Published in final edited form as:

Arch Biochem Biophys. 2015 April 15; 572: 81–88. doi:10.1016/j.abb.2015.01.004.

Dietary tomato powder inhibits alcohol-induced hepatic injury by suppressing cytochrome p450 2E1 induction in rodent models

Camilla P. Stice^{1,3}, Chun Liu¹, Koichi Aizawa¹, Andrew S. Greenberg^{2,3}, Lynne M. Ausman^{1,3}, and Xiang-Dong Wang^{1,3,*}

¹Nutrition and Cancer Biology Laboratory, Jean-Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA

²Obesity and Metabolism Laboratory, Jean-Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA

³Friedman School of Nutrition Science and Policy, Tufts University, Boston, MA, USA

Abstract

Chronic and excessive alcohol consumption leads to the development of alcoholic liver disease (ALD) and greatly increases the risk of liver cancer. Induction of the cytochrome p450 2E1 (CYP2E1) enzyme by chronic and excessive alcohol intake is known to play a role in the pathogenesis of ALD. High intake of tomatoes, rich in the carotenoid lycopene, is associated with a decreased risk of chronic disease. We investigated the effects of whole tomato (tomato powder, TP), partial tomato (tomato extract, TE), and purified lycopene (LYC) against ALD development in rats. Of the three supplements, only TP reduced the severity of alcohol-induced steatosis, hepatic inflammatory foci, and CYP2E1 protein levels. TE had no effect on these outcomes and LYC greatly increased inflammatory foci in alcohol-fed rats. To further support the protective effect of TP against ALD, TP was supplemented in a carcinogen (diethylnitrosamine, DEN)-initiated alcohol-promoted mouse model. In addition to reduced steatosis and inflammatory foci, TP abolished the presence of preneoplastic foci of altered hepatocytes in DEN-injected mice fed alcohol. These reductions were associated with decreased hepatic CYP2E1 protein levels, restored levels of peroxisome proliferator-activated receptor- α and downstream gene expression, decreased inflammatory gene expression, and reduced endoplasmic reticulum stress markers. These data provide strong evidence for TP as an effective whole food prevention strategy against ALD.

Keywords

tomato; lycopene; CYP2E1; alcohol; liver disease

© 2015 Published by Elsevier Inc.

*To whom correspondence should be addressed. Xiang-Dong Wang, MD, PhD, Nutrition and Cancer Biology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Room 514, Boston, MA 02111, Phone: 617-556-3130, Fax: 617-556-3344, xiang-dong.wang@tufts.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

Chronic and excessive alcohol consumption is commonplace in both the United States and worldwide and is known to cause alcoholic liver disease (ALD) [1]. The initial development of ALD is characterized by steatosis (fatty liver) followed by steatohepatitis. These lay the groundwork for progression to the more damaging and irreversible stages of fibrosis and cirrhosis [2], which increases the risk of hepatocellular carcinoma development by almost 5-fold [3].

The enzyme cytochrome p450 2E1 (CYP2E1) is activated when excessive alcohol is consumed. The metabolism of alcohol via CYP2E1 results in the production of reactive oxygen species and inflammation that are thought to contribute significantly to ALD and related carcinogenesis [4–9]. Indeed, the specific involvement of CYP2E1 in ALD has been demonstrated utilizing both CYP2E1 overexpression, knock out (KO), and knock in (KI) experimental models [5, 10, 11]. Additionally, many hallmark biomarkers of ALD, including inflammation, peroxisome proliferator-activated receptor α (PPAR α)-regulated steatosis, and endoplasmic reticulum (ER) stress have previously been associated with the increase in alcohol-induced CYP2E1 [4, 5, 12], pinpointing CYP2E1 as a possible target for dietary intervention in ALD models.

High consumption of tomatoes and tomato products, rich in the carotenoid lycopene (LYC), is associated with a decreased risk of chronic disease including many types of cancer [13–16]. Tomatoes are a valuable source of many micronutrients and phytochemicals [17], yet despite the myriad of nutrients present in the tomato, the health benefits associated with tomato consumption have mainly been attributed to its high LYC content [18]. However, it is important to note that the plasma LYC levels associated with these health benefits may serve only as a marker for tomato consumption and does not necessarily mean LYC itself is responsible for the observed effects.

Although extensive research has been done on the beneficial effects of LYC, we previously demonstrated that the combination of LYC supplementation and consumption of alcohol resulted in exacerbation of alcohol-induced inflammation in a rat model [19]. Additionally, in recent years there have been several studies demonstrating the superior protective effects of tomato powder (TP, representative of whole tomato) when compared to purified LYC in both epidemiologic studies and experimental animals models [15, 20–24].

The observed superior protective powers of whole tomato as compared with purified LYC combined with the negative interaction between purified LYC and alcohol observed in our previous study raises an important question: is supplementation with complete or partial extract of whole food more effective than purified compound (LYC) in regard to prevention of ALD? We addressed this question by supplementing Wistar rats with TP (representative of whole tomato), tomato extract (TE, lipid soluble tomato components), and purified LYC in a Lieber-DeCarli alcoholic diet rat model. We then further investigated the protective effects of TP in a diethylnitrosamine (DEN)-initiated Lieber-DeCarli alcoholic diet-promoted hepatic lesion mouse model.

2. MATERIALS AND METHODS

2.1 Diets, Carcinogen, and Supplementation

The Lieber-DeCarli liquid ethanol diet (EtOH diet, 36% and 27% of total calories as ethanol fed to rats and mice, respectively) and control diet (Ctrl diet, where ethanol is replaced by isocaloric amounts of maltodextrin) (Dyets, Inc.) were used as experimental diets for all animal protocols. In our rat protocol, both purified LYC (Lycovit, BASF Chemical Company, Germany, 10% water-soluble beadlets) and placebo (PBO, Lycovit, BASF Chemical Company, Germany, contains all contents of LYC supplement except LYC) were given at the dose of $1.1 \text{ mg.kg BW}^{-1}.\text{d}^{-1}$, which has previously been utilized by our laboratory [19]. Each dose of tomato supplement (TE and TP) was calculated to provide $1.1 \text{ mg.kg BW}^{-1}.\text{d}^{-1}$ LYC. TE, (Lycovit, Beer-Sheva, Israel) described previously [25], was given at the dose of $18.3 \text{ mg.kg BW}^{-1}.\text{d}^{-1}$. TP, (Kagome, Co., LTD, Japan) containing all nutrients found in the tomato, was given at $1.46 \text{ g.kg BW}^{-1}.\text{d}^{-1}$. In our mouse protocol, the liver-specific carcinogen DEN (Sigma, >99.9% purity) was used to initiate carcinogenesis. DEN was injected intraperitoneally at a dose of 25 mg.kg BW^{-1} in 0.2 ml sterile saline when mice were 14 days of age and TP was given at $3 \text{ g.kg BW}^{-1}.\text{d}^{-1}$. For all protocols, diets were made twice weekly, blended to ensure adequate distribution of the supplement (LYC, TE or TP), and stored in opaque bottles at 4°C.

2.2 Animals and Study Design

Experiment #1—Male Wistar rats, 8 weeks of age, (Charles River Laboratories, Inc., Wilmington, MA) were randomized to 8 groups, as follows: 1) Ctrl diet + PBO, $n = 12$, 2) Ctrl diet + LYC, $n = 6$, 3) Ctrl diet + TE, $n = 6$, 4) Ctrl diet + TP, $n = 6$, 5) EtOH diet + PBO, $n = 12$, 6) EtOH diet + LYC, $n = 12$, 7) EtOH diet + TE, $n = 12$, and 8) EtOH diet + TP, $n = 12$. Rats went through a dietary adaptation period including a one week adaptation to the liquid Ctrl diet followed by a gradual two week adaptation to the EtOH diet. More specifically, the EtOH content of the diet began at 5% total calories by ethanol and was increased by 5% every 2–3 days until the concentration reached the full 36% total calories by ethanol. Following adaptation, supplementation was added to the diet and animals were group pair-fed approximately 100 ml of EtOH diet (36% of total calories as ethanol) or Ctrl diet daily for a 4 week treatment period.

Experiment #2—Male C57BL/6 mice were used. As experimental procedures were initiated pre-weaning, pregnant female C57BL/6 mice (gestational age of 15 days) were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in our animal facilities for birthing and kept with their pups until weaning. Upon initiation of the study, mice were randomized into the following groups: 1) Sham injection + Ctrl diet, $n = 14$, 2) DEN injection + Ctrl diet, $n = 14$, 3) DEN injection + EtOH diet, $n = 14$, and 4) DEN injection + EtOH diet + TP supplementation, $n = 14$. At 14 days of age, mice were injected with $25 \text{ mg DEN.kg BW}^{-1}$ or sham (saline). Following weaning at 4 weeks of age, mice were given Ctrl diet for 2 weeks. At 6 weeks of age, mice in the EtOH diet groups began a gradual adaptation to the EtOH diet, as described in experiment #1. At 8 weeks of age, TP supplementation was given with the full 27% EtOH diet for an experimental period of 21

days. Mice were group pair-fed with DEN injection + EtOH diet as lead group for the duration of the animal protocol.

Throughout both animal protocols, animal growth was monitored via weekly weighing and dietary intake was assessed daily. As the Lieber-DeCarli liquid diets provide sufficient amounts of water, no extra fluids were given for the duration of the studies. Upon completion of the study timelines, animals were terminally exsanguinated under deep isoflurane anesthesia. The Institutional Animal Care and Use Committee at the USDA Human Nutrition Research Center on Aging at Tufts University (Boston, MA) approved all animal procedures.

2.3 Histological Analyses

Liver sections (5 mm) from rats and mice were formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosin (H&E, Sigma Aldrich) for histological analysis. 20 separate fields of view at 100X magnification were microscopically examined by two separate investigators blinded to treatment for pre-neoplastic foci of altered hepatocytes (FAH), steatosis, and inflammatory foci. Specifically, pre-neoplastic FAH were characterized by the presence of either basophilic or eosinophilic foci and presented as incidence and multiplicity. Inflammatory foci, characterized by clusters of recruited immune cells, were analyzed as previously described [19] and represented as the number of foci per cm² (each field of view represents 0.63 cm²). Steatosis (micro- and macro-vesicular) was quantified as previously described [26]. Briefly, a grading system based on the percentage of liver section that is occupied by fat vacuoles was used. The grading system is defined as follows: grade 0 = <5% steatosis; grade 1 = 5–25%; grade 2 = 26–50%; grade 3 = 51–75%; grade 4 = >75%.

2.4 Protein Isolation and Western Blotting

Approximately 50 mg of liver tissue was extracted and whole cell lysate samples containing 25–100 µg protein were run in SDS-polyacrylamide gels (Protogel, National Diagnostics), as previously described [27]. Specific antibodies used include CYP2E1 (Millipore AB1252), phosphorylated eIF2α (Cell Signaling #3398P), and total eIF2α (Cell Signaling #5324P). Proteins were detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and quantified using a densitometer (Bio-Rad GS-710, Bio-Rad Laboratories). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Millipore MAB374) was used to normalize protein levels.

2.5 RNA Extraction and Real Time-PCR

Hepatic mRNA levels were determined by real-time PCR. Liver RNA was extracted using TriPure Isolation Reagent (Roche Applied Science) as per the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse transcription PCR (M-MLV, Invitrogen). Real-time PCR was performed using SYBR green (Fast Start Universal SYBR Green Master, Roche) according to the manufacturer's instructions on a 7500 Real Time PCR System (Applied Biosystems). Specific primer sequences are listed in Supplemental Table 1. Relative changes in gene expression were determined using the -2^{-C_t} method and normalized to the housekeeping gene GAPDH.

2.6 HPLC

Quantitative analysis of LYC was performed utilizing HPLC techniques, as previously described [28]. LYC concentrations were measured at 472 nm and quantified utilizing the area under the curve respective to an appropriate standard curve. An internal control (retinoic acid) was added to ensure efficient extraction and all procedures were performed under red light.

2.7 Statistical Analyses

In our rat study, two-way ANOVA followed by Dunnett's test was performed for animal weights, inflammatory foci, mRNA, and protein data and two-way ANOVA followed by Tukey's HSD was used to analyze HPLC data. Kruskal-Wallis overall test followed by Wilcoxon rank-sum test was performed for steatosis grading in both studies as well as multiplicity of pre-neoplastic FAH in our mouse study. In our mouse study, one-way ANOVA with Tukey's HSD to adjust for multiple comparisons was performed to analyze animal weights, and inflammatory foci. One-way ANOVA followed by Dunnett's test or T-tests were done to compare mRNA and protein data. The Statistical Analysis System (SAS, version 9.2, SAS Institute, Inc.) was used for all statistical analysis. Results are presented as means $\pm$ standard error of the means (SEM), unless otherwise indicated, and significance was set at $P < 0.05$.

3. RESULTS

3.1 Effects of EtOH Diet and Supplementation on Rat Weights

Consumption of the EtOH diet had a significant effect on rat weights, resulting in decreased body weight and increased liver weight and liver weight as a percentage of body weight as compared with rats fed Ctrl diet (Table 1). Supplementation with LYC, TE, and TP resulted in no significant changes in final body weight, liver weight, or liver weight as a percentage of body weight in either Ctrl diet-fed animals with the exception of increased liver weight in Ctrl diet + TP rats and no changes in EtOH diet-fed animals.

3.2 Effects of EtOH Diet and Supplementation on Hepatic LYC Concentrations in Rats

LYC was detected in livers of animals receiving LYC, TE, and TP supplementation fed both Ctrl and EtOH diet utilizing HPLC techniques (Table 1). No LYC was detected in livers of animals receiving PBO supplementation. Consumption of the EtOH diet was a significant ($P < 0.0001$) main effect in the model, resulting in increased hepatic LYC concentrations for all supplements. In mice consuming Ctrl diet + TE, significantly lower hepatic LYC (2 ± 0.3 nmol/g) was detected as compared with hepatic LYC concentrations in mice consuming Ctrl diet + TP (5.3 ± 0.3 nmol/g). The hepatic LYC concentration in mice consuming Ctrl diet + LYC was detected at 4.1 ± 0.4 nmol/g. No significant differences were found between hepatic LYC concentrations of Ctrl diet + LYC supplemented animals and Ctrl diet + TE or Ctrl diet + TP fed animals. Interestingly, no significant differences were found between hepatic LYC concentrations in mice consuming EtOH diet with LYC, TE, or TP supplementation.

3.3 Effects of Supplementation on EtOH Diet-Induced Liver Injury Outcomes in Rats

Consumption of the EtOH diet significantly induced hepatic steatosis as compared to Ctrl diet (Table 1). TP supplementation significantly reduced EtOH diet-induced hepatic steatosis with 91.7% of animals scoring a grade of 0. Supplementation with TE and LYC had no significant effect on EtOH diet-induced steatosis. Rats consuming EtOH diet had significantly increased hepatic inflammatory foci as compared to rats on Ctrl diet (Table 1). Supplementation with TP significantly reduced the EtOH diet-induced hepatic inflammatory foci by 94%. Supplementation with TE had no effect on this outcome, and we observed a significant increase in hepatic inflammatory foci in rats consuming EtOH diet and supplemented with LYC (3.7 fold increase) compared with EtOH diet + PBO supplementation (Table 1).

3.4 Effects of Supplementation on EtOH Diet-Altered CYP2E1 in Rats

CYP2E1 protein levels were dramatically (>8 fold) increased in rats consuming the EtOH diet as compared with Ctrl diet (Table 1). Supplementation with TP significantly reduced this EtOH-induced expression by 36% while supplementation with TE and LYC had no effect on CYP2E1 protein levels. In contrast, EtOH diet significantly decreased *Cyp2E1* mRNA expression levels. Supplementation with LYC, TE, or TP had no effect on *Cyp2E1* mRNA expression levels in rats consuming either Ctrl diet or EtOH diet.

3.5 Effects of DEN Injection, EtOH Diet, and Dietary Tomato Powder on Mouse Weights and Hepatic LYC Concentrations

Injection with DEN as compared to Sham injection in Ctrl diet-fed animals resulted in no significant differences in final body weight or liver weight (Table 2). Analysis of liver weight as a percentage of body weight was significantly increased in DEN + Ctrl Diet mice as compared with Sham + Ctrl diet mice. Addition of the EtOH diet resulted in a significant decrease in final body weight in DEN injected mice as compared with both Sham and DEN + Ctrl diet animals. Liver weight was significantly increased as compared with Sham + Ctrl diet mice; however no significant difference was found between DEN + Ctrl diet and DEN + EtOH diet liver weights. Liver weight as a percentage of body weight in DEN + EtOH diet mice was significantly increased compared with both Sham and DEN + Ctrl diet animals. The addition of TP to DEN + EtOH diet mice had no effect on final body weight, liver weight, or liver weight as a percentage of body weight as compared with DEN + EtOH diet mice without supplemental treatment. A few of mice died in either Sham + Ctrl, DEN + EtOH and DEN + EtOH+TP group during the experiment period without any obvious pathology. It was unlikely due to the DEN injection, we cannot exclude the possibility that the death of these mice was due to the EtOH feeding. Hepatic lycopene concentrations were determined using HPLC analysis (Table 2). As expected, no lycopene was detected in the livers of mice without TP supplementation (Sham + Ctrl diet, DEN + Ctrl diet, and DEN + EtOH diet mice). Mice fed TP had hepatic LYC concentrations of $0.366 \pm 0.035 \text{ nmol.g}^{-1}$ liver tissue.

3.6 Tomato Powder Reduces Alcohol-Promoted Hepatic Pre-Neoplastic FAH in Mice

Hepatic pre-neoplastic FAH (Figure 1) were not found in Sham + Ctrl diet or DEN + Ctrl diet mice. Addition of the EtOH diet in DEN-injected mice resulted in a significant induction in both multiplicity (1.59 ± 0.54 FAH per cm^2) and incidence (53.85%). The addition of TP to DEN + EtOH diet completely abolished the presence of pre-neoplastic FAH as none of the animals supplemented with TP had any lesions (Table 2).

3.7 Tomato Powder Reduces Alcohol-Induced CYP2E1 Protein Levels

No significant changes were found in hepatic *Cyp2E1* mRNA expression levels in any of the experimental groups, regardless of DEN injection or EtOH diet. Hepatic CYP2E1 protein levels were very similar in DEN + Ctrl diet mice as compared with Sham + Ctrl diet mice, with relative levels of 0.63 ± 0.29 and 1.0 ± 0.47 , respectively. The addition of EtOH diet resulted in a striking 26 fold increase of hepatic CYP2E1 protein levels in DEN + EtOH diet mice as compared with DEN + Ctrl diet mice. Supplementation with TP significantly reduced EtOH diet-induced CYP2E1 protein levels by 55% (Figure 2).

3.8 Tomato Powder Reduces Alcohol-Induced Endoplasmic Reticulum Stress

No changes were observed in hepatic expression levels of X-box-binding protein 1 (*Xbp1*); however, the spliced and transcriptionally active form, spliced *Xbp1* (*sXbp1*), was significantly increased in DEN + EtOH diet mice (1.7 and 1.9 fold increase) as compared with Sham + Ctrl diet and DEN + Ctrl diet mice, respectively (Figure 3). Additionally, phosphorylated eukaryotic translation-initiation factor 2 α (p-eIF2 α) levels were significantly increased (2.6 and 1.9 fold) in DEN + EtOH diet mice as compared with Sham + Ctrl diet and DEN + Ctrl diet mice, respectively. Supplementation with TP significantly reduced both EtOH diet-induced ER stress markers. Specifically, dietary TP had no effect on *Xbp1* expression but resulted in a significant reduction (2.8 fold) in expression of *sXbp1* and a 2.3 fold decrease in p-eIF2 α protein levels as compared with DEN + EtOH diet mice receiving no supplementation.

3.9 Tomato Powder Reduces Alcohol-Induced Hepatic Steatosis and Restores Alcohol-Suppressed PPAR α and Related Target Genes

Steatosis (Figure 1) was not present in Sham + Ctrl diet mice, where 100% of mice had a score of 0 (<5% steatosis). In DEN + Ctrl Diet mice all but 1 mouse had a score of 0, with 1 mouse scoring 1 on the steatosis grade scale. This slight increase was not statistically significant. The addition of ethanol consumption in the DEN + EtOH diet group significantly increased hepatic steatosis scoring with all animals scoring a grade of either 1 or 2 (61.5% and 38.5%, respectively). TP supplementation significantly reduced hepatic steatosis. In this group, 30.8% of animals were scored a steatosis grade of 0 while 61.5% scored a grade of 1 and only 7.7% scored a grade of 2 (Table 2).

Consumption of the EtOH diet in DEN + EtOH diet mice resulted in a significant suppression of *PPAR α* mRNA expression. TP supplementation restored DEN + EtOH diet-suppressed *PPAR α* expression and significantly increased levels of *PPAR α* target genes

carnitine palmitoyltransferase-1 (*CPT-1*), acyl-CoA oxidase 1 (*ACOX1*), and acyl-CoA oxidase 3 (*ACOX3*) (Figure 4).

4.0 Tomato Powder Reduces Alcohol-Induced Hepatic Inflammatory Foci and Related Gene Expression

Analysis of hepatic inflammatory foci (Figure 1) showed no significant changes between Sham + Ctrl diet and DEN + Ctrl diet mice. The addition of alcohol in DEN + EtOH diet mice resulted in a significant 20 fold increase in hepatic inflammatory foci as compared with DEN + Ctrl diet mice. The addition of supplement in DEN + EtOH diet + TP mice resulted in a significant reduction (7.4 fold) in hepatic inflammatory foci as compared with DEN + EtOH diet alone. TP supplementation returned hepatic inflammatory foci levels almost completely back to those found in the Sham + Ctrl diet and DEN + Ctrl diet mice (Table 2). The reduction in inflammatory foci by supplementation with TP was associated with reduced levels of *IL-6*, interferon γ (*IFN* γ), *IL-1 β* , and NACHT, LRR and PYD domains-containing protein 3 (*NLRP3*) in DEN + EtOH diet + TP mice compared with DEN + EtOH diet mice fed no supplementation (Figure 4).

4. DISCUSSION

In our first study, we used an established rat model of ALD to demonstrate the protective effects of whole tomato (TP supplementation), but not partial extract (TE supplementation), against ALD via the proposed mechanism of CYP2E1 protein down-regulation. Additionally, our first study highlights the detrimental interaction of alcohol and supplementation with a single nutrient (LYC) in replace of whole food. We demonstrated that consumption of the EtOH diet resulted in increased steatosis, inflammatory foci, and CYP2E1 protein levels in the liver. LYC supplementation showed no significant alterations in steatosis or CYP2E1 protein levels but a dramatic 3.7-fold increase in hepatic inflammatory foci in EtOH diet-fed rats. This confirms our previous observation of increased hepatic inflammatory foci seen with the combination of EtOH diet and LYC supplementation [19]. This negative interaction represents an important public health message. Specifically, individuals consuming high amounts alcohol should be wary of supplementation with individual nutrients.

An important observation of this study was the difference between supplementation with TP (representative of whole tomato) compared with TE (extract containing only lipid soluble components of the whole tomato) against ALD. Interestingly, TP significantly reduced ethanol-induced hepatic injury while TE had no effect in our ALD rat model. Specifically, steatosis was reduced to an almost uniform (91.7%) grade 0 in rats supplemented with TP fed EtOH diet. Inflammatory foci were reduced by 88% with TP supplementation and there was a 38% reduction in hepatic CYP2E1 protein levels as compared with rats fed EtOH diet + PBO. As alcohol-induced CYP2E1 protein expression has been directly linked to the development of both steatosis and inflammatory foci [4, 5, 11, 29, 30], the observation that supplementation with TP significantly reduced CYP2E1 protein levels provides a possible mechanism of action to explain the associated reduction of steatosis and inflammatory foci by TP. Interestingly, no changes in *Cyp2E1* mRNA levels were found with the consumption of alcohol even with a dramatic increase in CYP2E1 protein levels. These observations point

to a posttranslational mechanism of CYP2E1 alteration. Indeed, a lack of change, and even reduced levels, in mRNA expression have been previously documented in alcohol-fed animals that have increased CYP2E1 protein [31, 32], thus supporting our findings.

Analysis showed that hepatic concentrations of LYC detected from LYC, TE, and TP supplements ($2.03\text{--}6.77\text{ nmol.g}^{-1}$) in rats and TP supplement ($0.366 \pm 0.035\text{ nmol.g}^{-1}$) in mice are within the normal range detected in human liver tissue ($0.1\text{--}20.7\text{ nmol.g}^{-1}$) [33, 34]. This indicates that the effects observed in both animal protocols are occurring at physiologically relevant concentrations. The LYC dosage chosen for our rat study ($1.1\text{ mg.kg BW}^{-1}.\text{d}^{-1}$) is equivalent to an intake of approximately 12.46 mg LYC per day for a 70-kg adult [35]. Due to the variation of lycopene content in food sources, it has been difficult to estimate lycopene daily intake and the ranges of 3.7 to 16.2 mg LYC per day have been reported for the United States [36]. While this dose is slightly higher than the current average intake, it is certainly an easily achievable dose based on whole food consumption. For example, in order to achieve this equivalent dose, one would need to consume approximately 4 medium sized tomatoes (calculated from 2.57 mg LYC per 100 g tomato and 1 medium tomato weighs approximately 125 g) or 1/3 cup of tomato sauce (15.15 mg LYC per 100 g tomato sauce and 1 cup = 245 g) per day [37]. The dose of $3\text{ g.kg BW}^{-1}.\text{d}^{-1}$ TP supplementation in our mouse study can also be calculated to an intake of approximately 4 medium sized tomatoes per day [37]. Our rat study clearly demonstrates that consumption of the EtOH diet significantly increases hepatic LYC concentrations compared with the same supplementation in Ctrl diet. At this point, the mechanism responsible for the observed increase in hepatic LYC with consumption of the EtOH diet is unclear; however, it is possible that ethanol increases intestinal absorption or tissue uptake of LYC thus leading to an increase in liver tissue levels. Although hepatic LYC concentrations were significantly lower in Ctrl diet + TE animals as compared with Ctrl diet + TP, no differences between supplementation with LYC, TE, or TP were found in rats fed EtOH diet. Therefore, it is clear that the observed differences in hepatic protection between LYC, TE, and TP supplements are not due to differences in LYC absorption, but rather other components found in the tomato.

The TP supplement is composed of all nutrients found in a tomato, including polyphenols, ascorbic acid, folate, α -tocopherol, quercetin, and carotenoids α - and β -carotene, lycopene, lutein, zeaxanthin, phytoene, and phytofluene. Although it is unclear what exact component or components of the TP are responsible for the observed hepatic protection, it is clear that all tomato components (or at least more than solely lipid soluble components found in TE) must be present to achieve this effect. Though we cannot be sure that a single compound found in TP and not TE is responsible, our study points to the collective beneficial effect given by whole food intervention. Whole food contains a complex collection of various nutrients. When separated, these nutrients may respond to different environments in an altered – or even contrasting – manner than when in the presence of their natural nutrient companions. It should be mentioned that the optimal placebo control in our study should be a tomato powder (or tomato extract) without lycopene. Unfortunately, it is not feasible to remove lycopene alone due to other fat-soluble components. Indeed, the weight gain of the animals fed with TE or TP were slightly higher than that of the control groups without TE or TP. However, since these animals were group pair-fed with the liquid diet, the contents of

alcohol, calories, and volume of the diets consumed were controlled in these animals. In addition, the supplements were in very small amount (with negligible caloric), relative to a large amount of liquid diet. We believe that the slightly body weight gain, which was not statistically significant, could be due to the protection of TP rather than caloric. Utilizing a whole food intervention approach provides multiple nutrients with a broad range of biological activities creating the potential for complementary, additive, or synergistic activities that are lacking when supplementation is given with single nutrients. This phenomenon could explain why superior protective effects are found from tomato supplementation versus purified LYC in some experimental models [15, 20–24].

To further support our findings that TP is capable of protecting against alcohol-induced hepatic injury, we then supplemented mice with TP in a DEN-initiated EtOH diet-promoted hepatic lesion model. In this model, pre-neoplastic FAH were only induced in mice given DEN injection + EtOH diet (53.85% incidence). No pre-neoplastic FAH were induced with DEN injection alone, most likely due to the small dose of DEN or short duration of the study. This demonstrates the crucial role that consumption of the EtOH diet plays in the promotion of pre-cancerous lesions in our model. The induction of pre-neoplastic FAH in DEN + EtOH diet mice was significantly correlated with hepatic injury including increased CYP2E1 protein levels, steatosis, and inflammatory foci. ER stress markers *sXbp1* expression and p-eIF2 α protein levels were also increased, indicating activation of both the protein kinase RNA –like ER kinase and inositol requiring 1 α branches of the unfolded protein response. Therefore, this newly established DEN-initiated EtOH diet-promoted hepatic lesion mouse model provides an ideal environment in which to further elucidate the mechanisms by which alcohol-promoted HCC development is initiated and investigate the potential protective effects of TP supplementation.

TP supplementation in our mouse model resulted in a complete eradication (0% incidence) of pre-neoplastic FAH in DEN + EtOH diet mice. The inhibition of pre-neoplastic FAH with TP supplementation was associated with a decrease in alcohol-induced CYP2E1 protein levels, further supporting CYP2E1 as a possible mechanistic target of TP. Indeed, the induction of CYP2E1 by alcoholic diet has not only been demonstrated as a key player in the development of alcohol-induced hepatic injury but also carcinogenesis [4, 5, 7, 8, 12].

Previous animal studies utilizing CYP2E1 KO and KI mouse models have demonstrated the connection between alcohol-induced CYP2E1 levels and a reduction of PPAR α leading to the development of steatosis observed with consumption of alcohol [4, 5]. Reduced PPAR α levels were associated with a reduction of ACOX, an enzyme essential for β -oxidation [5]. In our study, we observed a reduction in steatosis by TP supplementation. This reduction was associated with restored levels of EtOH diet-suppressed PPAR α expression and related fatty acid transport (*CPT-1*) and β -oxidation (*ACOX1*, and *ACOX3*) gene expression. These studies, including our own, suggest that the reduction in PPAR α seen with consumption of an alcoholic diet may be due, at least in part, to the induction of CYP2E1 in the alcohol models.

Alcohol-induced CYP2E1 expression has also been connected with the increase in inflammation associated with alcohol consumption. Treatment with a CYP2E1 inhibitor

resulted in the inhibition of alcohol-induced inflammatory cytokine expression in both Kupffer cell and rat models [29, 30]. Additionally, alcohol-induced ER stress has been associated with increased CYP2E1 protein levels in a micropig model [38]. Although no KO or chemical inhibitor studies have been done to demonstrate the causal role of CYP2E1 in alcohol-induced ER stress, it has been proposed in previous alcohol models [39]. In our study, the decrease in CYP2E1 by TP was associated a significant reduction in the severity of alcohol-induced inflammatory foci. Further, this reduction was associated with decreased inflammatory gene expression of *IL-6*, *IFN γ* , *IL-1 β* , and *NLRP3*. Alcohol-induced ER stress markers *sXbp1* mRNA expression and p-eIF2 α levels were also significantly reduced upon consumption of dietary TP.

Taken together, our studies clearly identify TP as a novel candidate for dietary prevention against alcohol-related hepatic injury through CYP2E1 protein reduction. Further, we stress the need for a whole food approach toward disease prevention and caution for the consumption of alcohol coupled with supplementation of isolated compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the USDA/ARS CRIS grant 1950-51000-074S and NIH/NCI CA176256 grant. Any opinions, findings, conclusions, and recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the sponsors. The authors would like to thank Dr. Donald Smith for his assistance in designing and conducting the animal protocols included in this manuscript. The authors would also like to thank Junrui Cheng for her assistance on this manuscript.

Abbreviations

ALD	alcoholic liver disease
CYP2E1	cytochrome p450 2E1
TP	tomato powder
TE	tomato extract
LYC	lycopene
DEN	diethylnitrosamine
KO	knockout
KI	knock in
PPARα	peroxisome proliferator-activated receptor α
ER	endoplasmic reticulum
EtOH	ethanol
Ctrl	control
H&E	hematoxylin and eosin

FAH	foci of altered hepatocytes
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Xbp1	X-box-binding protein 1
sXbp1	spliced Xbp1
CPT-1	palmitoyltransferase-1
ACOX1	acyl-CoA oxidase 1
ACOX3	acyl-CoA oxidase 3
IFNγ	interferon γ
NLRP3	NACHT, LRR and PYD domains-containing protein 3

References

- Williams R. Hepatology. 2006; 44:521–526. [PubMed: 16941687]
- Gao B, Bataller R. Gastroenterology. 2011; 141:1572–1585. [PubMed: 21920463]
- Hassan MM, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Hepatology. 2002; 36:1206–1213. [PubMed: 12395331]
- Cederbaum AI. Dig Dis. 2010; 28:802–811. [PubMed: 21525766]
- Lu Y, Wu D, Wang X, Ward SC, Cederbaum AI. Free Radic Biol Med. 2010; 49:1406–1416. [PubMed: 20692331]
- Wang Y, Millonig G, Nair J, Patsenker E, Stickel F, Mueller S, Bartsch H, Seitz HK. Hepatology. 2009; 50:453–461. [PubMed: 19489076]
- Tsuchishima M, George J, Shiroeda H, Arisawa T, Takegami T, Tsutsumi M. Dig Dis Sci. 2013; 58:1923–1933. [PubMed: 23371017]
- Ye Q, Lian F, Chavez PRG, Chung J, Ling W, Qin H, Seitz HK, Wang XD. HBSN. 2012; 1:5–18. [PubMed: 23543859]
- Seitz HK, Wang XD. Subcell Biochem. 2013; 67:131–143. [PubMed: 23400919]
- Morgan K, French SW, Morgan TR. Hepatology. 2002; 36:122–134. [PubMed: 12085356]
- Wu D, Wang X, Zhou R, Cederbaum A. Biochem Biophys Res Commun. 2010; 402:116–122. [PubMed: 20932821]
- Hoek JB, Pastorino JG. Alcohol. 2002; 27:63–68. [PubMed: 12062639]
- Etminan M, Takkouche B, Caamano-Isorna F. Cancer Epidemiology Biomarkers & Prevention. 2004; 13:340–345.
- Giovannucci E. Exp Biol Med (Maywood). 2002; 227:852–859. [PubMed: 12424325]
- Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. J Natl Cancer Inst. 2002; 94:391–398. [PubMed: 11880478]
- La Vecchia C. Exp Biol Med (Maywood). 2002; 227:860–863. [PubMed: 12424326]
- Canene-Adams K, Campbell JK, Zaripheh S, Jeffery EH, Erdman JW Jr. J Nutr. 2005; 135:1226–1230. [PubMed: 15867308]
- Basu A, Imrhan V. Eur J Clin Nutr. 2007; 61:295–303. [PubMed: 16929242]
- Veeramachaneni S, Ausman LM, Choi SW, Russell RM, Wang XD. J Nutr. 2008; 138:1329–1335. [PubMed: 18567756]
- Alshatwi AA, Al Obaid MA, Al Sedairy SA, Al-Assaf AH, Zhang JJ, Lei KY. Nutr Res. 2010; 30:66–73. [PubMed: 20116662]
- Boileau TW, Liao Z, Kim S, Lemeshow S, Erdman JW Jr, Clinton SK. J Natl Cancer Inst. 2003; 95:1578–1586. [PubMed: 14600090]
- Kim Y, DiSilvestro R, Clinton S. Phytomedicine. 2004; 11:152–156. [PubMed: 15070165]

23. Narisawa T, Fukaura Y, Hasebe M, Nomura S, Oshima S, Sakamoto H, Inakuma T, Ishiguro Y, Takayasu J, Nishino H. *Jpn J Cancer Res.* 1998; 89:1003–1008. [PubMed: 9849577]
24. Sesso HD, Liu S, Gaziano JM, Buring JE. *J Nutr.* 2003; 133:2336–2341. [PubMed: 12840203]
25. Wang Y, Ausman LM, Greenberg AS, Russell RM, Wang XD. *Int J Cancer.* 2010; 126:1788–1796. [PubMed: 19551842]
26. Wang Y, Seitz HK, Wang XD. *Alcohol Clin Exp Res.* 2010; 34:567–573. [PubMed: 20028348]
27. Chung J, Chavez PR, Russell RM, Wang XD. *Oncogene.* 2002; 21:1539–1547. [PubMed: 11896582]
28. Melendez-Martinez AJ, Nascimento AF, Wang Y, Liu C, Mao Y, Wang XD. *Hepatobiliary Surg Nutr.* 2013; 2:198–208. [PubMed: 24273751]
29. Fang C, Lindros KO, Badger TM, Ronis MJ, Ingelman-Sundberg M. *Hepatology.* 1998; 27:1304–1310. [PubMed: 9581684]
30. Ye Q, Wang X, Wang Q, Xia M, Zhu Y, Lian F, Ling W. *Hepato Res.* 2013; 43:1115–1123. [PubMed: 23421770]
31. Porter TD, Khani SC, Coon MJ. *Mol Pharmacol.* 1989; 36:61–65. [PubMed: 2747631]
32. Ronis MJ, Huang J, Crouch J, Mercado C, Irby D, Valentine CR, Lumpkin CK, Ingelman-Sundberg M, Badger TM. *J Pharmacol Exp Ther.* 1993; 264:944–950. [PubMed: 8437134]
33. Boileau TW, Boileau AC, Erdman JW Jr. *Exp Biol Med (Maywood).* 2002; 227:914–919. [PubMed: 12424334]
34. Schmitz HH, Poor CL, Wellman RB, Erdman JW Jr. *J Nutr.* 1991; 121:1613–1621. [PubMed: 1765826]
35. Reagan-Shaw S, Nihal M, Ahmad N. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2008; 22:659–661. [PubMed: 17942826]
36. Porrini M, Riso P. *J Nutr.* 2005; 135:2042S–2045S. [PubMed: 16046736]
37. USDA. 2012. [cited; Available from: <http://ndb.nal.usda.gov/ndb/search/list>]
38. Esfandiari F, Villanueva JA, Wong DH, French SW, Halsted CH. *Am J Physiol Gastrointest Liver Physiol.* 2005; 289:G54–63. [PubMed: 15705656]
39. Ji C, Kaplowitz N. *Gastroenterology.* 2003; 124:1488–1499. [PubMed: 12730887]

- Tomato powder is protective against alcohol-induced fatty liver disease.
- Tomato powder prevents the pre-neoplastic lesions in carcinogen-injected animals fed alcohol.
- Purified lycopene supplementation enhances alcohol-related liver injury.

Figure 1. Representative Histological Injury in Livers of DEN + EtOH Diet Mice
Representative H&E staining of A) basophilic foci, B) eosinophilic foci, C) inflammatory foci, and D) steatosis in DEN + EtOH diet mice. A ZEISS microscope with a PixeLINK USB 2.0 (PL-B623CU) digital Camera and PixeLINK μ Scope Microscopy Software was used for image capture.

Figure 2. Dietary TP Alleviates DEN + EtOH Diet-Induced CYP2E1 Protein Levels

CYP2E1 protein and *Cyp2E1* mRNA expression levels were measured in mouse livers. Addition of TP supplementation resulted in A) a significant decrease in DEN + EtOH diet-induced CYP2E1 protein levels with B) no change in *Cyp2E1* mRNA expression levels. Results are presented as means $\pm$ SEM (n = 10–14/per group). One-way ANOVA followed by Dunnett's test was used to determine statistical significance between treatment groups. * indicates $P < 0.05$.

Figure 3. Dietary TP Reduces DEN + EtOH Diet-Induced ER Stress Markers

Markers of the unfolded protein response pathways were examined to indicate activation of ER stress in the model. Dietary TP reduced DEN + EtOH diet-induced A) p-eIF2 and B) *sXBP1*, indicating the involvement of protein kinase RNA-like ER kinase and inositol requiring 1 α pathways, respectively. Results are presented as means $\pm$ SEM (n = 10–14/per group). One-way ANOVA followed by Dunnett's test was used to determine statistical significance between treatment groups * indicates $P < 0.05$.

Figure 4. Effects of Dietary TP on mRNA Expression of Lipid and Inflammatory Genes
 Dietary TP A) significantly reduced hepatic inflammatory gene expression of *IL-6*, *IFNγ*, *IL-1β*, and *NLRP3* and B) significantly increased expression levels of *PPARα*, *CPT1*, *ACOX1*, and *ACOX3* in DEN + EtOH Diet mice. Results are presented as means $\pm$ SEM (n = 10–14/per group). T-test was used to determine statistical significance between treatment groups. * indicates $P < 0.05$.

Table 1

Effects of EtOH Diet and Supplementation on Study Outcomes

	Ctrl Diet				EtOH Diet			
	PBO	LYC	TE	TP	PBO	LYC	TE	TP
Animal (n)	12	6	6	6	12	12	12	12
Body Wt.								
Initial Wt.(g) ¹	264±2.6	266±6.1	264.8±5.3	258.4±14.9	264.9±3.3	264.3±2.5	264.1±2.5	262.1±11.0
Final Wt.(g) ¹	379.4±4.5	366.7±6.8	393.3±8.5	398.7±7.6	362.7±6	362.1±8.1	379.3±5.1	378.2±6.1
% Wt. Change	+43%	+38%	+49%	+54%	+33%	+37%	+44%	+44%
Liver Wt. (g) ¹	12.1±0.3	11.5±0.3	12.9±0.3	13.9±0.6*	14.0±0.3	13.1±0.5	13.7±0.4	14.8±0.4
LW/FBW (%) ¹	3.2±0.1	3.1±0.1	3.3±0.1	3.5±0.1	3.9±0.1	3.6±0.1	3.6±0.1	3.9±0.1
Steatosis ²	0 (0–2) ^a	0 (0–1) ^a	0 (0–1) ^a	0 (0–1) ^a	1 (1–3) ^b	2 (2–4) ^b	1.5 (0–3) ^b	0 (0–1) ^a
Inflamm. Foci ¹	2.1±0.5	1.9±0.6	1.1±0.5	0±0	5.2±0.8	19.1±2.3 [†]	6.2±0.7	0.3±0.3 [†]
CYP2E1 (Relative Levels)								
Protein ¹	1.0±0.3	0.8±0.4	0.5±0.2	0.6±0.3	8.4±0.8	8.1±1.5	8.2±1.3	5.4±0.4 [†]
mRNA ¹	1.0±0.7	0.9±0.6	0.9±0.2	0.9±0.2	0.7±0.1	0.8±0.2	0.8±0.1	0.6±0.1
Hepatic LYC (nmol/g) ³	N.D.	4.1±0.4 ^{ab}	2±0.3 ^a	5.3±0.3 ^{bc}	N.D.	5.8±0.7 ^{bc}	4.9±0.4 ^{bc}	6.8±0.6 ^c

¹ Data are presented as means ± SEM. Two-way ANOVA followed by Dunnett's test was performed for each outcome. EtOH diet was a significant (P<0.01) main effect for all outcome models. For Ctrl diet-fed animals, * indicates a significant difference from Ctrl diet + PBO animals (P<0.05). For EtOH diet-fed animals, † indicates a significant difference from EtOH diet + PBO animals (P<0.05).

² Data are presented as median (grading range). Kruskal-Wallis overall test followed by Wilcoxon rank-sum test was performed. Data not sharing a common superscript letter are statistically significant from each other.

³ Data are presented as means ± SEM. Two-way ANOVA followed by Tukey's HSD was performed to determine statistical significance between groups. EtOH diet was a significant (P<0.0001) main effect in the model. Data not sharing a common superscript letter are statistically significant from each other.

For all data, significance was set at P<0.05. Abbreviations: Wt. – weight, LW/FBW – liver weight/final body weight, Inflamm. – inflammatory, N.D. – not detected.

Table 2

Effects of Dietary TP on Study Outcomes

	Animal (n)	Sham Ctrl Diet	DEN Ctrl Diet	DEN EtOH Diet	DEN EtOH Diet TP	P Value of Main Effect	
						DEN	TP
Body Weight ¹	11	14	13	10			
Initial Wt. (g)	21.5±0.5 ^a	20.6±0.7 ^a	20.3±0.3 ^a	20.3±0.3 ^a	0.2367	0.6301	0.9325
Final Wt. (g)	28.6±0.8 ^b	27.4±0.9 ^b	23.8±0.2 ^a	24.9±0.5 ^a	0.1946	0.0004	0.2824
Wt. Change (%)	+25±1.3 ^b	+25±1.0 ^b	+15±1.0 ^a	+17±1.6 ^a	0.8020	<.0001	0.0947
Liver Wt. (g) ¹	1.2±0.1 ^a	1.4±0.1 ^{ab}	1.4±0.1 ^b	1.4±0.1 ^b	0.0274	0.3846	0.8739
LW/FBW % ¹	4.3±0.1 ^a	5.0±0.1 ^b	6.0±0.1 ^c	5.8±0.2 ^c	0.0003	<.0001	0.2981
Inflamm. Foci (No./cm²) ¹	0.4±0.4 ^a	0.2±0.2 ^a	4.6±0.6 ^b	0.4±0.3 ^a	0.7255	<.0001	<.0001
Pre-neoplastic FAH ²							
Incidence (%)	0 ^a	0 ^a	53.8 ^b	0 ^a			
Multiplicity (No./cm²)	0±0 ^a	0±0 ^a	1.6±0.5 ^b	0±0 ^a			
Steatosis Grade (%)							
0	100	92.9	0	30.8			
1	0	7.1	61.5	61.5			
2	0	0	38.5	7.7			
3	0	0	0	0			
4	0	0	0	0			
Median Value ³	0 ^a	0 ^a	1 ^c	1 ^b			
Hepatic LYC (nmol/g)	N.D.	N.D.	N.D.	0.37±0.04			

¹ Data are presented as means ± SEM One-way ANOVA followed by Tukey's HSD was performed.

² Data are presented as means ± SEM and Kruskal-Wallis overall test followed by Wilcoxon rank-sum test was performed.

³ Data are presented as median (grading range). The degree of steatosis was graded 0–4 based on the area of liver section occupied by fat vacuole (grade 0 = <5% steatosis; grade 1 = 5–25%; grade 2 = 26–50%; grade 3 = 51–75%; grade 4 = >75%). Kruskal-Wallis overall test followed by Wilcoxon rank-sum test was performed.

For all rows, data not sharing a common superscript letter are statistically significant from each other (P<0.05). Abbreviations: Wt. – weight, LW/FBW – liver weight/final body weight, inflamm. – inflammatory, FAH – foci of altered hepatocytes.