at concentrations of 200-300 μM (Fig. 4B). This cytotoxicity was mediated by apoptosis as determined by Annexin V staining (not shown) and detecting a 4.5-fold increase in caspase 3/7 activation within 3 h of C18:0-al exposure (Fig. 4C). ADX-102 effectively prevented caspase 3/7 activation.

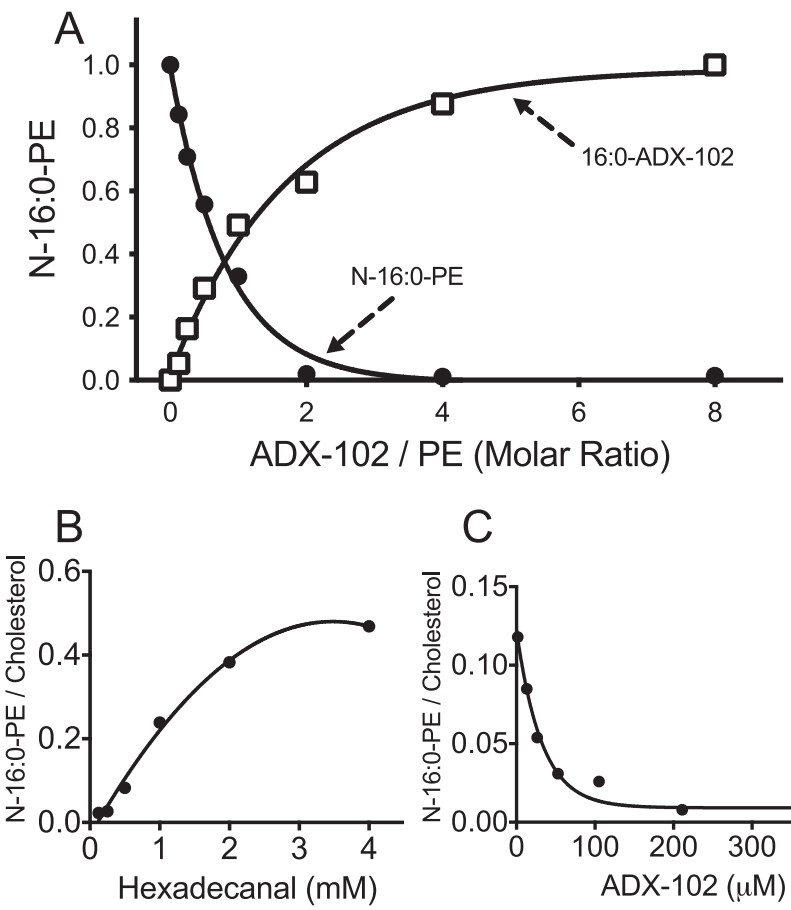

Fig. 3. Formation of NAPE. A. Chemical reaction of PE and hexadecanal (C16:0-al) in methanol forms NAPE (N-16:0-PE), which is competitively inhibited by ADX-102. The formation of N16:0-al-ADX-102 adduct is inversely correlated with decrease in N-16:0-PE. Data are expressed as the fraction of initial N-16:0-PE formed (100%) in the absence of ADX-102. B. NAPE (N-16:0-PE) formation in mouse liver microsomes (0.365 mg protein/reaction) is dependent on 16:0-al concentration. C. ADX-102 inhibits N-16:0-PE formation in liver microsomes in a concentration dependent manner. Microsomes were incubated with 0.5 mM 16:0-al. N-16:0-PE concentrations are normalized to cholesterol ($n = 2$).

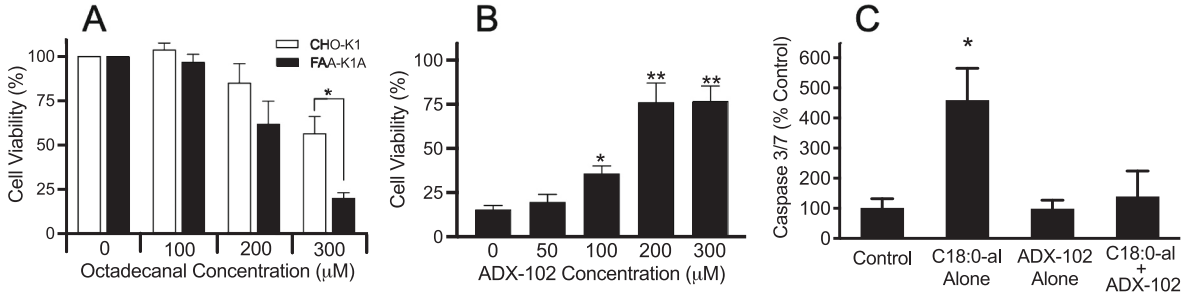

Fig. 4. ADX-102 protects Faldh-deficient FAA-K1A cells from C18:0-al toxicity. A. C18:0-al was added to CHO-K1 and FAA-K1A cells for 24 h at various concentrations. Viable treated cells are expressed as a percentage compared to untreated cells (100%). Open bars, wild-type CHO-K1 cells; black bars, FAA-K1A cells. Results are the mean ± SEM of 11 experiments. *, $p \leq 0.001$ comparing FAA-K1A and CHO-K1 cells using unpaired *t*-test. B. ADX-102 protects FAA-K1A cells from C18:0-al toxicity. Cells were exposed to media containing 300 μM C18:0-al plus varying concentrations of ADX-102 for 24 h. Results represent the mean ± SEM of 5 experiments. *, $p \leq 0.01$, or ** $p \leq 0.001$ comparing ADX-102 treated cells to cells receiving no ADX-102 using unpaired t-test. C. ADX-102 prevents C18:0-al-induced apoptosis. FAA-K1A cells were incubated in media containing 300 μM C18:0-al ± ADX-102 for 3 h. Cell caspase 3/7 activity in treated cells was expressed as the percentage measured in untreated cells (100%). Data are the mean ± SD ($n = 3$). *, $p \leq 0.01$ comparing untreated control cells to treated cells using an unpaired t-test.

3.4. ADX-102 blocks metabolism of C18:0-al to fatty alcohol

Most fatty alcohols are made from fatty acids by action of acyl-CoA reductase on fatty acyl-CoA as part of the fatty alcohol cycle (Fig. 1) [15]. However, fatty aldehydes added directly to cells [8] or generated from catabolism of precursor lipids, such as 1-*O*-alkylglycerol [7], can also be metabolized to fatty alcohols. We therefore incubated FAA-K1A cells with a low non-toxic concentration of C18:0-al (10 μM) for 18 h and observed a 300% increase in cellular octadecanol content (Fig. 5A), consistent with metabolism of C18:0-al to octadecanol. The C18:0-al dependent increase in octadecanol content, however, was blocked by ADX-102 (Fig. 5A). In support of this interpretation, ADX-102 itself had no effect on rates of cellular octadecanol oxidation (Fig. 5B), or its biosynthesis from fatty acid (Fig. 5C).

In contrast to these results with exogenously added C18:0-al, incubation of FAA-K1A cells with 50 μM ADX-102 alone for 4 days did not reduce the octadecanol content (- ADX-102: 19 ± 2 ng/mg protein; + ADX-102: 26 ± 7 ng/mg protein, $n = 3$). This suggests that production of C18:0-al from endogenous lipid catabolism and/or its conversion to fatty alcohol is not the major source of octadecanol in the CHO cells.

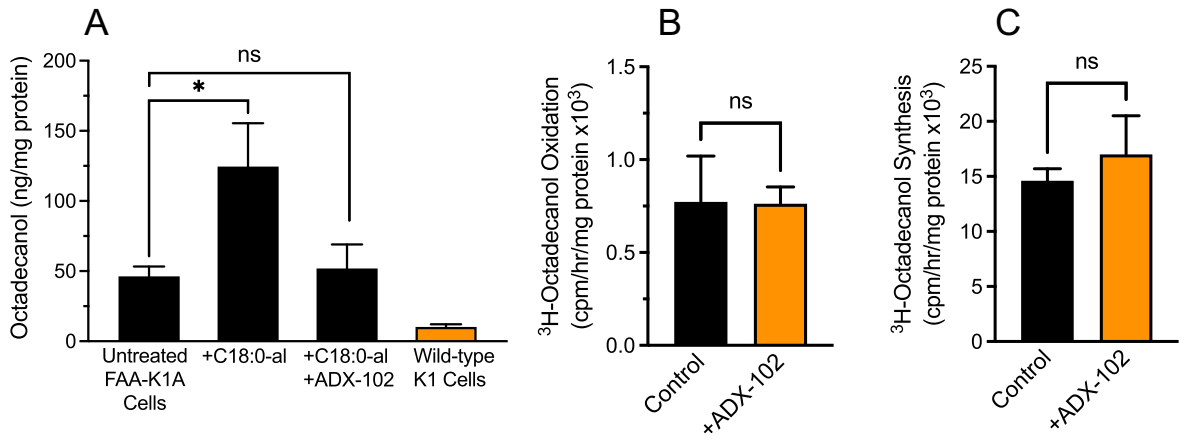

Fig. 5. Effects of ADX-102 on fatty aldehyde and alcohol metabolism in FAA-K1A cells. A. ADX-102 blocks metabolism of C18:0-al to octadecanol. FAA-K1A cells were incubated with 10 μM C18:0-al ± 50 μM ADX-102 for 18 h after which cellular octadecanol content was measured. The lower octadecanol content of untreated control CHO-K1 cells is shown for comparison. Data are mean ± SD ($n = 3$). B. ADX-102 does not inhibit ^{3}H-octadecanol oxidation to fatty acid in intact FAA-K1A cells. Cells were incubated with ^{3}H-octadecanol for 3 h. Data are mean ± SD ($n = 6$). C. ^{3}H-Octadecanol synthesis is unaffected by ADX-102. Cells were incubated with ^{3}H-stearic acid for 2 h and production of radioactive cellular octadecanol determined. Data represent the mean ± SD ($n = 4$). *, $p \leq 0.05$; ns = not significant.

3.5. ADX-102 decreases NAPE accumulation in FAA-K1A cells

It is possible that the effects of ADX-102 described in Sections 3.3 and 3.4 were due to its extracellular reaction with C18:0-al and prevention of aldehyde uptake by the cells.

To determine whether ADX-102 blocked fatty aldehydes generated from cellular metabolism, we looked for its effects on endogenous aldehyde adducts (NAPE). Under standard growth conditions, NAPE was detected in the FAA-K1A cells (Fig. 6). N-18:0-PE and N-16:0-PE comprised the major PE adducts detected and were present in approximately equal amounts. Compared to wild-type CHO-K1 cells, the mutant cells exhibited a 5-fold increase in total content of these two lipids (Fig. 6). When FAA-K1A cells were incubated with 20-50 μM ADX-102 for 4 days, the NAPE content of the cells was reduced to the level seen in untreated wild-type cells.

4. Discussion

We found that the aldehyde-reactive compound ADX-102 prevented C18:0-al-mediated cytotoxicity in Faldh-deficient FAA-K1A cells, blocked C18:0-al metabolism to fatty alcohol, and normalized NAPE content. ADX-102 itself did not appear to be toxic to CHO cells at concentrations up to 300 μM.

Free fatty aldehydes are not readily detected in cultured cells due to their ephemeral nature and reactivity with cellular components. Aldehyde adducts such as NAPE provide a surrogate measure of aldehyde accumulation. James and Zoeller [18] first reported NAPE in cultured SLS fibroblasts and CHO cells exposed to fatty aldehydes and suggested that these unusual phospholipids may be important in the pathogenesis of SLS. Subsequently, incubation of intact SLS fibroblasts with radioactive 1-*O*-[^{3}H]-octadecylglycerol, which generates radioactive fatty

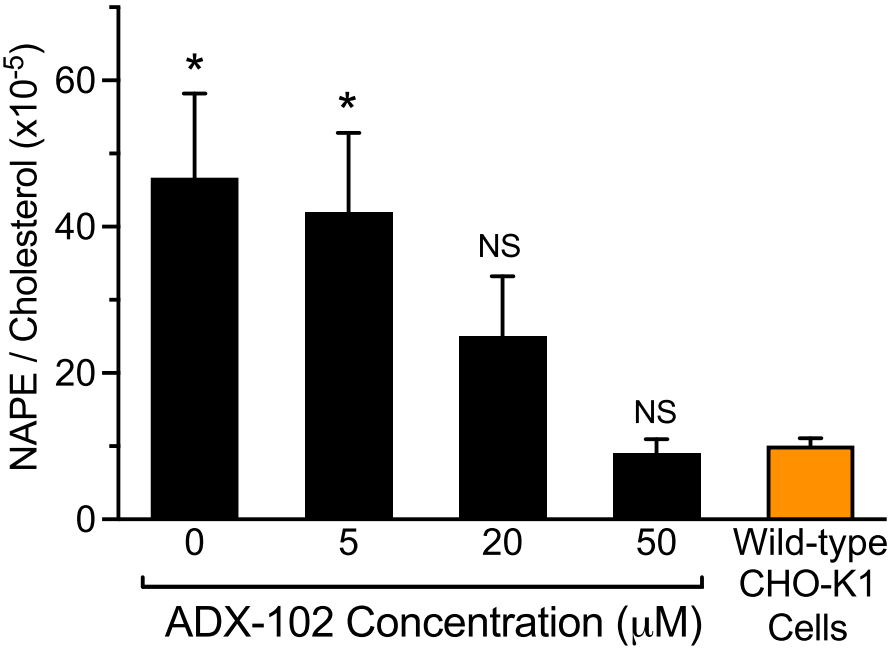

Fig. 6. Inhibition of NAPE accumulation in FALDH-deficient FAA-K1A cells grown for 4 days in the presence of ADX-102. Total NAPE represents the sum of N-16:0-PE + N-18:0-PE in the cells and is normalized to the amount of cellular cholesterol. Results represent the mean ± SD (n = 3). *, $p < 0.05$ comparing ADX-102-treated cells to wild-type CHO-K1 cells. NS, not significant.

aldehyde during its catabolism, resulted in formation of 4-fold more labeled NAPE compared to normal cells, demonstrating NAPE production from endogenous lipid metabolism [7]. Moreover, plasmalogen-derived long chain aldehydes generated during oxidative stress in brain homogenates also produced adducts with PE [24]. In these cases, saturated fatty aldehydes formed covalent Schiff base adducts with PE, which needed to be stabilized by chemically reducing the C═N double bond prior to detection. Schiff base formation with saturated fatty aldehydes is otherwise a potentially reversible reaction and must be captured in this manner.

Although we focused on saturated C16-C18 Schiff base adducts with PE, other types of adducts can form depending on the specific aldehyde structure and target molecule. Aliphatic aldehydes with α,β unsaturated carbon bonds vary in cytotoxic potential in a complex manner [25]. The unsaturated carbon bond significantly increases aldehyde electronegativity and enhances reactivity with nucleophiles. These fatty aldehydes have two reactive groups: the carbonyl moiety that participates in a Schiff base adduct, and the unsaturated carbon bond, which can form Michael addition products with a variety of cellular nucleophiles including proteins and DNA. Schumacher et al. [11] reported that the α,β unsaturated C16:1-al forms Michael adducts with glutathione, amino acids and proteins in HepG2 cell lysates. With glutathione, Michael adducts were much more abundant than Schiff base adducts, although this propensity varied with the nucleophile tested. Under in vitro conditions, most Michael adducts were not stable long term, with exception of 3-histidinyl-hexadecenal.

ADX-102 was effective in preventing accumulation of NAPE and protecting FAA-K1A cells from aldehyde cytotoxicity. ADX-102 has a free amino group that can compete with cellular targets for fatty aldehyde reactivity by acting as a "sacrificial lamb" and its hydrophobic nature suggests that it will be localized in membranes where fatty aldehydes accumulate. The unique structure of ADX-102 permits it to act as an aldehyde trap with certain aliphatic aldehydes, irrespective of chain length, by forming a Schiff base adduct with its amino group and reacting with its nearby hydroxyl group to irreversibly trap the aldehyde. Nevertheless, a variety of molecules with primary amines could theoretically mitigate fatty aldehyde toxicity by competing with more sensitive cellular target molecules. In a genetic mouse model of Stargardt disease, a degenerative eye condition, several small molecules with primary amino groups were shown to inhibit formation of retinal-PE adducts and protect the mice from intense light-induced eye damage [26]. In SLS, it may be speculated that tissues that are not pathologically affected produce endogenous metabolites, such as glutathione, that protect cellular targets from fatty aldehyde toxicity.

Proteins comprise a large group of potential aldehyde targets. C16:1-al is normally produced by catabolism of spingosine-1-phosphate and oxidized by FALDH [27]. In vitro studies using cell homogenates incubated with alkyne-tagged C16:1-al and C16:0-al identified more than 500 aldehyde adducted proteins [10]. Proteins modified by these two fatty aldehydes differed in their abundance and identity, suggesting some specificity in aldehyde targets and reactivity. One of the proteins modified by C16:1-al was Bax, a pro-apoptotic protein that is part of the JNK-mediated pathway leading to apoptosis. Alkyne-tagged C16:1-al also interacted with Bax in intact cells. Interestingly, C16:1-al is toxic to cultured cells through a JNK-dependent mechanism [28]. Whether C18:0-al toxicity in the FAA-K1A cells is mediated by Bax modification will need to be further explored.

The spectrum of aliphatic aldehydes that might contribute to disease mechanisms in SLS is not yet defined [2]. FALDH has a broad substrate specificity for aliphatic aldehydes ranging from C6-C24 with greatest activity against C14-C20 saturated, monounsaturated and branched-chain aldehydes [20]. Based on the C16-C18 species of NAPE detected in the FAA-K1A cells, we infer that the corresponding aldehydes accumulate, which is similar to the chain length of fatty alcohols that accumulate in SLS fibroblasts and plasma [22]. Saturated fatty aldehydes are less reactive molecules than unsaturated ones [29], due to their lower electrophilic properties and ability to only form Schiff base adducts. One of the most reactive aldehydes known is 4-hydroxy-2-nonenal (4-HNE), which is produced during lipid peroxidation and has been implicated in signal transduction, cell growth and apoptosis [30]. Overexpression of FALDH in cultured 3 T3-L1 cells and rat adipocytes has been shown to protect against adverse effects of 4-HNE [31,32]. In contrast, it is not yet established whether FALDH deficiency in SLS patients imparts a particular sensitivity to 4-HNE, since other aldehyde dehydrogenases, including ALDH3B1 [33] and ALDH3A1 [34,35], can also metabolize it, along with its elimination via non-enzymatic conjugation with glutathione [36].

The contribution of fatty aldehydes to the pathogenic mechanisms operating in SLS is probably complex. It may depend on the particular aldehydes that are generated and their molecular reactivity, the repertoire of ancillary aldehyde dehydrogenase isozymes or other detoxification mechanisms in a particular SLS tissue, and the critical targets for aldehyde adduct formation in a cell. Adding to the potential pathogenicity of fatty aldehydes in SLS is the emerging evidence that fatty alcohols and their metabolic products may also play a role [2]. Recent studies point to the accumulation of fatty alcohol-derived ether glycerolipids in SLS brain [6] and skin [5]. These lipids partition into membranes and likely alter membrane biophysical properties or protein-membrane interactions. In FAA-K1A cells, we saw that ADX-102 prevented the C18:0-al-dependent increase in octadecanol accumulation, but not in cells grown without addition of C18:0-al, suggesting that endogenous production of fatty aldehydes produced via lipid catabolism is not a major contributor to fatty alcohol levels. It is possible, however, that ADX-102 may impact fatty alcohol levels in specialized tissues, such as brain, that have more active turnover of fatty aldehyde precursor lipids such as plasmalogens and 2-OH-fatty acids. To the extent that fatty alcohols are derived from aldehyde metabolism in affected SLS tissues, ADX-102 may have a role in decreasing both fatty aldehydes and alcohols.

In summary, our studies demonstrate the ability of ADX-102 to block formation of long-chain aldehyde adducts with PE, prevent aldehyde cytotoxicity and inhibit aldehyde metabolism to fatty alcohol. These in vitro cellular findings have therapeutic implications for SLS and provide a compelling biochemical rationale for investigating aldehyde reactive agents in this disease.

Declaration of Competing Interest

None.

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