DEMONSTRATION OF THE EFFICACY OF SUBEROYLANILIDE HYDROXAMIC ACID IN THE TREATMENT OF X-LINKED ADRENOLEUKODYSTROPHY

An essay submitted in partial fulfillment of

the requirements for graduation from the

Honors College at the College of Charleston

with a Bachelor of Science in

Biochemistry and Chemistry

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May 2013

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DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

2013

Acknowledgements

The author would like to thank Dr. Inderjit Singh, Dr. Jaspreet Singh, and the Children's Research Institute at the Medical University of South Carolina for assistance in the planning and conduct of the research described. The author would also like to thank Dr. Jason Overby for assistance in essay preparation and review.

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ABSTRACT

This paper describes the research project that the author participated in during the summer of 2011 spent in Dr. Inderjit Singh's laboratory in the Darby Children's Research Institute at the Medical University of South Carolina. The author worked closely with Dr. Jaspreet Singh, a postdoctoral fellow in Dr. Singh's lab. The author was a research assistant for Dr. J. Singh and worked on his project exclusively. One of Dr. I. Singh's research focuses, which Dr. J. Singh was working on, is X-linked adrenoleukodystrophy (X-ALD). This genetic disease is due to one of several mutations of the ABCD1 gene; it is characterized by a defect in peroxisomal β-oxidation and the subsequent buildup of very long chain fatty acids (VLCFA, C>22) in the patient's plasma and tissues.

There are two most common forms of X-ALD: the more severe childhood cerebral ALD (cALD) manifests between ages 3 and 10, and a less severe adrenomyeloneuropathy (AMN), which manifests between the ages of 27 and 35. Both of these manifest only in boys since the mutation that causes the disease is on the X chromosome, though some heterozygous female carriers exhibit mild symptoms later in life. The project was an attempt to determine the efficacy of a compound, suberoylanilide hydroxamic acid (SAHA), for the treatment of X-ALD. SAHA was found to lower VLCFA levels in cultured X-ALD fibroblasts and to increase the β-oxidation of VLCFA by upregulating ABCD2, an analogous gene to ABCD1. SAHA also lowered the expression of ELOVL1, a gene which codes for an elongase responsible for the synthesis of both saturated (C26:0) and monounsaturated (C26:1) VLCFA.

INTRODUCTION

X-ALD is the most common genetic peroxisomal disorder (1). The ABCD1 gene, which is mutated in ALD, codes for a protein related to the ATP-binding cassette transporter which is responsible for transferring very long chain fatty acids to the peroxisomes (2), where they are degraded. The mutation in the ABCD1 gene makes the ATP-binding cassette unable to transport the VLCFA for proper degredation, which causes them to build up in the body. The buildup of VLCFA eventually leads to demyelination in cALD and in the range of other symptoms seen in AMN, though this mechanism is unknown at this time. ABCD1 mutation also seems to cause upregulation in the elongases responsible for producing VLCFA (3).

A satisfactory therapy for X-ALD remains elusive. Hematopoietic stem cell transplantation has been shown to halt the demyelination in cALD, but the transplant must be done at a very early stage of the disease to be effective (4). This is often impossible since generally the disease is too far advanced for stem cell transplantation to have any beneficial effects once the affected child starts to show symptoms. Gene therapy has been attempted using a retroviral vector to introduce a wild-type ABCD1 gene into the child's body (5), and has met with some success but this therapy is still in clinical trials. A pharmacological treatment for ALD is very attractive since it could have the potential to be administered prenatally, and thus give the best chance of survival for the infant. Histone-deacetylase (HDAC) inhibitors have been shown to have some positive effect on metabolic defects in neuroinflammatory disorders by upregulation of genes with overlapping function (6-8). Chemically-induced overexpression of the ABCD2 gene is an attractive target for therapeutic efforts since the overexpression of Abcd2 in Abcd1

knockout mice led to amelioration of metabolic disturbances. Several compounds including lovastatin, 4-phenylbutyrate, and fenofibrate have been shown to lower VLCFA levels in cultured ALD fibroblasts, but none of these have been able to slow or reverse neurodegeneration in mice.

The study in which the author participated demonstrates that treatment of fibroblasts from X-ALD patients with SAHA lowered VLCFA levels in the cells by increasing the β-oxidation activity. SAHA was also able to lower the expression of elongases associated with VLCFA synthesis. The author was mainly involved with cell culture, Western blotting and electrophoresis, and determination of gene regulation via RT-PCR.

RESULTS

It has been previously shown that upregulation of ABCD2 can compensate for a mutated ABCD1 gene (ref). The overexpression of ABCD2 was able to correct VLCFA β-oxidation in X-ALD fibroblast cultures and to prevent clinical symptoms in Abcd1$^{-/-}$ mice (refs). Given these previous results, ABCD2 is an attractive candidate for pharmacological gene therapy of X-ALD. The first step in the current study was to treat normal fibroblasts with SAHA at different doses and times to study the effect of SAHA on ABCD2 and ABCD3 expression in normal cells. Figure 1 shows the dose- and time-dependent upregulation of ABCD2 and ABCD3 when treated with SAHA. Both ABCD2 and ABCD3 expression were significantly induced by treatment with SAHA. A maximum dose of 5 μM of SAHA for three days induced a 7.2 and 5.7 fold induction of ABCD2 and ABCD3, respectively. Higher doses of SAHA and longer durations did not increase the expression of ABCD2 or ABCD3 significantly.

Since perturbation in the β-oxidation of VLCFA contributes significantly to the disease progression in X-ALD, the next step of this study focused on metabolism of VLCFA. Overexpression of ABCD2 in fibroblast cultures from Abcd1 knockout mice and X-ALD patients has been shown to reduce the VLCFA levels by restoring peroxisomal β-oxidation (refs). Working from these results, the effect of SAHA treatment on normal fibroblast β-oxidation of lignoceric acid (C24:0) and palmitic acid (C16:0) was studied next. These acids were chosen because lignoceric acid is a VLCFA and palmitic acid is a precursor to it. Control normal human fibroblasts were treated with SAHA with differing doses and durations. Figure 2 shows the effects of SAHA treatment on β-oxidation of the two fatty acids mentioned previously. Similar to the results of the effect

of SAHA on ABCD2/ABCD3 induction, the maximum effect on β-oxidation was seen with a 5 μM dose of SAHA for three days. This dose and duration stimulated lignoceric acid and palmitic acid β-oxidation by 45% and 55%, respectively. The increase in β-oxidation of both lignoceric and palmitic acid suggests that SAHA treatment stimulates overall fatty acid oxidation.

Once SAHA was shown to upregulate ABCD2/ABCD3 expression in normal fibroblasts, the next step was to test the effect of SAHA on X-ALD fibroblasts in order to determine the therapeutic potential of SAHA for X-ALD patients. Figure 3 shows the effect on ABCD2 mRNA expression of culturing four different lines of X-ALD fibroblasts in the presence of SAHA for three days. Figure 4 shows the same data for ABCD3 mRNA expression. A maximum dose of 5 μM SAHA for three days produced 11.8 (**A**), 6.0 (**B**), 2.8 (**C**), and 3.88 (**D**)-fold increases in ABCD2 mRNA in the respective cell lines. A similar response was seen with ABCD3 mRNA: 10 (**A**), 3.92 (**B**), 2.77 (**C**), and 3.45 (**D**)-fold upregulation was seen in the respective cell lines with the same SAHA treatment as the ABCD2 regulation study. The effect of SAHA on the X-ALD fibroblasts was quantified with RT-PCR.

To complement the RT-PCR determination of the effect of SAHA on ABCD2 and ABCD3 mRNA regulation, the amount of ALDRP and PMP70, two protein products of the ABCD2 and ABCD3 genes, was screened using Western blotting. The blots showed a significant dose-dependent increase in these two proteins in SAHA-treated cells. A similar screen for membrane protein $Na^{+/}K^{+}$-ATPase as an indicator of protein loading showed no significant difference between the cell samples used for ALDRP/PMP70

screening. Figure 5 shows the blots from cells screened for ABCD2 (**A and C**) and ABCD3 (**B and D**).

The peroxisomal β-oxidation of fatty acids is regulated by ALDP; for this reason, fatty acid metabolism is impaired in X-ALD patients. ALDRP has been shown to compensate for the loss of ALDP in the absence of the latter protein so the next experiment tested the effect of SAHA-regulated ALDRP on peroxisomal and mitochondrial β-oxidation using radio-labeled fatty acids. Four different X-ALD fibroblast cell lines were treated with 0.5 – 5 μM SAHA (40% - 55%) for three days. Lignoceric acid β-oxidation increased in a dose-dependent manner, as shown in Figure 6B. Figure 6A shows the dose-dependent increase in palmitic acid oxidation in cell cultures treated as described. The induction of both lignoceric and palmitic acid oxidation suggests that SAHA-regulated ALDRP affects both peroxisomal and mitochondrial fatty acid oxidation.

Although the precise mechanism by which ALDP is involved in peroxisomal β-oxidation is unknown at present, increased levels of VLCFA in X-ALD cells deficient in ALDP and their normalization following transfection with ALDP cDNA indicates a role of ALDP in β-oxidation. Also, an upregulation of ABCD2 results in a decrease in VLCFA in cultured X-ALD cells. Therefore we next determined if SAHA-induced expression of ABCD2 mRNA and protein would lead to a general decrease in VLCFA in cultured X-ALD fibroblasts. Hexacosanoic acid (C26:0) is often used to diagnose X-ALD and other peroxisomal disorders, so this was the first fatty acid level investigated. Without SAHA treatment C26:0 levels were 9-fold higher in X-ALD fibroblasts compared to normal cells, as shown in Figure 7A. Treatment with 5 μM SAHA for three

days resulted in a 37% reduction in VLCFA (C26:0) levels. SAHA treatment also led to decreased C26:1 levels in X-ALD human fibroblast cultures in a dose- and time-dependent manner (Figure 7B).

Elongases are enzymes responsible for synthesis and elongation of long-chain fatty acids. Two elongases, ELOVL1 and ELOVL3, have been identified as having chain-length specificity towards VLCFA. The mRNA levels of ELOVL1 and ELOVL3 are not significantly higher in X-ALD cells compared to normal cell cultures, indicating that substrate availability is their limiting factor. More long-chain fatty acids are present in X-ALD patients, giving ELOVLs 1 and 3 more substrate and compounding the problem of decreased β-oxidation. Because of the specificity towards VLCFA, knockdown of ELOVL1 in human X-ALD fibroblasts decreases the amount of C26:0 in the cells. Figure 8 shows that treatment with 5 μM SAHA decreased ELOVL1 expression by 81.55% for control cells and 63.35% for X-ALD cells. This shows the dual effect of SAHA in reducing the VLCFA concentration by stimulating β-oxidation of VLCFA and by inhibiting ELOVL1 expression and thus VLCFA elongation.

DISCUSSION

Pharmacological treatment for any genetic disease usually involves upregulation of redundant genes to compensate for lost gene function. According to a recent study by our lab, mouse astrocyte cultures silenced for both Abcd1 and Abcd2 showed an increase in VLCFA levels compared to single-knockout cultures. This observation, coupled with the observation in this study of an inverse correlation between ABCD2 expression and VLCFA levels in X-ALD human fibroblast cultures, presents ABCD2 as an attractive target for pharmacological therapy of X-ALD. Treatment with SAHA was able to increase ABCD2 and ABCD3 expression in both normal and X-ALD fibroblast cultures. Interestingly, the percent increase in ABCD2 mRNA expression was larger in normal cells than in X-ALD cells, perhaps due to a dominant negative effect of the ABCD1 mutation or deletion. Even so, SAHA treatment led to a significant increase in both ABCD2 mRNA and protein products.

Earlier studies from our lab have also shown that VLCFA are oxidized almost exclusively in the peroxisomes. SAHA treatment of X-ALD cells showed a significant decrease in both palmitic and lignoceric acid, suggesting that SAHA increases both peroxisomal and mitochondrial β-oxidation. However, SAHA treatment of X-ALD fibroblasts silenced for ABCD2 resulted in decreased C16:0 levels but had no effect on β-oxidation of C24:0. This indicates that the SAHA-stimulated increase of peroxisomal β-oxidation is dependent on the ABCD2 gene function. Therefore, SAHA-mediated induction of ABCD2 should compensate for the lack of ABCD1 and ameliorate the metabolic perturbations seen in X-ALD patients.

The substrate specificities of individual ABCD transporters are not known at the present time. However, previous studies have suggested that the substrate specificities of ALDP and ALDRP overlap, especially with regard to saturated and monounsaturated VLCFA. For example, one such study showed that 1 μM SAHA treatment led to a significant decrease in C26:1 levels with only a 2-fold increase in ABCD2. Since no significant change in C26:0 levels were observed it follows that the higher affinity of ABCD2 for C26:1 allows for a significant decrease in C26:1 at ABCD2 levels insufficient to change the levels of saturated VLCFA.

In addition to abnormal peroxisomal β-oxidation, heightened elongase activity in X-ALD patients also accounts for the VLCFA accumulation in X-ALD. To date, seven elongases have been identified in humans (ELOVL1-7). Of these, ELOVL1 and ELOVL3 have shown chain-length specificity towards VLCFA and are therefore the most important to the study of the role of elongases in X-ALD. A recent study by another lab showed that silencing human fibroblasts for ELOVL1 reduced the lengthening of C22:0 to C26:0 and thus decreased the concentration of C26:0. This implicates ELOVL1 in the biosynthesis of VLCFA. SAHA treatment of X-ALD fibroblasts resulted in a significant reduction in ELOVL1 expression. Interestingly, the opposite effect was observed on ELOVL3. This shows that the observed lowering of C26:0 and C26:1 levels in X-ALD cell cultures following SAHA treatment is due to a net effect of induction of peroxisomal and mitochondrial β-oxidation, decreased fatty acid chain elongation following repression of ELOVL1, and induction of ABCD2.

The neurodegeneration seen in X-ALD is believed by many to be caused by inflammation due to VLCFA buildup. For this reason, any effective pharmaceutical agent

for X-ALD must be able to cross the blood-brain barrier (BBB) to affect the VLCFA buildup in the brain and thus slow or stop the neurological symptoms associated with X-ALD. SAHA has been shown in several models to cross the BBB which makes it an attractive and potentially useful candidate drug. Our lab has shown previously that reduction of VLCFA in Abcd1/2-silenced mouse astrocyte cultures led to a decrease in inflammatory markers.

Recently, much attention has been drawn to the effect of gene expression alteration of disease pathology and on the possible role for histone deacetylase (HDAC) inhibitors as therapies for a variety of diseases. Studies from our lab and others have shown that phenylacetate (PA) and phenylbutyrate (PBA), both HDAC inhibitors, are somewhat successful in lowering the VLCFA in cultures. However, both PA and PBA needed millimolar concentrations to work, and their effects did not last long. SAHA, on the other hand, works at micromolar concentrations. SAHA has recently been shown to be an effective therapy in several animal models of inflammatory disease conditions such as lupus, graft-versus-host disease, ischemia, and sepsis. SAHA is already being used in clinical trials at the NIH and has shown good bioavailability.

CONCLUSIONS

Currently no good treatment for X-ALD exists. The neurodegeneration and associated disease progression are nearly unstoppable. Diagnosis of X-ALD at present is a death sentence for a child, and heartbreaking for parents. Gene therapies for X-ALD are still in trials, with no sign yet of being ready for general use. An effective pharmacological treatment is needed, and needed badly. SAHA has been shown in this study and others to be an ideal treatment for X-ALD. It reduces the VLCFA levels which cause the inflammation that leads to demyelination and neurodegeneration. It also increases the expression of a gene redundant to the one whose function is lost in X-ALD. SAHA has been administered in clinical trials for up to 37 months in doses ranging from 200 – 600 mg with no signs of toxicity. Since it crosses the BBB and is administered orally, it could in theory be given to mothers carrying X-ALD fetuses to ameliorate the disease even before birth. Also, SAHA has been approved by the FDA for treatment of T-cell lymphoma and is in trials for pediatric use. The dose of SAHA showing good results in animal models translates to only 120 mg dose per day for a 20 kg child, which is much lower than the maximum dose considered safe for pediatric use. Given these data, it is evident that SAHA is a strong candidate for the treatment and possible cure of X-ALD.

EXPERIMENTAL

Materials

For cell culture, Dulbecco's modified Eagle's medium (DMEM) and Hank's buffered salt aolution (HBSS) were purchased from Invitrogen Life Technologies (Grand Island, NY) and fetal bovine serum (FBS) was purchased from BioAbChem Inc. (Ladson, SC). The ALDP antibody was obtained from Chemicon International Inc. (Temecula, CA). ALDRP antibody was custom-made from ANASPEC (Fremont, CA) against the mouse 20-residue c-terminal sequence: 722 KILGEDSVLKTIQTPEKTS 741. Na^+K^+ATPase antibody for Western blot standardization was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL and nitrocellulose membranes were purchased from Amersham Biosciences (Pittsburgh, PA).

Human skin fibroblasts derived from normal (control; GM03348), X-ALD (GM04932, GM04934, and GM04904), and AMN (GM07531) patients were obtained from the NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (ccr.coriell.org/). A pool of three siRNA for human ABCD2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) [1-^{14}C]-labeled fatty acids (C24:0, lignoceric acid and C16:0, palmitic acid) were purchased from ARC (St. Louis, MO). The primer sets for use in RT-PCR were designed using the Oligoperfect™ designer by Invitrogen (Grand Island, NY) were purchased from Integrated DNA Technologies (Coralville, IA). Real time PCR was conducted using Bio-Rad iCycler (iCycler iQ Multi-Color Real Time PCR Detection System; Bio-Rad). The cDNA synthesis kit and SYBRGreen dye for PCR were purchased from Bio-Rad (Hercules, CA).

Methods

RNA Isolation and Quantitative Real-Time PCR: RNA was extracted from cultured fibroblasts using TRIzol purchased from Invitrogen and following the manufacturer's protocol. cDNA was synthesized from the total RNA using a Bio-Rad cDNA kit. To prepare samples for qRT-PCR, first a reaction mixture was made without cDNA in it to load the wells. 12.5μL SYBRGreen dye mix, 1μL of primer, and 10.5μL distilled water were used per well per sample. 24μL of this mixture was added to each well needed of a 96-well plate. 1μL of cDNA sample was added to each well and mixed by pipetting. The plates were then run on a BioRad Thermocycler using the procedure following: 95°C for five minutes, 95°C for one minute, 60°C for four minutes; steps two and three repeated 50 times. All gene expressions were normalized to GAPDH.

Western Blotting: Western blots were performed using 40 μg of protein extracted from cultured cells. Protein was resolved by electrophoresis using 4 – 20% polyacrylamide gels. Following transfer to nitrocellulose membranes, blots were incubated with antiserum against the protein of interest. They were then incubated with horseradish-peroxidase conjugated anti-rabbit or mouse IgG for one hour. Blots were visualized by autoradiography using ECL-plus from Amersham Biosciences following washing with TBST.

Fatty Acid Oxidation Assay: Total lipids were extracted from control and treated cells as described previously (ref). The fatty acid methyl esters (FAME) were analyzed by gas chromatography (GC; Shimadzu chromatograph GC-15A attached to a Shimadzu chromatopac C-R3A integrator) using a fused silica capillary column (25 M 007 series methyl silicone, 0.25 mm internal diameter, 0.25 μm film thickness) from

Quadrex Corporation (Woodbridge, CT) in a gas chromatograph GC-17A connected with a flame ionization detector from Shimadzu Corporation.

ABCD2/ABCD3 mRNA in Control Normal Human Fibroblasts with SAHA Treatment

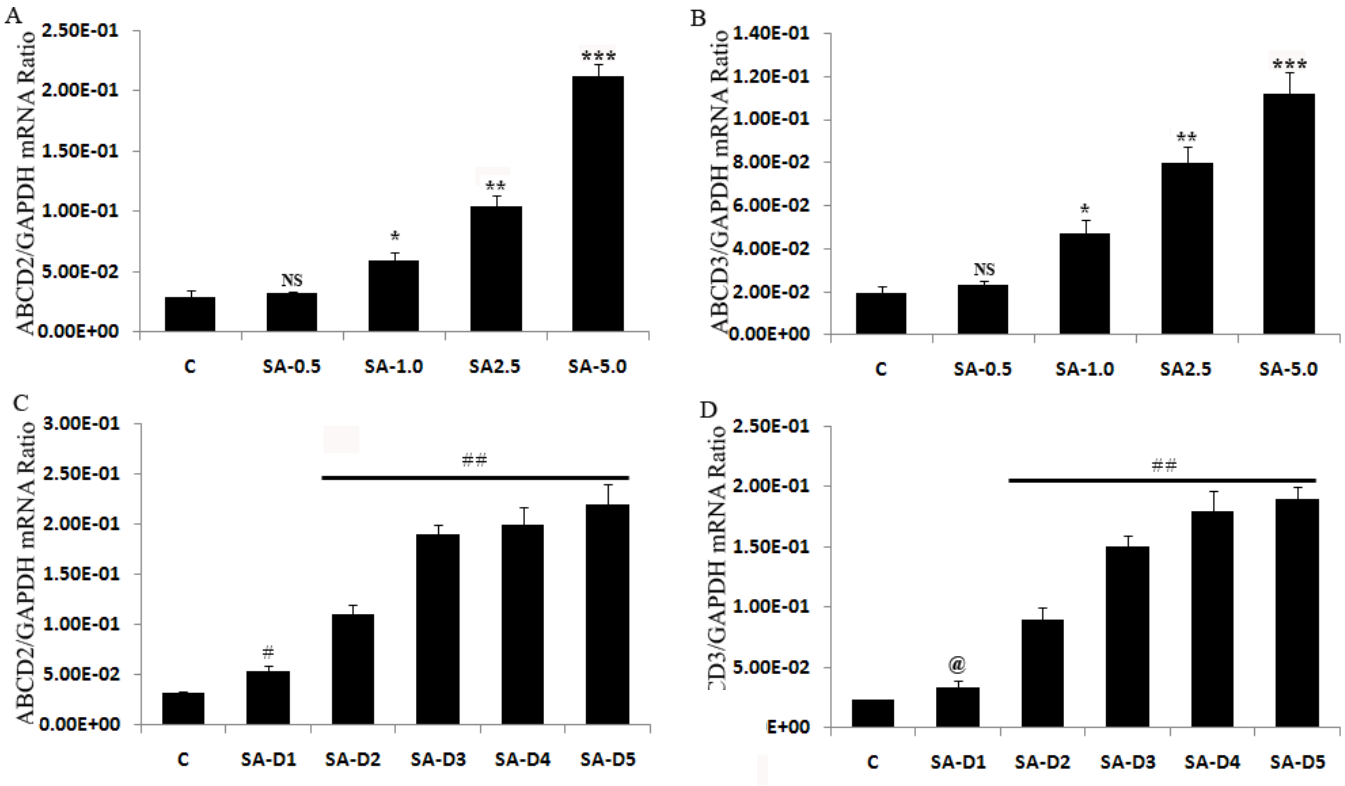

Figure 1

Figure 1: Treatment with SAHA significantly increases ABCD2 and ABCD3 mRNA expression in normal human fibroblasts. This effect is seen in normal fibroblasts when treated with SAHA in a dose (A and C) and time (B and D) dependent manner. ABCD2 and ABCD3 mRNA expressions were determined with qualitative RT-PCR and normalized to GAPDH. Data are represented as mean +/- standard deviation. * P<0.005 SAHA (1.0 μM) treatment compared with control; **P<0.001 SAHA (2.5 μM) treatment compared to control; ***P<0.001 SAHA (5.0 μM) treatment compared to control; # P<0.002 SAHA (5.0 μM, Day 1) treatment compared to control; @ P<0.02 SAHA (5.0 μM, Day 1) treatment compared to control; ## P<0.001 SAHA (5.0 μM, Day 2-5) treatment compared to control. NS, nonsignificant.

Fatty Acid Oxidation in Normal Human Fibroblasts with SAHA Treatment

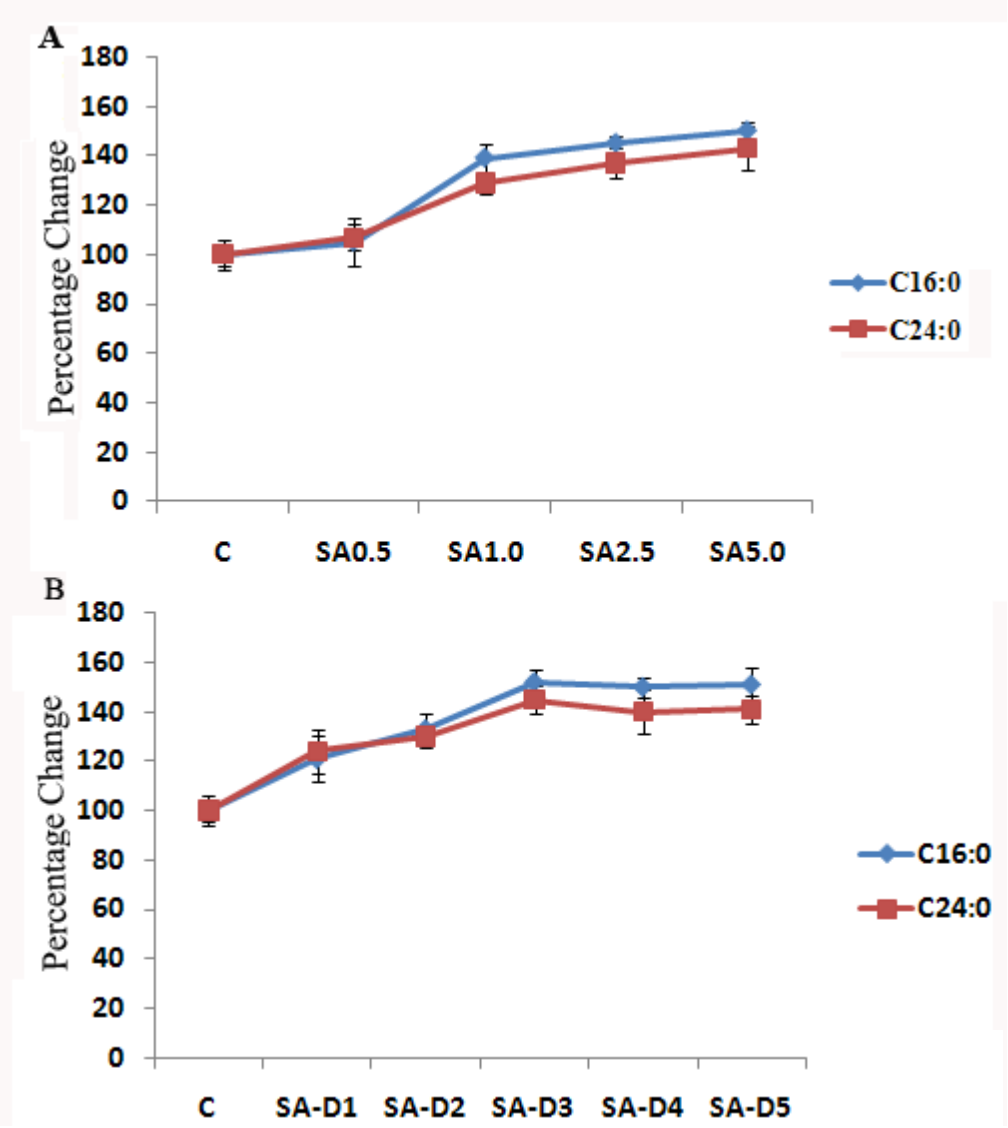

Figure 2

Figure 2: SAHA treatment increased the oxidation of palmitic and lignoceric acid in cultured normal human fibroblasts in a dose- and time-dependent manner. Cells were incubated with various concentrations of SAHA for three days (A) or with 5 μM SAHA for different lengths of time (B). Medium was replaced every 24 hours. Values given are mean +/- standard deviation of three different experiments.

Effect of SAHA on ABCD2 Expression in X-ALD Fibroblast Cell Cultures

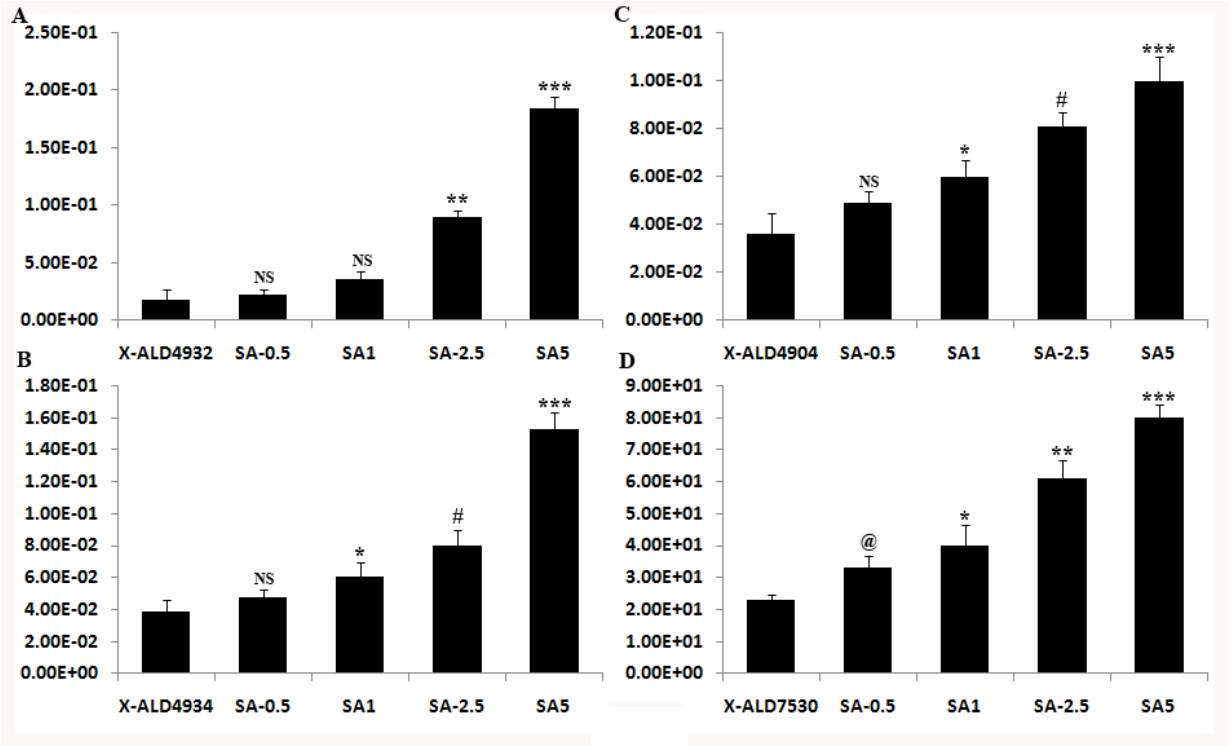

Figure 3

Figure 3: SAHA treatment induces ABCD2 expression in X-ALD fibroblasts. Human skin fibroblasts derived from X-ALD (GM04932 (A), GM04934 (B), and GM04904 (C)) and AMN (GM07530 (D)) treated with SAHA in a dose dependent manner and ABCD2 expression was measured. ABCD2 mRNA levels were determined by quantitative RT–PCR and normalized to GAPDH. Data are represented as mean ± standard deviation of three different experiments. *P<0.05 SAHA (1.0 μM) treatment compared with untreated X-ALD fibroblasts; **P<0.001 SAHA (2.5 μM) treatment compared with untreated X-ALD fibroblasts; ***P<0.002 SAHA (2.5 μM) treatment compared with untreated X-ALD fibroblasts; #P<0.005 SAHA (2.5 μM) treatment compared with untreated X-ALD fibroblasts; **@**P<0.02 SAHA (0.5 μM) treatment compared with untreated AMN fibroblasts. ***P<0.001 SAHA (5.0 μM) treatment compared with untreated X-ALD fibroblasts. NS, nonsignificant

SAHA Increases ABCD2/ABCD3 Protein Products in Control and X-ALD Fibroblasts

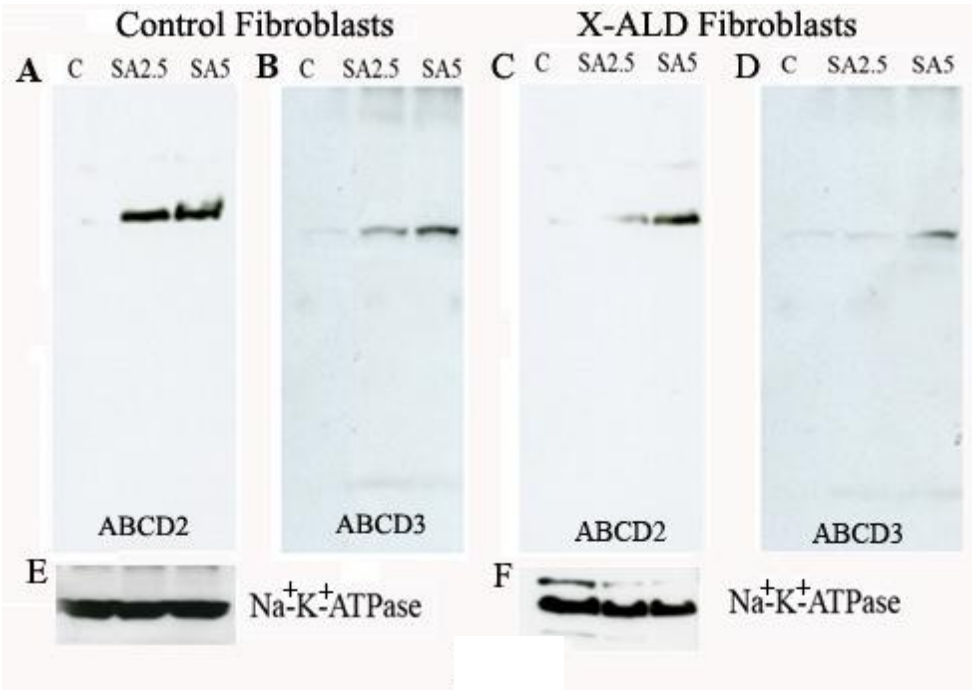

Figure 4

Figure 4: Treatment with SAHA upregulates ABCD2 and ABCD3 proteins in normal and X-ALD cells. Human control (GM07576) and X-ALD (GM04932) fibroblasts were cultured and used to analyze protein levels of the peroxisomal integral membrane proteins transporters ALDRP, coded by ABCD2 (A and C) and PMP70, coded by ABCD3 (B and D). Protein levels were analyzed by Western blot using cell membrane fractions containing integral membrane proteins. Na^+/K^+-ATPase, a constitutively expressed plasma membrane protein, was used as a control to indicate protein loading (E and F).

Changes in Fatty Acid Oxidation in X-ALD Fibroblasts with SAHA Treatment

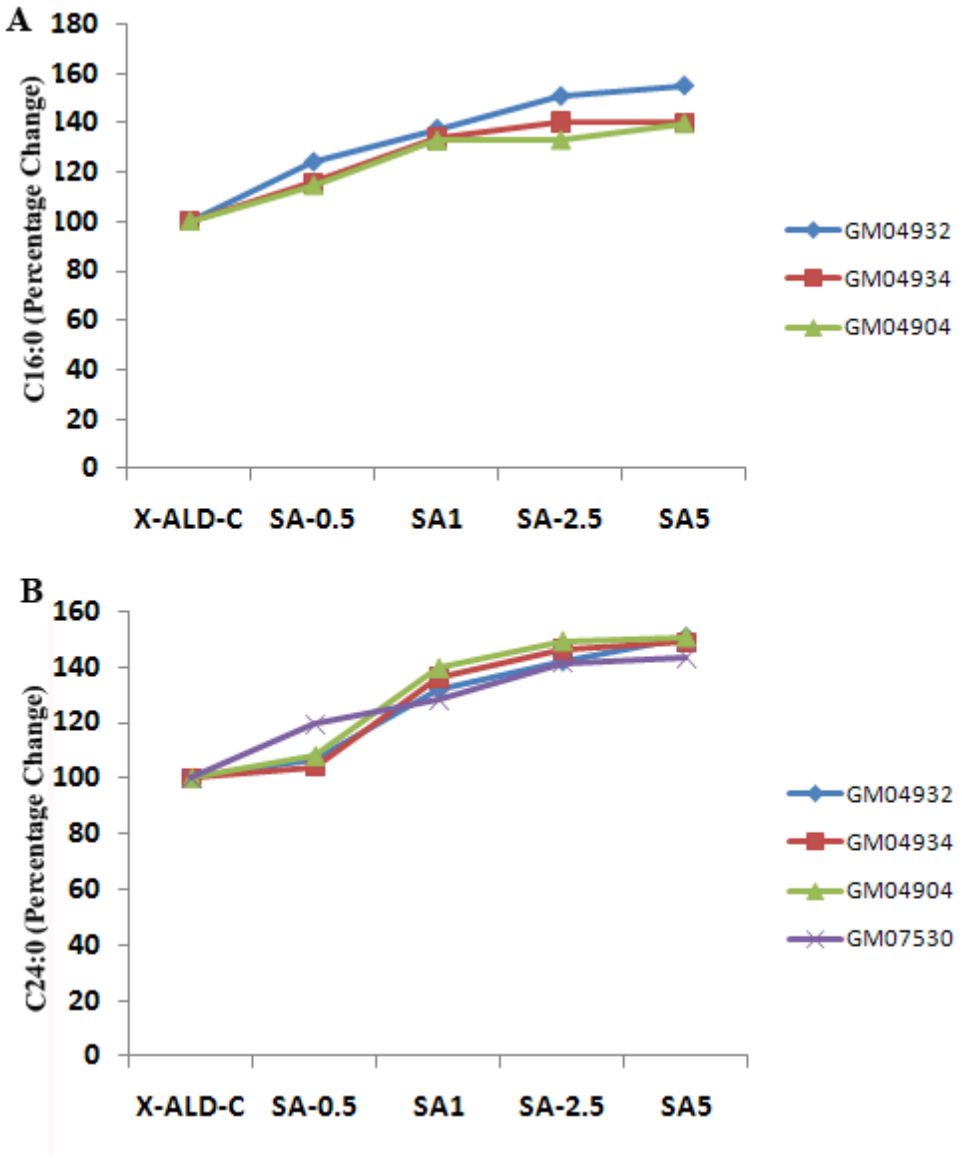

Figure 5

Figure 5: β-oxidation of lignoceric and palmitic acid is increased in X-ALD fibroblasts with SAHA treatment. Human skin fibroblasts from 3 X-ALD patients (GM04932, GM0493, and GM04904) and one AMN patient (GM07530) were incubated in serum-containing DMEM with different concentrations of SAHA for three days. B-oxidation activities of palmitic (A) and lignoceric acid (B) were then measured as described in the Methods section. Medium was replaced every 24 hours with the addition of fresh reagents. Values are mean of three different experiments.

Fatty Acids in Normal, X-ALD, and SAHA-treated Fibroblasts

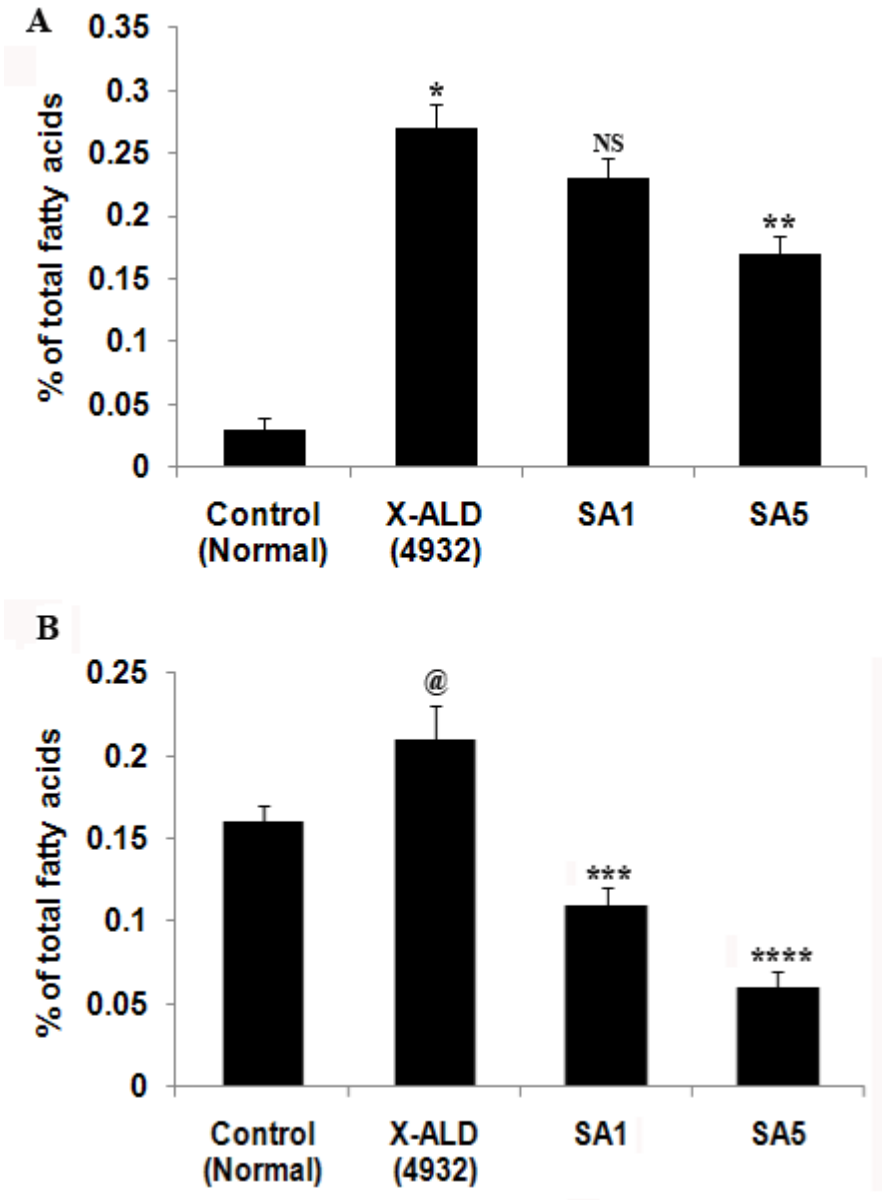

Figure 6

Figure 6: The effect of treatment with SAHA on VLCFA in normal and X-ALD human fibroblasts. Cells were incubated in DMEM with FBS and were treated with different concentrations of SAHA. Fatty acid methyl ester was prepared directly from cells as described in the Methods section. Fatty acids were analyzed by GC after adding C27:0 as an internal standard. Saturated (C26.0) and monounsaturated (C26:1) VLCFAs were measured and expressed as percent C26:0 (A) and C26:1 (B) of total FAs. Results represent the mean ± SD of duplicates from three different experiments. *P<0.001 percent C26:0 of total fatty acids in X-ALD compared to normal fibroblasts; **@**P<0.02 percent C26:1 of total fatty acids in X-ALD compared to normal fibroblasts; **P<0.002 SAHA (5.0μM) treatment compared with untreated X-ALD fibroblasts; ***P<0.001 SAHA (1.0μM) treatment compared with untreated X-ALD fibroblasts; ****P<0.001 SAHA (5.0μM) treatment compared with untreated X-ALD fibroblasts. NS, nonsignificant

ELOVL Activity Changes with SAHA Treatment

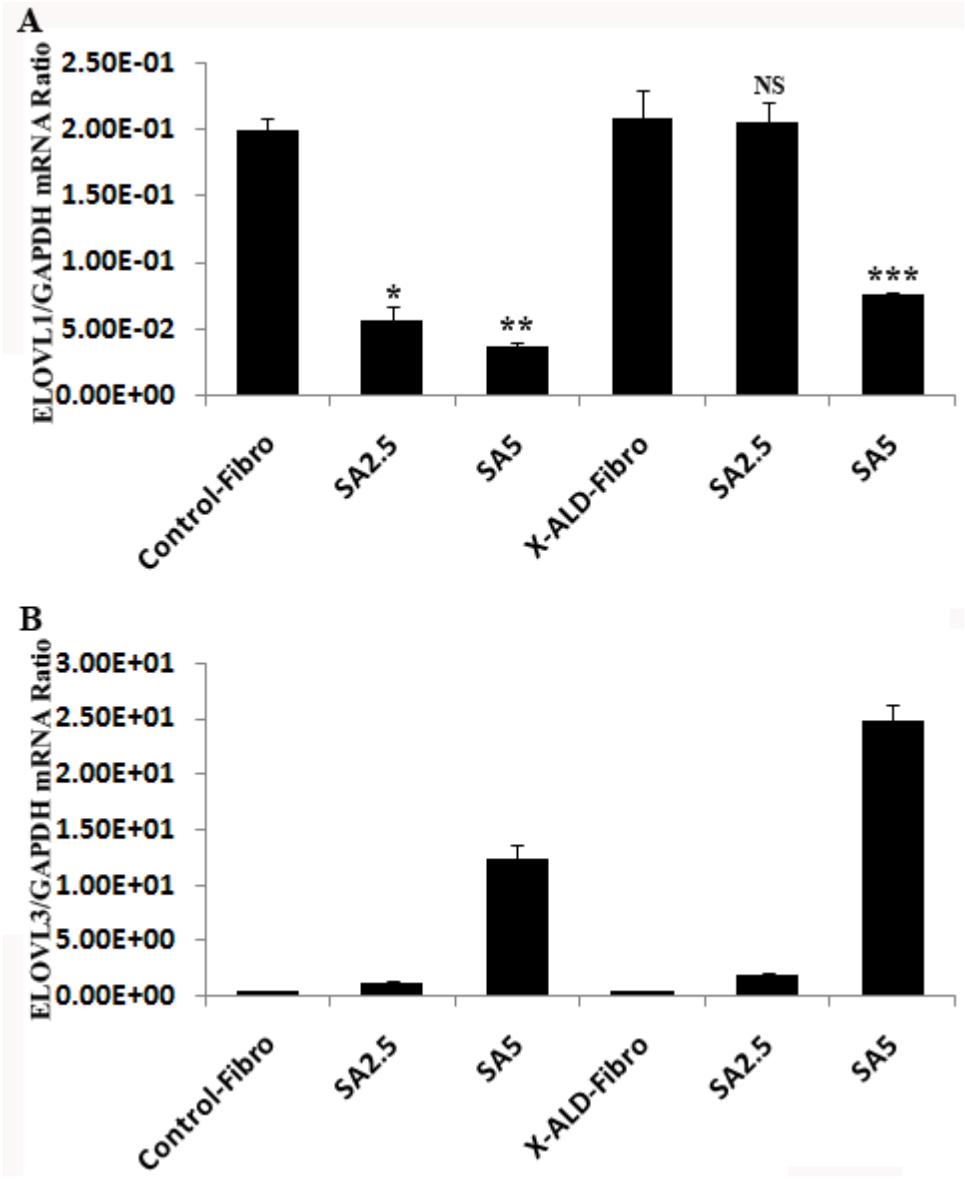

Figure 7

Figure 7: SAHA treatment lowers the mRNA expression of ELOVL1 in normal and X-ALD fibroblasts. Control and X-ALD human fibroblasts were treated with different doses of SAHA for 3 days and mRNA expression of ELOVL1 (A) and ELOVL3 (B) were quantified by RT-PCR. All samples were normalized to GAPDH. Data are represented as mean ± SD. *P<0.001 SAHA (2.5μM) treatment compared with untreated control fibroblasts; **P<0.001 SAHA (5.0μM) treatment compared with untreated control fibroblasts; ***P<0.001 SAHA (5.0μM) treatment compared with untreated X-ALD fibroblasts. NS, nonsignificant.

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