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Original Contribution

Cytochrome P450 2E1 potentiates ethanol induction of hypoxia and HIF-1α in vivo

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ABSTRACT

Ethanol induces hypoxia and elevates HIF-1α in the liver. CYP2E1 plays a role in the mechanisms by which ethanol generates oxidative stress, fatty liver, and liver injury. This study evaluated whether CYP2E1 contributes to ethanol-induced hypoxia and activation of HIF-1α in vivo and whether HIF-1α protects against or promotes CYP2E1-dependent toxicity in vitro. Wild-type (WT), CYP2E1-knock-in (KI), and CYP2E1 knockout (KO) mice were fed ethanol chronically; pair-fed controls received isocaloric dextrose. Ethanol produced liver injury in the KI mice to a much greater extent than in the WT and KO mice. Protein levels of HIF-1α and downstream targets of HIF-1α activation were elevated in the ethanol-fed KI mice compared to the WT and KO mice. Levels of HIF prolyl hydroxylase 2, which promotes HIF-1α degradation, were decreased in the ethanol-fed KI mice in association with the increases in HIF-1α. Hypoxia occurred in the ethanol-fed CYP2E1 KI mice as shown by an increased area of staining using the hypoxia-specific marker pimonidazole. Hypoxia was lower in the ethanol-fed WT mice and lowest in the ethanol-fed KO mice and all the dextrose-fed mice. *In situ* double staining showed that pimonidazole and CYP2E1 were colocalized to the same area of injury in the hepatic centrilobule. Increased protein levels of HIF-1α were also found after acute ethanol treatment of KI mice. Treatment of HepG2 E47 cells, which express CYP2E1, with ethanol plus arachidonic acid (AA) or ethanol plus buthionine sulfoximine (BSO), which depletes glutathione, caused loss of cell viability to a greater extent than in HepG2 C34 cells, which do not express CYP2E1. These treatments elevated protein levels of HIF-1α to a greater extent in E47 cells than in C34 cells. 2-Methoxyestradiol, an inhibitor of HIF-1α, blunted the toxic effects of ethanol plus AA and ethanol plus BSO in the E47 cells in association with inhibition of HIF-1α. The HIF-1α inhibitor also blocked the elevated oxidative stress produced by ethanol/AA or ethanol/BSO in the E47 cells. These results suggest that CYP2E1 plays a role in ethanol-induced hypoxia, oxidative stress, and activation of HIF-1α and that HIF-1α contributes to CYP2E1-dependent ethanol-induced toxicity. Blocking HIF-1α activation and actions may have therapeutic implications for protection against ethanol/CYP2E1-induced oxidative stress, steatosis, and liver injury.

Hypoxia-inducible factor (HIF) is activated by hypoxia and is a master regulator of oxygen homeostasis, as it regulates the expression of many genes involved in glycolysis, glucose transport, synthesis of nitric oxide and cytokines such as TNFα, blood flow, inflammation, and cell death [1–3]. Expression and activity of the HIF-1α subunit is regulated by cellular oxygen levels, primarily at the level of protein stability [4,5]. The HIF-1α protein is rapidly degraded under normoxic conditions, as oxygen-dependent proline hydroxylation of HIF-1α by proline hydroxylases promotes ubiquitination of HIF-1α followed by rapid proteasome-mediated degradation. Hypoxia enhances HIF-1α levels by inhibiting proline hydroxylation and therefore the degradation of HIF-1α [1–5]. The accumulated HIF-1α can dimerize with the aryl hydrocarbon receptor nuclear translocator, move to the nucleus, and bind to the hypoxia-responsive element in the promoter of its target genes [6–8]. HIF-1α has been implicated in the toxicity found in many models of liver injury, including alcohol-induced liver injury [9–11].

Chronic ethanol consumption by rats was shown to cause hypoxia due to increasing oxygen consumption; this reflected the requirement for oxygen to reoxidize reducing equivalents, NADH, produced by the oxidation of ethanol by alcohol dehydrogenase and the

Abbreviations: AA, arachidonic acid; ALT, alanine aminotransferase; BSO, l-buthionine sulfoximine; CYP2E1, cytochrome P450 2E1; GSH, reduced glutathione; HE, hematoxylin–eosin; 4-HNE, 4-hydroxynonenal; HIF, hypoxia-inducible factor; HPH-2, HIF prolyl hydroxylase 2; IHC, immunohistochemistry; KI, knock-in; KO, knockout; LDHA, lactate dehydrogenase A; MDA, malondialdehyde; 2-ME, 2-methoxyestradiol; 3-NT, 3-nitrotyrosine; PNP, *p*-nitrophenol; ROS, reactive oxygen species; WT, wild type; TBARS, thiobarbituric acid-reactive substances.

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oxidation of acetaldehyde by the low K_m mitochondrial aldehyde dehydrogenase [12–14]. Acute ethanol administration also produced hypoxia [14–16]. Chronic intake of ethanol by intragastric infusion caused hypoxia and oxidative stress in rat liver and pancreas [17–19]. The production of hypoxia by ethanol administration has been reviewed [20]. Because ethanol-induced hypoxia was most pronounced in the pericentral zone of the liver acinus reflecting the gradient of oxygen across the liver, the ethanol-induced hypoxia was hypothesized to play a role in the ethanol-induced liver injury [12,13,20]. Li et al. [21] showed that HIF-1α mRNA and protein were elevated after intragastric administration of ethanol to rats for 24 weeks. Recent studies have assessed the possible role of HIF-1α in ethanol-induced steatosis. Nath et al. [22] reported that feeding mice for 4 weeks with the Lieber–DeCarli diet increased HIF-1α mRNA, protein, and DNA-binding activity in wild-type mice and produced the typical fatty liver associated with ethanol consumption. Ethanol-induced fatty liver was intensified in mice engineered to express high levels of hepatic HIF-1α but was decreased in mice deficient in hepatic HIF-1α [22]. Nath et al. concluded that HIF-1α plays an important role in ethanol-induced fatty liver and liver injury [22]. Conversely, Nishiyama et al. [23] found that activation of HIF-1α suppresses ethanol-induced fatty liver. Using the same Lieber–DeCarli model of ethanol feeding, they found that hepatocyte-specific HIF-1α-knockout mice developed a more pronounced fatty liver and elevated triglycerides than did wild-type mice and concluded that HIF-1α is protective against ethanol induction of fatty liver. Possible reasons for these different conclusions were discussed [24] but remain unclear and additional studies on this are needed.

CYP2E1 metabolizes and activates many toxicologically important substrates to more toxic products [25–28]. Levels of CYP2E1 are elevated by ethanol [29] and during its catalytic reaction with molecular oxygen, reactive oxygen species such as superoxide and hydrogen peroxide are produced [30]. In some, but not all studies the ethanol-induced liver pathology correlated with CYP2E1 levels and the generation of ROS and oxidative stress [31–36]. We have shown that ethanol-induced steatosis and oxidative stress were lower in CYP2E1-knockout mice fed ethanol chronically compared to wild-type mice [37]. The fatty liver produced in wild-type mice was blunted by inhibitors of CYP2E1 [37]. Ethanol-induced fatty liver and oxidative stress was restored in CYP2E1-knock-in mice [38], in which the human CYP2E1 was expressed in the CYP2E1-knockout mouse. Ethanol and other pro-oxidants were more toxic to HepG2 cells expressing CYP2E1 than to control HepG2 cells not expressing CYP2E1 [31,39,40]. CYP2E1 seems to play an important role in the mechanisms by which ethanol generates oxidative stress and is hepatotoxic [31,39,40]. Given the differing results on the role of HIF-1α in ethanol-induced fatty liver, the goal of this study was to evaluate whether CYP2E1 contributes to ethanol-induced hypoxia and activation of HIF-1α and whether HIF-1α potentiates or prevents CYP2E1-dependent toxicity.

Materials and methods

In vivo mouse models

SV129 background CYP2E1-knockout (KO) mice [41] and humanized transgenic CYP2E1-knock-in (KI) mice [42,43] were kindly provided by Dr. Frank J. Gonzalez (Laboratory of Metabolism, National Cancer Institute, Bethesda, MD, USA) and breeding colonies of these mice were established at Mount Sinai. SV129 wild-type (WT) mice were purchased from Charles River Laboratory. All mice were housed in temperature-controlled animal facilities with 12-h light/12-h dark cycles. The mice received humane care, and experiments were carried out according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* and with approval of the Mount Sinai Animal Care and Use Committee.

WT, KO, and KI mice (total of 72 mice, 48 males, 24 females), 8–10 weeks of age, weighing 22–28 g, were initially fed the control liquid dextrose diet (Bio-Serv, Frenchtown, NJ, USA) for 3 days to acclimate them to the Lieber and DeCarli liquid diet [44]. Afterward, the mice were fed either the ethanol diet or the dextrose diet as follows. The content of ethanol was gradually increased every 7 days from 10% (1.77% (vol/vol)) of total calories to 20 (3.54% (vol/vol)), 30 (5.31% (vol/vol)), and finally 35% of total calories (6.2% (vol/vol)) for 4 weeks. The control mice were pair-fed the dextrose diet on an isoenergetic basis. The amounts of diet consumed by the knockout, the knock-in, and the wild-type mice were approximately the same. Mice were sacrificed between 3:00 and 5:00 PM on the 28th day of ethanol (6.2% vol/vol) feeding. For acute ethanol treatment, 24 male WT, KO, and KI mice, 6–8 weeks of age, with body weight of 20–25 g, were gavaged with 30% ethanol at a dose of 3 g/kg body wt, twice a day for 4 days or were gavaged with saline. Mice were fasted for 18 h before sacrifice after the fourth day of acute ethanol or saline administration.

At the end of treatment, the mice were sacrificed and serum and liver were collected. The liver was rapidly excised into small fragments and washed with cold saline. One aliquot of tissue was placed in 10% formalin solution for paraffin processing and one aliquot of tissue was placed in RNAlater solution for RNA isolation (Ambion, Grand Island, NY, USA). The remaining aliquots were stored at −80 °C for further assays. Liver homogenates were prepared in ice-cold 0.15 M KCl. Nuclear extract was freshly prepared according to a nuclear extract kit protocol (Active Motive, Carlsbad, CA, USA). All samples were stored at −80 °C in aliquots.

Serum alanine aminotransferase (ALT) and ethanol assay

Serum ALT levels were measured using a diagnostic kit (Pointe Scientific, Brussels, Belgium) and by kinetically following changes in absorbance at 340 nm using a UV-160 U recording spectrophotometer (Shimadzu, Kyoto, Japan). Serum ethanol was measured using an Abcam ethanol assay kit based on oxidizing ethanol by alcohol oxidase to generate H_2O_2, which then reacts with the provided probe to generate a specific chromophore color (λ_{max} 570 nm).

Organelle preparations and assays

Mitochondria were isolated after centrifugation of homogenates at 8500*g* for 30 min and suspended in 0.25 M sucrose–10 mM Tris, pH 7.4, buffer and washed once. The postmitochondrial supernatant was centrifuged at 100,000*g* at 4 °C for 60 min to obtain the microsomal pellets and the cytosolic supernatant fraction, respectively. The protein concentrations of the various fractions were determined using a protein assay kit based on the Lowry assay (Bio-Rad, Hercules, CA, USA). CYP2E1 activity was measured in liver microsomes by spectrophotometric analysis at 546 nm of the oxidation of *p*-nitrophenol to *p*-nitrocatechol in the presence of NADPH and oxygen [45]. The production of thiobarbituric acid-reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents, was assayed in liver mitochondrial fractions by the spectrophotometric analysis at 535 nm of the formation of thiobarbituric acid-reactive components. The concentration of malondialdehyde was calculated using an extinction coefficient of 156 × mmol/L/cm and expressed as picomoles per milligram of protein [46]. Reduced glutathione (GSH) was analyzed in liver mitochondria by a glutathione reductase assay [47].