Archives of Toxicology

10.1007/s00204-003-0526-z

Molecular Toxicology

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?

Valérie Wauthier[1], Roger K. Verbeeck[1] and Pedro Buc Calderon[1] Contact Information

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

Contact Information **Pedro Buc Calderon**
Email: calderon@pmnt.ucl.ac.be
Phone: +32-2-7647366
Fax: +32-2-7647359

Received: 27 May 2003 **Accepted:** 18 September 2003 **Published online:** 5 November 2003

Abstract Drug biotransformation and its therapeutic effect may be modified during ageing. Among different causative factors of ageing, the impairment of normal cellular functions by free radicals has been evoked as playing a critical role. The effect of age on the expression and activity of CYP2E1 and CYP3A was investigated in male Wistar rats of 3, 8, 11 and 18 months old. The total cytochrome P450 as well as the expression and the activity (midazolam oxidation) of CYP3A isoforms did not change until 18 months of age. Chlorzoxazone hydroxylation (CYP2E1 activity) increased from 3 to 8 months, remained constant between 8 and 11 months and then progressively decreased until 18 months. Interestingly, CYP2E1 microsomal protein followed the same enzyme activity profile from 3 to 8 months, but remained constant thereafter. The level of CYP2E1 mRNA did not change over the whole period. While the amount of proteins did not change after 8 months, their functionality may be affected by oxidative stress (increase in thiobarbituric acid reactive substances,

decrease in reduced glutathione level). However, no changes in carbonyl protein content were observed. The decrease in CYP2E1 activity in rats after 11 months is most probably due to post-translational modifications of CYP2E1 proteins. Indeed, it may be correlated with an accumulation of oxidative damage. Since no change was observed in CYP3A activity or in their protein and mRNA content, it seems that such isoforms should be less affected by oxidative stress.

Keywords Cytochrome P450 - Ageing - Oxidative stress - Proteasome

---

Introduction

Elderly people are an increasing part of the population. In the US for instance, persons over 65 years currently represent 15% of the population but by the year 2030 this will reach 20%. This age group consumes 33% of all prescription drugs and the cost of health care for the elderly is approaching 50% of the US healthcare budget (Schmucker *2001*). Since the elderly are the most medicated segment of society they are presumed to be more vulnerable to drug toxicity and side-effects. Indeed, 10% of all elderly admissions is related to adverse drug reactions. Changes in the pharmacodynamic and pharmacokinetic profiles of many compounds as well as other factors such as multiple medications, diseases and lack of compliance can explain such adverse reactions. Although the effect of ageing on pharmacokinetics has been extensively studied (Dodds *1995*; Tanira et al. *1997*; Espínola et al. *1999*; Balducci *2000*; Smorenburg et al. *2003*) its effect on drug metabolism still remains unresolved and, to some extent, controversial. A number of human studies have demonstrated an age-related decline in the clearance of drugs undergoing biotransformation by cytochrome P450 (CYP) enzymes, but conflicting results are reported when using human liver microsomes. Indeed, total hepatic CYP content as well as some CYP activities are either decreased (George et al. *1995*; Sotaniemi et al. *1997*) or remain unchanged (Hunt et al. *1990*; Schmucker et al. *1990*). It must be underlined that both in vivo and in vitro human studies may be complicated by epigenetic factors such as disease, medications, diet and tobacco or alcohol use, as well as genetic factors (Warrington et al. *2000*). Although the use of animal models can attenuate some of this variability, conflicting results have also been observed using rat liver microsomes. For instance, hepatic CYP levels have been reported to be either decreased (Schmucker and Wang *1981*; Rikans and Notley *1982*; Kamataki et al. *1985*; Rikans *1989*) or to remain unchanged (Birnbaum *1980*; Imaoka et al. *1991*) as a function of age. A major issue for such inconsistent results has been the use of non-specific CYP substrates and the measurement of single parameters, namely either enzyme activities or protein expression.

The aim of this work was to study the influence of ageing on the intrinsic

capacity of rat liver CYP enzymes to metabolize xenobiotics. CYP3A and CYP2E1 were selected as a model of phase I enzymes and they were studied with specific substrates in male rats at different stages of ageing, ranging from 3 to 18 months. The enzymatic activity of CYP3A was determined by measuring the formation of agr-hydroxy-midazolam and 4-hydroxy-midazolam, the main metabolites of midazolam, while CYP2E1 activity was measured by the formation of 6-hydroxy-chlorzoxazone, the main metabolite of chlorzoxazone (Kobayashi et al. *2002*). Since the enzymatic activity is mostly dependent on protein content, particular attention was given to the amount of apoprotein and its mRNA transcription level, which were assessed by Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. Whereas CYP3A isoforms are responsible for the biotransformation of many drugs (Guengerich *1999*), CYP2E1 is a constitutive expressed isoform that metabolizes a variety of structurally different chemicals, in particular small hydrophobic molecules that can be activated to cytotoxic and carcinogenic compounds (Ronis et al. *1996*). Moreover, CYP2E1 has the ability to convert dioxygen into reactive oxygen radicals (Ekström and Ingelman-Sundberg *1989*; Goasduff and Cederbaum *1999*), which suggests important toxicological implications for these enzymes. Therefore, it was hypothesised that putative changes in the activity of CYP enzymes might be closely associated with the changes in some markers of oxidative stress. Indeed, oxidative stress has been extensively studied in the etiology and as aggravating factor of ageing (Finkel and Holbrook *2000*; Stadtman *2002*; Szweda et al. *2002*). Reduced glutathione (GSH), the formation of carbonyl proteins and the extent of lipid peroxidation (thiobarbituric acid reactive substances, TBARS, and histochemical aldehyde detection) were utilized as markers of oxidative stress. To evaluate the capacity to degrade altered proteins, the chymotripsin-like activity of the proteasome was measured. A morphological study was also performed and liver histological sections were analysed by haematoxylin/eosin, periodic acid-Schiff (PAS) and Brachet staining under light microscopy.

Materials and methods

Animals

Male Wistar rats of 3 months of age were purchased from Iffa-Credo (Brussels, Belgium) and housed in individual cages in a temperature- and light-controlled room (12 h/12 h dark/light cycle). They received standard diet (A03; UAR, Epinay sur Orge, France) and water ad libitum. The rats were killed under pentobarbital anaesthesia (60 mg/kg i.p.) at the following ages: 3, 8, 11 or 18 months. The liver was removed; one part was used to prepare microsomes, another for the preparation of histological sections and the rest of the liver was

frozen in liquid nitrogen for subsequent assessment of mRNA content and oxidative stress markers.

Chemicals

Midazolam (Dormicum) and flunitrazepam were purchased from Roche (Grenzach-Wylhen, Germany). agr-Hydroxy-midazolam and 4-hydroxy-midazolam were provided by Hoffmann-La Roche (Basel, Switzerland). Chlorzoxazone, chlorzoxazolone, thiobarbituric acid, succinyl-leucyl-leucyl-valine-tyrosine-amino-4-methyl coumarin (Suc-LLVY-AMC), *N*-carbobenzoxy-Leu-Leu-leucinal (MG132) and dinitrophenylhydrazone (DNPH) were purchased from Sigma Chemicals (St. Louis, MO, USA). 6-Hydroxychlorzoxazone was purchased from Ultrafine Chemicals (Manchester, UK). Pararosaniline, charcoal, potassium bisulphite and sodium bisulphite used for the Shiffrsquos reagent preparation were obtained from Merck (Darmstadt, Germany). Rabbit polyclonal antibodies against CYP3A1 and CYP3A2 were from Chemicon (Temecula, CA, USA). Primers for RT-PCR were synthesized at the Laboratoire drsquoHématologie (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 by Leclerq 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*).

Cytochrome P450

Total cytochrome P450 was determined by changes in the absorbance at 450 nm of the reduced complex with CO as reported by Omura and Sato (*1964*).

Morphology of liver sections

Histological sections obtained from liver specimens were fixed in Carnoyrsquos fixative and embedded. The general morphology was observed after a haematoxylin/eosin colouration. Glycogen stores were visualised after PAS staining and nucleic acid was coloured by Brachet reagent.

Oxidative stress markers

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-coloured complex at 535 nm. In addition, aldehydes were further evaluated by using a direct Schiffrsquos reaction (Taper et al. *1988*). Protein carbonyls were measured by colourimetric formation of dinitrophenylhydrazone (Levine et al. *1990*). Finally, the amount of GSH 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 chymotripsin-like activity of the proteasome was measured as previously reported (Friguet et al. *1994*) with some modifications. Briefly, the mixture containing 10 µg protein from the cytosolic fraction, 50 mM Tris-HCl pH 8, 0.5 mM EDTA was incubated with 50 µM Suc-LLVY-AMC in a final volume of 100 µl. The reaction was stopped by adding 100 µl 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 µM. Chymotripsin-like activity was determined as the difference between total activity and the remaining activity of the cytosol in the presence of 20 µM proteasome inhibitor MG132.

Midazolam metabolism

The rate of midazolam hydroxylation by rat liver microsomes was quantified by measuring the formation of agr-hydroxy-midazolam and 4-hydroxy-midazolam (Eeckhoudt et al. *1998*). Briefly, 0.6 mg liver microsomal protein, 0.1 M phosphate buffer pH 7.4, 0.1 M KCl, 1 mM EDTA, 6 mM $MgCl_2.6H_2O$, 0.3 U isocitric dehydrogenase and 10 mM isocitric acid were pre-incubated at 37°C for 5 min with different concentrations of midazolam ranging from 1 to 100 µM. The incubation was started by the addition of a solution of 0.2 µM NADPH, 0.2 µM NADP. After 16 min, the reaction was terminated by 40 µl 1 *N* NaOH and was placed on ice. Flunitrazepam was added as internal standard. Midazolam and its metabolites were extracted with 4.5 ml cyclohexane–ether mixture (31:69). After mixing and centrifuging for 10 min at 2000 *g*, the organic layer was evaporated and conserved at 20°C. The residue was dissolved in water–acetonitrile (95:5) and injected on the high-performance liquid chromatography (HPLC) capillary column (150 mm×0.8 mm I.D.; LC Packings, Amsterdam, The Netherlands).

Chorzoxazone 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 Leclerq et al. (*1998*). Briefly, 0.3 mg liver microsomal protein, 5 mM $MgCl_2.6H_2O$, 0.7 µM NADPH, 0.1 M phosphate buffer pH 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 $ZnSO_4$ (15% w/v). Chlorzoxazolone was added as internal standard. After centrifugation, the supernatant was analysed by HPLC.

Western immunoblotting

Samples of 1.5 µg microsomal proteins for CYP3A2 and CYP2E1 and 3 µg for CYP3A1 were loaded alongside a known concentration (97.5 ng for CYP3A1, 360 ng for CYP3A2 and 375 ng for CYP2E1) of corresponding supersomes (Gentest, Woburn, MA, USA) and a molecular weight marker (NEN, Boston, MA, USA). The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% separating gel) followed by electroblot to a nitrocellulose membrane. 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 polyclonal rabbit anti-rat antibodies (1/16000 for CYP3A1, 1/8000 for CYP3A2 and 1/2500 for CYP2E1) at 25°C in a fresh solution of powdered milk (1% w/v) in TBS buffer. The membranes were washed and reprobed with goat anti-rabbit antibody coupled to horseradish peroxidase (1/10000) 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).

RT-PCR

Total RNA was extracted from approximately 30 mg rat liver using a SV Total RNA Isolation kit (Promega, Madison, WI, USA) according to the manufacturerrsquos 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 consisted of PCR buffer (200 Mm Tris-HCl, 500 mM KCl, pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of specific primers for CYP3A2 and CYP2E1 or primers for CYP3A1/3A23 (Table 1), 2 µl cDNA, and 1 unit Taq DNA polymerase (Invitrogen). PCR reactions 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 72°C for 1 min 30 s. Afterwards, the extension step was followed for 10 min at 72°C. beta-Actin mRNA was used as housekeeping gene to normalize the CYP mRNA content. PCR products were stored at 4°C and quantified using Picogreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands).

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

	Primers		PCR conditions	
	Forward	Reverse	No. of cycles	Annealing temperature (°C)
CYP3A1/3A23	GATGTTGAAATCAATGGTGTGT	TTCAGAGGTATCTGTTGTTTCC	23	55
CYP3A2	AGTAGTGACGATTCCAACATAT	TCAGAGGTATCTGTGTTT	22	54
CYP2E1	CTCCTCGTCATATCCATCTG	GCAGCCAATTCAGAAATGTGG	22	60
beta-Actin	CTGACCGAGCGTGGCTACAG	GGTGCTAGGAGCCAGGGCAG	23	61.8

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.

Statistical analysis

Each group was composed of at least four rats. The results presented in the figures correspond to the means ±SEM. Data were analysed using one-way analysis of variance to determine the statistical significance among the four age groups. The level of significance was set at $P<0.05$.

Results

Metabolic studies

The total cytochrome P450 content of liver microsomes from male Wistar rats did not change as a function of age but remained fairly constant at about 0.97±0.08 nmol/mg protein.

Figure 1 shows that ageing is influencing the CYP2E1 activity (Fig. 1a). The oxidation rate of chlorzoxazone is increased by 1.8-fold from 3 to 8 months of

age (3.15±0.24 to 5.55±0.43 nmol/min per mg protein) and then a progressive decrease was observed, reaching 3.00±0.26 nmol/min per mg protein at 18 months. It must be noted that K_m was not modified in rats from 3 to 18 months of age. A similar profile to that for the oxidation of chlorzoxazone was observed for the amount of CYP2E1 protein (an increase of 2.2-fold from 3 to 8 months) but at 18 months the amount of protein was similar to that of rats of 11 months (Fig. 1b,c). These results suggest that loss of enzyme activity is neither due to a change in protein turnover nor to impairment in the translation process. Although a decrease of about 20% was observed compared with that in rats of 8 months, this decrease was not statistically significant. The transcription level of CYP2E1 mRNA remained unchanged over the entire age range studied (Fig. 1d).

MediaObjects/s00204-003-0526-zfhb1.jpg

Fig. 1a–d Effect of aging on CYP2E1-mediated chlorzoxazone oxidation (**a**), amount of CYP2E1 proteins (**b,c**) and CYP2E1 mRNA levels (**d**). **a** Chlorzoxazone oxidation was quantified by recording (by HPLC) the formation of 6-hydroxy-chlorzoxazone after 20 min incubation of rat liver microsomes at 37°C. The values are expressed as V_{max} (nmol metabolite produced/min per mg protein). **P*<0.001 relative to animals 3 months of age; ***P*<0.001 relative to animals 8 months old. **b,c** The content of CYP2E1 protein was assessed by immunoblotting of CYP2E1 in the different age groups. In the representative Western blot showing CYP2E1 detection with polyclonal rabbit anti-rat CYP2E1 antibody (**b**), 1.5 µg liver microsomal protein per lane was loaded for rats of 3 (lane *1*), 8 (lane *2*), 11 (lane *3*) and 18 months of age (lane *4*). Values of CYP2E1 protein (**c**) are expressed as arbitrary units obtained after densitometry of the respective electrophoretic bands. **P*<0.001 relative to animals 3 months old. **d** The levels of CYP2E1 mRNA transcription were evaluated by RT-PCR and values are expressed arbitrary units obtained by the Picogreen method. In all bargraphs, values represent means ±SEM of at least four rats

---

The metabolism of midazolam, on the contrary, did not show any significant difference between the four age groups of rats (Fig. 2). Indeed, the mean values of V_{max} for both agr-hydroxy-midazolam and 4-hydroxy-midazolam formation were 0.42±0.10 and 0.99±0.27 nmol/min per mg protein, respectively (Fig. 2a). Moreover, the regioselectivity of midazolam oxidation was also retained since 4-hydroxy-midazolam was formed to a higher extent than agr-hydroxy-midazolam in all age groups. Figure 2b,c shows that no changes were observed in the amount of microsomal protein CYP3A1 and CYP3A2 or whatever CYP3A isoforms that the commercial antibodies can recognize (i.e. CYP3A9 or CYP3A18). Regarding mRNA levels, both CYP3A1/3A23 and CYP3A2 were unchanged (data not shown).

MediaObjects/s00204-003-0526-zfhb2.jpg

Fig. 2a–c Effect of aging on CYP3A-mediated midazolam oxidation (**a**), and on the amount of CYP3A proteins (**b,c**). **a** Midazolam oxidation was quantified by recording (by HPLC) the formation of 4-hydroxy-midazolzm (4-OH MDZ) and agr-hydroxy midazolam (agr-OH-MDZ) after 16-min incubation of rat liver microsomes at 37°C. The values are expressed as V_{max} (nmol metabolite produced/min per mg protein). **b,c** The content of CYP3A1 and CYP3A2 protein was assessed by immunoblotting. In representative Western blots showing CYP3A1 and CYP3A2 detection by polyclonal rabbit anti-rat CYP3A1 or polyclonal anti-rat CYP3A2 antibodies (**b**), 3 µg microsomal protein for CYP3A1 and 1.5 µg for CYP3A2 was loaded per lane for rats of 3 (lane *2*), 8 (lane *3*), 11 (lane *4*) and 18 months of age (lane *5*); results are also shown for 97.5 ng or 360 ng of protein of supersomes CYP3A1 or CYP3A2 (lane *1*). Values for CYP3A1 or CYP3A2 proteins are expressed as arbitrary units obtained after densitometry (**c**) of the respective electrophoretic bands. In all bargraphs, values represent means ±SEM of at least four rats

---

Proteolysis and oxidative stress studies

The proteasome chymotrypsin-like (CT-L) activity was measured in the cytosolic fraction of liver from all the different age groups of rats. The CT-L activity did not change as a function of age. It remained constant at about 87.3±8.6 nM/µg protein.

Figure 3 shows the influence of ageing on some oxidative stress markers, namely the amount of reduced GSH (Fig. 3a), the levels of protein carbonyls (Fig. 3b), and the aldehyde formation as detected by TBARS (Fig. 3c).

MediaObjects/s00204-003-0526-zfhb3.jpg

Fig. 3a–c Oxidative stress markers as a function of ageing. **a** Total reduced glutathione (GSH) was determined in liver homogenates after the formation of a fluorescent complex with *o*-phtalaldehyde and measurement at 345 nm excitation and 420 nm emission. Values are expressed in nmol GSH/mg protein. *P<0.05 relative to animals 8 months old. **b** Protein carbonyls were measured in liver homogenates by colourimetric formation of dinitrophenylhydrazone. Values are expressed in nmol carbonyl proteins/mg protein. **c** Thiobarbituric acid reactive substances (TBARS) were determined in liver homogenates following the formation of malonyldialdehyde (MDA), which reacts with thiobarbituric acid thus forming a pink-coloured complex at 535 nm. Values are expressed in nmol MDA-equivalents/mg protein. *P<0.05 relative to animals 3 months old. In all bargraphs, values represent means ±SEM of at least four rats

---

GSH content increased initially from 40.3±5.4 nmol GSH/mg protein (3 months) to 55.8±8.6 nmol GSH/mg protein (8 months). Thereafter, a slow but progressive decrease was observed,such that at 18 months it reached 36.9±8.3 nmol GSH/mg protein. It should be noted that the liver GSH content was significantly lower in rats of 18 months than in rats of 8 months. Such a

decrease in the amount of GSH is a reflection of an oxidative insult. Therefore, we looked for the formation of carbonyl derivatives, one of the most studied oxidative stress-induced modifications to proteins. The reactive carbonyl concentration in the liver did not change significantly among the experimental groups. The values of protein carbonyl levels were, indeed, around 1.31±0.31 nmol/mg protein.

Lipid peroxidation was estimated by following the formation of TBARS (thiobarbituric acid reactive substances). The TBARS content increased progressively from 3 to 18 months of age (0.46±0.06 to 1.32±0.33 nmol MDA-equivalents/mg protein) indicating that lipid peroxidation is enhanced with age. These results were further confirmed by the detection of isolated Schiff-positive foci indicating the accumulation of protein-containing pigments. These positive foci are denser and more frequent with age (data not shown).

Finally, none of the liver histological specimens, whatever staining was utilized (Brachet for nucleic acids, PAS for glycogen stores or haematoxylin/eosin for general morphology), showed any particular change in the four experimental groups due to ageing (data not shown).

Discussion

The influence of ageing on xenobiotic metabolism is still a matter of controversy for humans as well as for rats. Indeed, several authors reported a loss of hepatic P450 activity (Rikans and Notley *1982*; Rikans *1989*; Imaoka et al. *1991*; George et al. *1995*; Warrington et al. *2000*; Sotaniemi et al. *1997*), whereas some others reported that its activity was unchanged during ageing (Schmucker and Wang *1981*; Hunt et al. *1990*; Schmucker et al. *1990*). Most of these discrepancies are related to the experimental approach utilized by the different laboratories: namely, the animal models used, the time of observation, the isozyme studied and the specificity of the substrate used as a probe for enzymatic activity. Moreover, both in vivo and in vitro human studies are complicated by interfering factors such as disease, medications, diet, tobacco or alcohol use and the genetic composition of the individual (George et al. *1995*). The complex network of CYP450 enzymes makes it very difficult to select a ldquospecificrdquo substrate to be metabolized by only one CYP isoform. Allelic variant and redundant isozyme forms are naturally involved in the biotransformation of a given chemical, phenomena which in addition may change during ageing by either induction or by protein repression.

To examine the effect of age on in vitro CYP3A and CYP2E1 activity, protein and mRNA content, an ageing rat model was used. We demonstrated that total CYP450 did not change over the entire age range studied (i.e. 3–18 months).

Concerning the specific CYP3A isoforms, when using the specific primers reported by Mahnke et al. (*1997*), the PCR products for CYP3A1 were not detected whereas those for CYP3A23 were (data not shown). This result opens an interesting question about the individual CYP3A isoforms (CYP3A1 or CYP3A23) that are recognized by the commercial antibody. Indeed, other CYP3A isoforms such as CYP3A9 and CYP3A18 may also be involved in midazolam oxidation. Preliminary results obtained with liver microsomes from a female rat (10 weeks old), show that midazolam was 15 times less oxidized than in male rats of the same age (data not shown). Since CYP3A9 is strongly expressed in female rats and CYP3A2 is not (Mahnke et al. *1997*), it may be concluded that CYP3A9 is less involved in midazolam metabolism. On the other hand, no reports have been published about a possible role played by CYP3A18 on midazolam oxidation. In a previous report by Mahnke et al. (*1997*), they showed that levels of CYP3A2, CYP3A1/3A23 and CYP3A18 gene expression are not affected by puberty and did not change from 1 to 20 weeks of age. It may be hypothesized, therefore, that CYP3A18 may have a similar profile to that shown by CYP3A2 and CYP3A1/3A23. Thus, even if CYP3A18 gene expression and protein may be affected by ageing, this would have no influence on midazolam oxidation, a process that remained unchanged during ageing.

Concerning the other cytochrome P450 under study, we found that CYP2E1 activity (chlorzoxazone oxidation) is increased from 3 to 8 months of age, and thereafter decreased by 18 months, while the levels of mRNA did not change over the whole period. Interestingly, CYP2E1 proteins follow the same enzyme profile from 3 to 8 months of age but after 8 months CYP2E1 protein content remained constant. Regarding the increase of CYP2E1 between 3 and 8 months, previous studies on developmental issues show the following pattern. Thomas et al. (*1987*) observed a decrease of CYP2E1 content from 3 to 6 weeks of age, remaining constant up to 12 weeks (3 months) and followed by an increase at 48 weeks (12 months). Nakajima et al. (*1992*) also observed a strong decrease in CYP2E1 content in 18-week-old male rats compared to 3-week-old rats. These results are not necessarily differing from our data. Indeed, we also observed an increase in both CYP2E1 protein and chlorzoxazone oxidation from 12 weeks (3 months) to 32 weeks (8 months). In the absence of an intermediate time point, we cannot define with some precision at which time such a process starts.

On the other hand, it has been reported that changes during ageing only occurr in male-specific isoforms (Kamataki et al. *1985*; Imaoka et al. *1991*).These authors suggested that the decrease of testosterone level leads to a loss of protein content and enzyme activity only for male-specific CYP isoforms, a process that they called the feminization of overall cytochrome P450 composition. Our results are in disagreement with such studies: the CYP3A2 apoprotein, which is male-specific, remains constant and the activity of CYP2E1 (a non-male specific isoform) is decreased at 18 months of age.

What can explain the different sensitivities of these two CYP enzymes to ageing? Obviously, a loss of total CYP can not be evoked since, under our experimental conditions, its amount is not changing during ageing. It may be possible that loss of CYP2E1 enzyme activity is due to the accumulation of non-functional protein. Indeed, the unchanged mRNA levels indicated that the potential problem is not located at the level of either transcription or translation. While it has been reported that ageing may perturb CYP (in particular CYP2B1/2) enzymes at the transcription level (van Bezooijen et al. *1994*), we would like to suggest that CYP2E1 undergoes age-related post-translational modifications that diminish or inactivate its catalytic efficiency. Among such covalent modifications, protein phosphorylation represents a rapid way to inactivate CYP2E1 (Oesch-Bartlomowicz and Oesch *2003*). However, Eliasson et al. (*1992*) reported that CYP2E1 phosphorylation leads to its degradation, a conflicting result because under our experimental conditions CYP2E1 protein was unchanged during ageing. On the other hand, an increase in the concentration of damaged intracellular proteins as well as increase in the concentration of inactive or partially active forms of various enzymes in aging organisms has been generally linked to oxidative stress (Starke-Reed and Oliver *1989*; Stadtman *1992*; Bogdanov et al. *2000*; Levine and Stadtman *2001*). Impairment of protein function implies a change in shape or conformation (Stadtman *1992*; Levine and Stadtman *2001*). Although we were unable to show an increased level of intracellular protein carbonyls, such modifications are suggested by the increase in TBARS and the accumulation of protein-containing pigments such as lipofuscin and ceroid bodies. In that sense, proteins are recognized as major targets of oxidative modification, and the accumulation of oxidized proteins is a characteristic feature of aging cells, which has been reported in many experimental ageing models. In younger individuals, moderately oxidized soluble cell proteins appear to be selectively recognized and rapidly degraded by the proteasome. An age-related accumulation of oxidized proteins could, therefore, be the result of a declining activity of the proteasome. Although an age-related decline in the content and/or activity of the proteasome has been reported (Bulteau et al *2000*, *2002*; Grune et al *2001*), our results did not show any changes in the chymotripsin-like proteasome activity.

Ageing and oxidative stress are well correlated, although no clear cut evidence has been obtained to discriminate whether oxidant injury may be considered as a cause or an effect. Actually, it seems that loss of redox homeostasis is occurring during the process of ageing leading to a gradual cellular imbalance. Thus a progressive ldquodegenerativerdquo process makes the cell repair systems less and less efficient, leading to an accumulation of errors and abnormal macromolecules (Finkel and Holbrook *2000*). Among them, proteins play a key role as catalysts involved in both intermediary metabolism and drug biotransformation. Since markers of oxidative stress clearly indicate that cells are progressively exposed to an oxidizing environment, this oxidant insult may explain the loss of CYP2E1 activity. Moreover, it has been reported that

proteins become sensitive to oxidation by transcription mistakes or by improper folding (Dukan et al. *2000*). Since CYP2E1 generate high levels of reactive oxygen species (Ekström and Ingelman-Sundberg *1989*; Goasduff and Cederbaum *1999*), the particular susceptibility of CYP2E1 can be explained by the proximity of the source of oxidant generation. Moreover, the CYP active site is hydrophobic and sequestered from the action of the cytosolic antioxidant. Thus, contrary to the surface of the protein, the haem and adjacent amino acids are chronically exposed to radicals (Goasduff and Cederbaum *2000*). In addition, this possibility may also explain why CYP3A isoforms are not affected by such oxidative stress.

Acknowledgements We thank Nicole Maton and Véronique Allaeys for their excellent technical assistance. Animal care and experiments were performed according to Biosafety and Ethical rules in application in Belgium as adopted by the Bioethical Committee of the Université Catholique de Louvain.

References

Balducci L (2000) Geriatric oncology: challenges for the new century. Eur J Cancer 36:1741–1754

Birnbaum LS (1980) Altered hepatic drug metabolism in senescent mice. Exp Gerontol 15:259–267

Bogdanov M, Brown RH, Matson W, Smart R, Hayden D, OrsquoDonnell H, Flint Beal M, Cudkowicz M (2000) Increased oxidative damage to DNA in ALS patients. Free Radic Biol Med 29:652–658

Bulteau A-L, Petropoulos I, Friguet B (2000) Age-related alterations of proteasomes structure and function in aging epidermis. Exp Gerontol 35:767–777

Bulteau A-L, Szweda LI, Friguet B (2002) Age-dependant declines in proteasome activity in the heart. Arch Biochem Biophys 397:298–304

Dodds C (1995) Anaesthetic drugs in the elderly. Pharmacol Ther 66:369–386

Dukan S, Farewell A, Ballesteros M, Taddei F, Radman M, Nystrom T (2000) Protein oxidation in response to increased transcriptional or translational errors. Proc Natl Acad Sci USA 97:5746–5749

Eeckhoudt SL, Desager JP, Horsmans Y, De Winne AJ, Verbeeck RK (1998) Sensitive assay for midazolam and its metabolite 1prime-hydroxymidazolam in human plasma by capillary high-performance liquid chromatography. J Chromatogr B 710:165–171

Ekström G, Ingelman-Sundberg M (1989) Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). Biochem Pharmacol 38:1313–1319

Eliasson E, Mkrtchian S, Ingelman-Sunberg M (1992) Hormone- and substrate-regulated intracellular degradation of cytochrome P450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes. J Biol Chem 267:15765–15769

Espínola EB, Oliveira MGM, Carlini EA (1999) Differences in central and peripheral responses to oxotremorine in young and aged rats. Pharmacol Biochem Behav 62:419–423

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247

Friguet B, Szweda LI, Stadtman ER (1994) Susceptibility of glucose-6-phosphate dehydrogenase modified by 4-hydroxy-2-nonenal and metal catalyzed oxidation to proteolysis by multicatalytic protease. Arch Biochem Biophys 311:168–173

George J, Byth K, Farrell GC (1995) Age but not gender selectively affects expression of individual cytochrome P450 proteins in human liver. Biochem Pharmacol 50:727–730

Goasduff T, Cederbaum AI (1999) NADPH-dependent microsomal electron transfer increases degradation of CYP2E1 by the proteasome complex: role of reactive oxygen species. Arch Biochem Biophys 370:258–270

Goasduff T, Cederbaum AI (2000) CYP2E1 degradation by in vitro reconstituted systems: role of the molecular chaperone hsp90. Arch Biochem Biophys 379:321–330

Grune T, Shringarpure R, Sitte N, Davies K (2001) Age-related changes in protein oxidation and proteolysis in mammalian cells. J Gerontol 56:459–467

Guengerich FP (1999) Cytochrome P450 3A4: regulation and role in drug metabolism. Annu Rev Pharmacol Toxicol 39:1–17

Hissin PJ, Hilf R (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74:214–226

Hunt CM, Strater S, Stave GM (1990) Effect of normal aging on the activity of human hepatic cytochrome P450IIE1. Biochem Pharmacol 40:1666–1669

Imaoka S, Fujita S, Funae Y (1991) Age-dependent expression of cytochrome P-450s in rat liver. Biochim Biophys Acta 1097:187–192

Kamataki 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. J Pharmacol Exp Ther 233:222–228

Kobayashi K, Urashima K, Shimada N, Chiba K (2002) Substrate specificity for rat cytochrome P450 (CYP) isoforms: screening with cDNA-expressed systems of the rat. Biochem Pharmacol 63:889–896

Leclercq I, Saliez A, Wallemacq PE, Horsmans Y, Lambotte L (1997) The monoethylglycinexylide test does not correctly evaluate lidocaine metabolism after ischemic liver injury in the rat. Hepatology 26:1182–1188

Leclercq I, Horsmans Y, Desager JP (1998) Estimation of chlorzoxazone hydroxylase activity in liver microsomes and of the plasma pharmacokinetics of chlorzoxazone by the same high-performance liquid chromatographic method. J Chromatogr A 828:291–296

Levine RL, Stadtman ER (2001) Oxidative modification of proteins during aging. Exp Gerontol 36:1495–1502

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186:464–478

Lowry OH, Rosebrough NJ, Farr AL, Randall R (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mahnke A, Strotkamp D, Roos PH, Hanstein WG, Chabot GG, Nef P (1997) Expression and inducibility of cytochrome P450 3A9 (CYP3A9) and other members of the CYP3A subfamily in rat liver. Arch Biochem Biophys 337:62–68

Nakajima T, Wang RS, Katakura Y, Kishi R, Elovaara E, Park SS, Gelboin H, Vainio H (1992) Sex-, age- and pregnancy-induced changes in the metabolism of toluene and trichloroethylene in rat liver in relation to the regulation of cytochrome P450IIE1 and P450IIC11 content. J Pharmacol Exp Ther 261:869–887

Oesch-Bartlomowicz B, Oesch F (2003) Cytochrome-P450 phosphorylation as a functional switch. Arch Biochem Biophys 409:228–234

Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. II Solubilization, purification and properties. J Biol Chem 239:2370–2378

Rikans LE (1989) Hepatic drug metabolism in female Fischer rats as a function of age. Drug Metab Dispos 17:114–116

Rikans LE, Notley BA (1982) Age-related changes in hepatic microsomal drug metabolism are substrate selective. J Pharmacol Exp Ther 220:574–578

Ronis MJJ, Lindros KO, Ingelman-Sundberg M (1996) The CYP2E subfamily. In: Ioannides C (ed) Cytochromes P450: metabolic and toxicological aspects. CRC Press, Boca Raton, pp 211–239

Schmucker DL (2001) Liver function and phase I drug metabolism in the elderly: a paradox. Drugs Aging 18:837–851

Schmucker DL, Wang RK (1981) Effects of aging and phenobarbital on the rat liver microsomal drug-metabolizing system. Mech Ageing Dev 15:189–202

Schmucker DL, Woodhouse KW, Wang RK, Wynne H, James OF, McManus M, Kremers P (1990) Effects of age and gender on in vitro properties of human liver microsomal monooxygenases. Clin Pharmacol Ther 48:365–374

Smorenburg CH, ten Tije AJ, Verweij J, Bontenbal M, Mross K, van Zomeren DM, Seynaeve C, Sparreboom A (2003) Altered clearance of unbound paclitaxel in elderly patients with metastatic breast cancer. Eur J Cancer 39:196–202

Sotaniemi E A, Arranto AJ, Pelkonen O, Pasanen M (1997) Age and cytochrome P450-linked drug metabolism in humans: an analysis of 226 subjects with equal histopathologic conditions. Clin Pharmacol Ther 61:331–339

Stadtman ER (1992) Protein oxidation and aging. Science 257:1220–1224

Stadtman ER (2002) Importance of individuality in oxidative stress and aging. Free Radic Biol Med 33:597–604

Starke-Reed PE, Oliver CN (1989) Protein oxidation and proteolysis during aging and oxidative stress. Arch Biochem Biophys 275:559–567

Szweda PA, Friguet B, Szweda LI (2002) Proteolysis, free radicals, and aging. Free Radic Biol Med 33:29–36

Tanira MOM, Ali BH, Bashir AK (1997) Effect of endotoxin on gentamicin pharmacokinetics in old and young adult rats. Life Sci 60:413–424

Taper HS, Somer MP, Lans M, de Gerlache J, Roberfroid M (1988) Histochemical detection of the in vivo produced cellular aldehydes by means of direct Schiffrsquos reaction in CCl_4 intoxicated rat liver. Arch Toxicol 61:406–410

Thomas PE, Bandiera S, Maines SL, Ryan DE, Levin W (1987) Regulation of cytochrome P-450j, a high affinity *N*-nitrosodimethylamaine demethylase, in rat hepatic microsomes. Biochemistry 26:2280–2289

van Bezooijen RL, Wang RK, Lechner MC, Schmucker DL (1994) Aging effects on hepatic NADPH cytochrome P450 reductase, CYP2B1&2, and polymeric immunoglobulin receptor mRNAs in male Fischer 344 rats. Exp Gerontol 29:187–195

Warrington JS, Poku JW, von Moltke LL, Shader RI, Harmatz JS, Greenblatt DJ (2000) Effects of age on in vitro midazolam biotransformation in male CD-1 mouse liver microsomes. J Pharmacol Exp Ther 292:1024–1031