

Ageing is associated with increased expression but decreased activity of CYP2E1 in male Wistar rats

Valérie Wauthier, Véronique Schenten, Roger K. Verbeeck, Pedro Buc Calderon *

Unité de Pharmacocinétique, Métabolisme, Nutrition et Toxicologie (PMNT), Département des sciences pharmaceutiques, Université Catholique de Louvain, Avenue E. Mounier 73, 1200, Brussels, Belgium

Received 30 March 2006; accepted 13 June 2006

Abstract

The effect of ageing on CYP2E1 activity and its protein and mRNA contents was investigated in both adult (9 months) and senescent (24 months) male Wistar rats. The CYP2E1 activity (as measured by chlorzoxazone hydroxylation) was significantly decreased by 36% in senescent rats as compared to adult rats. However, this decrease of activity was not associated with a loss of protein content because the amount of both CYP2E1 protein and CYP2E1 mRNA did not decrease in senescent rats but rather increased, by 79% and 64% respectively, as compared to adult rats. Lipid peroxidation was increased significantly by 140% with ageing. The decrease in CYP2E1 activity could be explained by post-translational modification of CYP2E1 proteins, due to an increase in oxidative stress in senescent animals, leading to a loss of their functionality. However, no changes in the extent of protein carbonyls were observed in the adult versus senescent rats (16.2 ± 9.6 vs. 12.7 ± 7.3 nmol/mg prot) and the major proteasome activity remained unchanged. With regards to the increase of CYP2E1 expression, our results showed that the amount of hepatocyte nuclear factor 1 α mRNA, a transcription factor that positively regulates CYP2E1, was strongly increased (154%) in senescent rats. © 2006 Elsevier Inc. All rights reserved.

Keywords: CYP2E1; Ageing; Oxidative stress; HNF1 α

Introduction

Cytochrome P450s (CYPs) constitute a superfamily of heme-proteins that play an important role in the detoxification of numerous xenobiotics as well as endogenous compounds such as steroids, fatty acids, prostaglandins, and leukotrienes (Guengerich, 1990; Gonzalez, 1988; Nebert et al., 1991). In addition, they also play an important role in the maintenance of bile acid and cholesterol homeostasis (Chawla et al., 2000, 2001). The activity of these enzymes may be influenced by various genetic or non-genetic factors. Among the latter, ageing may affect these enzymes thus leading to alterations in the biotransformation of drugs and, consequently, their therapeutic efficacy and safety. The mechanisms underlying such effects are not totally elucidated.

To study the effect of ageing on P450 enzymes, we selected the ageing rat model as an experimental system and focused our

investigation on the CYP2E1 isoform. Indeed, different facts underline the important toxicological implication of this enzyme: CYP2E1 is constitutively expressed in several tissues; it shares high homology with the human isoform; it catalyses the oxidation of a wide variety of compounds including certain drugs, solvents and environmental pro-carcinogens (Tanaka et al., 2000; Fang et al., 1998); it is induced by ethanol (Clot et al., 1996; Fang et al., 1998) and it forms reactive oxygen species (Ekstrom and Ingelman-Sundberg, 1989; Goasduff and Cederbaum, 1999).

The regulation of P450 genes occurs at different levels via various mechanisms that are quite diverse and not well understood (Akiyama and Gonzalez, 2003). The analysis of promoter sequences contained in numerous genes that exhibit liver-specific expression reveals the presence of binding sites for liver-enriched transactivating factors, such as hepatocyte nuclear factor (HNF) 1, HNF3, HNF4, HNF6 or C/EBP α (Cereghini, 1996). In vitro transcription and transactivation studies have suggested that CYP2E1 is controlled in part by HNF1 α (Liu and Gonzalez, 1995; Ueno and Gonzalez, 1990).

* Corresponding author. Tel.: +32 2 7647366; fax: +32 2 7647359.

E-mail address: calderon@pmnt.ucl.ac.be (P.B. Calderon).

The aim of this work was to study the influence of ageing on CYP2E1 at the level of activity as well as its mRNA and protein content in both adult (9 months) and senescent (24 months) male Wistar rats. The oxidation of chlorzoxazone to 6-OH-chlorzoxazone was measured as an index of CYP2E1 enzyme activity (Kobayashi et al., 2003). The amount of both CYP2E1 proteins and mRNA was determined by immunoblotting and quantitative PCR, respectively. Due to the influence of oxidative stress in age-related processes, certain standard markers of an oxidant insult were measured; the levels of aldehydes (by both spectrophotometry and histochemistry), protein carbonyls (by both colorimetry and immunoblotting detection). The chymotrypsin-like proteasome activity was also recorded. Moreover, the activities of major antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHpx) and catalase (CAT) and the content of reduced glutathione (GSH) were also measured in both adult and senescent rats. Finally, since HNF1 α is involved in the expression of CYP2E1, we decided to investigate its potential role on the regulation of CYP2E1 by measuring its expression by RT-PCR.

Materials and methods

Animals

Animal care and experiments were performed according to the Biosafety and Ethical rules in application in Belgium as adopted by the Bioethical Committee of the Université Catholique de Louvain. Male Wistar rats 9 and 24 months of age were purchased from Harlan (Horst, The Netherlands) and housed in individual cages in a temperature- and light-controlled room (12 h/12 h dark/light cycle) during 1 week before their sacrifice. They received a standard diet (A03; UAR, Epinay sur Orge, France) and water ad libitum. The rats were killed under pentobarbital anesthesia (60 mg/kg i.p.). The liver was removed; one part was used to prepare microsomes, and the rest was frozen in liquid nitrogen for subsequent assessment of mRNA content and other parameters.

Chemicals

Chlorzoxazone (CZX), chlorzoxazolone, thiobarbituric acid, succinyl-leucyl-leucyl-valine-tyrosine-amino-4-methyl coumarin (Suc-LLVY-AMC), *N*-carboboxy-Leu-Leu-leucinal (MG132) and dinitrophenylhydrazine (DNPH) were purchased from Sigma Chemicals (St. Louis, MO, USA). 6-Hydroxy-chlorzoxazone was purchased from Ultrafine Chemicals (Manchester, UK). Pararosaniline, charcoal, potassium bisulphite and sodium bisulphite used for the preparation of Schiff's reagent were obtained from Merck (Darmstadt, Germany). Polyclonal antibodies against CYP2E1 were from Chemicon (Temecula, CA, USA). Rat CYP2E1 supersomes were from Gentest (Woburn, MA, USA). Primers for RT-PCR were synthesized at the Laboratoire d'Hématologie (Université catholique de Louvain, Brussels, Belgium). Solvents used were of HPLC grade and all other chemicals were of the purest quality available.

Preparation of liver microsomes

Hepatic microsomal fractions were prepared by differential centrifugation as described previously (Leclercq et al., 1997). Cytosolic fractions were used to measure the activity of the proteasome. The amount of protein was determined by the method of Lowry using bovine serum albumin as standard (Lowry et al., 1951). Total cytochrome P450 was determined in hepatic microsomes by changes in the absorbance at 450 nm of the reduced complex with CO as reported by Omura and Sato (1964).

Oxidative stress markers and antioxidant enzymes

Lipid peroxidation was estimated in frozen liver homogenate following the formation of malonyldialdehyde (MDA)-like substances, which react with thiobarbituric acid (TBA) thus forming a pink-colored complex at 535 nm. In addition, aldehydes were further histochemically evaluated by using a direct Schiff's reaction (Taper et al., 1988). The carbonyl groups in the protein side chains were either measured by colorimetric formation of DNPH (Levine et al., 1990) or derivatized to 2,4-dinitrophenylhydrazine (DNPH) by reaction with 2,4-dinitrophenylhydrazine using the Intergen OxyBlot kit according to the instructions of the manufacturer.

The activity of SOD was assessed by recording the reduction of nitro blue tetrazolium (McCord and Fridovich, 1969), CAT activity by using the TiSO₄ method (Baudhuin et al., 1964) and the GSHpx activity by following the NADPH oxidation (Wendel, 1981). The GSH content was determined after the formation of a fluorescent complex with *o*-phthalaldehyde (oPT) and measurement made at 345 nm excitation and 420 nm emission (Hissin and Hilf, 1976).

Proteasome assay

The chymotrypsin-like activity of the proteasome was measured as previously reported (Friguet et al., 1994) with some modifications. Briefly, the mixture containing 10 μ g of protein from the cytosolic fraction, 50 mM Tris-HCl pH 8, 0.5 mM EDTA was incubated with 50 μ M Suc-LLVY-AMC in a final volume of 100 μ l. The reaction was stopped by adding 100 μ l 100 mM sodium acetate pH 4. Fluorescence was determined by measuring the release of amino-methyl coumarin (AMC; excitation wavelength 365 nm, emission 450 nm). The concentration of liberated products was calculated using a standard curve of AMC ranging from 0 to 1.5 μ M. Chymotrypsin-like activity was determined as the difference between total activity and the remaining activity of the cytosol in the presence of 20 μ M proteasome inhibitor MG132.

Chlorzoxazone (CZX) metabolism

The rate of chlorzoxazone hydroxylation by rat liver microsomes was quantified by HPLC following the formation of its 6-hydroxy-derivative as previously reported by Leclercq et al. (1998). Briefly, 0.3 mg liver microsomal protein, 5 mM MgCl₂·6H₂O, 0.7 mM NADPH, 0.1 M phosphate buffer pH

Table 1
Gene-specific primers utilized and polymerase chain reaction (PCR) conditions

	Primers		PCR conditions	
	Forward	Reverse	No. of cycles	Annealing temperature (°C)
β-Actin	CTGACCGAGCGTGGCTACAG	GGTGCTAGGAGCCAGGGCAG	23	62
HNF1α	TTCTAAGCTGAGCCAGCTGCAGACG	GAAAGCAACGGGAGATCCTC	32	54

HNF1α: hepatocyte nuclear factor 1α.

7.4, 0.1 M KCl and 1 mM EDTA were incubated with different concentrations of chlorzoxazone ranging from 6 to 600 μM. After an incubation of 20 min at 37 °C the reaction was stopped by the addition of 200 μl ZnSO₄ (15% w/v). Chlorzoxazolone was added as internal standard and, after centrifugation, the supernatant was injected onto the HPLC system.

Western immunoblotting

Samples of 1.5 μg microsomal protein for CYP2E1 and 5 μg of derivatized cytosolic protein for the Oxyblot™ were loaded and proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% separating gel) followed by electroblot to a nitrocellulose membrane. Protein loading was assessed by staining the blots with Ponceau S. The membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS, pH 7.4) containing 5% w/v of powdered milk protein. Next, they were probed for 2 h with rabbit polyclonal antibodies against anti-rat CYP2E1 diluted at 1/2500 or with anti-DNP diluted at 1/150, at room temperature in TBS-Tween (0.1%). The membranes were washed and reprobed with the respective secondary antibodies coupled to horseradish peroxidase at room temperature. Immunodetection was performed using the ECL detection kit (Amersham, Little Chalfont, UK). The film was scanned and the density of the bands was calculated using the program Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Prior to the measurements of samples, linear conditions between protein concentrations and optical density were established. In addition, the band corresponding to CYP2E1 was checked by using CYP2E1 protein standards (rat CYP2E1 supersomes). The results are expressed as arbitrary optical density (O.D.) units.

RT-PCR

Total RNA was extracted from approximately 30 mg rat liver using an SV Total RNA Isolation kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cDNA was synthesized in a total volume of 20 μl reaction mixture containing 1 μg RNA in the RT reaction buffer (Invitrogen, Merelbeke, Belgium), 10 nmol/l dithiothreitol, 25 pg oligo(dT) primer, 0.5 mmol/l each deoxyribonucleoside triphosphate (dNTP), 200 units superscript II Rnase H⁻ Reverse Transcriptase (Invitrogen). Reactions were incubated for one cycle at 42 °C for 2 min and at 42 °C for 50 min, and were terminated by heating at 70 °C for 15 min and then chilled to 4 °C. The PCR reaction mixture consisted of PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 μM of

specific primers (Table 1) for HNF1α (Clotman et al., 2002), 2 μl cDNA, and 1 unit Taq DNA polymerase (Invitrogen). PCR reaction mixtures were incubated at 94 °C for 3 min followed by the respective number of cycles (Table 1) at 94 °C for 45 s, then for 30 s at their corresponding annealing temperatures (Table 1), and at 72 °C for 1 min 30 s. Afterwards, the extension step followed for 10 min at 72 °C. β-Actin mRNA was used as housekeeping gene to normalize the CYP mRNA content. The levels of mRNA are expressed as arbitrary units obtained by densitometry using the program Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) after normalization to β-actin mRNA levels.

Real-time PCR

The quantification of CYP2E1 mRNA in rat liver tissue was performed following a real-time RT-PCR method previously described (Haufrond et al., 2001). The only difference compared to the published method is an additional dilution step following reverse transcription and before the PCR reaction; cDNA was diluted 10 times for liver tissue. The commercial kit TaqMan® Gene Expression Assays (Applied Biosystems) was used according to the manufacturer's instructions. Results are expressed as a ratio of CYP2E1 cDNA copies with β-actin cDNA as a housekeeping gene.

Analysis of kinetic data

Kinetic data (V_{max} and K_m) were determined by a non-linear regression program (WinNonlin, SCI Software, Lexington, KY, USA) assuming single enzyme Michaelis–Menten kinetics.

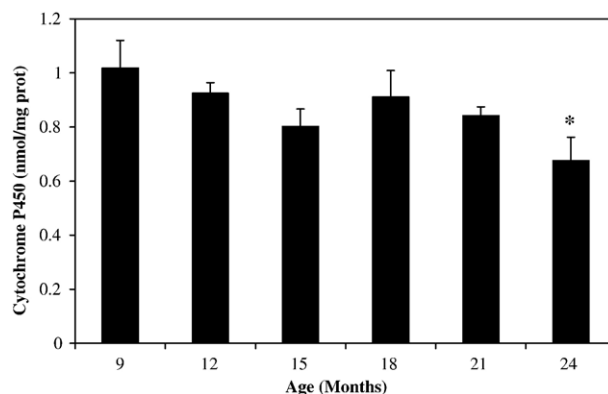


Fig. 1. The influence of ageing on the total cytochrome P450 content. Results are expressed as nmol/mg prot and represent the means±SEM of 3–6 rats. (*) ANOVA, $p < 0.05$ as compared to rats 9 months old.

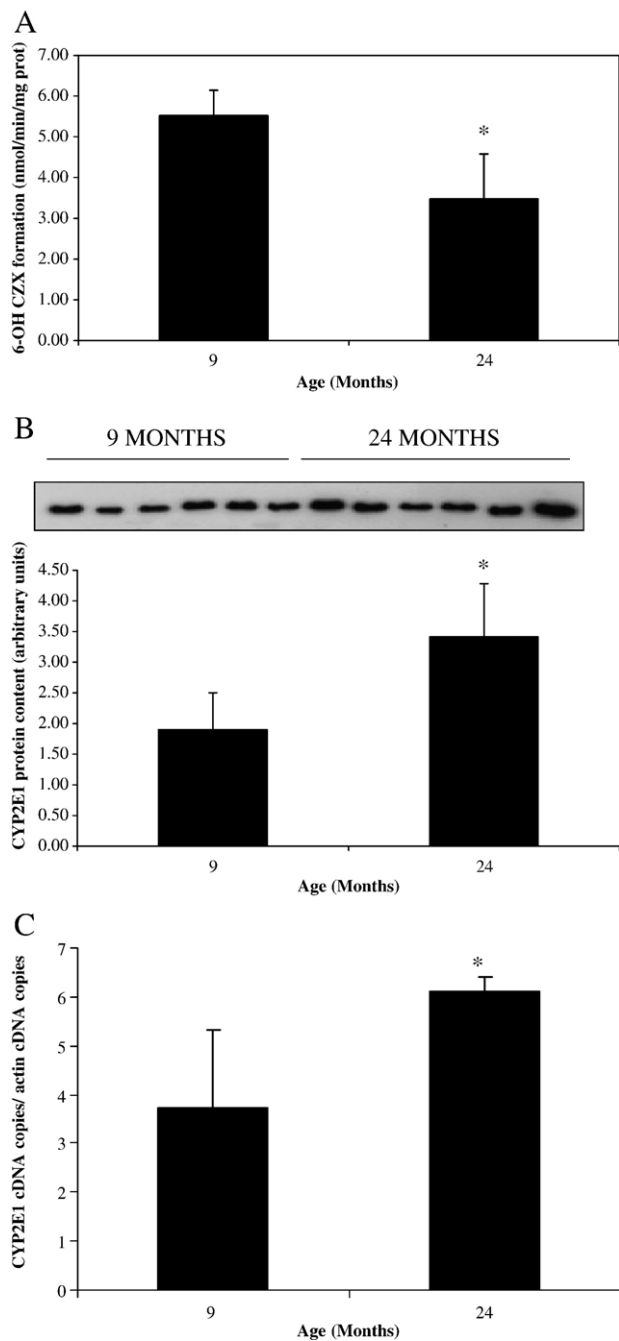


Fig. 2. The influence of ageing on CYP2E1 activity (A), on the amount of CYP2E1 protein (B), and on CYP2E1 mRNA level (C). (A) Chlorzoxazone oxidation (CYP2E1 activity) was measured by recording the formation of 6-hydroxy-chlorzoxazone (6-OH CZX) after 20 min incubation of rat liver microsomes at 37 °C. The results are expressed as V_{\max} (nmol/min/mg prot). (B) The amount of CYP2E1 proteins was determined by Western blots. Samples of 1.5 μ g liver microsomal liver protein per lane were loaded for rats 9 and 24 months of age. Values of CYP2E1 proteins are expressed as arbitrary O.D. units obtained by densitometry of the respective protein bands. The amount of protein loading in each lane was assessed by staining the blots with Ponceau S (data not shown). (C) The CYP2E1 mRNA content was assessed by quantitative PCR and values are expressed as the ratio of CYP2E1 cDNA copies to β -actin cDNA copies. The results represent the mean value \pm S.E.M. from 6 different animals. (*) $p < 0.05$ as compared to rats 9 months old.

Table 2
Antioxidant defences in rats during ageing

	9 months	24 months
SOD (U/mg prot)	12.4 \pm 3.5	10.4 \pm 2.9
CAT (U/mg prot)	0.9 \pm 0.1	0.9 \pm 0.2
GSHpx (U/mg prot)	0.26 \pm 0.04	0.29 \pm 0.08
GSH (nmol/mg prot)	40.3 \pm 8.3	34.6 \pm 3.5

The activity of the three antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and GSH peroxidase (GSHpx) was determined in liver homogenates as indicated under Materials and Methods. Total reduced glutathione (GSH) was determined after the formation of a fluorescent complex with *o*-phthalaldehyde and measurement at 345 nm excitation and 420 nm emission. Values represent the means \pm SEM of 6 rats.

Statistical analysis

Each group of adult and senescent rats was composed of at least six rats. The results presented in the figures correspond to the means \pm SEM. Data were analysed using either the one analysis of variance or Student's *t* test to determine the statistical significance among the different age groups. The level of significance was set at $P < 0.05$.

Results

In order to know the influence of ageing on a general index of drug metabolism, we determined the P450 content in male rats at different ages ranging from 9 to 24 months. Fig. 1 shows that the total cytochrome P450 content of liver microsomes from male Wistar rats remained relatively constant at about 0.8–1.0 nmol/mg protein between the ages of 9 to 21 months. A significant decrease of 35% was observed in rats of 24 months as compared to rats of 9 months. Indeed, the content of P450 decreased to 0.7 nmol/mg protein in 24 months old rats. Consequently, further experiments were carried out in at least 6 animals of either 9 months (adult) or 24 months (senescent rats).

Since in senescent rats, the total P450 content was decreased by 35%, we explored the effect of ageing on a specific P450 enzyme, i.e. CYP2E1. With regards to the oxidation of chlorzoxazone (CZX), a reaction mainly catalyzed by CYP2E1, the formation of 6-hydroxy-chlorzoxazone in liver microsomes was measured. The results obtained were expressed as V_{\max} (nmol 6-OH-CZX/min/mg protein). Fig. 2A shows that the oxidation of chlorzoxazone is significantly decreased (– 36%) in senescent

Table 3
Oxidative markers in rats during ageing

	9 months	24 months
TBARS (nmol/mg prot)	3.0 \pm 0.6	7.2 \pm 3.5 (*)
Protein carbonyl (nmol/mg prot)	16.2 \pm 9.6	12.2 \pm 7.3
Ch-T-like activity (nmol/min/mg protein)	31.7 \pm 7.0	36.8 \pm 17.5

Protein carbonyls were measured by colourimetric formation of dinitrophenyl-hydrazone. Thiobarbituric acid reactive substances (TBARS) were estimated following the formation of malondialdehyde (MDA), which reacts with thiobarbituric acid thus forming a pink-coloured complex at 535 nm. Chymotrypsin-like (Ch-T-like) activity was determined as the difference between total activity and the remaining activity of the cytosol in the presence of 20 μ M proteasome inhibitor MG132. Values represent means \pm SEM of 6 rats. (*) $p < 0.05$ relative to values of 9-month-old rats.

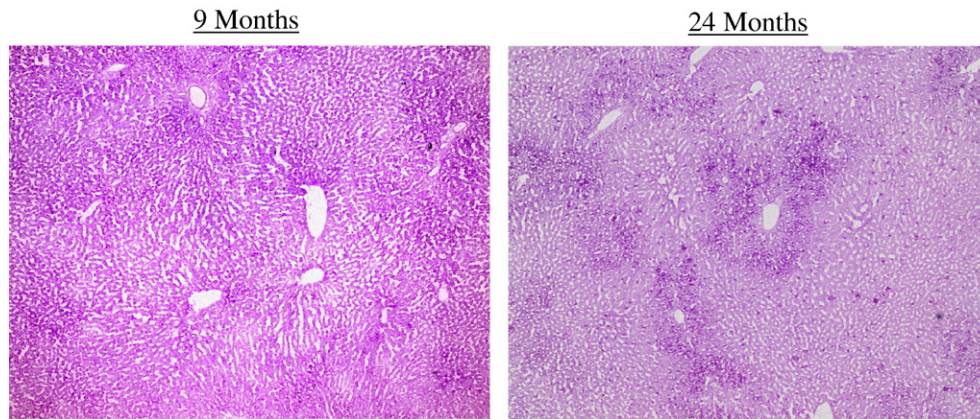


Fig. 3. The influence of ageing on the accumulation of aldehydes as shown by direct Schiff's reaction. Sections of hepatic tissues are shown for 9-month-old rats (a) and for 24-month-old rats (b). Both figures are representative for the hepatic tissues of the other animals in their age group.

rats as compared to adult rats. Indeed, in adult rats the V_{\max} was 5.5 ± 0.6 nmol/min/mg protein, whereas in senescent rats that value was of 3.5 ± 0.9 nmol/min/mg protein. It should be noted that the values of K_m were similar in both groups of rats (183.4 ± 31.1 and 157.5 ± 30.9 μM , for adult and senescent rats respectively) indicating that the affinity of the enzyme for the substrate is unaffected by ageing.

In order to see if this decrease of activity can be related to a decrease of CYP2E1 expression, we measured the CYP2E1 protein content and its mRNA level in both adult and senescent rats. Interestingly, while the microsomal oxidation of chlorzoxazone was decreased in senescent rats, the amount of the CYP2E1 protein, as measured by Western blot, was increased by approximately 79% (Fig. 2B). Indeed, in senescent rats, the amount of CYP2E1 protein was 3.4 ± 0.9 arbitrary O.D. units, whereas in adult rats it reached a value of only 1.9 ± 0.6 arbitrary O.D. units. This significant increase in the amount of CYP2E1 protein in senescent rats was associated with a 64% increase in the amount of CYP2E1 mRNA (as measured by quantitative PCR): in rats of 24 months the amount of CYP2E1 mRNA reached a value of 3.72 ± 1.62 , whereas in rats of 9 months this value was 6.10 ± 0.28 (Fig. 2C).

The impairment of enzyme activity in rats with high expression levels of CYP2E1 suggests that several processes occur during ageing. We looked first at some parameters related to oxidative stress and the antioxidant status. Table 2 shows the influence of ageing on the activity of the three major antioxidant

enzymes, namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHpx) and GSH as well. We observed that the antioxidant system was not impaired in senescent rats but rather it was maintained from 9 to 24 months.

Table 3 shows the influence of ageing on parameters usually associated to an oxidative stress: thiobarbituric acid reactive substances (TBARS), protein carbonyls and a proteasome-like activity. TBARS were significantly increased (140%) in senescent animals as compared to adult rats. This age-related increase of aldehydes was confirmed by the histochemical detection of isolated Schiff-positive foci in liver section. These positive foci, indicating the accumulation of protein-containing pigments, were denser and occurred more frequently in the livers of senescent rats (Fig. 3). Protein carbonyl content determined either by colorimetry or by immunodetection using oxyblot™ (Fig. 4) was similar in both adult and senescent rats. Regarding the chymotrypsin-like proteasome activity, no significant changes were observed during ageing.

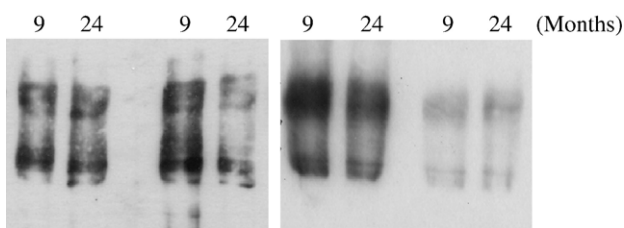


Fig. 4. Influence of ageing on the amount of protein carbonyls. Representative immunoblotting of cytosolic carbonylated proteins. The amount of protein loading in each lane was assessed by staining the blots with Ponceau S (data not shown).

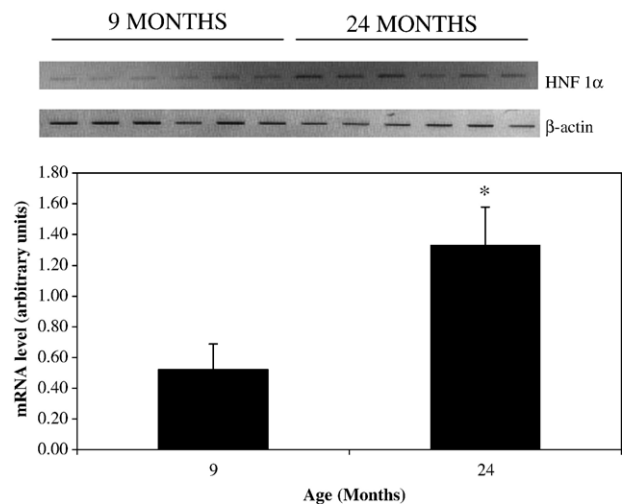


Fig. 5. The influence of ageing on the expression of hepatocyte nuclear factor 1 α (HNF-1 α) mRNA levels. The HNF-1 α mRNA contents were assessed by RT-PCR and values are expressed as arbitrary units obtained by densitometry and normalized to β -actin mRNA levels. The results represent the mean value \pm S.E. M. from 6 different animals. (*) $p < 0.05$ as compared to animals 9 months old.

Since HNF1 α is involved in the expression of CYP2E1 and in view of the increased CYP2E1 expression in senescent rats, we determined the level of HNF1 α . Fig. 5 shows that HNF1 α mRNAs is dramatically enhanced by 154% in senescent rats. Indeed, the amount of HNF1 α mRNA was 1.33 ± 0.25 arbitrary units in senescent rats. However, in adult rats, the amount of HNF1 α mRNA was only 0.52 ± 0.17 arbitrary units.

Discussion

CYP2E1 is a constitutively expressed isoform that has received much attention because of its importance in the activation of chemicals to cytotoxic products and its potential role in ethanol-induced liver toxicity. In agreement with previous reports showing age-related reductions in certain cytochrome P450 activities (Kamatani et al., 1985; Wauthier et al., 2004; Warrington et al., 2004a; Dhir and Shapiro, 2003), the results of the present study demonstrate a decreased CYP2E1 activity in senescent rats.

It may be argued that CYP2E1 is not the only P450 isoform metabolizing chlorzoxazone and that the decreased formation of 6-hydroxy-chlorzoxazone may be due to the decrease of other P450 isoforms. Previous inhibitory studies (using CYP2E1 inhibitors like 4-methylpyrazole, *trans*-1,2-dichloroethylene, diethyldithiocarbamate) have reported that chlorzoxazone oxidation is dependent to 80–85% on CYP2E1 (Mizuno et al., 2000; Tanaka, 2001; Poloyac et al., 2001, 2004). In accord with these studies, we observed a similar inhibitory effect of 4-methylpyrazole (10 μ M) in the formation of 6-OH chlorzoxazone (80%). Moreover, this inhibitory effect remains unchanged whatever the age of the rats from 3 to 24 months (data not shown). Recent studies have reported that CYP1A1 as well as CYP3A and 2C isoforms (to a lesser extent) are also involved in the metabolism of chlorzoxazone (Warrington et al., 2004a; Kobayashi et al., 2003). Although the estimation of their relative contributions is complicated to perform, they should not exceed 15–20%, as suggested by the inhibitory studies previously reported. Since CYP2E1 is the major isoform involved in the biotransformation of chlorzoxazone, and since CYP2E1 protein content is increased by 79% in senescent rats, the decrease of 37% in the formation of 6-hydroxy-chlorzoxazone due to other P450 isoforms seems unlikely.

On the other hand, the activity of a CYP isoform also requires adequate concentrations of NADPH, NADPH-reductase, flavo-protein and possibly cytochrome b5. Accordingly, it could be argued that the decreased metabolism of chlorzoxazone found in senescent rats can be due to an age-related decline in the availability of one or more of these cofactors as reported for NADPH cytochrome P450 reductase in liver of senescent rats (Warrington et al., 2004b). However, this possibility is unlikely because the oxidation of chlorzoxazone in the present study was assessed in rat liver microsomes containing a large excess of NADPH (and an NADPH-generating system). Consequently, under these conditions the limiting factor is not the NADPH reductase or the availability of cofactors but rather the CYP2E1 activity itself. In addition, if the availability of cofactors was the cause of a decreased metabolism during ageing, a general

decrease of P450 activities should be observed. However, this is not the case as seen for a CYP2C6 dependent activity that is unaffected by ageing (data not shown).

Interestingly, our results showed that ageing modifies CYP2E1 in two ways: by decreasing CYP2E1 activity without loss of protein content, suggesting post-translational modifications that diminish or inactivate its catalytic efficiency; and by an enhancement of CYP2E1 transcription, leading to an increased amount of CYP2E1 expression. Indeed, the regulation of CYP2E1 is unusually complicated and is exerted at different cellular levels: at transcription level, for example in fasting conditions (Johansson et al., 1990); by mRNA stabilization, such as in diabetes (Song et al., 1987); by change of translation efficiency, as done by the drug isoniazid (Park et al., 1993); or by enzyme stabilization, as observed with ethanol and acetone (Eliasson et al., 1990).

With regards to the decreased CYP2E1 activity, it is tempting to speculate that post-translational modifications occur by oxidative changes in CYP2E1 protein due to the increase of aldehyde formation (as revealed by both TBARS and Schiff assay). Indeed, proteins are recognized as major targets of oxidative modifications, and the cellular accumulation of oxidized proteins is a characteristic feature reported in many experimental ageing models (Levine and Stadtman, 2001; Bogdanov et al., 2000). Such oxidative modifications may involve protein cross-linking with aldehydes formed during lipid peroxidation. Since CYP2E1 generates high levels of reactive oxygen species (Tanaka et al., 2000; Clot et al., 1996; Ekstrom and Ingelman-Sundberg, 1989; Goasduff and Cederbaum, 1999), a particular susceptibility of CYP2E1 to be oxidized can be explained by the proximity of the source of oxidant generation. The impairment of protein function may also imply a change in shape or conformation (Levine and Stadtman, 2001). In addition, our results show that in senescent rats, the increased amount of CYP2E1 mRNA is accompanied by an increased amount of CYP2E1 proteins, without changes in the proteolytic activity. Therefore, we postulated that in senescent rats a progressive accumulation of modified CYP2E1 protein occurs leading to a loss of CYP2E1 enzyme activity.

In addition to the reduced CYP2E1 activity, our results showed that CYP2E1 expression is transcriptionally enhanced in senescent rats. In this regard, it should be noted that some cytochrome P450 genes, expressed in the liver of male rats, are transcriptionally regulated in a dual fashion by the sexually dimorphic secretion pattern of growth hormone (Shapiro et al., 1995). Thus, the increased CYP2E1 expression observed in senescent rats may occur by changes in the growth hormone (GH) secretion profile during ageing as reported by Dhir and Shapiro (2003). According to these authors, the GH secretion profile in old male rats becomes closer to the female secretion pattern leading to a feminization of cytochrome P450: for instance CYP2C12, a female-specific isoform, is expressed in old male rats, while CYP2C11, a male-specific isoform, is decreased. Since CYP2E1 expression is GH-dependent (Zhang et al., 2002; Chen et al., 1999) and CYP2E1 is more expressed in female than in male rats (Waxman et al., 1989), the feminization process observed during ageing could explain the increased expression of

CYP2E1 in senescent animals. In addition, the constitutive level of CYP2E1 is transcriptionally regulated by liver-enriched transcription factors. Among them, a role of HNF1 α in the positive regulation of CYP2E1 has been shown both by *in vitro* transcription and transactivation studies (Ueno and Gonzalez, 1990) and by studies using HNF1 α deficient mice, where a drastic decrease of CYP2E1 expression was observed (Cheung et al., 2003). Our results show a strong increase in the amount of HNF1 α mRNA in senescent male rats. Therefore, the increase of CYP2E1 expression in senescent rats may be due to the age-related increase of HNF1 α . The profile of GH secretion could modulate the expression of liver transcription factors. HNF6 and HNF3 β have been reported to be subject to sexual dimorphism (Lahuna et al., 1997). Thus, it may be possible that HNF1 α is also under the same type of regulation influencing in this way the CYP2E1 expression in senescent male rats.

In conclusion, in senescent male rats a loss of CYP2E1 activity was demonstrated in spite of an increased amount of CYP2E1 protein. This seemingly contradictory finding could be due to an increase with ageing of oxidative modifications leading to the formation of non-functional CYP2E1 protein. The increased amount of CYP2E1 protein and mRNA may be explained by the changes in the GH secretion profile occurring during ageing. The molecular mechanism underlying such an increase may imply that HNF1 α is upregulated during ageing thus inducing a transcriptional activity of CYP2E1 gene.

Acknowledgements

We thank Véronique Allaëys and Nicole Maton for their excellent technical assistance. The authors express their gratitude to Valérie Van Kerckhove and Vincent Haufroid (Unité TOXI, UCL) for their valuable contribution in the quantitative PCR determination of CYP2E1 mRNA.

References

- Akiyama, T.E., Gonzalez, F.J., 2003. Regulation of P450 genes by liver-enriched transcription factors and nuclear receptors. *Biochimica et Biophysica Acta* 1619, 223–234.
- Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P., deDuve, C., 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochemical Journal* 92, 179–188.
- Bogdanov, M., Brown, R.H., Matson, W., Smart, R., Hayden, D., O'Donnell, H., Flint Beal, M., Cudkovic, M., 2000. Increased oxidative damage to DNA in ALS patients. *Free Radical Biology & Medicine* 29, 652–658.
- Cereghini, S., 1996. Liver-enriched transcription factors and hepatocyte differentiation. *FASEB Journal* 10, 267–282.
- Chawla, A., Saez, E., Evans, R.M., 2000. Don't know much bile-ology. *Cell* 103, 1–4.
- Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. *Sciences* 294, 1866–1870.
- Chen, G.F., Ronis, M.J., Ingelman-Sundberg, M., Badger, T.M., 1999. Hormonal regulation of microsomal cytochrome P4502E1 and P450 reductase in rat liver and kidney. *Xenobiotica* 29, 437–451.
- Cheung, C., Akiyama, T.E., Kudo, G., Gonzalez, F.J., 2003. Hepatic expression of cytochrome P450s in hepatocyte nuclear factor 1-alpha (HNF1 α)-deficient mice. *Biochemical Pharmacology* 66, 2011–2020.
- Clot, P., Albano, E., Eliasson, E., Tabone, M., Arico, S., Israel, Y., Moncada, C., Ingelman-Sundberg, M., 1996. Cytochrome P4502E1 hydroxyethyl radical adducts as the major antigen in antibody formation among alcoholics. *Gastroenterology* 111, 206–216.
- Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2002. The oncut transcription factor HNF6 is required for normal development of the biliary tract. *Development* 129, 1819–1828.
- Dhir, R.N., Shapiro, B.H., 2003. Interpulse growth hormone secretion in the episodic plasma profile causes the sex reversal of cytochrome P450s in senescent male rats. *Proceedings of the National Academy of Sciences of the United States of America* 100, 15224–15228.
- Ekstrom, G., Ingelman-Sundberg, M., 1989. Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). *Biochemical Pharmacology* 38, 1313–1319.
- Eliasson, E., Johansson, I., Ingelman-Sundberg, M., 1990. Substrate-, hormone-, and cAMP-regulated cytochrome P450 degradation. *Proceedings of the National Academy of Sciences of the United States of America* 87, 3225–3329.
- Fang, C., Lindros, K.O., Badger, T.M., Ronis, M.J., Ingelman-Sundberg, M., 1998. Zonated expression of cytokines in rat liver: effect of chronic ethanol and the cytochrome P450 2E1 inhibitor, chlormethiazole. *Hepatology* 27, 1304–1310.
- Friguet, B., Stadtman, E.R., Szweda, L.I., 1994. Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. *Journal of Biological Chemistry* 269, 21639–21643.
- Goasduff, T., Cederbaum, A.I., 1999. NADPH-dependent microsomal electron transfer increases degradation of CYP2E1 by the proteasome complex: role of reactive oxygen species. *Archives of Biochemistry and Biophysics* 370, 258–270.
- Gonzalez, F.J., 1988. The molecular biology of cytochrome P450s. *Pharmacological Reviews* 40, 243–288.
- Guengerich, F.P., 1990. Enzymatic oxidation of xenobiotic chemicals. *Critical Reviews in Biochemistry and Molecular Biology* 25, 97–153.
- Haufroid, V., Toubeau, F., Clippe, A., Buyschaert, M., Gala, J.-L., Lison, D., 2001. Real-time quantification of cytochrome P4502E1 mRNA in human peripheral blood lymphocytes by reverse transcription-PCR: Method and practical application. *Clinical Chemistry* 47, 1126–1129.
- Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry* 74, 214–226.
- Johansson, I., Lindros, K.O., Eriksson, H., Ingelman-Sundberg, M., 1990. Transcriptional control of CYP2E1 in the perivenous liver region and during starvation. *Biochemical and Biophysical Research Communications* 173, 331–338.
- Kamatani, T., Maeda, K., Shimada, M., Kitani, K., Nagai, T., Kato, R., 1985. Age-related alteration in the activities of drug-metabolizing enzymes and contents of sex-specific forms of cytochrome P-450 in liver microsomes from male and female rats. *Journal of Pharmacology and Experimental Therapeutics* 233, 222–228.
- Kobayashi, K., Urashima, K., Shimada, N., Chiba, K., 2003. Selectivities of human cytochrome P450 inhibitors toward rat P450 isoforms: study with cDNA-expressed systems of the rat. *Drug Metabolism and Disposition* 31, 833–836.
- Lahuna, O., Fernandez, L., Karlsson, H., Maiter, D., Lemaigre, F.P., Rousseau, G.G., Gustafsson, J.-A., Mode, A., 1997. Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone. *Proceedings of the National Academy of Sciences of the United States of America* 94, 12309–12313.
- Leclercq, I., Saliez, A., Wallemacq, P.E., Horsmans, Y., Lambotte, L., 1997. The monoethylglycinexylidide test does not correctly evaluate lidocaine metabolism after ischemic liver injury in the rat. *Hepatology* 26, 1182–1188.
- Leclercq, I., Horsmans, Y., Desager, J.P., 1998. Estimation of chlorzoxazone hydroxylase activity in liver microsomes and of the plasma pharmacokinetics of chlorzoxazone by the same high-performance liquid chromatographic method. *Journal of Chromatography A* 828, 291–296.
- Levine, R.L., Stadtman, E.R., 2001. Oxidative modification of proteins during aging. *Experimental Gerontology* 36, 1495–1502.

- Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* 186, 464–478.
- Liu, S.Y., Gonzalez, F.J., 1995. Role of the liver-enriched transcription factor HNF-1 alpha in expression of the CYP2E1 gene. *DNA and Cell Biology* 14, 285–293.
- Lowry, O.H., Rosebrough, N.J., Farr, L., Randall, R., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265–275.
- McCord, J., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry* 244, 6049–6055.
- Mizuno, D., Tanaka, E., Tanno, K., Misawa, S., 2000. Chlorzoxazone: a probe drug whose metabolism can be used to monitor toluene exposure in rats. *Archives of Toxicology* 74, 139–144.
- Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., et al., 1991. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA and Cell Biology* 10, 1–14.
- Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. II Solubilization, purification, and properties. *Journal of Biological Chemistry* 239, 2379–2385.
- Park, K.S., Sohn, D.H., Veech, R.L., Song, B.J., 1993. Translational activation of ethanol-inducible cytochrome P450 (CYP2E1) by isoniazid. *European Journal of Pharmacology* 248, 7–14.
- Poloyac, S.M., Perez, A., Scheff, S., Blouin, R.A., 2001. Tissue-specific alterations in the 6-hydroxylation of chlorzoxazone following traumatic brain injury in the rat. *Drug Metabolism and Disposition* 29, 296–298.
- Poloyac, S.M., Tortorici, M.A., Przychodzin, D.I., Reynolds, R.B., Xie, W., Frye, R.F., Zemaitis, M.A., 2004. The effect of isoniazid on CYP2E1- and CYP4A-mediated hydroxylation of arachidonic acid in the rat liver and kidney. *Drug Metabolism and Disposition* 32, 727–733.
- Shapiro, B.H., Agrawal, A.K., Pampori, N.A., 1995. Gender differences in drug metabolism regulated by growth hormone. *International Journal of Biochemistry and Cell Biology* 27, 9–20.
- Song, B.J., Matsunaga, T., Hardwick, J.P., Park, S.S., Veech, R.L., Yang, C.S., Gelboin, H.V., Gonzalez, F.J., 1987. Stabilization of cytochrome P450j messenger ribonucleic acid in the diabetic rat. *Molecular Endocrinology* 1, 542–547.
- Tanaka, E., 2001. Chlorzoxazone: a probe drug the metabolism of which can be used to monitor one-point blood sampling in the carbon tetrachloride-intoxicated rat. *Human and Experimental Toxicology* 20, 381–385.
- Tanaka, E., Terada, M., Misawa, S., 2000. Cytochrome P450 2E1: its clinical and toxicological role. *Journal of Clinical and Pharmaceutical Therapeutics* 25, 165–175.
- Taper, H.S., Somer, M.P., Lans, M., de Gerlache, J., Roberfroid, M., 1988. Histochemical detection of the in vivo produced cellular aldehydes by means of direct Schiff's reaction in CCl4 intoxicated rat liver. *Archives of Toxicology* 61, 406–410.
- Ueno, T., Gonzalez, F.J., 1990. Transcriptional control of the rat hepatic CYP2E1 gene. *Molecular & Cell Biology* 10, 4495–4505.
- Warrington, J.S., Court, M.H., Greenblatt, D.J., von Moltke, L.L., 2004a. Phenacetin and chlorzoxazone biotransformation in aging male Fisher 344 rats. *Journal of Pharmaceutical and Pharmacology* 56, 819–825.
- Warrington, J.S., Greenblatt, D.J., von Moltke, L.L., 2004b. Age-related differences in CYP3A expression and activity in the rat liver, intestine, and kidney. *Journal of Pharmacology and Experimental Therapeutics* 309, 720–729.
- Wauthier, V., Verbeeck, R.K., Buc Calderon, P., 2004. Age-related changes in the protein and mRNA levels of CYP2E1 and CYP3A isoforms as well as in their hepatic activities in Wistar rats. What role for oxidative stress? *Archives of Toxicology* 78, 131–138.
- Waxman, D.J., Morrissey, J.J., LeBlanc, G.A., 1989. Female-predominant rat hepatic P-450 forms j (IIE1) and 3 (IIA1) are under hormonal regulatory controls distinct from those of the sex-specific P-450 forms. *Endocrinology* 124, 2954–2966.
- Wendel, A., 1981. Glutathione peroxidase. *Methods in Enzymology* 77, 325–333.
- Zhang, S., Cawley, G.F., Eyer, C.S., Backes, W.L., 2002. Altered ethylbenzene-mediated hepatic CYP2E1 expression in growth hormone-deficient dwarf rats. *Toxicology and Applied Pharmacology* 179, 74–82.