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PROTEASOME INHIBITOR TREATMENT REDUCED FATTY ACID, TRIACYLGLYCEROL AND CHOLESTEROL SYNTHESIS

Joan Oliva, Samuel W. French, Jun Li, and Fawzia Bardag-Gorce

Dept. of Pathology, LA BioMed at Harbor-UCLA Medical Center, Torrance, CA 90502, U.S.A

Abstract

In the present study, the beneficial effects of proteasome inhibitor treatment in reducing ethanol-induced steatosis were investigated. A microarray analysis was performed on the liver of rats injected with PS-341 (Bortezomib, Velcade®), and the results showed that proteasome inhibitor treatment significantly reduced the mRNA expression of SREBP-1c, and the downstream lipogenic enzymes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis. ELOVL6, which is responsible for fatty acids long chain elongation, was also significantly down regulated by proteasome inhibitor treatment. Moreover, PS-341 administration significantly reduced the expression of acyl-glycerol-3-phosphate acyltransferase (AGPAT), and diacylglycerol acyltransferase (DGAT), enzyme involved in triacylglycerol (TAG) synthesis. Finally, PS-341 was found to down regulate the enzymes 3-hydroxy-3-methylglutaryl-CoenzymeA synthase (HMG-CoA synthase) that is responsible for cholesterol synthesis. Proteasome inhibitor was also found to play a role in intestinal lipid adsorption because apolipoproteins A (apoA-I, apoAII, apoA-IV and ApoCIII) were down regulated by proteasome inhibitor treatment, especially ApoA-II that is known to be a marker of alcohol consumption. Proteasome inhibitor treatment also decreased apobec-1 complementation factor (ACF) leading to lower level of editing and production of ApoB protein. Moreover apolipoprotein C-III, a major component of chylomicrons was significantly down regulated. However, lipoprotein lipase (Lpl) and High density lipoprotein binding protein (Hdlbp) mRNA levels were increased by proteasome inhibitor treatment. These results suggested that proteasome inhibitor treatment could be used to reduce the alcohol-enhanced lipogenesis and alcohol-induced liver steatosis. A morphologic analysis, performed on the liver of rats fed ethanol for one month and treated with PS-341, showed that proteasome inhibitor treatment significantly decreased ethanol-induced liver steatosis. SREBP-1c, FAS and ACC were increased by ethanol feeding alone, but were significantly decreased when proteasome inhibitor was administered to rats fed ethanol. Our results also show that both mRNA and protein levels of these lipogenic enzymes, up regulated by ethanol, were then down regulated when proteasome inhibitor was administered to rats fed ethanol. It was also

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Corresponding Author: Fawzia Bardag-Gorce, Ph.D., LA BioMed at Harbor-UCLA Medical Center, Department of Pathology, 1124 W. Carson St, Torrance, CA 90502, Tel.: 310-222-2483, Fax: 310-222-5333, fgorce@labiomed.org.

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confirmed that alcohol feeding caused an increase in AGPAT and DGAT, which was prevented by proteasome inhibitor treatment of the animal fed ethanol. Chronic alcohol feeding did not affect the gene expression of HMG-CoA synthase. However, PS341 administration significantly reduced the HMG-CoA synthase mRNA levels, confirming the results obtained with the microarray analysis. C/EBP transcription factors alpha (CCAAT/enhancer-binding protein alpha) has been shown to positively regulate SREBP-1c mRNA expression, thus regulating lipogenesis. Proteasome inhibition caused a decrease in C/EBP alpha mRNA expression, indicating that C/EBP down regulation may be the mechanism by which proteasome inhibitor treatment reduced lipogenesis. In conclusion, our results indicate that proteasome activity is not only involved in down regulating fatty acid synthesis and triacylglycerol synthesis, but also cholesterol synthesis and intestinal lipid adsorption. Proteasome inhibitor, administered at a non-toxic low dose, played a beneficial role in reducing lipogenesis caused by chronic ethanol feeding and these beneficial effects are obtained because of the specificity and reversibility of the proteasome inhibitor used.

Keywords

Fatty acid; Triacylglycerol and Cholesterol Synthesis; Proteasome inhibitor

INTRODUCTION

Steatosis is the initial pathology associated with early stage of alcoholic liver disease, which is characterized by the accumulation of triglycerides and fat in the liver. Alcoholic steatosis is considered clinically benign because it is reversible when the causative agent, i.e., alcohol syndrome is removed. However, chronic exposure to ethanol causes the progression of alcoholic steatosis to alcoholic steatohepatitis (ASH), which is the key step in the development of alcoholic liver disease (ALD).

Fat accumulation could result in the liver from a dysfunction in the lipid synthesis, lipid oxidation, or peripheral lipid transport [Sanal, 2008]. One of the well documented mechanisms of alcoholic fatty liver is the activation of lipogenic transcription factor sterol regulatory element-binding protein 1c (SREBP1c) [You et al., 2002, 2004]. SREBPs are a family of transcription factors that activate the entire program of cholesterol and fatty acid synthesis. In the liver, these transcription factors regulate the production of lipids for export into the plasma and into the bile as lipoproteins and micelles, respectively. Transgenic mice overexpressing SREBP-1 develop a massive fatty liver engorged with both cholesterol and triglycerides [Shimano et al., 1996]. Studies on transgenic mice expressing the active fragments of SREBP suggest that SREBP-1 regulates positively the transcription of genes involved in hepatic fatty acids synthesis (e.g., those encoding acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1), adenosine triphosphate (ATP)-citrate lyase, and malic enzyme) [Carrasco et al., 2001, 2002]. As a result of SREBP-1 activation by ethanol, lipogenic genes that are regulated by SREBP-1, which include FAS, SCD-1, and ACC, are up regulated in the liver, thereby contributing to increased hepatic lipid synthesis [Ji et al., 2006].

To date, several molecular approaches have been reported to decrease ethanol-induced liver steatosis. S-adenosylmethionine and betaine, both methyl donors, have been the most investigated because of their beneficial role in decreasing the ethanol-induced hyperhomocysteinemia [Bardag-Gorce et al., 2010a, 2010b, Kharbanda, 2009]. Only SAME has been investigated in a clinical trial, and its specific beneficial effect and the mechanism in respect to attenuate liver steatosis still needs to be determined [Lucey, 2009]. However, betaine [Ji et al., 2006] and pioglitazone (an anti-diabetic agent) [Tomita et al., 2004] has been reported to down regulate SREBP-1c in the liver of rats fed ethanol, and partially decrease hepatic lipogenesis, mainly by decreasing homocysteine levels, ER stress, and activation of PPAR gamma.

PS-341 (Bortezomib, Velcade®) is an antitumor drug approved by the FDA, and is used to treat patients with multiple myeloma and other cancers [Bold, 2004]. In our model, we treated rats fed ethanol chronically with PS-341 to investigate the beneficial effects of PS-341 prevention of ethanol-induced liver steatosis. In the present study, we report and claim the beneficial effects of proteasome inhibitor drug treatment in reducing fatty acid synthesis by reducing SREBP-1c gene expression, and thus in reducing the downstream lipogenic enzymes gene expression. We also report the beneficial effects of proteasome inhibitor in down regulating the gene expression of key enzymes involved in triglycerides and cholesterol synthesis. In addition, we have discovered that proteasome activity is involved in intestinal lipids adsorption, because proteasome inhibitor treatment changed markedly the levels of several apolipoproteins, such as apoA-I, apoAII, apoA-IV, ApoCIII and apobec-1 complementation factor (ACF). Our data indicated the great potential that proteasome inhibitor treatment may have to prevent hypercholesterolaemia, and alcoholic and non-alcoholic liver steatosis.

MATERIAL AND METHODS

Animals

Male Wistar rats from Harleco (Hollister, CA), weighing between 250–300 g, were used. They were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences and published by the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council.

Ethanol Feeding

Rats were continuously fed intragastrically a liquid diet for 4 weeks. This diet contained ethanol (13 g/kg body weight/day). Controls were pair-fed isocaloric dextrose. Details of the diet and the intragastric model have already been described by [Bardag-Gorce et al., 2002].

PS-341 Treatment

Rats were given proteasome inhibitor PS-341 (LC Laboratories, Woburn, MA) intraperitoneal (IP) at 0.5 mg/kg body weight one time, and were sacrificed 24h later. In the groups of rats fed alcohol for one month, the first group was given PS-341 at 0.5 mg/kg once a week 4 times for 4 weeks along with continuous ethanol feeding and then sacrificed 4 hours post last injection, according to [Bardag-Gorce et al., 2004]. The 2nd group was given

PS-341 at 0.125 mg/kg twice a week for 4 weeks, along with ethanol feeding and sacrificed 24h later.

Proteasome chymotrypsin-like Activity Measurement

1 to 5 µg of total protein from the liver, homogenated by ultra-Turrax, was used. The reaction mixture contained 50 mmol/L Tris-HCl pH 8, 1 mmol/L DTT, and 40 mmol/L Suc-LLVY-AMC substrate for chymotrypsin-like activity. The mixture was incubated for 30 min. at 37°C, and the reaction was then stopped by adding 100 mmol/L monochloroacetate and 30 mmol/L sodium acetate (pH=4.3). Fluorescence was determined by measuring the release of AMC (λ excitation: 370 nm, λ emission: 430 nm), using a Perkin Elmer LS 30 spectrofluorometer.

Microarray Analysis

Fast frozen rat liver tissue were subjected to microarray analysis. Total liver RNAs were extracted with Ultraspec™ RNA Isolation Systemic (Biotecx Laboratories, Houston, TX), and cleaned with Rneasy columns (Qiagen, Valencia, CA). Five micrograms of total RNA were used for preparing biotin-labeled cRNA. Labeled and fragmented cRNA was subsequently hybridized to Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). Labeling, hybridization, image scanning, and initial data analysis were performed at the Microarray Core at the Los Angeles Biomedical Research Institute. Sample preparation and loading, hybridization, staining, and microarray data analysis were then performed according to [Bardag-Gorce et al., 2006].

mRNA Analysis by qRT-PCR

Quantitative RT-PCR was performed to measure the levels of antioxidative enzymes. Total liver RNAs was extracted with Trizol Plus RNA Purification kit (Invitrogen, Carlsbad, CA). Synthesis of cDNAs was performed with 5 µg total RNA, and 50 ng random hexamer primers using SuperScriptIII RNase H-Reverse Transcriptase (Invitrogen). PCR primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA), and have been tested in our lab. The primers for rat GSS, GSR, and GPX2, SOD2 are shown in Table 1. Quantitative PCR was achieved using the SYBR Green JumpStart™ Tag ReadyMix (Sigma, St. Louis, MO) on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Thermal cycling consists of an initial step at 50°C for 2 min., followed by a denaturation step at 95°C for 10 min., then 40 cycles at 95°C for 15 s and 60°C for 1 min. A single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Each data point was repeated three times. The target mRNA abundance in each sample was normalized to its 18S level as $DC_t = C_{t_{\text{target gene}}} - C_{t_{18S}}$. For each target gene, the highest DC_t was assigned as $DC_{t_{\text{max}}}$.

Western Blots

Proteins from the cytosolic fraction were separated by SDS-PAGE electrophoresis using 12% polyacrylamide gels. Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1h in 25 mM Tris-HCl (pH 8.3), glycine 192 mM and 20% methanol. Antibodies against SREBP-1c (Thermo Scientific, Rockford, IL) and fatty acid synthase

(Novus Biology Littleton, CO) and Beta-actin (Sigma, St. Louis, MO) were used. Goat anti-mouse and goat anti rabbit antibody (Bio-Rad, Hercules, CA) were used as the second antibody. Immunodetection was done using an alkaline phosphatase kit from Bio-Rad or ECL plus (Amersham Bioscience Corp., Piscataway, NJ). Densitometric measurements of the bands were done using the GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

Statistical Analysis

Data were obtained from at least three separate experiments. Bars represent mean values $\pm$ SEM. P values are determined by one-way ANOVA and Student-Newman Keuls for multiple group comparisons (Sigma-Stat software, San Francisco, CA). Statistical significance is set at $p < 0.05$.

RESULTS

Microarray analysis was performed on the liver of rats injected with PS-341. A KEGG functional pathway analysis showed that proteasome inhibitor drug treatment significantly down regulated fatty acid metabolism, biosynthesis, and fatty acid elongation [Oliva et al., 2009]. Data mining of this microarray analysis showed that proteasome inhibitor treatment down regulated the gene expression of sterol regulatory element binding protein 1 (SREBP-1c) and that of the downstream lipogenic enzyme (Table 2).

This microarray analysis was the starting point of the hypothesis of proteasome inhibitor treatment may have beneficial effects in reducing liver steatosis and cholesterol synthesis. The intragastric alcohol infusion rat model was used to verify our hypothesis [French, 2001; Tsukamoto et al., 1990]. PS-341 treatment was done using two different dosing protocols. The first group of rats, fed ethanol, was given PS-341 once a week at 0.5 mg/kg bw for 4 weeks, and sacrificed 4 h later after the last injection. The 2nd group, fed ethanol, was given PS-341 twice a week every 72 hours at 0.125 mg/kg bw for 4 weeks. These two protocols generated the same proteasome inhibition and the proteasome activity was recovered 48 hours to activated 72 hours post the last injection [Bardag-Gorce et al., 2010a, 2010c]. The relevance of the two dosing protocols was to determine the non toxic low dose of proteasome inhibitor that showed beneficial effects. We obtained the same beneficial effects in reducing liver steatosis with either one dose (0.5mg/kg) or two lower doses (0.125 mg/kg) of PS-341 per week. Lower doses than 0.125 mg/kg were not effective and higher doses, as well as more frequent administrations were not tolerated by the animals fed alcohol. Our results also showed that the beneficial effects of the drug come from its specificity and its reversibility.

Figure 1(A and B) show that ethanol caused an increase in liver weight and that PS-341 treatment prevented this increase. The body weight however was decreased by PS-341 for both dextrose and ethanol fed animals (Figure 1C). These results indicated that PS-341 prevented the ethanol-induced increase in the liver weight, probably by decreasing ethanol-induced steatosis. In addition proteasome inhibitor treatment decreased significantly the levels of plasma ALT (Figure 1D) and ER stress marker Grp78 (Figure 1 E) indicating the beneficial effects of proteasome inhibitor.

The histology analysis showed that chronic ethanol feeding caused fat deposition in the liver, as shown by macrovesicular steatosis formation (Figure 2B) compared to the control (Figure 2A). PS-341 administration, for one month, once a week at 0.5 mg/kg bw, prevented liver macrovesicular steatosis formation (Figure 2C). The administration of PS-341, at a lower dose (0.125 mg/kg) twice a week, also attenuated the ethanol-induced fat deposition (Figure 2E) compared to the animal fed ethanol alone (Figure 2D).

To confirm the results obtained from the microarray analysis done on the liver of a rat treated with PS-341, we performed an RT-PCR to analyze the mRNA levels of SREBP-1c and the down stream lipogenic enzymes. Figure 3 shows that SREBP1 gene expression was significantly down regulated when the animals were treated with PS-341 and sacrificed 4 hours later. An even greater decrease of SREBP-1c was found when the animal were sacrificed 24 hours later (Figure 3A). This greater decrease was also found in the gene expression of the enzyme ACC (ACAB) (Figure 3B), fatty acid synthase (FASN) (Figure 3C), and stearoyl-Coenzyme A desaturase 1 (SCD1) (Figure 3D).

These lipogenic enzymes are all involved in fatty acid synthesis and their down regulation by proteasome inhibitor PS-341 explains the reduction of fat accumulation in the liver of rats fed ethanol.

Protein levels of SREBP-1c and fatty acid synthase enzyme were also down regulated by proteasome inhibitor treatment (Figure 4).

The levels of ELOVL5 and ELOVL6, elongation of very long chain fatty acids family member 5 and 6, were found significantly decreased by proteasome inhibitor treatment (Figure 5A and B, respectively), confirming the effects of the proteasome inhibitor in reducing fatty acid synthesis.

In addition to reducing gene expression of fatty acid synthesis, proteasome inhibitor treatment was efficient in reducing the gene expression of fatty acid elongation factors.

Proteasome inhibitor treatment also decreased the gene expression of key enzymes involved in triglycerides synthesis (TAGs). Acylglycerol-3-phosphate acyltransferase (AGPAT) is responsible for glycerol -3-phosphate acylation with fatty acyl-CoA up stream in diacylglycerol (DAG) synthesis. The gene expression of this enzyme was significantly down regulated in the liver of rats treated with PS-341 (Figure 6A). It was also found that mRNA levels of diacylglycerol acyltransferase (DGAT), which is responsible for converting DAG into TAG in the final step of TAG synthesis, was significantly decreased by proteasome inhibitor treatment (Figure 6B). We also analyzed the effects of proteasome inhibitor treatment on cholesterol synthesis. Results showed that 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (HMG-CoA synthase) gene expression was markedly decreased by proteasome inhibitor treatment (Fig. 6C).

Ethanol feeding did not change cholesterol synthesis enzyme RNA levels. However, PS-341 treatment significantly decreased the mRNA levels of HMG-CoA synthase 24 hours post PS-341 administration, which confirmed the microarray results obtained from the group of rats treated with PS-341 alone. Our results are the first evidence that proteasome inhibitor

treatment reduces the levels of the enzyme responsible for cholesterol synthesis. HMG-CoA synthase catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form hydroxymethylglutaryl-CoA (HMG-CoA), which is the first step of cholesterol synthesis that precedes HMG-CoA reductase, a well known target enzyme of the widely available cholesterol-lowering statins drugs. These results confirmed the microarray data and demonstrate the reproducible effects of proteasome inhibitor treatment in decreasing fatty acid, triglycerides synthesis and cholesterol synthesis. The microarray data mining analysis also showed that enzymes involved in intestinal fat adsorption were down regulated by proteasome inhibitor treatment (Table 3). ApoA2, known to be a marker of alcohol consumption, was markedly decreased by PS-341. Hdlbp, a high density lipoprotein-binding protein that specifically binds HDL molecules and may function in the removal of excess cellular cholesterol, was also found increased by PS-341 treatment (Table 3).

Results of microarray analysis showed that PS-341 significantly decreased ACF gene expression, which is involved in hepatic apoB mRNA editing for translation and production (Figure 7A). Apolipoprotein C-III (Apoc3) was also found decreased by PS-341 treatment (Figure 7B). Apoc3 is a major component of chylomicrons and VLDL involved in the catabolism of triglycerides-rich lipoproteins. An increase in Apoc3 levels induces the development of hypertriglyceridemia [Ruixing et al., 2010]. However, PS-341 significantly increased the levels of lipoprotein lipase (Lpl), an enzyme that hydrolyzes triglycerides in lipoproteins. 24 hours after the last injection, Lpl was not increased by PS-341 (Figure 7C) which may be explained by the reversibility of PS-341.

In an attempt to understand the mechanistic role played by the proteasome in regulating the gene expression of fatty acid biosynthesis, we first analyzed the mechanism of SREBP-1c gene expression. Recently, Payne et al. [Payne et al., 2009] showed that C/EBP transcription factors (CCAAT/enhancer-binding protein a/b/d) directly regulated SREBP-1c gene expression, and that C/EBPbeta and delta regulated SREBP-1c expression in early adipocytes maturation [Payne et al., 2009]. C/EBPalpha regulates SREBP-1c expression in mature adipocytes. Our microarray analysis showed a significant increase in C/EBPbeta and delta levels (2 and 11.3 fold change, respectively) and a significant decrease in C/EBPalpha (-3.2 fold change), indicating that C/EBPalpha expression inhibition was involved in SREBP-1c expression down regulation. In addition to the microarray analysis, we found that proteasome inhibitor treatment significantly reduced the C/EBPalpha increase by ethanol feeding (Figure 8).

DISCUSSION

Alcoholic steatosis is characterized by fat deposition, which leads to liver steatohepatitis and cirrhosis. This fat accumulation results from a dysfunction in the signaling of at least three important regulatory molecules: PPARalpha, AMPK, and SREBP-1c. PPARalpha and AMPK ethanol-induced inhibition reduces the ability of the liver to oxidize and export fatty acids [Crabb et al., 2006]. Several therapeutic approaches have been designed to block the effects of ethanol and prevent fatty liver. Pioglitazone, a PPARgamma agonist, has been reported to prevent alcohol-induced liver steatosis in rodents through the up-regulation of adiponectin levels, which was associated with marked increase in the rates of fatty acid oxidation,

preventing hepatic fatty acid accumulation [Tomita et al., 2004]. Taurine supplementation has also been reported to restore the concentration of adiponectin and attenuate ethanol-induced steatosis in the liver of rats fed ethanol [Chen et al., 2009]. The ethanol-induced inhibition of AMPK activity causes an increase in ACC activity, the rate-limiting enzyme in lipid biosynthesis. This activity is associated with malonyl-CoA production that decreases mitochondrial fatty acid oxidation by potentially inhibiting the carnitine palmitoyltransferases [You et al., 2004b; Crabb et al., 2006]. AMPK activators, such as sauchinone [Kim et al., 2010], resveratrol [Ajmo et al., 2008], AICAR [Greenberg et al., 1992], and metformin [Bergheim et al., 2006], have been the subject of extensive research to block the AMPK ethanol-induced inhibition. This was shown to decrease ethanol-induced liver steatosis.

S-adenosylmethionine (SAME) has also been proposed as a treatment for alcoholic liver disease [Mato et al., 1999]. However, it has not become standard therapy. Only Disulfiram, an antagonist of acetaldehyde dehydrogenase, has the longest period of approval for use in treating alcoholism [Mutschler et al., 2008], but no research as been done on Disulfiram beneficial roles in reducing liver steatosis. Interestingly, Disulfiram has been shown to inhibit proteasome activity and, thus, has been used as an antitumor drug [Chen et al., 2006; Cvek et al., 2008; Rickardson et al., 2007]. This supports our hypothesis of the beneficial effects of proteasome inhibitor PS-341 in alcoholic liver steatosis. Our results showed that PS-341 proteasome inhibitor treatment significantly down regulated the expression of both genes *Acaca* (ACC1) and *Acacb* (ACC2), indicating that the proteasome inhibitor treatment played a key role in fatty acid biosynthesis and fatty acid oxidation at the gene expression level. The expression of these two genes is regulated by the transcriptional factor SREBP-1c, which was also significantly down regulated by proteasome inhibitor treatment. These results indicate that the beneficial effects of the proteasome inhibitor in reducing ethanol-induced liver steatosis, originate from the down regulation of SREBP-1c gene expression and from programming the liver of rats fed ethanol for a low level of lipogenesis rather than stimulating fatty acid oxidation to utilize the accumulated fat.

The present study is the first to report the effects of proteasome inhibitor treatment on cellular lipogenesis, particularly, the regulatory effects of proteasome inhibitor on SREBP-1c transcriptional levels. SREBP-1c is a major player in ethanol-induced liver steatosis because it stimulates the gene expression of several enzymes responsible for fatty acid synthesis. Its activation represents the major cause of lipid accumulation in the liver of animals fed ethanol. Proteasome inhibitor treatment down regulated mRNA levels of SREBP-1c, which coincided with the down regulation of lipogenic gene expression, as well as a decrease in fatty acid droplets in the hepatocytes, as shown by morphology analysis. The underlying molecular mechanisms that control SREBP-1c mRNA expression is, however, still not fully determined. The C/EBP family of transcription factors (CCAAT/enhancer-binding proteins α , β and δ) plays a critical role in both the early induction of SREBP1c and the maintenance of its expression in maturing adipocytes [Payne et al., 2009]. These authors demonstrated the direct binding of these transcriptional factors with the SREBP-1c promoter and that the loss of any of these C/EBP factors significantly impaired the induction of SREBP1c. Specifically, these authors demonstrated that when the transcriptional level of C/EBP α is down regulated, the SREBP-1c transcriptional level is also down regulated in mature adipocytes. Our results showed a decrease in mRNA levels of

C/EBPalpha in the liver of rats treated with PS-341, which is associated with SREBP-1c mRNA decrease. Ethanol has been shown to increase C/EBP alpha in our model and this increase was significantly down regulated by treating the ethanol fed rats with the proteasome inhibitor.

There is growing evidence for a role of ER stress caused by hyperhomocysteinemia in alcoholic fatty liver. It triggers the unfolded protein response (UPR) and SREBP-1c activation [Kaplowitz and Ji, 2006]. Betaine supplementation stimulates betaine--homocysteine S-methyltransferase1 (BHMT) activity and reduces homocysteine by its conversion to methionine. This is the mechanism by which betaine supplementation corrects the levels of homocysteine and lowers ER stress, as well as SREBP-1c activation [Purohit et al., 2007]. When used to treat multiple myeloma, the proteasome inhibitor treatment, at a highly toxic dose, induces apoptosis caused by excessive endoplasmic reticulum (ER) stress by activating the terminal unfolded protein response (UPR) [Nawrocki et al., 2005; Obeng et al., 2006], particularly ATF4 [Rzymiski et al., 200]. In our experimental model, we did not observe an increase in ER stress because the treatment was done using a low dose of proteasome inhibitor, and because we gave the animal enough time to recover between the dose injections. Our results even show that proteasome activity rebound probably enhanced the clearance of unfolded protein that might results from enhanced ER stress. PS-341 is a reversible drug and proteasome activity was increased at 48 hours to 72 hours post injection [Bardag-Gorce et al., 2010c]. This rebound may be the key to the beneficial effects of proteasome inhibitor treatment.

Triglyceride synthesis plays a significant role in the prevalence of metabolic diseases, such as obesity, hyperlipidemia, hepatic steatosis, and insulin resistance. Triglycerides and phospholipids are synthesized through the glycerol phosphate and the monoacylglycerol pathways. The glycerol phosphate pathway is responsible for the majority of de novo biosynthesis of triglycerides in most cell types, while the monoacylglycerol pathway is very important in triglyceride synthesis in the small intestine. Triglycerides synthesis is catalyzed sequentially by enzymes that have multiple isoforms including 1-acylglycerol-3-phosphate acyltransferase (AGPAT) and diacylglycerol acyltransferase (DGAT). It has been shown that mutation and genetic alteration of AGPAT [Agarwal and Garg, 2010] caused a marked decrease in adipose tissue. Inhibition of DGAT1 is already considered as a potential therapeutic target in a treatment of obesity [Matsuda et al., 2007, 2010; Yamamoto et al., 2011]. Our results showed that the proteasome inhibitor significantly decreases the mRNA levels of DGAT2, specific enzymes for the liver and liver steatosis. The advantage of proteasome inhibitor PS-341 is that it is a reversible drug. The animals exposed to PS-341 recover from its effects and DGAT2 down regulation rebounds to normal levels within 72 hours.

Cholesterol synthesis is well documented in the literature because of the well know effects of Statins in lowering cholesterol synthesis. Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). Studies have also reported that statins and the proteasome inhibitor have similar beneficial effects in vascular cells [Ludwig et al., 2005]. We discovered that the PS-341 proteasome inhibitor is also an inhibitor of the enzymes 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS), which precedes

HMGR in cholesterol synthesis pathway. This indicates that statins and proteasome inhibitors at least partially affect the same pathways, but from independent initial points.

Finally, we discovered that proteasome is also involved in lipid adsorption and that proteasome inhibition causes a down regulation of apolipoproteins apoA-I, apoAII, apoA-IV, ApoCIII and apobec-1 complementation factor (ACF). The literature mainly addresses the role of proteasome degradation of apolipoprotein-B100 (ApB100), a primary apolipoprotein of low-density lipoproteins (LDL) [Rutledge et al., 2009]. There has been no data reported on the effect of proteasome inhibition on the apolipoproteins A. *Our study is the first to report that proteasome inhibition down regulates the mRNA levels for apolipoproteins A.*

In conclusion, we discovered that proteasome inhibitor treatment lead to a marked reduction of fat accumulation and liver steatosis because it down regulated the fatty acid, triglyceride, and cholesterol synthesis. These discovery is the indication that the proteasome inhibitor treatment could be beneficial against alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH), which have various phenotypic manifestations, i.e., overweight/obesity, visceral adiposity, type 2 diabetes, hypertriglyceridemia and hypertension.

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Figure 1.

A. Liver weight; B. Liver weight/body weight ratios. Note that PS-341 treatment given with ethanol feeding significantly decreased the liver weight and the liver weight/body weight ratios compared with animals fed ethanol alone. C. Body weight measurements. Note that PS-341 also decreased body weight for both ethanol and dextrose rats. (means $\pm$ SE, $n = 3-4$). D. ALT levels in the plasma of rats treated with proteasome inhibitor PS-341 at 0.125 mg/kg bw. E. Proteasome inhibitor treatment prevented the ethanol-induced Grp78.

Figure 2.

Proteasome inhibitor was administrated 4 times, once a week at 0.5 mg/kg along with ethanol feeding (A, B, C) and twice a week at 0.125mg/kg along with ethanol (D and E). Liver sections were stained with hematoxylin and eosin. Hepatocytes from rats fed dextrose (Control, A) showed normal liver without steatosis. Hepatocytes from rats fed ethanol (B and D), showed macrovesicular steatosis. However, hepatocytes from rats fed ethanol + PS-341 (C) showed less macrovesicular steatosis.

Figure 3. qRT-PCR analysis of SREBP1, ACAB, FASN, and SCD1 gene expression showed that proteasome inhibitor treatment caused a significant decrease of mRNA levels of the lipogenic enzymes. Mean $\pm$ SEM, n = 4.

Figure 4. Western blot analysis of SREBP-1 (A) and fatty acid synthase (B) in the liver of rats fed ethanol, and rats fed ethanol and treated with PS-341, at 0.5 mg/kg b.w. once a week for 4 weeks. Beta-actin stain as a loading control (C). Mean $\pm$ SEM, $n = 3$

Figure 5. qRT-PCR analysis of ELOVL5 and ELOVL6 gene expression showed that proteasome inhibitor treatment caused a significant decrease of mRNA levels of the elongation of very long chain fatty acids genes. Mean $\pm$ SEM, $n = 4$.

Figure 6. qRT-PCR analysis showed that proteasome inhibitor treatment decreased the gene expression of both enzymes AGPAT (A) and DGAT responsible for TAG Synthesis. (C) qRT-PCR analysis showed that proteasome inhibitor PS-341 down regulated HMG-CoA synthase.

Figure 7.

qRT-PCR analysis of ACF, Apoc3, and Lpl gene expression showed that proteasome inhibitor treatment caused a significant decrease in the mRNA levels of intestinal lipid adsorption enzyme. However, proteasome inhibitor treatment significantly increased the levels of Lpl 4 hours after the last PS341 injection. (Mean $\pm$ SEM, $n = 3$).

Figure 8.

qRT-PCR analysis of C/EBP alpha gene expression showed that ethanol feeding slightly increased mRNA levels of C/EBP alpha, while proteasome inhibitor treatment of rats fed ethanol caused a significant decrease of the mRNA levels of c/EBP alpha.

Table 1

Primers Sequence

Gene Name	Gene ID		Sequence
SREBP-1c	XM_213329.4	Forward	CGCTACCGTTCCTCTATCAATGA
		Reverse	GTGCTGTAAGAAGCGGATGTAGTC
ACC (ACAB)	NM_053922.1	Forward	GAATTGTGACCCGCTTTGG
		Reverse	TGGAGCGCATCCACTTGA
FASN	NM_017332.1	Forward	GGACATGGTCACAGACGATGAC
		Reverse	GTGGAAGTTGGACAGATCCTTCA
SCD1	NM_139192.2	Forward	CCGTGGCTTTTCTTCTCTCA
		Reverse	CTCTTTGACAGCCGGGTGT
ELOVL 5	NM_134382.1	Forward	CTGGTCCAGTTGTGCTGACA
		Reverse	AGGAGCACGGCCAGATGA
C/EBP alpha	NM_012524.2	Forward	GGCGGGAA CGCAACAAC
		Reverse	TTGCGCTGTTGGCTTTATCT
3HMG-CoA (Hmgs 1)	NM_017268.1	Forward	GCTTTCCCAGAGTCCATTGC
		Reverse	AACGCTCCGTGCCAACA
Lipoprotein Lipase (Lpl)	NM_012598.2	Forward	TGGGCCTAACTTTGAGTATGCA
		Reverse	GACATCTACGAAATCCGCATCA
Apolipoprotein CIII	NM_012501.1	Forward	CTTGCCCCGAGCTGATGAG
		Reverse	TTGTTCCATGTAGCCCTGCAT
AGPAT	NM_001107821.1	Forward	AGGAATGGGCAACGCATTAC
		Reverse	GCCTGGTTCCTGAATGTTTGTGTC
DGAT	NM_001012345.1	Forward	AGGCCTTGATGGTTTCTATCCA
		Reverse	GCTGCCCTTCCCAATTAAC

Table 2

Data Mining of Microarray Analysis Comparing Lipogenic Enzymes mRNA Levels in the Livers of Rat Given PS-341 and Control

Gene expression analysis comparison: PS-341-treated rat with its control		
Gene Title	Gene Symbol	Ratio
Sterol regulatory element binding factor 1	Srebf1	-7
Acyl-CoA synthetase long-chain family member 1	Acs11	-17.1
ATP citrate lyase	Acly	-4.3
Acetyl-Coenzyme A carboxylase beta (ACC2)	Acacb	-3.5
Acetyl-coenzyme A carboxylase alpha (ACC1)	Acaca	-2.8
Fatty acid synthase	Fasn	-6.1
Stearoyl-Coenzyme A desaturase 1	Scd1	-26
ELOVL family member 5, elongation of long chain fatty acids	Elovl5	-4.6
ELOVL family member 6, elongation of very long chain fatty acids	Elovl6	-5.7
1-acylglycerol-3-phosphate O-acyltransferase 2	Agpat2	-2.8
diacylglycerol O-acyltransferase homolog 2	Dgat2	-8.6
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Hmgcs1	-13.9
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	-776
C/EBP transcription factors alpha (CCAAT/enhancer-binding protein alpha)	C/EBP	-4

Table 3

Data Mining of Microarray Analysis Comparing Intestinal Fat Adsorption Enzymes mRNA Levels in the Livers of Rat Given PS-341 and Control

Gen title	Gene Symbol	Fold change
Apolipoprotein A-I	Apoa1	-2.5
Apolipoprotein A-II	Apoa2	-27,9
Apolipoprotein A-IV	Apoa4	-2.5
Apolipoprotein C-III	Apoc3	-4.3
Apobec-1 complementation factor	ACF	-5,7
Lipoprotein lipase	Lpl	9.2
High density lipoprotein binding protein	Hdlbp	2