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Authors

Sinha-Hikim, Indrani
Sinha-Hikim, Amiya P.
Shen, Ruqing
et al.

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A novel cystine based antioxidant attenuates oxidative stress and hepatic steatosis in diet-induced obese mice

Indrani Sinha-Hikim^{a,b,*}, Amiya P. Sinha-Hikim^{a,b}, Ruoqing Shen^a, H. Kim^c, Samuel W. French^d, Nostrola D. Vazari^c, Albert Crum^e, Tripathi B. Rajavashisth^{a,b}, and Keith C. Norris^{a,b}

^aDepartment of Internal Medicine, Charles R. Drew University, Los Angeles, CA, USA

^bDavid Geffen School of Medicine at University of California, Los Angeles, CA, USA

^cDivision of Nephrology and Hypertension, University of California, Irvine, CA, USA

^dDepartment of Pathology, Harbor-UCLA Medical Center, Torrance, CA, USA

^eProimmune®, Rhinebeck, NY, USA

Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common form of liver pathologies and is associated with obesity and the metabolic syndrome. Here, we investigated the molecular mechanisms by which a novel cystine based glutathione precursor with added selenomethionine (F1) prevents hepatic steatosis in a moderate high fat dietary model of NAFLD. Adult (8 weeks old), male apolipoprotein E (ApoE)^{-/-} mice were fed with a normal diet (ND) or high fat diet (HFD), consisting of 21% fat and 0.21% cholesterol, with or without dietary supplementation of F1 (3 g/kg food) for 16 weeks. Compared with ApoE^{-/-} mice fed with ND with or without F1, ApoE^{-/-} mice fed with HFD exhibited significant weight gain, hepatomegaly, and increased serum cholesterol and triglycerides levels with no change in serum albumin levels. High resolution light and electron microscopy revealed micro- and macro-vesicular steatosis in ApoE^{-/-} mice fed on a HFD. HFD-induced obesity also led to increased lipogenesis, oxidative stress, activation of c-Jun-NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), perturbation of the BAX/BCL-2 rheostat, hepatocyte apoptosis, and activation of caspases 9 and 3. F1 fully prevented the adverse effects of HFD on serum triglyceride levels, body and liver weights, and hepatic steatosis and substantially attenuated HFD-induced increase in lipogenesis, oxidative stress, kinase activation, apoptotic signaling, and hepatocyte ultrastructural abnormalities. These results demonstrate that administration of F1, a glutathione precursor, ameliorates HFD-induced hepatic steatosis in ApoE^{-/-} mice and emphasizes the role of oxidative stress in diet-induced obesity and hepatic steatosis.

Keywords

Antioxidant; Oxidative stress; Apoptosis; High fat diet; Hepatic steatosis; Lipogenesis

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*Corresponding author at: Department of Internal Medicine, Charles R. Drew University of Medicine and Science, 1731 E. 120th Street, Los Angeles, CA 90059, United States. Fax: +1 323 563 9352., indranisinhahikim@cdrewu.edu (I. Sinha-Hikim).

Conflict of interest

The authors declare that there are no conflicts of interest.

Introduction

Obesity and the metabolic syndrome have become a worldwide epidemic. Nonalcoholic fatty liver disease (NAFLD) is the most common of liver pathologies and is associated with obesity, diabetes, and metabolic syndrome (Farrel et al., 2008; Postic and Girard, 2008; Trauner et al., 2010). NAFLD includes the whole spectrum of fatty liver, ranging from simple steatosis to steatohepatitis (NASH), which can progress to liver cirrhosis and hepatocellular carcinoma (Farrel et al., 2008; Postic and Girard, 2008; Trauner et al., 2010). Fatty liver occurs at a higher rate in individuals with obesity and type 2-diabetes and is considered the hepatic component of the metabolic syndrome (Schindhelm et al., 2006). Currently, 34% of the general population and over 75% of obese and extremely obese individuals are estimated to have fatty liver (Browning and Horton, 2004). Hispanics have the highest prevalence of hepatic steatosis followed by Caucasians and then African-Americans (Browning and Horton, 2004).

The precise molecular mechanisms and pathogenesis of steatosis and its progression to NASH is not well understood. Steatosis is believed to prime the liver to more severe liver pathologies when individuals are exposed to subsequent metabolic and/or environment stressors. The prevalent hypothesis is the “two-hit” hypothesis, with the “first-hit” being the accumulation of triglycerides in hepatocytes (steatosis) followed by the “second-hit” that triggers the progression of steatosis to NASH (Trauner et al., 2010; Mantena et al., 2008). Insulin resistance appears to be the “first-hit”, which induces fat accumulation in hepatocytes and renders the liver more susceptible to injury or diseases. Examples of “second-hit” include environmental stressors like high fat diet, cigarette smoke, drugs, or pollutants and metabolic stressors such as obesity, diabetes, hypertension, hypertriglyceridemia, and hypercholesterolemia. Molecular mechanisms underlying how steatosis predisposes liver to transition from simple fatty liver to NASH are not well understood. Oxidative stress coupled with hepatocyte apoptosis is believed to play a pivotal role in pathogenesis of NAFLD (Trauner et al., 2010; Kojima et al., 2007). In fact, emerging data now suggest that hepatocyte apoptosis may be a key component of the “second hit” involved in the progression of simple steatosis to NASH.

With the increased prevalence of NAFLD/NASH worldwide and in the United States (Farrel et al., 2008; Postic and Girard, 2008; Trauner et al., 2010), novel and safe treatments to prevent these diseases are urgently needed. Currently, effective therapeutic strategies for the prevention of disease progression in patients with NAFLD are lacking (Mantena et al., 2008; Tilg and Kaser, 2005). Earlier studies, using NASH patients as well as murine models of NASH, have suggested some beneficial role of antioxidants, including Vitamin E, N-acetylcysteine (NAC), and S-adenosylmethionine (SAME) in NAFLD (Fusai et al., 2005; Oz et al., 2006; Thong-Ngam et al., 2007; Baumgardner et al., 2008; Li et al., 2008; de Oliveira et al., 2008). The most abundant endogenous antioxidant in eukaryotic cells is the tripeptide glutathione (L- γ -glutamyl-L-cysteinyl-glycine; GSH), which is critical for the maintenance of the cellular redox state (Wu et al., 2004; Han et al., 2006). In fact, early depletion of GSH is a common feature of apoptosis triggered by a variety of stimuli (Franco and Cidlowski, 2009). However, oral glutathione is limited in its ability to increase intracellular glutathione levels. In the gastrointestinal tract, glutathione is largely converted into constituent amino acids (including the highly oxidizable L-cysteine), which cannot be re-constituted into glutathione extracellularly as its re-assimilation requires two cytosolic, ATP-dependent enzymes. Thus, the processes that achieve glutathione synthesis require special conditions that are only present within the cell (Jones, 2006). Intravenous glutathione administration is expensive, short-lived, and impractical, as the transient rise in plasma concentration is largely ineffective in improving intracellular glutathione activity, where much of its redox actions take place. However, a glutathione precursor with cystine as the physiologic cysteine

carrier could enhance the Cys/CySS redox state to attenuate oxidative stress to a greater intracellular delivery of substrate. In vascular smooth muscle cells (VSMC), we recently demonstrated that FT061452/RE39734TM or F1, a cystine based GSH precursor, is more effective than glutathione or NAC in preventing spermine (a uremic toxin)-induced oxidative stress, impairment of glucose metabolism, and apoptosis (Sinha-Hikim et al., 2010a, 2010b).

The objective of this study is to determine whether administration of a novel glutathione precursor, F1, can prevent hepatic steatosis in a mouse model of dietary steatosis. In the process of doing so, we also characterized the key signal transduction pathways involved in F1-mediated protection of hepatic steatosis. Our results show that F1 prevents high fat diet (HFD)-induced hepatic steatosis through reduction in lipogenesis together with suppression of oxidative stress, kinase activation, and mitochondria-dependent intrinsic pathway signaling.

Materials and methods

Antioxidant

F1 (FT061452/RE39734TM), a GSH precursor with cystine replacing cysteine and selenomethionine added was obtained from Proim-mune®, Rhinebeck, NY (Crum, 2007). The formulation (per 500 mg) contains L-cystine—99.68 mg, glycine—199.39 mg, selenomethionine —1.54 mg, and L-glutamine—199.39 mg. This compound is tasteless and odorless.

Animals

Adult (8 weeks old), male ApoE^{-/-} mice on a C57BL/6 background were used. These mice were obtained from the breeding colonies established in our animal facility at Charles R. Drew University of Medicine and Science. We elect to use ApoE^{-/-} mice, as these animals, even when fed with Western diet, as opposed to very high fat diet with 60% calories derived from fat, develop hepatic steatosis (Guillen et al., 2009; Ferre et al., 2009; Lohmann et al., 2009; Martinez-Clemente et al., 2010). Furthermore, because NAFLD has been identified as an independent risk factor of atherosclerosis and cardiovascular disease (Targher et al., 2005; Hemaguchi et al., 2007), an additional advantage of this model is that it would be an ideal model for linking obesity, atherosclerosis, and hepatic steatosis. Animals were housed under controlled temperature (22 °C) and photoperiod (12-h light and 12-h dark cycle). Groups of six ApoE^{-/-} mice fed either with a normal chow diet (ND) or a high fat diet (HFD) consisting of 20% protein, 50% carbohydrate, 21% fat, and 0.21% cholesterol (D12079BB; Research Diets, New Brunswick, NJ) with or without supplementation of F1 (3 mg/kg food) for 16 weeks. Body weight and food intake were measured weekly. Food intake was measured per cage with 3 mice per cage to avoid the stress of individual housing (de Meijer et al., 2010). All mice were fasted overnight before euthanized with a lethal injection of sodium pentobarbital (200 mg/kg BW). Blood samples were collected from each animal by cardiac puncture immediately after death, and serum was separated and stored at -20 °C for subsequent measurements of cholesterol, triglyceride, and serum albumin levels. These parameters were measured in the core laboratories at the Division of Nephrology and Hypertension, University of California at Irvine. Livers were removed and weighed. Portions of liver were snap frozen in liquid N₂ and stored frozen for subsequent analysis by western blotting and by enzyme immunometric assay (EIA). The remaining portions of liver were either fixed in 2.5% glutaraldehyde for high resolution light and electron microscopy or 4% paraformaldehyde for routine histological and immunohistochemical or immunofluorescence studies. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by

the Charles R. Drew University School of Medicine and Science animal care and use review committee.

Measurements of GSH/GSSG ratio and oxidative DNA damage

GSH/GSSG ratio in liver samples was measured using a commercial kit (BIOXYTECH® GSH/GSSG-412™ assay kit (OXISResearch™, A division of Oxis Health Product, Inc., Portland, OR), as described previously (Sinha-Hikim et al., 2010b). This assay, using different sample preparations, measures both reduced (GSH) and oxidized (GSSG) concentrations and the GSH/GSSG ratio. The omission or addition of 1-methyl-2-vinylpyridinium trifluoromethanesulfonate allows the measurement of GSH and GSSG, respectively. The GSH/GSSG ratio is inversely related to reactive oxygen species (ROS) levels.

We also measured the formation of 8-hydroxydeoxyguanosine (8-OHdG), an additional marker of oxidative stress in livers, using Oxiselect™ Oxidative DNA Damage ELISA kit (Cell Biolabs, Inc., San Diego, CA). In brief, the unknown 8-OHdG samples or 8-OHdG standards are first added to an 8-OHdG/BSA conjugate preabsorbed EIA plate. After a brief incubation, an anti-8-OHdG monoclonal antibody is added, followed by an HRP conjugated secondary antibody. The 8-OHdG content in unknown samples is determined by comparison with predetermined 8-OHdG standard curve.

Measurements of JNK and p38 MAPK activation

Activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun-NH₂-terminal kinase (JNK) in liver lysates were measured by TiterZyme EIA kit (Assay Designs Inc., Ann Arbor, MI), as described previously (Brown et al., 2009; Jia et al., 2009; Kovacheva et al., 2010).

Assessment of apoptosis

In situ detection of cells with DNA strand breaks was performed in paraformaldehyde-fixed, paraffin-embedded liver sections by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) technique (Sinha-Hikim et al., 2007, 2010a; Brown et al., 2009; Jia et al., 2009; Kovacheva et al., 2010; Braga et al., 2008) using an ApopTag-peroxidase kit (Chemicon International, Inc., San Francisco, CA). Enumeration of TUNEL-positive nuclei was carried out in liver sections using an American Optical Microscope with an X40 objective and a pair of X10 eyepieces. Methyl green was used as a counterstain to detect non apoptotic nuclei. A square grid fitted within one eyepiece provided a reference of 62,500 μm^2 . The rate of liver cell apoptosis was expressed as the percentage of the TUNEL-positive apoptotic nuclei per total nuclei (apoptotic plus non apoptotic) present within the reference area (Kovacheva et al., 2010; Sinha-Hikim et al., 2007; Braga et al., 2008).

Liver pathology

Liver pathology was evaluated using conventional histological analysis on hematoxylin and eosin stained sections. Further evaluation of pathology was achieved by high-resolution light microscopy using glutaraldehyde fixed, osmium tetroxide post-fixed, epoxy embedded, and toluidine blue stained sections (Deng et al., 2005) and electron microscopy. Accumulation of intracellular fat was quantified by computerized densitometry using the ImagePro Plus software coupled to an Olympus BHS microscope equipped with a VCC camera as described previously (Sinha-Hikim et al., 2010a; Kovacheva et al., 2010). For electron microscopic studies, thin sections from selected tissue blocks were sectioned with an LKB ultramicrotome, stained with uranyl acetate, and examined in a Hitachi 600 electron

microscope (Hitachi, Indianapolis). Micrographs were taken by an observer who was unaware of the treatment assignment. From each treatment group, 40 micrographs were selected for ultrastructural analysis.

Immunohistochemical and immunofluorescence analyses

Paraformaldehyde-fixed, paraffin-embedded liver sections were immunostained as described previously (Sinha-Hikim et al., 2007, 2010a; Brown et al., 2009; Jia et al., 2009; Kovacheva et al., 2010; Braga et al., 2008). Primary antibody included a mouse monoclonal 4-hydroxynonenal protein adducts (4-HNE) antibody (25 mg/ml; Oxis International Inc., Foster City, CA). Immunoreactivity was detected using biotinylated anti-mouse IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex, and visualized with diaminobenzidine tetrahydrochloride (DAB) as per the manufacturer's instructions (VECTASTAIN Elite ABC Mouse IgG kit, Burlingame, CA, USA). Slides were counterstained with hematoxylin. Negative control was run for every assay and was processed in an identical manner, except the primary antibody was substituted by the mouse IgG. Immunoreactivity was quantified by computerized densitometry using the ImagePro Plus software coupled to an Olympus BHS microscope equipped with a VCC camera as described previously (Sinha-Hikim et al., 2010a; Kovacheva et al., 2010).

For immunofluorescence staining, liver sections were incubated overnight in a humidified chamber at 4 °C with a mouse monoclonal 4-HNE antibody followed by goat-anti-mouse Texas Red-labeled secondary antibody for 45 min at room temperature, washed, and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, inc., Burlingame, CA). For controls, sections were treated only with secondary antibody, and no signals were detected. Sections were viewed using a Zeiss Axioscop 40 fluorescence microscope.

Activation of the executioner caspase 3 in hepatocytes undergoing apoptosis was detected by confocal microscopy using double immunostaining for the active caspase 3 (red) and DNA fragmentation (green) as previously described (Sinha Hikim et al., 2003; Vera et al., 2006). In situ detection of cells with DNA strand breaks was performed in paraformaldehyde fixed, paraffin-embedded liver sections using an ApopTag-fluorescein kit (Chemicon International). In brief, after deparaffinization and rehydration, tissue sections were incubated with proteinase K for 15 min at room temperature, washed in PBS, and then incubated with a mixture containing digoxigenin-conjugated nucleotide and TdT in a humidified chamber at 37 °C for 1 h and subsequently treated with antidigoxigenin-fluorescein for 30 min in the dark. After fluorescein staining, slides were washed in PBS and incubated with blocking serum for 20 min to reduce non-specific antibody binding. Slides were then incubated in a humidified chamber for overnight at 4 °C with a rabbit polyclonal active caspase 3 antibody (1:50; Cell Signaling Technology, Beverly, MA) followed by goat-anti-rabbit Texas Red-labeled secondary antibody for 45 min at room temperature, washed and mounted in Vectashield mounting medium. For controls, sections were treated only with secondary antibody, and no signals were detected. Confocal imaging was performed using a Leica TCS-SP-MP confocal microscope.

Subcellular fractionation and Western blotting

Nuclear and cytoplasmic fractions were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL). Western blotting was performed using liver lysates or subcellular fractions as described previously (Brown et al., 2009; Jia et al., 2009; Kovacheva et al., 2010; Sinha Hikim et al., 2003, 2007; Braga et al., 2008; Vera et al., 2006). In brief, proteins (50–80 µg) were separated on a 4–12% SDS-polyacrylamide gel with MES or MOPS buffer purchased from Invitrogen (Carlsbad, CA,

USA) at 200 V. Gel was transferred on an Immuno-blot PVDF Membrane (Bio-Rad, Hercules, CA) overnight at 4 °C. Membranes were blocked in blocking solution (0.3% Tween 20 in Tris-buffered saline and 10% nonfat dry milk) for 1 h at room temperature then probed using rabbit polyclonal phospho-adenosine-5-monophosphate (AMP)-activated protein kinase (1:400; AMPK), phospho-JNK (1:200), phospho-p38 MAPK (1:200), nuclear factor-erythroid-2-related factor 2 (Nrf2; 1:200), Kelch-like ECH-associated protein 1 (Keap1; 1:200), and BAX (1:200) from Santa Cruz Biotechnology Santa Cruz, CA); BCL-2 (1:200), cleaved caspase 9 (1:200), cleaved caspase 3 (1:200) from Cell Signaling, Technology; and fatty acid synthase (FAS; Upstate, New York, NY) for 1 h at room temperature or overnight at 4 °C with constant shaking. Following 3×10-min washes in TBS-T buffer, membranes were then incubated in anti-rabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For immuno-detection, membranes were washed three times in TBS-T wash buffer, incubated with ECL solutions per the manufacturer's specifications (Amersham Biosciences), and exposed to Hyper film ECL. The membranes were stripped and reprobed with a rabbit polyclonal GAPDH (1:2000), actin (1:2000), or histone H1 for normalization of the loading. Band intensities were determined using Quantity One software from Bio-Rad (Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed using the SigmaStat 2.0 Program (Jandel Cooperation, San Rafael, CA). Data are presented as mean±SE unless otherwise indicated. We used one-way ANOVA to compare group differences. If overall ANOVA revealed significant differences, post hoc (pairwise) comparisons were performed using Tukey's test. Differences were considered significant if $P < 0.05$.

Results

F1 prevents HFD-induced weight gain, hepatomegaly, increased serum triglyceride levels, and liver pathology

Compared with ApoE^{-/-} mice on ND with or without F1, ApoE^{-/-} mice fed with HFD exhibited significant weight gain as early as within 2 weeks of feeding (Fig. 1A). These mice when fed with HFD for 16 weeks became markedly obese (Fig. 1B) and showed hepatomegaly (Fig. 1B), and significant ($P < 0.05$) hypercholesterolemia and hypertriglyceridemia with no change in serum levels of albumin (Table 1). The adverse effects of HFD on body and liver weights, and serum triglyceride levels were fully prevented by dietary supplementation of F1 (Figs. 1A and B and Table 1). There were no differences in food intake among various experimental groups. In fact, mean daily food intake (g/day/mouse) was essentially similar between mice fed with HFD (3.61 ± 0.15) and HFD +F1 (3.57 ± 0.16).

We next examined the effects of dietary supplementation of F1 on mitigation of HFD-induced liver pathology. Hematoxylin and eosin stained liver sections from ApoE^{-/-} mice fed with ND with or without F1 had apparently normal morphology (Fig. 1C, upper panels). In contrast, ApoE^{-/-} mice fed with HFD displayed micro- and macro-vesicular steatosis (Fig. 1C, upper panels). These results were further substantiated by high resolution light microscopy, using glutaraldehyde fixed, osmium tetroxide post-fixed, epoxy embedded, and toluidine blue stained liver sections (Fig. 1C, lower panels). Glutaraldehyde fixation followed by osmium post-fixation allows retention of fat that would have been normally washed out during tissue processing. This also provides optimum preservation of intracellular lipids necessary for morphometric analysis. Dietary supplementation of F1 fully prevented such HFD-induced hepatic steatosis (Fig. 1C, upper and lower panels) in ApoE^{-/-}

– mice. Image analysis of toluidine blue stained liver sections confirmed the visual inspection and revealed a significant ($P<0.01$) increase in intracellular lipid content ($373\pm48\ \mu\text{m}^2$) in ApoE $^{-/-}$ mice fed with HFD compared with ApoE $^{-/-}$ mice fed with ND ($143\pm20\ \mu\text{m}^2$) or ND +F1 ($171\pm9\ \mu\text{m}^2$). Treatment with F1 attenuated such HFD-induced increase in intracellular fat accumulation to levels identical to that seen in ApoE $^{-/-}$ fed with ND ($148\pm18\ \mu\text{m}^2$ versus $143\pm20\ \mu\text{m}^2$).

We also performed electron microscopy to further substantiate our light microscopic observations. Hepatocytes from mice fed with ND exhibited normal ultrastructure, characterized by numerous mitochondria, well developed Golgi complexes, smooth- and rough-endoplasmic reticulum (ER) and glycogen deposition. In contrast, hepatocytes from mice fed with HFD revealed a striking increase in lipid deposition of varying sizes along with a marked decrease in the amount of glycogen, smooth and rough ER compared to those fed with ND with or without antioxidant (Fig. 1D). HFD-induced accumulation of lipid droplets was also noted in stellate cells (Fig. 1D; HFD-S). Such alterations in cellular ultrastructure were fully prevented by dietary supplementation of F1 (Fig. 1D).

Given that hepatocyte apoptosis is believed to play a pivotal role in the pathogenesis of NFLD (Trauner et al., 2010; Mantena et al., 2008), we next analyzed the incidence of hepatocyte apoptosis in ApoE $^{-/-}$ mice fed with HFD with or without antioxidant treatment (Fig. 1E). Minimal apoptosis was observed in mice fed with ND with or without the antioxidant. Apoptotic index in liver of mice fed with HFD was about 9% but was significantly ($P<0.05$) reduced in mice fed with HFD plus F1 (Fig. 1F).

HFD-induced oxidative stress in obese mice are suppressed by antioxidant treatment

To determine whether increased oxidative stress is a common feature of HFD-induced hepatic steatosis, we measured the GSH/GSSG ratio, which is inversely related to ROS levels, in liver samples of all experimental groups (Fig. 2A). ApoE $^{-/-}$ mice fed with HFD had greater oxidative stress, indicated by low GSH/GSSG ratio, relative to mice on ND. We also examined oxidative DNA damage in liver samples of various treatment groups. Levels of 8-OHdG were significantly ($P<0.05$) increased in liver from mice on HFD compared to that of mice fed with ND (Fig. 2B). No significant changes in the GSH/GSSG ratio or in the levels of 8-OHdG were noted between mice fed with ND and ND + antioxidant (Figs. 2A and B). Treatment with F1 significantly ($P<0.05$) prevented this HFD-induced increase in oxidative stress.

We next compared the in vivo expression of a lipid peroxidation product 4-HNE, a biomarker of oxidative stress (Kohen and Nysks, 2002; Tam et al., 2003) in liver sections. As shown in Fig. 2C, we found a marked increase in 4-HNE expression in mice fed with HFD relative to mice on ND. The increase in 4-HNE expression in mice fed with HFD was prevented by antioxidant treatment. This was further corroborated by image analysis of changes in the staining intensity (Fig. 2D).

Dietary supplementation of F1 activates Nrf2 but suppresses lipogenesis through inactivation of AMPK

Nrf2, a redox sensitive transcription factor (Motohashi and Yamamoto, 2004; Nguyen et al., 2009), which plays an important role in the pathogenesis of NAFLD (Chowdhry et al., 2010; Zhang et al., 2010). We used immunoblot analysis to examine the potential role of the Nrf2-Keap1 signaling pathway in F1-mediated protection of HFD-induced hepatic steatosis in liver samples (Figs. 3A–C) We found greater nuclear accumulation of Nrf2 in mice fed with HFD compared to mice fed on ND (Fig. 3A). A further increase in nuclear abundance of Nrf2 was detected after combined treatment with F1 and HFD (Fig. 3A). In contrast,

compared with mice on ND with or without F1, mice fed with HFD had elevated cytosolic Keap1 (Fig. 3B). Dietary supplementation of F1 prevented HFD-induced cytosolic accumulation of Keap1 (Fig. 3B). These findings were further corroborated by densitometric analysis (Fig. 3C).

AMPK, a central regulator of cellular energy homeostasis, plays an important role in fatty acid metabolism through its ability to regulate key fatty acid biosynthetic pathway (Postic and Girard, 2008; Zhang et al., 2009) and can also be modulated by oxidative stress (Zou et al., 2002; An et al., 2007). Thus to investigate whether the HFD-induced hepatic steatosis and obesity is associated with inactivation of APMK, we measured the levels of phospho-AMPK in liver by immunoblotting. Compared with mice on ND with or without F1, mice fed with HFD had decreased levels of phospho-AMPK and that could be partially but significantly ($P<0.05$) restored by dietary supplementation of F1 (Fig. 3D). Given that AMPK can also modulate FAS, which catalyzes the last step in the fatty acid biosynthetic pathway and is believed to be a determinant of the maximal capacity of liver to synthesize fatty acid by de novo lipogenesis (Postic and Girard, 2008; Zhang et al., 2009), we next measured the levels of FAS in liver by immunoblotting. As expected, FAS levels were increased in ApoE^{-/-} mice fed with HFD but were effectively suppressed by dietary supplementation of F1 (Fig. 3D).

F1 prevents activation of JNK and p38 MAPK, perturbation of BAX/BCL-2 ratio, and caspase activation in livers in mice fed with HFD

Since oxidative stress can promote activation of both p38 MAPK and JNK, which through mitochondria-dependent intrinsic pathway signaling promotes apoptosis in various cell types (Franco and Cidlowski, 2009), we examined the contribution of these kinases in HFD-induced hepatic steatosis and its intervention by dietary supplementation of an antioxidant. EIA revealed a significant ($P<0.05$) increase in both phospho-JNK and phospho-p38 MAPK levels in livers from mice fed with HFD compared with that of mice on ND (Fig. 4A). Treatment with F1 significantly ($P<0.05$) prevented HFD-induced activation of these stress kinases (Fig. 4A). Kinase activation was further ascertained by Western blotting. As shown in Fig. 4B, immunoblot analysis revealed increased levels of both phospho-JNK and phospho-p38 MAPK in livers from mice fed with HFD compared with that of mice on ND. The increased levels of both phospho-JNK and phospho-p38 MAPK in livers of mice fed with HFD were markedly suppressed by antioxidant therapy.

Because the ratio of anti-apoptotic and pro-apoptotic BCL-2 family members such as BCL-2/BAX constitutes a rheostat that sets the thresholds for susceptibility to apoptosis in the intrinsic pathway signaling (Danial and Korsmeyer, 2004), we examined the expression of profiles of BAX and BCL-2 by immunoblotting. We found an increase in BAX and a decrease in BCL-2 in mice fed with HFD compared with that of mice on ND (Fig. 4B). Densitometric analysis further revealed a significant ($P<0.05$) increase in BAX by 1.9-fold and a decrease in BCL-2 levels by 33.7% in liver lysates from mice on HFD compared to that of mice fed with ND. Dietary supplementation of F1 significantly ($P<0.05$) suppressed HFD-induced up-regulation of BAX (Fig. 4B).

Perturbation of the BAX/BCL-2 ratio in mice fed with HFD was further associated with activation of both the initiator caspase 9 and the executioner caspase 3, as evidenced by immunoblotting (Fig. 4B). We also found activation of caspase 3 in hepatocytes undergoing apoptosis, as evidenced by double immunofluorescence staining of active caspase 3 and TUNEL (Fig. 4C). Dietary supplementation of F1 effectively suppressed HFD-induced activation of these caspases (Fig. 4C).

Discussion

ApoE^{-/-} mice have been used as a model for studying diet-induced hepatic steatosis, since these mice, even when fed with Western diet, develop hepatic steatosis (Guillen et al., 2009; Ferre et al., 2009; Lohmann et al., 2009; Martinez-Clemente et al., 2010). In this study, using ApoE^{-/-} mice, we elucidated the molecular mechanisms by which a cystine based GSH precursor with added selenomethione (F1) prevents HFD-induced oxidative stress and hepatic steatosis in obese mice. We showed that ApoE^{-/-} mice, when fed with Western diet consisting of 21% fat for 16 weeks, become obese and exhibit hepatomegaly, have increased serum cholesterol and triglyceride levels, augmented lipogenesis, enhanced generation of oxidative stress in the liver, and micro- and macro-vesicular steatosis. We previously demonstrated that in human VSMC, F1 prevents spermine-induced oxidative stress, impaired glucose metabolism, and apoptosis through suppression of JNK-mediated mitochondria-dependent intrinsic pathway signaling (Sinha-Hikim et al., 2010a, 2010b). The results of the present study confirm and extend our in vitro data by demonstrating that F1 fully prevented the adverse effects of HFD on serum triglyceride levels, body and liver weights, and hepatic steatosis and markedly reduced HFD-induced increase in lipogenesis, oxidative stress, kinase activation, apoptotic signaling, and hepatocyte ultrastructural abnormalities.

Induction of high levels of ROS subjects the cells to a state of oxidative stress, which is believed to play a pivotal role in pathogenesis of NAFLD (Trauner et al., 2010; Mantena et al., 2008; Kojima et al., 2007). Consistent with a role for oxidative stress, in the present study, we found ApoE^{-/-} mice fed with HFD had greater oxidative stress, as evidenced by a low GSH/GSSG ratio. Dietary supplementation of F1 significantly prevented the HFD-induced increase in oxidative stress. Thus, it is likely that increased ROS generation could contribute to HFD-induced liver lesions through the formation of a reactive and biologically active lipid peroxidation product such as 4-HNE and oxidative DNA damage (Trauner et al., 2010; Mantena et al., 2008; Kojima et al., 2007; Serviddio et al., 2010). The observed increase in levels of 4-HNE and 8-OhdG in livers of mice fed with HFD is consistent with this view. Dietary supplementation of F1 is effective in mitigating HFD-induced liver damage caused by oxidative stress and lipid peroxidation.

F1 is further capable of activation of Nrf2 and preventing HFD-induced inhibition of AMPK and in the expression of lipogenic enzymes such as FAS. Nrf2 serves as master regulator of a cellular defense system against oxidative stress (Motohashi and Yamamoto, 2004; Nguyen et al., 2009). Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Keap1, which facilitates its ubiquitination and proteasomic degradation. Upon exposure to oxidative stress, the sequestration complex breaks down and the dissociated Nrf2 translocates into the nucleus, where it binds to cis-acting antioxidant response elements (AREs) and promotes the transcription of a large number of cytoprotective genes (Kensler et al., 2007; de Vries et al., 2008). Our Western blot data clearly show that HFD-induced oxidative stress is associated with activation of Nrf2, as evidenced by a significant ($P < 0.05$) elevation of Nrf2 in the nuclear fractions. Dietary supplementation of F1 markedly prevented HFD-induced cytosolic accumulation of Keap1 and promoted further translocation of Nrf2 into the nucleus. Thus, F1 could protect HFD-induced oxidative stress mediated hepatic steatosis through the Nrf2-Keap1 signaling pathway. This concept is supported by another line of evidence showing that genetic ablation of Nrf2 markedly exacerbates nonalcoholic steatohepatitis (Chowdhry et al., 2010). Conversely, enhanced expression of Nrf2 in mice bearing a hepatocyte-specific knockdown of Keap1 attenuates the fatty liver induced by a methionine- and choline-deficient diet (Zhang et al., 2010).

We are also intrigued by the observations that dietary supplementation of F1 is capable of preventing HFD-induced hypertriglyceridemia, inactivation of AMPK and increased expression of lipogenic enzymes such as FAS. These results are consistent with earlier reports linking inhibition of AMPK with NAFLD. AMPK is a central regulator of lipid homeostasis and mediates suppression of lipogenic gene expression such as acetyl-CoA-carboxylase (ACC) and FAS through inhibition of sterol regulatory element binding protein-1C (SREB1-c) (Zhang et al., 2009). For example, sustained activation of AMPK inhibits transcriptional stimulation of ACC and FAS induced by insulin or glucose in primary hepatocytes (Ferre et al., 2003). Mice fed with high sucrose diet develop hepatic steatosis in association with inhibition of AMPK coupled with increased lipogenesis and this can be attenuated by betaine, a naturally occurring metabolite of choline and an essential biochemical component of the methionine-homocysteine cycle, treatment (Song et al., 2007). The increase in activated AMPK with F1 supplementation was further associated with a decrease in FAS levels, which catalyzes the last step in the fatty acid biosynthetic pathway and is believed to be a determinant of the maximal capacity of liver to synthesize fatty acid by de novo lipogenesis (Postic and Girard, 2008; Zhang et al., 2009). Of further interest, there have been both in vitro as well as in vivo studies indicating that oxidative stress can lead to triglyceride accumulation in hepatocytes through upregulation of lipogenic transcription factors and enzymes involved in fatty acid synthesis, and that can, be effectively prevented by antioxidants such as NAC (Sekiya et al., 2008; Bettzieche et al., 2008; Lin et al., 2004). Collectively, these data indicate that F1 may reduce lipid accumulation in liver by directly reducing the oxidative stress coupled with activation of AMPK, and, in turn, reducing de novo lipid synthesis.

Oxidative stress has been implicated in apoptotic signaling in various cell types, including hepatocytes (Trauner et al., 2010; Mantena et al., 2008; Serviddio et al., 2010). One possible mechanism by which oxidative stress can induce hepatocyte apoptosis in response to HFD is through stimulation of p38 MAPK and JNK signaling, resulting in the activation of the mitochondria-dependent intrinsic pathway signaling (Franco and Cidlowski, 2009). Indeed, in the present study, we found activation of both of these kinases in livers of mice fed with HFD compared to that of mice on ND. It is pertinent to note here that JNK is activated in various animal models of obesity and also in patients with NASH and its deletion results in attenuation of fatty liver (Trauner et al., 2010). Thus, the observed activation of JNK in liver in HFD-induced obese mice is consistent with its pivotal role in NASH. Data reported herein also provide preliminary evidence of involvement of p38 MAPK in HFD-induced hepatic steatosis. Activation of JNK and p38 MAPK is further associated with stimulation of the mitochondria-dependent apoptotic pathway characterized by the perturbation of the BAX/BCL-2 rheostat and activation of the initiator caspase 9 and the executioner caspase 3. Most importantly, we further show that dietary supplementation of F1 significantly prevented such HFD-induced hepatocyte apoptosis through suppression of JNK and p38 MAPK-mediated mitochondria-dependent pathway. This is consistent with our previous in vitro studies demonstrating that F1-mediated protection of spermine-induced VSMC apoptosis involved suppression of JNK-mediated mitochondria-dependent apoptotic pathway (Sinha-Hikim et al., 2010a). However, it remains possible that death receptor pathway such as Fas-mediated extrinsic pathway signaling may also be involved in HFD-induced hepatocyte apoptosis.

The reason for less weight gain in mice fed with HFD plus F1 is unclear but given similar dietary intake, could be due to two broad categories, increased physical activity and/or increased basal metabolic rate. We are unable to address the issue of differences in activity as we did not collect data on the physical activity/inactivity of the groups inside their cages. We found AMPK, a central regulator of cellular energy homeostasis was inactivated in mice fed with HFD but was activated by dietary supplementation with F1. Oxidative stress can

also affect metabolism through pathways such as the induction of unfolded protein response signaling pathways that could lead to obesity, metabolic dysfunction, and fatty liver (Oyadomari et al., 2008). Activating transcription factor 4 (ATF4) is an important regulator for evolutionarily conserved mammalian stress response pathways that regulate thermogenesis and energy expenditure. ATF4-null mice are lean, have increased energy expenditure, and resist age-related and diet-induced obesity (Seo et al., 2009). They also resist HFD-induced hepatic steatosis (Seo et al., 2009). Thus, the possibility that F1 could confer resistance to HFD-induced obesity and hepatic steatosis through down regulation of ATF4 cannot be excluded.

The F1 formulation (per 500 mg) contains L-cystine—99.68 mg, glycine—199.39 mg, selenomethionine—1.54 mg, and L-glutamine—199.39 mg. At present we do not have comparable data tested with other oxidants. However, in a recent study, we have shown that, compared to F1, F2 (N-acetylcysteine fortified with same amount of selenomethionine like F1) is less effective in ameliorating spermine-induced reduction in GSH levels and perturbation in cellular morphology (Sinha-Hikim et al., 2010b). Thus, it is conceivable that cystine replacing cysteine in F1 formulation may play an important role in F1-mediated protection of VSMC against spermine-induced injury. A priori, it seems paradoxical that cystine, the oxidized form of cysteine can lead to an enhanced redox state. This seeming paradox is unraveled in the revelation of the pleiotropic nature of cystine/cysteine with cystine, functioning as a cysteine carrier, whereby cysteine in the plasma undergoes auto-oxidation to a stable and relatively inert cystine. The “stable cysteine” now in its oxidized form as cystine, can be taken up from the extracellular space and decoupled intracellularly back to two cysteine, via substrate-specific enzymes oxidoreductase and thioltransferases, supplying the intracellular cysteine necessary for glutathione and protein biosynthesis (Zhu et al., 2008). In this context, it is worth noting that in an in vitro setting, we found F1 is even more effective than NAC in preventing spermine-induced VSMC apoptosis through suppression of JNK and nitric oxide-mediated intrinsic pathway signaling (Sinha-Hikim et al., 2010a). Thus, we speculate that cystine replacing cysteine in F1 formulation may play an important role in F1-mediated protection of HFD-induced fatty liver.

In summary, we have provided insights into the molecular mechanisms by which dietary supplementation of F1 ameliorates HFD-induced hepatic steatosis in ApoE^{-/-} mice and emphasizes the suitability of this model for investigating the mechanisms of diet-induced obesity and hepatic steatosis. A deeper understanding of the F1-mediated signaling cascades in preventing hepatic steatosis could also lay the foundation for designing new therapies involving this novel GSH precursor in attenuating diet-induced obesity as well as other oxidative stress-induced medical conditions such as premature cardiovascular disease and chronic kidney disease.

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Abbreviations

AMPK	adenosine-5-monophosphate (AMP)-activated protein kinase
APOE	apolipoprotein A
EIA	enzyme immunometric assay

4-HNE	4-hydroxy-nonenal protein adducts
HFD	high fat diet
JNK	c-Jun-NH ₂ -terminal kinase
Nrf2	nuclear factor-erythroid-2-related factor 2
ND	normal diet
NAFLD	nonalcoholic fatty liver disease
Keap1	kelch-like ECH-associated protein 1
p38 MAPK	p38 mitogen-activated protein kinase
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling

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Fig. 1.

Novel glutathione precursor (F1) prevents high fat diet (HFD)-induced weight gain, hepatomegaly, and liver pathology in ApoE^{-/-} mice. A: Effect of F1 on HFD-induced body weight gain. B: Representative gross appearance of livers in various treatment groups. The overall liver morphology of mice fed with HFD +F1 is essentially similar to that of mice fed with normal diet (ND). C (upper panels): Representative hematoxylin and eosin-stained liver sections from ApoE^{-/-} mice fed with ND with or without F1 exhibit normal histological appearance. In contrast, liver sections from ApoE^{-/-} mice fed with HFD show micro- and macro-vesicular steatosis. Dietary supplementation of F1 fully attenuates such HFD-induced hepatic steatosis in ApoE^{-/-} mice. Scale bar=50 μ m. C (lower panels): Representative light microscopic images of glutaraldehyde fixed, osmium tetroxide post-fixed, epoxy-embedded, and toluidine-blue stained liver sections from different treatment groups show varying degrees of intracellular fat deposition (shown as yellow) in hepatocytes of mice fed with HFD, which can be fully attenuated by dietary supplementation of F1. Scale bar=25 μ m. D: typical ultrastructural appearance of liver cells from ApoE^{-/-} mice fed with ND, HFD, or HFD+F1. Portion of a hepatocyte from a mouse fed with ND exhibit normal ultrastructure, characterized by numerous mitochondria, well developed Golgi complexes, smooth- and rough-ER and glycogen deposition. A representative hepatocyte from an ApoE^{-/-} mouse fed with HFD shows a striking increase in lipid accumulation of varying sizes along with a marked decrease in the amount of glycogen as well as smooth and rough ER. HFD-induced accumulation of lipid droplets is also noted in stellate cells (D, HFD-S). The overall morphology of the majority of hepatocytes after F1 supplementation is essentially similar to that of mice on ND. E: In situ detection of hepatocyte apoptosis detected by TUNEL assay. Compared to mice fed with ND with or without F1, in which no apoptosis is detected, a distinct increase in hepatocyte apoptosis (arrow) is evident in mice fed with HFD and which can be fully prevented by F1 treatment. Scale bar=25 μ m. F: Quantitation of hepatocyte apoptosis in various treatment groups reveals that dietary supplementation of F1 significantly ($P < 0.05$) prevents such HFD-induced liver cell apoptosis. Values are means $\pm$ SEM of six animals per group. Means with unlike superscripts are significantly different.

Fig. 2.

Dietary supplementation of F1 suppresses HFD-induced increase in oxidative stress in liver. A and B: ApoE^{-/-} mice fed with HFD exhibit greater oxidative stress, as evidenced by low GSH/GSSG ratio (A) and higher levels of 8-OHdG, compared to mice on ND with or without F1. F1 treatment fully normalizes such HFD-induced increase in oxidative stress. Values are means $\pm$ SEM of six animals per group. Means with unlike superscripts are significantly different. C: Immunohistochemical analysis of 4-HNE expression in various treatment groups. Compared with mice fed with ND, where little or no expression of 4-HNE is detected, a marked increase in 4-HNE immunoreactivity is noted in liver of mice fed with HFD. F1 treatment effectively prevents such HFD-induced increase in 4-HNE levels. DAPI was used as a counter stain. Scale bar=25 μ m. D: Computerized densitometric analysis shows significant ($P<0.05$) suppression of HFD-induced increase in 4-HNE immunoreactivity by dietary supplementation of F1. Values are means $\pm$ SEM of six animals per group. Means with unlike superscripts are significantly different.

Fig. 3.

F1 activates Nrf2 but suppresses lipogenesis through inactivation of AMPK. A: Western blot analysis shows greater nuclear accumulation of Nrf2 in mice fed with HFD compared to mice fed with ND. A further increase in nuclear abundance of Nrf2 can be detected after combined treatment with F1 and HFD. B: Compared with mice on ND with or without F1, mice fed with HFD have elevated cytosolic Keap1. F1 treatment prevents HFD-induced cytosolic accumulation of Keap1. The gels are representative of two animals in each group from one of three separate experiments. Histone H1 and actin in the immunoblot are shown as loading controls for nuclear and cytoplasmic fractions, respectively. C: Densitometric analysis shows a significant ($P < 0.05$) increase in nuclear abundance of Nrf2 and a significant reduction in cytoplasmic Keap1 in mice fed with HFD plus F1. Values are means $\pm$ SEM of six animals per group. Means with unlike superscripts are significantly different. D: Western blot analysis shows decreased levels of phospho-AMPK (inactivation) but increased levels of FAS in livers of mice fed with HFD when compared with that mice fed on ND or ND+F1. Dietary supplementation of F1 effectively suppresses such HFD-induced inactivation of AMPK and augmented lipogenesis. The gels are representative of two animals in each group from one of three separate experiments. GAPDH in the immunoblot is shown as a loading control.

Fig. 4.

F1 treatment prevents activation of JNK and p38 MAPK, perturbation of BAX/BCL-2 ratio, and caspase activation in livers in mice fed with HFD. A: EIA reveals significantly ($P < 0.05$) increased levels of p-JNK (open bar) and p-p38 MAPK (closed bar) in livers of mice fed with HFD when compared with that mice fed on ND or ND+F1 and that can be fully prevented by F1 treatment. Values are means $\pm$ SEM of six animals per group. Means with unlike superscripts are significantly different. B: Western blot analysis shows increased levels of phospho-JNK (p-JNK), phospho-p38 MAPK (p-p38 MAPK) in livers of mice fed with HFD when compared with that mice fed on ND or ND+F1. HFD-induced activation of JNK and p38 MAPK is further associated with an increase in BAX expression and a decrease in BCL-2 expression and activation of caspase 9 and caspase 3 as detected by immunoblotting. Dietary supplementation of F1 effectively suppresses such HFD-induced changes in kinase activation, the BAX/BCL-2 ratio and activation of caspases. The gels are representative of two animals in each group from one of three separate experiments. GAPDH in the immunoblot is shown as a loading control. C: Activation of caspase 3 in hepatocytes undergoing apoptosis. Confocal images of liver cells show TUNEL (green), active caspase 3 (red) and colocalization of TUNEL and active caspase 3 (yellow) in HFD-induced apoptosis. Scale bar=25 μ m.

Table 1

Liver weights and serum parameters.

Parameter	ND	ND+F1	HFD	HFD+F1
Liver weight (g)	1.24±0.9 ^a	1.58±0.7 ^a	2.64±0.3 ^b	1.81±0.3 ^c
Cholesterol (mg/dL)	610±35 ^a	719±93 ^a	1194±210 ^b	1076±71 ^b
Triglyceride (mg/dL)	83.9±12.3 ^a	93.7±271 ^a	158.1±38.9 ^b	64.8±10.0 ^a
Albumin (g/dL)	3.8±0.3 ^a	4.3±0.5 ^a	3.3±0.2 ^a	3.4±0.3 ^a

Values are given as mean±SD (N=6). Means with superscripts 'a' are significantly (P<0.05) different from means with superscripts 'b' or 'c'.