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Fructose Promotes Leaky Gut, Endotoxemia, and Liver Fibrosis Through Ethanol-Inducible Cytochrome P450-2E1–Mediated Oxidative and Nitrative Stress

Young-Eun Cho,[1,2*] Do-Kyun Kim,[3*] Wonhyo Seo,[4] Bin Gao,[4] Seong-Ho Yoo,[5] and Byoung-Joon Song[1]

Fructose intake is known to induce obesity, insulin resistance, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD). We aimed to evaluate the effects of fructose drinking on gut leakiness, endotoxemia, and NAFLD and study the underlying mechanisms in rats, mice, and T84 colon cells. Levels of ileum junctional proteins, oxidative stress markers, and apoptosis-related proteins in rodents, T84 colonic cells, and human ileums were determined by immunoblotting, immunoprecipitation, and immunofluorescence analyses. Fructose drinking caused microbiome change, leaky gut, and hepatic inflammation/fibrosis with increased levels of nitroxidative stress marker proteins cytochrome P450-2E1 (CYP2E1), inducible nitric oxide synthase, and nitrated proteins in small intestine and liver of rodents. Fructose drinking significantly elevated plasma bacterial endotoxin levels, likely resulting from decreased levels of intestinal tight junction (TJ) proteins (zonula occludens 1, occludin, claudin-1, and claudin-4), adherent junction (AJ) proteins (β-catenin and E-cadherin), and desmosome plakoglobin, along with α-tubulin, in wild-type rodents, but not in fructose-exposed *Cyp2e1*-null mice. Consistently, decreased intestinal TJ/AJ proteins and increased hepatic inflammation with fibrosis were observed in autopsied obese people compared to lean individuals. Furthermore, histological and biochemical analyses showed markedly elevated hepatic fibrosis marker proteins in fructose-exposed rats compared to controls. Immunoprecipitation followed by immunoblot analyses revealed that intestinal TJ proteins were nitrated and ubiquitinated, leading to their decreased levels in fructose-exposed rats. *Conclusion:* These results showed that fructose intake causes protein nitration of intestinal TJ and AJ proteins, resulting in increased gut leakiness, endotoxemia, and steatohepatitis with liver fibrosis, at least partly, through a CYP2E1-dependent manner. (Hepatology 2021;73:2180-2195).

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Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome, and the increased incidence of NAFLD is a major public health concern in the Western, developed countries.[1] Indeed, NAFLD encompasses a spectrum of liver disease ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis/cirrhosis, and progression to hepatic cancer in some patients.[2] Recent studies suggest that overconsumption of Western-style high-fat diet and/or

Abbreviations: 3-NT, 3-nitrotyrosine; Acy-His3, acetyl-histone-3; AFLD, alcoholic fatty liver disease; AJ, adherent junction; ALT, alanine aminotransferase; AMPK, adenosine 5′ monophosphate-activated protein kinase; Bcl-2, B-cell lymphoma 2–associated X protein; BMI, body mass index; CLOCK, clock circadian regulator; Col, collagen; CYP2E1, ethanol-inducible cytochrome P450-2E1; EU/mL, endotoxin units per milliliter; FITC-D4, fluorescein isothiocyanate–labeled 4-kDa dextran; H&E, hematoxylin and eosin; HF, hepatic fibrosis; HSC, hepatic stellate cell; IHC, immunohistochemistry; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; kDa, kilodalton; LF, liver fibrosis; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NLRP3, NLR family pyrin domain-containing 3; p-, phosphorylated; PER-2, period circadian regulator 2; PGC1-α, proliferator-activated receptor-γ coactivator 1 alpha; PPARα, peroxisome proliferator-activated receptor alpha; ROS, reactive oxygen species; Sirt1, sirtuin-1; α-SMA, α-smooth muscle actin; SREBP, sterol regulatory element-binding protein; TEER, transepithelial electrical resistance; TG, triglyceride; TGF-β, transforming growth factor beta; TIMP-1, tissue inhibitor of metalloproteinase-1; TJ, tight junction; TLR-4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha; WT, wild type; ZO-1, zonula occludens 1.

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high-fructose corn syrup, primarily in the form of soft drinks, is a major causal factor for the development of NAFLD/NASH.[2,3] Increased fructose intake causes fatty liver in animals along with increased fat mass and obesity,[2,3] *de novo* lipogenesis,[3] and inflammation[2] and induces insulin resistance and metabolic syndrome,[4] particularly in overweight animals and individuals.[2]

Recent studies of NAFLD/NASH in both rodent models and in humans have shown that dietary habits can influence gut flora, leading to increased gut permeability (leaky gut) and circulating endotoxin levels.[5,6] An n-6 fatty-acid–enriched high-fat and high-fructose–containing diet offers a potential explanation for the increased prevalence of intestinal bacterial overgrowth in patients with cirrhosis, leading to NAFLD/NASH.[7] In fact, Bergheim et al. previously reported that fructose alone, either in drinking water or solid diet, can cause insulin resistance, obesity, and NAFLD with inflammation.[8-10] These conditions, accompanied with leaky gut, contribute to increased levels of serum bacterial endotoxin lipopolysaccharide (LPS) and advanced liver disease. However, in our opinion, the underlying mechanisms of fructose-mediated gut leakiness are poorly understood with respect to the role of increased oxidative and nitrative (nitroxidative) stress in stimulating protein modifications of intestinal proteins, including various tight junction (TJ) and adherent junction (AJ) proteins, and apoptosis of enterocytes.

Therefore, this study was aimed to investigate the roles of the intestinal and hepatic ethanol-inducible cytochrome P450-2E1 (CYP2E1) and inducible nitric oxide synthase (iNOS) in promoting nitroxidative stress, contributing to leaky gut and inflammatory liver disease in fructose-exposed rats and mice. We further studied the roles of microbiome change, apoptosis of intestinal enterocytes, and protein modifications of gut TJ/AJ proteins in increased gut leakiness and advanced liver disease in rats and wild-type (WT) versus *Cyp2e1*-null mice. The effects of fructose on levels of cell death and TJ proteins were also investigated in T84 colonic cells as a model to strengthen our rodent results and conduct the mechanistic studies on fructose-mediated epithelial barrier dysfunction. Finally, we analyzed ileums and livers from autopsied obese people with NASH compared to those of nonobese individuals to validate the results observed with the experimental models.

Materials and Methods

HUMAN SUBJECTS

This study, with the protocol to collect ileums and body fluids from autopsied people, was approved by the Human Subjects Institutional Review Board of

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**These authors equally contributed for the report.*

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ARTICLE INFORMATION:

From the [1]Section of Molecular Pharmacology and Toxicology, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, NIH, Bethesda, MD; [2]Department of Food and Nutrition, Andong National University, Andong, South Korea; [3]Mast Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; [4]Laboratory of Liver Diseases, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD; [5]Department of Forensic Medicine, Seoul National University College of Medicine, Seoul, South Korea.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Byoung-Joon Song, Ph.D.
Laboratory of Membrane Biochemistry and Biophysics
National Institute on Alcohol Abuse and Alcoholism
9000 Rockville Pike
Bethesda, MD 20892-9410
E-mail: bj.song@nih.gov
Tel.: +1-301-496-3985

TABLE 1. Clinical Data of Autopsied Human Subjects With NASH

Sample No.	Sex	Age	BMI (kg/m^2)	Group*	Liver Histology
1	Female	26	17.8	Nonobese	Normal
2	Male	74	17.8	Nonobese	Normal
3	Female	46	15.9	Nonobese	Normal
4	Male	29	21.8	Nonobese	Normal
5	Male	60	16.7	Nonobese	Normal
6	Male	58	18.9	Nonobese	Normal
7	Female	61	25.5	Obese	NASH/fibrosis
8	Male	62	30.8	Obese	NASH/fibrosis
9	Male	57	29.1	Obese	NASH/fibrosis
10	Male	54	30.7	Obese	Not analyzed
11	Male	50	29.7	Obese	Not analyzed

*Classification of obesity as recommended by the Asia-Pacific Task Force.

the Seoul National University Medical College (Seoul, Korea). Clinical characteristics of autopsied obese and nonobese individuals are presented in Table 1.

ANIMAL TREATMENTS

All animal experimental procedures were carried out by following the National Institutes of Health guidelines for small animal experiments and approved by the National Institute on Alcohol Abuse and Alcoholism Institutional Animal Care and Use Committee, as described.[11] Age-matched 7-week-old female Fischer 344 WT rats (n ≥ 8/group) as well as approximately 6- to 8-week-old female WT and *Cyp2e1*-null mice on Svj129 background (n = 6/group)[11] were exposed to tap water (control) or 30% (w/v) fructose in drinking water for 8 weeks *ad libitum*. Food intake, weight gain, and liver weight in rats or mice are listed in Supporting Table S1. However, we do not know the reason(s) why fructose exposure significantly increased liquid intake and decreased chow intake in rats, but not in mice.

HISTOLOGICAL ANALYSIS AND MEASUREMENTS OF PLASMA ALANINE AMINOTRANSFERASE AND ENDOTOXIN

After exposure to fructose or regular tap water (control) for 8 weeks, each rat or mouse was briefly sedated to carbon dioxide gas followed by decapitation to immediately collect trunk blood, small intestine, and liver. Paraffin-embedded blocks of formalin-fixed individual liver or small intestine sections were cut at 4 microns and stained with hematoxylin and eosin (H&E) or Sirius Red by American Histolabs, Inc. (Gaithersburg, MD). In addition, plasma alanine aminotransferase (ALT) and endotoxin levels were determined by using the standard endpoint colorimetric assay kit (TECO Diagnostics, Anaheim, CA) and the commercially available endpoint LAL Chromogenic Endotoxin Quantitation Kit with a concentration range of 0.015-1.2 endotoxin units per milliliter (EU/mL; Thermo Fisher Scientific, Waltham, MA), respectively, as described.[11]

TRIGLYCERIDE DETERMINATION IN LIVER

Hepatic triglyceride (TG) levels were assessed by using a commercially available kit (Thermo Fisher Scientific), as described.[11]

Please see Supporting Materials and Methods for details.

STASTISTICAL ANALYSIS

The experiments were conducted at least twice, unless otherwise stated. Statistical significance was determined by using two-tailed t-test. ANOVA and Dunnet's multiple comparison post-test were used to compare the means of multiple groups by using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA).

Results

INCREASED ENDOTOXIN, OXIDATIVE STRESS PROTEINS WITH DECREASED INTESTINAL TJ AND AJ PROTEINS, AND LIVER FIBROSIS IN AUTOPSIED OBESE PEOPLE WITH NASH

Recent studies suggest that excessive consumption of fructose is linked to the development and severity of NAFLD/NASH, especially in overweight individuals.[3] In addition, high blood LPS levels are found in fructose- and/or Western-style high-fat-diet–induced obesity. Therefore, we evaluated LPS levels in body fluids of autopsied obese versus nonobese people, whose body mass index (BMI) and other characteristics, including NAFLD/NASH and fibrosis, are summarized in Table 1. Liver histology revealed that obese people (BMI ≥25) had significantly greater levels of hepatic fat and inflammatory cells (Supporting Fig. S1) with fibrosis (Supporting Fig. S2) than nonobese individuals (BMI <25). LPS levels were significantly elevated in body fluids of obese people with NASH (Fig. 1A).

Several reports showed that hepatic CYP2E1 is increased in NASH patients compared to normal individuals.[12] Increased levels of nitroxidative stress, as reflected by elevated levels of CYP2E1, iNOS, and nitrated proteins (Fig. 1B), were observed in ileums of obese people with NASH compared to nonobese controls. Amounts of the indicated intestinal TJ (Fig. 1C), AJ proteins, and plakoglobin (Fig. 1D) were markedly decreased in obese people with NASH. In addition, apoptosis marker proteins, such as phosphorylated (p)-JNK (c-Jun N-terminal kinase), B-cell lymphoma 2–associated X protein (Bax), and cleaved caspase-3, were significantly increased in obese people (Fig. 1E). All these results suggest elevated leaky gut and endotoxemia,

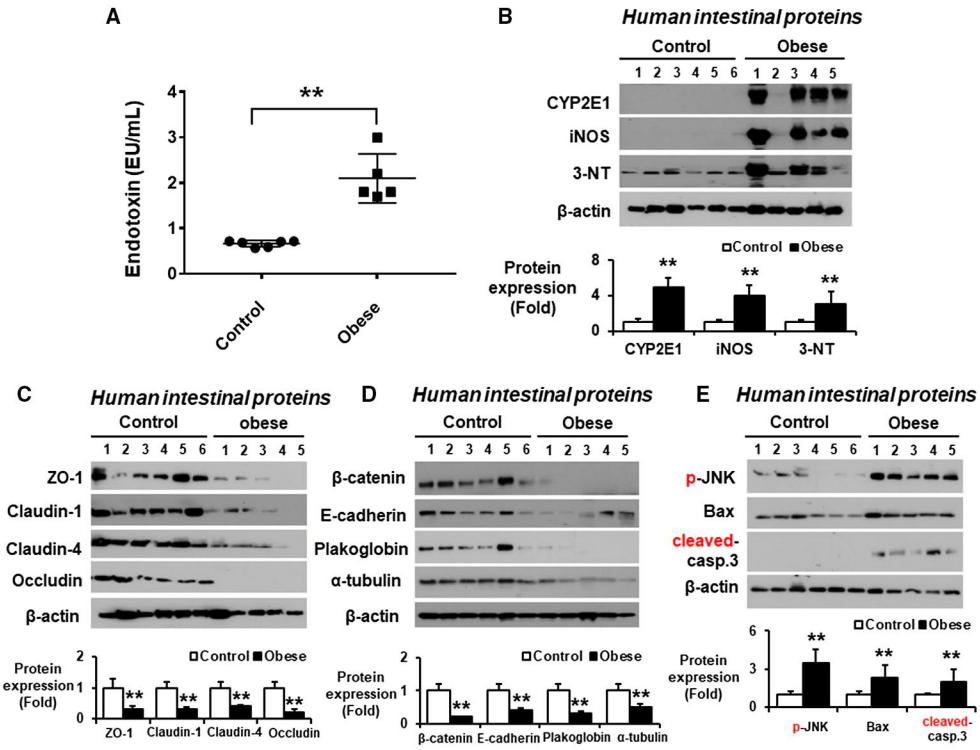

FIG. 1. Increased levels of endotoxin and oxidative stress marker proteins with decreased intestinal TJ and AJ proteins in autopsied obese people. (A) Plasma endotoxin levels and (B) amounts of immunoreactive CYP2E1, iNOS, or nitrated proteins detected by anti-3-NT antibody in gut homogenates and densitometric quantitation for each protein relative to β-actin are shown for the indicated groups. (C) Immunoblotting results of the intestinal TJ proteins, ZO-1, claudin-1, claudin-4, and occludin, (D) the intestinal AJ proteins (β-catenin, E-cadherin), desmosome plakoglobin, and α-tubulin, and (E) apoptosis-associated proteins and their amounts relative to β-actin are presented for the indicated groups. $^{**}P < 0.01$.

resulting in hepatic inflammation (NASH) and fibrosis in obese people.

INCREASED PLASMA ENDOTOXIN, INTESTINAL CYP2E1, AND iNOS PROTEINS IN FRUCTOSE-EXPOSED RATS

We also evaluated the histological change in small intestines and plasma endotoxin levels in fructose-exposed rats, given that fructose was shown to cause NASH through leaky gut and endotoxemia.[8,13] Histological analysis revealed markedly increased loss, blebbing of the laminar propria, and inflammatory cell infiltration/activation in small intestines and colons in fructose-exposed rats (Fig. 2A and Supporting Fig. S3). Consistently, levels of plasma endotoxin (Fig. 2B) and *Escherichia coli* mRNA in fresh liver tissues (Fig. 2C) were elevated in fructose-exposed rats compared to water controls. Sequencing analyses of fecal DNA

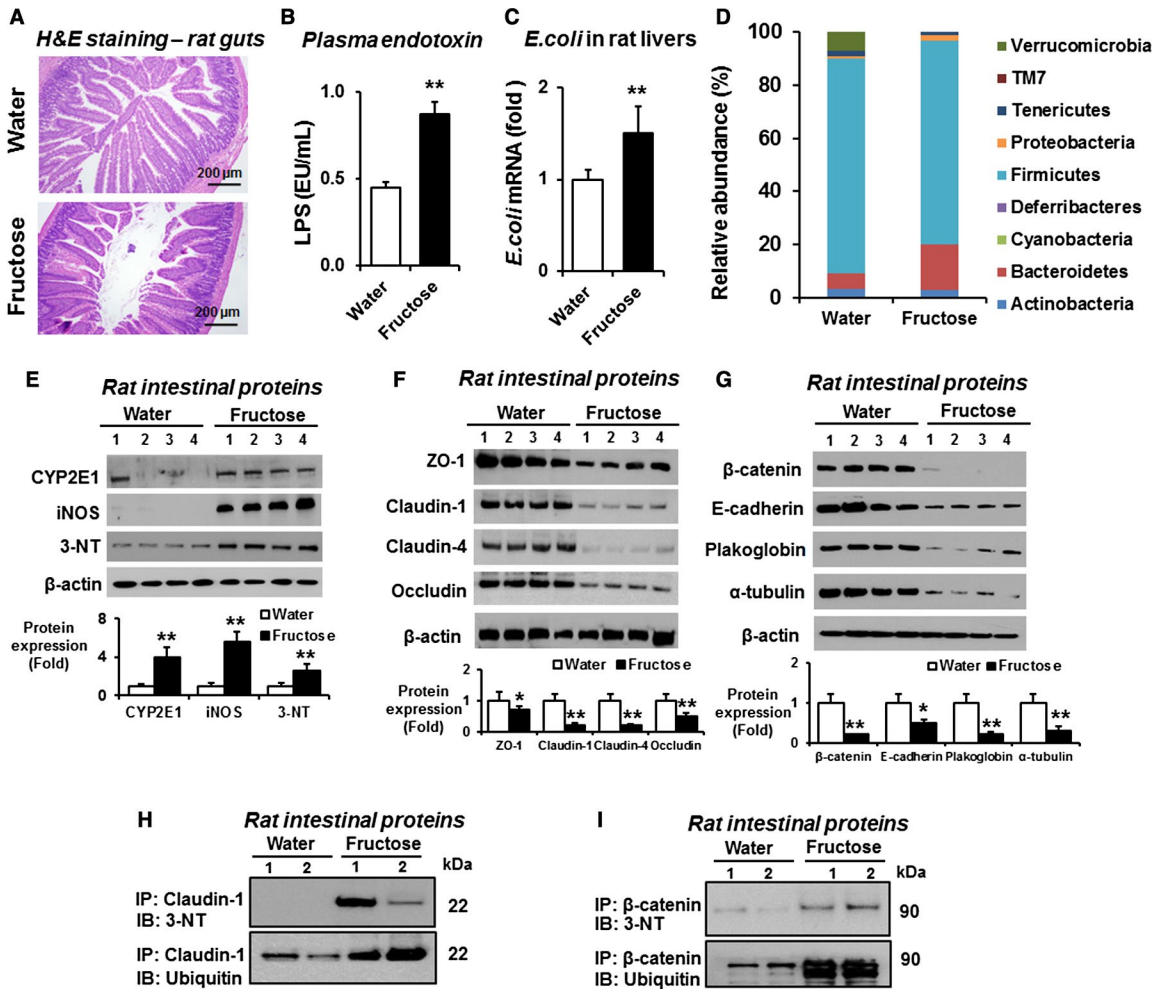

FIG. 2. Fructose drinking stimulated gut leakiness with elevated plasma endotoxin and intestinal CYP2E1 and other oxidative/nitrative stress markers in rats. Age-matched Fischer F344 female rats were exposed to water or 30% (w/v) fructose in drinking water for 8 weeks (n ≥ 8/group). (A) Representative H&E staining of formalin-fixed small intestine sections, (B) plasma LPS levels, and (C) amounts of *E.* coli mRNA transcripts in fresh liver extracts determined by real-time RT-PCR are presented for the indicated rat groups. (D) Comparison of the taxonomic abundance bacteria phyla. (E) Immunoblotting results for CYP2E1, iNOS, or nitrated proteins detected by anti-3-NT antibody in gut homogenates and densitometric quantitation for each protein relative to β-actin, used as a loading control, are shown from the indicated groups. (F) Immunoblotting results of the intestinal TJ proteins, ZO-1, claudin-1, claudin-4, and occludin, and (G) the intestinal AJ proteins (β-catenin, E-cadherin), desmosome plakoglobin, and α-tubulin and their amounts relative to β-actin are presented. (H,I) Same amounts of intestinal proteins from water control or fructose-exposed rats were immunoprecipitated with the specific antibody to each target protein claudin-1 or β-catenin and then subjected to immunoblotting analysis with the specific antibody to 3-NT or ubiquitin. $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations: IB, immunoblotting; IP, immunoprecipitation.

revealed that nine gut bacteria phyla were predominantly detected in water- or fructose-exposed rats (Fig. 2D). The increased abundance of Bacteroidetes and Proteobacteria in fructose-exposed rats was mainly explained by elevated levels of *Bacteroides* and *Escherichia* (Supporting Fig. S4). In addition, the decreased abundance of Firmicutes and Verrucomicrobia in the fructose-exposed group could be explained by the reduced amounts of *Lactobacillus* and *Akkermansia* (Supporting Fig. S4). These gut microbiota changes in the fructose-exposed group might also contribute to leaky gut, as similar to the earlier report on gut microbiome status in NASH patients.[14]

CYP2E1 is critically important in causing alcoholic liver injury through increased oxidative stress and gut leakiness in WT mice given that *Cyp2e1*-null mice were protected despite the extreme doses of binge alcohol.[11] Consistently, amounts of CYP2E1 and iNOS proteins, markers of oxidative and nitrative stress, respectively, were significantly elevated in small intestines of fructose-exposed rats (Fig. 2E).

ROLE OF NITRATION AND UBIQUITIN-DEPENDENT PROTEIN DEGRADATION OF INTESTINAL TJ AND AJ PROTEINS IN FRUCTOSE-EXPOSED RATS

Amounts of intestinal TJ proteins zonula occludens 1 (ZO-1), occludin, claudin-1, and claudin-4, critically important in the integrity and function of the intestinal barrier, were significantly decreased in fructose-exposed rats compared to water controls (Fig. 2F). Levels of AJ proteins (β-catenin and E-cadherin), desmosome plakoglobin, and α-tubulin were also significantly decreased in fructose-exposed rats (Fig. 2G). Additionally, apoptosis marker proteins, such as p-JNK, Bax, cleaved caspase-3, and caspase-3 activity, were significantly increased in fructose-exposed rats (Supporting Fig. S5). Immunoprecipitation with the specific antibody to the selected TJ or AJ protein, followed by immunoblotting analysis with anti-3-NT (3-nitrotyrosine) or anti-ubiquitin antibody, revealed that nitration and ubiquitin conjugation of claudin-1 (Fig. 2H) or β-catenin (Fig. 2I) were significantly increased in fructose-exposed rats, suggesting ubiquitin-dependent proteolytic degradation of the nitrated TJ and AJ proteins, contributing to leaky gut, endotoxemia, and inflammatory liver disease.

ROLE OF CYP2E1 AND OXIDATIVE STRESS IN DECREASED GUT JUNCTIONAL PROTEINS IN FRUCTOSE-EXPOSED MICE

We further determined the critical role of CYP2E1 in fructose-mediated intestinal barrier dysfunction and endotoxemia in fructose-exposed WT and *Cyp2e1*-null mice. Histological analysis revealed markedly increased loss, blebbing of the laminar propria, and inflammatory cell infiltration in small intestines and colons in fructose-exposed WT, but not in corresponding *Cyp2e1*-null, mice (Fig. 3A and Supporting Fig. S6). Consistently, levels of plasma endotoxin (Fig. 3B), *in vivo* permeability (Fig. 3C), and the *E. coli* mRNA transcripts in liver (Fig. 3D) were significantly elevated in fructose-exposed WT, but not in corresponding *Cyp2e1*-null, mice. In addition, amounts of intestinal CYP2E1, iNOS, and nitrated proteins were markedly elevated in fructose-exposed WT (Fig. 3E). Consistently, amounts of immunoreactive intestinal TJ proteins ZO-1, occludin, claudin-1, and claudin-4 were markedly decreased in fructose-exposed WT mice (Fig. 3F). Levels of intestinal AJ proteins, desmosome plakoglobin, and α-tubulin were also markedly decreased in fructose-exposed WT (Fig. 3G). Immunohistochemistry (IHC) of ZO-1 also showed decreased levels in fructose-exposed WT compared to water controls or fructose-exposed *Cyp2e1*-null mice (Fig. 3H). Additionally, apoptosis marker proteins, such as p-JNK, Bax, cleaved caspase-3 and caspase 3 activity (Supporting Fig. S7A,B), as well as terminal deoxynucleotidyl transferase dUTP nick end labeling–positive apoptotic enterocytes (Supporting Fig. S7C), were significantly increased in fructose-exposed WT mice. These results indicate the similar mechanisms of fructose-induced gut leakiness in rats and WT mice through decreased gut TJ/AJ proteins with increased apoptosis of intestinal enterocytes in a CYP2E1-dependent manner. Significantly elevated plasma ethanol concentrations with little changes in uric acid concentrations were observed in fructose-exposed rats and mice (Supporting Fig. S8A,B, respectively). Levels of plasma reactive oxygen species

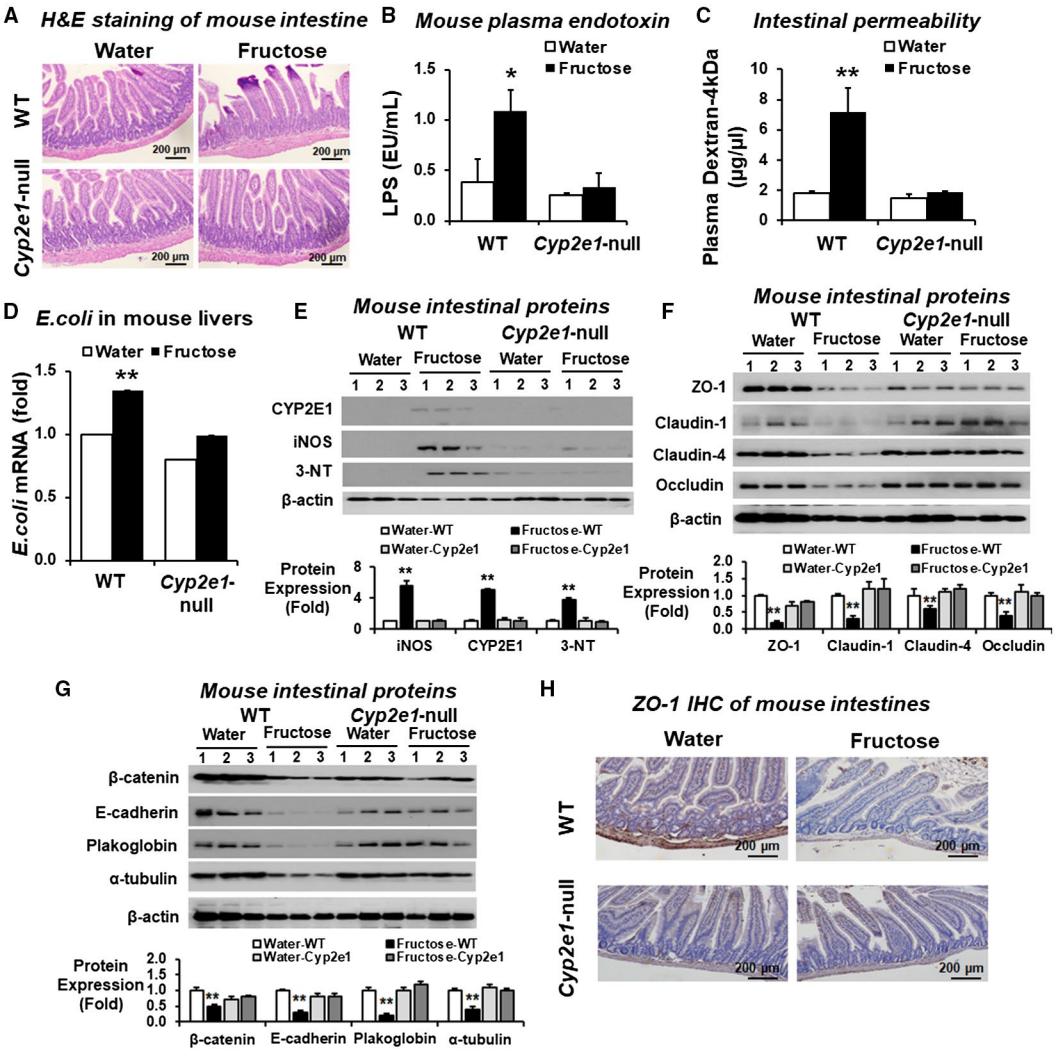

FIG. 3. Fructose drinking increased endotoxemia and nitroxidative stress proteins with decreased TJ and AJ proteins in WT compared to *Cyp2e1*-null mice. Levels of (A) representative H&E staining (B) plasma endotoxin, (C) intestinal permeability, (D) *E. coli* mRNA transcripts in liver extracts assessed by real-time RT-PCR, (E) gut nitroxidative stress marker proteins, (F) gut TJ proteins, and (G) AJ and associated proteins and a loading control β-actin for the indicated mouse groups (n ≥ 6/group) are presented. $^{*}P < 0.05$; $^{**}P < 0.01$. (H) Representative IHC staining for ZO-1 in formalin-fixed small intestine sections of the indicated mouse groups.

(ROS) were significantly increased in fructose-drinking rats and WT mice compared to those of water controls or fructose-exposed *Cyp2e1*-null mice (Supporting Fig. S9). Furthermore, levels of decreased TJ and AJ proteins appeared to be correlated with those of the circadian clock proteins, clock circadian regulator (CLOCK) and period circadian regulator 2 (PER-2), and their respective mRNAs in fructose-exposed rats and WT mice (Supporting Fig. S10). In contrast, amounts of CLOCK and PER-2 proteins and their mRNA transcripts were unchanged in fructose-exposed *Cyp2e1*-null mice (Supporting Fig. S10C,D, respectively).

DISRUPTION OF TJ PROTEINS WITH ELEVATED APOPTOSIS SIGNALS IN FRUCTOSE-EXPOSED T84 COLON CELLS

To further study the underlying mechanisms by which fructose causes intestinal barrier dysfunction, T84 colon cells were used as a model and treated with

fructose at 0, 2.5, and 5 mM for 24 hours. Amounts of ZO-1 were significantly decreased in fructose-exposed T84 cells (Supporting Fig. S11A). Levels of apoptosis-related marker proteins (p-JNK, Bax, and cleaved-caspase 3) were increased in fructose-treated T84 cells (Supporting Fig. S11B). In addition, fructose exposure significantly reduced transepithelial electrical resistance (TEER) and increased permeation of fluorescein isothiocyanate–labeled 4-kilodalton (kDa) dextran (FITC-D4; Supporting Fig. S12A,B, respectively). However, cotreatment with the specific CYP2E1 inhibitor, chlormethiazole, or the specific iNOS inhibitor, 1400W, efficiently prevented epithelial cell permeability in fructose-exposed T84 cells whereas these inhibitors significantly decreased levels of CYP2E1 and iNOS, respectively (Supporting Fig. S12C). These biochemical and confocal image analysis results clearly support an important role of nitroxidative stress in redistribution of ZO-1 and increased apoptosis of fructose-exposed T84 colon cells, contributing to epithelial cell barrier dysfunction.

INCREASED AMOUNTS OF HEPATIC FAT, PROINFLAMMATORY CYTOKINES, CYP2E1, AND iNOS PROTEINS IN FRUCTOSE-EXPOSED RATS

Liver histology and IHC revealed ballooned hepatocytes with increased fat accumulation, CYP2E1, and iNOS contents in fructose-exposed rats compared to water controls (Fig. 4A). Fructose drinking significantly increased the levels of plasma ALT (Fig. 4B), hepatic TG (Fig. 4C), and hepatic tumor necrosis factor alpha (TNF-α; Fig. 4D). Additionally, real-time RT-PCR analyses showed increased amounts of hepatic monocyte chemoattractant protein 1 (MCP-1), TNF-α, interleukin (IL)-6, and arginase-1 (ARG-1) mRNA transcripts in fructose-drinking rats compared to water controls (Fig. 4E). Hepatic CYP2E1 contents (Fig. 4F) and activity (Fig. 4G) as well as levels of iNOS and nitrated proteins (Fig. 4F)

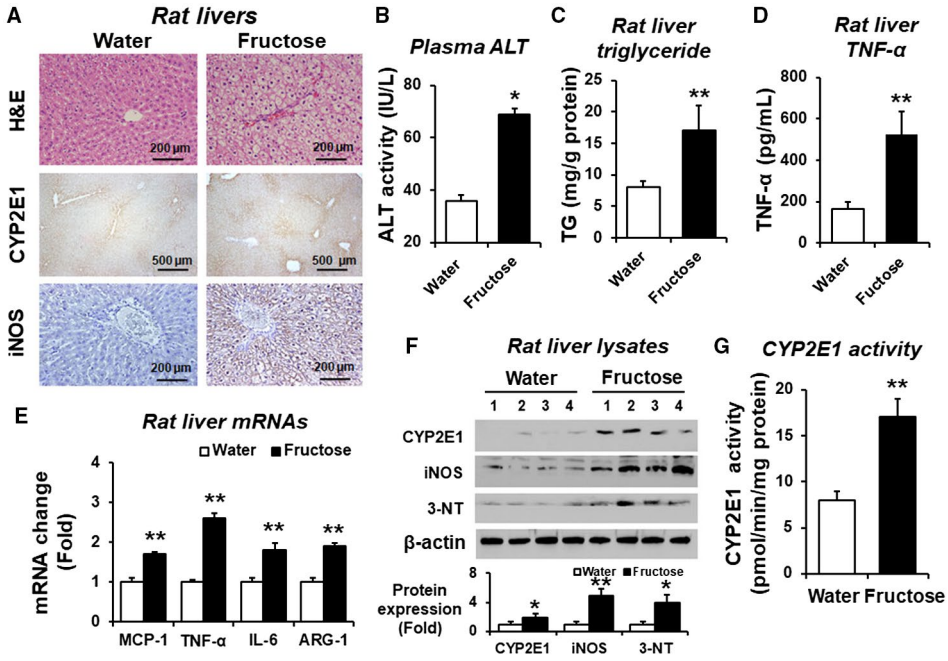

FIG. 4. Fructose drinking elevated fatty liver, oxidative stress, and hepatic inflammation in rats. (A) Representative H&E staining and IHC staining for CYP2E1 or iNOS in formalin-fixed liver sections of the indicated rat groups. (B) Plasma ALT levels, (C) hepatic TG levels, and (D) amounts of hepatic TNF-α measured by ELISA (n ≥ 6/group) are shown for the indicated groups. (E) Relative amounts of the mRNA transcripts of hepatic inflammatory markers as determined by real-time RT-PCR and (F) the immunoreactive levels of hepatic CYP2E1, iNOS, and nitrated proteins detected with anti-3-NT antibody are shown. Densitometric quantitation of each protein relative to β-actin, used as a loading control, is shown. (G) CYP2E1 activity in each indicated group was measured by hydroxylation of *p*-nitrophenol and expressed as pmol/min/mg of protein. $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviation: ELISA, enzyme-linked immunosorbent assay.

were significantly elevated in fructose-exposed rats. These results suggest that fructose drinking significantly increased the proinflammatory cytokines and hepatic contents of nitroxidative stress marker proteins CYP2E1, iNOS, and nitrated proteins in rats. Consistently, the hepatic levels of these oxidative stress marker proteins were significantly increased in fructose-exposed WT mice (Supporting Fig. S13).

ROLE OF CYP2E1 IN ELEVATED HEPATIC FAT CONTENTS, PROINFLAMMATORY CYTOKINES, AND MACROPHAGES INFILTRATION IN FRUCTOSE-EXPOSED MICE

Histological analysis showed numerous lipid droplets, necrotic hepatocytes, and infiltration/activation of macrophages determined by F4/80 marker in liver (Fig. 5A and Supporting Fig. S14). Plasma ALT and hepatic total TG levels (Fig. 5B,C, respectively) were significantly elevated in fructose-exposed WT mice compared to corresponding *Cyp2e1*-null mice or water controls. Fructose drinking increased mRNA amounts of hepatic NLR family pyrin domain-containing 3 (NLRP3), TNF-α, and IL-1β in WT mice (Fig. 5D). Amounts of hepatic cytokine (TNF-α) and chemokine (MCP-1) proteins were significantly increased in fructose-exposed WT (Fig. 5E,F, respectively). These results suggest that fructose intake increased hepatic levels of lipids, proinflammatory cytokines, and infiltration of macrophages in WT mice, reflecting NAFLD/NASH in a CYP2E1-dependent manner.

INCREASED LIVER FIBROSIS IN FRUCTOSE-EXPOSED RATS

Sirius Red staining showed greater areas of liver fibrosis (LF) in fructose-exposed rats compared to water controls, suggesting that fructose can promote hepatic fibrosis (HF) in rats (Fig. 6A). Consistently, fructose exposure significantly elevated hepatic amounts of α-smooth muscle actin (α-SMA), collagen (Col)-1, Col-4, and transforming growth factor beta (TGF-β) in rats (Fig. 6B). In addition, expressed levels of hepatic α-SMA and TGF-β mRNA transcripts were significantly elevated in fructose-exposed rats (Fig. 6C).

Hepatic sirtuin-1 (Sirt1), a cytosolic NAD^+-dependent protein deacetylase, plays a major role in ameliorating steatosis and inhibiting fibrosis in alcoholic fatty liver disease (AFLD).[15] Levels of hepatic Sirt1 and p-Sirt1 proteins significantly decreased whereas acetyl-histone-3 (Acy-His3; Fig. 6D) were significantly elevated in fructose-exposed rats. Furthermore, hepatic amounts of proliferator-activated receptor-γ coactivator 1 alpha (PGC1-α), peroxisome proliferator-activated receptor alpha (PPARα), and p-AMPK (adenosine 5′ monophosphate-activated protein kinase) proteins were significantly decreased, whereas sterol regulatory element-binding protein (SREBP)-1 levels were increased, in fructose-drinking rats (Fig. 6E). Similarly, hepatic levels of PGC1-α and PPARα mRNA transcripts were significantly decreased in fructose-exposed rats (Supporting Fig. S15). Immunoprecipitation with the specific antibody to Sirt1, followed by immunoblotting analysis with anti-3-NT or antiubiquitin antibody, revealed that nitration and ubiquitination of Sirt-1 were markedly increased in fructose-exposed rats (Fig. 6F). Taken together, fructose drinking stimulated LF, at least partly, through the decreased amount of Sirt1 in rats.

However, Sirius Red staining did not show obvious signs of LF in fructose-exposed WT and *Cyp2e1*-null mice (Supporting Fig. S16A). Consistently, amounts of hepatic α-SMA, Col-1, and TGF-β proteins were not significantly increased in fructose-exposed WT and *Cyp2e1*-null mice compared to their respective water controls (Supporting Fig. S16B). These results showed that fructose drinking only stimulated HF in rats, but not in mice, as reported.[8]

CYP2E1-DEPENDENT ACTIVATION OF HEPATIC STELLATE CELLS BY LPS

Increased gut permeability with elevated LPS can activate hepatic stellate cells (HSCs), contributing to LF.[16] Therefore, we determined whether LPS or fructose can activate LX2 human HSCs and primary mouse HSCs. Confocal microscope analysis showed that α-SMA protein expression was markedly increased in LPS-treated LX2 cells or primary mouse HSCs compared to those treated with fructose or water control (Fig. 7A,D, respectively). Amounts of α-SMA, Col-1, tissue inhibitor

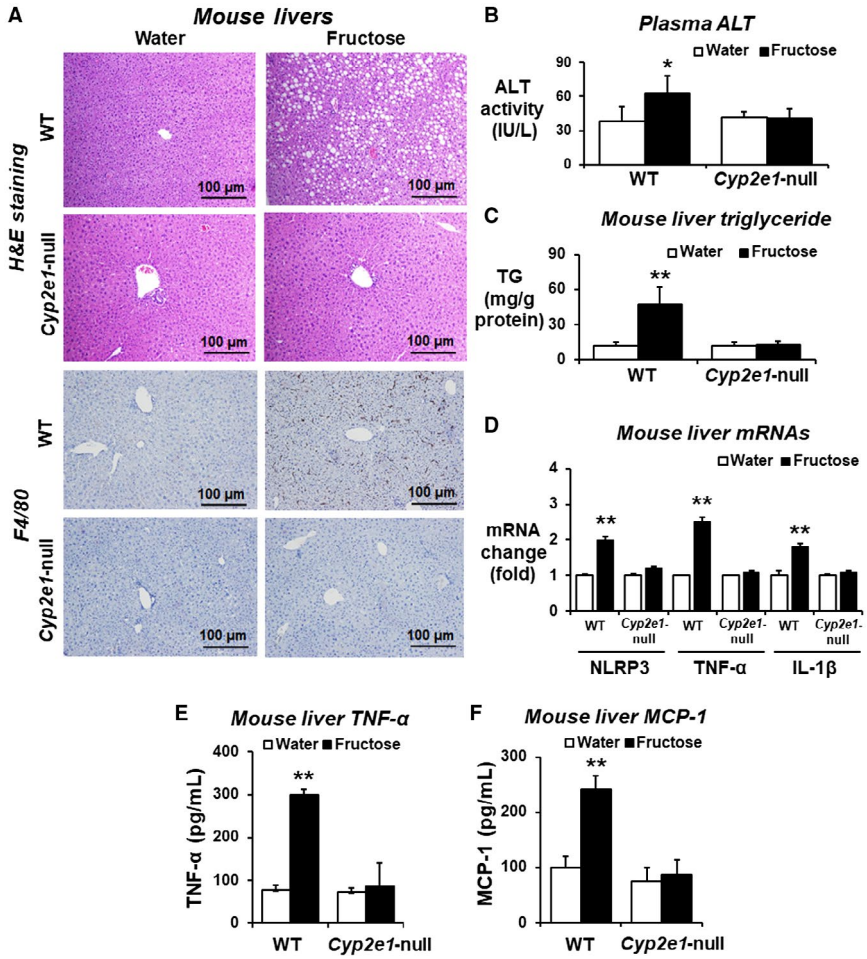

FIG. 5. Fructose drinking significantly increased the levels of inflammatory marker proteins in WT mice. (A) Representative H&E staining and F4/80 immunohistochemistry image of formalin-fixed mouse liver for the indicated groups (n ≥ 6/group). Levels of (B) plasma ALT, (C) hepatic TG, and (D) relative levels of inflammatory marker gene NLRP3, TNF-α, or IL-1β in fructose-exposed WT or *Cyp2e1* -null mice and their respective water controls are shown. $^{*}P < 0.05$, $^{**}P < 0.01$. (E,F) Amounts of the TNF-α or MCP-1 protein in the indicated mouse groups. $^{**}P < 0.01$.

of metalloproteinase-1 (TIMP-1), TGF-β, and Toll-like receptor 4 (TLR-4) proteins were significantly elevated in LPS-treated LX2 cells (Fig. 7B). Expressed levels of α-SMA, COL1, and TGF-β mRNAs were also increased in LPS-treated LX2 cells (Fig. 7C) or primary mouse HSCs (Fig. 7E). However, fructose alone did not increase expression of these fibrosis-marker proteins and their mRNA transcripts in HSCs. In addition, the LF marker proteins in primary mouse HSCs isolated from *Cyp2e1*-null mice were unchanged by the same LPS treatment (data not shown). These results strongly indicate that the elevated LPS, resulting from fructose-induced leaky gut but not fructose *per se*, is likely to activate HSCs, contributing to liver inflammation/fibrosis in a CYP2E1-dependent manner, emphasizing the critical roles of CYP2E1, gut leakiness, and endotoxemia in promoting inflammatory liver disease through the gut-liver axis.

Discussion

Consumption of soft drinks in the United States has increased by >40-fold since the 1990s and has been implicated as a major risk factor of the development NAFLD/NASH,[17,18] which can also be caused by a variety of agents including a high-fat Western

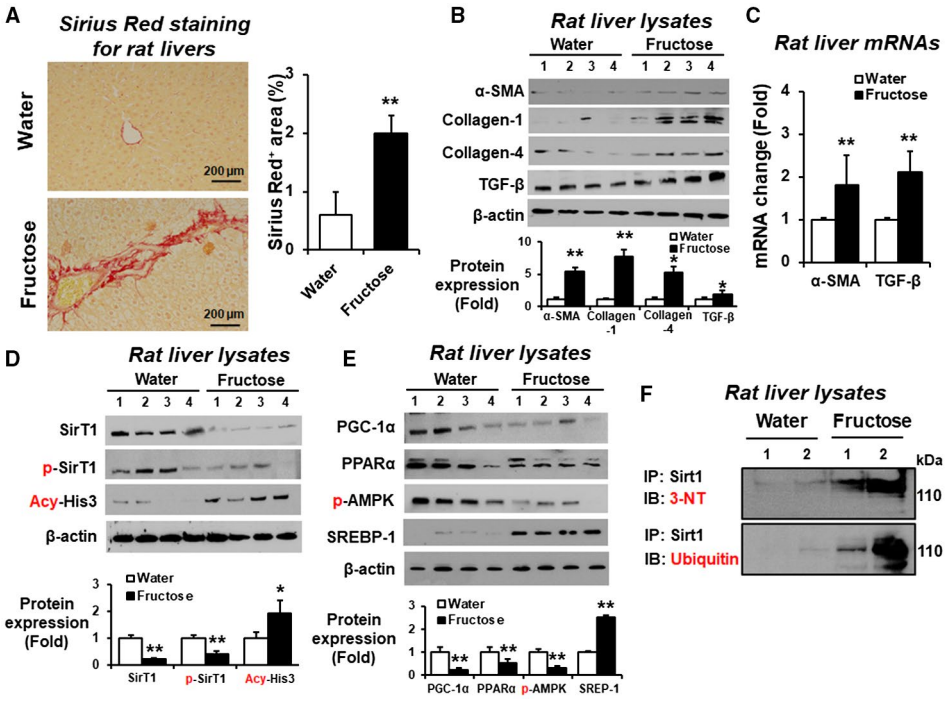

FIG. 6. Fructose drinking elevated HF markers in rats. (A) Representative Sirius Red staining of formalin-fixed liver sections for the indicated rat groups and densitometric quantitation are shown. (B) Immunodetection of the HF marker proteins (α-SMA, Col-1, Col-4, and TGF-β) from the indicated groups. Densitometric quantitation of each protein relative to β-actin, used as a loading control, is shown. (C) Relative amounts of the mRNA transcripts of HF markers (α-SMA or TGF-β) were determined by real-time RT-PCR and are shown for the indicated rat groups. (D) Immunoblotting results for hepatic Sirt1, p-Sirt1, or Acy-His3 and (E) hepatic PGC1-α, PPARα, p-AMPK, and SREBP-1 relative to β-actin are shown. (F) Same amounts of liver lysates from rats exposed to water or fructose were immunoprecipitated with the specific antibody to Sirt1 protein and then subjected to immunoblotting analysis with the antibody to 3-NT or ubiquitin protein, as indicated. $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations: IB, immunoblotting; IP, immunoprecipitation.

diet containing n-6 fatty acids and cholesterol. Several randomized trials of sugar-containing soft drinks versus low-calorie or calorie-free beverages show that either sugar, 50% of which is fructose, or fructose alone increased serum TGs, body weight, visceral adipose tissue, muscle fat, and liver fat in humans.[17] Thus, our experimental models exposed to fructose and mechanistic studies are potentially implicated in human settings where many people, who consume fructose-containing soft drinks, show signs of obesity and NAFLD/NASH.

Recent reports showed that fructose drinking or solid diets can induce inflammatory hepatic injury (NAFLD/NASH) directly or indirectly through alterations in gut microbiota, barrier dysfunction, and loss of TJ proteins with elevated serum LPS,[13] which activates the hepatic TLR-4.[19] In fact, Bergheim et al. reported that dietary fructose decreased the amounts of ZO-1 and occludin in mouse duodenums.[5] However, the roles of CYP2E1 and increased nitroxidative stress in fructose-induced gut leakiness and advanced liver disease and the underlying mechanisms for gut leakiness have been poorly understood. Because the contributing roles of CYP2E1, protein modifications of TJ and/or AJ proteins, and apoptosis of enterocytes in intestinal permeability and inflammatory liver disease in fructose or other nonalcoholic substances should be studied systematically, this study was aimed to evaluate whether fructose drinking can induce liver inflammation and fibrosis through elevated plasma endotoxin resulting from the loss of TJ and AJ protein integrity with elevated apoptosis of enterocytes in rats, mice, T84 colon cells, and autopsied human ileum specimens. In our experimental conditions, fructose (30% [w/v] in drinking water) raised intestinal CYP2E1 levels with elevated oxidative and nitrative stress, which can decrease the amounts of TJ proteins (e.g., ZO-1, claudin-1, claudin-4, and occludin), AJ

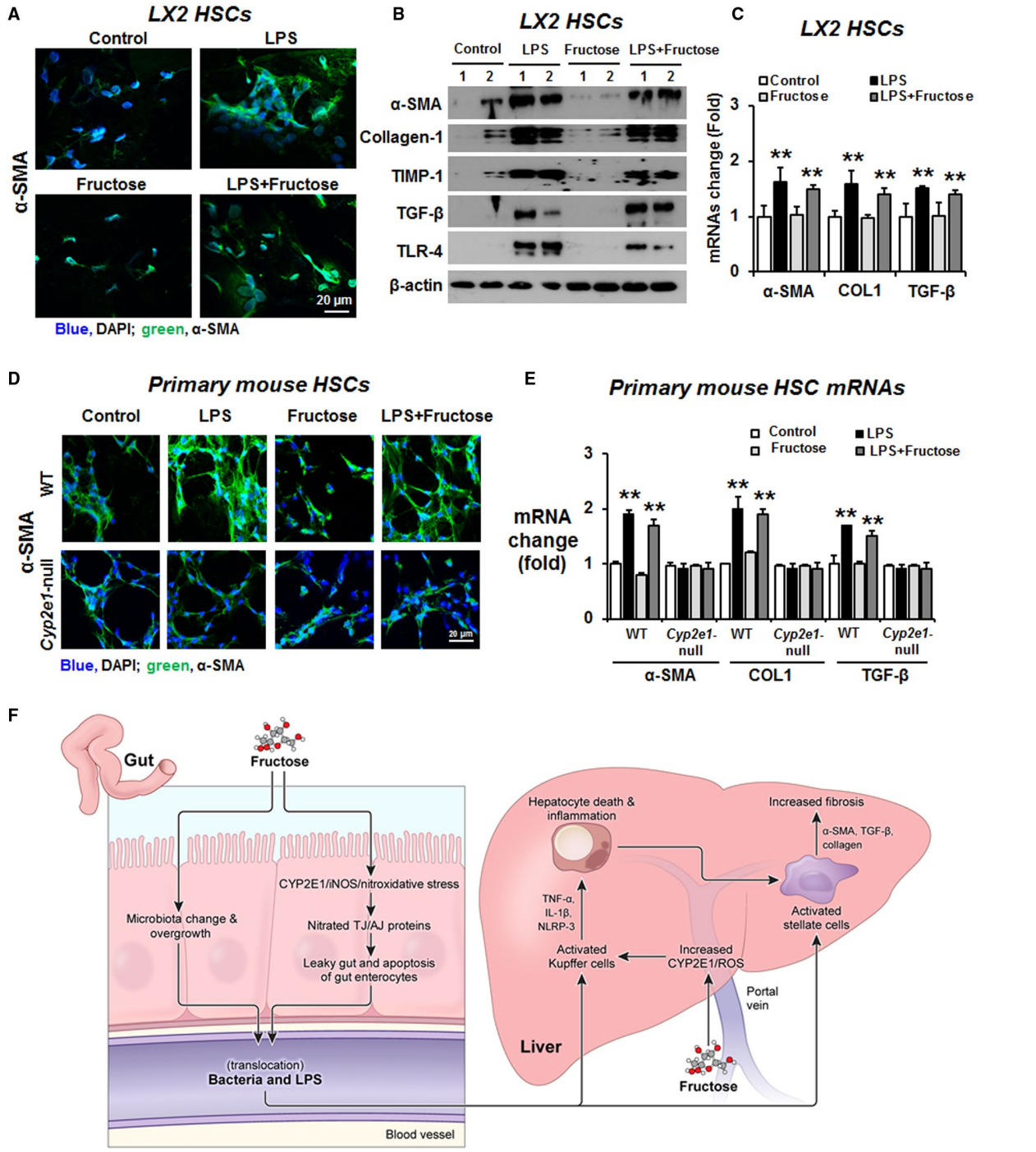

FIG. 7. LPS activated HSCs. (A) LX2 cells were treated with water, LPS, fructose (2.5 mM), or LPS + fructose for 24 hours. The confocal image shows up-regulation of α-SMA in LPS-exposed LX2 cells. Nuclei of LX2 cells were counterstained with DAPI. (B) Immunoblotting results for fibrosis marker proteins α-SMA, Col-1, TIMP-1, TGF-β, and TLR-4 in LX2 cells. (C) Real-time PCR results for fibrosis marker mRNAs α-SMA, COL1, and TGF-β in LX2 cells. (D) The primary HSCs from WT or *Cyp2e1*-null mice were treated with LPS, fructose, or LPS + fructose for 24 hours. The confocal image shows increased α-SMA in LPS-exposed mouse primary HSCs from WT mice. Nuclei of mouse primary HSCs were counterstained with DAPI. (E) Real-time PCR results for fibrosis marker mRNAs α-SMA, COL1, and TGF-β in mouse primary HSCs. $^{**}P < 0.01$. (F) The proposed mechanisms of fructose-induced gut leakiness and LF. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

proteins (e.g., β-catenin and E-cadherin), desmosome plakoglobin, and α-tubulin with increased apoptosis of gut enterocytes, contributing to increased levels of blood LPS and steatohepatitis (NASH) in rats, mice, and autopsied obese people with NASH and/or fibrosis. The decreased levels of intestinal TJ/AJ proteins in fructose-exposed rats and WT mice were likely caused by nitration followed by ubiquitin-dependent protein degradation,[20,21] leading to elevated leaky gut. In contrast, *Cyp2e1*-null mice were resistant to these changes despite the same amount of fructose exposure, confirming an important role of CYP2E1 in gut leakiness and hepatic inflammation (NASH), similar to the intestinal and hepatic injury caused by binge alcohol exposure[11,22] and Western-style high-fat-diet–mediated gut leakiness and NASH.[23] Consistent with the results with rodent models, *in vitro* mechanistic studies with T84 colonic cells also showed disorganization or disruption of ZO-1 TJ protein and elevated apoptosis-related marker proteins after fructose exposure. In this case, the effects of oxidative and nitrative stress on fructose-mediated epithelial barrier dysfunction, determined by TEER and FITC-D4 transport, were significantly blocked by treatment with the specific inhibitor of CYP2E1 or iNOS. Although we have not studied nitration and ubiquitin conjugation of many TJ and AJ proteins, it is likely that other junctional complex proteins might undergo similar types of modifications followed by proteolytic degradations, resulting in their decreased levels. Nonetheless, these results indicate that both apoptosis of intestinal enterocytes and decreased TJ/AJ proteins contribute to epithelial cell barrier dysfunction and leaky gut, leading to inflammatory liver disease. Furthermore, our results with cultured HSCs underscore a contributing role of gut leakiness (with elevated endotoxins) in stimulating LF, given that fructose alone did not activate human and mouse HSCs, whereas these cells were activated by LPS.

We observed that fructose did not cause additional weight gain in WT mice compared to controls, similar to the report of little body weight change in fructose-exposed mice compared to control mice.[8] However, as expected, fructose markedly increased the levels of plasma ALT and hepatic TG, which is reflected by ballooned hepatocytes with numerous lipid droplets, possibly through *de novo* lipogenesis.[3] A recent report suggested that TNF-α plays a casual role in onset of fructose-induced NAFLD/NASH and insulin resistance in mice.[10] Consistent to the histological and biochemical data, levels of hepatic TNF-α and MCP-1 were significantly elevated in fructose-exposed rats and WT mice, but not in corresponding *Cyp2e1*-null counterparts.

Miele et al. demonstrated that gut dysbiosis can cause intestinal barrier dysfunction, leading to development of obesity-related NAFLD/NASH, and patients with NAFLD/NASH were known to have intestinal bacterial overgrowth and increased intestinal permeability.[7] Our study also revealed unique composition, ecological diversity, and enterotyping patterns of gut microbiomes in fructose-exposed rats, possibly through adaptation, as reviewed.[24] Furthermore, the patterns of intestinal bacterial composition in fructose-exposed rats were similar to the changes in gut microbiome in NASH patients.[14] For instance, the increased abundance of *Bacteroides*[25,26] and *Escherichia*[14] with decreased levels of *Lactobacillus* and *Akkermansia*[27] in fructose-exposed rats may also stimulate intestinal permeability change, contributing to leaky gut, liver inflammation, and/or fibrosis.

The gut microbiome changes in fructose-exposed rodents may also be responsible for the endogenous ethanol production, although we do not know how fructose exposure would alter the gut microbiomes. Altered nuclear farnesoid and G-protein–coupled receptors with decreased enterohepatic circulation of bile acids are known to regulate gut microbiome population, which, in turn, modulates the rates of bile acid production.[28] In addition, Schnabl et al. recently reported that chronic alcohol exposure disrupted the enterohepatic circulation of bile acids and reduced the intestinal farnesoid X receptor (FXR)/fibroblast growth factor (FGR) 15/19 axis, which could, in turn, change the gut microbiome in mice with alcoholic liver disease.[29] Whether the altered bile acids and FXR/FGR15 pathway are also responsible for the gut microflora changes in fructose-exposed rodents needs further investigations.

Keshavarzian et al. recently reviewed that CYP2E1 is the major mediator of alcohol-induced gut leakiness.[30] In this report, elevated CYP2E1 following alcohol exposure was suggested to regulate the circadian clock proteins, CLOCK and PER-2, both of which are important in alcohol-induced epithelial barrier dysfunction and leaky gut in ethanol-exposed Caco-2 colon cells and mice, respectively, through increased oxidative and nitrative stress. Suppression of CYP2E1 function with a specific small interfering RNA or

treatment with an antioxidant decreased expression of CLOCK and PER-2 proteins, leading to normalization of the intestinal permeability and liver injury. Interestingly, our results showed increased CYP2E1 in the intestines and livers of fructose-exposed rats and WT mice, possibly attributed to enhanced protein stabilization in the presence of elevated plasma ethanol concentration.[31] Consistent with the previous report with the alcohol-exposed models,[30] our quantitative real-time RT-PCR and immunoblotting analyses showed significantly elevated mRNA and protein amounts of CLOCK and PER-2 in fructose-exposed rats or WT mice. These results indicate that increased oxidative stress is likely to regulate the circadian rhythm proteins, CLOCK and PER-2, which also play a contributing role in fructose-induced leaky gut and inflammatory liver injury in both rats and WT mice in a CYP2E1-dependent manner.

Increased hepatic CYP2E1 is known to directly cause fatty liver disease by elevating oxidative/nitrative stress and various forms of posttranslational modification of cellular proteins, contributing to endoplasmic reticulum stress, mitochondrial dysfunction, and apoptosis of hepatocytes.[32] Furthermore, intestinal CYP2E1 can cause liver inflammation indirectly through promoting posttranslational modifications of the TJ/AJ proteins and apoptosis of enterocytes, contributing to leaky gut and endotoxemia, as demonstrated in binge-alcohol–exposed rodents.[11] Based on these facts, it is likely that both intestinal and hepatic CYP2E1 increased in fructose-exposed rats, WT mice, and T84 cells could regulate the circadian proteins, elevate oxidative/nitrative stress, and promote posttranslational modifications of the junctional complex proteins and apoptosis of enterocytes, resulting in epithelial barrier dysfunction. Consequently, elevated LPS accelerates inflammatory liver disease in a CYP2E1-dependent manner, given that *Cyp2e1*-null mice were resistant to all these changes.

In the present study, IHC and immunoblotting analyses showed that hepatic amounts of CYP2E1, iNOS, nitrated proteins, and CYP2E1 activity were significantly elevated in fructose-exposed rats and WT mice compared to their respective controls. These results are consistent with the earlier reports that hepatic CYP2E1 levels were increased in NASH patients[33] and in animals fed a high-fat diet containing n-6 fatty acids[34] or fructose.[35] CYP2E1 induction or activation is usually associated with an increased production of ROS, as observed in the fructose-exposed rats and WT mice compared to the corresponding *Cyp2e1*-null mice or water controls. Elevated ROS and reactive nitrogen species through increased iNOS could be involved in the up-regulation of the intestinal circadian clock proteins and nitration of TJ/AJ proteins, leading to fructose-mediated leaky gut and liver disease, similar to those observed in alcohol-exposed cells or rodents.[30] In fact, several studies indicate that iNOS also plays a critical role in the progression of AFLD[36] and NAFLD, given that iNOS-null mice were protected from AFLD and NAFLD/NASH[37] possibly through preventing endotoxin and TLR-4 interactions, as shown in fructose-induced NAFLD/NASH.[38] In this study, we also demonstrated the important role of CYP2E1 in fructose-mediated gut leakiness and NASH by comparing the results between WT and *Cyp2e1*-null mice as well as using a specific inhibitor of CYP2E1 in T84 cells. We believe that fructose-mediated epithelial barrier dysfunction and leaky gut are likely caused by CYP2E1-mediated oxidative and nitrative stress.

In NAFLD/NASH patients, daily fructose ingestion is associated with reduced hepatic steatosis but increased HF.[39] In the present study, we found that classical fibrosis marker proteins α-SMA, Col-1, Col-4, and TGF-β were elevated in fructose-exposed rats. Similar results were reported for mice with HF after exposure to a diet containing saturated fat, fructose, and cholesterol.[40] Although liver injury was associated with significantly increased mucosal inflammation, TJ disruption, and intestinal epithelial permeability to bacterial endotoxins, the underlying mechanisms of decreased TJ proteins and apoptosis of enterocytes were not studied. Our current study not only confirmed the contributing role of leaky gut in advanced liver inflammation/fibrosis, but also demonstrated the important role of CYP2E1 in causing intestinal barrier dysfunction in fructose-exposed WT rodents and T84 colon cells. Furthermore, we demonstrated the underlying mechanisms of the decreased amounts of several TJ/AJ proteins as well as other desmosome proteins associated with the junctional complex[41] by protein nitration followed by ubiquitin conjugation.

Recently, sirtuin isoforms (Sirts 1-7) have been shown to play important roles in the pathophysiology of various metabolic diseases, including NAFLD.[42] Sirt1 has received great attention because it increases expression of antioxidant proteins and decreases

apoptosis and inflammation. Indeed, liver-specific deletion of Sirt1 or Sirt1 down-regulation worsened hepatic steatosis, inflammation, and endoplasmic reticulum stress in mouse models.[43] The amounts of hepatic Sirt1 were also decreased in fructose-exposed rats.[44] We further demonstrated a mechanism of decreased levels of Sirt1 through nitration and ubiquitin conjugation in fructose-exposed rats. The current study also suggests that fructose-mediated Sirt1 inhibition can directly regulate PGC1-α signaling, which is critically important in mitochondrial biogenesis and other functions.[45] Taken together, decreased Sirt1 is also likely to contribute to the development of steatohepatitis and HF in fructose-exposed rats.

In summary, our data demonstrate that fructose intake causes gut microbiome change, apoptosis of intestinal enterocytes, and tyrosine nitration of intestinal TJ and AJ proteins through increased gut CYP2E1 and nitroxidative stress. These changes result in elevated intestinal barrier dysfunction and endotoxemia, eventually contributing to activation of hepatic TLR-4 and development of NAFLD/NASH with HF in rats. Most of the results of increased CYP2E1, nitroxidative stress markers, apoptosis-associated proteins, epithelial cell barrier dysfunction with decreased gut TJ/AJ complex proteins, and elevated liver inflammation were also observed in fructose-exposed WT mice (and T84 cells), but not in corresponding *Cyp2e1*-null mice. These results support the important role of CYP2E1 in promoting leaky gut and the development of NASH.[11,22,23,30] Finally, the results observed in experimental models were also consistently observed in the ileums of autopsied obese people, suggesting the existence of conserved mechanisms of the gut-liver interactions among different species.

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Author names in bold designate shared co-first authorship.

Supporting Information

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