Ethanol increases mitochondrial cytochrome P450 2E1 in mouse liver and rat hepatocytes

Marie-Anne Robin*, Ingrid Sauvage, Thomas Grandperret, Véronique Descatoire, Dominique Pessayre, Bernard Fromenty

INSERM Unité 481, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France

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Abstract Enhanced hepatic levels of cytochrome P450 2E1 (CYP2E1) may play a key role in the pathogenesis of some liver diseases because CYP2E1 represents a significant source of reactive oxygen species. Although a large fraction of CYP2E1 is located in the endoplasmic reticulum, CYP2E1 is also present in mitochondria. In this study, we asked whether ethanol, a known inducer of microsomal CYP2E1, could also increase CYP2E1 within mitochondria. Our findings indicated that ethanol increased microsomal and mitochondrial CYP2E1 in cultured rat hepatocytes and in the liver of lean mice. This was associated with decreased levels of glutathione, possibly reflecting increased oxidative stress. In contrast, in leptin-deficient obese mice, ethanol administration did not increase mitochondrial CYP2E1, nor it depleted mitochondrial glutathione, suggesting that leptin deficiency hampers mitochondrial targeting of CYP2E1. Thus, ethanol intoxication increases CYP2E1 not only in the endoplasmic reticulum but also in mitochondria, thus favouring oxidative stress in these compartments.

Keywords: Cytochrome P450 2E1; Ethanol; Mitochondria; Oxidative stress; Steatosis

1. Introduction

Cytochrome P450 2E1 (CYP2E1) metabolises many endogenous or exogenous small molecules such as acetone, glycerol, ethanol, acetaminophen, carbon tetrachloride, halothane or nitrosamines [1–3]. Hepatic expression of the CYP2E1 mRNA and/or expression of the CYP2E1 protein are increased in different physiological or pathological conditions, such as fasting, a high fat diet, diabetes, obesity or ethanol intoxication [1,4]. Because CYP2E1 generates large amounts of reactive oxygen species (ROS), increased hepatic expression of CYP2E1 in obese, diabetic or alcoholic patients is believed to play a significant role in the pathogenesis of non-alcoholic steatohepatitis (NASH) and alcoholic liver disease (ALD) [3,5,6]. Interestingly, it has been shown that ROS overproduction due to increased CYP2E1 expression can oxidatively damage liver DNA [7] and contribute to hepatic fibrosis [8].

A large fraction of cellular CYP is located in the endoplasmic reticulum. This fraction will be henceforth referred to as 'microsomal CYP'. However, CYP enzymes can also be found in significant amounts in other cell compartments, including plasma membrane, Golgi apparatus and mitochondria [9–11]. Interestingly, the presence of CYPs on the plasma membrane could be involved in some liver diseases, such as drug-induced immunoallergic and/or autoimmune hepatitis [12,13].

CYP2E1 is one of the several CYP enzymes that are partly located within liver mitochondria [9,14–16]. Targeting of CYP2E1 to liver mitochondria depends on a cryptic signal at sequence 21–31 of the protein, which is activated by cAMP-dependent phosphorylation of Ser-129 by protein kinase A (PKA) [16]. The mitochondrial expression of CYP2E1 in the liver can be enhanced in rats treated with pyrazole [9] or in rats made diabetic by streptozotocin administration [17]. Moreover, it has been demonstrated that accumulation of CYP2E1 within mitochondria is associated with an increased generation of ROS by mitochondria [17]. Thus, increased mitochondrial levels of CYP2E1 could have deleterious consequences in some circumstances.

Liver mitochondria are key targets of ethanol toxicity, and mitochondrial dysfunction is known to play a major role in ALD [18–20]. One of the mechanisms whereby ethanol could injure mitochondria is via increased generation of ROS and lipid peroxidation products, which could damage key mitochondrial components such as cytochrome *c* oxidase and mitochondrial DNA [19,21]. Importantly, oxidative damage to mitochondria can occur whenever the major antioxidant defences (i.e., glutathione, glutathione peroxidase-1 and manganese superoxide dismutase) are overwhelmed within these organelles [20].

The overproduction of ROS during ethanol intoxication seems to occur in different cell compartments, including cytosol and mitochondria. During ethanol intoxication, increased ROS generation within liver mitochondria could be the consequence of respiratory chain dysfunction [18,20]. Yet another possible mechanism might be an increased expression of CYP2E1 within mitochondria. Thus, the goal of this study was to determine whether ethanol intoxication could increase mitochondrial expression of CYP2E1 in vitro in rat hepatocytes but also in vivo in mouse liver. Moreover, during our

*Corresponding author. Fax: +33 1 44 85 92 79.
E-mail address: robin@bichat.inserm.fr (M.-A. Robin).

Abbreviations: ALD, alcoholic liver disease; CPR, NADPH CYP reductase; CYP2E1, cytochrome P450 2E1; GSH, reduced glutathione; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; PKA, protein kinase A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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in vivo investigations, we asked whether CYP2E1 expression in liver mitochondria differs between lean and leptin-deficient obese ob/ob mice. To the best our knowledge, these issues have never been addressed before.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley CHChR rats (180–220 g) and Swiss CR1:CD1-ICR BR mice (30–31 g) were purchased from Charles River (L'arbresle, France). Male ob/ob mice (C57BL/6-J-Rj-ob), weighing 49–56 g, and their lean male +/+ littermates (referred to as lean mice), weighing 26–30 g, were purchased from Janvier (Le-Genest-St-Isle, France). All experiments were performed according to national guidelines for the use of animals in biomedical research. Mice were fed ad libitum on a normal diet (A04 biscuits, UAR, Villemoisson-sur-Orge, France).

2.2. Animal treatment

Swiss mice were intoxicated either by daily gastric intubation for 4 days, or by ethanol added to the drinking water for 4 weeks. For the short-term intra-gastric treatment, ethanol was diluted in water (50:50; v/v), and was administered once daily by gastric intubation at the dose of 5 g/kg body weight for 4 consecutive days, as previously described [19]. Using this protocol, serum ethanol concentrations (means ± S.E.M. for 7 mice) were 4.9 ± 0.7 g/l two hours after the last intoxication [19]. Control mice only received water. Intoxicated Swiss mice ate less and did not gain weight. After 4 days of treatment, their mean body weight was 8.8% less than in control mice. All mice were sacrificed 2 h after the last administration of water or ethanol. For the prolonged treatment, ethanol was added to the drinking water for 4 weeks as previously described, with minor modifications [22]. In our protocol, ethanol was given at a concentration of 5% v/v during the first week, 10% v/v during the second week and 15% v/v for the last two weeks. Ethanol was left in the drinking water until sacrifice. During the treatment, there was no difference in food intake between control and intoxicated mice and the mean body weight of intoxicated mice was increased by 9.2% compared to control mice, although this difference was not significant.

Ob/ob mice and their lean littermates (lean mice) also received ethanol by gastric intubation, but at the dose of 2.5 g/kg body weight daily for 4 days, as previously described [23]. Control mice received water. In all experiments, mice were sacrificed 2 h after the last administration of ethanol or water. Like intoxicated Swiss mice, intoxicated lean or obese mice ate less and failed to gain weight. At the end of the experiment, the mean body weight was 4.6% less in intoxicated than naïve lean mice, and 6.3% less in intoxicated than naïve obese mice.

2.3. Primary culture of rat hepatocytes

Rats were anaesthetized with pentobarbital and hepatocytes were isolated by two-step in situ perfusion of collagenase, as described previously [24]. Hepatocytes (7×10^6) were seeded in 100-mm culture dishes containing William's E medium supplemented with 0.1 mg/ml insulin (Sigma Chemical Co, St. Louis, MO), 100 IU/ml penicillin (Invitrogen, Cergy-Pontoise, France), 0.1 mg/ml streptomycin (Invitrogen), 0.07 mmol/l hydrocortisone (Sigma), and 10% fetal calf serum (VWR International S.A.S., Fontenay-sous-bois, France). Hepatocytes were maintained in an incubator at 37 °C under 5% CO_2 in air for 3 h. After plating, the medium was replaced by fresh medium without fetal calf serum, and was supplemented or not with 100 mmol/l ethanol. Cells were kept for various lengths of time (from 3 to 72 h), while the medium was changed each day. The lack of ethanol toxicity was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, based on the mitochondrial reduction of the tetrazolium salt to an aqueous insoluble formazan product by the succinic dehydrogenase [25].

2.4. Isolation of microsomes and mitochondria

Unless otherwise indicated, hepatic sub-cellular fractions (mitochondria, microsomes and cytosol) were isolated as previously described [17] with some modifications. Livers were homogenized in 220 mmol/l mannitol, 70 mmol/l sucrose, 2 mmol/l HEPES, and 0.1 mmol/l EDTA (pH 7.4), and the homogenate was centrifuged at $600 \times g$ for 10 min at 4 °C. The supernatant (liver tissue homogenate), was centrifuged at $8000 \times g$ for 20 min at 4 °C. The $8000 \times g$ supernatant was centrifuged at $100\,000 \times g$ for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 50 mmol/l Tris–HCl buffer, pH 7.4, containing 1.15% KCl, 10% glycerol, 0.1 mmol/l dithiothreitol, 0.1 mmol/l EDTA and 0.1 mmol/l PMSF, and was stored at −80 °C. The supernatant (cytosol) was kept at −80 °C. The $8000 \times g$ pellet (containing mitochondria) was loaded onto a 28% (v/v) Percoll medium in order to purify the mitochondrial fraction. After centrifugation at $27\,000 \times g$ for 45 min at 4 °C, mitochondria formed a band at the bottom of the tube. This band was diluted in 5 volumes of sucrose/mannitol buffer and centrifuged at $12\,000 \times g$ for 20 min. The resultant loose pellet was washed twice, resuspended in sucrose/mannitol buffer and stored at −80 °C.

In order to prepare sub-cellular fractions from cultured rat hepatocytes, cells were scraped, washed and homogenized in the sucrose/mannitol buffer, and the same procedure as for liver was followed, except for mitochondrial purification. The $8000 \times g$ pellet of mitochondria was resuspended in sucrose/mannitol buffer, loaded on 1 M sucrose cushion and centrifuged at $10\,000 \times g$ for 20 min at 4 °C. The pellet was again resuspended, washed three times and stored at −80 °C.

To assess the microsomal contamination of purified mitochondria, the protein expression and activity of NADPH–cytochrome P450 reductase were measured in mitochondrial and microsomal fractions [26]. We found that microsomal contamination was less than 4% in the mitochondria isolated from rat hepatocytes, and was comprised between 5% and 10% in the mitochondria isolated from mouse livers.

Cytosolic and mitochondrial samples for reduced glutathione (GSH) measurement were precipitated with 10% trichloroacetic acid, centrifuged and stored at −80 °C.

2.5. Assessment of CYP2E1 activity and protein levels

To assess CYP2E1 expression, mitochondrial (100 μg) and microsomal (50 μg) proteins were subject to SDS–10% polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane, CYP2E1 was revealed with a rabbit polyclonal antibody (Euromedex, Souffelweyersheim, France). This antibody recognizes both mitochondrial and microsomal CYP2E1, since the primary structure of both forms is identical [9]. To assess equal protein loading, blots were stripped and incubated either with an anti-calregulin antibody (Tebu-bio, Le Perray en Yvelines, France) or an anti-NADPH CYP reductase (CPR) antibody (Interchim, Montluçon, France) for the microsomal fraction and with an anti-porin antibody (Calbiochem, La Jolla, Ca) for the mitochondrial fraction. Protein bands were quantified by densitometry.

CYP2E1 activity was determined in mitochondrial and microsomal fractions by the hydroxylation of aniline into *p*-aminophenol [27]. Briefly, reactions were carried out in 500 μl final volumes in a 100 mmol/l phosphate buffer (pH 7.4) containing 1 mmol/l $MgCl_2$, 5 mmol/l aniline, and 500 μg of proteins as the enzyme source. Reactions were initiated by the addition of 2.5 mmol/l NADPH, and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by adding an equal volume of 20% trichloroacetic acid. Precipitated proteins were removed by centrifugation at $10\,000 \times g$ for 10 min and 500 μl of supernatant were added to 500 μl of a solution containing 2% phenol and 0.5 mol/l NaOH. After 45 min incubation, the *p*-aminophenol was converted into a blue complex with an absorption peak at 630 nm.

2.6. Assessment of GSH levels

GSH levels were determined in both mitochondrial and cytosolic fractions by a method adapted from Tietze et al. [28].

2.7. Statistical analysis

Differences between control and treated cultured hepatocytes or between control and intoxicated Swiss mice were assessed by a Student's *t* test for independent data. Differences between naïve and intoxicated lean and obese C57BL/6-J mice were assessed using analysis of variance and Fischer exact test.