Studying fatty aldehyde metabolism in living cells with pyrene labeled compounds

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Running foot line:
Fatty aldehyde metabolism of cultured cells

Abbreviations: SLS, Sjögren Larsson Syndrome; SD, standard deviation; FALDH, fatty aldehyde dehydrogenase; AGMO, alkylglycerol monooxygenase; ADH, alcohol dehydrogenase; CHO-K1, Chinese hamster ovary cells
ABSTRACT

Lack of fatty aldehyde dehydrogenase function in Sjögren Larsson Syndrome patient cells not only impairs the conversion of fatty aldehydes into their corresponding fatty acid, but has also an effect on connected pathways. Alteration of the lipid profile in these cells is thought to be responsible for severe symptoms such as ichthyosis, mental retardation and spasticity. Here we present a novel approach to examine fatty aldehyde metabolism in a time dependent manner by measuring pyrene labeled fatty aldehyde, fatty alcohol, fatty acid and alkylglycerol in the culture medium of living cells using HPLC separation and fluorescence detection. Our results show that in fibroblasts from Sjögren Larsson Syndrome patients fatty aldehyde is not accumulating, but is converted readily into fatty alcohol. In control cells, in contrast, exclusively the corresponding fatty acid is formed. Sjögren Larsson Syndrome patient cells did not display a hypersensitivity towards hexadecanal or hexadecanol, but 3-fold lower concentrations of the fatty alcohol than the corresponding fatty aldehyde were needed to induce toxicity in Sjögren Larsson Syndrome patient and in control cells.

SUPPLEMENTARY KEYWORDS

Sjögren Larsson Syndrome; SLS; fatty aldehyde metabolism; long chain fatty aldehyde dehydrogenase; FALDH; ALDH3A2; SLS, E.C. 1.2.1.48;
The autosomal, inherited disease Sjögren Larsson Syndrome (SLS, OMIM: 270200) is caused by mutations in the ALDH3A2 gene (1). This gene codes for the enzyme fatty aldehyde dehydrogenase (FALDH, [EC 1.2.1.48]) which catalyses the irreversible, NAD-dependent conversion of a wide range of different fatty aldehydes into their corresponding fatty acids (2, 3). This enzyme is active as a homodimer (4) and is bound to membranes by its hydrophobic carboxy-terminus (5). So far, more than 70 mutations are known that impair fatty aldehyde dehydrogenase function (6), thereby having a severe impact on the metabolism of lipids in cells (7). Accumulating fatty aldehydes in SLS patients are suspected to cause a wide range of symptoms such as ichthyosis, mental retardation and spasticity (8), by chemical interaction with free amino-groups of lipids and proteins (9).

The crucial role of fatty aldehyde dehydrogenase in multiple lipid pathways is not only emphasized by the diversity of symptoms, but also by the targeted localization of fatty aldehyde dehydrogenase splice variants to the endoplasmatic reticulum and to peroxisomes, respectively (5), and its role in diabetes induced lipid peroxidation (10, 11). Fatty aldehydes are produced in fatty alcohol metabolism (2). They are also formed in the enzymatic cleavage of ether lipids by alkylglycerol monooxygenase (12) and in leucotriene B4 degradation (13).

So far, the role of fatty aldehyde dehydrogenase in lipid metabolism was studied by quantification of different lipid species in SLS patient cells by organic phase extraction of cell pellets and analysis with thin layer chromatography and high performance thin layer
chromatography (7, 9, 14). Another approach was to use radiolabeled substrates which are quantified by scintillation spectrometry, subsequent to organic phase extraction, separation by thin layer chromatography, identification of bands of interest and scraping off selected bands (7, 9, 14).

Here, we present a sensitive method that allowed us to follow the fate of four different fluorescent labeled substrates in the culture medium of living cells. We used this method to monitor the metabolism of fatty aldehyde and fatty alcohol in SLS patients and in control fibroblasts.
MATERIALS AND METHODS

Materials
Pyrenedecanol was synthesized from pyrenedecanoic acid and 1-O-pyrenedecyl-sn-glycerol (1-O-pyrenedecylglycerol) was synthesized from pyrenedecanol and glycerol as described in (Werner 2007). Pyrenedecanal was purchased from Ramidus AB (Lund, Sweden).

Synthesis of n-hexadecanal
2.0 g n-Hexadecanol was dissolved in 30 ml of dry CH₂Cl₂. Then, 1.3 ml dry DMSO was added and mixture was cooled to 0°C in an ice bath. 2.35 g P₂O₅ was inserted and the mixture stirred for 2 h without cooling. After completion of the reaction (TLC control) the mixture was cooled again to 0°C and 4.25 ml of triethylamine was added drop wise, followed by 1 h stirring at room temperature. Then, the reaction mixture was mixed with 20 ml of water and 18% (w/v) HCl solution was added drop wise until pH 2 was obtained. The phases were separated, the organic layer was dried over Na₂SO₄, filtered and the solvent was removed under reduced pressure. The crude product was purified by a short column chromatography (silica gel) using a solvent mixture of hexane/ethylacetate 10:1 (v:v). After removing the solvents a colorless solid was obtained (15, 16). Yield, 1.8 g (92%); mp., 32°C. Product quality was confirmed by ¹H- and ¹³C-NMR.

Cells
Fibroblasts from three different SLS patients were provided by the Laboratory of Prof. J.A. Ronald Wanders (Genetic Metabolic Diseases, Academic Medical Center at the University of...
Amsterdam, The Netherlands) (13, 17). The patients displayed the typical symptoms of SLS. Lack of enzymatic activity had been confirmed by measurement of FALDH activity in fibroblasts and the mutations in the ALDH3A2 gene were identified. Additional information on the Sjögren Larsson Syndrome patient cells can be found in Table 1. Human dermal fibroblasts from three healthy individuals served as controls and were kindly provided by Christine Heufler (Department of Dermatology, Innsbruck Medical University). Informed consent and institutional approval of the studies has been obtained at the respective institutions. Chinese hamster ovary K1 cells (CHO-K1) used for transfection experiments were purchased from LGC Promochem (Wesel, Germany).

Cell culture conditions

If not stated otherwise, all cells were grown at 37°C, 100% humidity and in an atmosphere of 5% CO₂. Normal human fibroblasts and Sjögren Larsson Syndrome patient fibroblast cells were maintained in 75 cm² flasks in Dulbecco’s Modified Eagle Medium (DMEM, containing GlutaMAX I, 1,000 mg/l D-glucose and sodium pyruvate; Gibco, Invitrogen, Carlsbad, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (PAN Biotech, Aidenbach, Germany). CHO-K1 cells were grown in F12-K medium with 10% (v/v) fetal calf serum and Penicillin-Streptomycin (Sigma, Vienna, Austria).

Quantification of fluorescent lipid metabolites in the culture medium of living cells

Fibroblasts were plated in 24-well plates at a density of 10⁵ cells per well in 2 ml medium and allowed to adhere for 24 h. The cells were incubated with one of the fluorescent compounds: pyrenedecanal, pyrenedecanol, pyrenedecanoic acid or 1-O-pyrenedecylglycerol, which were added to the medium from a sterile 100-fold stock solution in ethanol:water (1:7; v:v) resulting in a final concentration of 5 µM.
After 24 h of incubation, 10 µl of the culture medium was transferred into a 1.5 ml tube and 30 µl of methanol was added. The mixture was mixed and centrifuged at 13,000 x g and 4°C for 10 minutes. 10 µl of the mixture were injected into the HPLC system and fluorescent compounds were quantified as described below. Peaks were identified by spiking of samples with standard solutions of the fluorescent compounds. The quantification was done by referring to the total fluorescence of initially applied substrate.

For time course experiments, a 10 µl sample was taken under sterile conditions after 5 min, 30 min, 1 h, 4 h, 10 h and 24 h. The samples were analyzed as described above.

Quantification of fluorescent metabolites in cell pellets

After having taken the sample for the culture medium assay, the remaining medium was removed by suction. Adherent cells were washed with 1 ml Dulbecco’s PBS buffer (Serva, AL-Labortechnik, Amstetten, Austria) and then detached with 500 µl trypsin/EDTA (Sigma, Vienna, Austria) for 5 min at 37°C. Subsequently, 500 µl PBS were added and the cell suspension was transferred into a 1.5 ml tube. Cells were pelleted by centrifugation for 10 minutes at 5,000 x g. 30 µl of methanol was added to the pellets followed by shaking at 900 rpm and room temperature for 1 h in order to lyse the cells and to extract the fluorescence labeled lipids. The mixture was then centrifuged at 13,000 x g for 10 minutes and 10 µl of the supernatant was injected into the HPLC system.

Since lipids were extracted from cell pellets, quantification was not possible by referring to the initial substrate amount, instead data were normalized by the total fluorescence of
pyrene-labeled lipids in each sample. Controls showed that there was no carry-over of fluorescent metabolites by adherence on plasticware.

Cytotoxic effects of long chain fatty aldehydes, alcohols and acids on SLS and control cells

Cytotoxic effects were studied with a method adapted from (9). Cells were plated in 96-well plates at a density of 500 cells/well in 200 µl of DMEM medium. They were allowed to adhere for 24 h. 0, 25, 62.5, 125, 187.5, 250, 500, 750 and 1250 µM of hexadecanal, hexadecanol or hexadecanoic acid or 5 µM of pyrene labeled aldehyde, alcohol, alkylglycerol and fatty acid were added from a 40x stock solution in ethanol (triplicates for each condition). The percentage of ethanol added to the individual wells was 2.5% (v/v) which had no influence on cell viability, as was shown by comparison of ethanol-only treated controls with untreated cells.

Cells were incubated with the compounds for 24 h, then the medium was replaced with compound-free DMEM and cells were allowed to grow out for another 96 h. Cell viability was measured using the MTT assay (Promega, Mannheim, Germany). Ethanol-only treated samples served as reference.

Solubility of hexadecanoic acid in DMEM medium containing 2.5% (v/v) ethanol was limited to concentrations below 750 µM, for hexadecanal and hexadecanol to concentrations below 1250 µM as judged by visual inspection of turbidity. Therefore, the analysis was only performed in those concentrations where compounds were soluble (up to 500 µM).
Fluorescent substrates and their metabolites were quantified on an Agilent 1200 Series HPLC system using a Zorbax XDB-C8 USP-L7 column (Agilent Technologies, Vienna, Austria) as described in (4). Briefly, after treating the sample with a 3-fold excess of methanol, 10 µl of the mixture was injected into the HPLC system and eluted with 10 mM potassium phosphate, pH 6.0, containing 81.25% (v/v) methanol at a flow rate of 1.0 ml/min. After 8 min, a linear gradient to 100% methanol at 8.5 min was applied. From 12.5 to 13 min the initial mobile phase composition was restored. Fluorescent detection was performed with excitation at 340 nm and emission at 400 nm. The identity of pyrene labeled compounds was confirmed by spiking with a standard solution of the respective compound. For fatty aldehyde dehydrogenase activity measurements, peaks were quantified via their peak area in comparison to an external 100 nM standard solution of synthetic pyrenedecanoic acid.

Transfection of CHO-K1 cells

Expression plasmids of candidate genes were obtained from OriGene Technologies Inc. (Rockville, MD, USA; ALDH1A1: SC321535; ALDH2: SC119703; ALDH3A1: SC321516; ALDH3B1: SC119707; ALDH3B2: SC119708). CHO-K1 cells were grown in F12-K medium (Gibco, Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum and Penicillin-Streptomycin. For transfection, cells were plated in 6-well plates with a density of 10^6 cells/well. Plasmids (3 µg per well) were transfected to CHO cells using ExGen 500 (Fermentas, St. Leon-Rot, Germany) in presence of 1 µM of the tetrahydrobiopterin precursor sepiapterin (Schircks Laboratories, Jona, Switzerland). Cells were cultivated for 24 h, harvested and fatty aldehyde dehydrogenase activities were determined.
Fatty aldehyde dehydrogenase activity measurement

For activity measurement, monolayers of transfected CHO-K1 were collected by trypsinization, washed with Dulbecco’s PBS buffer (Serva) and resuspended in distilled water containing 0.5% CHAPS (Roth, Lactan, Graz Austria) and 1% (v/v) of a protease inhibitor mixture (GE Healthcare, Vienna, Austria). Cells were opened by rapid freeze thawing.

Fatty aldehyde dehydrogenase activity was measured as published in (4). Briefly, the assay mix contained 20 mM sodium pyrophosphate, pH 8.0, 1 % Triton-X100 (v/v, reduced form, Serva), 50 μM pyrenedecanal and 1 mM NAD (Sigma). The reaction was started by the addition of the reagent mixture to 3 μl of sample, corresponding to 15-23 μg of total protein. Samples were incubated in air tight tubes at 37°C in a total volume of 10 μl for 10 min. No loss of volume was observed under these conditions. Then the reaction was terminated by addition of 30 μl methanol. The amount of pyrenedecanoic acid formed was determined by HPLC with fluorescence detection as described above. To control for variations in the extraction efficiency and for errors in sampling, the total area of all fluorescent peaks was monitored. This parameter was stable. Typical variation coefficients observed were 3.1 % within run, 11.9 %. day to day.

Statistical analysis

Unless otherwise stated, data are presented as means ± standard deviation (SD). Statistical significance was determined by 2-way ANOVA for the quantification of fluorescent labeled metabolites or one-way ANOVA for transfection experiments with Bonferroni as posthoc analysis using GraphPad Prism 5.01 (GraphPad Software, Inc., San Diego, CA, USA).
RESULTS

HPLC-based quantification of lipid metabolites formed from fluorescent labeled compounds in living cells

Our aim was to study the fatty aldehyde and fatty alcohol metabolism in living cells. Therefore we established a novel protocol that allows us to follow the fate of different fluorescent labeled compounds in the culture medium of cells. We used the following four pyrene labeled substrates (see Figure 1): 1-O-pyrenedecylglycerol ([1], peak 1), which is converted into pyrenedecanal ([2], peak 2) by alkylglycerol monooxygenase (AGMO) (12). The formed fatty aldehyde is further metabolized into pyrenedecanoic acid ([3], peak 3) by fatty aldehyde dehydrogenase (FALDH) (4) or into pyrenedecanol ([4], peak 4) by a fatty alcohol dehydrogenase (ADH) (18). Several further unidentified fluorescent peaks appeared in the chromatogram in addition to the four metabolites quantified (Figure 1B). The four metabolites, however, accounted for a major portion (74.5 ± 10.5 %, mean ± SD, n = 44) of the total area of all fluorescent peaks detected.

Sjögren Larsson Syndrome patient fibroblasts (SLS cells, see Table 1) of three different individuals were incubated with one of the fluorescent compounds at a time. Fibroblast from three healthy individuals served as controls. Our HPLC method was used to quantify the metabolites which are formed during incubation. Figure 1B shows typical chromatograms for the incubation of normal human fibroblast (upper trace) and Sjögren Larsson Syndrome patient fibroblasts (lower trace) with pyrenedecanal. Comparison of the chromatograms shows that there are big differences in the pyrene-labeled metabolite composition of SLS
and control cells. However, the original substrate pyrenedecanal (peak 2) is metabolized to a large extend in both SLS and control cells.

The detection limit for pyrenedecanoic acid was 10 fmoles. Due to the longer retention times and broadening of peaks the detection limits of pyrenedecanal, pyrenedecanol and 1-O-pyrenedecylglycerol were 10 times higher (100 fmoles). The 5 µM of fluorescent substrates we used were not cytotoxic to the cells. Viable cell numbers as determined by the MTT assay in presence of 5 µM pyrene-labeled aldehyde, alcohol, alkylglycerol and acid in comparison to solvent - only treated cells (in percent, mean ± SD, n = 3) were for control fibroblasts 107.5 ± 17.5, 106.9 ± 17.5, 101.6 ± 21.7, and 97.5 ± 15.4, respectively. For SLS patient fibroblast the corresponding viable cell numbers were 110.9 ± 12.3, 108.3 ± 13.0, 99.6 ± 12.9, and 96.1 ± 7.4, respectively.

Fluorescent metabolite composition in the culture medium of living fibroblasts

Figure 2A shows the metabolite composition in the culture medium of cells after incubation with pyrenedecanal. Both, control and SLS cells were able to readily metabolize the fatty aldehyde. Under our experimental conditions, more than three quarters of the added pyrenedecanal were metabolized. Control cells converted it almost exclusively into the corresponding fatty acid and formed only marginal amounts of fatty alcohol. In contrast, in SLS cells the most prominent species found was the fatty alcohol. Fatty acid was also formed to some extent by SLS cells, yielding up to about one-third of the levels found in the culture medium of control cells. We detected no accumulation of pyrene-labeled alkylglycerol in SLS or control cells, although fatty alcohols are precursors for etherlipid synthesis. This may be due to conversion of alkylglycerols to ether phospholipids in the cells.
The ratio of fatty acid to fatty alcohol after incubation with pyrenedecanal was a reliable marker for the ability of cells to oxidize fatty aldehydes to the corresponding acid. In the culture medium of SLS patient cells the ratio was $R_{\text{acid/alcohol}} = 0.6 \pm 0.2$ (n = 17, range: 0.2 – 1.0). For control cells the measured mean ratio was about 46-fold higher: $R_{\text{acid/alcohol}} = 28 \pm 11$ (n = 17, range: 14-50, when no fatty alcohol was detectable the detection limit was used for data analysis). $R_{\text{acid/alcohol}}$ was not dependent on incubation time as was shown by time course experiments.

The metabolite composition in the culture medium of living cells after the incubation with pyrenedecanol is shown in Figure 2B. Control cells were able to convert approximately half of the added fatty alcohol into the corresponding fatty acid. SLS cells, in contrast, metabolized less than a quarter of the added fatty alcohol. In addition, less than 15 % of pyrenedecanoic acid was formed by SLS as compared to control cells. These differences were highly significant (P < 0.001). Interestingly, neither in control nor in SLS fibroblasts an accumulation of pyrene-labeled fatty aldehyde was observed.

The metabolite compositions after incubation with pyrenedecanoic acid and 1-O-pyrenedecylglycerol are shown in Figures 2C and 2D, respectively. Both substances were not metabolized to compounds detectable in our chromatograms in control and in SLS cells. A major part of the added compounds was still present in the culture media after the incubation period.
Fluorescent metabolite composition in control and SLS cell pellets

We measured not only metabolites in the culture medium, but we also collected the respective cell pellets, extracted the lipid components and analyzed them with the same HPLC method (see material and methods section). The results for these measurements are presented in Figure 3. Incubation with pyrenedecanal yielded very similar relative fluorescent metabolite compositions in the culture media (Figure 2A) and the pellets (Figure 3A). While in control cells mainly the corresponding fatty acid was formed, fatty alcohol was the major product in SLS cells (P < 0.001). Also the relative amounts of other pyrene labeled metabolites in the cell pellets (Figures 3B-D) have high similarities to their culture medium counterpart (compare Figures 2B-D). From the total amount of the pyrene labeled lipids added (100%), the pellet accounted for less than one percent and most of the pyrene label (65 - 85%) appeared as fluorescent metabolites in the culture medium.

Time course experiments

Due to the low volume requirements (10 µl) for analysis of fluorescent compounds in the culture medium it was possible to incubate cells with the fatty aldehyde pyrenedecanal and to follow the change of metabolite composition in the same well over a period of 24 h (see Material and Methods). Figure 4A shows the steady decrease of fatty aldehyde in the medium and a simultaneous formation of fatty acid in control cells. Fatty alcohol was not detectable at any time point. In contrast, fatty alcohol increased linearly together with some fatty acid in SLS cells when incubated with pyrenedecanal (Figure 4B).
Cytotoxic effects of hexadecanal, hexadecanol and hexadecanoic acid on control and SLS patient fibroblasts

Since the results of the fluorescent metabolite composition experiments suggested that fatty aldehydes are readily metabolized into fatty alcohols in SLS cells, we compared the cytotoxic effect of fatty aldehyde, fatty alcohol and fatty acid. We measured the LD₅₀ values of hexadecanal, hexadecanol and hexadecanoic acid (see Table 2). We found no significant difference between the cytotoxic effect of these compounds on control and SLS cells (P > 0.05). Surprisingly, LD₅₀ values for hexadecanol (SLS cells: 45.7 ± 1.5 μM; controls: 38.6 ± 6.6 μM) were about three times lower than the values for hexadecanal (SLS cells: 128 ± 10 μM; controls: 122 ± 15 μM) (P < 0.001).

Substrate specificity of fatty aldehyde dehydrogenase

Our results show that SLS cells are to some extend able to convert fatty aldehydes into fatty acids. To check whether aldehyde dehydrogenases other than fatty aldehyde dehydrogenase are able to metabolize pyrenedecanal, we transfected different aldehyde dehydrogenases into CHO-K1 cells to measure their capability to catalyze this reaction. The results are shown in Figure 5. One-way ANOVA analysis revealed that only fatty aldehyde dehydrogenase shows a significantly higher enzymatic activity than the green fluorescent protein transfected controls (P < 0.05). However, transfection of the closely related ALDH3A1 (68% protein sequence homology, NCBI, blastp suite, NP_000373.1 versus Homo sapiens RefSeq protein) results in an observable but not significant increase of pyrenedecanal degradation rate (Figure 5). No difference to the controls was found for ALDH3B1 (55% protein sequence homology), ALDH3B2 (51% protein sequence homology), ALDH1A1 (28% protein sequence homology) and ALDH2 (28% protein sequence homology).
DISCUSSION

In previous work we showed that pyrenedecanal can be used to quantify fatty aldehyde dehydrogenase activity in cell and tissue homogenates (4). The method we present here extends the range of application of this compound to monitor fatty aldehyde metabolism in the culture media of living cells. Our approach enables measurement of the accumulation or depletion of metabolites formed from pyrenedecanal, pyrenedecanol, pyrenedecanoic acid and 1-O-pyrenedecyl glycerol in a time dependent manner in cells grown in a 24 well culture dish. We demonstrate that the four quantified fluorescent metabolites appear in comparable relative amounts in the cell pellets and in the culture medium. This shows that the transport of the added substrates and the quantified metabolites through the cell membrane is not a limiting factor. Further metabolites such as esterified compounds (e.g. triglycerides, phospholipids, cholesterol esters) might be formed from the fluorescent labeled compounds and might not elute from our column at 100 % methanol. Balance calculations indicate that we recover 65 - 85 % of the initially added fluorescent label in the chromatograms. Thus, the four metabolites we measure appear to constitute a major portion of the metabolites formed under our experimental conditions.

For our measurements we utilized 5 µM of fluorescent substrate which was not cytotoxic to the cells. Since only small amounts of the culture medium and no cell material are required this analysis can be easily combined with other assays.

Our experiments demonstrate that relative amount of fluorescent labeled metabolites measured after incubation of cultivated, living SLS patient and control cells with pyrenedecanal and pyrenedecanol differ strongly. Lack of fatty aldehyde dehydrogenase
function in SLS patient cells gave rise to the accumulation of fatty alcohols from fatty aldehydes, whereas in control cells almost exclusively the non toxic fatty acid was formed. While control cells were able to convert fatty alcohols into the corresponding fatty acid, they remained unmetabolized by SLS patient cells. These results are in line with previous findings by Rizzo et al. (7, 19) who detected elevated fatty alcohol levels in serum and fibroblasts of SLS patients and decreased ability of SLS patient cells to oxidize long chain fatty alcohols to acid.

Inability of patient cells to metabolize fatty aldehydes and the formation of Schiff base adducts with lipids and proteins is suspected to be responsible for symptoms of SLS (9). Surprisingly, we found in our time course experiments that control and SLS cells were both able to metabolize fatty aldehydes at the same rate. Despite the sensitive fluorescent detection system we found no accumulation of fatty aldehydes in all cells at any time point when incubated with fatty alcohol, fatty acid or alkylglycerol. Although we cannot exclude that the failure to find pyrene-labeled fatty aldehyde when cells were incubated with pyrenedecanol could be due to its unmeasured stable Schiff base formation with phosphatidylethanolamine, more alcohol remains unmetabolized in SLS as compared to control fibroblasts (Figure 2B). Thus the deficiency to oxidize the aldehyde to the acid in SLS fibroblasts apparently impairs the metabolism of the alcohol. As is evident from the results of experiments feeding pyrenedecanal to SLS fibroblasts, the equilibrium between aldehyde and alcohol lies on the alcohol side in these cells (Figure 2A).

In contrast to previous findings in which SLS patient fibroblasts are described to be hypersensitive towards fatty aldehydes (9), we could not detect significantly different LD50
values for hexadecanal, hexadecanol or hexadecanoic acid in SLS patient and control cells. Cells were tolerant towards the fatty acid up to the highest soluble concentrations and a LD$_{50}$ value was not assessable. Interestingly, in our setting fatty alcohols were more toxic than fatty aldehydes in SLS and in control cells, as is reflected by the 3 times lower LD$_{50}$ value for hexadecanol. Our data suggest that particular attention should be paid to the biochemical action and toxicity of fatty alcohols when interpreting SLS symptoms.

Beside the accumulation of fatty alcohols in SLS patient fibroblasts we also observed the simultaneous formation of fatty acids. To determine to what extend other aldehyde dehydrogenases are responsible for the residual fatty aldehyde dehydrogenase activity, we transfected FALDH, ALDH1A1, ALDH2, ALDH3A1, ALDH3B1 and ALDH3B2 into CHO-K1 cells. FALDH was found to be the only aldehyde dehydrogenase able to significantly increase the conversion of pyrenedecanal to pyrenedecanoic acid when compared to controls. However, there was a clear trend that transfected ALDH3A1 was able to partially take over this reaction. The results of a gene expression analysis (fibroblasts from 18 individuals; http://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-1719, (20)) suggest that FALDH, ALDH3B2 and ALDH2 are about equally expressed in human dermal fibroblasts, whereas the signals of ALDH3A1, ALDH3B1 and ALDH1A1 were 5, 10 and 20 fold lower. This suggests that in SLS patient fibroblasts ALDH3A1 could only partially substitute for the fatty aldehyde dehydrogenase function. The ability of ALDH3A1 to metabolize fatty aldehydes may play a more prominent role in other SLS patient tissues with high ALDH3A1 expression levels. So far ALDH3A1 has been mainly studied in cornea (21), but also shows high expression in lung, esophagus and stomach (22). For a complete understanding of the interplay between different aldehyde dehydrogenase enzymes, further investigations are needed.
Since in many tissues fatty aldehydes are produced by cleavage of alkylglycerols (18), we were also interested in the ability of fibroblasts to degrade this lipid species. With the method we describe here, we monitored alkylglycerol metabolism in living SLS patient and control cells. We detected only marginal amounts of fatty acid and no fatty aldehyde after incubation with 1-O-pyrenedecyl glycerol. The only enzyme known to be able to cleave the ether bond of alkylglycerols is alkylglycerol monooxygenase. However, we were able to measure alkylglycerol monooxygenase activities in other cell lines such as RAW 264.7 (data not shown) in which the enzyme is expressed (12). Also Rizzo et al. (14) by the use of radioactive labeled alkylglycerols described the formation of only minute levels of fatty acids (1.4%) in normal human fibroblasts. Altogether, our data suggest that in fibroblasts alkylglycerols may play only a minor role as fatty aldehyde source.

We present an easy to handle approach to monitor fatty aldehyde dehydrogenase activity in intact cultured cells by analyzing 10 µl of the cell culture medium. The pyrene label is an attractive alternative to radiolabeling of lipids since it is widely accepted by enzymes metabolizing long aliphatic side chains (23), and can be detected with high sensitivity due to its intense fluorescence. As with other labeling techniques, however, also our method presented here gives no information on endogenous levels of the respective lipids.
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REFERENCES


**Table 1:** Sjögren Larsson Syndrome patient fibroblast cells used for this study.

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<th>Cells</th>
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<th>allele 2 nucleotide</th>
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**Table 2. Cytotoxicity of hexadecanal, hexadecanol and hexadecanoic acid towards control and SLS patient fibroblasts**

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Figure Legends

Figure 1. **Monitoring of fatty alcohol and aldehyde metabolism with fluorescent labeled substrates.** A) 1-O-pyrenedecylglycerol [1] is cleaved by alkylglycerol monooxygenase (AGMO), forming pyrenedecanal [2] and a glycerol derivative. The fatty aldehyde is then either converted into pyrenedecanoic acid [3] by fatty aldehyde dehydrogenase (FALDH) or into pyrenedecanol [4] by a fatty alcohol dehydrogenase (ADH). B) Living fibroblasts were incubated with 5 µM of one of the fluorescent substrates [1-4]. For the chromatograms shown here pyrenedecanal [2] was used. After 24 h, 10 µl of the culture medium were mixed with 30 µl methanol, centrifuged and fluorescent compounds were quantified using HPLC and fluorescent detection. The metabolite composition in normal human fibroblasts is shown in the upper trace. The lower trace depicts the composition in SLS patient fibroblasts. Two representatives of 32 chromatograms are shown.

Figure 2. **Fluorescent metabolites in the culture medium of living cells.** 5 µM of one of the fluorescent substrates was added to cultured fibroblasts. After 24 h the metabolite composition in the culture medium was measured by HPLC with fluorescence detection (see materials and methods section for more details). Control fibroblasts are shown in white bars, Sjögren Larsson Syndrome patient cells in black bars. The grey bars indicate the individual substrate used: A) pyrenedecanal, B) pyrenedecanol, C) pyrenedecanoic acid and D) 1-O-pyrenedecyl glycerol. Values are shown as mean ± S.D. Means of experiments with cells from 3 controls and from 3 SLS patients, each with 4-6 replicates are shown.

Figure 3. **Fluorescent metabolites in pellets of cells.** 5 µM of one of the fluorescent substrates was added to cultured fibroblasts. After 24 h the cells were collected, washed and...
treated with 30 μl methanol. The metabolite composition was measured by HPLC and fluorescence detection (see materials and methods section for more details). Control fibroblasts are shown in white bars, Sjögren Larsson Syndrome patient cells in black bars. The grey bars indicate the initial substrate: A) pyrenedecanal, B) pyrenedecanol, C) pyrenedecanoic acid and D) 1-O-pyrenedecyl glycerol. Data are normalized to the total fluorescence in each sample. Values are shown as mean ± SD. Means of experiments with cells from 3 controls and from 3 SLS patients, each with 4-6 replicates are shown.

Figure 4. Fatty aldehyde consumption and metabolite release of Sjögren Larsson Syndrome patient and control cells. 5 μM of pyrenedecanal was added to cultured fibroblasts and the metabolite composition in the culture medium was measured after 5 min, 30 min, 1 h, 4 h, 10 h and 24 h with HPLC and fluorescence detection (see materials and methods section for more details). A) Incubation of control cells, B) Sjögren Larsson Syndrome patient cells. Values are shown as mean ± SD, n=3.

Figure 5. Capability of FALDH, ALDH1A1, ALDH2, ALDH3A1, ALDH3B1 and ALDH3B2 to metabolize the long chain fatty aldehyde pyrenedecanal. CHO-K1 cells were transfected with mammalian expression vectors for human FALDH, ALDH1A1, ALDH2, ALDH3A1, ALDH3B1 and ALDH3B2 (see materials and methods). The ability to convert the pyrenedecanal substrate into pyrenedecanoic acid was measured in the cell pellet as described in (4). Values are shown as mean ± SD, n=6 for FALDH and control, n=3 for all other transfections.
Figure 1

A

1-O-pyrenedecyl glycerol

AGMO → pyrenedecanal

R = [1]

FALDH

pyrenedecanoic acid

ADH

pyrenedecanol

B

control cells

SLS cells

fluorescence (arbitrary units)

0 4 8 12

t (min)

1 2 3 4
Figure 2

A

B

C

D

control
SLS cells

concentration (µM)

acid
aldehyde
alcohol
alkylglycerol

concentration (µM)

acid
aldehyde
alcohol
alkylglycerol

concentration (µM)

acid
aldehyde
alcohol
alkylglycerol

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Figure 5

aldehyde dehydrogenase activity (pmol min$^{-1}$ mg$^{-1}$)

FALDH, ALDH3A1, ALDH3B1, ALDH3B2, ALDH1A1, ALDH2, control