DISSERTATION

NOVEL THERAPIES FOR NAFLD

Submitted by

Angela Marie Nivala

Department of Food Science and Human Nutrition

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2011

Doctoral Committee:

Advisor: Michael Pagliassotti

Jorge Vivanco
Melinda Frye
Jairam Vanamala

ABSTRACT

NOVEL THERAPIES FOR NAFLD

BACKGROUND/AIMS

Non Alcoholic Fatty Liver Disease (NAFLD) is a chronic liver disease often associated with metabolic disorders like type 2 diabetes, cardiovascular disease, obesity, and metabolic syndrome. It is characterized by hepatic fat accumulation (steatosis) that is at or above 5% of liver weight in the absence of excessive alcohol consumption (< 20 g/day). Current treatment for NAFLD focuses on reducing body weight and improving insulin action. The intent of this thesis was to identify therapies that targeted the liver. In the first aim, we examined whether secretions from plant roots contained compounds that restricted lipid accumulation or improved insulin action. The second aim examined whether taurine could prevent characteristic features of disease progression. Specifically, we hypothesized that (1) onion root exudates will prevent or reduce lipid accumulation and improve insulin signaling and (2) taurine will prevent or reduce endoplasmic reticulum (ER) stress, oxidative stress and liver injury.

METHODS

To examine these hypotheses, liver cells and dietary models of NAFLD were employed. Analyses focused on hepatic triglycerides, insulin signaling, ER stress, oxidative stress and inflammation using basic biochemical methods such as western blotting, Real Time PCR, immunohistochemistry and enzyme-linked assays.

RESULTS

Onion root exudates prevented fatty acid-mediated lipid accumulation and enhanced insulin signaling in H4IIE liver cells. Onion root exudates reduced plasma glucose, free fatty acids (FFA), and improved insulin action in rats fed a high fat diet.

Taurine mitigated palmitate-mediated caspase-3 activity, cell death, ER stress, and oxidative stress in H4IIE liver cells and primary hepatocytes. In rats fed a high sucrose diet, taurine supplementation significantly reduced hepatic lipid accumulation, liver injury, inflammation, plasma triglycerides and insulin levels. The high sucrose diet resulted in an induction of multiple components of the unfolded protein response (UPR) in the liver consistent with ER stress which was ameliorated by taurine supplementation. Treatment of mice with the ER stress inducing agent tunicamycin resulted in liver injury, UPR induction and hepatic lipid accumulation, and this was significantly ameliorated by supplementation with taurine.

CONCLUSION

Both onion root exudates and taurine reduced metabolic abnormalities associated with NAFLD. Onion root exudates appear to exert this effect through reduced

lipid accumulation and enhanced insulin sensitivity in the liver, while taurine reduced hepatic steatosis, ER stress, oxidative stress and liver injury. Overall, onion root exudates and taurine show promise as novel therapies for the treatment of NAFLD.

ACKNOWLEDGEMENTS

The author would like to thank her advisor, Dr. Michael Pagliassotti for all of the support, generosity, encouragement, patience, and understanding throughout this doctoral process. Thank you for providing such a great and nurturing environment that has not only given me confidence as a scientist, but also hope that a career in science is something worth striving for. "Leaving something better than how you found it" can apply to people as well as things; my confidence, skills, and overall outlook on life has changed profoundly for the better since becoming a part of your lab. I don't have the words to describe how grateful I am for this opportunity. Thank you to my committee members Dr. Jorge Vivanco, Dr. Melinda Frye, and Dr. Jairam Vanamala for taking time to participate and add valuable input into my development as a scientist. Your support throughout this process is greatly appreciated. Thank you Dr. Yuren Wei and Dr. Dong Wang for all that you do in the lab, especially in your contributions to the onion exudates and taurine projects; this thesis would not exist if not for your amazing abilities and hard work. Thank you Dr. Chris Gentile for all of your contributions, especially in the taurine chapter. Your advice has been invaluable. Thank you Vivanco Lab, Jennifer Kemp, Dr. Michael Bizeau, Kale Flory, and Jessica Stover for your contributions to the onion exudates project. Thank you McLean Lab, Peterson Lab, Dr. Kyle Pfaffenbach, and Jon Gonzalez for their contributions to the taurine project. Thank

you Rebecca Cox and Lauren Reese for your help in the lab. Thank you to my husband Peter for your mad formatting skills and all that you do, you are loved and appreciated more than you know. Thank you to my daughter Lily, for not only reminding me to make time for the important things in life but to never give up on them either.

TABLE OF CONTENTS

CHAPTERS

CHAPTER 1

INTRODUCTION

Non Alcoholic Fatty Liver Disease (NAFLD) is an emerging disease associated with metabolic dysfunction. NAFLD ranges from simple steatosis, or steatosis accompanied by inflammation and fibrosis (steatohepatitis or NASH) that can further develop into cirrhosis or hepatocellular carcinoma (HCC). The progression of simple steatosis to NASH is thought to occur via the "two-hit hypothesis";[1] hepatic fat accumulation acts as a predisposing factor (1st hit) to further insults (2nd hits) such as oxidative stress, cytokines, bacterial endotoxin, or endoplasmic reticulum (ER) stress. In addition, fat accumulation in the liver is associated with insulin resistant states, which is a common feature among individuals with obesity, type 2 diabetes, and/or metabolic syndrome.[2-10] In fact, NAFLD has been estimated to affect up to 30% of the population. There is a substantial positive correlation between the rising obesity trend and NAFLD, which has also been observed in obese children.[2,11-15] There is no exclusive treatment for NAFLD, and as a result it is often treated with therapies intended for obesity and diabetes.[16] These treatments include weight loss, insulin sensitizers, lipid-lowering drugs, and/or antioxidants, which have a number of side effects and are not designed to treat specific characteristics of NAFLD such as hepatic steatosis, liver insulin resistance or the later

stage features that include inflammation and fibrosis.[16] Our intent is to identify therapies that can decrease steatosis, improve insulin action and/or ameliorate oxidative stress and ER stress in the liver.

PLANT ROOT EXUDATION

Plant bioactive compounds can affect characteristics of NAFLD, in particular insulin resistance and inflammation. As most studies have focused on compounds that arise from the above ground portion of plants, that which rests below ground is largely unstudied in the context of potential therapeutic sources. Plant roots have the ability to secrete (exude) a variety of compound into their surrounding environment, known as the "rhizosphere". The composition of these "exudates" is dependent on temperature, age and species of the plant, biotic, and abiotic factors.[17] Plant roots exudates have a variety of purposes, from communication and/or symbiosis with surrounding plants and microbes, altering nutrients into forms able to be taken up by the plant, and allelopathy.[17-19] The potency of exudates is enough to alter gene regulation of plants and microbes living in the surrounding rhizosphere, in some cases altering pathways involved in carbohydrate and lipid metabolism.[20] As these pathways are potentially dysfunctional in NAFLD, the bioactive potential of plant root exudates is promising. Preliminary studies done by our lab suggest onion root exudates contain bioactive compounds that can prevent lipid accumulation and enhance insulin signaling in vitro, thus warranting further investigation into their role as potential therapies for NAFLD.

TAURINE

Taurine is a small amino sulphonic acid which can be obtained through the diet or synthesized endogenously from methionine and cysteine. Taurine aids in numerous physiological processes, including bile salt formation, osmoregulation, central nervous system function, and calcium homeostasis. Taurine deficiency has been observed in some studies involving individuals with obesity, type 2 diabetes and cirrhosis. In the studies involving patients with obesity and type 2 diabetes, supplementation of taurine improved characteristics of these diseases, although the mechanisms by which they do so remain unclear. Since NAFLD is observed in up to 80% of the cases of obesity and type 2 diabetes, the improvements observed with taurine treatment might involve pathways that also play a role in the progression of liver disease in NAFLD. In models of NASH, a progressive stage of NAFLD, oxidative stress, ER stress, inflammation and liver injury are key characteristics observed along with hepatic steatosis and insulin resistance. Increasing evidence suggests that taurine may have beneficial effects on NAFLD.[21] Taurine supplementation prevents alcoholic fatty liver disease in experimental animals, and mice characterized by hetero- or homozygous knockout of the taurine transporter develop chronic liver disease characterized by fibrosis, inflammation and hepatocyte apoptosis.[22,23] Interestingly, the beneficial effects of taurine are often accompanied by reductions in ER stress, suggesting a link between the therapeutic properties of taurine and restoration of ER homeostasis.[24-26] The above studies give justification for further examination of taurine as a potential therapy for NAFLD.

BIBLIOGRAPHY

1. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology. 1998;114(4):842-5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9547102 [Accessed August 31, 2010].

2. Festi D, Colecchia A, Sacco T, et al. Hepatic steatosis in obese patients: clinical aspects and prognostic significance. *Obes Rev*. 2004;5(1):27-42. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14969505.

3. Bernard S, Touzet S, Personne I, et al. Association between microsomal triglyceride transfer protein gene polymorphism and the biological features of liver steatosis in patients with type II diabetes. *Diabetologia*. 2000;43(8):995-999. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10990076.

4. Petersen KF, Dufour S, Befroy D, et al. Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes*. 2005;54(3):603-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15734833.

5. Kursawe R, Eszlinger M, Narayan D, et al. Cellularity and Adipogenic Profile of the Abdominal Subcutaneous Adipose Tissue From Obese Adolescents: Association With Insulin Resistance and Hepatic Steatosis. *Diabetes*. 2010;59(9):2288-2296. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2927952&tool=pmcentrez&rendertype=abstract [Accessed August 31, 2010].

6. Fabbrini E, Mohammed BS, Magkos F, et al. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology*. 2008;134(2):424-31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2705923&tool=pmcentrez&rendertype=abstract [Accessed January 4, 2011].

7. Nakamuta M, Kohjima M, Higuchi N, et al. The significance of differences in fatty acid metabolism between obese and non-obese patients with non-alcoholic fatty liver disease. *International journal of molecular medicine*. 2008;22(5):663-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18949388 [Accessed October 9, 2010].

8. Bertola A, Bonnafous S, Anty R, et al. Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. *PloS one*. 2010;5(10):e13577. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2962651&tool=pmcentrez&rendertype=abstract [Accessed December 6, 2010].

9. Fabbrini E, Magkos F, Mohammed BS, et al. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(36):15430-5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2741268&tool=pmcentrez&rendertype=abstract.

10. Donnelly KL, Smith CI, Schwarzenberg SJ, et al. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*. 2005;115(5):1343-1351. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15864352.

11. Lin Y-C, Chang P-F, Hu F-C, et al. A Common Variant in the PNPLA3 Gene is a Risk Factor for Non-Alcoholic Fatty Liver Disease in Obese Taiwanese Children. *The Journal of pediatrics*. 2010. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21168155 [Accessed January 2, 2011].

12. Ehtisham S, Barrett TG. The emergence of type 2 diabetes in childhood. *Annals of clinical biochemistry*. 2004;41(Pt 1):10-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14713381 [Accessed December 21, 2010].

13. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology*. 2003;37(5):1202-1219. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12717402.

14. Angulo P. Nonalcoholic fatty liver disease. *New England Journal of Medicine*. 2002;346(16):1221. Available at: http://content.nejm.org/cgi/content/extract/346/16/1221 [Accessed November 1, 2010].

15. Zhu L, Baker SS, Liu W, et al. Lipid in the livers of adolescents with nonalcoholic steatohepatitis: combined effects of pathways on steatosis. *Metabolism: clinical and experimental*. 2010. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21075404 [Accessed December 29, 2010].

16. Tolman KG, Dalpiaz AS. Treatment of non-alcoholic fatty liver disease. *Therapeutics and clinical risk management*. 2007;3(6):1153-63. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2387293&tool=pmcentrez&rendertype=abstract.

17. Badri DV, Vivanco JM. Regulation and function of root exudates. *Plant, Cell & Environment*. 2009;32(6):666-681. Available at: http://blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2009.01926.x [Accessed September 23, 2010].

18. Steinkellner S, Lendzemo V, Langer I, et al. Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules (Basel, Switzerland)*. 2007;12(7):1290-306. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17909485.

19. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol*. 2006;57:233-266. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16669762.

20. Pava-Ripoll M, Angelini C, Fang W, et al. The rhizosphere-competent entomopathogen Metarhizium anisopliae expresses a specific subset of genes in plant root exudate. *Microbiology (Reading, England)*. 2011;157(Pt 1):47-55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20947574 [Accessed January 18, 2011].

21. Chen SW, Chen YX, Shi J, Lin Y, Xie WF. The restorative effect of taurine on experimental nonalcoholic steatohepatitis. *Dig Dis Sci*. 2006;51(12):2225-2234. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17080243.

22. Chen X, Sebastian BM, Tang H, et al. Taurine supplementation prevents ethanol-induced decrease in serum adiponectin and reduces hepatic steatosis in rats. *Hepatology*. 2009;49(5):1554-1562. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19296466.

23. Warskulat U, Borsch E, Reinehr R, et al. Chronic liver disease is triggered by taurine transporter knockout in the mouse. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20(3):574-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16421246 [Accessed December 11, 2010].

24. Zulli A, Lau E, Wijaya BP, et al. High dietary taurine reduces apoptosis and atherosclerosis in the left main coronary artery: association with reduced

CCAAT/enhancer binding protein homologous protein and total plasma homocysteine but not lipidemia. *Hypertension*. 2009;53(6):1017-1022. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19398656.

25. Nonaka H, Tsujino T, Watari Y, Emoto N, Yokoyama M. Taurine prevents the decrease in expression and secretion of extracellular superoxide dismutase induced by homocysteine: amelioration of homocysteine-induced endoplasmic reticulum stress by taurine. Circulation. 2001;104(10):1165-1170. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11535574.

26. Men X, Han S, Gao J, et al. Taurine protects against lung damage following limb ischemia reperfusion in the rat by attenuating endoplasmic reticulum stress-induced apoptosis. *Acta orthopaedica*. 2010;81(2):263-7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2895349&tool=pmcentrez&rendertype=abstract [Accessed November 1, 2010].

CHAPTER 2

LITERATURE REVIEW

NAFLD

Metabolic disorders related to excess nutrient intake have been on the rise in recent decades. Increased incidences of metabolic syndrome, obesity and type 2 diabetes have also lead to an increased awareness of the significance of comorbidities associated with these diseases, one of which is nonalcoholic fatty liver disease (NAFLD). NAFLD is a metabolic-related disorder characterized by fat infiltration of the liver in the absence of chronic alcohol consumption. Clinically, NAFLD encompasses a broad spectrum of hepatic derangements ranging from fat accumulation (steatosis) to severe inflammation and fibrosis (NASH, nonalcoholic steatohepatitis) that can lead to cirrhosis (Figure 1). Recent estimates suggest that up to 30% of the general population have some form of NAFLD and up to 75% of patients with obesity and diabetes mellitus have NAFLD. The increasing prevalence of NAFLD in children has made it a common pediatric disease, affecting 3-9% of all children and up to 50% of obese children.[1] Approximately 90% of patients with NAFLD have at least one characteristic of the metabolic syndrome, which is characterized by a patient having any three of the following: abdominal obesity (waist circumference in men greater or equal to 40 inches, in women greater or equal to

35 inches), triglyceride levels greater than 150 mg/dl, HDL concentrations less than 40 mg/dl in men and 50 mg/dl in women, blood pressure >130/>85 mmHg, and fasting glucose levels greater than 110 mg/dl.[2,3] More recent studies have indicated that NAFLD is associated with an increased risk of cardiovascular disease.[4]

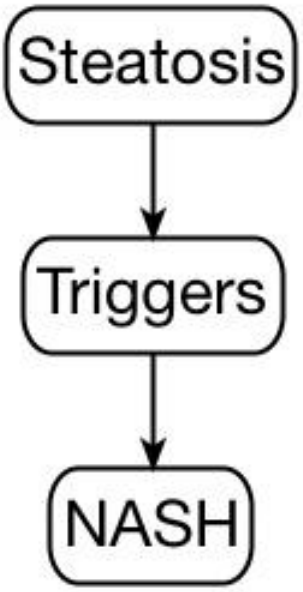


Figure 1. Progression of NAFLD. Fat accumulation (steatosis) occurs as a consequence of either increased import of triglyceride into hepatocytes or decreased export of triglyceride from hepatocytes. Steatosis predisposes the liver to susceptibility to a second "hit" trigger that can lead to inflammation, fibrosis, and hepatocellular injury characterized by NASH. If NASH further progresses, it can develop into cirrhosis of the liver.

ETIOLOGY OF NAFLD

The initial stage of NAFLD involves accumulation of triglycerides (TG) in the liver. Hepatic lipids are derived from three potential sources: the diet, de novo lipogenesis (DNL) and circulating fatty acids released from adipose tissue (Figure 2*).*[5] A study done by Donnelly et al. used stable isotopes to label and track serum

nonesterified fatty acids (NEFA), dietary fatty acids, and those derived from DNL in adult humans with NAFLD over a 4 day period.[6] NEFAs contributed 59.0%±9.9%, DNL contributed 26.1%±6.7% and diet contributed 14.9%±7.0% to the triacylglycerol present in the liver.[6] Studies performed using animal models generally support the role of diet, lipogenesis and circulating NEFA in the development of hepatic steatosis. For example, Larter et al. fed a high fat diet to mice genetically predisposed to obesity and diabetes and demonstrated that dietary lipid was partitioned to the liver, diabetes was induced and adiponectin secretion reduced.[7] These events preceded the development of steatohepatitis and fibrosis in these mice.[7] DNL involves the conversion of carbohydrate-derived acetyl coenzyme A to fatty acid. Once synthesized, these fatty acids can be incorporated into triglyceride and stored in or released by the liver. Normally in the fasted state, the contribution of DNL to the hepatic triglyceride pool is very small; however it appears to be elevated in insulin resistant states and in NAFLD.[8,9] Hepatic overexpression of diacylglycerol acyltransferase (DGAT2, the final committed step in triglyceride synthesis) promotes hepatic steatosis in mice, however this steatosis was insufficient to cause insulin resistance.[10] The implications of this study are twofold; first, upregulation of critical proteins involved in lipid synthesis may contribute to the development of hepatic steatosis, and second, hepatic steatosis can occur in the absence of insulin resistance, and thus may be an early event in NAFLD. Although inhibition of DGAT2 reduced hepatic steatosis in mice with diet-induced NASH, these mice were characterized by exacerbation of liver damage and fibrosis.[11] Finally, experimental suppression of circulating NEFAs improved insulin action and reduced

hepatic steatosis and liver damage in both human populations and animal models.[12] Thus, impairments in any of the three pathways that contribute to the input of lipid to the liver can contribute to the development of hepatic steatosis.

The liver can also dispose of lipids via oxidation or release as very low density lipoprotein (VLDL) triglyceride (Figure 2). Acetyl CoA carboxylase (ACC) is a lipogenic enzyme that catalyzes the synthesis of malonyl CoA, an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT1). CPT1 regulates the transfer of long chain acyl-CoAs from the cytosol into the mitochondria for oxidation. Savage et al. demonstrated that suppression of ACC1 and 2 reversed both diet-induced hepatic steatosis and hepatic insulin resistance in rats via mechanisms that involved enhanced hepatic fat oxidation in the fed state.[13] Minehira et al. demonstrated that inhibition of VLDL secretion, through hepatic deletion of microsomal triglyceride transfer protein (MTTP), resulted in hepatic steatosis in the absence of changes in peripheral lipid stores and insulin sensitivity in mice.[14] Therefore, imbalances in lipid input relative to output can also promote hepatic steatosis.

The accumulation of lipids, in particular lipids characterized by increased saturated fatty acids, can be toxic to cells, a condition termed lipotoxicity. Lipotoxicity is not a result of triglyceride accumulation itself, but rather the lipid-derived metabolites that can activate inflammatory and ROS generating pathways.[15] Saturated fatty acids such as palmitate are poorly incorporated into triglyceride and readily cause apoptosis

in human and rat hepatocytes.[16-18] Unsaturated fatty acids such as oleate exhibit a protective role against palmitate-induced apoptosis by promoting palmitate incorporation into triglyceride rather than into pro-apoptotic pathways.[19] In the event that triglyceride accumulation is impaired or exhausted, free fatty acids may no longer be safely incorporated into triglyceride pools and instead act to increase susceptibility to liver injury and second hit triggers leading to NASH development.

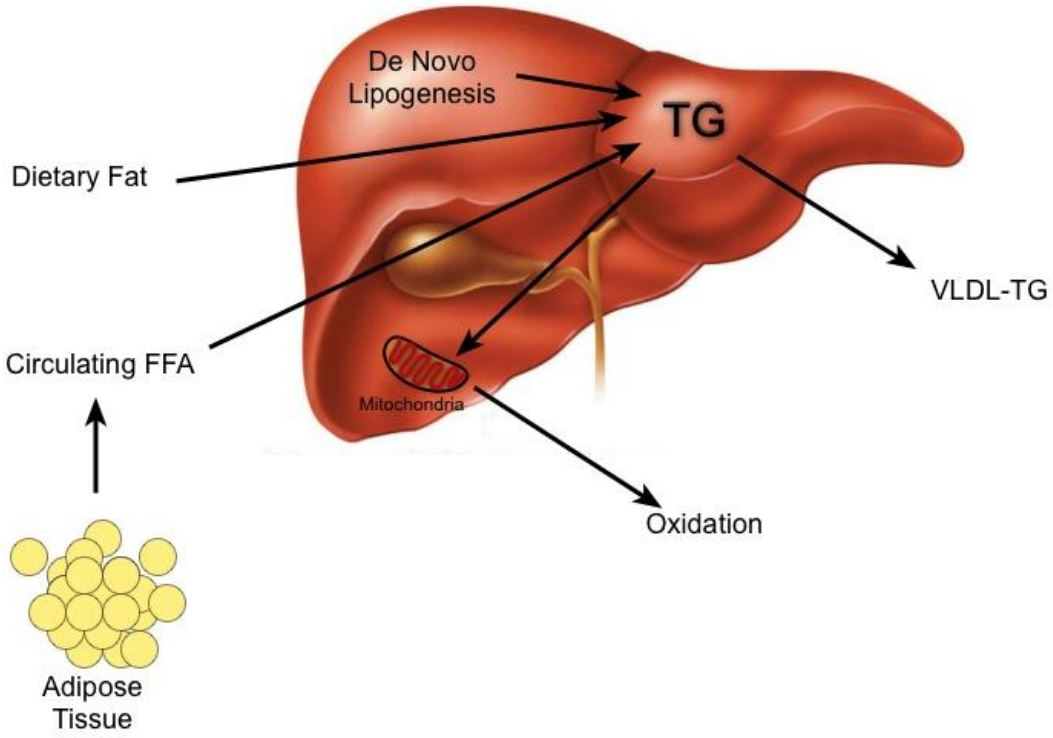


Figure 2. Input and output pathways of lipid to and from the liver. Lipids can enter the liver either through diet, FFA, or lipogenesis. Once inside the liver, lipids can be esterified with glycerol into TG, while lipogenesis involves the conversion of precursors like glucose to FFA, where they are esterified with glycerol to produce TG. Liver TG can be broken down to FFA and exported via mitochondrial oxidation, or packaged into VLDL for export to various other tissues. In a normal liver, this TG flux is in balance so the net amount of TG stored in the liver is very low, however in states of nutrient excess or dysfunction of one or more of these pathways, TG accumulation can occur leading to metabolic impairments.

CONSEQUENCES OF HEPATIC STEATOSIS

Lipid accumulation in insulin sensitive tissues has been linked to insulin resistance in both human and animal studies.[20-23] Thus one potential consequence of hepatic steatosis is the development of insulin resistance. Samuel et al., using a short term, high fat diet in rats, demonstrated that hepatic fat accumulation was followed by hepatic insulin resistance.[24] In this study, hepatic fat accumulation decreased insulin activation of glycogen synthase and increased gluconeogenesis. [24] These changes were associated with activation of proteins, protein kinase C-e and c-Jun terminal kinase, which interfere with tyrosine phosphorylation of IRS-1 and IRS-2 and therefore insulin signaling.[24] Several studies have demonstrated that hepatic steatosis is more closely linked to hepatic insulin resistance than factors such as whole body obesity, visceral obesity or circulating fatty acids in humans. [21,25-28]

Fat accumulation in the liver leads to a variety of consequences, namely systemic insulin resistance and impaired insulin extraction by the liver. Hwang et al. sought to elucidate what lipid depot (i.e. intramyocellular lipid (IMCL), intrahepatic triglyceride (IHTG), visceral fat (VF), and/or deep abdominal subcutateous fat (SF)) was correlated with insulin resistance.[25] Using 1H-magnetic resonance spectroscopy and magnetic resonance imaging, they were able to show significant inverse correlation between IHTG and whole body Rd.[25] This correlation suggests hepatic triglyceride accumulation can have systemic consequences involving impaired insulin sensitivity in non-hepatic tissues. Recent studies have emphasized the role of liver fat on hepatic insulin clearance. In non-

diabetic subjects, 50-70% of the insulin secreted by the pancreas is removed by the liver during first-pass transit.[29-36] In advanced liver disease, hepatic insulin clearance is reduced, which is considered to be a major cause of hyperinsulinemia in liver cirrhosis.[37-40] Recent work by Kotronen et al has demonstrated that increased liver fat is associated with impaired insulin clearance in nondiabetic human subjects.[29] Thus, it is possible that the link between liver fat and non-hepatic insulin resistance involved a sequence of events that include reduced hepatic insulin clearance, increased systemic insulin concentrations, and down regulation of insulin receptors and insulin signaling.

TRIGGERS INDUCING NASH DEVELOPMENT

NASH is characterized by steatosis and the induction of inflammation, fibrosis, and hepatocellular injury. It is interesting that not all of individuals with hepatic steatosis develop NASH. It has been estimated that 15-25% of cases of early stage NAFLD progress to NASH, and 15-20% of NASH cases further develop into cirrhosis.[41] As a result of this data, it has been suggested that steatosis increases susceptibility of the liver to various other triggers, and those individuals characterized by the appropriate combination of triggers will be at highest risk for development of NASH (Figure 3). This concept has experimental support. In a study by Zhang et al., mice were fed a high fat diet (60% daily caloric intake) for 8 months to induce steatosis, obesity, insulin resistance and dyslipidemia.[42] There was no evidence of significant liver damage or inflammation produced by the diet.[42] Hepatocytes were then isolated; steatotic hepatocytes had increased production of intracellular ROS and were more susceptible to TNF-a induced apoptosis than nonsteatotic hepatocytes.[42] Studies such as these imply

that a combination of steatosis and triggers such as increased cytokines may promote the development of NASH.

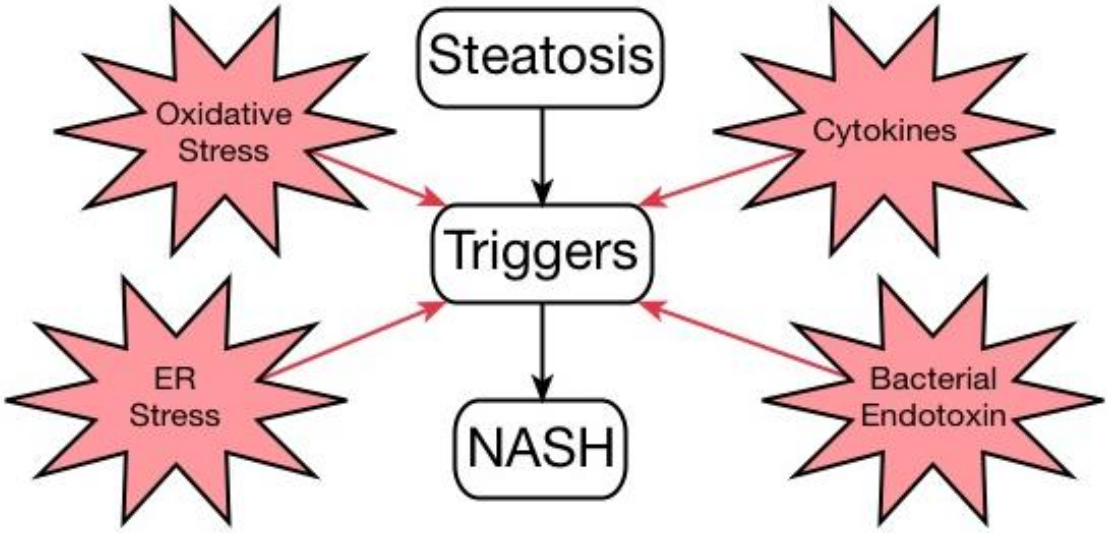


Figure 3. Types of triggers in the progression of NAFLD. Steatosis predisposes the liver to injury from 2nd hit triggers, which can include oxidative stress, cytokines, bacterial endotoxin, or ER stress.

Several so-called second "hit" triggers, in addition to cytokines, have been proposed including oxidative stress, bacterial endotoxin, and/or ER stress (Figure 3). While simple steatosis occurs primarily in hepatocytes, the development of inflammation and fibrosis (characteristic features of NASH) involve Kupffer cells and stellate cells, respectively (Figure 4). Thus, the development of NASH is much more complex than the development of steatosis and involves multiple triggers and cell types within the liver.

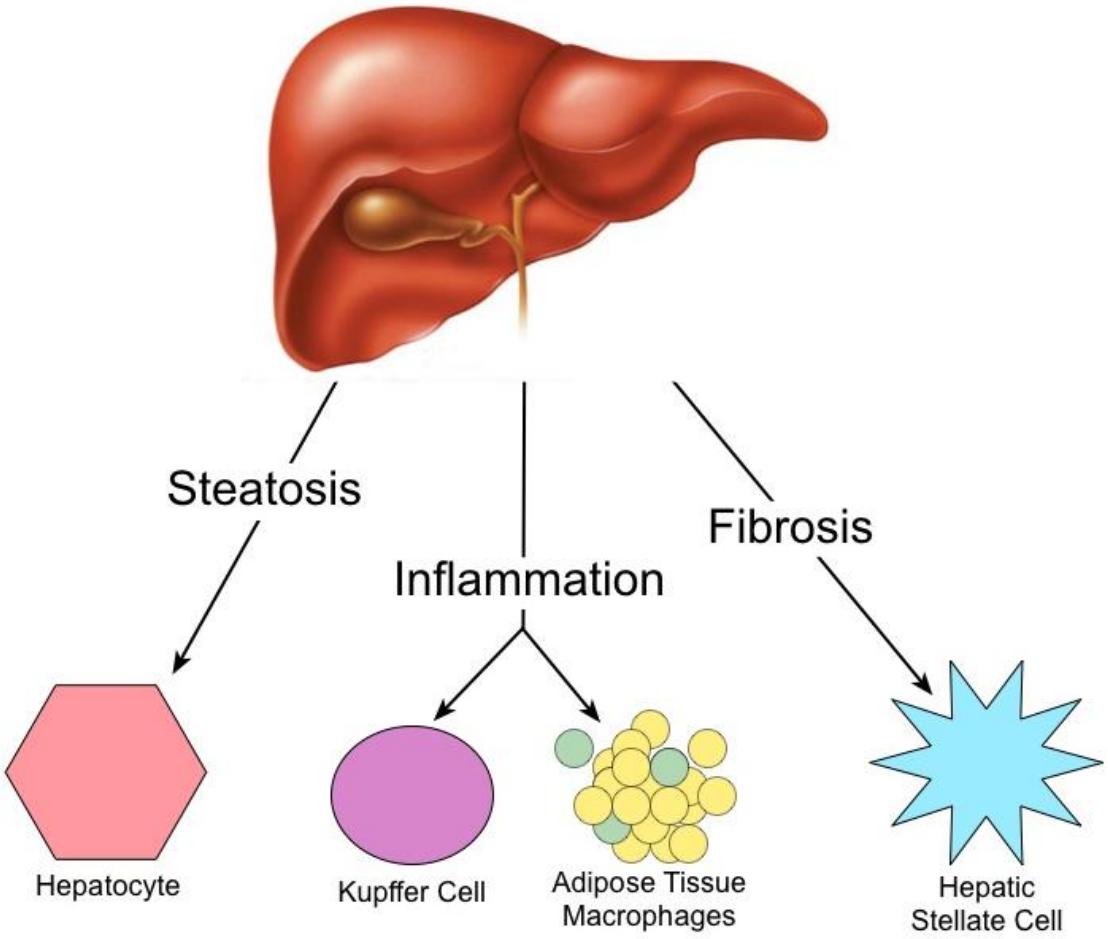


Figure 4. Liver cell types involved in the progression of NAFLD. Lipid accumulation occurs primarily in hepatocytes, while inflammation occurs as a result of cytokine release from kupffer cells and adipose tissue. Inflammation leads to hepatic stellate cell activation and the induction of fibrosis.

OXIDATIVE STRESS

Oxidative stress occurs when reactive oxygen species (ROS) are produced in excess of antioxidant defenses.[43-44] ROS is a collective term that includes both oxygen radicals such as singlet oxygen and the superoxide anion, and certain nonradicals that are oxidizing agents and/or are easily converted into radicals, such as hydrogen peroxide and peroxynitrite.[45] Unresolved oxidative stress can interfere with normal cell

metabolism and can cause damage leading to cell death. In the liver, oxidative stress can be generated through mechanisms involving mitochondrial dysfunction (leading to progressive impairment of b-oxidation, respiratory chain, and ATP synthesis), increased fatty acid oxidation in either peroxisomes (β-oxidation) by acyl-CoA oxidase or in the endoplasmic reticulum by CYP-2E1 and CYP4A isoforms or ER protein folding.[44,46]

MITOCHONDRIA

FFA oxidation in mitochondria involves electron generation through redox reactions of the cofactors NAD^+ and FAD to/from NADH and $FADH_2$.[47,48] These electrons are transferred to the electron transport chain where they generate an electrochemical potential required for oxidative phosphorylation and the generation of ATP.[47,48] Normally electrons travel through the electron transport chain to cytochrome C oxidase, where they combine with oxygen and protons to form water.[47,48] Sometimes, a fraction of these electrons "leak" from the electron transport chain and react directly with oxygen to form the ROS superoxide.[47,48] Manganese superoxide dismutase (Mn-SOD) converts superoxide into peroxide, which is further converted to water by mitochondrial glutathione peroxidase (which requires reduced glutathione to function).[49,50] A dysfunctional mitochondrial environment where either the endogenous antioxidant enzymes Mn-SOD or glutathione peroxidase are downregulated or lacking in substrate to function properly promotes ROS formation.[50-52] Ectopic expression of cytochrome P450 2E1 (CYP2E1) in mitochondria can also lead to ROS expression.[53-59]

Mitochondrial structure can also play a role in its dysfunction; in NASH patients, at least 40% of mitochondria are structurally abnormal.[60] These abnormalities (enlarged mitochondria, loss of mitochondrial cristae and paracrystalline inclusions) impair the electron transport chain enzyme activity and lead to uncoupling of oxidation from phosphorylation and production of ROS.[50,61,62] Enhanced mitochondrial ROS formation has been shown in obese mice and hepatocyte and rat models of obesity and NASH.[53,63,64] NASH patients have been reported to have enhanced hepatic levels of CYP2E1; studies in streptozotocin-induced diabetic rats showed increased levels and activity of mitochondrial CYP2E1 in the livers and other tissues, providing another potential source of ROS.[53]

PEROXISOMES/MICROSOMES

Fatty acid β-oxidation or ω-oxidation can also occur in peroxisomes and microsomes, respectively; however, hydrogen peroxide is generated without the coupling of oxidative phosphorylation.[65-68] The endogenous antioxidant enzyme of peroxisomes is catalase, which converts hydrogen peroxide to water. If the amount or activity of catalase is insufficient to reduce the hydrogen peroxide produced through β-oxidation, the hydrogen peroxide can react with cell components, particularly lipids, and causing cell damage and death.[65-68] As peroxisomal FFA β-oxidation is viewed as a protective response to fatty acid overload in the liver mediated by PPAR-α, recent studies have shown PPAR-α expression in NAFLD patients is reduced.[69-74] Gene expression studies in NAFLD patients show enhanced expression of DNL, fatty acid uptake, fatty acid oxidation (however, reduced PPAR-α) and antioxidant genes,

indicating antioxidant gene expression was adjusted to deal with the excess ROS generated from fatty acid oxidation.[72] A study done in sodium valproate induced steatotic rats showed that, in addition to structural and functional alterations in hepatic mitochondria, increased lipid peroxidation was evident in hepatic peroxisomes and microsomes when compared to nonsteatotic control rats.[75] These studies suggest there are impairments in fatty acid oxidation that generates ROS in NAFLD livers, making them more susceptible to lipotoxic conditions.

ENDOPLASMIC RETICULUM

The endoplasmic reticulum (ER) is an organelle responsible for many cell processes, including oxidative processes like protein folding that can generate ROS. Protein folding is an essential function of the ER mediated by protein disulphide isomerases (PDI), FAD dependent oxidases ERO1p, ERV2p and Fmo1p, with the final electron transfer being from ERO1p to 0_2 with peroxide and superoxide as minor electron acceptors from ERO1p.[76] Uncoupling of ERO1p, e.g. during ER stress (disruption of ER function leading to complex signaling cascades that attempt to ameliorate the stress), can lead to generation of ROS.[76]

The cytochrome P450 family of proteins are a diverse group of enzymes, most of which are involved in catalyzing the oxidation of organic substances such as lipids, steroids, and xenobiotics. Two cytochrome P450 enzymes, CYP2E1 and CYP4A, are found in the ER and are responsible for a variety of detoxification reactions. CYP2E1 catalyzes the ω-1 hydroxylation of long chain fatty acids, while CYP4A catalyzes the ω

and ω-1 hydroxylation of medium chain fatty acids (C_6-C_{12}).[77] CYP2E1 catalyzes the NADH-dependent reduction of oxygen leading to lipid peroxidation.[78,79] CYP2E1 expression has been shown to be increased in both rat dietary models of steatohepatitis as well as in the livers of patients with NASH.[78,80] In mice fed an methionine choline deficient diet (MCD diet), steatohepatitis was induced as well as CYP2E1 expression and catalysis of lipid peroxides by hepatic microsomes.[78] This study showed that CYP2E1 can act as an initiator of oxidative stress in steatotic livers, however when CYP2E1 knockout mice were fed a MCD diet, steatohepatitis and lipid peroxidation were still induced, suggesting there are other catalysts to lipid peroxidation involved in the progression of steatohepatitis.[78] One such alternative catalyst is CYP4A, which was discovered in vitro to play a role in lipid peroxidation in the absence of CYP2E1.[78] Thus targeting a specific enzyme involved in lipid peroxidation may be futile due to the redundant nature of microsomal enzyme expression in lipid store management under conditions of NAFLD.

Oxidative stress can induce other forms of stress that further propagate the progression of NAFLD. Hanada et al. used human hepatoma cells and hepatocytes to show that oxidative stress coupled with limited proteosome inhibition induced ER dysfunction and inclusion formation.[81] Inclusion formations (or Mallory bodies) in hepatocytes are significant markers of many liver diseases, including NASH. Prevention or alleviation of oxidative stress may prevent or reduce Mallory body formation and ER dysfunction in NAFLD.

CYTOKINES/ADIPOKINES

Cytokines are pleiotropic regulatory peptides that can be made by virtually all nucleated cells in the body.[82] Types of cytokines include interferons, interleukins (IL), tumor necrosis factors (TNF), colony stimulating factors (CSF), transforming growth factors (TGF), erythropoietin, and thymopoietin. When the immune system is activated, cytokines activate and signal immune cells such as T-cells and macrophages to travel to the site of infection. Activated immune cells also release cytokines, propagating the immune response signal. Cytokines can be either pro-inflammatory (i.e. tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6)) or anti-inflammatory (i.e. IL-1 receptor antagonist, interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13)). Pro-inflammatory cytokines can upregulate the synthesis of secondary mediators and pro-inflammatory cytokines by macrophages and mesenchymal cells, stimulate production of acute phase proteins, attract inflammatory cells, or act as endogenous pyrogens (elevate thermoregulatory setpoint of the hypothalamus). Anti-inflammatory cytokines counteract inflammation through direct inhibition of pro-inflammatory cytokines or through other means. In the context of liver injury, TNF-a production occurs early and triggers production of other cytokines that recruit inflammatory cells, kill hepatocytes, and initiate fibrogenesis.[83]

The liver plays a secretory role in the development of NASH in the context of cytokine release from Kupffer cells (resident hepatic macrophages).[84] TNF-a production occurs early and triggers production of other cytokines that recruit inflammatory cells,

kill hepatocytes, and initiate fibrogenesis. From a positional standpoint, both the liver and visceral adipose tissue share proximal associations between metabolic cells (hepatocytes and adipocytes, respectively) and secretory cells such as immune cells (NK and NKT cells), Kupffer cells, hepatic stellate cells, endothelial cells or macrophages, with each tissue having immediate access to an extensive network of blood vessels for continuous or dynamic immune and metabolic responses.[84,85] Data in the fatty livers of Zucker diabetic (fa/fa) rats and leptin deficient ob/ob mice showed abnormal basal cytokine production and/or after a Kupffer cell-activating LPS challenge, which resulted in the development of severe NASH.[86,87] The significance of these studies was that a cytokine (TNFα, released by many types of cells but predominately Kupffer cells in the context of LPS-induced lipotoxicity) was shown to be dysfunctionally released in conditions of hepatic steatosis and contributed to the severity of NASH. TNFα has consistently shown to be increased in patients with NASH, and correlates with NASH severity.[88,89]

A subcategory of cytokines specific (but not exclusive) to adipose tissue is termed adipokines. White adipose tissue serves three known functions, energy storage, hydrolysis of triglyceride to free fatty acids, and release of adipokines, since adipose tissue contains adipokine-producing cells such as macrophages, fibroblasts and infiltrating monocytes.[90] In the context of NAFLD, obesity contributes to a higher prevalence of cirrhosis, suggesting that adipose tissue is a significant factor in the progression of the disease.[91]

Visceral adipose tissue seems to play an important role in the development of NASH by secreting FFA, hormones and adipokines.[84] Visceral adipose tissue, like the liver, shares proximal associations between metabolic cells (adipocytes) and secretory cells such as immune cells (NK and NKT cells), endothelial cells or macrophages; interspersed networking of blood vessels allows for expedited immune and metabolic responses.[84,85] Different adipokines are expressed in subcutaneous versus visceral adipose tissue. Subcutaneous adipose tissue predominantly expresses leptin, adiponectin, retinol binding protein-4 (RBP-4), and acylation stimulating protein, which are all involved in metabolic control.[92] Visceral adipose tissue predominantly expresses inflammatory proteins, such as tumor necrosis factor-α (TNF-α), interleukin-6, interleukin-8, adipsin, and resistin; proteins involved in tissue repair such as plasminogen activator inhibitor-1 and angiotensinogen, and proteins involved in regulation of metabolism, such as visfatin.[84,93] Ectopic fat, such as visceral adipose or epicardial or mediastinal adipose tissue of the heart, is more closely linked to impairments in metabolic control and inflammation in obesity-related disorders.[94] In NAFLD, adiponectin is inversely correlated with hepatic fat content.[92,95-98] RBP-4 appears to be positively correlated with peripheral insulin resistance and hepatic fat content; however, since RBP-4 is produced by both the liver and adipose tissue, it is unclear whether changes in RBP-4 occur as a result of changes in liver and/or adipose tissue production.[90,92,99,100]

BACTERIAL ENDOTOXIN

Bacterial endotoxin, such as lipopolysaccharides (LPS) derived from gram-negative bacteria, are glycolipids that have the ability to induce an inflammatory response in infected organisms. The liver functions to restrict the entry of LPS from the GI tract into the systemic circulation. The activation of Kupffer cells in the liver plays a critical role in the hepatic clearance of LPS. Specifically, this activation is mediated through LPS binding protein (LBP), CD14, and Toll-like receptor-4 (TLR-4). LPS binds to LBP in the serum, which transfers LPS to the peripheral monocyte membrane bound CD14. In Kupffer cells, CD14 expression is relatively low normally but is rapidly upregulated upon exposure to agents such as LPS. TLR-4 is a downstream component of CD14 and required for Kupffer cell activation. Upon activation, Kupffer cells release cytokines such as TNF-a, IL-1, and reactive oxygen species (ROS).[101] This inflammatory response can induce liver injury, perhaps not via a single cytokine alone but rather through disruption of the balance between pro and anti-inflammatory factors.[102]

The composition of gut microflora can have a profound impact on caloric intake in both mice and humans.[103-105] There are two predominant populations of microbiota in both the mouse and human, Firmicutes and Bacteroidetes. In obese individuals, the proportion of Firmicutes is higher than that of lean individuals.[104,105] The significance of this observation lies in the ability of Firmicutes to encode enzymes that can break down otherwise indigestible dietary polysaccharides, thus increasing caloric absorption. When chronic, this enhanced caloric absorption may lead to increased body weight.[106]

Alterations in the composition of gut microflora can influence the integrity of the liver. In one study, Sprague-Dawley rats received either saline, probiotics, Escherichia coli, Salmonella enteritidis, or gentamicin orally for 7 days.[107] On the 8th day, acute liver damage was induced by intraperitoneal injection of D-galactosamine in all but the control saline group.[107] The probiotic, E. coli and gentamicin groups had attenuated liver damage, decreased bacterial translocation, and decreased levels of TNF-a, IL-6, IL-10, and IL-12.[107] Overall, the study showed that alterations in gut microflora acted through modifications of bacterial translocation, local gut cytokine expression, and endotoxin to prevent (e.g. probiotic, E. coli and gentimicin) or exacerbate (e.g. S. enteritidis) acute liver injury.[107] The composition of gut microbiota can also influence gut permeability.[108] In a study done by Cani et al., ob/ob mice were treated with a control diet or a control diet fortified with either prebiotic or nonprebiotic carbohydrates.[108] Prebiotic treatment involved incorporation of fermentable dietary fiber (oligofructose), whereas nonprebiotic treatment involved incorporation of nonfermentable dietary fiber (cellulose). Prebiotic treated mice exhibited lower circulating LPS, cytokines, and decreased hepatic expression of inflammatory and oxidative stress biomarkers when compare to nonprebiotic controls.[108]

ER Stress

The endoplamic reticulum (ER) is one of the largest cellular organelles and is responsible for a variety of functions, depending on the domain of ER involved (nuclear envelope, smooth ER, or rough ER).[109] Its functions include translocation of secretory proteins across the ER membrane, integration of proteins into the membrane, folding

and modification of proteins in the ER lumen, synthesis of phospholipids and steroids, detoxification, storage of calcium ions in the ER lumen and their release in the cytosol, and segregation of nuclear contents from the cytoplasm.[110,111] In the rough ER, protein assembly and folding homeostasis is monitored by a series of signaling pathways collectively known as the unfolded protein response (UPR, Figure 5). Stressors such as loss of the luminal oxidizing environment, disruption of calcium homeostasis or impaired N-glycosylation can disrupt protein folding and lead to protein aggregate accumulation in the ER lumen, termed ER stress. The UPR attempts to alleviate the protein folding burden on the ER through activation of pathways that can decrease general translation and increase proteins and enzymes involved in protein folding or degradation (i.e. chaparones or proteases). If this effort is unsuccessful, the UPR can initiate apoptosis through mitochondrial dependent or independent mechanisms.[112]

ER stress has been implicated in a wide variety of diseases, including obesity, diabetes and cardiovascular disease.[113,114] The involvement of ER stress in NAFLD is less studied; however, evidence exists that it too plays a part in the pathogenesis of this disease.[115-123] Gregor et al. followed 11 obese men and women before and 1 year post gastric bypass surgery.[120] ER stress markers in both subcutaneous adipose and liver tissue were upregulated before surgery and subsequently reduced after, suggesting a relationship between obesity-related ER stress and metabolic dysfunction.[120] Puri et al. compared metabolic syndrome patients with normal liver histology to those with steatosis and/or NASH and found steotic or NASH liver biopsies had increased

phosphorylation of eIF2a (PERK associated signaling pathway of UPR), yet there was failure in activation of downstream recovery pathways of the UPR (ATF4, CHOP, GADD34).[123] Thus, in patients with NAFLD, there appears to be activation of some components of the UPR and perhaps impairments in others.

Diet composition may play a role in the development of ER stress and subsequent liver injury in NAFLD. Male Wistar rats were fed diets enriched with starch (STD), sucrose (HSD), polyunsaturated fat (HPUFA), or saturated fat (HSAT) for 1, 4 or 24 weeks.[124] Liver triglycerides were increased to a similar extent in HSD, HPUFA, and HSAT compared with STD at 4 and 24 weeks; however, saturated fatty acid content of triglycerides and microsomal membranes was increased in HSD and HSAT compared with HPUFA.[124] HSD and HSAT were also characterized by hepatic ER stress that preceded the manifestation of liver injury.[124] Importantly, increased liver injury in these dietary models was observed independently of differences in cytokines and insulin action.[124] These data suggest that diet composition and perhaps the composition of fatty acids delivered to and stored within the liver are important determinants of ER homeostasis and liver cell integrity.

It is important to note that ER stress can affect various cell types in the liver. A study done by Yang et al. demonstrated that ER stress related decreases in hepatocyte CD1d, a lipid antigen presenter, contributes to natural killer T cell (NKT) dysfunction in the steatotic liver.[125] This occurs because under normal conditions, the ER is responsible

for CD1d trafficking to plasma membranes; however, hepatic steatosis induces ER stress and prevents this trafficking from occurring and results in less CD1d on the plasma membrane surface. CD1d is necessary for appropriate interaction with NKT cells, and in its absence, NKT cells cannot be properly activated or matured to proliferate more NKT cells. The liver is the primary reservoir for NKT cells in adults, and their depletion may have many harmful effects including increased susceptibility to obesity-associated diseases like NAFLD.[125]

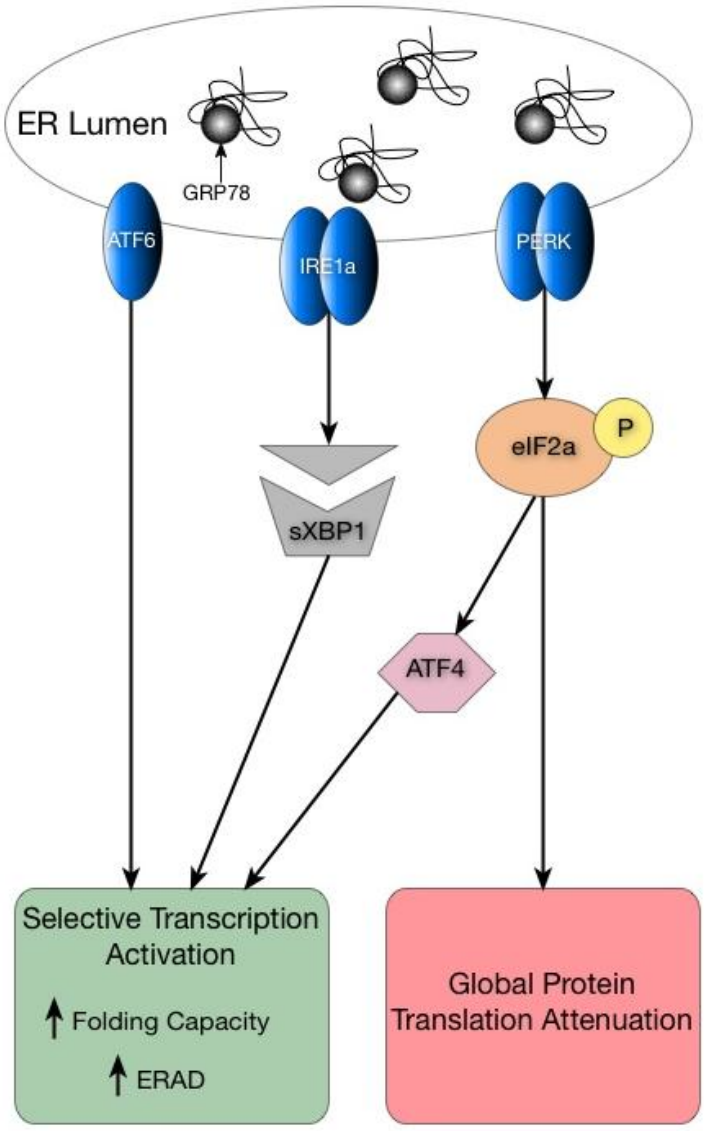


Figure 5. UPR activation. Upon accumulation of proteins in the ER, three proximal signaling arms are activated: activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1), and RNA-activated protein kinase (PKR)-like ER kinase (PERK). ATF6, IRE1a (through splicing of X box-binding protein 1 (XBP1)), and PERK (through activating transcription factor 4 (ATF4)) can induce transcriptional activation of chaparones for protein folding or degradation proteins to alleviate the protein load in the ER lumen. PERK, through phosphorylation of eiF2a, can attenuate global translation of proteins to further decrease the protein load in the ER lumen.

THERAPEUTICS

In treating NAFLD, potential targets for intervention include metabolic pathways that contribute to the development of hepatic steatosis and/or amelioration of second hit triggers like oxidative stress, ER stress, cytokines, or bacterial endotoxin. Currently, there is no established treatment specific to NAFLD. Weight loss, insulin sensitizers such as thiazolidinediones (TZDs) and metformin, and other therapies that treat obesity and components of the metabolic syndrome are often prescribed since they can indirectly, via reductions in body weight and fat mass, attenuate hepatic steatosis and disease progression in NAFLD. TZDs are thought to act through adipose tissue PPARγ to increase peripheral insulin sensitivity.[126] Metformin acts through its activation of AMPK to suppress hepatic glucose production.[127] Rapid weight loss (greater than 1.6 kg/week) can cause portal inflammation and fibrosis,[128] while weight loss in general can have a high rate of recidivism[129] and thus only be a temporary solution to NAFLD progression. Although some studies have shown that antidiabetic agents such as metformin and insulin-sensitizing thiazolidinediones may improve NAFLD, the long term effects are unclear, are ineffective in advanced states of NAFLD (i.e. due to fibrosis), cause weight gain, or are potentially hepatotoxic in some patients.[130,131] Historically, plants have been used to prevent or treat many diseases. Diabetes, both insulin dependent and independent, has been recognized and treated with medicinal plants by Chinese and Ayurvedic physicians for millennia.[132] In fact, over 1000 species of plants have been reported to have been used to treat diabetes or have antidiabetic activity.[133-135] A study by Zhang et al. in rats showed that puerarin, a major isoflavanoid compound isolated

from the Chinese vine *Pueraria lobata*, could reverse chemical-induced liver fibrosis.[136] Elevated liver enzymes, a plasma marker of liver injury, were decreased with puerarin treatment, while apoptosis of activated hepatic stellate cells was increased.[136] Bcl-2 (an inhibitor of apoptosis) mRNA was also downregulated with puerarin.[136] Immunohistochemical staining showed a decrease in pathological characteristics of fibrosis (marked fatty degeneration, portal inflammation and necrosis, collagen deposition, hepatocyte loosening, perihepatocyte fibrosis, and slight confluence).[136] Thus, medicinal plants may provide a novel source of bioactive compounds that may be effective in the treatment of NAFLD.

ONION

Onions are a staple annual crop grown across the country, particularly in Colorado. Dry storage onions make up 47% of the vegetable crops grown in Colorado, representing a 43 million dollar value annually.[137] In addition to its agricultural importance, onions have been used medicinally for centuries.[138] In a study done by Gabler et al., pigs were fed a high fat diet (25% w/w) for 2 weeks followed by supplementation of a low dose of onion (human equivalent to half an onion/day), a high dose (human equivalent of 1.5 onions/day), or no supplementation.[139] Onion supplementation, regardless of dosage, provided a reduction in postprandial plasma triglycerides by 15%, antioxidant protection against lipoprotein oxidation, immune stimulation, and haematological responses.[139] Although there was no statistical effect on the plasma cholesterol profile, previous studies done by the authors on different varieties of onions demonstrated that onions with higher amounts of organosulfur

compounds and flavonoids have a dose dependent effect on the lipoprotein profile.[139,140] Numerous studies have reported hypolipidemic and hypoglycemic effects of onions or onion extracts in diabetic rats,[141] rabbits,[142] and humans.[143,144] Kumari et al. showed that the S-methyl cysteine sulfoxide (SMCS) extract of onion contributed to reductions in serum and tissue cholesterol, triglyceride, and phospholipids in high cholesterol (1%) fed rats.[145] Also, adipose tissue lipoprotein lipase was downregulated, leading to a decrease in circulating free fatty acids.[145] Overall, SMCS caused a decrease in endogenous lipogenesis as well as an increase in lipid catabolism and excretion.[145] SMCS also has a stimulatory effect on insulin secretion by the pancreas.[146] The above studies illustrate that onion contains a variety of bioactive compounds, such as organo-sulfur compounds like SMCS, which may have ameliorating effects on the hyperlipidemic, pro-oxidant, insulin resistant features of NAFLD. The problem with using onions therapeutically is that, from a dietary standpoint, a patient would have to consume 0.5 to 1.5 raw onions per day in order to show the effects observed in the pig study by Gabler et al. Extracting compounds like SMCS may be therapeutically beneficial, however there may be other bioactive compounds left behind or destroyed during the extraction process that could have beneficial effects on patients with metabolic disorders.

Plant Root Exudation as a Source of Therapeutic Compounds

Most bioactivity studies done on plants have focused on the part of the plant above the ground, thus allowing the plant root to go unnoticed. Plant roots provide additional function to their mechanical support and water/nutrient uptake roles;

notably, they can synthesize, accumulate, and secrete a wide range of compounds. Root branching and root system architecture (RSA), type of root cell (e.g. root cap, epidermal cells and root hairs), nutrient availability, age of plant, species of plant and the surrounding biotic and abiotic environment can all contribute to the specificity, quality, and quantity of compounds exuded by the root into the surrounding rhizosphere.[147,148] Organic compounds such as mucilage, polysaccharides, proteins, organic acids, amino acids, simple sugars and secondary metabolites are the major components of root exudation.[149] Three pathways are thought to passively mediate plant root exudation: diffusion, ion channels, and vesicle transport.[148] More recently, active transport of root exudates has been shown to occur through root-specific ABC transporters (ATP dependent; e.g. fatty acids, flavonoids).[148,150-152]

While diurnal variations in metabolites are commonplace among plants, a recent study in Arabidopsis revealed root exudation of phytochemicals was predominantly not influenced by diurnal rhythms.[151] A study done on root exudates from ginseng found that nitrogen and potassium deficiency caused enhanced exudation of a variety of organic and phenolic acids.[153] Soil microorganisms also have the ability to influence the type of chemical exuded by the plant root; microbial products from the soil microorganisms Pseudomonas bacteria and Fusarium fungi enhanced amino acid efflux from the roots of alfalfa, maize, and wheat.[154] Through secretion (or exudation) of compounds into the surrounding soil (rhizosphere), roots can regulate the surrounding environment to promote symbiosis, allelopathy, discourage herbivory and alter the

chemical composition of the soil.[155] The exudates produced by plants may be contained within the plant, or be unique to the root cells. To date, there are no studies published pertaining to the therapeutic effect of plant root exudates on human metabolic diseases. A study done by Bao et al. analyzed the effect of pretreatment of the turmeric rhizome (the underground stem of a plant where roots and shoots originate) derived bioactive constituent curcumin on oxidative stress induced by ethanol in hepatocytes.[156] Curcumin pretreatment alleviated hepatocyte damage through redox enzyme regulation and through a dose and time dependent induction of heme oxygenase-1, which is the rate limiting enzyme in the breakdown of heme into carbon monoxide, iron, and bilirubin.[156] Another study demonstrated that rhizome extracts of *Solidago chilensis* had potent anti-inflammatory properties in pleural cavity inflammation in mice.[157] This effect was much more potent than that of other above ground parts of the plant. With more exploration into its therapeutic potential, plant root exudation may be a novel, renewable source of bioactive compounds that may be used in the treatment of metabolic diseases like NAFLD.

TAURINE

Taurine is an endogenous amino sulfonic acid involved in intracellular osmoregulation, bile acid formation, the central nervous system, retinal function, cardiac function, inhibition of protein phosphorylation and development. It is found in high intracellular concentrations in animal tissues and some algae. Taurine is crucial in transforming cholesterol to water soluble bile salts for cholesterol excretion, and is primarily synthesized from cysteine in the liver. Although taurine can be endogenously

made, it is also taken up through taurine-rich sources such as seafood or shellfish, and this dietary uptake dictates plasma levels of taurine since vegans have taurine plasma levels that are only 50% that of omnivore levels.[158] In high fat diet induced and genetically obese mice, as well as high fructose diet induced insulin resistant rats, plasma taurine concentrations were notably decreased compared to their respective controls.[159,160] Dietary taurine supplementation prevented high fat diet induced obesity. While mice fed a high fat diet have decreased resting energy expenditure compared to high carbohydrate diet controls, this decrease was prevented with taurine supplementation.[159,160] In high fructose diet fed rats, the characteristic hyperglycaemia, hyperinsulinaemia, and insulin resistance were all reduced with taurine supplementation. Taurine deficiency in humans has also been documented; obese patients had significantly lower plasma taurine levels than healthy controls (48±4 versus 85±6 umol/liter, respectively) and patients with diabetes had significantly lower plasma taurine levels than in nondiabetic controls (32.1±1.9 versus 48.6±4.9 umol/liter, respectively).[160-162] In a study done by Xiao et al., six overweight or obese nondiabetic male volunteers underwent 48 hour intravenous infusion of saline, intralipid and heparin to simulate chronic elevation of plasma NEFA, and intralipid and heparin with concurrent oral taurine supplementation.[163] These conditions were randomized and spaced 4-6 weeks apart.[163] Oral taurine supplementation ameliorated lipid induced impairments in insulin sensitivity and beta cell function.[163]

Gestational taurine deficiency has been linked to impairments in the development of the pancreas. Female rats fed a normal diet plus taurine produce offspring that have weak glucose intolerance and islet cells are more sensitive to cytokines.[164] Fetal islets from offspring of dams that were provided either a normal diet, a low protein diet, or a low protein diet supplemented with taurine during gestation, insulin secretion was normalized in the taurine supplemented group when compared to the low protein diet group, providing evidence that taurine is an essential nutrient in islet development.[165] These results are supportive of the "thrifty phenoptype" hypothesis that links intrauterine growth retardation and the development of type 2 diabetes later in life.[166] Intrauterine growth retardation has also been linked to adult development of coronary heart disease, stroke, hypertension, and obesity.[167] Thus, maternal taurine deficiency may contribute to the development of obesity and diabetes later in life.

Taurine supplementation may also be directly beneficial in the treatment of NASH. In a study by Chen et al., rats were either fed a standard control diet, a high fat diet, or a high fat diet with concomitant subcutaneous injection of taurine (for 8 weeks) for 12 weeks.[168] Taurine treatment resulted in improved lipid and glucose metabolism, decreased TNF-α and TNF-β1 synthesis, increased synthesis of adiponectin, and had a restorative effect on indices of experimental NASH.[168]

NAFLD is on the rise, mirroring the increase in obesity in the United States. Treatments for this disease are non-specific and fraught with the same drawbacks as treatments aimed at obesity. We hypothesize that onion plant root exudates and taurine may provide a therapeutic tool for the treatment and/or prevention of NAFLD.

SPECIFIC AIMS

1. To determine whether onion plant root exudates can reduce hepatic fat accumulation and improve insulin signaling in vitro and/or in vivo.

2. To determine whether taurine can reduce markers of oxidative stress, ER stress, lipid accumulation, inflammation and liver injury in vitro and/or in vivo.

BIBLIOGRAPHY

1. Papandreou D, Rousso I, Mavromichalis I. Update on non-alcoholic fatty liver disease in children. *Clin Nutr*. 2007;26(4):409-415. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17449148.

2. Comar KM, Sterling RK. Review article: Drug therapy for non-alcoholic fatty liver disease. *Aliment Pharmacol Ther*. 2006;23(2):207-215. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16393299.

3. Marchesini G, Bugianesi E, Forlani G, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*. 2003;37(4):917-923. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12668987.

4. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis*. 2007;191(2):235-240. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16970951.

5. Postic C, Girard J. The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes & metabolism*. 2008;34(6 Pt 2):643-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19195625 [Accessed February 20, 2011].

6. Donnelly KL, Smith CI, Schwarzenberg SJ, et al. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*. 2005;115(5):1343-1351. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15864352.

7. Larter CZ, Yeh MM, Van Rooyen DM, et al. Roles of adipose restriction and metabolic factors in progression of steatosis to steatohepatitis in obese, diabetic mice. *J Gastroenterol Hepatol*. 2009;24(10):1658-1668. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19788606.

8. Schwarz JM, Linfoot P, Dare D, Aghajanian K. Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr*. 2003;77(1):43-50. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12499321.

9. Timlin MT, Parks EJ. Temporal pattern of de novo lipogenesis in the postprandial state in healthy men. *Am J Clin Nutr*. 2005;81(1):35-42. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15640457.

10. Monetti M, Levin MC, Watt MJ, et al. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell Metab*. 2007;6(1):69-78. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17618857.

11. Yamaguchi K, Yang L, McCall S, et al. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology*. 2007;45(6):1366-1374. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17476695.

12. Rigazio S, Lehto H-R, Tuunanen H, et al. The lowering of hepatic fatty acid uptake improves liver function and insulin sensitivity without affecting hepatic fat content in humans. *American journal of physiology. Endocrinology and metabolism*. 2008;295(2):E413-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18505832 [Accessed August 11, 2010].

13. Savage DB, Choi CS, Samuel VT, et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J Clin Invest*. 2006;116(3):817-824. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16485039.

14. Minehira K, Young SG, Villanueva CJ, et al. Blocking VLDL secretion causes hepatic steatosis but does not affect peripheral lipid stores or insulin sensitivity in mice. *Journal of lipid research*. 2008;49(9):2038-44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18515909 [Accessed October 18, 2010].

15. Cusi K. Role of insulin resistance and lipotoxicity in non-alcoholic steatohepatitis. *Clin Liver Dis*. 2009;13(4):545-563. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19818304.

16. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J Biol Chem*. 2001;276(18):14890-14895. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11278654.

17. Wei Y, Wang D, Pagliassotti MJ. Saturated fatty acid-mediated endoplasmic reticulum stress and apoptosis are augmented by trans-10, cis-12-conjugated linoleic acid in liver cells. *Mol Cell Biochem*. 2007;303(1-2):105-113. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17426927.

18. Wei Y, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab*. 2006;291(2):E275-81. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16492686.

19. Listenberger LL, Han X, Lewis SE, et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A*. 2003;100(6):3077-3082. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12629214.

20. Krssak M, Falk Petersen K, Dresner A, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia*. 1999;42(1):113-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10027589 [Accessed February 20, 2011].

21. Fabbrini E, Magkos F, Mohammed BS, et al. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(36):15430-5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2741268&tool=pmcentrez&rendertype=abstract.

22. Gavrilova O, Marcus-Samuels B, Graham D, et al. Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *The Journal of clinical investigation*. 2000;105(3):271-8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=377444&tool=pmcentrez&rendertype=abstract [Accessed January 11, 2011].

23. Koyama K, Chen G, Lee Y, Unger RH. Tissue triglycerides, insulin resistance, and insulin production: implications for hyperinsulinemia of obesity. *The American journal of physiology*. 1997;273(4 Pt 1):E708-13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9357799 [Accessed February 20, 2011].

24. Samuel VT, Liu ZX, Qu X, et al. Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J Biol Chem*. 2004;279(31):32345-32353. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15166226.

25. Hwang JH, Stein DT, Barzilai N, et al. Increased intrahepatic triglyceride is associated with peripheral insulin resistance: in vivo MR imaging and spectroscopy studies. *Am J Physiol Endocrinol Metab*. 2007;293(6):E1663-9. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17911339.

26. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, et al. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocrinol Metab*. 2002;87(7):3023-3028. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12107194.

27. Kelley DE, McKolanis TM, Hegazi RAF, Kuller LH, Kalhan SC. Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. *American journal of physiology. Endocrinology and metabolism*. 2003;285(4):E906-16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12959938 [Accessed February 20, 2011].

28. Kotronen A, Seppala-Lindroos A, Bergholm R, Yki-Jarvinen H. Tissue specificity of insulin resistance in humans: fat in the liver rather than muscle is associated with features of the metabolic syndrome. *Diabetologia*. 2008;51(1):130-138. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18008059.

29. Kotronen A, Vehkavaara S, Seppälä-Lindroos A, Bergholm R, Yki-Järvinen H. Effect of liver fat on insulin clearance. *American journal of physiology. Endocrinology and metabolism*. 2007;293(6):E1709-15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17895288 [Accessed February 20, 2011].

30. Bratusch-Marrain PR, Waldhäusl WK, Gasić S, Hofer A. Hepatic disposal of biosynthetic human insulin and porcine C-peptide in humans. *Metabolism: clinical and experimental*. 1984;33(2):151-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6141519 [Accessed February 20, 2011].

31. Saccà L, Orofino G, Petrone A, Vigorito C. Direct assessment of splanchnic uptake and metabolic effects of human and porcine insulin. *The Journal of clinical endocrinology and metabolism*. 1984;59(2):191-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6145721 [Accessed February 20, 2011].

32. Meistas MT, Margolis S, Kowarski AA. Hyperinsulinemia of obesity is due to decreased clearance of insulin. *The American journal of physiology*.

1983;245(2):E155-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6349380 [Accessed January 14, 2011].

33. Gower BA, Granger WM, Franklin F, Shewchuk RM, Goran MI. Contribution of insulin secretion and clearance to glucose-induced insulin concentration in african-american and caucasian children. *The Journal of clinical endocrinology and metabolism*. 2002;87(5):2218-24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11994367 [Accessed January 14, 2011].

34. Faber OK, Christensen K, Kehlet H, Madsbad S, Binder C. Decreased insulin removal contributes to hyperinsulinemia in obesity. *The Journal of clinical endocrinology and metabolism*. 1981;53(3):618-21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7021583 [Accessed January 14, 2011].

35. Cerutti F, Sacchetti C, Bessone A, et al. Insulin secretion and hepatic insulin clearance as determinants of hyperinsulinaemia in normotolerant grossly obese adolescents. *Acta paediatrica (Oslo, Norway : 1992)*. 1998;87(10):1045-50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9825970 [Accessed January 14, 2011].

36. Kim SP, Ellmerer M, Kirkman EL, Bergman RN. Beta-cell "rest" accompanies reduced first-pass hepatic insulin extraction in the insulin-resistant, fat-fed canine model. *American journal of physiology. Endocrinology and metabolism*. 2007;292(6):E1581-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17284579 [Accessed January 14, 2011].

37. Iwasaki Y, Ohkubo A, Kajinuma H, Akanuma Y, Kosaka K. Degradation and secretion of insulin in hepatic cirrhosis. *The Journal of clinical endocrinology and metabolism*. 1978;47(4):774-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/400732 [Accessed February 20, 2011].

38. Letiexhe MR, Scheen AJ, Gérard PL, et al. Insulin secretion, clearance, and action on glucose metabolism in cirrhotic patients. *The Journal of clinical endocrinology and metabolism*. 1993;77(5):1263-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8077319 [Accessed February 20, 2011].

39. Manchanayake J, Chitturi S, Nolan C, Farrell GC. POST PRANDIAL HYPERINSULINEMIA IS UNIVERSAL IN NON-DIABETIC PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE. *Journal of gastroenterology and hepatology*. 2010. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21155882 [Accessed January 14, 2011].

40. Kimura Y, Hyogo H, Ishitobi T, et al. Postprandial insulin secretion pattern is associated with the histological severity in nonalcoholic fatty liver disease patients without prior known diabetes mellitus. *Journal of gastroenterology and hepatology*. 2010. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21054523 [Accessed January 14, 2011].

41. Greenfield V, Cheung O, Sanyal AJ. Recent advances in nonalcholic fatty liver disease. *Curr Opin Gastroenterol*. 2008;24(3):320-327. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18408460.

42. Zhang W, Kudo H, Kawai K, et al. Tumor necrosis factor-alpha accelerates apoptosis of steatotic hepatocytes from a murine model of non-alcoholic fatty liver disease. *Biochem Biophys Res Commun*. 391(4):1731-1736. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20043871.

43. Robertson G, Leclercq I, Farrell GC. Nonalcoholic steatosis and steatohepatitis. II. Cytochrome P-450 enzymes and oxidative stress. *American journal of physiology. Gastrointestinal and liver physiology*. 2001;281(5):G1135-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11668021 [Accessed August 31, 2010].

44. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*. 2004;114(2):147-152. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15254578.

45. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol*. 2004;142(2):231-255. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15155533.

46. Santos CXC, Tanaka LY, Wosniak J, Laurindo FRM. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxidants & redox signaling*. 2009;11(10):2409-27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19388824 [Accessed October 19, 2010].

47. Pessayre D. Role of mitochondria in non-alcoholic fatty liver disease. *Journal of gastroenterology and hepatology*. 2007;22 Suppl 1:S20-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17567459 [Accessed July 29, 2010].

48. Fromenty B, Pessayre D. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. *Pharmacology & therapeutics*. 1995;67(1):101-54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7494860 [Accessed January 16, 2011].

49. Pessayre D, Mansouri A, Fromenty B. Nonalcoholic steatosis and steatohepatitis. V. Mitochondrial dysfunction in steatohepatitis. *American journal of physiology. Gastrointestinal and liver physiology*. 2002;282(2):G193-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11804839 [Accessed January 16, 2011].

50. Begriche K, Igoudjil A, Pessayre D, Fromenty B. Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion*. 2006;6(1):1-28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16406828.

51. Haouzi D, Lekehal M, Tinel M, et al. Prolonged, but not acute, glutathione depletion promotes Fas-mediated mitochondrial permeability transition and apoptosis in mice. *Hepatology (Baltimore, Md.)*. 2001;33(5):1181-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11343247 [Accessed January 16, 2011].

52. Fernandez-Checa JC, Kaplowitz N. Hepatic mitochondrial glutathione: transport and role in disease and toxicity. *Toxicology and applied pharmacology*. 2005;204(3):263-73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15845418 [Accessed September 6, 2010].

53. Raza H, Prabu SK, Robin M-A, Avadhani NG. Elevated mitochondrial cytochrome P450 2E1 and glutathione S-transferase A4-4 in streptozotocin-induced diabetic rats: tissue-specific variations and roles in oxidative stress. *Diabetes*. 2004;53(1):185-94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14693714 [Accessed January 16, 2011].

54. Chitturi S, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. *Seminars in liver disease*. 2001;21(1):27-41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11296694 [Accessed November 4, 2010].

55. Robin M-A, Anandatheerthavarada HK, Biswas G, et al. Bimodal targeting of microsomal CYP2E1 to mitochondria through activation of an N-terminal chimeric signal by cAMP-mediated phosphorylation. *The Journal of biological chemistry*. 2002;277(43):40583-93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12191992 [Accessed September 30, 2010].

56. Robin MA, Anandatheerthavarada HK, Fang JK, et al. Mitochondrial targeted cytochrome P450 2E1 (P450 MT5) contains an intact N terminus and requires mitochondrial specific electron transfer proteins for activity. *The Journal of biological chemistry*. 2001;276(27):24680-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11325963 [Accessed January 16, 2011].

57. Robin M-A, Sauvage I, Grandperret T, et al. Ethanol increases mitochondrial cytochrome P450 2E1 in mouse liver and rat hepatocytes. *FEBS letters*. 2005;579(30):6895-902. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16337197 [Accessed January 9, 2011].

58. Chalasani N, Gorski JC, Asghar MS, et al. Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. *Hepatology (Baltimore, Md.)*. 2003;37(3):544-50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12601351 [Accessed January 16, 2011].

59. Weltman MD, Farrell GC, Hall P, Ingelman-Sundberg M, Liddle C. Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology (Baltimore, Md.)*. 1998;27(1):128-33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9425928 [Accessed January 16, 2011].

60. Pessayre D, Fromenty B. NASH: a mitochondrial disease. *Journal of hepatology*. 2005;42(6):928-40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15885365 [Accessed December 14, 2010].

61. Sanyal AJ, Campbell-Sargent C, Mirshahi F, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology*. 2001;120(5):1183-92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11266382 [Accessed August 3, 2010].

62. Malaguarnera M, Di Rosa M, Nicoletti F, Malaguarnera L. Molecular mechanisms involved in NAFLD progression. *J Mol Med*. 2009;87(7):679-695. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19352614.

63. Guo W-X, Pye QN, Williamson KS, et al. Mitochondrial dysfunction in choline deficiency-induced apoptosis in cultured rat hepatocytes. *Free radical biology & medicine*. 2005;39(5):641-50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16085182 [Accessed January 16, 2011].

64. Yang S, Zhu H, Li Y, et al. Mitochondrial adaptations to obesity-related oxidant stress. *Archives of biochemistry and biophysics*. 2000;378(2):259-68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10860543 [Accessed September 6, 2010].

65. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *Journal of Clinical Investigation*. 2004;114(2).

66. Johnson EF, Palmer CN, Griffin KJ, Hsu MH. Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1996;10(11):1241-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8836037 [Accessed January 18, 2011].

67. Kersten S, Seydoux J, Peters JM, et al. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *The Journal of clinical investigation*. 1999;103(11):1489-98. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=408372&tool=pmcentrez&rendertype=abstract [Accessed August 23, 2010].

68. Berson A, De Beco V, Lettéron P, et al. Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes.

Gastroenterology. 1998;114(4):764-74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9516397 [Accessed January 18, 2011].

69. Puri P, Baillie RA, Wiest MM, et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology (Baltimore, Md.)*. 2007;46(4):1081-90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17654743 [Accessed January 18, 2011].

70. Bass NM. Lipidomic dissection of nonalcoholic steatohepatitis: moving beyond foie gras to fat traffic. *Hepatology (Baltimore, Md.)*. 2010;51(1):4-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20034031 [Accessed January 18, 2011].

71. Hall D, Poussin C, Velagapudi VR, et al. Peroxisomal and microsomal lipid pathways associated with resistance to hepatic steatosis and reduced pro-inflammatory state. *The Journal of biological chemistry*. 2010;285(40):31011-23. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2945592&tool=pmcentrez&rendertype=abstract [Accessed August 8, 2010].

72. Kohjima M, Enjoji M, Higuchi N, et al. Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *International journal of molecular medicine*. 2007;20(3):351-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17671740 [Accessed October 9, 2010].

73. Nakamuta M, Kohjima M, Higuchi N, et al. The significance of differences in fatty acid metabolism between obese and non-obese patients with non-alcoholic fatty liver disease. *International journal of molecular medicine*. 2008;22(5):663-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18949388 [Accessed October 9, 2010].

74. Puri P, Wiest MM, Cheung O, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology (Baltimore, Md.)*. 2009;50(6):1827-38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19937697.

75. Natarajan SK, Eapen CE, Pullimood AB, Balasubramanian KA. Oxidative stress in experimental liver microvesicular steatosis: role of mitochondria and peroxisomes. *J Gastroenterol Hepatol*. 2006;21(8):1240-1249. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16872304.

76. Schröder M, Kaufman RJ. ER stress and the unfolded protein response. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 2005;569(1-2):29–63. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0027510704003719 [Accessed November 1, 2010].

77. Enriquez A, Leclercq I, Farrell GC, Robertson G. Altered expression of hepatic CYP2E1 and CYP4A in obese, diabetic ob/ob mice, and fa/fa Zucker rats. *Biochem Biophys Res Commun*. 1999;255(2):300-306. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10049703.

78. Leclercq IA, Farrell GC, Field J, et al. CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. *J Clin Invest*. 2000;105(8):1067-1075. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10772651.

79. Gómez-Lechón MJ, Jover R, Donato MT. Cytochrome p450 and steatosis. *Current drug metabolism*. 2009;10(7):692-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19702532.

80. Weltman MD, Farrell GC, Hall P, Ingelman-Sundberg M, Liddle C. Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology*. 1998;27(1):128-133. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9425928.

81. Hanada S, Harada M, Kumemura H, et al. Oxidative stress induces the endoplasmic reticulum stress and facilitates inclusion formation in cultured cells. *J Hepatol*. 2007;47(1):93-102. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17434230.

82. Tilg H, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. *N Engl J Med*. 2000;343(20):1467-1476. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11078773.

83. Valenti L, Ludovica Fracanzani A, Fargion S. The immunopathogenesis of alcoholic and nonalcoholic steatohepatitis: two triggers for one disease? In: *Seminars in immunopathology*.Vol 31. Springer; 2009:359–369. Available at: http://www.springerlink.com/index/mu77831h78873g7p.pdf [Accessed November 1, 2010].

84. Wree A, Kahraman A, Gerken G, Canbay A. Obesity affects the liver - the link between adipocytes and hepatocytes. *Digestion*. 2011;83(1-2):124-33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21042023 [Accessed December 14, 2010].

85. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444(7121):860-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17167474 [Accessed January 9, 2011].

86. Diehl AM. Nonalcoholic steatosis and steatohepatitis IV. Nonalcoholic fatty liver disease abnormalities in macrophage function and cytokines. *American journal of physiology. Gastrointestinal and liver physiology*. 2002;282(1):G1-5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11751151 [Accessed August 30, 2010].

87. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM. Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(6):2557-62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=20127&tool=pmcentrez&rendertype=abstract [Accessed January 5, 2011].

88. Wigg AJ, Roberts-Thomson IC, Dymock RB, et al. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. *Gut*. 2001;48(2):206-11. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1728215&tool=pmcentrez&rendertype=abstract [Accessed January 5, 2011].

89. Diehl AM. Tumor necrosis factor and its potential role in insulin resistance and nonalcoholic fatty liver disease. *Clinics in liver disease*. 2004;8(3):619-38, x. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15331067 [Accessed January 5, 2011].

90. Marra F, Bertolani C. Adipokines in liver diseases. *Hepatology*. 2009;50(3):957-969. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19585655.

91. Marchesini G, Moscatiello S, Di Domizio S, Forlani G. Obesity-associated liver disease. *J Clin Endocrinol Metab*. 2008;93(11 Suppl 1):S74-80. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18987273.

92. Perseghin G, Lattuada G, De Cobelli F, et al. Serum retinol-binding protein-4, leptin, and adiponectin concentrations are related to ectopic fat accumulation. *J Clin Endocrinol Metab*. 2007;92(12):4883-4888. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17986645.

93. Qureshi K, Abrams G a. Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. *World journal of*

gastroenterology : WJG. 2007;13(26):3540-53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17659704.

94. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature*. 2006;444(7121):881-887. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17167477.

95. Bugianesi E, Pagotto U, Manini R, et al. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. *J Clin Endocrinol Metab*. 2005;90(6):3498-3504. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15797948.

96. Matikainen N, Manttari S, Westerbacka J, et al. Postprandial lipemia associates with liver fat content. *J Clin Endocrinol Metab*. 2007;92(8):3052-3059. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17488790.

97. Westerbacka J, Corner A, Tiikkainen M, et al. Women and men have similar amounts of liver and intra-abdominal fat, despite more subcutaneous fat in women: implications for sex differences in markers of cardiovascular risk. *Diabetologia*. 2004;47(8):1360-1369. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15309287.

98. Bajaj M, Suraamornkul S, Piper P, et al. Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients. *J Clin Endocrinol Metab*. 2004;89(1):200-206. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14715850.

99. Stefan N, Hennige AM, Staiger H, et al. High circulating retinol-binding protein 4 is associated with elevated liver fat but not with total, subcutaneous, visceral, or intramyocellular fat in humans. *Diabetes Care*. 2007;30(5):1173-1178. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17259477.

100. Wu H, Jia W, Bao Y, et al. Serum retinol binding protein 4 and nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract*. 2008;79(2):185-190. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17904683.

101. Su GL. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol*. 2002;283(2):G256-65. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12121871.

102. Nanji AA, Jokelainen K, Rahemtulla A, et al. Activation of nuclear factor kappa B and cytokine imbalance in experimental alcoholic liver disease in the rat. *Hepatology*. 1999;30(4):934-943. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10498645.

103. Turnbaugh PJ, Ley RE, Mahowald MA, et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. 2006;444(7122):1027-1031. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17183312.

104. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature*. 2006;444(7122):1022-1023. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17183309.

105. Bajzer M, Seeley RJ. Physiology: obesity and gut flora. *Nature*. 2006;444(7122):1009-1010. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17183300.

106. Hill JO. Understanding and addressing the epidemic of obesity: an energy balance perspective. *Endocr Rev*. 2006;27(7):750-761. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17122359.

107. Li YT, Wang L, Chen Y, et al. Effects of gut microflora on hepatic damage after acute liver injury in rats. *J Trauma*. 2010;68(1):76-83. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20065761.

108. Cani PD, Possemiers S, Van de Wiele T, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut*. 2009;58(8):1091-1103. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19240062.

109. Sitia R, Meldolesi J. Endoplasmic reticulum: a dynamic patchwork of specialized subregions. *Mol Biol Cell*. 1992;3(10):1067-1072. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1421566.

110. Goetz JG, Nabi IR. Interaction of the smooth endoplasmic reticulum and mitochondria. *Biochemical Society transactions*. 2006;34(Pt 3):370-3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16709164.

111. Voeltz GK, Rolls MM, Rapoport TA. Structural organization of the endoplasmic reticulum. *EMBO Rep*. 2002;3(10):944-950. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12370207.

112. Zhao L, Ackerman SL. Endoplasmic reticulum stress in health and disease. *Curr Opin Cell Biol*. 2006;18(4):444-452. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16781856.

113. Vasa-Nicotera M. The new kid on the block: the unfolded protein response in the pathogenesis of atherosclerosis. *Cell death and differentiation*. 2004;11 Suppl 1:S10-1. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15243579 [Accessed November 1, 2010].

114. Harding HP, Ron D. Endoplasmic reticulum stress and the development of diabetes: a review. *Diabetes*. 2002;51 Suppl 3:S455-61. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12475790.

115. Han KL, Choi JS, Lee JY, et al. Therapeutic potential of peroxisome proliferators--activated receptor-alpha/gamma dual agonist with alleviation of endoplasmic reticulum stress for the treatment of diabetes. *Diabetes*. 2008;57(3):737-745. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18065517.

116. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. 2004;306(5695):457-461. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15486293.

117. Sreejayan N, Dong F, Kandadi MR, Yang X, Ren J. Chromium alleviates glucose intolerance, insulin resistance, and hepatic ER stress in obese mice. *Obesity*. 2008;16(6):1331-1337. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18388893.

118. Rahman SM, Schroeder-Gloeckler JM, Janssen RC, et al. CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. Hepatology. 2007;45(5):1108-1117. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17464987.

119. Kammoun HL, Chabanon H, Hainault I, et al. GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice. *J Clin Invest*. 2009;119(5):1201-1215. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19363290.

120. Gregor MF, Yang L, Fabbrini E, et al. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes*. 2009;58(3):693-700. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19066313.

121. Foretz M, Guichard C, Ferre P, Foufelle F. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc Natl Acad Sci U S A*. 1999;96(22):12737-12742. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10535992.

122. Eberle D, Hegarty B, Bossard P, Ferre P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie*. 2004;86(11):839-848. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15589694.

123. Puri P, Mirshahi F, Cheung O, et al. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology*. 2008;134(2):568-76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18082745.

124. Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology*. 2006;147(2):943-951. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16269465.

125. Yang L, Jhaveri R, Huang J, Qi Y, Diehl AM. Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. *Lab Invest*. 2007;87(9):927-937. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17607300.

126. Kahn CR, Chen L, Cohen SE. Unraveling the mechanism of action of thiazolidinediones. *The Journal of clinical investigation*. 2000;106(11):1305-7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=387251&tool=pmcentrez&rendertype=abstract [Accessed January 22, 2011].

127. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. *Annals of internal medicine*. 2002;137(1):25-33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12093242 [Accessed January 22, 2011].

128. Andersen T, Gluud C, Franzmann MB, Christoffersen P. Hepatic effects of dietary weight loss in morbidly obese subjects. *J Hepatol*. 1991;12(2):224-229. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2051001.

129. Festi D, Colecchia A, Sacco T, et al. Hepatic steatosis in obese patients: clinical aspects and prognostic significance. *Obes Rev*. 2004;5(1):27-42. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14969505.

130. Calamita G, Portincasa P. Present and future therapeutic strategies in non-alcoholic fatty liver disease. *Expert Opin Ther Targets*. 2007;11(9):1231-1249. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17845148.

131. Tolman KG, Dalpiaz AS. Treatment of non-alcoholic fatty liver disease. *Therapeutics and clinical risk management*. 2007;3(6):1153-63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2387293&tool=pmcentrez&rendertype=abstract.

132. Ackerknecht EH. *A short history of medicine (Revised edition)*. Baltimore/London: Johns Hopkins University Press; 1982.

133. Marles RJ FNR. Antidiabetic plants and their active constituents. *Phytomedicine*. 1995;2:137-189.

134. Eddouks M, Maghrani M, Lemhadri A, Ouahidi ML, Jouad H. Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). *J Ethnopharmacol*. 2002;82(2-3):97-103. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12241983.

135. Afifi FU, with Hamdan II. Studies on the in vitro and in vivo hypoglycemic activities of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine. *J Ethnopharmacol*. 2004;93(1):117-121. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15182916.

136. Zhang S, Ji G, Liu J. Reversal of chemical-induced liver fibrosis in Wistar rats by puerarin. *J Nutr Biochem*. 2006;17(7):485-491. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16426832.

137. Anon. www.coloradoonion.com.

138. Anon. http://www.onions-usa.org/about/history.php.

139. Gabler NK, Osrowska E, Imsic M, et al. Dietary onion intake as part of a typical high fat diet improves indices of cardiovascular health using the mixed sex pig model. *Plant Foods Hum Nutr*. 2006;61(4):179-185. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17123162.

140. Ostrowska E, Gabler NK, Sterling SJ, et al. Consumption of brown onions (Allium cepa var. cavalier and var. destiny) moderately modulates blood lipids, haematological and haemostatic variables in healthy pigs. *Br J Nutr*. 2004;91(2):211-218. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14756906.

141. El-Demerdash FM, Yousef MI, El-Naga NI. Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. *Food Chem Toxicol*. 2005;43(1):57-63. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15582196.

142. Augusti KI, Roy VC, Semple M. Effect of allyl propyl disulphide isolated from onion (Allium cepa L.) on glucose tolerance of alloxan diabetic rabbits. *Experientia*. 1974;30(10):1119-1120. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4435106.

143. Sharma K, Gupta R, Gupta S. Antihyperglycemic effect of onion: effect on fasting blood sugar and induced hyperglycemia in man. *The Indian journal of medical research*. 1977;65(3):422-429. Available at: http://www.ncbi.nlm.nih.gov/pubmed/336527 [Accessed November 1, 2010].

144. Augusti KT, Benaim ME. Effect of essential oil of onion (allyl propyl disulphide) on blood glucose, free fatty acid and insulin levels of normal subjects. *Clinica chimica acta; international journal of clinical chemistry*. 1975;60(1):121-3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1126028 [Accessed December 20, 2010].

145. Kumari K, Augusti KT. Lipid lowering effect of S-methyl cysteine sulfoxide from Allium cepa Linn in high cholesterol diet fed rats. *J Ethnopharmacol*. 2007;109(3):367-371. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16987625.

146. Kumari K, Augusti KT. Antidiabetic and antioxidant effects of S-methyl cysteine sulfoxide isolated from onions (Allium cepa Linn) as compared to standard drugs in alloxan diabetic rats. *Indian J Exp Biol*. 2002;40(9):1005-1009. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12587728.

147. B. Prithiviraj Jorge M. Vivanco MWP. Root Communication: The Role of Root Exudates . *Encyclopedia of Plant and Crop Science*. 2006.

148. Badri DV, Vivanco JM. Regulation and function of root exudates. *Plant, Cell & Environment*. 2009;32(6):666-681. Available at: http://blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2009.01926.x [Accessed September 23, 2010].

149. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol*. 2006;57:233-266. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16669762.

150. Loyola-Vargas VM, Broeckling CD, Badri D, Vivanco JM. Effect of transporters on the secretion of phytochemicals by the roots of Arabidopsis thaliana. *Planta*. 2007;225(2):301-10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16868775 [Accessed December 12, 2010].

151. Badri DV, Loyola-Vargas VM, Broeckling CD, Vivanco JM. Root secretion of phytochemicals in Arabidopsis is predominantly not influenced by diurnal rhythms. *Molecular plant*. 2010;3(3):491-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20154222 [Accessed January 22, 2011].

152. Sugiyama A, Shitan N, Yazaki K. Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-Rhizobium symbiosis. *Plant physiology*. 2007;144(4):2000-8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1949875&tool=pmcentrez&rendertype=abstract [Accessed November 1, 2010].

153. Li Y, Huang XF, Ding WL. [Effects of nutrient deficiency on principal components of ginseng root exudates]. *Ying Yong Sheng Tai Xue Bao*. 2008;19(8):1688-1693. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18975743.

154. Phillips DA, Fox TC, King MD, Bhuvaneswari TV, Teuber LR. Microbial products trigger amino acid exudation from plant roots. *Plant Physiol*. 2004;136(1):2887-2894. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15347793.

155. Walker TS, Bais HP, Grotewold E, Vivanco JM. Root exudation and rhizosphere biology. *Plant Physiol*. 2003;132(1):44-51. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12746510.

156. Bao W, Li K, Rong S, et al. Curcumin alleviates ethanol-induced hepatocytes oxidative damage involving heme oxygenase-1 induction. *Journal of ethnopharmacology*. 2010;128(2):549-53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20080166 [Accessed November 1, 2010].

157. Goulart S, Moritz MI, Lang KL, et al. Anti-inflammatory evaluation of Solidago chilensis Meyen in a murine model of pleurisy. *J Ethnopharmacol*. 2007;113(2):346-353. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17686594.

158. Stapleton PP, Charles RP, Redmond HP, Bouchier-Hayes DJ. Taurine and human nutrition. *Clin Nutr*. 1997;16(3):103-108. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16844580.

159. Nandhini ATA, Thirunavukkarasu V, Anuradha CV. Stimulation of glucose utilization and inhibition of protein glycation and AGE products by taurine. *Acta physiologica Scandinavica*. 2004;181(3):297-303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15196090 [Accessed January 20, 2011].

160. Tsuboyama-Kasaoka N, Shozawa C, Sano K, et al. Taurine (2-aminoethanesulfonic acid) deficiency creates a vicious circle promoting obesity. *Endocrinology*. 2006;147(7):3276-3284. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16627576.

161. Jeevanandam M, Ramias L, Schiller WR. Altered plasma free amino acid levels in obese traumatized man. *Metabolism*. 1991;40(4):385-390. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2011079.

162. De Luca G, Calpona PR, Caponetti A, et al. Taurine and osmoregulation: platelet taurine content, uptake, and release in type 2 diabetic patients. *Metabolism*. 2001;50(1):60-64. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11172476.

163. Xiao C, Giacca A, Lewis GF. Oral taurine but not N-acetylcysteine ameliorates NEFA-induced impairment in insulin sensitivity and beta cell function in obese and overweight, non-diabetic men. *Diabetologia*. 2008;51(1):139-146. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18026714.

164. Merezak S, Reusens B, Renard A, et al. Effect of maternal low-protein diet and taurine on the vulnerability of adult Wistar rat islets to cytokines. *Diabetologia*. 2004;47(4):669-675. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15298344.

165. Cherif H, Reusens B, Ahn MT, Hoet JJ, Remacle C. Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. *J Endocrinol*. 1998;159(2):341-348. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9795376.

166. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*. 1992;35(7):595-601. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1644236.

167. Barker DJP. *Mothers, Babies and Health in Later Life 2nd ed.* Edinburgh, U.K. Churchill Livingstone; 1998.

168. Chen SW, Chen YX, Shi J, Lin Y, Xie WF. The restorative effect of taurine on experimental nonalcoholic steatohepatitis. *Dig Dis Sci*. 2006;51(12):2225-2234. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17080243.

CHAPTER 3

ONION PLANT ROOT EXUDATES ENHANCE HEPATIC INSULIN SIGNALING AND GLUCOSE HOMEOSTASIS

INTRODUCTION

NAFLD is a metabolic disease that varies from simple steatosis to steatohepatitis (NASH), and can further progress to advanced fibrosis and cirrhosis. NAFLD is highly prevalent in cases of diabetes, obesity, cardiovascular disease and the metabolic syndrome, although it can exist in their absence.[1-3] Recent estimates suggest that up to 30% of the general population have some form of NAFLD, up to 75% of patients with obesity and diabetes mellitus, and 80% of patients with metabolic syndrome have NAFLD. Alarmingly, the recent increase in childhood obesity has resulted in a surge of type 2 diabetes and NAFLD in this population.[4-7] There is no known recommended treatment specific to NAFLD; therapies such as gradual weight reduction, insulin sensitizers, antioxidants, and lipid-lowering agents are prescribed to treat diabetes, obesity, cardiovascular disease and/or metabolic syndrome and can improve NAFLD to various degrees. However, these therapies have a number of side effects and are not designed to treat the specific characteristics of NAFLD, such as hepatic steatosis and liver insulin resistance. Also, access to, trust and compliance with

such treatments is not easily achieved in “susceptible populations” such as Native Americans, Hispanic and Latino Americans, and African Americans.[8-15] A targeted therapeutic approach specific to the metabolic abnormalities of NAFLD and feasible for incorporation into the culture and lifestyle of these populations is crucial in preventing and treating development of NAFLD.

A potential source of therapeutic compounds for NAFLD may exist in plants. Worldwide, over 1200 species of plants have been identified as traditional medicine for diabetes, but few have been experimentally evaluated.[16] Several plant-based spices, such as fenugreek, garlic, onion, turmeric, and cumin have been shown to lower glucose levels in animals, and in the case of fenugreek and onion, in humans.[17-19] Unfortunately, the quantities required to achieve a glucose lowering effect appear to be excessive.

In addition to bioactive compounds present within plants, plant roots secrete (exude) an enormous range of large and small molecular weight compounds into the rhizosphere, the soil zone that surrounds and is influenced by the roots of plants.[20-22] Compound synthesis and secretion by specialized root epidermal cells can be elicited by biotic and abiotic factors, and these compounds may or may not be present in the whole plant.[20-23] Plant root exudates may constitute a novel frontier in the search for natural, sustainable, and alternative therapies for disease prevention and treatment.

AIM

To determine whether onion plant root exudates can reduce hepatic fat accumulation and improve insulin signaling in vitro and/or in vivo.

METHODS

PROTOCOL FOR GROWING ONION PLANTS AND EXUDATE COLLECTION

Onion seeds were surface sterilized with regular laundry bleach for one minute and subsequently washed with sterile double distilled water by vortexing four times before plating the seeds in Murashige and Skoog (MS, supplemented with 3% sucrose) agar plates. Surface sterilized seeds were plated on MS (with 3% sucrose) agar plates with the help of sterile forceps and plates were sealed with parafilm. After plating the seeds, plates were incubated in a growth chamber (Percivel Scientific Co.) at 25±2 ^{o}C with photoperiodism of 16 hours light and 8 hours dark for germination. Twelve-day-old seedlings from the MS agar plates were transferred in to MS liquid media (containing 3% sucrose) (25 seedlings per 250 ml MS liquid media) and incubated in an orbital shaker at 25±3 ^{o}C with a photoperiodism of 16 hours light and 8 hours dark under cool white fluorescent light. Thirty days after transferring in to Liquid MS media, exudates were collected, filtered with Whatman filter paper and subsequently filter sterilized with 0.45µm membrane filters to remove all the root sheathing cells and debris. The pH of the exudates was determined by using a Metler Toledo pH meter. Filter sterilized exudates were freeze-dried (Labconco). Onion seeds (Evergreen Long White Bunching, *hybridia* granex) were obtained from W. Atlee Burpee & Co., PA. MS (Murashige &

Skoog) salts were obtained from Caisson Laboratories, Inc., Product No. MSP0509. Membrane filters (0.45µm) were obtained from Millipore, Product No. HVLP04700.

Freeze-dried onion root exudates were reconstituted in Dulbecco's Modified Eagle's Medium (DMEM) for cell experiments and saline for intraperitoneal injection into rats. The dosage used in cell-based experiments was the freeze-dried equivalent of 1 ml of original exudate. In rats, this same quantity was provided in each injection.

Cell Culture

The rat hepatoma liver cell line, H4IIE, (American Type Culture Collection, Manassas, VI) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 8 mM glucose and supplemented with 10% fetal bovine serum, penicillin and streptomycin sulfate. Experiments were performed at 80-100% cell confluence. To test the effects of onion root exudates on hepatic insulin signaling, H4IIE liver cells were treated overnight in the presence or absence of insulin (10 nM) in 6 ml of growth media (S) or 6 ml of growth media containing 1 ml reconstituted onion root exudate (E) per 100mm plate. To test the effects of onion root exudates on hepatic lipid accumulation, H4IIE liver cells were treated for 24 hours in the presence or absence of oleate (500 uM) in 6 ml of growth media (S) or 6 ml of growth media containing 1ml reconstituted onion root exudate (E) per 100 mm plate. Oleate (Sigma Chemical Co., St. Louis, MO) was complexed to bovine serum albumin (BSA) at a 2:1 molar ratio prior to treatment.

Lipid Measurement in H4IIE Cells

Triglyceride concentration. Total lipids were extracted using the methods of Bligh and Dyer.[24] Total triglyceride content was determined enzymatically (Sigma Company, St. Louis, MO).

Lipid extraction and methylation. All glass tubes and pipettes were soaked in dilute HCl for 24 hours and ddH2O for 24 hours before each experiment. Methanol:chloroform (2:1) was added to samples immediately after harvesting. The mixture was gassed with nitrogen and let stand for 2 hours while vortexing every thirty minutes. The suspension was then filtered using Whatman paper #54 into a clean glass tube. The original tube was washed with 1.5 ml methanol:chloroform:water (2:1:0.8). One ml chloroform and 1 ml ddH2O was added to the new tube, gassed with nitrogen, and shaken vigorously to mix. The samples were centrifuged at 2500 rpm for 30 minutes. The lower layer was carefully transferred into a new tube. The samples were evaporated to dryness under nitrogen and suspended with a small amount of chloroform:methanol (2:1). The samples were stored at -20 °C under nitrogen. Samples were methylated by adding 0.5 ml of 0.5 N methanolic NaOH and vortexed. Samples were heated at 70 °C for 10 minute and cooled to room temperature. Three ml of 12.5% BF3:MeOH was added to each tube and heated for 30 minutes at 70 °C. Samples were cooled to room temperature and 0.5 ml hexane and 1 ml ddH2O were added. Samples were then vortexed and allowed to separate. The upper layer was removed and placed into a glass vial for later fatty acid analysis by gas chromatography.

*Analysis of Fatty Acid Composition***.** Samples were analyzed using an Agilent 6890 Gas Chromatograph using helium as the gas carrier and a flame ionization detector.

ANIMALS

Male Wistar Crl(WI)BR rats (Charles River, Wilmington, MA) weighing approximately 180 g (7-8 weeks of age upon arrival) were used for the study. They were housed individually in a temperature and humidity-controlled environment under 12 hour light and dark cycles. Food and water were provided *ad libitum*. Bodyweight and food and water intake were monitored weekly. Male Wistar rats were provided a high fat diet (HFD, Research Diets, New Brunswick, NJ, 45% of energy from corn oil, 35% from cornstarch, 20% from casein) or control diet (STD, Research Diets, New Brunswick, NJ, 12% of energy from corn oil, 68% from corn starch, 20% from casein) for 4 wks accompanied by a daily IP injection of exudate (STDE or HFDE, n=9 per group, approximately 10.7 mg dried exudate in 200 ul sterile saline) or saline (STDS or HFDS, n=8). After 4 wks, overnight fasted rats were anesthetized (sodium pendtobarbital, 50 mg/kg), and blood and tissue samples obtained and processed for analysis. Protocols for animal experimentation and maintenance were carried out according to institutional guidelines and approved by the Colorado State University Animal Care and Use Committee.

REAL TIME RT-PCR

Total RNA (2 ug) was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Purified RNA was treated with DNAse (RQ1, Promega, Madison, WI) and reverse transcription (RT) was performed using Superscript II reverse transcriptase (Life Technologies, Carlsbad, CA). Transcribed cDNA was then subjected to polymerase chain reaction (PCR) amplification with IQ-SYBR green master mix (Bio-Rad, Hercules, CA, USA) using primers (Integrated DNA Technologies, Coralville, IA unless otherwise noted) specific for the genes listed below. All PCR reactions were run in duplicate with two individual preparations of reverse-transcribed cDNA. DNAse-treated RNA that had not been subjected to RT acted as a negative control.[25,26]

Insulin Receptor Substrate-1 (IRS1, GenBank NM_012969),

5'-GCCTGTCCTCTCCTACTACTC and 3'-CAGTTGCGGTATAGCGAAGG;

Insulin Receptor Substrate-2 (IRS2, GenBank NM_001081212),

5'-CTCTCCAGAGGCTTCATCCC-3' and 3'-CCTCTGGATATGGCGGACG;

V-Akt Murine Thymoma Viral Oncogene Homolog 1 (Akt1, NM_033230,

5'-GCAGGAGGAGGAGACGATGG and 3'-CATGGTCACACGGTGCTTGG;

Glucose-6-Phosphatase Catalytic Subunit (G6Pase; GenBank NM_013098),

5'-GTGGGTCCTGGACACTGACT and 3'-AATGCCTGACAAGACTCCA;

Phosphoenolpyruvate Carboxykinase (PEPCK; GenBank NM_198780),

5'-GCTGGCAGCATGGGGTGTTTGTAGG and 3'-GACCTCACCTACAAGC;

ß2-Microglobulin (used as a control; GenBank NM_012512),

5'-GGTGACCGTGATCTTTCTGGTG and 3'-GGATGGCGAGAGTACACTTGAATT.

PLASMA MEASURES

Glucose was measured with an automated analyzer (Beckman Instruments, Fullerton, CA). Insulin was analyzed by ELISA (Linco Research, St. Charles, MO). Free fatty acid levels were determined using the WAKO NEFA-C kit (Richmond, VA).

WESTERN BLOTTING

Liver tissue or cells were homogenized using a lysis buffer containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM sodium vanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 uM β-glycerophosphate, 3 mM benzamidine, 10 uM leupeptin, 5 uM pepstatin, and 10 ug/ml aprotinin. Total protein was measured according to the methods of Lowry et al.[27] Equivalent amounts of protein (50-100 ug) was separated using SDS-PAGE, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA), blocked and incubated with antibodies against the proteins of interest. The following antibodies

were used for protein detection: pAKT (serine 473, Cell Signaling), AKT (Cell Signaling), and actin (Sigma). Detection of proteins was done using horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, Santa Cruz Biotechnology), an enhanced chemiluminescence reagent (Santa Cruz Biotechnology), and a UVP bioimaging system (Upland, CA, USA) for optical density quantification.

Data Analysis

Statistical analysis was done using one way ANOVA where statistical significance was set at $p<0.05$. All data are reported as means ± standard deviation.

Results

Triglyceride Accumulation in H4IIE Liver Cells.

In the presence of 0.5 mM oleate incubation, H4IIE liver cells accumulated triglyceride. This accumulation was prevented when cells were co-incubated with onion exudate (Figure 1A). Similar results were observed with free fatty analysis by GC (data not shown).

Insulin Signaling in H4IIE Liver Cells.

Upon incubation with 10 nM insulin, H4IIE liver cells displayed increased insulin-mediated phosphorylation of AKT, a protein involved in insulin signaling. This insulin-mediated phosphorylation of AKT was enhanced in the presence of onion exudates (Figure 1B). In the absence of insulin, no increase in phosphorylation of AKT was observed in the presence of onion exudate.

IN VIVO EFFECTS OF ONION EXUDATES.

Both HFDS and HFDE rats had significantly greater initial body weight than STDS rats (Table 1). Food intake was not significantly different among groups. Bodyweight gain was significantly greater in HFDS compared to STDS and HFDE (Table 1). Epididymal fat pad weight was significantly greater in HFDS than that of STDS rats, but not different from HFDE (Table 1). Retroperitoneal weights were not different between groups.

Plasma glucose levels were significantly lower in HFDE compared to STDS and HFDS (Figure 2A). Plasma insulin levels were significantly lower in HFDE compared to HFDS (Figure 2B). Plasma FFA levels were significantly reduced in HFDE rats with respect to HFDS (Figure 2C). Phosphorylation of AKT, a protein in the insulin signaling cascade, when normalized to AKT and insulin, was significantly increased in HFDE compared to HFDS (Figure 2D). Hepatic G6Pase (a gene that encodes the glucose-6-phosphatase protein, a protein that promotes release of glucose by the liver) mRNA was lower in HFDE compared to HFDS, and AKT mRNA was lower in HFDE compared to STDS (Figure 3).

DISCUSSION

The aim of this study was to examine the therapeutic potential of onion root exudates in NAFLD using both in vitro and in vivo models. Onion root exudates reduced fatty acid-mediated triglyceride accumulation and enhanced insulin-induced phosphorylation of AKT in H4IIE liver cells. Onion root exudates also reduced plasma

FFA, glucose, and insulin in rats fed a HFD. In addition, phosphorylation of AKT relative to insulin levels was normalized in the liver of rats fed a HFD and treated with onion root exudates. Overall, these results imply that onion root exudates contain bioactive compounds that may reduce hepatic steatosis from free fatty acids and improve insulin action.

Onion contains a wide range of bioactive compounds that have been found to have potential therapeutic effects in vitro and in vivo. Of significance to features of NAFLD, several studies have shown hypoglycemic, hypolipidemic, antioxidant, and anti-inflammatory effects of onion in various models of diabetes, obesity and cardiovascular disease.[28-44] Unfortunately, the amount of onion required to achieve these effects is quite high and therefore likely unrealistic from a dietary standpoint. Onion root exudation may provide a source of bioactive compounds that is renewable and effective at relatively low concentrations.

In our study, oleate exposure in H4IIE liver cells caused intracellular accumulation of lipids, which was prevented with co-incubation of onion root exudates. Incubation of HepG2 liver cells with S-propyl cysteine, an extract of onion, reduced cellular triacylglycerol (TAG) and cholesterol ester synthesis from [^{14}C]oleate, without affecting cellular TAG synthesis from [^{14}C]acetate, suggesting that S-propyl cysteine reduced the transport of exogenous fatty acids into cells or fatty acid esterification.[45] If the compounds present in onion root exudates contain cysteine derivatives like S-propyl

cysteine, reduced transport of fatty acids into cells could be one possible mechanism by which triglyceride accumulation was prevented in liver cells; however, FFA disappearance from the media was not decreased in the presence of onion root exudate. A reduction in cellular TAG could also result from increased release of TAG by hepatocytes; however we were unable to detect any TAG in the media. Therefore, we hypothesize that increased FFA oxidation may be the mechanism by which exudates prevent lipid accumulation in liver cells.

Onion root exudates also enhanced insulin signaling in H4IIE liver cells. Although the mechanisms leading to this enhancement are unknown, several possibilities can be considered. These include insulin mimetic effects, increased interaction of insulin with the insulin receptor or downstream signaling proteins to their respective targets, or by alterations in the activity of proteins involved in the propagation of the insulin signal (e.g. kinases) or proteins involved in turning the insulin signal off (e.g. phosphatases).[46-51] It is unlikely that the increase in phosphorylation of AKT in the presence of exudates was due to an insulin mimetic effect by the exudates, since there was no exudate-derived signal enhancement in the absence of insulin (i.e. in the absence of insulin exudates did not increase pAKT). It is possible compounds present in onion root exudates could somehow enhance the ability of insulin to reach the insulin receptor (mobility effects). While mobility effects could be present, the rapid nature of the insulin signal under normal conditions and the increased expression of pAKT caused by the exudates even after 18 hours suggests enhancement of the "on" signaling proteins

and/or attenuation of those proteins associated with the "off" signal. Pyridazine analogues have been shown to be noncompetitive reversible inhibitors of PTP1b (protein tyrosine phosphatase 1 beta, which dephosphorylates many proteins including pAKT), resulting in increased insulin-stimulated insulin receptor phosphorylation in vitro.[50] Additionally, the synthetic vanadium compound TSAG0101 selectively inhibits PTP1b and resulted in blood glucose lowering effects in rats.[46] Thus, since compounds exist that act on proteins involved in insulin signaling, a natural compound with similar properties is plausible.

In rats fed a high fat diet, onion root exudates decreased fasting insulin levels and therefore improved whole body insulin action. In addition, onion root exudates increased pAKT relative to insulin in the liver. Improved pAKT is consistent with our in vitro data and suggests a similar mechanism of action. The observed decrease in plasma insulin levels implies that exudates may have insulin sensitizing properties in tissues other than the liver. Related to this, onion root exudates decrease plasma FFA levels. This implies that bioactive compounds present in these exudates influence adipose tissue lipolysis or FFA removal from plasma, and/or subsequent increased oxidation or storage. Epididymal and retroperitoneal fat pad weights were not significantly different in HFD rats provided saline or exudate. If reduced adipose tissue lipolysis was the mechanism by which exudates caused the observed reduction of FFA, one might expect an increase in adipose tissue mass. It is also possible that the reduction of lipolysis was not sufficient to expand fat pad mass, or that other fat depots, such as mesenteric fat,

that were not measure were increased. It is also unlikely that decreased FFA were the result of increased plasma removal of FFA and subsequent storage in non-adipose tissue. This notion is generally incompatible with improved whole body insulin sensitivity. Food intake was not different among groups; however, HFDS rats had significantly greater body weight gain than STDS and HFDE. This may be due to the fact that the HFD had a higher caloric density than the STD. Energy expenditure resulting in increased oxidation is another possible mechanism by which exudates reduced fasted FFA and overall bodyweight. Regardless, improved insulin action was observed in both H4IIE liver cells and rat liver treated with onion root exudates and mechanistically may involve enhanced signal longevity, increased activity-induced fat oxidation, or suppression of adipose tissue lipolysis.

Fasting plasma glucose levels were decreased in rats provided HFD and onion root exudates. Improved insulin action in the liver could account for this via enhanced insulin suppression of glucose production. We examined genes associated with the insulin signaling and gluconeogenic pathways in the liver. Hepatic gene expression of insulin signaling (IRS1, IRS2, AKT) and gluconeogenic (G6Pase, PEPCK) enzymes failed to show a statistically significant perturbation induced by the exudates. These data imply that onion root exudates are most likely not acting at the level of transcription, however since these measurements represent only a "snapshot", exudates may act transcriptionally at a different time point than was measured. Exudate treatment tended to lower G6Pase mRNA (protein expression could not be measured due to the

lack of available antibodies) and may be a mechanism by which exudates lower fasting plasma glucose levels. The liver is the primary site for glucose production and G6Pase is the final enzymatic step converting glucose-6-phosphate to glucose. It is well established that insulin potently inhibits G6Pase transcription.[52] Thus, exudate-mediated improvements in insulin signaling in the liver may explain the decrease in G6Pase mRNA. Further, if these changes also occur at the protein level, a reduction in glucose release by the liver could result.

Augusti et al. demonstrated that s-methyl cysteine sulfoxide treatment of alloxan diabetic rats reduced hyperglycemia. This effect was postulated to be due to a subtle insulin secretagogue effect on pancreatic β-cells, release of insulin from bound forms, or enhanced transport of blood glucose to peripheral tissue.[54-57] Since our results indicate that onion root exudates reduce both insulin and glucose in rats provided a HFD, a compound distinct from s-methyl cysteine sulfoxide is likely involved.

Future studies involving onion root exudates may elucidate the specific compounds and mechanisms by which they exert their bioactive effects. Additionally, manipulations to the growth conditions of the onion plants may allow for increased preferential exudation of these bioactive compounds so that the overall potency of the resulting exudates is enhanced. We have successfully demonstrated that onion root exudates elicit physiologic responses when injected; however, the efficacy of orally administered compounds is presently unknown. Unfortunately, before any future

studies can be attempted, there are a few major problems that must be addressed. First, since the initial in vitro and in vivo studies were from a pilot batch of exudates, documentation of the growth conditions and any manipulations of the exudates post harvest was lacking. Experiments on subsequent batches of exudates have provided variable results. At present, the reasons for this variability are unclear. It is possible that the bioactive compounds in onion root exudates can vary diurnally.[58] Until this is resolved, experiments to understand the significance and mechanisms of these initial observations cannot proceed.

Onion root exudates display therapeutic effects on indices of NAFLD, namely reduced oleate-mediated lipid accumulation in H4IIE liver cells, improved insulin sensitivity and reductions in fasting glucose and FFA levels. While some of these effects have been observed with onion extracts, others, such as the reduction of plasma insulin, may be unique to onion root exudates. If true, onion root exudates could provide a novel source of compounds for the treatment of NAFLD.

BIBLIOGRAPHY

1. Marchesini G, Bugianesi E, Forlani G, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*. 2003;37(4):917-923. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12668987.

2. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis*. 2007;191(2):235-240. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16970951.

3. Marchesini G, Moscatiello S, Di Domizio S, Forlani G. Obesity-associated liver disease. *J Clin Endocrinol Metab*. 2008;93(11 Suppl 1):S74-80. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18987273.

4. Barshop NJ, Francis CS, Schwimmer JB, Lavine JE. Nonalcoholic fatty liver disease as a comorbidity of childhood obesity. *Pediatric health*. 2009;3(3):271-281. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2885801&tool=pmcentrez&rendertype=abstract [Accessed December 21, 2010].

5. Pacifico L, Poggiogalle E, Cantisani V, et al. Pediatric nonalcoholic fatty liver disease: A clinical and laboratory challenge. *World journal of hepatology*. 2010;2(7):275-88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21161009 [Accessed December 21, 2010].

6. Libman IM, Arslanian SA. Prevention and treatment of type 2 diabetes in youth. *Hormone research*. 2007;67(1):22-34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17008794 [Accessed December 21, 2010].

7. Ehtisham S, Barrett TG. The emergence of type 2 diabetes in childhood. *Annals of clinical biochemistry*. 2004;41(Pt 1):10-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14713381 [Accessed December 21, 2010].

8. Schraer CD, Mayer AM, Vogt AM, et al. The Alaska Native diabetes program. *International journal of circumpolar health*. 2001;60(4):487-94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11768423 [Accessed December 21, 2010].

9. Narayanan ML, Schraer CD, Bulkow LR, et al. Diabetes prevalence, incidence, complications and mortality among Alaska Native people 1985-2006. *International*

journal of circumpolar health. 2010;69(3):236-52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20501061 [Accessed December 21, 2010].

10. Kurian AK, Cardarelli KM. Racial and ethnic differences in cardiovascular disease risk factors: a systematic review. *Ethnicity & disease*. 2007;17(1):143-52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17274224 [Accessed December 21, 2010].

11. Hoehner CM, Williams DE, Sievers ML, et al. Trends in heart disease death rates in diabetic and nondiabetic Pima Indians. *Journal of diabetes and its complications*. 20(1):8-13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16389161 [Accessed December 21, 2010].

12. Schulz LO, Bennett PH, Ravussin E, et al. Effects of traditional and western environments on prevalence of type 2 diabetes in Pima Indians in Mexico and the U.S. *Diabetes care*. 2006;29(8):1866-71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16873794 [Accessed June 23, 2010].

13. Oubré AY, Carlson TJ, King SR, Reaven GM. From plant to patient: an ethnomedical approach to the identification of new drugs for the treatment of NIDDM. *Diabetologia*. 1997;40(5):614-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9165233 [Accessed December 21, 2010].

14. Young TK, Reading J, Elias B, O□Neil JD. Type 2 diabetes mellitus in Canada□s first nations: status of an epidemic in progress. *CMAJ : Canadian Medical Association journal = journal de l□Association medicale canadienne*. 2000;163(5):561-6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=80466&tool=pmcentrez&rendertype=abstract [Accessed December 21, 2010].

15. Scott JD, Garland N. Chronic liver disease in Aboriginal North Americans. *World journal of gastroenterology : WJG*. 2008;14(29):4607-15. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2738784&tool=pmcentrez&rendertype=abstract [Accessed December 21, 2010].

16. Marles RJ FNR. Antidiabetic plants and their active constituents. *Phytomedicine*. 1995;2:137-189.

17. Srinivasan K. Plant foods in the management of diabetes mellitus: spices as beneficial antidiabetic food adjuncts. *Int J Food Sci Nutr*. 2005;56(6):399-414. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16361181.

18. Saxena A, Vikram NK. Role of selected Indian plants in management of type 2 diabetes: a review. *J Altern Complement Med*. 2004;10(2):369-378. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15165418.

19. Campos KE, Diniz YS, Cataneo AC, et al. Hypoglycaemic and antioxidant effects of onion, Allium cepa: dietary onion addition, antioxidant activity and hypoglycaemic effects on diabetic rats. *Int J Food Sci Nutr*. 2003;54(3):241-246. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12775373.

20. Walker TS, Bais HP, Grotewold E, Vivanco JM. Root exudation and rhizosphere biology. *Plant Physiol*. 2003;132(1):44-51. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12746510.

21. Badri DV, Vivanco JM. Regulation and function of root exudates. *Plant, Cell & Environment*. 2009;32(6):666-681. Available at: http://blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2009.01926.x [Accessed September 23, 2010].

22. B. Prithiviraj Jorge M. Vivanco MWP. Root Communication: The Role of Root Exudates . *Encyclopedia of Plant and Crop Science*. 2006.

23. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol*. 2006;57:233-266. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16669762.

24. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*. 1959;37(8):911-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13671378 [Accessed October 21, 2010].

25. Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology*. 2006;147(2):943-951. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16269465.

26. Gonzales JC, Gentile CL, Pfaffenbach KT, et al. Chemical induction of the unfolded protein response in the liver increases glucose production and is activated during insulin-induced hypoglycaemia in rats. *Diabetologia*. 2008;51(10):1920-9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2597049&tool=pmcentrez&rendertype=abstract [Accessed December 20, 2010].

27. Lowry O, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *The Journal of biological chemistry*. 1951;193(1):265-75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14907713 [Accessed December 20, 2010].

28. Gabler NK, Osrowska E, Imsic M, et al. Dietary onion intake as part of a typical high fat diet improves indices of cardiovascular health using the mixed sex pig model. *Plant foods for human nutrition (Dordrecht, Netherlands)*. 2006;61(4):179-85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17123162 [Accessed November 1, 2010].

29. Augusti KI, Roy VC, Semple M. Effect of allyl propyl disulphide isolated from onion (Allium cepa L.) on glucose tolerance of alloxan diabetic rabbits. *Experientia*. 1974;30(10):1119-1120. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4435106.

30. El-Demerdash FM, Yousef MI, El-Naga NIA. Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2005;43(1):57-63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15582196 [Accessed November 1, 2010].

31. Kumari K, Augusti KT. Lipid lowering effect of S-methyl cysteine sulfoxide from Allium cepa Linn in high cholesterol diet fed rats. *J Ethnopharmacol*. 2007;109(3):367-371. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16987625.

32. Kumari K, Augusti KT. Antidiabetic and antioxidant effects of S-methyl cysteine sulfoxide isolated from onions (Allium cepa Linn) as compared to standard drugs in alloxan diabetic rats. *Indian J Exp Biol*. 2002;40(9):1005-1009. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12587728.

33. Augusti KT, Benaim ME. Effect of essential oil of onion (allyl propyl disulphide) on blood glucose, free fatty acid and insulin levels of normal subjects. *Clin Chim Acta*. 1975;60(1):121-123. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1126028.

34. Adamu I, Joseph PK, Augusti KT. Hypolipidemic action of onion and garlic unsaturated oils in sucrose fed rats over a two-month period. *Experientia*. 1982;38(8):899-901. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7128726.

35. Augusti KT. Effect of alloxan diabetes of allyl propyl disulphide obtained from onion. *Naturwissenschaften*. 1974;61(4):172-173. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4833622.

36. Augusti KT. Therapeutic values of onion (Allium cepa L.) and garlic (Allium sativum L.). *Indian journal of experimental biology*. 1996;34(7):634-40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8979497 [Accessed December 20, 2010].

37. Augusti KT, Mathew PT. Effect of long-term feeding of the aqueous extracts of onion (Allium cepa Linn.) and garlic (Allium sativum Linn.) on normal rats. *Indian J Exp Biol*. 1973;11(3):239-241. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4782629.

38. Bobboi A, Augusti KT, Joseph PK. Hypolipidemic effects of onion oil and garlic oil in ethanol-fed rats. *Indian J Biochem Biophys*. 1984;21(3):211-213. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6519678.

39. Han S-young, Hu Y, Anno T, Yanagita T. S-propyl cysteine reduces the secretion of apolipoprotein B100 and triacylglycerol by HepG2 cells. *Nutrition (Burbank, Los Angeles County, Calif.)*. 2002;18(6):505-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12044824.

40. Helen A, Krishnakumar K, Vijayammal PL, Augusti KT. Antioxidant effect of onion oil (Allium cepa. Linn) on the damages induced by nicotine in rats as compared to alpha-tocopherol. *Toxicol Lett*. 2000;116(1-2):61-68. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10906423.

41. Helen A, Rajasree CR, Krishnakumar K, Augusti KT, Vijayammal PL. Antioxidant role of oils isolated from garlic (Allium sativum Linn) and onion (Allium cepa Linn) on nicotine-induced lipid peroxidation. *Vet Hum Toxicol*. 1999;41(5):316-319. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10509436.

42. Hounsome N, Hounsome B, Tomos D, Edwards-Jones G. Plant metabolites and nutritional quality of vegetables. *J Food Sci*. 2008;73(4):R48-65. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18460139.

43. Islam MS, Choi H, Loots du T. Effects of dietary onion (Allium cepa L.) in a high-fat diet streptozotocin-induced diabetes rodent model. *Ann Nutr Metab*. 2008;53(1):6-

12. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18772584.

44. Jung Y-S, Kim MH, Lee SH, et al. Antithrombotic effect of onion in streptozotocin-induced diabetic rat. *Prostaglandins, leukotrienes, and essential fatty acids*. 2002;66(4):453-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12054917 [Accessed November 1, 2010].

45. Han SY, Hu Y, Anno T, Yanagita T. S-propyl cysteine reduces the secretion of apolipoprotein B100 and triacylglycerol by HepG2 cells. *Nutrition*. 2002;18(6):505-509. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12044824.

46. Scior T, Guevara-García JA, Melendez FJ, et al. Chimeric design, synthesis, and biological assays of a new nonpeptide insulin-mimetic vanadium compound to inhibit protein tyrosine phosphatase 1B. *Drug design, development and therapy*. 2010;4:231-42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2948933&tool=pmcentrez&rendertype=abstract [Accessed December 17, 2010].

47. Daisy P, Balasubramanian K, Rajalakshmi M, Eliza J, Selvaraj J. Insulin mimetic impact of Catechin isolated from Cassia fistula on the glucose oxidation and molecular mechanisms of glucose uptake on Streptozotocin-induced diabetic Wistar rats. *Phytomedicine : international journal of phytotherapy and phytopharmacology*. 2010;17(1):28-36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19931438 [Accessed December 17, 2010].

48. Rao YK, Lee M-J, Chen K, et al. Insulin-mimetic Action of Rhoifolin and Cosmosiin Isolated from Citrus grandis (L.) Osbeck Leaves: Enhanced Adiponectin Secretion and Insulin Receptor Phosphorylation in 3T3-L1 Cells. *Evidence-based complementary and alternative medicine : eCAM*. 2009. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20008903 [Accessed December 17, 2010].

49. Schlein M, Ludvigsen S, Olsen HB, et al. Properties of small molecules affecting insulin receptor function. *Biochemistry*. 2001;40(45):13520-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11695899 [Accessed December 17, 2010].

50. Liljebris C, Martinsson J, Tedenborg L, et al. Synthesis and biological activity of a novel class of pyridazine analogues as non-competitive reversible inhibitors of protein tyrosine phosphatase 1B (PTP1B). *Bioorganic & medicinal chemistry*. 2002;10(10):3197-212. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12150865 [Accessed December 17, 2010].

51. Jung SH, Ha YJ, Shim EK, et al. Insulin-mimetic and insulin-sensitizing activities of a pentacyclic triterpenoid insulin receptor activator. *The Biochemical journal*. 2007;403(2):243-50. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1874232&tool=pmcentrez&rendertype=abstract [Accessed October 21, 2010].

52. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of clinical investigation*. 2002;109(9):1125-31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=150968&tool=pmcentrez&rendertype=abstract [Accessed July 4, 2010].

53. Streeper RS, Eaton EM, Ebert DH, et al. Hepatocyte nuclear factor-1 acts as an accessory factor to enhance the inhibitory action of insulin on mouse glucose-6-phosphatase gene transcription. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95(16):9208-13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=21317&tool=pmcentrez&rendertype=abstract [Accessed December 20, 2010].

54. Kumari K, Mathew BC, Augusti KT. Antidiabetic and hypolipidemic effects of S-methyl cysteine sulfoxide isolated from Allium cepa Linn. *Indian J Biochem Biophys*. 1995;32(1):49-54. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7665195.

55. Hellman B, Lernmark A, Sehlin J, Soderberg M, Taljedal IB. On the possible role of thiol groups in the insulin-releasing action of mercurials, organic disulfides, alkylating agents, and sulfonylureas. *Endocrinology*. 1976;99(5):1398-1406. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=186256.

56. Jain RC, Vyas CR. Garlic in alloxan-induced diabetic rabbits. *Am J Clin Nutr*. 1975;28(7):684-685. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1146717.

57. Chang ML, Johnson MA. Effect of garlic on carbohydrate metabolism and lipid synthesis in rats. *J Nutr*. 1980;110(5):931-936. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6989965.

58. Badri DV, Loyola-Vargas VM, Broeckling CD, Vivanco JM. Root secretion of phytochemicals in Arabidopsis is predominantly not influenced by diurnal rhythms. *Molecular plant*. 2010;3(3):491-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20154222 [Accessed January 22, 2011].

FIGURE LEGENDS

Figure 1. Onion root exudates prevent lipid accumulation and enhance insulin signaling in liver cells. H4IIE liver cells were grown in 6 ml of growth media (S) or exudate-reconstituted growth media (E) per plate. Cells were treated overnight in the presence or absence of (A) oleate (500uM) or (B) insulin (10nM). Data are presented as the mean ± SDEV for n=3 (A) or n=8 (B). *, significantly ($p<0.05$) different from low-glucose controls; +, significantly ($p<0.05$) different from oleate or insulin controls.

Figure 2. Onion root exudates decrease glucose, insulin, and FFA levels and increase pAKT. Plasma (A) glucose, (B) insulin, (C) free fatty acid (FFA), and (D) insulin signaling (phosphorylation of AKT relative to plasma insulin levels) in overnight fasted rats following 4 wks of ad libitum exposure to a starch control diet (STD) or high fat diet (HFD) and daily injections of either saline (S) or onion exudates (E). Data are presented as the mean ± SDEV for n=8-9. *, significantly ($p<0.05$) different from STDS. #, significantly ($p<0.05$) different from HFDS. $, significantly ($p<0.05$) different from HFDE.

Figure 3. Hepatic expression of select insulin signaling and gluconeogenic genes. Real Time PCR analysis of hepatic IRS1, IRS2, AKT, G6Pase, and PEPCK. Data are presented as the mean ± SDEV for n=8. *, significantly ($p<0.05$) different from STDS. #, significantly ($p<0.05$) different from HFDS.

Table 1: General and Metabolic Characteristics

VARIABLE	STDS	HFDS	HFDE
Daily Food Intake, g	19.73±0.61	21.24±1.43	20.07±0.86
Daily Exudate Intake, mg/rat	0	0	10.7+
Initial Body Weight, g	137.0±4.1	163.1±12.9*	159.8±6.0*
Weight Gain, g	223.1±12.3	256.9±20.3*	236.0±22.4#
Epidydymal Fat, g	6.12±0.68	7.96±1.21*	7.41±2.00
Retroperitoneal Fat, g	5.12±1.06	7.32±1.47	7.17±2.05

Table 1. General and metabolic characteristics of rats fed either Starch Control Diet with daily saline intraperitoneal injection (STDS, n=6), High Fat Diet with daily saline intraperitoneal injection (HFDS, n=8), or High Fat Diet with daily exudate intraperitoneal injection (HFDE, n=8). +, the amount of exudate is estimated to be 10.7 mg/rat/day. *, significantly ($p<0.05$) different from STDS. #, significantly ($p<0.05$) different from HFDS.

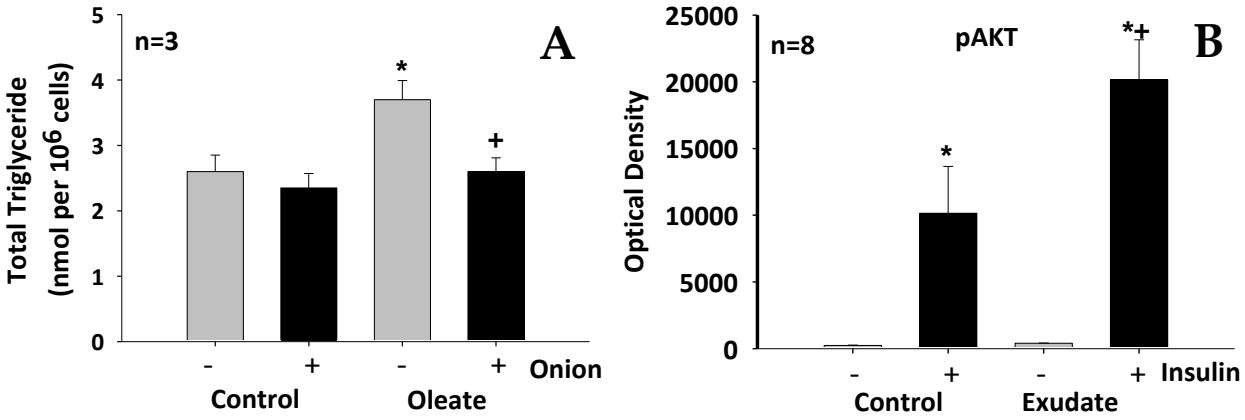

n=3
A
Total Triglyceride (nmol per 10^6 cells)
5
4
3
2
1
0
*
+
- + - + Onion
Control
Oleate
n=8
pAKT
B
*+
Optical Density
25000
20000
15000
10000
5000
0
*
- + - + Insulin
Control
Exudate

Figure 1.

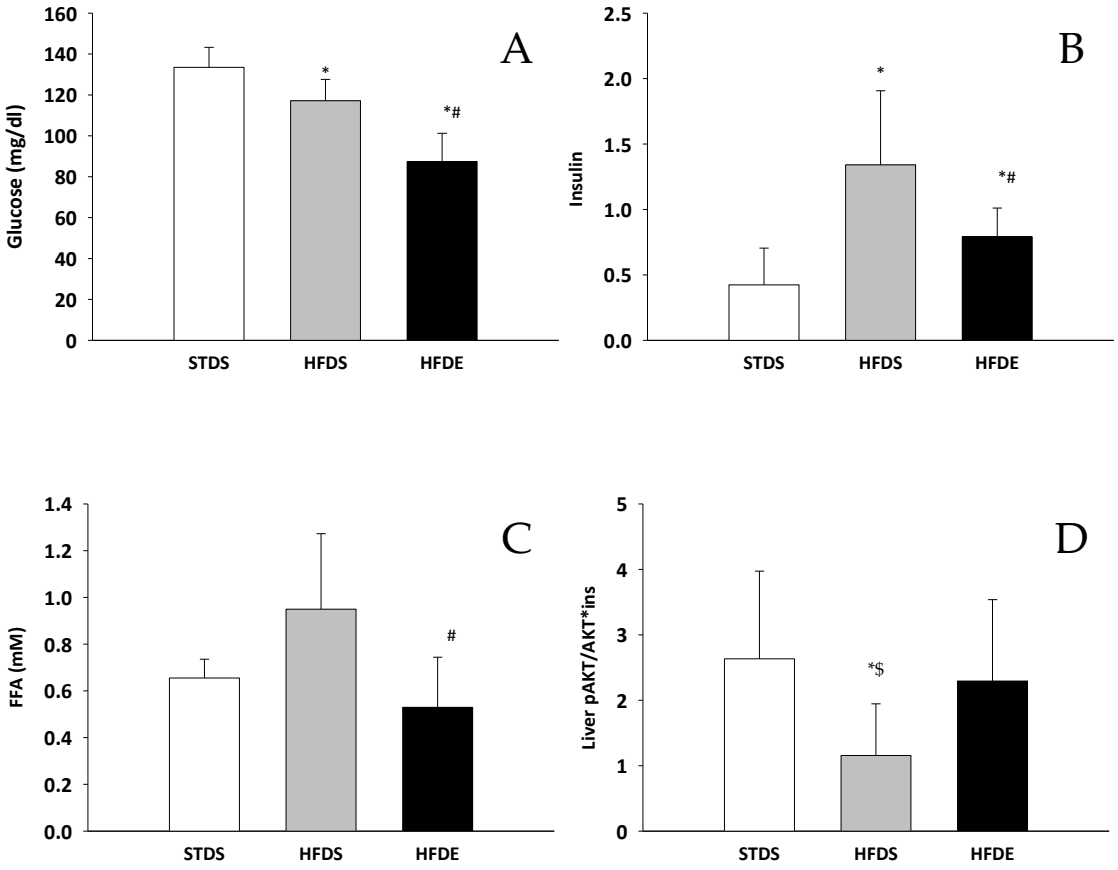

A
Glucose (mg/dl)
160
140
120
100
80
60
40
20
0
*
*#
STDS
HFDS
HFDE
B
Insulin
2.5
2.0
1.5
1.0
0.5
0.0
*
*#
STDS
HFDS
HFDE
C
FFA (mM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
STDS
HFDS
HFDE
D
Liver pAKT/AKT*ins
5
4
3
2
1
0
*$
STDS
HFDS
HFDE

Figure 2.

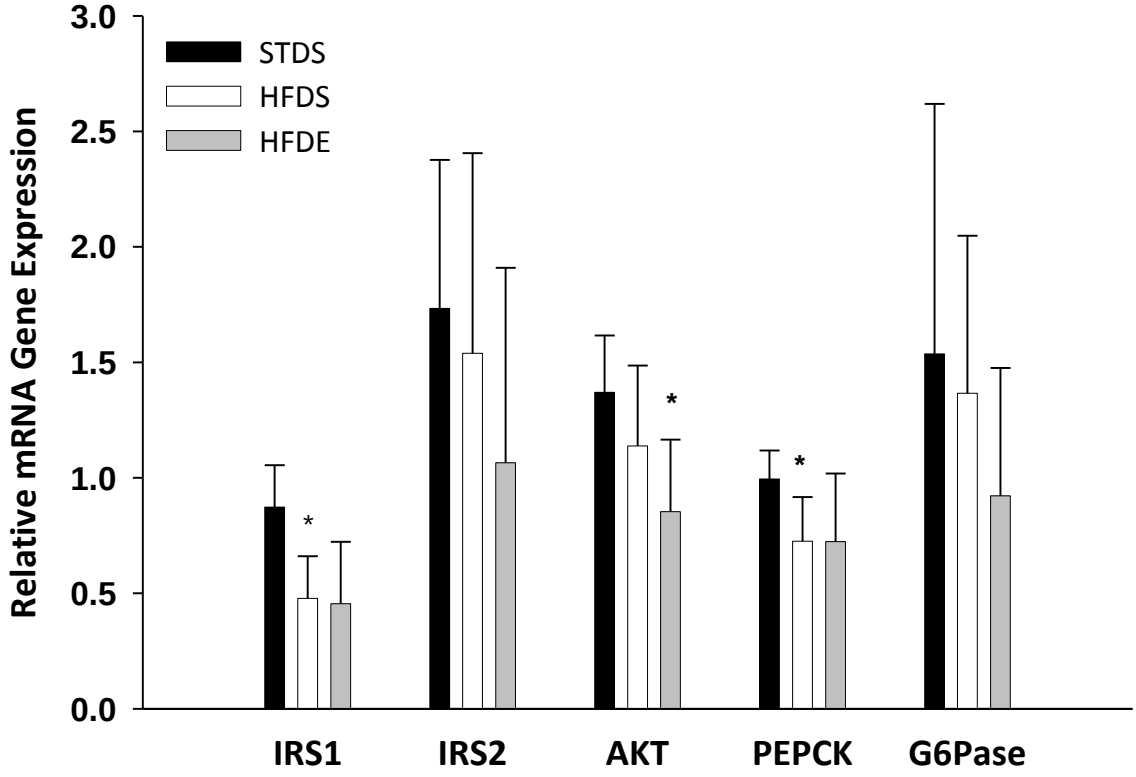

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative mRNA Gene Expression
STDS
HFDS
HFDE
*
*
*
IRS1
IRS2
AKT
PEPCK
G6Pase

Figure 3.

CHAPTER 4

THE PROTECTIVE EFFECTS OF TAURINE ON NUTRIENT-INDUCED HEPATIC ENDOPLASMIC STRESS, OXIDATIVE STRESS AND CELL DAMAGE

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is a burgeoning metabolic disorder characterized by fatty infiltration of the liver (steatosis) with progression, in some, to non-alcoholic steatohepatitis (NASH) and liver failure.[1,2] Presently, NAFLD is estimated to affect upwards of 24% of the general population, as well as 3–9% of all children in the United States.[3,4] Of great concern are statistics indicating that, within 10 years of diagnosis, nearly 20% of the patients presenting with NASH progress to cirrhosis.[5-7] Recent data also suggest that NAFLD is independently associated with the development of cardiovascular disease and overall-obesity-related mortality.[8-11] Thus, in light of these data, there is a critical need to elucidate the mechanisms that mediate the development and progression of NAFLD, and to identify potential therapies for the disease.

Although the pathogenesis of NAFLD remains uncertain, it has been suggested that various factors, including oxidative stress, pro-inflammatory cytokines, gut-derived bacterial endotoxin, and disruptions in lipid metabolism may contribute to the

progression of the disease.[12] Accumulation of lipids in non-adipose tissues can lead to cell dysfunction such as insulin resistance and cell death, a phenomenon known as lipotoxicity.[13] Experimental and clinical data indicate that excess fatty acids, in particular long chain saturated fatty acids, are an important determinant of liver cell integrity, liver function, and potentially, an independent risk factor for the progression from NAFLD to NASH.[14-16]

An accumulating body of literature suggests that an important factor linking excess fatty acids to liver damage is dysfunction of the endoplasmic reticulum (ER). One of the largest subcellular organelles, the ER plays an important role in the correct assembly of proteins destined for intracellular organelles and the cell surface.[17] We and others have demonstrated that saturated fatty acids induce markers of ER stress, an indicator of disrupted ER homeostasis, in liver cells.[18,19] We have also demonstrated that diets which produce hepatic steatosis characterized by increased saturated fatty acids (e.g. high saturated fat or high sucrose diets) lead to ER stress and liver injury.[14]

Furthermore, multiple markers of ER stress have been observed in livers taken from genetic models of obesity[20] and in livers and adipose tissue from obese patients with NAFLD.[21,22] There is no proven, effective therapy for NAFLD; thus, lifestyle modifications, similar to those recommended for obesity, remain the primary treatment option.[23] However, this option remains fraught with problems of compliance and there

is a pressing need for therapeutic strategies that might prevent NAFLD in the continuing presence of high-fat and/or high sugar diets.

Taurine, 2-aminoethanesulfonic acid, is a major intracellular amino sulfonic acid with diverse physiological functions. Taurine supplementation may be beneficial in preventing various metabolic disorders, including obesity, insulin resistance, and atherosclerosis.[24,26] Increasing evidence also suggests that taurine may have beneficial effects on NAFLD.[27] Also, taurine supplementation prevents alcoholic fatty liver disease in experimental animals,[28] and mice characterized by hetero- or homozygous knockout of the taurine transporter develop chronic liver disease characterized by fibrosis, inflammation and hepatocyte apoptosis.[29] Plasma taurine levels are also decreased in various forms of liver cirrhosis.[30,31] Interestingly, the beneficial effects of taurine are often accompanied by reductions in ER stress, suggesting a link between the therapeutic properties of taurine and restoration of ER homeostasis.[32-34]

The present study was undertaken to examine the effects of taurine supplementation on cell and animal models of NAFLD with a view to assessing its potential as a preventative treatment. Our findings demonstrate that taurine mitigates palmitate-induced ER stress and apoptosis in primary hepatocytes and H4IIE liver cells. Taurine reduced ER and oxidative stress in sucrose-fed rats and ER stress and lipid accumulation in tunicamycin-treated mice. Collectively, our results indicate that part of the protective effects of taurine in diet-induced NAFLD are related to the amelioration

of ER stress and that dietary supplementation with this compound offers significant potential as a preventative treatment for this disease.

METHODS

EXPERIMENTAL AGENTS

Fatty acids (Sigma Chemical Co., St. Louis, MO) were complexed to bovine serum albumin at a 2:1 molar ratio.[35] Taurine and tunicamycin were purchased from Sigma.

CELL CULTURE

The rat hepatoma liver cell line, H4IIE, (American Type Culture Collection, Manassas, VI) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 8 mM glucose and supplemented with 10% fetal bovine serum, penicillin and streptomycin sulfate. Experiments were performed at 80-100% cell confluence. In preparation for primary cell culture, hepatocytes were isolated from male, Wistar rats (Charles River Laboratories, Wilmington, MA) by collagenase perfusion.[36,37] All procedures involving rats were reviewed and approved by the Colorado State University Institutional Animal Care Committee. Cells were first incubated in Roswell Park Memorial Institute media (RPMI; HyClone, Logan, UT) containing 11 mM glucose, 10^{-7} M dexamethasone, and 10^{-7} M insulin on Matrigel- coated plates (for RNA) or on collagen-coated plates containing 5% fetal bovine serum (for protein) for 4h (attachment period). The medium was then changed to one containing RPMI, 8 mM glucose, 10^{-7} M dexamethasone, and 10^{-8} M insulin. The following morning experimental treatments were performed using RPMI that contained 8 mM glucose and 10^{-7} M dexamethasone

(control low glucose medium, LG). Each independent experiment was performed in triplicate.

ANIMALS

Male Wistar Crl(WI)BR rats (Charles River, Wilmington, MA) weighing 180 g (7–8 wk of age) on arrival were provided free access to a purified high-starch diet (68% of energy from cornstarch, 12% from corn oil, and 20% from casein; Research Diets, New Brunswick, NJ)[38] and water for 1 wk. Rats were housed individually in a temperature- and humidity-controlled environment with a 12-h light, 12-h dark cycle. All procedures were reviewed and approved by the Colorado State University institutional animal care committee. After the 1 wk acclimation period, rats were fed either the high starch diet (STD) or a high sucrose diet (HSD; 68% of energy from sucrose, 12% from corn oil, 20% from casein; Research Diets, New Brunswick, NJ)[38] for 4 weeks. Dietary groups were randomly assigned to either normal drinking water (n=6 per diet) or to drinking water that contained taurine (2% w/v; n=6 per diet). Water and food intake were measured every other day and body weight was measured weekly.

Male, C57BL/6J mice (7-11 wks) were housed in colony cages and maintained on a 12-hour light/dark cycle. Mice were randomly assigned to one of four treatment groups (n=6 for each group): 1) control, IP injected with PBS; 2) taurine, mice were provided taurine (2.0% w/v added to the drinking water) for one week prior to sham injection with PBS; 3) tunicamycin, IP injected with tunicamycin) and 4) tunicamycin + taurine, mice were provided taurine (2.0% w/v added to the drinking water) for one

week prior to IP injection with tunicamycin. Previous work has shown that the dose of tunicamycin used (0.5 mg/kg body weight) results in lassitude, lack of grooming, weight loss, liver injury and hepatic steatosis that peaks between day 4 and 5 post-injection.[39] In groups 2 and 4, taurine treatment was maintained until sacrifice at four days post vehicle/tunicamycin injection. Four days post injection, all mice were anaesthetized (isoflourane/oxygen) and sacrificed by decapitation, and blood samples were collected for plasma analysis. Livers were removed, weighed and then samples were either immersion-fixed overnight in 4% paraformaldehyde in PBS (pH 7.3) for subsequent histological analysis or were snap frozen in liquid nitrogen and examined for morphological, histological and biochemical analyses. The animal care and procedures were approved by the Animal Care and Use Committee of the University of Colorado at Denver Health Sciences Center and the Guide for the Care and use of Laboratory Animals prepared by the National Academy of Sciences.

RNA Isolation and Analysis

Total RNA was extracted with TRIzol reagent using the manufacturer's protocol (Invitrogen, Carlsbad, CA). For analysis of XBP1 splicing, a two-step protocol was used for reverse transcription polymerase chain reaction (PCR) using Superscript II reverse transcriptase and Taq polymerase.[36] For Real Time PCR, reverse transcription was performed using 0.5 µg of DNase-treated RNA, Superscript II RnaseH- and random hexamers. PCR reactions were performed in 96 well plates using transcribed cDNA and IQ-SYBR green master mix (Bio Rad, Hecula, CA). Primer sets can be found in a previous publication.[35] PCR efficiency was between 90-105% for all primer and probe sets and

linear over 5 orders of magnitude. The specificity of products generated for each set of primers was examined for each fragment using a melting curve and gel electrophoresis. Reactions were run in triplicate and data calculated as the change in cycle threshold (ΔCT) for the target gene relative to the ΔCT for β2-microglobulin and cyclophilin (control genes) according to the procedures of Muller et al.[40] Results were similar regardless of the control gene; therefore data in the results section are reported using β2-microglobulin and normalized to 18S mRNA.

Immunoblot Analysis

Cells and liver tissue were processed as described previously.[14] Equivalent amounts of protein (50μg) were subjected to SDS-PAGE, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and the membranes incubated with antibodies against glucose regulated protein 78 (GRP78; Stressgen, Ann Arbor, MI), phospho-eukaryotic initiation factor 2-α (p-eIF2α; Cell Signaling, Waverly, MA), eIF2α (Cell Signaling), C/EBP homologous protein (CHOP; Santa Cruz Biotechnology, Santa Cruz, CA) and/or beta-actin (Sigma). Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence reagent (Pierce, Rockford, IL). Density was quantified using a UVP Bioimaging system (Upland, CA).

Determination of Caspase Activity and Cell Death

Activity of the caspase-3 class of cysteine proteases was determined with the Colorimetric Caspase-3 Activation Assay, which uses a caspase-specific peptide that is

conjugated to the color reporter molecule p-nitroanaline (R & D Systems, Minneapolis, MN). Caspase activity was normalized to cell lysate protein concentration. Cell death was examined using the Cell Death Detection ELISA kit (Roche Diagnostics, Penzberg, Germany). The assay is based on the quantitative sandwich-enzyme immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones. This allows specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates.

DCF Fluorescence and Protein Carbonyl Formation

Oxidative stress was estimated using 2,7- dichlorofluorescein di-acetate (DCFH-DA).[41] This assay is based on the ability of DCFH-DA to diffuse across the cell membrane and, following enzymatic hydrolysis to DCFH, oxidized to the fluorescent compound DCF (2',7'-dichlorodihydrofluorescein). Following treatments, cells were loaded with 5 µM DCFH-DA (Molecular Probes), using serum free media, for 45 min at 37ºC.[42] Fluorescence was monitored with excitation and emission wavelengths of 490 and 535 nm, respectively. Data are reported as the fold increase in median fluorescence over control cells. Protein carbonyls were measured in H4IIE liver cells using an ELISA-based kit (Biocell, New Zealand). Briefly, protein samples were reacted with dinitrophenylhydrazine (DNP) at room temperature for 45 min, after which time samples were rinsed and a blocking solution was added for 1 h. Anti-DNP antibody and, subsequently, a secondary antibody, were both added for 1 h. Finally, substrate buffer was added for 10 min, and absorbance was measured at 405 nm.

HISTOLOGY

Paraffin embedded sections were stained with hematoxylin and eosin to evaluate steatosis and inflammatory infiltrate.

IMMUNOHISTOCHEMISTRY

Paraffin embedded liver samples were cut as 4-µm-thick serial sections. The unfolded protein response (UPR) was analysed using an anti-KDEL monoclonal antibody (SPA-827), which recognizes both glucose regulated protein (GRP)78/BiP and GRP94 (StressGen Biotechnologies Corp. Ann Arbor, MI). The anti-CHOP polyclonal antibody (sc-575) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Anti-KDEL was diluted 1/50 and used without antigen retrieval. Rabbit anti-CHOP polyclonal antibody was diluted 1/40 and applied after heat retrieval in Retrieve-all 2 (Signet Laboratories) at 95°C for 30 minutes and a 10-minute incubation in 0.1% Triton X. Sections were deparaffinized, and the endogenous peroxidase activity was blocked with 0.5% H2O2 in methanol for 10 minutes. After blocking with 5% normal goat serum, sections were incubated with primary antibody for 1 hour at room temperature, followed by goat antirabbit, or rabbit anti-mouse biotinylated secondary antibodies (Vector Laboratories), diluted 1/500 in 0.05 mol/L Tris buffer, pH 7.5, for 30 minutes, and streptavidin-peroxidase (Zymed Laboratories), diluted 1/20, for 5 minutes. Sections were developed in Nova Red peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin. Nonspecific immunostaining was not detected in sections stained with nonimmune IgG as the primary antibody or with the secondary antibody alone.

HEPATIC LIPIDS

Liver lipid was extracted using the procedure of Bligh and Dyer.[43] Triglyceride concentration was determined using a kit (Sigma).

PLASMA MEASURES

Glucose was measured with an automated analyzer (Beckman Instruments, Fullerton, CA). Insulin and C-reactive protein (CRP) were analyzed by ELISA (Linco Research, St. Charles, MO and Helica, Fullerton, CA, respectively). Plasma tumor necrosis factor (TNF)-alpha levels were determined by ELISA (R and D systems). In order to assess the level of oxidative stress, plasma levels of reactive aldehydes formed by lipid peroxidation were determined using a thiobarbituric acid reactive substances (TBARS) assay. TBARS determinations were carried out with an OXI-TEK TBARS Assay Kit (Alexis Biochemicals, San Diego, California, USA) according to the manufacturer's standard protocol with modifications as described previously.[44] Results were expressed as nmol malondialdehyde (MDA) equivalents/mL for plasma samples. Plasma triglycerides were determined enzymatically (Sigma Company, St. Louis, MO) and free fatty acid levels were determined using the WAKO NEFA-C kit (Richmond, VA). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed using kits (ThermoDMA, Arlington, TX).

Data Analysis and Statistics

Statistical comparisons were calculated using analysis of variance and post-hoc comparisons among means using the Scheffe's or Tukey's test. Statistical significance was set at $p<0.05$. All data are reported as the means ± SDEV.

Results

Effects of Taurine on Palmitate-Mediated Cell Death in Liver Cells

We have previously reported that saturated fatty acids, but not unsaturated fatty acids, cause liver cell death.[35,45] Palmitate- mediated cell death appears to be caspase-dependent and is consistently present after 16 h but not 6 h incubations.[35,45] In the present study, we first examined the effects of taurine on palmitate mediated caspase-3 activity and liver cell death. The unsaturated fatty acid oleate was used as a negative control. Taurine significantly reduced palmitate-mediated induction of caspase-3 activity and cell death in H4IIE liver cells and primary hepatocytes following 16 h incubations at palmitate concentrations of 500 µM, but not at 250 µM (Figure 1). Taurine did not significantly affect the cellular utilization of glucose or free fatty acids based on the net loss of these nutrients from the medium (data not shown).

Effects of Taurine on Palmitate-Mediated ER Stress in Liver Cells

Disruption of ER homeostasis, collectively termed ER stress, activates the UPR.[46] The UPR is initiated by three ER transmembrane proteins, inositol requiring ER-to-nucleus signaling protein 1α (IRE1α), RNA dependent protein kinase-like ER eukaryotic initiation factor-2α kinase (PERK) and activating transcription factor-6 (ATF6).[47]

Activation of IRE1α promotes the splicing of X-box-binding protein-1 (Xbp1) mRNA and subsequent transcription of molecular chaperones (e.g. GRP78) and genes involved in ER-associated degradation.[48] PERK activation leads to phosphorylation of the α-subunit of the translation initiation factor eIF2 and subsequent attenuation of translation initiation, as well as increased expression of GRP78, CHOP, a pro-apoptotic gene, and growth arrest and DNA damage-inducible protein 34 (GADD34).[48,49] GADD34 mediates dephosphorylation of eIF2α and therefore reversal of translational attenuation.[47] Activation of ATF6 can also lead to increased expression of both molecular chaperones and CHOP. Taurine reduced, but did not prevent, the palmitate-mediated increase in gene and protein markers of ER stress and UPR activation in H4IIE liver cells (Figure 2) and primary hepatocytes (Figure 3). Taurine had little or no effect on palmitate-mediated Xbp1 splicing in either cell type (Figures 2 and 3).

Effects of Taurine on Palmitate-Mediated Oxidative Stress

Reduced oxidative stress has been identified as a potential mechanism by which taurine exerts its protective effects. The anti-oxidant properties of taurine include the ability to scavenge reactive oxygen species and reduce lipid peroxidation.[50,51] Thus, we next examined the effects of taurine on palmitate-mediated oxidative stress. Palmitate, but not oleate, increased ($p<0.05$) DCF fluorescence in both H4IIE liver cells (Figure 4A) and primary hepatocytes (Figure 4B), and these effects were prevented by co-incubation with taurine. Time course studies revealed that the palmitate-mediated increase in DCF fluorescence was observed after 2 h and was maximal after 6 h (data not shown). Dose-response studies demonstrated that 0.25 and 0.5% taurine did not effectively reduce

DCF fluorescence (data not shown). Additionally, palmitate, but not oleate, increased protein carbonyls, and the presence of taurine significantly reduced this effect in H4IIE liver cells (Figure 4C).

Effects of Taurine on High Sucrose Diet-Induced Hepatic Steatosis, ER Stress, Inflammation and Liver Injury

To date, the role of dietary fat and blood lipids in obesity and NAFLD/NASH have probably attracted the majority of attention. However, it is important to realize that carbohydrate, the macronutrient that typically tends to increase as a percentage of total caloric intake when fats are restricted, may not be a totally desirable nutrient either.[14,52] Previous work in our laboratory has shown that a high sucrose diet induces hepatic steatosis characterized by increased saturated fatty acids, ER stress, liver injury and insulin resistance in rats.[14,53,54] It is possible that excess dietary fat and excess dietary sucrose exert similar pathogenic effects in the liver but through functionally distinct mechanisms. In order to further investigate the therapeutic potential of taurine in NAFLD/NASH, we examined the effects of taurine supplementation in a rat model of high sucrose diet-induced NAFLD. Taurine supplementation in the drinking water of rats provided the high sucrose diet significantly ($p<0.05$) reduced plasma insulin, and plasma triglycerides (Table 1). Hepatic triglycerides were reduced by 21% (Table 1; and the neutral lipid pool was clearly reduced based on oil-red O staining, Supplemental Figure 1). Taurine also reduced gene and protein markers of ER stress (Figure 5, Supplemental Figure 2), oxidative stress (plasma TBARS (Fig. 6A)), and inflammation (TNFα (Figure 6B), CRP (Figures 6C and D). Taurine conferred significant protective effects with regard to

liver injury as assessed by plasma ALT and AST levels (Figures 6E and F) in rats provided the high sucrose diet compared to the relevant controls. Collectively, this data indicates that taurine supplementation has significant therapeutic potential in preventing NAFLD and liver injury induced by excessive consumption of sucrose.

Effects of Taurine on High Sucrose Diet-Induced Hepatic Gene Expression

In an effort to determine potential targets of taurine under conditions of high sucrose diet, we examined a number of genes whose protein products affect pathways implicated in NAFLD. The following representative genes were screened: ER/UPR stress (GRP78, GADD34, Chop), inflammation (SAP, CRP), lipogenesis (FAS, ACCα, ACCβ, SREBP1c, SCD1, ChREBP), fatty acid oxidation (CPT1α, PGC1α, PPARα) and fatty acid transport (FABP1, FABP5). Of these, ACCα (Figure 7B), CRP (Figure 6C), GRP78 (Figure 5A), GADD34 (Figure 5B), and Chop (Figure 5C) were significantly reduced in HSD fed rats treated with taurine. Although FAS followed this trend (Figure 7A) it was not statistically significant.

Effects of Taurine on Tunicamycin Induced Hepatic Steatosis, Liver Injury and UPR Induction

The experiments described above indicate that taurine may exert protective effects in diet induced NAFLD by ameliorating oxidative stress, inflammation and ER stress. A major complication to our understanding of the protective mechanisms of taurine supplementation is the fact that these pathogenic features are interrelated phenomena, with each capable of inducing the other.[55,56] In an effort to dissect the protective mechanisms of taurine in NAFLD, we used an animal model of tunicamycin

induced steatosis and liver injury as described in the materials and methods. Tunicamycin causes ER stress and induction of the UPR by inhibiting UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-P transferase, and thereby blocking protein N-glycosylation. In this model, ER stress is the primary initiating factor and any resultant oxidative stress and/or inflammation will be downstream consequences of the treatment. Tunicamycin injection resulted in hepatic steatosis and concomitant increases in liver weight, plasma ALT and liver lipid content (Figure 8). Hepatic lipid accumulation and liver injury were significantly attenuated by taurine supplementation (Figure 8). The effect of taurine upon tunicamycin mediated induction of the UPR was analyzed by immunohistochemistry. This analysis revealed that taurine supplementation reduced the induction of proteins containing the KDEL signal sequence and completely ablated tunicamycin induced expression of CHOP (Supplemental Figure 3). These results are consistent with the possibility that taurine exerts hepatoprotective effect in NAFLD at least in part by reducing ER stress.

DISCUSSION

This study provides insight into the therapeutic role of taurine in metabolic dysfunction associated with hepatic steatosis, which is often present in conditions of obesity, type 2 diabetes, and the metabolic syndrome. Taurine is a small amino sulphonic acid which can be obtained through the diet or synthesized endogenously from methionine and cysteine.[57] Taurine aids in numerous physiological processes, including bile salt formation, osmoregulation, central nervous system function, and calcium homeostasis.[58] The present study demonstrates that taurine reduces nutrient-

(palmitate, high sucrose diet) and chemically-(tunicamycin) induced hepatic steatosis, ER stress, inflammation and injury. Importantly, the beneficial effects of taurine were consistent across four models: palmitate incubation of isolated primary hepatocytes and H4IIE liver cells, long-term (4-wk) high sucrose feeding in rats and acute tunicamycin injection in mice.

Other studies have also shown protective effects of taurine in models of diabetes, atherosclerosis, obesity, and a variety of forms of liver injury including steatosis. This study adds to the literature in suggesting possible mechanisms by which taurine exerts its beneficial effects in preventing dysfunction. For example, substantial evidence indicates that taurine protects a wide variety of cells from oxidative damage by increasing antioxidant defense systems,[33,59] decreasing the formation of ROS,[60,61] and interfering with ROS activity.[62,63] In the present study, taurine reduced 1) palmitate-mediated protein carbonyl formation in H4IIE liver cells, which is elevated in patients suffering from NAFLD or type 2 diabetes;[64-66] 2) palmitate-mediated DCF fluorescence in H4IIE liver cells and primary hepatocytes; and 3) reduced plasma TBARS in high sucrose diet fed rats. Collectively these results suggest that taurine, directly or indirectly, can mitigate oxidative stress in the liver.

The ER provides a unique oxidizing environment for protein folding and disulfide bond formation. Each disulfide bond formed during oxidative protein folding produces a single reactive oxygen species. It has been estimated that secretory cells produce 3-6

million disulfide bonds per minute, thus it has been postulated that protein folding in the ER is intimately linked to oxidative stress.[56,67,68] Indeed, oxidants not only provoke ER stress but also are signals generated by misfolded proteins in the ER that then activate the UPR and can lead to cell death.[56,67,68] In the present study, taurine reduced or prevented palmitate-, sucrose-, and tunicamycin-mediated ER stress and UPR activation. These results are consistent with recent reports indicating that taurine mitigates ER stress in lung tissue and vascular cells following various insults,[32-34] and suggest that attenuation of ER stress may be an important mechanism by which taurine leads to reduced inflammation, liver injury and cell death.

High sucrose diets are characterized by their ability to induce hepatic steatosis primarily through lipogenesis. Interestingly, this diet also results in the induction of the UPR, which has recently been linked to lipid biosynthesis control, fatty acid oxidation, and lipoprotein secretion.[69-72] A recent study showed that taurine reduced the expression of genes involved in de novo lipogenesis (e.g. Scd2, Elovl6, ACCa, and FAS) in ob/ob mice.[73] Our high sucrose diet study may further support this observation in that initial RNA screening showed a significant reduction in ACCa mRNA and a trend towards reduction in FAS mRNA. In the livers of high carbohydrate diet fed mice, activation of the IRE1a/XBP-1 UPR branch directly controlled expression of genes involved in fatty acid biosynthesis, including ACC.[74,75] It is therefore possible that the ability of taurine to reduce hepatic steatosis is due to attenuation of ER stress and therefore UPR activation.

Chronic ER stress and activation of the UPR has been linked to impairments in insulin action, activation of inflammatory cascades and the innate immune response, and apoptosis.[76-78] Thus, it is attractive to suggest that the ability of taurine to reduce palmitate-mediated cell death, inflammation (based on TNFα and CRP) and liver injury results from a centralized effect on ER homeostasis. However, a few observations from the present study suggest that the effects of taurine may be more complex. First, in liver cells taurine reduced ER stress markers at both concentrations of palmitate but significant reductions in caspase-3 activity and cell death were only observed at the higher concentration of palmitate. Thus, chronic ER stress and UPR activation are clearly not the only mediators of palmitate-mediated cell death. Second, taurine did not reduce palmitate-mediated Xbp1 splicing. Although this may be due to the inability to detect small changes in Xbp1 splicing it suggests that the IRE1 branch of the UPR may remain active in the presence of taurine. The ability to maintain activation of this branch of the UPR has been linked to cell survival,[79] thus the maintenance of Xbp1 splicing in cells may be linked to reduced cell death.

Despite dietary and lifestyle recommendations, nutrient excess-related disorders such as obesity and NAFLD are on the rise. Given the therapeutic potential of taurine to alleviate metabolic impairments associated with these diseases, supplementation could have advantages on the health of those afflicted, with the additional benefit of reduced healthcare costs. Taurine is already supplemented in infant total parenteral nutrition (TPN) due to its ability to reduce impairments in liver, brain and retinal

development.[80,81] Adult supplementation, in particular to those at risk for metabolic diseases, does not seem so farfetched in that it is inexpensive, nontoxic, and can be orally administered.

There are some limitations of the current study that should be noted. In regards to DCF fluorescence as a marker of oxidative damage, we cannot rule out the possibility that reactive species that do not react with DCFH may remain operative in the presence of taurine and therefore we have underestimated the contribution of oxidative stress to palmitate-mediated cell damage and death. Also, the use of this probe to estimate oxidative stress during apoptosis should be approached with caution, as cytochrome c is a powerful catalyst of DCFH oxidation.[42] A high fat diet could have also been used to further examine taurine's effects on lipotoxicity, however Wistar rats are resistant to high fat diet-induced steatohepatitis.[82] Finally, although the results of the current experiments suggest that the beneficial effects of taurine are accompanied by reductions in oxidative stress and ER stress, the methods employed cannot determine causality, nor can they delineate the respective contribution of each stress.

BIBLIOGRAPHY

1. Matteoni CA, Younossi ZM, Gramlich T, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*. 1999;116(6):1413-1419. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10348825.

2. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology*. 2003;37(5):1202-1219. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12717402.

3. Papandreou D, Rousso I, Mavromichalis I. Update on non-alcoholic fatty liver disease in children. *Clin Nutr*. 2007;26(4):409-415. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17449148.

4. Yu AS, Keeffe EB. Nonalcoholic fatty liver disease. *Rev Gastroenterol Disord*. 2002;2(1):11-19. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12122975.

5. Powell EE, Cooksley WG, Hanson R, et al. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology*. 1990;11(1):74-80. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2295475.

6. Clark JM, Brancati FL, Diehl AM. The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol*. 2003;98(5):960-967. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12809815.

7. Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology*. 1994;107(4):1103-1109. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7523217.

8. Ong JP, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. *Journal of hepatology*. 2008;49(4):608-12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18682312 [Accessed December 10, 2010].

9. Söderberg C, Stål P, Askling J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology*. 2010;51(2):595-602. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20014114 [Accessed December 10, 2010].

10. Targher G, Bertolini L, Poli F, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes*. 2005;54(12):3541-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16306373 [Accessed December 10, 2010].

11. Targher G, Bertolini L, Rodella S, et al. Nonalcoholic fatty liver disease is independently associated with an increased incidence of cardiovascular events in type 2 diabetic patients. *Diabetes care*. 2007;30(8):2119-21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17519430 [Accessed December 10, 2010].

12. Harrison SA, Kadakia S, Lang KA, Schenker S. Nonalcoholic steatohepatitis: what we know in the new millennium. *Am J Gastroenterol*. 2002;97(11):2714-2724. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12425538.

13. Unger RH, Orci L. Lipoapoptosis: its mechanism and its diseases. *Biochim Biophys Acta*. 2002;1585(2-3):202-212. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12531555.

14. Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology*. 2006;147(2):943-951. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16269465.

15. Toshimitsu K, Matsuura B, Ohkubo I, et al. Dietary habits and nutrient intake in non-alcoholic steatohepatitis. *Nutrition*. 2007;23(1):46-52. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17140767.

16. Klein-Platat C, Drai J, Oujaa M, Schlienger JL, Simon C. Plasma fatty acid composition is associated with the metabolic syndrome and low-grade inflammation in overweight adolescents. *Am J Clin Nutr*. 2005;82(6):1178-1184. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16332649.

17. Pahl HL. Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol Rev*. 1999;79(3):683-701. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10390516.

18. Malhi H, Bronk SF, Werneburg NW, Gores GJ. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *J Biol Chem*. 2006;281(17):12093-12101. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16505490.

19. Gentile CL, Pagliassotti MJ. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *J Nutr Biochem*. 2008;19(9):567-576. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18430557.

20. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. 2004;306(5695):457-461. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15486293.

21. Puri P, Mirshahi F, Cheung O, et al. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology*. 2008;134(2):568-76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18082745.

22. Das SK, Chu WS, Mondal AK, et al. Effect of pioglitazone treatment on endoplasmic reticulum stress response in human adipose and in palmitate-induced stress in human liver and adipose cell lines. *Am J Physiol Endocrinol Metab*. 2008;295(2):E393-400. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18544642.

23. Calamita G, Portincasa P. Present and future therapeutic strategies in non-alcoholic fatty liver disease. *Expert Opin Ther Targets*. 2007;11(9):1231-1249. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17845148.

24. Haber CA, Lam TK, Yu Z, et al. N-acetylcysteine and taurine prevent hyperglycemia-induced insulin resistance in vivo: possible role of oxidative stress. *Am J Physiol Endocrinol Metab*. 2003;285(4):E744-53. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12799318.

25. Petty MA, Kintz J, DiFrancesco GF. The effects of taurine on atherosclerosis development in cholesterol-fed rabbits. *Eur J Pharmacol*. 1990;180(1):119-127. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2364997.

26. Yamori Y, Murakami S, Ikeda K, Nara Y. Fish and lifestyle-related disease prevention: experimental and epidemiological evidence for anti-atherogenic potential of taurine. *Clin Exp Pharmacol Physiol*. 2004;31 Suppl 2:S20-3. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15649278.

27. Chen SW, Chen YX, Shi J, Lin Y, Xie WF. The restorative effect of taurine on experimental nonalcoholic steatohepatitis. *Dig Dis Sci*. 2006;51(12):2225-2234. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17080243.

28. Chen X, Sebastian BM, Tang H, et al. Taurine supplementation prevents ethanol-induced decrease in serum adiponectin and reduces hepatic steatosis in rats. *Hepatology*. 2009;49(5):1554-1562. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19296466.

29. Warskulat U, Borsch E, Reinehr R, et al. Chronic liver disease is triggered by taurine transporter knockout in the mouse. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20(3):574-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16421246 [Accessed December 11, 2010].

30. Yamamoto S. Plasma taurine in liver cirrhosis with painful muscle cramps. *Advances in experimental medicine and biology*. 1996;403:597-600. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8915399 [Accessed December 11, 2010].

31. Weisdorf SA, Freese DK, Fath JJ, Tsai MY, Cerra FB. Amino acid abnormalities in infants with extrahepatic biliary atresia and cirrhosis. *Journal of pediatric gastroenterology and nutrition*. 6(6):860-4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3681571 [Accessed December 11, 2010].

32. Zulli A, Lau E, Wijaya BP, et al. High dietary taurine reduces apoptosis and atherosclerosis in the left main coronary artery: association with reduced CCAAT/enhancer binding protein homologous protein and total plasma homocysteine but not lipidemia. *Hypertension*. 2009;53(6):1017-1022. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19398656.

33. Nonaka H, Tsujino T, Watari Y, Emoto N, Yokoyama M. Taurine prevents the decrease in expression and secretion of extracellular superoxide dismutase induced by homocysteine: amelioration of homocysteine-induced endoplasmic reticulum stress by taurine. *Circulation*. 2001;104(10):1165-1170. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11535574.

34. Men X, Han S, Gao J, et al. Taurine protects against lung damage following limb ischemia reperfusion in the rat by attenuating endoplasmic reticulum stress-induced apoptosis. *Acta orthopaedica*. 2010;81(2):263-7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2895349&tool=pmcentrez&rendertype=abstract [Accessed November 1, 2010].

35. Wei Y, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab*. 2006;291(2):E275-81. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16492686.

36. Wang D, Wei Y, Schmoll D, Maclean KN, Pagliassotti MJ. Endoplasmic reticulum stress increases glucose-6-phosphatase and glucose cycling in liver cells. *Endocrinology*. 2006;147(1):350-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16223860 [Accessed November 26, 2010].

37. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol*. 1969;43(3):506-520. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4900611.

38. Pagliassotti MJ, Shahrokhi KA, Moscarello M. Involvement of liver and skeletal muscle in sucrose-induced insulin resistance: dose-response studies. *Am J Physiol*. 1994;266(5 Pt 2):R1637-44. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8203644.

39. Zinszner H, Kuroda M, Wang X, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & development*. 1998;12(7):982-95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=316680&tool=pmcentrez&rendertype=abstract [Accessed December 11, 2010].

40. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques*. 2002;32(6):1372-1374,1376,1378-1379. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12074169.

41. Guthrie HC, Hatrick A, Gerrard DJ. Traumatic variation of popliteal artery entrapment syndrome. *Journal of the Royal Army Medical Corps*. 2006;152(3):161-2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17295014 [Accessed December 11, 2010].

42. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol*. 2004;142(2):231-255. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15155533.

43. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*. 1959;37(8):911-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13671378 [Accessed October 21, 2010].

44. Lapenna D, Ciofani G, Pierdomenico SD, Giamberardino MA, Cuccurullo F. Reaction conditions affecting the relationship between thiobarbituric acid reactivity and lipid peroxides in human plasma. *Free radical biology & medicine*. 2001;31(3):331-5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11461770 [Accessed December 11, 2010].

45. Wei Y, Wang D, Pagliassotti MJ. Saturated fatty acid-mediated endoplasmic reticulum stress and apoptosis are augmented by trans-10, cis-12-conjugated linoleic acid in liver cells. *Mol Cell Biochem*. 2007;303(1-2):105-113. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17426927.

46. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev*. 1999;13(10):1211-1233. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10346810.

47. Rutkowski DT, Kaufman RJ. A trip to the ER: coping with stress. *Trends Cell Biol*. 2004;14(1):20-28. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14729177.

48. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. *Mutat Res*. 2005;569(1-2):29-63. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15603751.

49. Kaufman RJ. Orchestrating the unfolded protein response in health and disease. *J Clin Invest*. 2002;110(10):1389-1398. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12438434.

50. Parildar-Karpuzoglu H, Mehmetcik G, Ozdemirler-Erata G, et al. Effect of taurine treatment on pro-oxidant-antioxidant balance in livers and brains of old rats. *Pharmacol Rep*. 2008;60(5):673-678. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19066413.

51. Erman F, Balkan J, Cevikbaş U, Koçak-Toker N, Uysal M. Betaine or taurine administration prevents fibrosis and lipid peroxidation induced by rat liver by ethanol plus carbon tetrachloride intoxication. *Amino acids*. 2004;27(2):199-205. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15338317 [Accessed December 11, 2010].

52. Parks EJ, Hellerstein MK. Carbohydrate-induced hypertriacylglycerolemia: historical perspective and review of biological mechanisms. *The American journal of clinical nutrition*. 2000;71(2):412-33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10648253 [Accessed December 11, 2010].

53. Pagliassotti MJ, Kang J, Thresher JS, Sung CK, Bizeau ME. Elevated basal PI 3-kinase activity and reduced insulin signaling in sucrose-induced hepatic insulin resistance. *American journal of physiology. Endocrinology and metabolism*. 2002;282(1):E170-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11739098 [Accessed December 11, 2010].

54. Thresher JS, Podolin DA, Wei Y, Mazzeo RS, Pagliassotti MJ. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2000;279(4):R1334-40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11004002 [Accessed December 11, 2010].

55. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants & redox signaling*. 2007;9(12):2277-93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17979528 [Accessed July 2, 2010].

56. Malhotra JD, Miao H, Zhang K, et al. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(47):18525-30. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2587584&tool=pmcentrez&rendertype=abstract [Accessed December 11, 2010].

57. Franconi F, Loizzo A, Ghirlanda G, Seghieri G. Taurine supplementation and diabetes mellitus. *Curr Opin Clin Nutr Metab Care*. 2006;9(1):32-36. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16444816.

58. Hansen SH. The role of taurine in diabetes and the development of diabetic complications. *Diabetes Metab Res Rev*. 2001;17(5):330-346. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11747139.

59. Vohra BP, Hui X. Taurine protects against carbon tetrachloride toxicity in the cultured neurons and in vivo. *Archives of physiology and biochemistry*. 2001;109(1):90-4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11471076 [Accessed December 11, 2010].

60. Li Y, Arnold JM, Pampillo M, Babwah AV, Peng T. Taurine prevents cardiomyocyte death by inhibiting NADPH oxidase-mediated calpain activation. *Free Radic Biol Med*. 2009;46(1):51-61. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18950702.

61. Schaffer SW, Azuma J, Mozaffari M. Role of antioxidant activity of taurine in diabetes. *Canadian journal of physiology and pharmacology*. 2009;87(2):91-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19234572 [Accessed December 11, 2010].

62. Merezak S, Hardikar AA, Yajnik CS, Remacle C, Reusens B. Intrauterine low protein diet increases fetal beta-cell sensitivity to NO and IL-1 beta: the protective role of taurine. *The Journal of endocrinology*. 2001;171(2):299-308. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11691650 [Accessed December 11, 2010].

63. Hamaguchi T, Azuma J, Schaffer S. Interaction of taurine with methionine: inhibition of myocardial phospholipid methyltransferase. *Journal of cardiovascular pharmacology*. 1991;18(2):224-30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1717783 [Accessed December 11, 2010].

64. Dalle-Donne I, Aldini G, Carini M, et al. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med*. 2006;10(2):389-406. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16796807.

65. Telci A, Cakatay U, Kayali R, et al. Oxidative protein damage in plasma of type 2 diabetic patients. *Horm Metab Res*. 2000;32(1):40-43. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10727013.

66. Martin-Gallan P, Carrascosa A, Gussinye M, Dominguez C. Biomarkers of diabetes-associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. *Free Radic Biol Med*. 2003;34(12):1563-1574. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12788476.

67. Santos CXC, Tanaka LY, Wosniak J, Laurindo FRM. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxidants & redox signaling*. 2009;11(10):2409-27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19388824 [Accessed October 19, 2010].

68. Shimizu Y, Hendershot LM. Oxidative folding: cellular strategies for dealing with the resultant equimolar production of reactive oxygen species. *Antioxidants & redox signaling*. 2009;11(9):2317-31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2819804&tool=pmcentrez&rendertype=abstract [Accessed December 11, 2010].

69. Lee A-H, Scapa EF, Cohen DE, Glimcher LH. Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science*. 2008;320(5882):1492-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18556558.

70. Rutkowski DT, Wu J, Back S-H, et al. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Developmental cell*. 2008;15(6):829-40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19081072 [Accessed August 1, 2010].

71. Ota T, Gayet C, Ginsberg HN. Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. *J Clin Invest*. 2008;118(1):316-332. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18060040.

72. Flowers MT, Keller MP, Choi Y, et al. Liver gene expression analysis reveals endoplasmic reticulum stress and metabolic dysfunction in SCD1-deficient mice fed a very low-fat diet. *Physiological genomics*. 2008;33(3):361-72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18381840 [Accessed August 31, 2010].

73. Yang J-S, Kim JT, Jeon J, et al. Changes in hepatic gene expression upon oral administration of taurine-conjugated ursodeoxycholic acid in ob/ob mice. *PloS one*. 2010;5(11):e13858. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2974643&tool=pmcentrez&rendertype=abstract [Accessed December 4, 2010].

74. Lee AH, Scapa EF, Cohen DE, Glimcher LH. Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science*. 2008;320(5882):1492-1496. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18556558.

75. Glimcher LH, Lee AH. From sugar to fat: How the transcription factor XBP1 regulates hepatic lipogenesis. *Ann N Y Acad Sci*. 2009;1173 Suppl:E2-9. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19751410.

76. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*. 2010;140(6):900-17. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20303879.

77. Malhotra JD, Kaufman RJ. The endoplasmic reticulum and the unfolded protein response. *Seminars in cell & developmental biology*. 2007;18(6):716-31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2706143&tool=pmcentrez&rendertype=abstract [Accessed December 11, 2010].

78. Ozcan U, Yilmaz E, Ozcan L, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science*. 2006;313(5790):1137-40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16931765 [Accessed July 16, 2010].

79. Lin JH, Li H, Yasumura D, et al. IRE1 signaling affects cell fate during the unfolded protein response. *Science (New York, N.Y.)*. 2007;318(5852):944-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17991856 [Accessed December 11, 2010].

80. Moran JM, Salas J, Botello F, Macià E, Climent V. Taurine and cholestasis associated to TPN. Experimental study in rabbit model. *Pediatric surgery international*. 2005;21(10):786-92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16172874 [Accessed December 28, 2010].

81. Chesney RW, Helms RA, Christensen M, et al. The role of taurine in infant nutrition. *Advances in experimental medicine and biology*. 1998;442:463-76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9635063 [Accessed December 28, 2010].

82. Romestaing C, Piquet M-A, Bedu E, et al. Long term highly saturated fat diet does not induce NASH in Wistar rats. *Nutrition & metabolism*. 2007;4:4. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1805500&tool=pmcentrez&rendertype=abstract [Accessed December 30, 2010].

FIGURE LEGENDS

Figure 1. Fatty Acid-Mediated Cell Death in H4IIE Liver Cells and Primary Hepatocytes. (A) Caspase-3 activity; (B) ELISA-based cell death in H4IIE liver cells and primary hepatocytes. Cells were incubated for 16 h in control media (LG) or LG supplemented with albumin-bound oleate at 250 µM (O250), oleate at 500 µM (O500), palmitate at 250 µM (P250) or palmitate at 500 µM (P500) in the absence (no additions) or presence of taurine (1% w/v). Values are means ± SD for n=6-8 independent experiments. * = significantly different from same condition without taurine.

Figure 2. Fatty Acid-Mediated ER Stress in H4IIE Liver Cells. (A) CHOP and GADD34 mRNA relative to 18S; (B) Unspliced (u) and spliced (s) Xbp1 mRNA; and (C) GRP78, phosphorylated eIF2α (p-eIF2α), total eIF2α and actin protein. Incubations were exactly as described in figure 1 with the exception that the duration of the incubations was 6 h. In graphs, values are means ± SD for n=6-8 independent experiments and gels are representative of n=6-8. Note the different order of treatments between blot and graph for eIF2α. * = significantly different from same condition without taurine.

Figure 3. Fatty Acid-Mediated ER Stress in Primary Hepatocytes. (A) CHOP and GADD34 mRNA relative to 18S; (B) Unspliced (u) and spliced (s) Xbp1 mRNA; and (C) GRP78, phosphorylated eIF2α (p-eIF2α), total eIF2α and actin protein. Incubations were exactly as described in figure 1 with the exception that the duration of the incubations was 6 h. Values in graphs are means ± SD for n=6 independent experiments and gels are representative of n=6. Note the different order of treatments between blot and graph for GRP78 and eIF2α. * = significantly different from same condition without taurine.

Figure 4. Fatty Acid-Mediated Oxidative Stress in H4IIE Liver Cells and Primary Hepatocytes. (A) DCF fluorescence in H4IIE liver cells; (B) DCF fluorescence in primary hepatocytes; (C) protein carbonyl formation in H4IIE liver cells. Incubations were exactly as described in figure 1 with the exception that the duration of the incubations was 6 h. Values are means ± SD for n=3-6 independent experiments. * = significantly different from same condition without taurine.

Figure 5. The Effects of Taurine on Gene Markers of ER Stress in Rats Provided a High Starch or High Sucrose Diet. (A) GRP78 mRNA, (B) GADD34 mRNA; (C) Chop mRNA relative to 18S. STD, high starch diet for 4 wks; STD+T, high starch diet and drinking water with 2% (w/v) taurine for 4 wks; HSD, high sucrose diet for 4 weeks; HSD+T, high sucrose diet for 4 weeks and drinking water with 2% taurine (w/v). Values are means ± SD for n=4-6 per group. * = significantly different from all other groups.

Figure 6. The Effects of Taurine on Markers of Oxidative Stress, Inflammation and Liver Enzymes in Rats Provided a High Starch or High Sucrose Diet. (A) Plasma thiobarbituric acid reactive substances, (TBARS); (B) Plasma tumor necrosis factor-α, (TNFα); (C) C-reactive protein mRNA relative to 18S, (CRP); (D) Plasma CRP; (E) Plasma alanine aminotransferase, (ALT); (E) Plasma aspartate aminotransferase (AST). STD, high starch diet for 4 wks; STD+T, high starch diet and drinking water with 2% (w/v) taurine for 4 wks; HSD, high sucrose diet for 4 weeks; HSD+T, high sucrose diet for 4 weeks and drinking water with 2% taurine (w/v). Values are means ± SD for n=4-6 per group. * = significantly different from all other groups.

Figure 7. The Effects of Taurine on Lipogenic Gene Expression in Livers of Rats Provided High Starch or High Sucrose Diet. (A) FAS; (B) ACCα relative to 18S. STD, high starch diet for 4 wks; STD+T, high starch diet and drinking water with 2% (w/v) taurine for 4 wks; HSD, high sucrose diet for 4 weeks; HSD+T, high sucrose diet for 4 weeks and drinking water with 2% taurine (w/v). Values are means ± SD for n=4-6 per group. * = significantly different from all other groups.

Figure 8. The Effects of Taurine in Mice Injected with Tunicamycin. (A) Liver weight; (B) H&E stain and liver triglycerides; (C) alanine aminotransferase (ALT). Con, carrier injected; Con + Tau, carrier injected and provided taurine in drinking water; Tun, tunicamycin injected; Tun + Tau, tunicamycin injected and provided taurine in drinking water. Values are means ± SD n=6/group. * = significantly different from all other groups.

Table 1: General and Metabolic Characteristics

VARIABLE	STD	STD+T	HSD	HSD+T
Daily Food Intake, kcal	93.5±4.4	99.9±10.4	100.0±10.0	105.1±5.7
Daily Taurine Intake, g[1]	0	0.8±0.1	0	0.9±0.2
Weight Gain, g	166.8±15.3	191.7±29.1	185.2±22.5	188.8±16.9
Epididymal Fat, g	7.2±1.4	8.5±1.9	9.9±2.2†	7.4±0.9
Retroperitoneal Fat, g	6.1±3.0	8.2±3.7	8.0±2.4	6.2±0.9
Plasma Glucose, mg/dl	139.0±6.0	145.0±4.0	153.0±6.0	149.0±6.0
Plasma Insulin, ng/ml	0.9±0.2	1.0±0.2	2.0±0.3*	1.5±0.1
Plasma FFA, mM	0.30±0.04	0.32±0.03	0.33±0.04	0.37±0.03
Plasma TG, mM	0.39±0.09	0.48±0.09	1.10±0.08*	0.55±0.10
Liver TG, umol/g	6.4±0.7	7.1±0.7	12.4±0.9#	9.8±0.4#

Values are mean ± SEM for n = 6 per group; [1] = Taurine intake estimated based on daily water consumption; * = significantly different from all other groups, # = significantly different from STD and STD+T, † = significantly different from STD; FFA=free fatty acids; TG=triglycerides

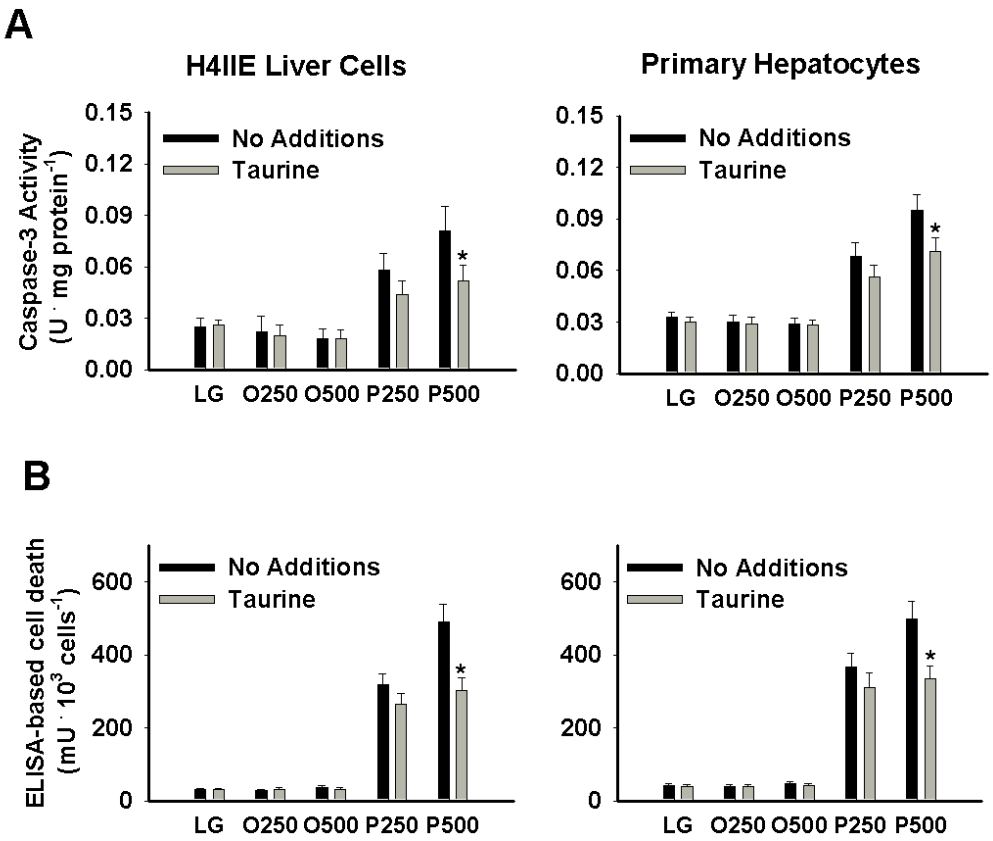

A
H4IIE Liver Cells
Primary Hepatocytes
Caspase-3 Activity
(U · mg protein-1)
No Additions
Taurine
0.15
0.12
0.09
0.06
0.03
0.00
LG O250 O500 P250 P500
B
ELISA-based cell death
(mU · 10³ cells-1)
600
400
200
0

Figure 1

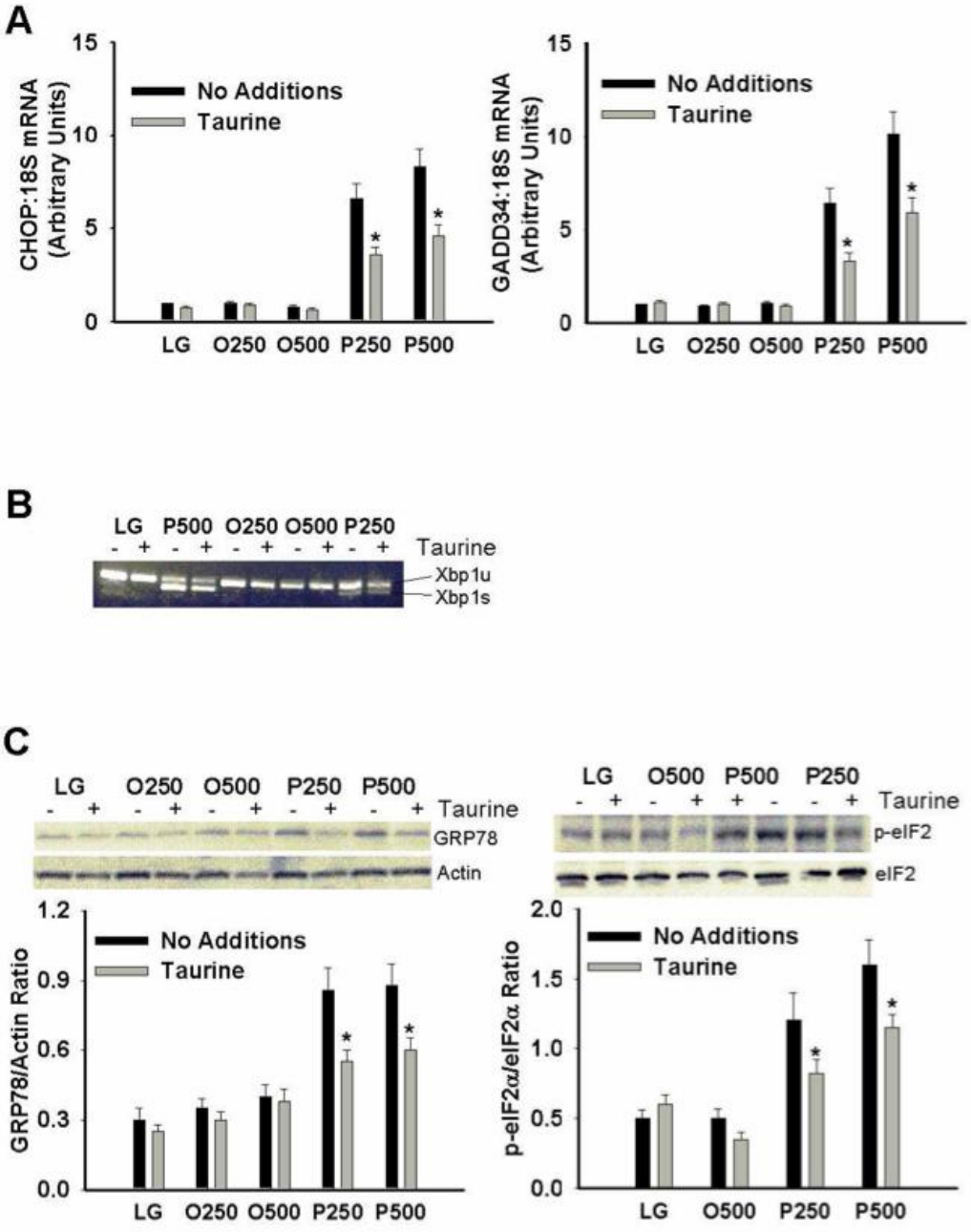

A
CHOP:18S mRNA (Arbitrary Units)
No Additions
Taurine
LG O250 O500 P250 P500
GADD34:18S mRNA (Arbitrary Units)
B
LG P500 O250 O500 P250
Taurine
Xbp1u
Xbp1s
C
LG O250 O500 P250 P500
Taurine
GRP78
Actin
GRP78/Actin Ratio
LG O500 P500 P250
p-eIF2
eIF2
p-eIF2α/eIF2α Ratio

Figure 2

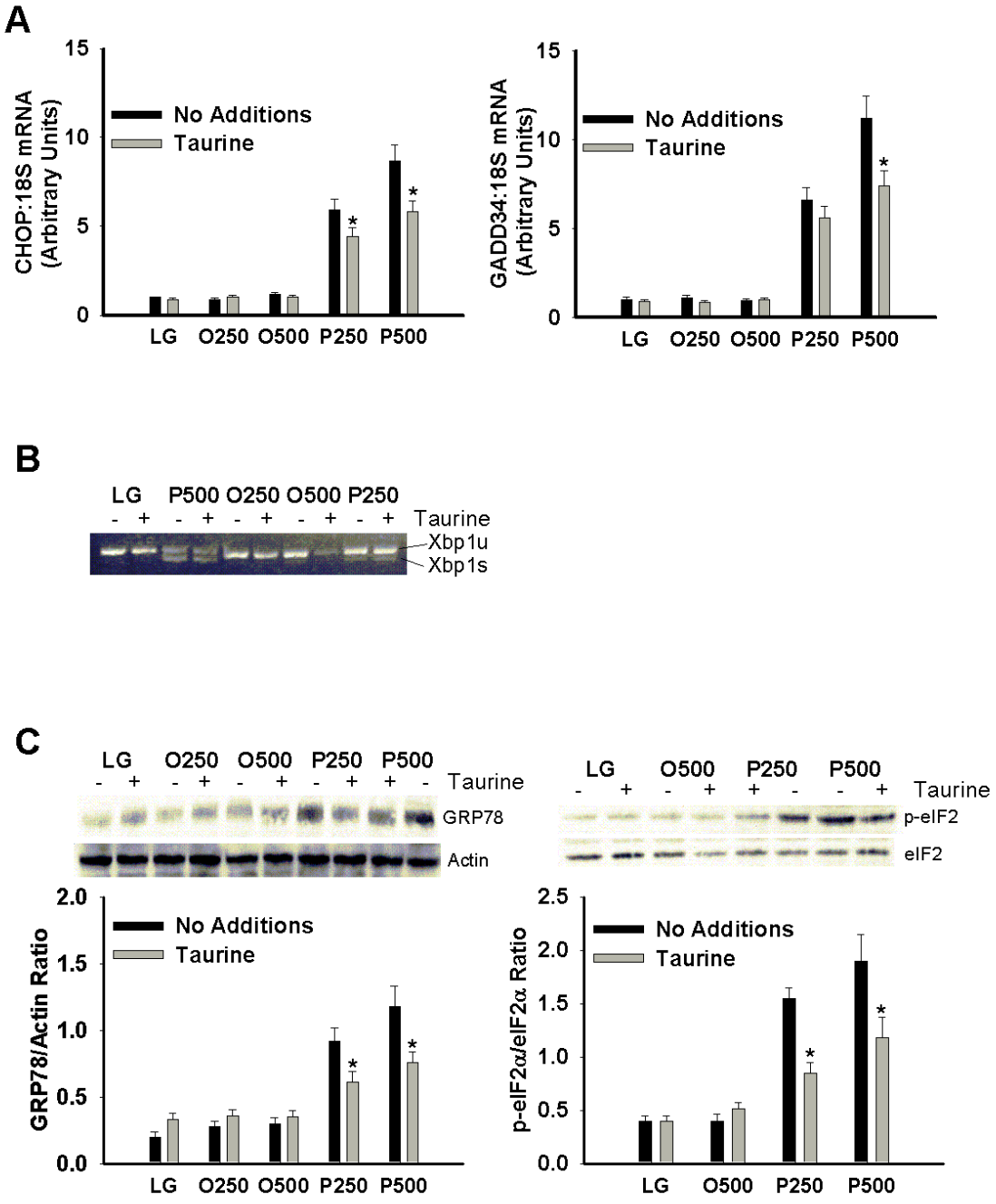

A
CHOP:18S mRNA (Arbitrary Units)
No Additions
Taurine
LG O250 O500 P250 P500
GADD34:18S mRNA (Arbitrary Units)
B
LG P500 O250 O500 P250
Taurine
Xbp1u
Xbp1s
C
LG O250 O500 P250 P500
Taurine
GRP78
Actin
LG O500 P250 P500
p-eIF2
eIF2
GRP78/Actin Ratio
p-eIF2α/eIF2α Ratio

Figure 3

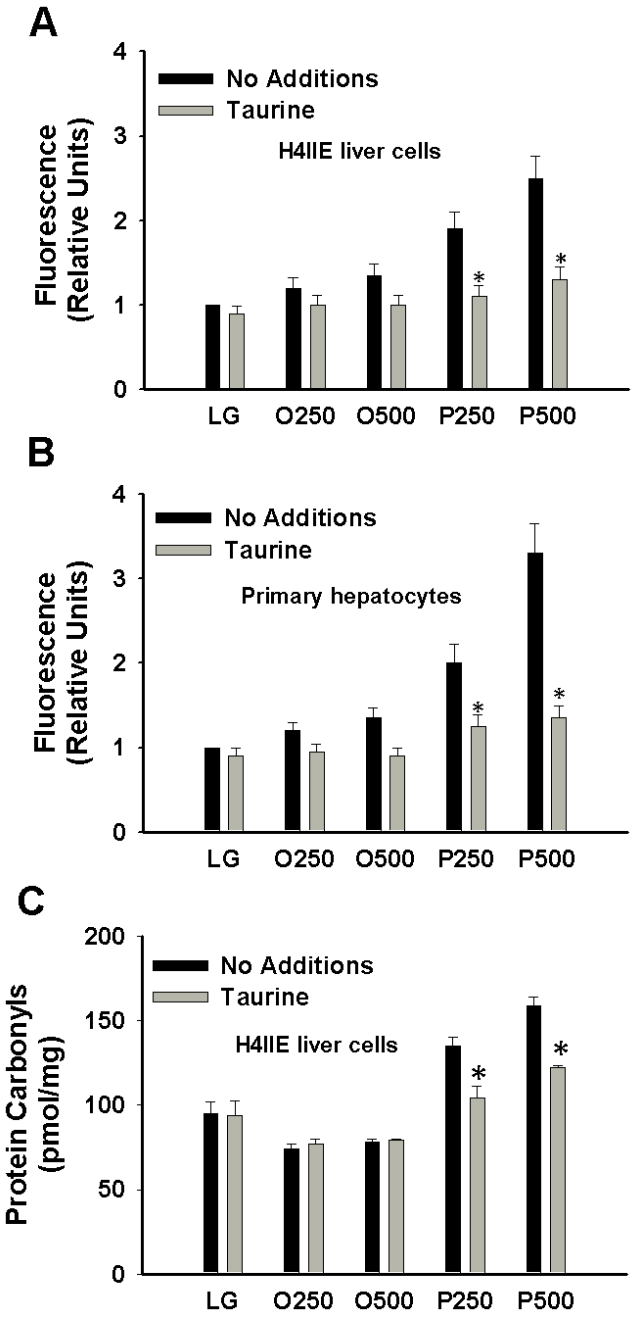

A
Fluorescence (Relative Units)
No Additions
Taurine
H4IIE liver cells
LG
O250
O500
P250
P500
B
Fluorescence (Relative Units)
No Additions
Taurine
Primary hepatocytes
LG
O250
O500
P250
P500
C
Protein Carbonyls (pmol/mg)
No Additions
Taurine
H4IIE liver cells
LG
O250
O500
P250
P500

Figure 4

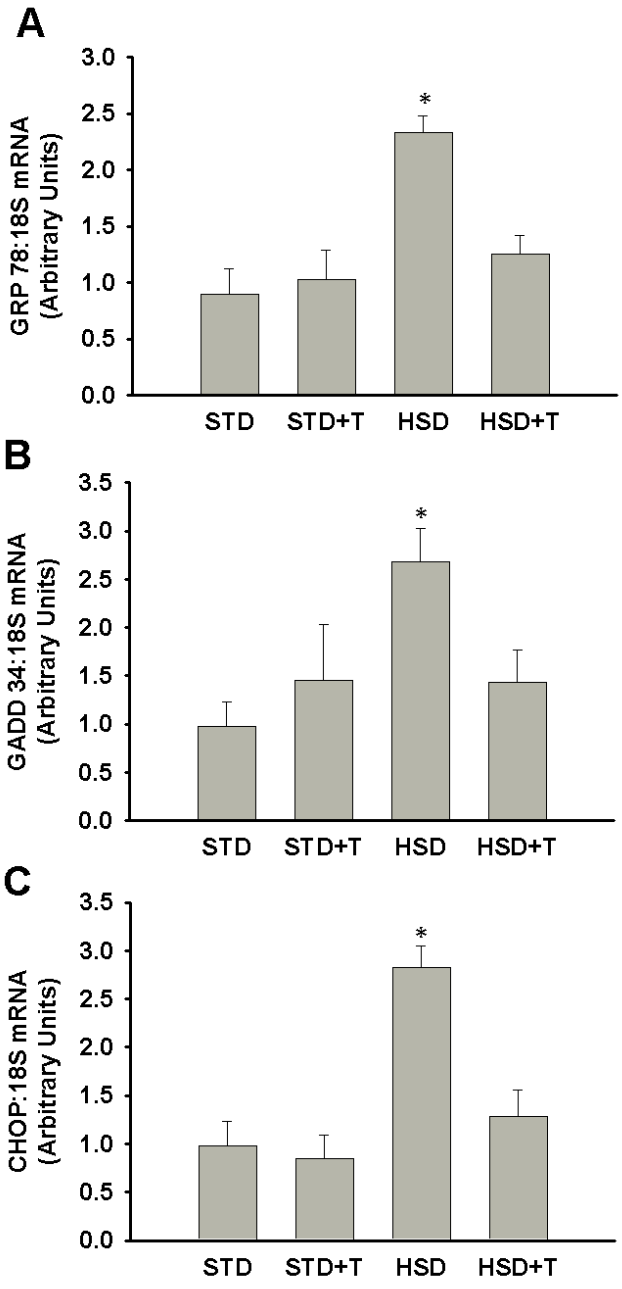

A
GRP 78:18S mRNA (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
STD
STD+T
HSD
HSD+T
B
GADD 34:18S mRNA (Arbitrary Units)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
STD
STD+T
HSD
HSD+T
C
CHOP:18S mRNA (Arbitrary Units)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
STD
STD+T
HSD
HSD+T

Figure 5

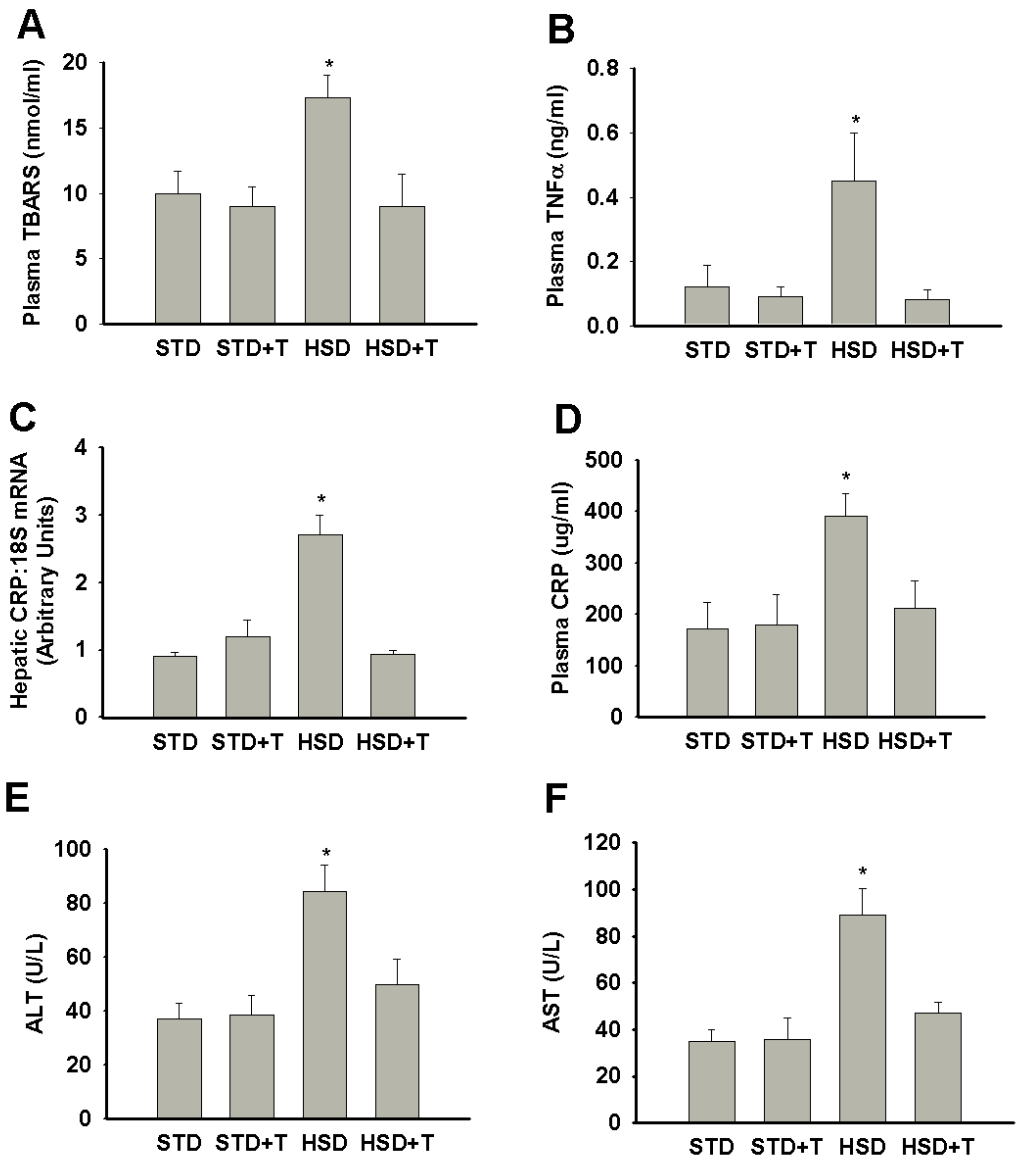

A
Plasma TBARS (nmol/ml)
20
15
10
5
0
*
STD STD+T HSD HSD+T
B
Plasma TNFα (ng/ml)
0.8
0.6
0.4
0.2
0.0
*
STD STD+T HSD HSD+T
C
Hepatic CRP:18S mRNA (Arbitrary Units)
4
3
2
1
0
*
STD STD+T HSD HSD+T
D
Plasma CRP (ug/ml)
500
400
300
200
100
0
*
STD STD+T HSD HSD+T
E
ALT (U/L)
100
80
60
40
20
0
*
STD STD+T HSD HSD+T
F
AST (U/L)
120
100
80
60
40
20
0
*
STD STD+T HSD HSD+T

Figure 6

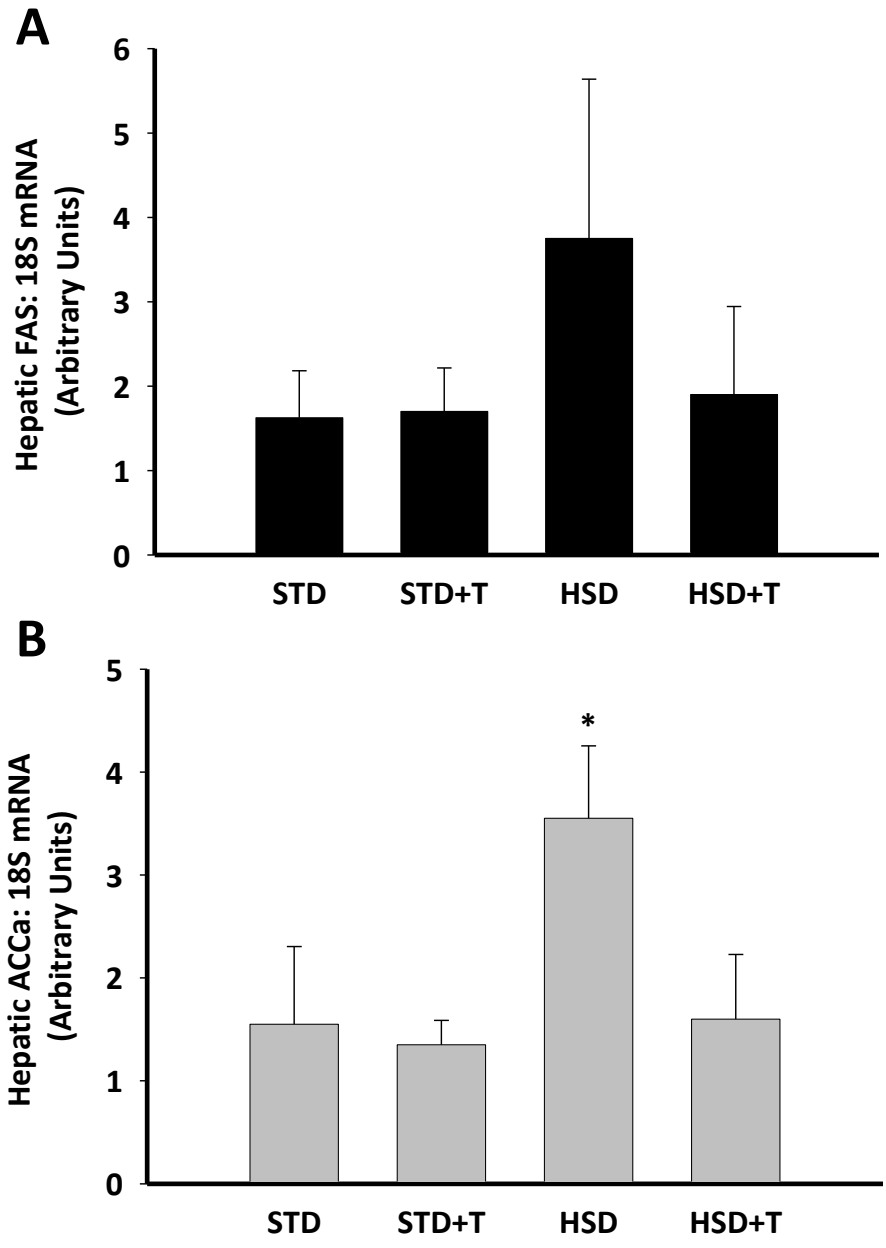

A
Hepatic FAS: 18S mRNA (Arbitrary Units)
6
5
4
3
2
1
0
STD
STD+T
HSD
HSD+T
B
Hepatic ACCa: 18S mRNA (Arbitrary Units)
5
4
3
2
1
0
*
STD
STD+T
HSD
HSD+T

Figure 7

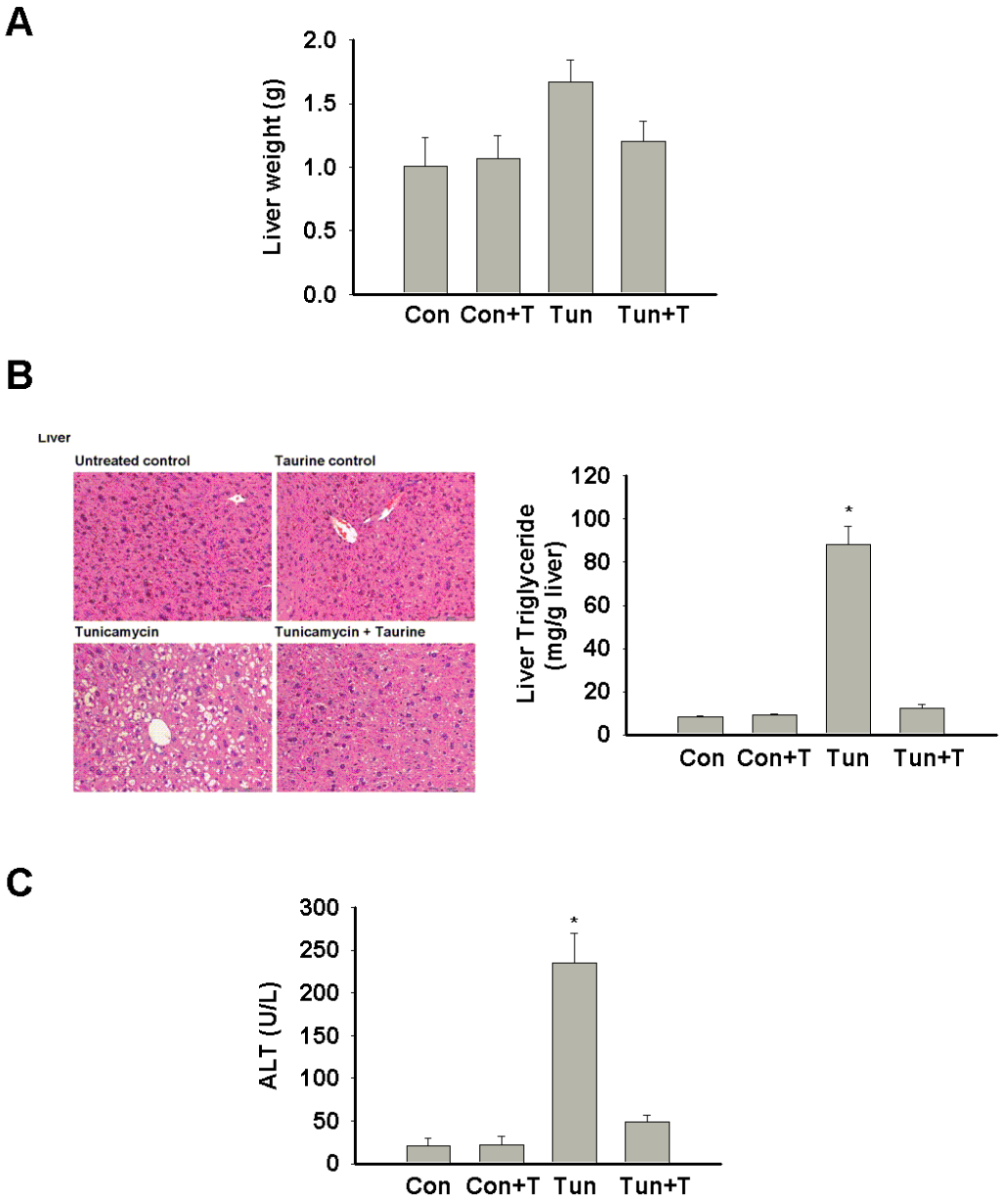

A
Liver weight (g)
2.0
1.5
1.0
0.5
0.0
Con Con+T Tun Tun+T
B
Liver
Untreated control
Taurine control
Tunicamycin
Tunicamycin + Taurine
Liver Triglyceride (mg/g liver)
120
100
80
60
40
20
0
*
Con Con+T Tun Tun+T
C
ALT (U/L)
300
250
200
150
100
50
0
*
Con Con+T Tun Tun+T

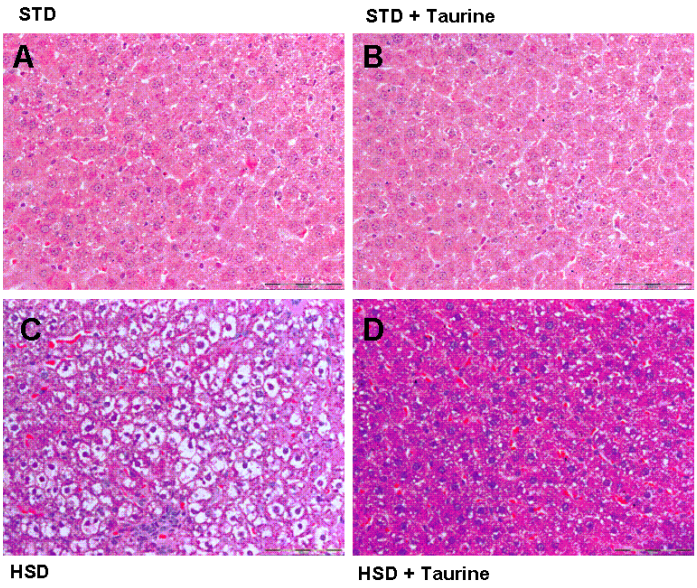
Figure 8

SUPPLEMENTAL DATA

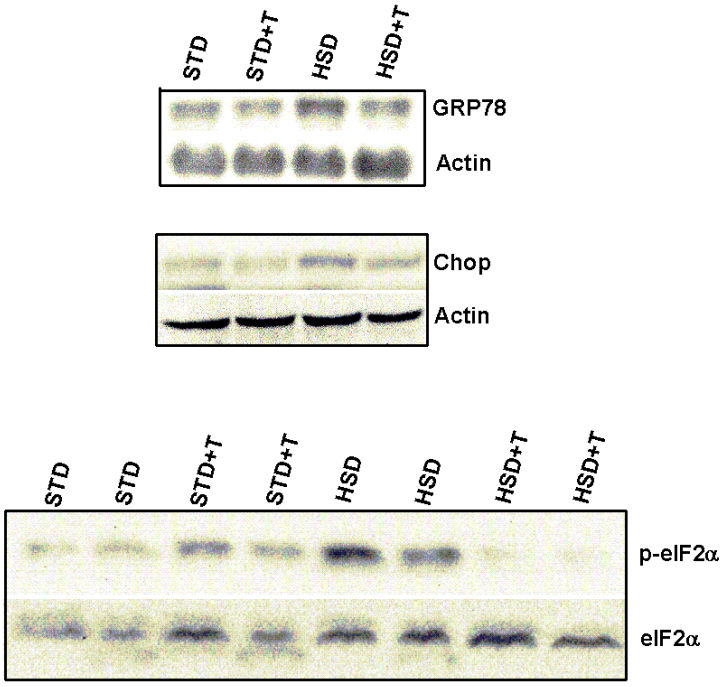
Supplemental Figure 1. H&E Staining in Rats Provided a High Starch or High Sucrose Diet. (A) High starch diet (STD); (B) high starch diet with taurine in drinking water (STD + Taurine); (C) high sucrose diet (HSD); (D) high sucrose diet with taurine in drinking water (HSD + taurine). Data is representative of n=4-6 per group.

Supplemental Figure 2. Western Blot Analysis in Rats Provided a High Starch or High Sucrose Diet. Representative western blots for GRP78, Chop, phosphorylated eIF2α, total eIF2α and actin. STD,high starch diet for 4 wks; STD+T, high starch diet and drinking water with 2% (w/v) taurine for 4 wks; HSD, high sucrose diet for 4 weeks; HSD+T, high sucrose diet for 4 weeks and drinking water with 2% taurine (w/v).

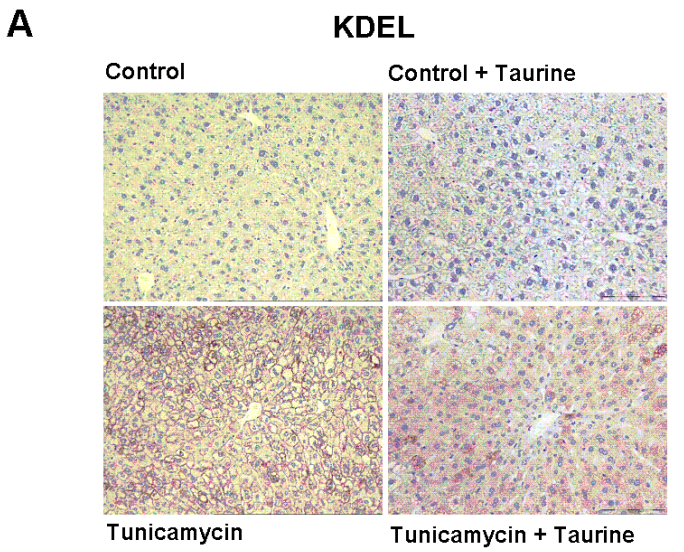
Supplemental Figure 3. Amelioration of Tunicamycin-Mediated Induction of the UPR in Mice by Taurine Supplementation. Liver sections were prepared from C57BL/6 mice four days after treatment with a single IP injection of either tunicamycin (0.5 mg/kg body weight) or PBS. Representative images are shown from each experimental group immunostained with (A), anti-KDEL and (B), anti-CHOP antibodies as described in the materials and methods section. Scale bar denotes 200 micrometers.

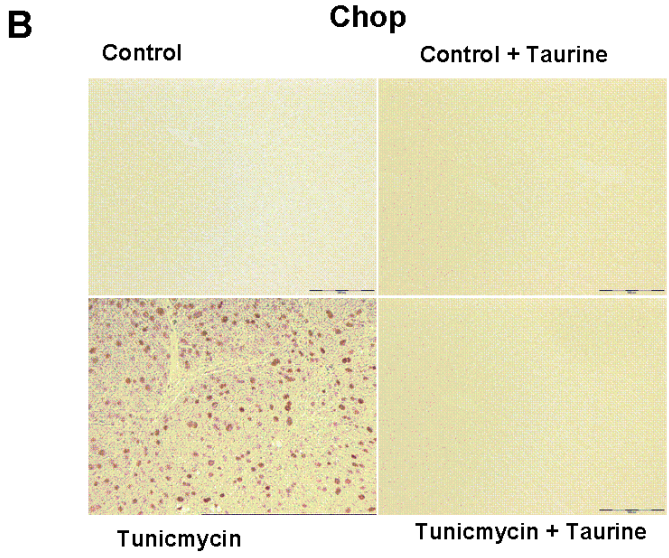


Supplemental Figure 1



Supplemental Figure 2





Supplemental Figure 3

CHAPTER 5

FUTURE DIRECTIONS

ONION EXUDATES

While there is potential for onion exudates as a therapeutic tool for NAFLD, many issues need to be addressed before this can happen. Determining why the presence of bioactivity, specifically activity that influences insulin action and fat accumulation, is variable among batches is of utmost importance. Possible sources of variability could include onion growth media inconsistencies, degradation of the relevant compounds, and/or calculation errors with regards to mass of lyophilized exudates. Evaluation of the constituents of various media used to grow plants in the laboratory demonstrated a wide variability. We have examined a few of these MS media (i.e. Caisson labs, Sigma, Phytotechnologies) and have observed that some of these media have effects on insulin-dependent phosphorylation of AKT. It is also possible that these media will elicit a different composition of exudates upon interaction with the plant root. Thus, a systemic study that evaluates onion root exudates grown under identical conditions but in different growth media is required. Degradation is another potential issue that could explain our variability in onion exudates bioactivity. Our initial batch of onion exudates that elicited positive results in vivo were stored at

4°C covered in foil and used within a month for the pilot in vivo study. A second successful in vivo study was done using the same batch a few months later. Thus, it was assumed that the bioactive compounds were stable. It is possible that subsequent batches of exudates were less stable and were characterized by a loss of the relevant bioactive compounds. Future studies would be handled in a way to minimize this risk by avoiding heat, light, and potentially oxidation. Freezing samples post lyophilization at -80°C and minimizing freeze/thaw cycling may help to preserve the bioactive compounds present. After onion exudates were collected, they were sterile filtered and lyophilized to a powder; this powder contains a mixture of growth media components and compounds exuded by the plant roots. This powder was then reconstituted in saline for intraperitoneal injections for in vivo studies, or in MEM growth media for in vitro studies. The calculation for reconstitution was based on the mass of lyophilized onion exudates present; it is possible that mass measurements do not correlate with the amount of bioactive compounds present. Also, post-lyophilization, the majority of mass comes from the 3% sucrose in the onion growth media; if any moisture remains mixed with the sucrose, the lyophilized onion exudates mass will not be accurate and could contribute to bioactive variability. Ideally, removal of the sucrose would not only aid in mass measurements but also would prevent its interference in qualitative/quantitative HPLC screening of onion root exudates.

Assuming the above issues and bioactivity variability can be eliminated, the next step would be to identify the relevant bioactive compound classes through relevant

extractions and subsequent screening in liver cells or rats for bioactivity retention. For example, a recent study performed an aqueous extraction of *Belamcanda chinensis* leaves followed by precipitation with 80% ethanol; the supernatant was freeze-dried, reconstituted in distilled water, and either further extracted with petroleum ether, chloroform, acetic ester, or n-butanol or eluted using a macroporous resin HPLC column and a gradient of water to 95% ethanol solvent, to isolate compound classes.[1] These isolates were tested in male Wistar rats for hypoglycemic effects; isolates having the strongest hypoglycemic effects were further analyzed using NMR, MS, and UV spectral analysis and found to be isoflavones.[1] Since onion root exudates show bioactivity both in vitro and in vivo, it would be much easier to test isolates in liver cells first, and then progress to rats for verification.

It is not necessary to identify the exact compounds that are bioactive in onion root exudates in order to explore their bioactivities. For example, crude bean root exudates were shown to promote germination of the insect pathogen *Metarhizium anisopliae*, in part through upregulation of genes involved in carbohydrate metabolism, lipid metabolism, cofactors and vitamins, energy metabolism, proteolysis, extracellular matrix/cell wall proteins, transport proteins, DNA synthesis, the sexual cycle and stress response.[2] Extractions and isolations followed by bioactivity screening serve to further expand on the various physiologic roles of compound fractions. A study done on various Indian Ayurvedic medicinal plants found that three isopropanol extracts had potent inhibition of pancreatic a-amylase, which controls post prandial hyperglycemia through

prevention of starch breakdown.[3] In the case of onion root exudates, once an isolate is determined to be maximally bioactive in terms of promoting insulin action and/or preventing fat accumulation, it can be used in order to determine potential details in its mechanism(s) of action. As onion root exudates proved to have a potent stimulatory effect on pAKT, possible mechanisms of action could be through inhibition of protein tyrosine phosphatase 1β (PTP1β, involved in dephosphorylation of pAKT; liver-specific PTP1β knockout mice show improved hepatic insulin signaling[4]), proteaomal proteins involved in degradation of insulin signaling proteins (which has been shown to occur in adipocytes with proteosome inhibitors MG132 and lactacystin and led to prolonged insulin signaling and glucose transport[5]), or via stimulatory effects on upstream insulin signaling proteins or interactions. The ability of onion root exudates to prevent fatty acid-mediated hepatic lipid accumulation could be further examined in studies that directly investigated fatty acid transport and oxidation. Giving liver cells and rats bioactive isolates of onion root exudates followed by genomic, proteomic and metabolomic analyses would provide a systems approach to showing global effects induced by the compounds of interest, either directly or indirectly.

Once the above methods have been successfully implemented, they can be used to test other varieties of onion or related plant exudates for similar or more potent bioactivity towards NAFLD characteristics. Much further down the road, methods to enrich the secretion or potency of the bioactive compounds in onion root exudates (or other root exudates) would be desired. Modification of external stressors can affect the

quality and quantity of root exudates;[6] an example of this is a study involving induced biotic attack of the barley root system which resulted in root secretion of phenolic compounds with antimicrobial properties.[7] If the target bioactive components of onion root exudates are phenolic and the barley and onion root systems are similar, perhaps a similar biotic induction would enrich the surrounding growth media.

TAURINE

As taurine has a diverse physiological role, determination of its mechanism of action in the context of treating or preventing NAFLD characteristics is the next step. In our study, taurine reduced nutrient-(palmitate, high sucrose diet) and chemically-(tunicamycin) induced hepatic steatosis, ER stress, inflammation and injury across four models of NAFLD. A high throughput method of analyzing gene expression, protein expression and interactions, and metabolic alterations induced by taurine would provide potential targets for further analysis. Genomics, proteomics, and metabolomics are three exciting new areas of bioinformatics that could satisfy the need to look at multiple pathways and interactions to which taurine may directly or indirectly affect. Genomics utilizes vast arrays of cDNA probes that can be customized to desired genes or pathways. As the resulting analysis produces an enormous amount of data, proper bioinformatics software is necessary in order to elucidate any significant findings. Proteomics involves gel separation of proteins followed by digestion, separation and identification using an LC/MS or variant of. Proper software is, again, crucial since massive libraries must be used to piece together fragmented peptide sequences into the identity (and potentially the quantity) of the original protein/s. Some applications of

proteomics can also show protein interactions. Metabolomics uses LC/MS analysis of fluids such as plasma or urine in order to identify and quantify small molecules including metabolites.

With respect to taurine treatment, one could envision looking at lipogenic, oxidative stress, ER stress, or inflammatory pathways and their respective signaling components to see what perturbations are induced. Many studies have examined the antioxidant potential of taurine, however recently it has been implicated as having a role in calcium homeostasis in the brain, specifically through altering the presence of the calcium-binding proteins calbindin-D28k, calretinin, and parvalbumin, and reducing CaMKII activity.[8] As calcium homeostasis is important in all cells, measuring taurine's effect on calcium binding or signaling proteins in the liver may be a mechanism by which taurine reduces characteristics of NAFLD. Numerous studies have shown Tauroursodeoxycholate (TUDCA, a chemical chaperone that alleviates ER stress through enhanced protein folding) reduces hepatic steatosis, apoptosis, inflammation in adipocytes, hyperglycemia, and systemic insulin resistance in various experimental models.[9-14] Taurine may act similarly, either through conjugation to form TUDCA or an active metabolite of TUDCA, and is thus a warranted mechanism to explore. Additionally, since taurine is endogenously made, one could look at differences in its amount at various stages of NAFLD to see if a deficiency could be a component to the onset or severity of the disease.[15-18]

BIBLIOGRAPHY

1. Chen Y, Wu C-M, Dai R-J, et al. Combination of HPLC chromatogram and hypoglycemic effect identifies isoflavones as the principal active fraction of Belamcanda chinensis leaf extract in diabetes treatment. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2010. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21239237 [Accessed January 30, 2011].

2. Pava-Ripoll M, Angelini C, Fang W, et al. The rhizosphere-competent entomopathogen Metarhizium anisopliae expresses a specific subset of genes in plant root exudate. *Microbiology (Reading, England)*. 2011;157(Pt 1):47-55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20947574 [Accessed January 18, 2011].

3. P S, Zinjarde SS, Bhargava SY, Ravikumar A. Potent alpha-amylase inhibitory activity of Indian Ayurvedic medicinal plants. *BMC complementary and alternative medicine*. 2011;11(1):5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21251279 [Accessed January 26, 2011].

4. Delibegovic M, Zimmer D, Kauffman C, et al. Liver-specific deletion of protein-tyrosine phosphatase 1B (PTP1B) improves metabolic syndrome and attenuates diet-induced endoplasmic reticulum stress. *Diabetes*. 2009;58(3):590-9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2646057&tool=pmcentrez&rendertype=abstract [Accessed January 13, 2011].

5. Rondinone CM, Kramer D. Proteasome inhibitors regulate tyrosine phosphorylation of IRS-1 and insulin signaling in adipocytes. *Biochemical and biophysical research communications*. 2002;296(5):1257-63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12207909 [Accessed January 30, 2011].

6. Badri DV, Vivanco JM. Regulation and function of root exudates. *Plant, Cell & Environment*. 2009;32(6):666-681. Available at: http://blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2009.01926.x [Accessed September 23, 2010].

7. Lanoue A, Burlat V, Henkes GJ, et al. De novo biosynthesis of defense root exudates in response to Fusarium attack in barley. *The New phytologist*. 2010;185(2):577-88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20699651 [Accessed September 7, 2010].

8. Junyent F, Romero R, Lemos L de, et al. Taurine treatment inhibits CaMKII activity and modulates the presence of calbindin D28k, calretinin, and parvalbumin in the brain. *Journal of neuroscience research*. 2010;88(1):136-42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19658200 [Accessed September 29, 2010].

9. Lee YY, Hong SH, Lee YJ, et al. Tauroursodeoxycholate (TUDCA), chemical chaperone, enhances function of islets by reducing ER stress. *Biochemical and biophysical research communications*. 2010;397(4):735-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20541525 [Accessed October 20, 2010].

10. Yang J-S, Kim JT, Jeon J, et al. Changes in hepatic gene expression upon oral administration of taurine-conjugated ursodeoxycholic acid in ob/ob mice. *PloS one*. 2010;5(11):e13858. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2974643&tool=pmcentrez&rendertype=abstract [Accessed December 4, 2010].

11. Amaral JD, Viana RJS, Ramalho RM, Steer CJ, Rodrigues CMP. Bile acids: regulation of apoptosis by ursodeoxycholic acid. *Journal of lipid research*. 2009;50(9):1721-34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2724780&tool=pmcentrez&rendertype=abstract [Accessed September 4, 2010].

12. Jiao P, Ma J, Feng B, et al. FFA-Induced Adipocyte Inflammation and Insulin Resistance: Involvement of ER Stress and IKKβ Pathways. *Obesity (Silver Spring, Md.)*. 2010;(July 2010):1-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20829802 [Accessed September 14, 2010].

13. Ozcan U, Yilmaz E, Ozcan L, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science*. 2006;313(5790):1137-40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16931765 [Accessed July 16, 2010].

14. Zhou L, Zhang J, Fang Q, et al. Autophagy-mediated insulin receptor down-regulation contributes to endoplasmic reticulum stress-induced insulin resistance. *Molecular pharmacology*. 2009;76(3):596. Available at: http://molpharm.aspetjournals.org/content/76/3/596.full [Accessed November 15, 2010].

15. Stapleton PP, Charles RP, Redmond HP, Bouchier-Hayes DJ. Taurine and human nutrition. *Clin Nutr*. 1997;16(3):103-108. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16844580.

16. Tsuboyama-Kasaoka N, Shozawa C, Sano K, et al. Taurine (2-aminoethanesulfonic acid) deficiency creates a vicious circle promoting obesity. *Endocrinology*.

2006;147(7):3276-3284. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16627576.

17. Aerts L, Van Assche FA. Taurine and taurine-deficiency in the perinatal period. *J Perinat Med*. 2002;30(4):281-286. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12235714.

18. Warskulat U, Borsch E, Reinehr R, et al. Chronic liver disease is triggered by taurine transporter knockout in the mouse. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20(3):574-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16421246 [Accessed December 11, 2010].