Toxicology and Applied Pharmacology 286 (2015) 102–111

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Inhibition of soluble epoxide hydrolase attenuates hepatic fibrosis and endoplasmic reticulum stress induced by carbon tetrachloride in mice

Todd R. Harris [a], Ahmed Bettaieb [b], Sean Kodani [a], Hua Dong [a], Richard Myers [c], Nipavan Chiamvimonvat [c], Fawaz G. Haj [b,d], Bruce D. Hammock [a,*]

[a] Department of Entomology and Comprehensive Cancer Center, University of California, Davis, CA 95616, USA
[b] Department of Nutrition, University of California, Davis, CA 95616, USA
[c] Department of Internal Medicine: Cardiovascular, University of California, Davis, CA 95616, USA
[d] Department of Internal Medicine: Endocrinology, Diabetes and Metabolism, University of California, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 3 January 2015
Revised 18 March 2015
Accepted 20 March 2015
Available online 28 March 2015

Keywords:
Soluble epoxide hydrolase
Endoplasmic reticulum stress
Carbon tetrachloride
Fibrosis

ABSTRACT

Liver fibrosis is a pathological condition in which chronic inflammation and changes to the extracellular matrix lead to alterations in hepatic tissue architecture and functional degradation of the liver. Inhibitors of the enzyme soluble epoxide hydrolase (sEH) reduce fibrosis in the heart, pancreas and kidney in several disease models. In this study, we assess the effect of sEH inhibition on the development of fibrosis in a carbon tetrachloride (CCl_4)-induced mouse model by monitoring changes in the inflammatory response, matrix remolding and endoplasmic reticulum stress. The sEH inhibitor 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) was administered in drinking water. Collagen deposition in the liver was increased five-fold in the CCl_4-treated group, and this was returned to control levels by TPPU treatment. Hepatic expression of Col1a2 and 3a1 mRNA was increased over fifteen-fold in the CCl_4-treated group relative to the Control group, and this increase was reduced by 50% by TPPU treatment. Endoplasmic reticulum (ER) stress observed in the livers of CCl_4-treated animals was attenuated by TPPU treatment. In order to support the hypothesis that TPPU is acting to reduce the hepatic fibrosis and ER stress through its action as a sEH inhibitor we used a second sEH inhibitor, *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (*t*-TUCB), and sEH null mice. Taken together, these data indicate that the sEH may play an important role in the development of hepatic fibrosis induced by CCl_4, presumably by reducing endogenous fatty acid epoxide chemical mediators acting to reduce ER stress.

Introduction

Liver fibrosis is a pathological condition that results from a normally beneficial wound healing process (Hernandez-Gea and Friedman, 2011). Damage to the liver triggers the inflammatory response, which initiates recruitment of macrophages and fibrocytes and remodeling of the extracellular matrix (ECM) in order to clear damaged cells and preserve the architecture of the liver (Bataller and Brenner, 2005). However, repeated injury, such as occurs in chronic alcoholism, exposure to environmental toxins, and viral infection can lead to a cascade of inflammatory system-related changes in the liver that result in abnormal deposition and composition of collagens and other elements of the ECM, as well as extensive damage to the tissue (Iredale, 2007). This pathological condition is called fibrosis.

Fibrosis is facilitated by a number of signaling molecules that modulate the immune response such as the cytokines IL-6 and IL-1β and the growth factor TGFβ (Wynn and Ramalingam, 2012). Alongside these commonly studied protein inflammatory mediators, bioactive lipids play an important role (Funk, 2001; Stables and Gilroy, 2011). There is growing evidence of the importance of lipid signaling in liver fibrosis. The eicosanoids are a class of lipid mediators that include the leukotrienes, prostaglandins and epoxyeicosatrienoic acids (EETs), produced through the activity of lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome P450 enzymes, respectively (Funk, 2001; Stables and Gilroy, 2011). The leukotrienes and prostaglandins have been implicated in the progression of liver fibrosis in studies employing leukotriene receptor antagonists and COX inhibitors. A leukotriene receptor antagonist, montelukast, attenuates liver damage and fibrosis in a bile duct ligation (BDL) and resection rat model (El-Swefy and Hassanen, 2009). A COX inhibitor, meloxicam, reduces fibrosis in this same model (Kim et al., 2008), although contradictory results have been reported using celecoxib (Yu et al., 2009).

A third class of arachidonic acid-derived mediators, the EETs, display anti-inflammatory and anti-fibrotic properties (Stables and Gilroy, 2011). The EETs are metabolized by soluble epoxide hydrolase (sEH), producing dihydroxy molecules that are less lipophilic and more readily

* Corresponding author at: Department of Entomology and Comprehensive Cancer Center, University of California, Davis, 1 Shields Avenue, Davis, CA 95616, USA.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).

http://dx.doi.org/10.1016/j.taap.2015.03.022
0041-008X/

conjugated, leading to their removal from the site of action (Morisseau and Hammock, 2013). sEH inhibitors modulate the levels of epoxy fatty acids (EpFAs) in tissues and dramatically reduce acute systemic inflammation in LPS-induced models as well as attenuate fibrosis and inflammation in the heart, kidney and pancreas (Li et al., 2008; Iyer et al., 2012; Kompa et al., 2013; Sirish et al., 2013). In diabetic models, sEH inhibitors display organ protective properties, reducing islet cell apoptosis in the pancreas and tissue damage in the liver (Iyer et al., 2012). It has been hypothesized that these inhibitors act by blocking the major route of metabolism of organ protective and anti-inflammatory EpFA (Iyer et al., 2012). Given these reported findings, we evaluated a sEH inhibitor in a murine model of liver fibrosis.

Once activated by metabolism, carbon tetrachloride (CCl_4) causes damage to the liver through lipid peroxidation, resulting in an upregulation of pro-inflammatory pathways and eventual fibrosis (Iredale, 2007). EpFAs have been implicated in this model. Both COX-2 and 5-LOX inhibitors have been found to reduce fibrosis after CCl_4 treatment, and when used in combination, reduce both inflammation and necrosis (Horrillo et al., 2007), though there are contradictory results when different COX-2 inhibitors are employed, as in the BDL model of fibrosis (Hui et al., 2006). Dietary supplementation with arachidonic acid and the omega-3 docosahexaenoic acid (DHA) has been found to attenuate fibrosis in an ethanol-induced model of liver damage (Song et al., 2008).

A growing body of evidence indicates that endoplasmic reticulum (ER) stress is a contributor to fibrotic diseases (Mollica et al., 2011; Lenna and Trojanowska, 2012; Tanjore et al., 2012). Directly relevant to this study, recent reports have demonstrated an increase in ER stress in rodents injected with CCl_4 (Ji et al., 2011; Lee et al., 2011; Jin et al., 2013). However, the link between the development of hepatic fibrosis and ER stress is still poorly understood. The ER is highly responsive to nutrients and to the energy state of the cell. It plays an important role in folding of newly synthesized proteins. When the folding capacity of the ER is exceeded, misfolded proteins accumulate and lead to ER stress (Schroder and Kaufman, 2005). Cells use adaptive mechanisms to counter the deleterious effects of ER stress known as the unfolded protein response (Kaufman et al., 2002). The unfolded protein response consists of three major branches that are controlled by the ER transmembrane proteins PKR-like ER-regulated kinase (PERK), inositol requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (Hotamisligil, 2010; Hummasti and Hotamisligil, 2010; Ron and Walter, 2007). In particular, the IRE1α sub-arm of ER stress signaling is critical for the unfolded protein response in fibrotic tissues (Chiang et al., 2011; Martino et al., 2013). The various unfolded protein response sub-arms synergize to attenuate stress by increasing the folding capacity of the ER (Schroder and Kaufman, 2005). However, if the compensatory mechanisms fail to facilitate the adaptation of cells to ER stress, induction of the unfolded protein response can lead to elimination of stressed cells by apoptosis (Zinszner et al., 1998; Nishitoh et al., 2002). We recently demonstrated that sEH deficiency or pharmacological inhibition in mice attenuated chronic high fat diet-induced ER stress in liver and adipose tissue in a cell autonomous manner (Bettaieb et al., 2013).

Based on the above information we examined markers of ER stress in our murine model of liver fibrosis and asked if they could be reduced by treatment with sEH inhibitors.

Materials and methods

Reagents. TPPU (1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea) (Rose et al., 2010) and *t*-TUCB (*trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid), (Hwang et al., 2007) were synthesized and the physical properties were assessed as previously reported. Antibodies for pPERK (Thr980), PERK, peIF2α (Ser51), eIF2α, sXBP1, ATF6, IRE1α, and BiP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while phospho-p38 (Thr180/Tyr182), p38, pJNK (Thr183/Tyr185), JNK and cleaved caspase3 antibodies were from Cell Signaling (Beverly, MA). Antibodies for pIRE1α (Ser724) were purchased from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from BioResources International (Carlsbad, CA). Unless otherwise indicated, all other chemicals were purchased from Sigma (St. Louis, MO).

Animals. All animal studies were approved by the University of California Davis Animal Use and Care Committee and were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Male C57BL (20 g) were obtained from Charles River Laboratories (Wilmington, MA). A colony of C57BL mice with a disruption in the sEH gene resulting in a functional knockout is maintained by the UC Davis Mouse Biology Program (Luria et al., 2007). Mice were divided into four groups of 8: The Control group was given intraperitoneal injections (I.P.) of sterile filtered Neobee M-5 (Fisher Scientific, Houston, TX) every five days for five weeks, for a total of 7 injections. They also received drinking water containing 1% PEG-400 (Fisher Scientific). The CCl_4-only group was given I.P. injections of 197 mg/kg CCl_4 (Sigma) mixed 1:7 in sterile filtered Neobee M-5 every five days for five weeks, for a total of 7 injections. This dose and route of administration was chosen since it has been shown to induce robust fibrosis in C57BL mice (Constandinou, et al., 2005). They received drinking water containing 1% PEG-400 (Fisher Scientific). The CCl_4 + TPPU group was treated with CCl_4 similar to the CCl_4-only group and received drinking water with 10 mg/L TPPU and 1% PEG-400, prepared as follows. TPPU was dissolved in PEG-400 to give a 1000 mg/L clear solution. This solution was then added to warm drinking water with rapid stirring to give the 10 mg/L solution of TPPU in 1% PEG-400 in drinking water. The water was provided ad lib. The TPPU-only group was given I.P. injections of sterile filtered Neobee M-5 like the Control group, and received drinking water containing TPPU similar to the CCl_4 + inhibitor groups. In the experiment with the second sEH inhibitor, *t*-TUCB was solubilized in PEG-400 and then added to drinking water, resulting in a final concentration of 10 mg/L TPPU and 1% PEG-400. Based on estimated daily water consumption, a concentration of 10 mg/L inhibitor in drinking water will result in a dose of approximately 1.7 mg/kg/day. The mice were euthanized three days after the final injection.

Histology. Liver samples were embedded, sectioned and stained by the UC Davis Veterinary Medical Teaching Hospital Anatomic Pathology Service (Davis, CA). The slides were imaged using a Nikon Diaphot inverted microscope and quantified using ImageJ (NIH, Bethesda, MD).

Protein analysis. Mouse tissues were dissected and immediately frozen in liquid nitrogen. For zymography, liver samples were homogenized in cold 25 mM Tris–HCl buffer using 3 × 30 s bursts of a roto-stator homogenizer with one minute intervals in between. Zymography was performed using Novagen pre-cast gelatin zymography gels (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and quantified using ImageJ (NIH). For Western blots, tissues were ground in the presence of liquid nitrogen and lysed using radio-immunoprecipitation assay (RIPA) buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors). Lysates were clarified by centrifugation at 10,000 ×*g* for 10 min and protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblots were performed with the relevant antibodies. Proteins were visualized using Luminata™ Forte (Millipore, Billerica, MA). For quantitation purposes, pixel intensities of immuno-reactive bands from blots that were in the linear range of loading and exposure were quantified using FluorChem 9900 (Alpha Innotech, San Lenardo, CA).