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The use of precision-cut liver slices from male Wistar rats as a tool to study age related changes in CYP3A induction and in formation of paracetamol conjugates

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Abstract

Precision-cut liver slices (PCLS) offer a lot of advantages because all heterogeneity and cell-cell interactions within the original tissue matrix are maintained. This in vitro model was used to study the effect of ageing on certain aspects of drug metabolism and liver function in young (3 months), adult (9 months) and old (24 months) Wistar male rats. Protein synthesis, an important liver function, was not modified in young, adult and old rats, suggesting that ageing does not impair liver functionality but it affects some specific targets. Among them, a decrease in total P450 in liver microsomes and the loss of CYP3A23 inducibility in PCLS were clearly observed in old rats as compared to adult rats. Finally, the amount of total paracetamol conjugates was not modified between 9 and 24 months but in old rats, sulfoconjugation of paracetamol, its major route of elimination, was decreased.

Keywords: CYP3A-ageing; P450 induction; Rat liver slices; Paracetamol

1. Introduction

It is well know that ageing may lead to alterations in the biotransformation of drugs, and therefore their therapeutic efficacy and safety. A number of studies in male rats have documented significant age-related declines in the amounts, specific activities and rates of induction of liver microsomal mono-oxygenases (Birnbaum and Baird, 1978; McMartin et al., 1980; Schmucker and Wang, 1981), as well as an increase in inter-individual variability in most liver functions (Schmucker, 2001). To learn more about the underlying causes of this age-related alteration in drug metabolism numerous in vivo studies have been carried out in animals, especially rats. However, experiments in animals to investigate the possible causes of these alterations and especially the interpretation of the results of in vivo studies are complicated by a number of problems. Indeed, the in vivo drug metabolic clearance is not only affected by intrinsic metabolic capacity but also by organ blood flow and binding to plasma proteins, factors which may also be altered due to the increased incidence of functional abnormalities in a number of organs with ageing (Barnett et al., 1974; Messineo et al., 1983; van Bezooijen, 1984). Moreover, it is highly unlikely that ageing is affecting the various isoforms of drug metabolizing enzymes to the same extent. Although an approach looking for simultaneous measurements of different P450 isoforms can be conducted by using "special mixtures", the possible contribution of extrahepatic tissues to the overall metabolism complicates the interpretation of the results.

In order to solve some of these inconveniences, we decided to use the in vitro model of precision-cut liver slices (PCLS) to investigate the influence of ageing on drug metabolism in rats. PCLS present a lot of advantages by maintaining cell heterogeneity and cell-cell interactions within the original tissue matrix. Indeed, PCLS have been shown to be a suitable model to study survival and cellular metabolism, drug toxicity

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and xenobiotic metabolism (Morales et al., 1998; Vanhulle et al., 2001; Evdokimova et al., 2001a, 2002; Rekka et al., 2001). In the present study, some parameters related to drug biotransformation were investigated in PCLS prepared from young (3-month), adult (9-month) and old (24-month) male Wistar rats. They include the total content of P450, the capacity of CYP3A23 to be induced under in vitro conditions, and a phase II-related activity, namely the formation of paracetamol conjugates (glucuronide, sulfate and glutathione) together with SULT1A1 protein content. In addition, protein synthesis ability (a major metabolic activity of hepatocytes), was measured to assess the metabolic competence of the slices.

2. Materials and methods

2.1. Animals

Male Wistar rats of 3, 9 and 24 months were purchased from Harlan (The Netherlands) and housed in individual cages in a temperature- and light- controlled room (12 h dark /light cycle). They received a standard diet (A03 UAR, France) and water ad libitum. After one week of acclimation, rats were weighed and killed under pentobarbital anesthesia (60 mg/kg i.p.). The liver was removed. A part was used to prepare liver microsomes and the other part for the preparation of PCLS.

2.2. Chemicals

Williams' medium E (WME) and fetal calf serum (FCS) were purchased from Gibco BRL (Middlesex, UK). Gentamicin sulfate, leupeptine, PMSF, antipain, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), dexamethasone-21-phosphate (Dex), Tween 20 and Triton X-100 were obtained from Sigma Chemicals (St Louis, MO, USA). Paracetamol (Janssen Pharmaceutica, Beerse, Belgium), 2-acetamidophenol (Aldrich Chemicals Co., Dorset, UK), and insulin (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark) were commercially available. Paracetamol glucuronide, sulfate and gluthatione conjugates were isolated from human urine and purified by HPLC. Polyclonal sheep antibodies raised against rat SULT1A1 were kindly provided by Dr. M. Coughtrie (University of Dundee, Scotland, UK). Rabbit antibody to rat CYP3A1 and goat anti-rabbit antibody was purchased from Chemicon (Temecula, USA), donkey anti-goat antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A western immunoblotting kit employing enhanced chemiluminescence detection was obtained from Pierce (Rockford, Illinois). Solvents used were of HPLC grade and all other chemicals were of the highest quality available.

2.3. Preparation of PCLS

Rat surgical procedures were carried out under pentobarbital (60 mg/kg) anesthesia. PCLS (250–300 μm thickness) were prepared by using the Krumdieck tissue slicer according to procedures previously described (Evdokimova et al., 2001b). They were kept for 30 min at 4 °C in WME containing FCS (10%), glutamine (2 mM) and insulin (100 nM). After preincubation, PCLS were transferred to vials containing WME (one slice in 2 ml) supplemented with glutamine (2.4 mM), insulin (100 nM) and 50 μg/ml gentamicin sulfate (only when slices were incubated for 24 h). PCLS were incubated in a shaking water-bath (100 cycles/min) at 37 °C under a continuous flow of O_2/CO_2, (95%/5%) for the indicated times. For the study of CYP3A induction, liver slices were incubated for 24 h in the absence or in the presence of 10 μM dexamethasone.

2.4. Protein synthesis

Protein synthesis was estimated by measuring the incorporation of [^{14}C]-leucine (specific activity: 94 μCi/mmol, 0.57 mM unlabelled leucine) into the protein pellet obtained after perchloric acid precipitation as described by Seglen (1976). After a preincubation of 30 min, PCLS were rinsed in saline, further incubated for 2 h in a fresh medium containing unlabelled leucine in the presence of [^{14}C]-leucine. The results are expressed as pmol leucine incorporated per mg of protein. The amount of protein was determined by the Lowry method (Lowry et al., 1951) by using bovine serum albumin as standard.

2.5. Cytochrome P450

Total cytochrome P450 was determined in fresh liver microsomes prepared according to Leclercq et al. (1998). The change in the absorbance was recorded at 450 nm of the reduced complex with CO as reported by Omura and Sato (1964).

2.6. Western immunoblotting

Liver slices were sonicated in Tris HCl buffer (pH 7.4) containing PMSF 0.1 mM, leupeptine 2 μg/ml, antipaine 2 μg/ml, DTT 2 mM, EDTA 0.1 mM and triton 0.4%. After centrifugation, the supernatant was stored at −30 °C. 2.5 μg of proteins were subjected to (SDS)-polyacrylamide gel electrophoresis (10% separating gel) followed by electroblot to nitrocellulose membrane. The membranes were blocked for 1 h at room temperature in TBS buffer (pH 7.4) containing 5% (w/v) of powdered milk protein. Subsequently, they were probed for 2 h with polyclonal rabbit anti-rat antibody for CYP3A1 (1/16000) or with polyclonal goat anti-rat antibody for

SULT1A1 (1/10000) at 25 °C in a solution of TBS buffer containing 0.1% of Tween 20. The membranes were washed and incubated with goat anti-rabbit antibody or with donkey anti-goat antibody coupled to horseradish peroxidase (1/10000) at room temperature. Immunodetection was performed using the Pierce ECL system, the film was scanned and the density of the bands was calculated using the program Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands).

2.7. Paracetamol metabolism in PCLS

Paracetamol conjugates (glucuronide, sulfate and glutathione) were quantified by using a reverse-phase HPLC according to the procedure of Lau and Critchley (1994). PCLS were incubated for 2 h in medium supplemented with 5 mM paracetamol (a concentration close to in vivo conditions and that does not saturate conjugation enzymes). At the end of the incubation, aliquots of incubation medium were stored at −20 °C. After centrifugation, 25 μl of samples were injected on a Nova-Pak C18 column. 2-Acetamidophenol was used as internal standard. The mobile phase (0.1 M KH_2PO_4/0.1% acetic acid/0.75% propan-2-ol) was delivered to the column at a flow rate of 1.5 ml/min. The results are expressed as μg metabolites/mg protein.

2.8. Statistical analysis

Each group was composed of 4 rats. The results are mean values ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) to determine the statistical significance among the different age groups. The level of significance was set at $p < 0.05$.

3. Results

Table 1 shows the effect of ageing on the ability of PCLS to synthesize proteins and on the amount of cytochrome P450 in liver microsomes. The capacity to synthesize new proteins by liver slices was measured by the incorporation of [^{14}C]-leucine into proteins and expressed as pmol leucine incorporated/mg protein/min. Although a slight decrease is observed in old rats, no statistical differences were observed in protein synthesis rates in PCLS from rats of 3, 9 and 24 months. In adult rats the amount of total cytochrome P450 reached about 0.8–1.0 nmol/mg protein (an increase of 25% as compared to young rats), and remained at such a value until 21 months (data not shown), afterwards it decreased by about 35% in old rats.

As illustrated in Fig. 1, the percentage increase in the anti-rat CYP3A1 reactive protein (CYP3A23) between untreated and dexamethasone-treated slices changed significantly with ageing. While in PCLS from young rats the induction was about 410%, the induction capacity was decreased to 31% in PCLS from old animals ($p = 0.007$). Concerning the CYP3A isoform we detected, the commercial antibody we employed is thought to specifically recognize CYP3A1, but, we were unable to detect CYP3A1 mRNA in any of the

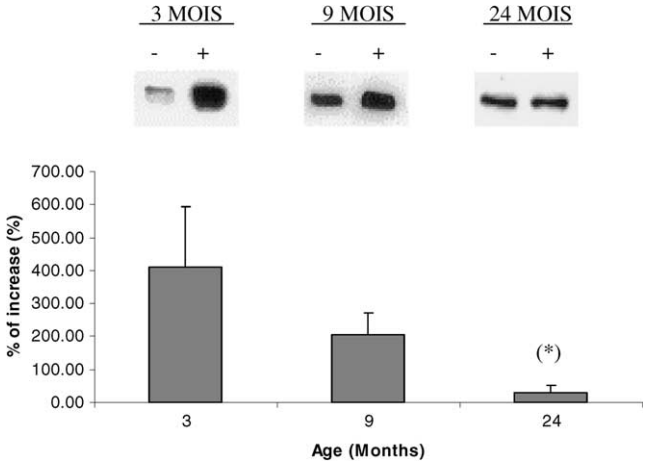

Fig. 1. Effect of age on CYP3A23 apoprotein induction by dexamethasone in rat precision-cut liver slices. For CYP3A23 induction, liver slices were incubated for 24 h in the presence of 10 μM of dexamethasone. Precision-cut liver slices were homogenized and immunoblots were performed as described in materials and methods. Representative Western blots showing levels of CYP3A23 in untreated (−) and dexamethasone-treated (+) liver slices of rats of 3, 9 and 24 months are presented. CYP3A23 content in the blots was measured by densitometry and the results are expressed as percentage increase. Results represent the mean ± S.E.M ($n = 4$). *$p < 0.01$ as compared to animals 3 months old.

Table 1
Effect of age on protein synthesis and total Cytochrome P450

	Protein synthesis rates (pmol Leu/min/mg prot)	P450 content (nmol/mg prot)
PCLS-young rats	50.1 ± 8.7	0.77 ± 0.04
PCLS-adult rats	44.6 ± 10.3	1.02 ± 0.10[a]
PCLS-old rats	38.0 ± 12.2	0.67 ± 0.09[b]

Protein synthesis rates were measured after 2-h incubation of PCLS in the presence of radiolabeled leucine. Results are mean values ± SEM and expressed in pmol of leucine incorporated per min and mg of protein. Total P450 was determined in liver microsomes by changes in the absorbance at 450 nm of the reduced complex with CO. Results are mean values ± SEM and expressed in nmol of P450 per mg of protein.

[a] $p < 0.01$ as compared to rats of 3 months old.

[b] $p < 0.001$ as compared to rats of 9 months old.

dexamethasone treated slices (data not shown) whatever the age of the animals (Wauthier et al., 2004). Therefore, due to the high homology between CYP3A1 and CYP3A23 (97%) we assume that the protein band we detected is probably CYP3A23.

Phase II paracetamol metabolites produced by rat liver slices were measured in the medium after a 2 h incubation time in the presence of paracetamol (Fig. 2). In preliminary experiments, the formation rates of the glucuronide, sulfate and glutathione conjugates had been shown to be linear during a 2 h incubation period at a paracetamol concentration of 5 mM. Fig. 2 shows a significant increase of 53% in total conjugates (sum of glucuronide, sulfate and glutathione conjugates) between animals of 3 and 9 months ($p = 0.008$). A progressive increase of Paracetamol glucuronidation reaching 68% between 3 and 24 months was observed ($p = 0.003$). Both in young and adult rats, the paracetamol sulfate was the predominant form of drug elimination (50–60% of total conjugates), while paracetamol glucuronide represented about 35–40% of total conjugates. Interestingly, between 9 and 24 months the total conjugates level was rather constant but in old animals, a shift in conjugates occurred: the route of paracetamol glucuronidation became the predominant form of drug elimination reaching more than 60% of the total conjugates whereas paracetamol sulfation only represented 32%. The variability in glutathione conjugation of paracetamol as a function of age was without statistical significance with values around 1 μg/mg protein.

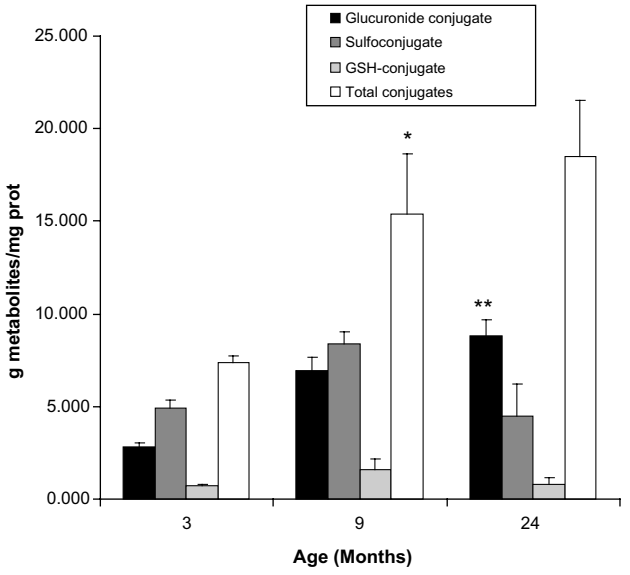

Fig. 2. Effect of age on the formation of paracetamol conjugates by PCLS. The formation of paracetamol conjugates by PCLS was measured in the medium after 2 h incubation in the presence of 5 mM paracetamol. Values are expressed as μg of metabolite per mg of protein ±SEM ($n = 4$ rats). *$p < 0.01$ as compared to animals 3 months old. **$p < 0.005$ as compared to animals 3 months old.

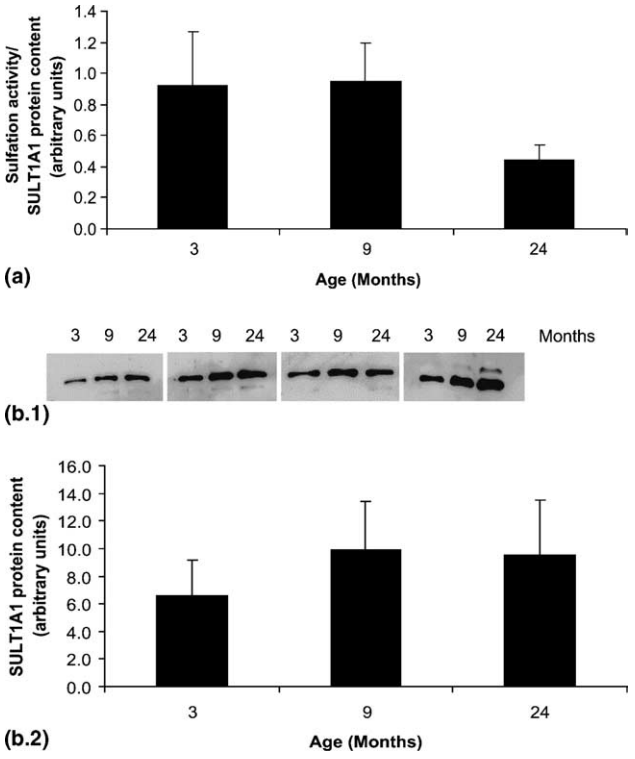

Fig. 3. Effect of age on the ratio between paracetamol sulfation activity and the amount of SULT1A1 proteins (a) and on SULT1A1 protein content (b). (a) Values of paracetamol sulfation activity for each group were divided by the amount of SULT1A1 protein. Values are expressed as arbitrary units and represent the mean ± SEM ($n = 4$). (b) The content of SULT1A1 protein was assessed by immunoblotting in the different groups. Western blots showing individual amount of SULT1A1 proteins in rats of 3, 9 and 24 months are presented (b.1). Values are expressed as arbitrary units obtained after densitometry of the respective electrophoretic bands. Results represent the mean-± S.E.M ($n = 4$) (b.2).

Fig. 3a shows the ratio between paracetamol sulfation activity and the amount of SULT1A1 proteins in PCLS from young, adult and old rats. No difference in the rate of paracetamol sulfation relative to the SULT1A1 protein content was observed between animals of 3 and 9 months but a decrease of 55% was observed at 24 months compared to 9 months. However, this difference did not reach statistical significance ($p = 0.072$). The amount of SULT1A1 protein detected by Western immunoblotting in young, adult and old rats are presented in Fig. 3b. Although an increase of 33% in SULT1A1 protein content was observed between 3 and 9 months, this difference was not significant. After 9 months the amount of SULT1A1 remained constant.

4. Discussion

The results of the present study are consistent with the findings of previous investigations that reported a

decrease in total amount of P450 during ageing (Schmucker and Wang, 1981; Kamataki et al., 1985). Regarding the isoform that may be affected by ageing we focused our study on CYP3A because it is an important sub-family in critical tissues such as the gastrointestinal tract and liver in both rat and man and it is involved in the oxidative biotransformation of numerous clinically useful therapeutic agents in humans (Dorne et al., 2003). CYP3A activity can also be readily modulated by many inducers like rifampicin, dexamethasone, and several anticonvulsant agents, and potent inhibitors exist such as azole antifungal agents and macrolide antibiotics (Jurima-Romet et al., 1994; Luo et al., 2002). Accordingly, a high probability of drug interactions exists between these drugs as well as other CYP3A substrates, when given concomitantly. Although in rats the major P450 isoform is the CYP2C11, the CYP3A isoforms accounted for about 30-40% of the total P450 (Waxman et al., 1985). Among the different CYP3A isoforms, CYP3A23 was selected to study the influence of ageing on in vitro induction by dexamethasone. This choice is explained by the fact that after 24 h incubation, the CYP3A2 protein is not detected anymore (Rekka et al., 2002). Moreover, Hoen et al. (2000) reported that in rat hepatocytes, neither CYP3A2 nor CYP3A18 and CYP3A9 mRNA levels were affected by dexamethasone after three days of incubation. Since the commercial antibody we employed is thought to recognize CYP3A1, but CYP3A1 is not expressed in male Wistar rats (Wauthier et al., 2004), we concluded that the protein band we detected is indeed CYP3A23.

Our results show that 24 h after dexamethasone addition the transcriptional induction of CYP3A23 is followed by a significant increase in CYP3A23 protein in liver slices of both young and adult rats (410% and 207% respectively). Nevertheless, the induction was only of 31% in old rats. This result is in agreement with in vivo studies on the induction ability of various types of rat liver P450 when administering phenobarbital or dexamethasone (Groen et al., 1994; Horbach et al., 1990a,b). Nevertheless, a recent in vivo study reported no change in the ability of induction of CYP3A1 by dexamethasone in old (24 months) Sprague Dawley rats (Agrawal and Shapiro, 2003). These contradictory results may be to the fact that we used Wistar rats, and as the authors quoted in their paper, the effect of strain is a critical factor influencing age-associated changes in drug metabolism. The induction of CYP3A23 seems to proceed in two stages through both the glucocorticoid and pregnane X receptor (PXR). Moreover, nuclear receptors COUP-TF and HNF-4 are essential to get a full response (Huss and Kasper, 2000). The loss of induction ability observed in old animals may be explained by a decrease in glucocorticoid receptor concentration as shown by Djordjevic-Markovic et al. (1999). Nevertheless, no reports have been published concerning possible changes in PXR or hepatic nuclear factor HNF-4 with age. The identification of liver transcription factors involved in the induction process during ageing was not part of the present investigation but merits to be investigated. Interestingly, preliminary reports indicated that the expression of mRNA HNF-4α did not change with age (Wauthier et al., manuscript in preparation).

To characterize the phase-II biotransformation of drugs during ageing, PCLS from young (3-month), adult (9-month) and old (24-month) rats were prepared and paracetamol was used as model substrate. The results observed in young and adult animals are in agreement with the observations made by several authors (Price and Jollow, 1982; Miller et al., 1993; Oddy et al., 1997) indicating that sulfation is the major and predominant route of paracetamol biotransformation. In young and adult rats, sulfates, glucuronides and glutathione conjugates represent about 50–60%, 35–40% and 4–10%, respectively. These values are close to the ones reported by Miller et al. (1993). Nevertheless, in PCLS from old animals, a shift in the profile of conjugation was observed since paracetamol glucuronidation was twice as high compared to sulfate conjugation. These results are consistent with both in vitro and in vivo studies that reported changes in sulfation of paracetamol or others drugs during ageing (Galinsky and Corcoran, 1986; Galinsky et al., 1990). Although sulfation of paracetamol has been characterized as a high-affinity and low-capacity conjugation reaction in rats (Moldeus, 1978), this fact is unlikely explaining the major proportion of glucuronide formed in old animals because the same dose of paracetamol was used. The increase in paracetamol sulfation activity between 3 and 9 months can be explained by the increase in SULT1A1 protein content during that period. However, because SULT1A1 protein content remained constant between 9 and 24 months, the decrease in paracetamol sulfation activity during this period must be dependent on others factors than the amount of SULT1A1 proteins. This decrease of sulfation activity may be due to either a change in the protein itself or in the availability of the required enzymatic cofactors. In a previous study, we have shown that some markers of oxidative stress were enhanced in old rats (Wauthier et al., 2004). Therefore, the decrease in paracetamol sulfation might be related to a partial loss in catalytic efficiency by post-translational modifications of proteins. A lot of studies on ageing reported loss of protein activity generally associated to oxidative stress (Starke-Reed and Oliver, 1989; Stadtman, 1992). On the other hand, sulfation may be limited by the availability of its cofactor, the 3′-phosphoadenasine-5′-phosphosulfate (PAPS), which in turn is dependent on its synthesis, degradation and ultimately its utilization in the sulfation reaction itself. PAPS synthesis is dependent on the availability of sulfate and on the activity of the 2 enzymes of its synthesis, ATP-sulfurylase and APS-kinase (Klaassen

and Boles, 1997). An effect of ageing in any of these steps may alter PAPS availability and lead to decreased sulfation.

Finally, with regard to the influence of ageing on the ability of hepatocytes to synthesize proteins, our results agree with previous reports indicating that protein synthesis rates in the liver are maintained during ageing (Mays et al., 1991; Mosoni et al., 1993). Since protein synthesis is a complex process requiring the availability of cofactors, high energy donors and the retention of the ribosome ultrastructure, this result suggests that ageing does not result in overall impairment of liver function.

PCLS represent an interesting tool that may be employed in studies of ageing by applying a multiple and complex approach including the measurement of phase I and phase II xenobiotic biotransformation enzymes, standard biochemical markers of cell viability, and phenotypic markers dealing with specific liver functions such as gluconeogenesis, urea and/or protein synthesis.

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