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Fenretinide induces ubiquitin-dependent proteasomal degradation of stearoyl-CoA desaturase in human retinal pigment epithelial cells

William Samuel¹, R. Krishnan Kutty¹, Todd Duncan¹, Camasamudram Vijayasathy², Bryan C. Kuo[†], Krysten M. Chapa[#], and T. Michael Redmond¹

¹Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892-0608

²National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Maryland 20892-0608

[†]Summer Internship Program of National Eye Institute, National Institutes of Health

[#]Community College Summer Enrichment Program, National Institutes of Health

Abstract

Stearoyl-CoA desaturase (SCD, SCD1), an endoplasmic reticulum (ER) resident protein and a rate-limiting enzyme in monounsaturated fatty acid biosynthesis, regulates cellular functions by controlling the ratio of saturated to monounsaturated fatty acids. Increase in SCD expression is strongly implicated in the proliferation and survival of cancer cells, whereas its decrease is known to impair proliferation, induce apoptosis, and restore insulin sensitivity. We examined whether fenretinide, (*N*-(4-hydroxyphenyl)retinamide, 4HPR), which induces apoptosis in cancer cells and recently shown to improve insulin sensitivity, can modulate the expression of SCD. We observed that fenretinide decreased SCD protein and enzymatic activity in the ARPE-19 human retinal pigment epithelial cell line. Increased expression of BiP/GRP78, ATF4 and GADD153 implicated ER stress. Tunicamycin and thapsigargin, compounds known to induce ER stress, also decreased the SCD protein. This decrease was completely blocked by the proteasome inhibitor MG132. In addition, PYR41, an inhibitor of ubiquitin activating enzyme E1, blocked the fenretinide-mediated decrease in SCD. Immunoprecipitation analysis using anti-ubiquitin and anti-SCD antibodies and the blocking of SCD loss by PYR41 inhibition of ubiquitination further corroborate that fenretinide mediates the degradation of SCD in human RPE cells via the ubiquitin-proteasome dependent pathway. Therefore, the effect of fenretinide on SCD should be considered in its potential therapeutic role against cancer, type-2 diabetes, and retinal diseases.

Keywords

SCD; Fenretinide; 4HPR; Ubiquitin; Proteasome; Endoplasmic reticulum stress; Retinal pigment epithelium

Introduction

Stearoyl-CoA desaturase (SCD, SCD1 and delta-9-desaturase) is a membrane bound enzyme that catalyzes the rate-limiting step in the biosynthesis of unsaturated fatty acids. Palmitate and stearate, preferred substrates for this enzyme, are converted to palmitoleate and oleate, respectively (Enoch et al., 1976). The ratio of saturated to unsaturated fatty acids is thought to control the structural integrity and fluidity of membranes, which in turn could modulate cell growth and differentiation (Ntambi, 1995; Ntambi, 1999). Saturated fatty acids, in particular palmitate, can induce apoptosis, and play a pivotal role in many age-related human diseases including cardiovascular disease, cancer, and type-2 diabetes (Ford, 2010). The unsaturated fatty acids oleate and palmitoleate have been shown to exert protective effects against the toxic effects of saturated fatty acids (Staiger et al., 2006). Since SCD plays a crucial role in maintaining the intracellular equilibrium of saturated fatty acids and unsaturated fatty acids that has a distinct impact on cellular function, a new therapeutic strategy has been emerged in recent years to target the expression of SCD (Brown and Rudel, 2010). Increase in SCD expression has been strongly implicated in the proliferation, invasiveness, and survival of cancer cells from saturated fatty acid-mediated lipotoxicity (Falvella et al., 2002; Scaglia and Igal, 2005; Watson et al., 2009). Targeted deletion or inhibition of SCD by genetic or pharmacological manipulations results in impaired *de novo* lipid synthesis, decreased cell proliferation, and increased apoptosis in cancer cells (Fritz et al., 2010; Hess and Igal, 2011; Igal, 2010). These studies suggest that targeting *de novo* lipogenesis through SCD expression might serve as a possible new therapeutic strategy to arrest oncogenesis, type-2 diabetes and cardiovascular diseases in humans.

Several studies have demonstrated that palmitate-derived ceramide production mediate the pro-apoptotic effect of palmitate, and accumulation of both palmitate and ceramide plays a key role in insulin resistance, obesity, and lipid metabolism (Holland et al., 2011; Holland et al., 2007; Hu et al., 2011). Apart from potentiating insulin resistance, increased ceramide generation has been shown to induce endoplasmic reticulum (ER) stress, which plays a fundamental role in the pathogenesis of several diseases such as diabetes, cancer and neurodegenerative disorders (Salminen et al., 2010; Schonthal, 2012). A recent study has shown that fenretinide (N-(4-hydroxyphenyl)retinamide, 4HPR) a synthetic derivative of all-*trans* retinoic acid originally developed as a chemotherapeutic agent, improved insulin sensitivity in mouse liver and muscle cells by blocking the formation of ceramide due to its ability to inhibit dihydroceramide desaturase (Des1) (Bikman et al., 2012; Rahmaniyan et al., 2011). Fenretinide has been shown to activate the expression of alkaline ceramidase 2 (ACER2), an enzyme that catalyzes the hydrolysis of dihydroceramides to generate dihydrosphingosine (Mao et al., 2010). It also been shown to increase the activity of serine palmitoyl transferase (SPT), which catalyzes the first rate-limiting step in the *de novo* synthesis of ceramides involving the condensation of L-serine with palmitate (Wang et al., 2001). The synthesis of ceramide from saturated fatty acids such as palmitate has been shown to increase the activity of SPT, while silencing the expression of SPT decreases palmitate-driven ceramide synthesis, and curbs lipid-induced insulin resistance (Watson et al., 2009). Interestingly, deleting *Scd1* expression has been shown to decrease ceramide synthesis by down-regulating SPT expression in mice skeletal muscle (Peter et al., 2009).

Furthermore, *Scd1* deficiency increased insulin sensitivity in mice, whereas increased SCD activity contributed to the insulin resistance in humans and animals (Dobrzyn et al., 2010; Garcia-Serrano et al., 2011; Gutierrez-Juarez et al., 2006; Peter et al., 2009; Rahman et al., 2003). Thus, it is possible that SCD could play an important role in mediating the effects of fenretinide on apoptosis and insulin signaling. However, the effect of fenretinide on SCD expression is not yet known.

Retinal pigment epithelium (RPE) is a single layer of epithelial cells located between the light-sensing photoreceptor cells and the choriocapillaris. A normally functioning RPE is indispensable for vision, and any disruption or RPE cell death could hasten retinal degenerative diseases such as retinitis pigmentosa and age-related macular degeneration (AMD) (Sparrow et al., 2010). Indeed fenretinide has been proposed as a treatment for the geographic atrophy form of AMD (Mata et al., 2012). We have shown earlier that fenretinide induces apoptosis in cultured human RPE cells (Samuel et al., 2006). We have also reported that SCD is expressed in RPE cells and that its expression is regulated by all-*trans* retinoic acid (Samuel et al., 2001; Samuel et al., 2002). The present work is undertaken to study the potential regulation of SCD during fenretinide-induced apoptosis in ARPE-19 cells, a human RPE cell line. We show that fenretinide-induced ER stress decreased the SCD protein and enzymatic activity in RPE cells via an ubiquitin-dependent proteasomal pathway.

Materials and Methods

Materials

Fenretinide, MG132, PSI, lactacystin, mono- and polyubiquitinated antibody, mouse anti-actin and anti- α -tubulin antibodies were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). D3-stearate and D3-palmitate were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). PYR41, inhibitor of ubiquitin activating enzyme E1, was from LifeSensors, Inc. (Malvern, PA). Monoclonal anti-SCD antibody was obtained from Kamiya Biomedical Company (Seattle, WA), and OriGene Technologies (Rockville, MD). Rabbit polyclonal BiP/GRP78 antibody was from Abcam (Cambridge, MA). The enhanced chemiluminescence (ECL) detection system and peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from GE Healthcare Life Sciences (Piscataway, NJ).

Cells and culture conditions

Human retinal pigment epithelial cells (ARPE-19 cells) obtained from ATCC (Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing nutrient mixture F12 (Cellgro, Herdon, VA) supplemented with 5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml) as described previously (Samuel et al., 2001). Cells were seeded onto tissue culture plates at a density of 2×10^5 cells/ml in complete medium and allowed to grow overnight. The culture medium was replaced the next day with fresh serum-free medium containing penicillin (100 U/ml) and streptomycin (100 μ g/ml) before treating with fenretinide, tunicamycin or thapsigargin. MG132, PSI and PYR41 were added 30 minutes prior to the addition of fenretinide. Treatments were performed under subdued light and other conditions as reported previously (Samuel et al., 2001). Fenretinide,

MG132, PSI, thapsigargin, tunicamycin and PYR-41 were dissolved in DMSO before adding to the cell culture medium. The controls received the same amount of DMSO. The cells were maintained at 37 °C in a humidified environment of 5% CO₂ in air.

Apoptosis ELISA

Detection of apoptosis in human RPE cells was performed by quantitative enzyme-linked immunosorbent assay (ELISA) using mouse monoclonal antibodies directed against DNA and histones (Cell Death Detection Elisa kit, Roche). This technique estimates the amount of cytoplasmic histone-associated DNA fragments, which are known to increase in cells during apoptosis. Briefly, ARPE-19 cells cultured in 24 well plates were treated with or without fenretinide for 24 h, and the cells were lysed by adding lysis buffer (250 µl/well). The cell lysates were then centrifuged at 250 × g for 10 min, and 10 µl of supernatants were incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase antibodies. The immunoreactivity was developed using 2, 2'-azino-di- [3-ethyl-benzthiazoline sulfonate] diammonium salt (ABTS) as substrate, and was quantitated by measuring the absorbance at 405 nm using Victor² Multilabel Counter (Perkin Elmer).

Quantitative Real-Time RT-PCR

For quantitative real-time RT-PCR, 2 µg of total RNA extracted from ARPE-19 cells with RNeasy Protect Mini Kit (Qiagen) was reverse transcribed using High Capacity cDNA Archive Kit (Applied Biosystems). After reverse transcription, 5 µl of cDNA preparations were used as templates for quantitative real-time PCR performed on an Applied Biosystems 7500 Real-Time PCR System using TaqMan Universal PCR Master Mix and other reagents from Applied Biosystems following manufacturer's default thermal cycling conditions. Each PCR reaction was set up in 50 µl using validated TaqMan probe and primer for ATF4 (assay identification number Hs00909568_g1), and GADD153 (assay identification number Hs00358796_g1). Human GAPDH gene (catalog number 4326317E) was used as endogenous control. The gene specific probe was labeled with reporter dye FAM, and the endogenous control GAPDH was labeled with a different reporter dye VIC at the 5' end. Gene amplification data were analyzed with an Applied Biosystems 7500 System Sequence Detection Software version 1.2.3. The results were expressed as *n*-fold induction or inhibition in gene expression relative to endogenous control calculated using the C_T method.

Ubiquitination Assay

Ubiquitin Enrichment Kit obtained from Thermo Fisher Scientific (Rockford, IL) was used for detecting ubiquitinated proteins. Briefly, cells were treated with fenretinide in the presence or absence of various proteasome inhibitors such as MG132, and the cell lysates were (0.1 mg total protein) applied to a spin column containing 20 µl of polyubiquitin affinity resin. The spin column containing both sample and a positive control were incubated at 4°C overnight on an end-over-end rotator. After collecting the flow through by centrifuging the spin column at 5000 × g for 15 seconds, the column was washed three times with 300 µl of wash buffer. Then the proteins bound to the column was eluted by incubating the spin column at 70°C with 50 µl of SDS-PAGE sample loading buffer followed by

centrifugation at $5000 \times g$ for 30 seconds. The ubiquitin-enriched eluate was then analyzed by western blotting using mono- and polyubiquitin polyclonal antibody.

Immunoprecipitation and Western Blot Analysis

The cultured human RPE cells treated with fenretinide in the presence or absence of proteasome inhibitor, MG132 or PSI, were washed twice with phosphate-buffered saline and cell extracts were prepared using cell lysis buffer according to manufacturer's recommendation (Cell Signaling, Danvers, MA). Cell lysates were also prepared under denaturing condition to examine whether SCD is modified by polyubiquitin chains. The cell pellets were vortexed vigorously in 100 μ l lysis buffer (50 mM Tris-HCl buffer (pH 7.5) containing 2% SDS and 5 mM β -mercaptoethanol) and incubated at 95°C for 10 min. The lysates were then diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 1% Triton X-100 and 5 mM β -mercaptoethanol) and kept on ice for 10 min. The DNA was sheared by sonication to reduce the viscosity and the samples were clarified by centrifugation at $14,000 \times g$ for 10 min. Protein concentrations of the cell extracts were quantified using Bio-Rad DC™ protein assay reagent (Bio-Rad, Hercules, CA).

Cell lysates containing equal amounts of protein were precleared by incubating with Protein A/G-agarose beads for 20 min at 4°C. Following a brief centrifugation at $14,000 \times g$, the supernatant was used for immunoprecipitation. Universal Magnetic Co-IP kit (Active Motif, Carlsbad, CA) was used for the immunoprecipitation analysis. In brief, cell lysates (400 μ g of protein) prepared under denaturing or nondenaturing conditions were mixed with either biotinylated mouse monoclonal SCD antibody (5 μ g) prepared using ChromaLink™ Biotin Antibody Labeling Kit (Solulink, San Diego, CA) or mono- and polyubiquitin polyclonal antibody, and then incubated overnight at 4°C on a rotator. A total of 25 μ l Dynabeads® M-280 Streptavidin Magnetic Beads (Invitrogen) or Protein G Magnetic Beads (Active Motif) was added to each tube and incubated for an additional 1 hour. Immunoprecipitates were washed 4 times with complete Co-IP/Wash Buffer using a magnet, and the bead pellets were resuspended in 20 μ l of 4X reducing NuPAGE® LDS sample buffer (Invitrogen). For western blot analysis, equal amounts of bead pellets in sample buffer or total protein (50 μ g) from cell extracts were subjected to SDS-polyacrylamide gel electrophoresis using 4-12% NUPAGE Bis-Tris gels and then transferred to a nitrocellulose membrane using iBlot® Dry Blotting System (Invitrogen, Carlsbad, CA). After blocking in 5% non-fat milk in Tris-buffered saline (TBS) containing 0.01% Tween 20 for 1 h, the membranes were incubated overnight at 4°C with mouse monoclonal anti-SCD antibody, anti-rabbit BiP/GRP78 or mono- and polyubiquitinated peroxidase-conjugated rabbit polyclonal antibody. Peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibody was used as secondary antibody. Immunocomplexes were visualized by a chemiluminescence method using the ECL Prime western blotting Detection Kit (GE Healthcare Life Sciences). The blots were then stripped and reprobed with mouse monoclonal anti- α -tubulin antibody.

Cell Lysis and Fatty acid Extraction

Cells were treated with either 5 or 10 μ M of fenretinide for 16 h, and then incubated with 50 μ M fatty acid triply deuterated (D3) on the methyl group (either D3-stearate or D3-

palmitate) for an additional 5 h before harvesting them. Cell pellets washed with PBS were resuspended in 450 μ l cell lysis buffer (Cell Signaling Technology, Danvers, MA), and then sonicated while keeping the tube on ice. An aliquot (50 μ l) of the dispersed cell suspension was used for protein quantitation, and the remaining 400 μ l was used for lipid extraction as follows. Samples were spiked with a small volume of pentadecanoic acid (in CHCl_3) as an internal standard and then were subjected to saponification for 15 minutes at 72°C by addition of an equal volume of 0.6 M methanolic KOH. Following saponification, samples were acidified by addition of 100 μ l formic acid. The acidified mixture was extracted twice with 1 ml chloroform to recover free fatty acids. The chloroform extracts were pooled, solvent evaporated under argon at 37°C and the residue was resolubilized in 100 μ l chloroform/methanol (1:4).

Liquid Chromatography Mass Spectrometry

Lipid extracts were analyzed by liquid chromatography mass spectrometry (LC-MS) using a modification of the procedures of Dillon et al. (Dillon et al., 2008). The LC-MS system consisted of a 1200 Series Capillary LC (Agilent, Santa Clara, CA) coupled to a Q-ToF micro mass spectrometer (Waters Corp., Milford, MA) equipped with an electrospray ionization source. Separation of fatty acids was achieved by isocratic elution on a PLRP-S polymeric reversed-phase column (100 Å, 3 μ m, 2.1 $\times$ 150 mm; Agilent) heated to 65°C. A mobile phase consisting of 25% acetonitrile:chloroform (1:1), 40% methanol, 25% water, 10% 10 mM ammonium acetate was run at a flow rate of 0.1 ml/min. The mass spectral analysis of the fatty acids was performed in the negative ion mode using the following instrument settings: capillary voltage, 1600; sample cone voltage, 40; cone gas flow, 40 L/hr; desolvation gas flow, 400 L/hr; source temperature, 80°C; desolvation temperature, 100°C. Data was then analyzed using Masslynx software v4.1 (Waters) by extracting the ion chromatograms of the labeled and non-labeled stearic, palmitic, oleic, and palmitoleic acid peaks and measuring peak area.

Statistical Analysis

All values have been expressed as mean $\pm$ SD, $n = 4$. For statistical significance, paired Student's t-test in Excel was used. $P < 0.05$ denotes statistically significant differences. The results shown are representative of 3 or more independent experiments.

Results

SCD protein decreases during fenretinide-induced apoptosis

Previously, we have shown that all-*trans* retinoic acid treatment increased SCD mRNA expression with a corresponding increase in its activity in ARPE-19 cells (Samuel et al., 2001). Therefore, we investigated whether fenretinide treatment modulates the expression of SCD in ARPE-19 cells. We treated ARPE-19 cells with 5 or 10 μ M fenretinide for 24 h, and measured the viability and cell death of these cells by phase-contrast microscopy and apoptosis *ELISA*, respectively. Cells treated with 5 or 10 μ M fenretinide appeared to shrink from their normal shape, and detach from the surface and started to float in the culture medium when viewed under microscope (Fig. 1A), similar to our earlier observation. As shown in Fig. 1B, treating ARPE-19 cells with fenretinide resulted in a dose-dependent

increase in the generation of DNA fragments, as expected. Apoptosis increased with increasing concentration of fenretinide, and more than a 10-fold increase over the control was observed with 5 μ M fenretinide at 24 h, supporting the light microscopy findings. We did not observe any apoptotic cell death in untreated cells used as controls at various time points (data not shown). Therefore, we have used this treatment condition, treating ARPE-19 cells with 5 and 10 μ M fenretinide for 24 h, for subsequent studies.

To determine the SCD protein content, the cells were treated with 5 or 10 μ M fenretinide, and cell lysates analyzed by western blotting. Fig. 1C shows that the SCD protein (37 kDa) was decreased with fenretinide treatment compared to control.

Fenretinide treatment decreases SCD activity

We investigated whether the decrease in SCD protein results in reduced SCD enzyme activity. Both control and fenretinide treated (16 h) ARPE-19 cells were treated with D3-stearic acid or D3-palmitic acid for 5 h, and the formation of D3-oleic acid or D3-palmitoleic acid were analyzed by an LC/MS method as an index of SCD enzyme activity. As shown in Fig. 2A and B, fenretinide treatment resulted in a concentration dependent decrease in SCD activity as evident by the decrease in the formation of D3-oleic acid and D3-palmitoleic acid. More than 80% decrease in SCD activity over the control was observed with 10 μ M fenretinide. Fig. 2C shows the effect of fenretinide on the ratio of unsaturated fatty acid to saturated fatty acid in ARPE-19 cells. Briefly, the peak areas were obtained for D3-oleic acid and D3-palmitoleic acid (shown in Figs. 2A and B) and for D3-stearic acid and D3-palmitic acid from their respective extracted ion chromatograms (m/z 286.3 and 258.4, respectively; data not shown) using Masslynx software. The fenretinide treatment altered the ratio of oleate to stearate, and palmitoleate to palmitate in a dose-dependent manner. Thus, both SCD enzyme activity and SCD protein decreased during apoptotic cell death induced by fenretinide.

Fenretinide-induced decrease in SCD protein is proteasome-dependent

Proteasomes, large multi-subunit protease complexes, play a major role in the degradation of many proteins involved in apoptosis (Hershko and Ciechanover, 1998). Therefore, we examined whether the fenretinide-induced decrease in SCD protein was mediated by the proteasomal degradation pathway using the proteasomal inhibitor MG132. The cells were pretreated with MG132 before adding fenretinide, and after incubation the cell lysate were analyzed for SCD protein using western blotting. SCD protein decreased after fenretinide treatment in the RPE cells, as expected (Fig. 3). The MG132 pretreatment blocked the fenretinide-dependent decrease in SCD protein. MG132 at higher concentration increased SCD protein in control. We have also tested whether the blockade of fenretinide-induced decrease in SCD protein by MG132 is associated with corresponding change in SCD activity in ARPE-19 cells. However, we observed that MG132 pretreatment did not block the fenretinide-induced decrease in SCD activity (data not shown). This could possibly be due to the inhibitory effect of fenretinide on SCD activity. Fenretinide has been shown to inhibit enzymes involved in lipid metabolism such as the SCD-related enzyme dihydroceramide desaturase (DES1) (Rahmaniyan et al., 2011). To evaluate whether other proteasome inhibitors were also able to block the degradation, we treated the cells with PSI, a cell-

permeable inhibitor of the multicatalytic proteinase complex 20S proteasome. PSI also blocked the degradation of SCD protein induced by fenretinide (Fig. 3). We have also observed similar results when lactacystin, an irreversible proteasome inhibitor, was used (data not shown). In addition, MG132, as with all other proteasome inhibitors, is toxic to cells and tissues, and will cause cell death at high concentrations or after prolonged treatment. The concentration that we have used (2.5 μ M) is much less than the concentration (>100 μ M) used by Acharya *et al* in their study to inhibit the proteasomal dependent turnover of CYP3A in cultured primary rat hepatocytes (Acharya et al., 2009). We did not observe any morphological changes in ARPE-19 cells treated with 2.5 μ M MG132 when examined using light microscopy. However, we have noticed an additional protein band of lower molecular weight than SCD in cells treated with proteasome inhibitors alone, the intensity of which depends on the concentration of the proteasome inhibitor used.

Fenretinide-induced decrease of SCD protein mediated by ER stress

To examine whether fenretinide-induced SCD degradation in RPE cells is associated with ER stress, the mRNA expression of ATF4 (activating transcription factor 4) and GADD153 (growth arrest and DNA damage-inducible protein 153), and protein expression of BiP/GRP78, all known markers for ER stress (Lee, 2005; Szegezdi et al., 2006), were analyzed by RT-PCR and immunoblotting, respectively. The mRNA expression of ATF4 and GADD153 were increased with fenretinide treatment in a concentration dependent manner (Fig. 4A & B). We also observed that fenretinide treatment induced a substantial but dose-dependent increase in the expression of BiP/GRP78 protein in RPE cells (Fig. 4C). The level of BiP/GRP78 protein expression was small but significant after 24 h with 5 μ M fenretinide, and the level was markedly increased over the control when the cells were treated for 24 h with 10 μ M fenretinide. Despite the accumulation, pretreatment of cells with MG132 did not block the increase in BiP/GRP78 protein expression induced by fenretinide. Interestingly, the proteasome inhibitor MG132 by itself highly increased the amount of BiP/GRP78 protein in ARPE-19 cells. This result is in accordance with accumulating evidence indicating that the proteasome inhibitor MG132 is a potent inducer of ER stress (Park et al., 2011; Wang et al., 2007).

ER stress inducers decreases SCD protein

To test if ER stress plays an important role in the degradation of SCD protein, we tested chemicals that can induce ER stress such as tunicamycin, which suppresses glycosylation, and thapsigargin, which disturbs calcium homeostasis (Schonthal, 2012). ARPE-19 cells were treated with tunicamycin and thapsigargin in the presence or absence of MG132, and the SCD protein was analyzed by western blotting. Results show that exposure of ARPE-19 cells to tunicamycin or thapsigargin for 24 h also resulted in a decrease in SCD protein (Fig. 5), suggesting that degradation of SCD protein was due, at least in part, to activation of ER stress or is an ER stress-mediated cellular response. To further determine whether the degradation of SCD in this case was through the proteasomal pathway, cells were treated with these agents in the presence or absence of MG132 and SCD protein in cell lysates was analyzed by immunoblotting after 24 h of treatment. The decrease in SCD protein was also blocked by the proteasomal inhibitor MG132, indicating the proteasomal pathway plays a role in regulating the ER stress-induced degradation of SCD protein.

Fenretinide induces polyubiquitination of proteins in ARPE-19 cells

Since proteins destined for proteasomal degradation are often ubiquitinated, we examined whether fenretinide treatment induces the accumulation of ubiquitinated proteins in ARPE-19 cells (Fig. 6A). RPE cells were pretreated with or without MG132 before adding fenretinide, and the cell lysate prepared after 24 h of incubation were passed through a polyubiquitin affinity resin column to enrich ubiquitinated protein. The fraction recovered from the column was analyzed by western blotting using anti-ubiquitin antibody. As shown in Figure 6A, fenretinide treatment induced a marked increase in the accumulation of polyubiquitinated proteins in RPE cells when compared to control. This *in vivo* increase in polyubiquitination of protein is also dependent on the concentration of fenretinide as evident from the greater accumulation of ubiquitinated proteins at the higher fenretinide concentration.

Once a protein is ubiquitinated, it is delivered to the proteasome for degradation (Hershko and Ciechanover, 1998). Since proteasome inhibitors turn off the proteasome function and allow the ubiquitinated proteins to accumulate in the cell (Lee and Goldberg, 1998), we examined whether MG132 treatment can cause polyubiquitinated proteins to accumulate in RPE cells. As shown (Fig.6A), MG132 caused an accumulation of ubiquitinated proteins, as expected. We used cell lysate from HeLa cells treated with epoxomicin as a positive control in our western blot analysis. These results suggest that fenretinide elicits polyubiquitination of proteins, a prerequisite for their degradation by proteasomes.

We then examined whether SCD is modified by ubiquitination. Denaturing conditions were employed for immunoprecipitation in order to exclude the possibility that SCD is coimmunoprecipitated with other ubiquitinated proteins. The RPE cells treated with fenretinide in the presence or absence of MG132 for 24 h were lysed under denaturing condition in a buffer containing SDS. The cell lysates were subjected to immunoprecipitation using biotinylated mouse monoclonal anti-SCD antibody. The immunoprecipitates were analyzed by western blotting using an anti mono- and polyubiquitin polyclonal antibody (Fig. 6B). We did not observe the high molecular weight immunoreactive band indicative of polyubiquitinated SCD in extracts from cells treated with fenretinide, suggesting that ubiquitinated SCD was rapidly degraded by proteasomes. But, an immunoreactive high molecular weight band was detected with SCD antibody in the fenretinide treated cell lysate in the presence of MG132 indicating that MG132 blocks the proteasome which in turn causes accumulation of ubiquitinated SCD. The immunoprecipitates from control cell lysate did not have such signals. This result clearly indicates that fenretinide treatment induced ubiquitinylation of SCD prior to its degradation via an ubiquitin-proteasome pathway. Polyubiquitinated SCD was also detected in the MG132 treated cell lysates. At this time we do not know why MG132, a well-known proteasome inhibitor, by itself induced the polyubiquitination of SCD. However, it has been reported that MG132 could induce ER stress, which in turn activate the downstream ubiquitin-proteasome system as the protective and compensatory mechanism during ER stress (Ding et al., 2007). In addition, Kato et al (Kato et al., 2006) have also observed similar increase in polyubiquitinated SCD in the presence of MG132 in CHO-K1 cells transiently expressing HA-tagged SCD1.

Fenretinide induces ubiquitination of SCD in ARPE-19 cells

We also investigated the possibility that SCD is polyubiquitinated prior to its degradation by proteasomes using immunoprecipitation analysis under non-denaturing conditions. Cells preincubated with or without MG132 were treated with fenretinide for 24 h, and the cell lysates were analyzed by immunoprecipitation using biotinylated mouse monoclonal anti-SCD antibody. The immunoprecipitates were analyzed by western blotting using anti-mono- and polyubiquitin polyclonal antibody (Fig. 7A). The blot showed high molecular weight bands, possibly indicating the formation of polyubiquitinated SCD in extracts from cells treated with fenretinide in the presence of proteasome inhibitor MG132. This could be due to fenretinide-induced ubiquitination of SCD protein and the inhibition of its consequent proteasomal degradation by MG132. We did observe polyubiquitinated SCD protein bands in cells treated with MG132 alone, similar to that observed for immunoprecipitation done under denaturing conditions, shown in Fig.6B. The western blot analysis of cell lysates prior to immunoprecipitation shows that fenretinide decreased SCD protein and this decrease was blocked by MG132, as expected (Fig.7B). Therefore, our results indicate that the degradation of SCD induced by fenretinide could be mediated through the ubiquitin-proteasome system.

Ubiquitin-proteasome pathway mediates fenretinide-induced degradation of SCD

The conjugation of ubiquitin molecules to protein substrates is carried out by the E1-E2-E3 cascade of enzymes (Hershko and Ciechanover, 1998). E1, ubiquitin activating enzyme, catalyzes the first common step in the ubiquitination process, and inhibition of this enzyme, in principle, blocks ubiquitination and the ubiquitin-proteasome system. Therefore, we evaluated whether an inhibitor of E1 could block the degradation of SCD protein induced by fenretinide. The RPE cells were pretreated with or without PYR41, a cell permeable and irreversible inhibitor of E1, prior to fenretinide treatment. The cell lysates were subjected to SDS-PAGE, and the immunoreactive SCD was identified by western blotting. As shown (Fig. 8), fenretinide decreased SCD protein, as expected, and this effect is blocked by PYR41 at the different concentrations tested. A near maximal effect was evident at the lowest concentration used (1 μ M). Thus, it appears that fenretinide treatment elicits polyubiquitination of SCD prior to proteasomal degradation. However, PYR41 treatment alone (lane 7 and 8) showed a higher level of SCD than fenretinide plus PYR41 (lane 3 to 6). At this time, we do not know why PYR41 by itself increases SCD protein content. In addition, we have also tested whether the blockade of fenretinide-induced decrease in SCD protein by PYR41 is associated with corresponding change in SCD activity in ARPE-19 cells. However, we observed that PYR41 pretreatment did not block the fenretinide-induced decrease in SCD activity (data not shown). This may be due to the inhibitory effect of fenretinide on SCD activity, in common with the related dihydroceramide desaturase (DES1) (Rahmaniyan et al., 2011).

Discussion

In this study we show that the expression of SCD protein decreased drastically in human RPE cells during fenretinide-induced apoptosis. The observed decrease in SCD protein correlates well with the decrease in SCD enzymatic activity seen by the decreased formation

of unsaturated fatty acids oleate and palmitoleate from saturated fatty acids stearate and palmitate, respectively, in treated cells. This decrease was associated with increased expression of ER stress markers. Furthermore we found that compounds known to increase ER stress also decreased the SCD protein. We also found that proteasomal inhibitors blocked fenretinide-mediated decrease in SCD. The intimate role of the ubiquitin pathway in fenretinide-mediated SCD inhibition was shown by increased ubiquitination of SCD and the fact that SCD decrease was blocked by an inhibitor of the ubiquitin-activating enzyme E1. Taken together, we suggest that this fenretinide-mediated reduction in SCD protein must be taken into consideration, especially as fenretinide is being tested for use in cancer and type-2 diabetes, in both of which SCD plays an important role, and in AMD, in which no role for SCD is yet known.

Accumulation of stearate and palmitate can alter cell membrane fluidity which in turn controls cell proliferation, differentiation, and apoptosis (Igal, 2010; Ntambi, 1995). Increased palmitate concentration in the membrane bilayer has been shown to reduce the membrane fluidity in HepG2 cells resulting in impaired cellular functions and/or cell death (Leekumjorn et al., 2008). In addition, both stearate and palmitate are capable of inducing apoptosis in various types of cells (Ulloth et al., 2003). An increase in the activity of SCD has been shown to protect human arterial endothelial cells from the toxic effects of saturated fatty acids (Peter et al., 2008). SCD has been found to be overexpressed in a variety of cancers including colon, esophageal and hepatocellular carcinomas (Li et al., 1994). A decrease in SCD activity has been shown to reduce cell proliferation, and make the cells more sensitive to apoptotic cell death (Scaglia and Igal, 2005). Also, the inhibition of SCD activity by CVT-11127, a small molecule inhibitor of SCD, has been shown to inhibit cell cycle progression and induce apoptosis in lung cancer cells (Hess et al., 2010). The apoptotic effect of fenretinide on cancer cells (Di Vinci et al., 1994; Oridate et al., 1996; Oridate et al., 1997; Supino et al., 1996) may stem from its ability to decrease the SCD protein content as observed in the present study.

ER stress may play an important role in decreasing the SCD protein during fenretinide-induced apoptosis of human RPE cells. ER is responsible for synthesis, maturation of membrane and secretory proteins as well as metabolizing fatty acids, steroids and carbohydrates and is quite sensitive to changes in redox status, calcium homeostasis and glycosylation (Schonthal, 2012). Here we observed that the expression of GRP78 (BiP/GRP78), a marker for ER stress, and the expression of ATF4 (activating transcription factor 4) and GADD153 (growth arrest and DNA damage-inducible protein 153), were increased by fenretinide in ARPE-19 cells. Both ATF4 and GADD153 (also known as CHOP), are known to increase in response to ER stress (Schonthal, 2012). Our observation that the SCD protein was decreased by tunicamycin and thapsigargin, known inducers of ER stress (Schonthal, 2012), and that this could be blocked by MG132, corroborated the hypothesis that ER stress was implicated. To our knowledge, this is the first report of SCD regulation by ER stress.

With ER stress, the intracellular signaling pathway known as unfolded protein response (UPR), which plays a central role in regulating the half-lives and activity of many cellular proteins (Hershko and Ciechanover, 1998), may be activated to restore ER homeostasis

(Schonthal, 2012). Here, we show that the proteasome-specific inhibitor MG132 blocked the decrease in SCD protein content induced by fenretinide in ARPE-19 cells. The proteasomal inhibition resulted in accumulation of SCD which is in line with the observation that inhibition at the level of proteasome leads to accumulation of intracellular proteins (Lee and Goldberg, 1998). Furthermore, other proteasome inhibitors including PSI and lactacystin also blocked SCD degradation. These data suggest that SCD is regulated by degradation through the proteasome pathway. Also, it appears that a stimulus such as ER stress is prerequisite for the proteasomal degradation of SCD by this pathway. When the cells were treated with proteasome inhibitors alone, we noticed one additional band of lower molecular size apart from the 37 kDa band of SCD. At present we do not know how this lower molecular weight band is generated. But, Kato *et al* have also observed one additional band of lower molecular size apart from the 37 kDa band of SCD in the presence of MG132 in CHO-K1 cells transiently expressing HA-tagged SCD1 probed with anti-HA antiserum (Kato et al., 2006). Furthermore, the lower molecular weight band could also possibly be produced by proteolysis of SCD by a proteasome-independent pathway as observed by Ozols and collaborators (Heinemann and Ozols, 1998; Ozols, 1997). Treatment of cancer cells with proteasome inhibitors has been reported to generate bands of lower sizes in the case of kruppel-like factor 5 (Chen et al., 2005).

Covalent attachment of the small protein tag ubiquitin is a prerequisite for the degradation of many proteins by the proteasome (Glickman and Ciechanover, 2002). Using cells treated with fenretinide, we did detect immunoreactive band indicative of polyubiquitinated SCD protein in the human RPE cells in the presence of proteasome inhibitor MG132. However, we also observed polyubiquitinated SCD in extracts from cells treated with MG132 alone. At this time we do not know why MG132 by itself induced the accumulation of polyubiquitinated SCD. MG132, apart from being a well-known proteasome inhibitor, is also a potent inducer of ER stress (Park et al., 2011; Wang et al., 2007). In our study we observed that the expression of GRP78 (BiP/GRP78), a marker for ER stress, was also increased by MG132 in ARPE-19 cells, which is similar to the effect of fenretinide in RPE cells. Thus, an increase in ubiquitinated SCD could be expected with MG132, whether fenretinide is present or not. Polyubiquitinated protein accumulation has been observed in human epidermal carcinoma A431 cells as well as in U937 human leukemic cells pretreated with MG132 (Melikova et al., 2006; Parcellier et al., 2003). Thus, we conclude that SCD protein is degraded by the ubiquitin-dependent proteasomal pathway. It has been reported earlier that proteasome inhibitors increased the half-life of SCD1 when overexpressed in CHO-K1 cells (Kato et al., 2006). Also, these authors showed the involvement of ubiquitin-proteasome-dependent ERAD system in the constitutive degradation of mouse SCD1 endogenously expressed in adipocyte-differentiated NIH 3T3-L1 cells. On the other hand *in vitro* experiments using isolated rat liver microsomes have shown that the degradation of SCD1 proceeds via a non-proteasomal pathway (Heinemann and Ozols, 1998; Ozols, 1997). Our result shows for the first time that fenretinide induces the degradation of native SCD protein via an ubiquitin-dependent proteasomal pathway.

Ubiquitination is a posttranslational modification catalyzed by sequential action of three enzymes, a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and a

ubiquitin ligase (E3) (Hershko and Ciechanover, 1998). Here we observed that PYR41, an E1 inhibitor blocked the degradation of SCD during fenretinide-induced apoptosis in ARPE-19 cells further supporting our observation that SCD is ubiquitinated prior to proteasomal degradation. Inhibiting the E1 enzyme has been shown to increase the abundance of short half-life proteins such as p53 and cyclin D3 in cancer cells (Xu et al., 2010), and attenuates cytokine-mediated activation of nuclear factor-kappa B by preventing the ubiquitination and downstream proteasomal degradation of I κ B α (Yang et al., 2007).

A common side effect associated with long-term administration of fenretinide is night blindness caused by decreased plasma retinol levels and decreased retinol binding protein (RBP4) (Camerini et al., 2001). The decrease in RBP4 by fenretinide has been reported to increase insulin sensitivity (Koh et al., 2012; Yang et al., 2005). A recent study from our laboratory suggests that fenretinide could also induce night blindness by inhibiting β -carotene 15, 15'-monooxygenase (BCMO1) (Poliakov et al., 2012). Fenretinide has been shown to decrease the formation of lipofuscin-fluorophores in RPE of ABCA4 knockout mice (Radu et al., 2005), suggesting that fenretinide has the therapeutic potential for treating nonexudative AMD (Mata et al., 2012). However, a recent study showed that fenretinide treatment increases lesion size and leakage in laser induced choroidal neovascularization in mice (Sreekumar et al., 2008). In the present study we have shown that fenretinide targets SCD for proteasomal degradation in human RPE cells in culture. The role of SCD in the physiology and pathology of eye is not yet known. However, *Scd1*^{ab-J} and *Scd1*^{ab-2J} mouse strains carrying a naturally occurring mutation of *Scd1* gene exhibit small eyes with closed eye lids. These mice also exhibit hypoplasia of the sebaceous glands, and their hepatic cholesterol ester and triglyceride synthesis is deficient and cannot be restored through diet (Paton and Ntambi, 2009). In the skin of *Scd1* knockout mice the levels of retinol and retinoic acid as well as retinoic-acid responsive genes were markedly elevated indicating that retinol metabolism is one of the major metabolic pathways affected by the absence of *Scd1* (Flowers et al., 2011). The RPE plays a central role in retinoid metabolism in the eye, and the decrease in SCD protein induced by fenretinide in human RPE cells could affect not only lipid metabolism but also retinoid metabolism in RPE.

Studies have shown that fenretinide can elicit its action by modulating ceramide metabolism (Kravaka et al., 2007; Wang et al., 2001), and that it inhibits dihydroceramide desaturase (DES1) (Kravaka et al., 2007; Rahmaniyan et al., 2011), thereby increasing insulin sensitivity (Bikman et al., 2012). Also, it has been shown that dihydrosphingosine (DHS) not dihydroceramide (DHC) mediates the cytotoxicity of fenretinide as it activates alkaline ceramidase 2 (ACER2) (Mao et al., 2010). It is interesting to note that *Scd1* deficiency has been reported to decrease ceramide synthesis in mouse (Dobrzyn et al., 2005). However, the role of SCD in mediating the effects of fenretinide has not been investigated. A number of studies have suggested that fenretinide regulates the growth, differentiation and apoptosis of cells by modulating sphingolipid metabolism (Kravaka et al., 2007; Wang et al., 2008; Wang et al., 2001). This correlates well with the role of SCD, as any change in the expression or activity of SCD has a wide ranging effect on cell proliferation, differentiation, insulin sensitivity, metabolic rate, atherosclerosis, obesity and cancer (Igal, 2010; Ntambi, 1995; Ntambi, 1999; Paton and Ntambi, 2009). Lack of SCD expression protects mice from

diet-induced obesity, reduces *de novo* fatty acid synthesis, and increases insulin sensitivity (Jiang et al., 2005; Miyazaki et al., 2009; Rahman et al., 2003). In recent years much effort has gone into developing small molecule inhibitors of SCD to treat type-2 diabetes mellitus, metabolic disorders and cancer (Brown and Rudel, 2010; Liu, 2009). Given our observations that fenretinide decreases SCD protein, it is possible that some of the effects of fenretinide on apoptosis, insulin sensitivity and lipogenesis are mediated by its effect on SCD.

In summary, we present data demonstrating that SCD protein and its enzymatic activity are decreased during fenretinide-induced apoptosis in human RPE cells, and is mediated via the ubiquitin-dependent proteasomal pathway in response to ER stress induced by fenretinide. Finally, this effect of fenretinide on SCD should be taken into consideration when developing this compound as therapeutic agent in treating cancer, type-2 diabetes and age-related macular degeneration.

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Abbreviations used are

SCD, SCD1	stearoyl-CoA desaturase
Fenretinide	(N-(4-hydroxyphenyl)-retinamide, 4HPR
RPE	Retinal Pigment Epithelium
ER	Endoplasmic Reticulum
BiP/GRP78	immunoglobulin binding protein/glucose regulated protein 78KDa
ATF4	activating transcription factor 4
GADD153	growth arrest- and DNA damage-inducible gene 153
Des1	dihydroceramide desaturase 1
ACER2	alkaline ceramidase 2
SPT	serine palmitoyl transferase
AMD	age-related macular degeneration
DHS	dihydrosphingosine
DHC	dihydroceramide

Fig. 1. SCD protein is decreased during fenretinide induced apoptosis in human RPE cells

ARPE-19 cells were treated with indicated concentrations of fenretinide (4HPR) for 24 h. *Panel A*, Phase-contrast microscopic analysis. *Panel B*, Fenretinide induced apoptosis. Mono-and oligonucleosomes formation was analyzed by *ELISA*. *Panel C*, Fenretinide treatment decreases SCD protein. Cell lysates were analyzed by western blotting using antibody against SCD. α -Tubulin shows that the amount of protein used in different samples were similar.

* $P < 0.01$ compared to control. The values are mean $\pm$ SD, $n=4$.

Fig. 2. SCD enzyme activity is decreased by fenretinide in ARPE-19 cells

ARPE-19 cells were treated with or without fenretinide (4HPR) for 16 h before incubating with 50 μ M D3-stearate or 50 μ M D3-palmitate for 5h. Lipids were extracted then analyzed by LCMS. *Panel A*, Extracted ion chromatograms of D3-oleic acid, m/z 284.3. *The peak denotes the ^{13}C isotope of naturally occurring stearic acid in the cell. *Panel B*, Extracted ion chromatograms of D3-palmitoleic acid, m/z 256.4. *The peak denotes the ^{13}C isotope of naturally occurring palmitic acid in the cell. *Panel C*, The histogram shows the effect of fenretinide on the ratio of unsaturated fatty acid to saturated fatty acid.

Fig. 3. Proteasome inhibitors blocks fenretinide-induced decrease in SCD protein

ARPE-19 cells were treated with fenretinide (4HPR) in the presence or absence of proteasome inhibitors MG132 and PSI, and analyzed by western blotting using SCD antibody. α-Tubulin expression shows that the amount of protein used in different samples were similar.

Fig. 4. Fenretinide induces ER stress in ARPE-19 cells

ARPE-19 cells were treated with indicated concentrations of fenretinide (4HPR) for 24 h in the presence or absence of MG132.

Panel A, RT-PCR analysis of ATF4 expression. *Panel B*, RT-PCR analysis of GADD153 expression. *Panel C*, Fenretinide treatment increases BiP/GRP78 protein expression when analyzed by western blotting. α -Tubulin expression shows that the amount of protein used in different samples were similar.

* $P < 0.01$ compared to control. The values are mean $\pm$ SD, $n=4$.

Fig. 5. Endoplasmic reticulum stress inducers decrease SCD protein

ARPE-19 cells were treated with tunicamycin and thapsigargin in the presence or absence of MG132, and SCD protein was analyzed by western blotting. The decrease in SCD protein induced by tunicamycin (Tun) and thapsigargin (Thaps) was blocked by MG132. α -Tubulin expression shows that the amount of protein used in different samples are similar.

Fig. 6. Fenretinide induces the accumulation of polyubiquitinated proteins

ARPE-19 cells were treated with fenretinide (4HPR) in the presence or absence of MG132. The cell lysates were used for ubiquitin enrichment as described under Materials and Methods, and then analyzed by western blotting using *anti-ubiquitin* antibody. *Panel A*, Fenretinide induced the accumulation of polyubiquitinated proteins in the presence of MG132. Cell lysate from epoxomicin-treated HeLa cells was used as a positive control (P.C). *Panel B*, Polyubiquitinated SCD accumulates in the presence of MG132. ARPE-19 cells were treated with fenretinide (4HPR) in the presence or absence of MG132, and the cells were solubilized under denaturing conditions with a buffer containing SDS (see Materials and Methods) and subjected to

immunoprecipitation using mouse monoclonal anti-SCD antibody. The immunoprecipitates were then analyzed by western blotting using rabbit polyclonal anti-mono- and polyubiquitin antibody.

Fig. 7. Fenretinide induces ubiquitination of SCD protein in ARPE-19 cells

ARPE-19 cells were treated with fenretinide (4HPR) in the presence or absence of MG132. The cell lysates prepared under non-denaturing conditions were used for the immunoprecipitation analysis using biotinylated mouse monoclonal SCD antibody. The immunoprecipitates were then analyzed by western blotting using anti-mono- and polyubiquitin antibody. *Panel A*, Western blot analysis of mono- and polyubiquitinated proteins in the cell lysates after immunoprecipitating with biotinylated mouse monoclonal anti-SCD antibody. *Panel B*, Western blot analysis of SCD in cell lysate prior to immunoprecipitation with anti-SCD mouse monoclonal antibody. α -Tubulin expression shows that the amount of protein used in different samples were similar.

Fig. 8. Fenretinide induces SCD protein degradation by the ubiquitin-proteasome system

PYR41, an inhibitor of ubiquitin activating enzyme E1, inhibited fenretinide-induced degradation of SCD. ARPE-19 cells were treated with fenretinide (4HPR) in the presence or absence of PYR41, and then analyzed by western blotting using antibody specific to SCD. α -Tubulin expression shows that the amount of protein used in different samples were similar.