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Characterization of the Cytochrome P450 epoxyeicosanoid pathway in non-alcoholic steatohepatitis

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ABSTRACT

Non-alcoholic steatohepatitis (NASH) is an emerging public health problem without effective therapies. Cytochrome P450 (CYP) epoxygenases metabolize arachidonic acid into bioactive epoxyeicosatrienoic acids (EETs), which have potent anti-inflammatory and protective effects. However, the functional relevance of the CYP epoxyeicosanoid metabolism pathway in the pathogenesis of NASH remains poorly understood. Our studies demonstrate that both mice with methionine-choline deficient (MCD) diet-induced NASH and humans with biopsy-confirmed NASH exhibited significantly higher free EET concentrations compared to healthy controls. Targeted disruption of *Ephx2* (the gene encoding for soluble epoxide hydrolase) in mice further increased EET levels and significantly attenuated MCD diet-induced hepatic steatosis, inflammation and injury, as well as high fat diet-induced adipose tissue inflammation, systemic glucose intolerance and hepatic steatosis. Collectively, these findings suggest that dysregulation of the CYP epoxyeicosanoid pathway is a key pathological consequence of NASH *in vivo*, and promoting the anti-inflammatory and protective effects of EETs warrants further investigation as a novel therapeutic strategy for NASH.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a rapidly growing public health concern that is prevalent in approximately 30% of the United States population and fueled by the diabetes and obesity epidemic [1,2]. Progression from hepatic steatosis to non-alcoholic steatohepatitis (NASH) occurs in approximately 10–20% of cases, and is characterized by progressive hepatic inflammation, injury, and fibrosis; however, the mechanisms that underlie the development and progression of this syndrome remain poorly understood [2]. Furthermore, there are currently no treatments approved for the prevention or treatment of NASH [1,2]. In order to develop novel therapeutic strategies for NASH, an improved understanding of the key pathways that regulate its development and progression is needed.

Cytochrome P450 (CYP) enzymes are expressed abundantly in the liver where they are essential for the oxidative biotransformation of xenobiotics. In parallel to cyclooxygenases (COX) and lipoxygenases (LOX), certain CYP isoforms metabolize arachidonic acid to biologically active eicosanoids. Notably, CYP epoxygenase enzymes from the CYP2J and CYP2C subfamilies metabolize arachidonic acid to bioactive epoxyeicosatrienoic acids (EETs) [3]. However, EETs are rapidly hydrolyzed by soluble epoxide hydrolase (sEH, *EPHX2*) to their corresponding dihydroxyeicosatrienoic acids (DHETs), which are generally less biologically active [4]. CYP epoxygenase-derived EETs elicit cellular and organ protective effects in various preclinical models, including hypertension, ischemia-reperfusion injury and chemotherapy-induced organ

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injury, via attenuating inflammation, apoptosis and fibrosis [4–6]. More recently, it has been reported that promoting the effects of EETs elicits protective effects in obesity-associated metabolic disease and in the atherogenic diet model of NAFLD/NASH in preclinical models [7–12]. In addition, altered circulating CYP-derived DHET concentrations have been observed in humans diagnosed with NAFLD/NASH [13]. However, the impact of NASH on EET concentrations in humans is unknown, and the functional relevance of the CYP epoxyeicosanoid metabolism pathway in the development and progression of NASH remains poorly understood.

Therefore, the objective of this study was to (1) evaluate whether EET levels are significantly altered following experimental induction of NASH in mice and in humans with biopsy-confirmed NASH; and, (2) determine whether promoting the effects of CYP epoxygenase-derived EETs attenuates the development and progression of NASH in mice.

2. Materials and methods

2.1. Reagents

Reagents were obtained from ThermoFisher Scientific (Waltham, MA) unless otherwise indicated.

2.2. Animals

All experiments were performed in adult mice on a C57BL/6J background (age 8–20 weeks). Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). A colony of mice with targeted disruption of *Ephx2* (*Ephx2*$^{-/-}$) were backcrossed onto a C57BL/6J genetic background for more than 10 generations, as described [14,15]. All mice had free access to food and water and were housed with littermates (one to four mice per cage) in temperature and humidity controlled rooms using a 12 h light/dark cycle. All studies were completed in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals*, and were approved by the Institutional Animal Care and Use Committee at the University of North Carolina-Chapel Hill (UNC) and the National Institute of Environmental Health Sciences.

2.3. Experimental induction of NAFLD/NASH in mice

The first series of experiments evaluated the impact of experimental induction of NASH on hepatic and circulating CYP-derived eicosanoid concentrations in WT mice. Male WT mice were fed a commercially available methionine-choline deficient (MCD) diet (D518810, Dyets Inc., Bethlehem, PA; n = 14) or a composition-matched methionine-choline replete control diet (D518754; n = 10) for 4 weeks. Dietary depletion of methionine and choline leads to hepatic steatosis and oxidative stress, and subsequent liver injury, inflammation and fibrosis, and is a widely used preclinical model of NASH [16,17]. The second series of experiments evaluated the effect of disrupting sEH-mediated EET hydrolysis on MCD diet induced hepatic steatosis, injury and inflammation in male and female *Ephx2*$^{-/-}$ (n = 27 [male: n = 14, female: n = 13]) and corresponding WT control (n = 24 [male: n = 8, female: n = 16]) mice. A parallel group of WT mice were fed the control diet for reference (n = 15 [male: n = 6, female: n = 9]).

The MCD diet is limited by a lack of significant weight gain and glucose intolerance [16,17]. Thus, a third series of experiments was completed to evaluate the effect of disrupting sEH-mediated EET hydrolysis on the development of obesity-associated hepatic steatosis. Male and female *Ephx2*$^{-/-}$ (n = 41 [male: n = 26, female: n = 15]) and corresponding WT control (n = 46 [male: n = 33, female: n = 13]) mice were fed a commercially available high-fat diet (HFD; D12492 [60% kcal fat], Research Diets Inc., New Brunswick, NJ) for 8 weeks. A parallel group of WT mice were fed a composition-matched low-fat diet (LFD; D12450B [10% kcal fat]) for reference (n = 28 [male: n = 16, female: n = 12]).

Body weight was measured in each mouse weekly. Food consumption was measured weekly in each cage by weighing the food at the beginning and end of each week. At the termination of each experiment, blood was collected via cardiac puncture, plasma was separated by centrifugation, and liver and epididymal white adipose tissue (eWAT) were harvested. One part of each tissue was snap-frozen in liquid nitrogen and stored at −80 °C. The remainder was either fixed in 4% paraformaldehyde and embedded in paraffin or embedded in Tissue-Tek O.C.T. compound and snap-frozen in liquid nitrogen for subsequent histological analysis.

2.4. Human NASH case: control study

Human samples were obtained from a single-center, case:control study of male and female patients with biopsy-confirmed NASH (n = 7) and corresponding healthy volunteer controls (n = 15) [18,19]. The inclusion and exclusion criteria have been described in detail previously [18]. Briefly, patients with biopsy-confirmed non-cirrhotic NASH (defined as a NAFLD activity score (NAS) > 3) and a BMI ≤45 kg/m^2 were recruited from the UNC hepatology clinic. In parallel, healthy volunteers with no history of hepatic or metabolic disease and a BMI ≤30 kg/m^2 were recruited from the local community. Written informed consent was obtained from all participants. The study protocol was approved by the UNC Biomedical Institutional Review Board.

Study participants fasted overnight prior to initiation of the study visit at the UNC Clinical and Translational Research Center. A blood sample was collected from an indwelling catheter at baseline and every 30 min for 2 h after administration of a standardized meal containing 509 kcal (27.2 g protein, 23.9 g fat, 53.3 g carbohydrates), as described [18,19]. Serum was separated by centrifugation, aliquoted and stored at −80 °C until analysis.

2.5. Quantification of eicosanoid concentrations

Free eicosanoid metabolite concentrations were quantified from mouse and human samples using a targeted liquid chromatography-tandem mass spectrometry (LC/MS/MS) method with optimized sensitivity and specificity for EET quantification, as previously described [11,12,20]. Briefly, plasma/serum (0.25 mL) and homogenized liver (20 mg) and eWAT (50 mg) tissue were diluted in 0.1% acetic acid/5% methanol solution containing 0.009 mM butylated hydroxytoluene (BHT), and internal standards were added. The samples were processed by liquid–liquid extraction to isolate lipids, and then dried. Following reconstitution, free eicosanoid metabolites were quantified by LC/MS/MS, as described [11].

Data were acquired and concentrations were quantified with Analyst software (v1.5, Applied Biosystems) using metabolite and internal standard peaks for each sample. Tissue concentrations were normalized to tissue weight. In the human study, detectable metabolite concentrations that were <0.5× the lower limit of quantitation or >1.5× the upper limit of quantitation were imputed as such. Metabolites with more than 50% of the values outside of this range were not included in the analysis. Among the panel of 34 CYP-, COX- and LOX-derived metabolites evaluated, 24 metabolites met the criteria for analysis (Table S2). Twenty-one of the 24 metabolites (88%) had <10% of their values outside of the quantitation range.

Due to significant correlations among the EET and DHET regioisomers [11,12,20], the sum of the EET regioisomers (sum EETs) and DHET regioisomers (sum DHETs) were calculated to minimize redundancy. The sum of the EET and DHET regioisomers (sum