CYTOCHROME P-450 2E1 ACTIVITY AND OXIDATIVE STRESS IN ALCOHOLIC PATIENTS†

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Abstract — As cytochrome P-450 2E1 (CYP2E1) induction was related to oxidative stress in experimental models, the aim of this study was to investigate the relationship between CYP2E1 activity and markers of oxidative stress in 40 alcoholic patients entering a rehabilitation programme. Plasma oxidized proteins, lipid peroxides (LPO) and antibodies against hydroxyethyl radical (HER) or malondialdehyde (MDA) adducts were assessed as markers of the production of free radicals, whereas vitamin E levels were evaluated as a marker of the antioxidant defence. CYP2E1 activity was determined by using the 6-hydroxychlorzoxazone:chlorzoxazone blood metabolic ratio, 2 h after drug intake. This ratio was increased by 4-fold in alcoholics, compared to non-alcoholic patients, and was correlated with daily intake of ethanol, carbohydrate-deficient transferrin, and blood alcohol level at the time of admission to hospital. Plasma levels of LPO and oxidized proteins were slightly increased (20%) in alcoholic patients when compared with the control group, whereas those of vitamin E were found to be slightly decreased (by 18%). Antibodies against HER or MDA adducts showed a very significant increase. However, when alcoholic patients were divided into two groups according to low or high CYP2E1 induction, no significant difference was observed in the variation of these parameters, except for anti-HER adducts antibodies. Therefore, our study confirms the main involvement of CYP2E1 in HER production. By contrast, CYP2E1 does not appear to be the main factor responsible for the oxidative stress occurring during human chronic alcoholism. Free radicals from other sources may therefore contribute significantly to the generation of this oxidative stress.

INTRODUCTION

Ethanol consumption is able to induce an oxidative stress in the liver and in extrahepatic tissues, linked to an imbalance between the pro-oxidant and the antioxidant systems in favour of the former (Nordmann, 1994; Kurose et al., 1996). Peroxidation of lipids leads to by-products which have been shown to promote collagen production (Parola et al., 1993; Tsukamoto et al., 1995) and to form adducts with proteins. They have been suggested to be initiators of inflammatory response and fibrosis and therefore suspected to play an important role in alcoholic liver disease (Ingelman-Sundberg et al., 1993). Recent studies have shown that cytochrome P-450 2E1 (CYP2E1), which is induced by administration of a large ethanol dose or after chronic ethanol ingestion, has a high oxidase activity and plays a crucial role in the microsomal generation of reactive oxygen species which have the capability of initiating membranous lipid peroxidation (Eckström and Ingelman-Sundberg, 1989). CYP2E1 is also involved in the production of ethanol-derived free radicals during ethanol oxidation (Albano et al., 1995). These free radicals have the property to bind covalently to proteins forming adducts able to induce autoantibodies which are found in rats treated with ethanol (Albano et al., 1995) and in human alcoholics (Clot et al., 1995). These antibodies may represent markers of the production of ethanol-derived free radical adducts and contribute to the hepatotoxicity of ethanol in promoting immune mechanisms of liver injury (Clot et al., 1997).

In experimental conditions, Morimoto et al. (1995) showed that CYP2E1 induction was related to alcoholic liver disease possibly by a link involving oxidative stress, because CYP2E1 inhibitors reduced both alcoholic liver disease and oxidative stress. In a previous study (Dupont et al., 1998), we showed that CYP2E1 activity greatly influenced the formation of hydroxyethyl radicals in alcoholics but no information was available on the oxidative stress in these patients. Therefore, the aim of the present study was to investigate in a new population of alcoholic patients the relationship between CYP2E1 activity, as assessed by chlorzoxazone metabolism (Girre et al., 1994) and some blood markers of oxidative stress, namely oxidized proteins, and lipid peroxides (LPO). Antibodies against the hydroxyethyl radical (HER) or malondialdehyde (MDA) adducts were also evaluated as markers of the production of free radicals, and vitamin E levels were also used as a marker of antioxidant defences.

PATIENTS AND METHODS

Subjects

Forty chronic alcoholic inpatients (mean ± SD age 43 ± 8 years), who had just entered a rehabilitation programme, participated in the study. They exhibited no clinical or biological evidence of hepatic insufficiency. None suffered from malnutrition. All patients consumed excessive amounts of alcohol until hospitalization. Daily tobacco smoking and alcohol consumption were established by interview. Eighteen healthy controls (mean ± SD age 38 ± 8 years; alcohol consumption <20 g/day, non-smoking subjects) were recruited within, and through contacts of, the research staff. All subjects gave their informed consent to this study, which conformed to the ethical guidelines of the 1975 declaration of Helsinki and was approved by the ethical committee of CHU Morvan (CCPPRB, Brest, France). The laboratory and other biological characteristics of the patients are given in Table 1.

Biochemical analysis

Blood samples. Venous blood samples were collected after an overnight fast into EDTA-containing or dry tubes. The blood was immediately centrifuged at 1000 g for 10 min and
plasma or serum samples respectively were protected from light and promptly stored at –80°C until analysis.

**CYP2E1 activity.** This was assessed using chlorozoxazone metabolism as previously described (Girre et al., 1994). Briefly, after a 12 h fast, subjects were administered a 500 mg tablet of chlorozoxazone (Lemmon Company, Sellersville, PA, USA). A blood sample was withdrawn 2 h later to measure chlorozoxazone and 6-hydroxyclozoaxzone using a high power liquid chromatography (HPLC) method (Lucas et al., 1993). The 6-hydroxyclozoaxzone:chlorozoxazone concentration ratio (CHZ-MR, or chlorozoxazone metabolic ratio) has been shown to reflect the rate of chlorozoxazone hydroxylation and thus CYP2E1 activity. In the days preceding the study, subjects had taken no medication which might interfere with CYP2E1 activity, such as paracetamol, disulfiram or isoniazid.

Alcoholics were tested for chlorozoxazone metabolism on the day following their admission to hospital. Blood ethanol was determined on their arrival and before each chlorozoxazone test to verify that they had no ethanol in the blood because of the possible competition between ethanol and chlorozoxazone for metabolism by CYP2E1. Control subjects were not submitted to the CHZ test as normal values were already available in our laboratory (Lucas et al., 1995b).

**Parameters of oxidative stress.** These were assessed in the samples of controls and alcoholic patients. Blood samples were taken after an overnight fast, just before the CHZ test for the alcoholic patients. Plasma lipid peroxides (LPO) were estimated by haemoglobin-catalysed oxidation of 10-N-methyl-carbamoyl-3,7-dimethylamino-10-phenothiazine after treatment with lipoprotein lipase using cumene hydroperoxide as standard, using a kit supplied by Kamiya Biomedical Co. (Seattle, WA, USA). Oxidized proteins were determined by the 2,4-dinitrophenylhydrazine procedure according to Levine et al. (1990). All reagents were of analytical grade and were purchased from Sigma (St Quentin Fallavier, France).

**Vitamin E.** This was measured using a reversed-phase HPLC method as described by Ganiere-Monteil et al. (1994). Determination of α-tocopherol in plasma was performed after extraction by hexane. γ-Tocopherol was added as internal standard. Tocopherol was detected by its native fluorescence (excitation 295 nm; emission 340 nm). α- and γ-Tocopherols were purchased from Sigma.

All other biochemical parameters were determined using a multiparametric routine automat (Synchron Clinical System Myosotis, Beckman Instruments, Brea, USA). Reagents were used according to the manufacturer’s recommendations for determining alkaline phosphatase, gamma-glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, cholesterol and triglycerides levels. Carbohydrate-deficient transferrin (CDT) was assessed using a kit supplied by Biorad (Ivy sur Seine, France).

**Detection of antibodies against protein adducts.** Antigens consisting of HER and MDA adducts with bovine serum albumin (BSA) were immobilized on microplates as previously described (Dupont et al., 1998). Circulating IgGs against epitopes (HER or MDA) were determined in serum samples using an enzyme-linked immunosorbent assay (ELISA) method. Data were expressed as the ratio between the absorbance readings in the wells containing HER-BSA or MDA-BSA and those containing unmodified protein to avoid non-specific reactions to different antigen used. Ratios of ~1 indicate the lack of these antibodies.

**Statistical analysis**

Results were expressed as means ± SD and were compared between controls and alcoholics by Student’s t-test. Linear regression analysis was performed to assess the relationship between CYP2E1 activity and other parameters (Graphpad Instat, Graphpad Software, San Diego, CA, USA).

## RESULTS

The goal of this study was to examine the relationship between CYP2E1 activity and oxidative stress in alcoholic patients who were hospitalized for rehabilitation. These patients displayed no clinical or biological evidence of severe liver disease, but showed laboratory evidence of heavy alcohol consumption (Table 1).

**Induction of CYP2E1 in chronic alcoholics**

CYP2E1 activity was assessed using the CHZ-MR 2 h after intake of 500 mg CHZ. The CHZ-MR was increased by 4-fold in alcoholics, compared to non-alcoholics (Table 2) as previously shown in other studies (Lucas et al., 1995a). A weak but significant correlation was observed between CYP2E1 induction and the mean amount of alcohol ingested per day ($r = 0.31$, $P < 0.05$), the CDT level ($r = 0.33$, $P < 0.05$) and the blood alcohol level (BAL) at the time of admission to hospital ($r = 0.41$, $P < 0.001$).

**Oxidative stress in alcoholics**

Production of free radicals leads to oxidative lipid and protein damage, which can be evaluated in terms of plasma...
liperoxide (LPO) and oxidized proteins, respectively. These parameters were increased by 20% in alcoholics when compared with the control group \((P < 0.05, \text{Table 2)}\). Free radicals also contribute to the formation of protein adducts, such as MDA or HER adducts, which gives rise to antibodies, and these were also measured using ELISA. Data reported in Table 2 also show a highly significant increase of these antibodies in alcoholic patients \((P < 0.001)\). As a decrease in the antioxidant blood content of alcoholic patients has often been reported, plasma levels of vitamin E were assessed in the present work and were found to be decreased by 18% \((P < 0.05)\) (Table 2).

### Relationship between CYP2E1 and parameters of the oxidative stress

When the alcoholic population was divided into two groups according to the characteristics given in Table 3, one group with low CYP2E1 induction (CHZ-MR <0.69, mean ± 3 SD of the control population) and the other with high induction (CHZ-MR >0.69) parameters of oxidative stress, expressed as percentage of variation vs controls, were not significantly different between both groups, except for anti-HER adduct antibodies (Figure 1). As both groups differed very significantly by their CYP2E1 activity \((P < 0.0001)\) (Table 3), this suggests that CYP2E1, although contributing to the oxidative stress, is only one of the factors involved in the oxidation of lipids or proteins by contrast with its main role in the formation of HER adducts. Accordingly, no significant correlation was found between CYP2E1 activity and parameters of oxidative stress, except the anti-HER adducts \((r = 0.45, P < 0.001)\).

### DISCUSSION

Several mechanisms by which ethanol could promote oxidative stress have been suggested (Cederbaum, 1989) including increased generation of superoxide anion and ethanol-derived free radicals at the microsomal level, especially through the involvement of CYP2E1 (Nordmann, 1994). Therefore, we have attempted to define the contribution of CYP2E1 in the oxidative stress observed in alcoholic subjects hospitalized for voluntary detoxification treatment.

CYP2E1 activity can be estimated in humans using chlorozoxazone metabolism. Recent pharmacokinetic studies validated a single point chlorozoxazone measure at 2 h as an acceptable phenotypic index of the 6-hydroxychlorozoxazone formation clearance (Digler et al., 1997; Frye et al., 1998) and the selectivity of this probe for CYP2E1 in humans has recently been reassessed (Lucas et al., 1999). Regulation of CYP2E1 by ethanol may involve protein stabilization which appeared dependent upon blood-alcohol levels (BAL) in the rat and increased mRNA synthesis at high ethanol levels (Badger et al., 1993; Ronis et al., 1993; Roberts et al., 1994) or by long-term ethanol consumption (Takahashi et al., 1993). In our study, we observed for the first time a weak but significant correlation between CYP2E1 activity and the mean daily alcohol consumption, assessed by using a face-to-face interview, the CDT level (a biological marker of alcohol consumption) or BAL at admission to hospital. BAL might also regulate CYP2E1 activity in humans, but the poor correlation observed could be explained by the fact that level at admission does not necessarily reflect the mean daily BAL.

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### Table 2. Cytochrome P-450 2E1 (CYP2E1) activity and markers of oxidative stress

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>CYP2E1 activity (\mu\text{M})</th>
<th>Lipoperoxides (\mu\text{M})</th>
<th>Oxidized proteins (\text{nmol/mg})</th>
<th>Vitamin E (\mu\text{M})</th>
<th>Anti-HER Ab</th>
<th>Anti-MDA Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholics ((n = 40))</td>
<td>1.32 ± 0.097</td>
<td>3.28 ± 0.92</td>
<td>0.28 ± 0.06</td>
<td>17 ± 5.9</td>
<td>1.24 ± 0.31</td>
<td>1.68 ± 0.49</td>
</tr>
<tr>
<td>Controls ((n = 18))</td>
<td>0.30* ± 0.13</td>
<td>2.70 ± 0.70</td>
<td>0.23 ± 0.03</td>
<td>20.7 ± 4.6</td>
<td>1.01 ± 0.06</td>
<td>1.06 ± 0.29</td>
</tr>
<tr>
<td>Statistical significance ((P))</td>
<td>&lt;0.001 &lt;0.05</td>
<td>&lt;0.01 &lt;0.05</td>
<td>&lt;0.05 &lt;0.05</td>
<td>&lt;0.001 &lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data were compared using Student’s \(t\)-test. CYP2E1 activity was expressed as the 6-hydroxycoloroxazone/chloroxazone blood concentration ratio 2 h after intake of 500 mg of chloroxazone.

*Controls were from Lucas et al. (1995b). Anti-HER Ab (antibodies against hydroxyethyl radical adducts) and Anti-MDA Ab (antibodies against malondialdehyde adducts), are expressed as the ratio calculated for absorbance readings in an enzyme-linked immunosorbent assay for IgG against HER/bovine serum albumin (BSA) and MDA/BSA vs unmodified protein (BSA). Lipoperoxides, oxidized proteins and vitamin E levels were determined in plasma.

### Table 3. Biological characteristics of alcoholic patients with low and high CYP2E1 induction

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Age (years)</th>
<th>CYP2E1 activity (\mu\text{M})</th>
<th>Ethanol intake (\text{g/day})</th>
<th>Tobacco (\text{cig/day})</th>
<th>CDT (%)</th>
<th>PAL (\text{UI/l})</th>
<th>GGT (\text{UI/l})</th>
<th>ALT (\text{UI/l})</th>
<th>AST (\text{UI/l})</th>
<th>Bilirubin (\mu\text{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-induced</td>
<td>43</td>
<td>0.39 ± 0.16</td>
<td>149 ± 66</td>
<td>19 ± 9</td>
<td>7.8 ± 2.7</td>
<td>65 ± 25</td>
<td>124 ± 153</td>
<td>37 ± 37</td>
<td>48 ± 43</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>Alcoholics ((n = 14))</td>
<td>± 11</td>
<td>1.80*** ± 0.80</td>
<td>195* ± 76</td>
<td>26 ± 17</td>
<td>10.5* ± 4.4</td>
<td>74 ± 40</td>
<td>148 ± 201</td>
<td>43 ± 59</td>
<td>59 ± 18</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>High-induced</td>
<td>42</td>
<td>1.80*** ± 0.80</td>
<td>195* ± 76</td>
<td>26 ± 17</td>
<td>10.5* ± 4.4</td>
<td>74 ± 40</td>
<td>148 ± 201</td>
<td>43 ± 59</td>
<td>59 ± 18</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Alcoholics ((n = 26))</td>
<td>± 7</td>
<td>1.80*** ± 0.80</td>
<td>195* ± 76</td>
<td>26 ± 17</td>
<td>10.5* ± 4.4</td>
<td>74 ± 40</td>
<td>148 ± 201</td>
<td>43 ± 59</td>
<td>59 ± 18</td>
<td>18 ± 6</td>
</tr>
</tbody>
</table>

Values represent the means ± SD. \(*P < 0.05, ***P < 0.001\) (Student’s \(t\)-test). CYP2E1 activity was expressed as the 6-hydroxycoloroxazone/chloroxazone blood concentration ratio 2 h after intake of 500 mg chloroxazone.

CYP2E1, carboxydrate-deficient transferrin; PAL, alkaline phosphatase; GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; cig, cigarettes.
Because of the rapid turnover of the enzyme (2.5 days in humans, Lucas et al., 1995a), CYP2E1 activity was determined on the day following the entry, as it was necessary to wait for the elimination of ethanol from the blood. However, CYP2E1 activities have been shown to differ greatly between individuals, at the basal level as after ethanol intoxication (Lucas et al., 1995b). Known genetic polymorphism reported for 2E1 (Hayashi et al., 1991; Hirvonen et al., 1993) does not appear to be involved (Lucas et al., 1995a; Carriere et al., 1996), whereas environmental factors, such as physiological or hormonal status and diet, might be more determinant (Lucas et al., 1998). McCarver et al. (1998) recently reported a new genetic polymorphism in the regulatory sequences of CYP2E1 associated with an increased hydroxylation of CHZ in the presence of obesity or ethanol intake. The frequency of this polymorphism was estimated to be 7% in Caucasians.

Ethanol administration has been shown to induce an oxidative stress either by enhancing the production of oxygen reactive species and/or by decreasing the level of endogenous antioxidants. In our study, blood markers of oxidative stress, such as oxidized proteins and lipid peroxides, were increased by ~20% in alcoholic patients, whereas plasma concentrations of α-tocopherol, a marker of antioxidant defence, were depressed. These results are consistent with several previous reports showing increased lipoperoxidation and reduced plasma vitamin E levels in alcoholics entering a rehabilitation programme (Girre et al., 1990; Lecomte et al., 1994). Depletion in vitamin E might reflect the pro-oxidant action of alcohol in the liver and facilitate the development of lipid peroxidation in some patients, as supplementation of diet with vitamin E is known to reduce on-going in vivo lipid peroxidation and ethanol-induced liver damage (Bondy et al., 1996). However, increased lipid peroxidation and decreased vitamin E levels are far from being constant after chronic ethanol administration (Fernandez-Solà et al., 1998) and during the initial period, an adaptive enhancement in some elements of the antioxidant defence, such as an increase in glutathione peroxidase activity, may prevent the occurrence of oxidative stress and decrease in vitamin E. Tobacco smoking, which is very often associated with alcohol consumption, also produces free radicals and thus participates in the generation of oxidative stress (Khan et al., 1998).

In order to analyse the relationship between CYP2E1 activity and oxidative stress in alcoholics, blood parameters of oxidative stress were compared in patients with low and high induction of CYP2E1. Patients with low CYP2E1 induction drank significantly less alcohol and had lower CDT levels than those with high CYP2E1 induction, but did not differ significantly by their smoking habits. Although low-induced patients displayed lower mean values than high-induced patients for all parameters, no significant difference was observed except for autoantibodies raised against HER adducts. CYP2E1 has been shown to be the major target protein involved in HER adducts (Clot et al., 1996) and generation of 1-hydroxyethyl radicals from ethanol involves at least two pathways: one with ·OH radicals produced in a Fenton-type reaction from endogenously formed hydroperoxide, and the other is cytochrome P-450-mediated and apparently independent from ·OH (Albano et al., 1991; Reinke et al., 1997). Thus, CYP2E1 may play a key role in the formation of these adducts and this is evidenced in our study. By contrast, no significant difference was observed with anti-MDA adduct antibodies, which reflect MDA formation, or the other parameters measuring oxidative stress, namely oxidized proteins, lipid hydroperoxides and vitamin E.
During ethanol intoxication, pro-oxidant species are generated in hepatic tissue at various subcellular sites: in the endoplasmic reticulum by CYP2E1, in the cytosol by xanthine oxidase and in the mitochondria by the respiratory chain (Nordmann, 1994). In addition, phagocytes recruited and activated by tissue injury will also contribute to this production. Many reports show that various experimental conditions of CYP2E1 induction may lead to overt lipid peroxidation. For example, combined treatment with chronic intragastric ethanol feeding and unsaturated fat-rich diets results in dramatically increased CYP2E1 activity and associated lipid peroxidation which can be prevented by CYP2E1 inhibitors in rats (Morimoto et al., 1995). Due to its potential for free radical generation, CYP2E1 is thought to contribute to overall production of reactive oxygen species more significantly than do other enzymes. However, our study, which is the first to examine CYP2E1 activity in connection with oxidative stress in humans, does not show evident correlation between CYP2E1 induction and different parameters of oxidative stress. Our data thus suggest that CYP2E1 does not play a prominent role in the oxidative stress which occurs during ethanol intoxication, even if its participation is undoubted.

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