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Induction of CYP2C12 expression in senescent male rats is well correlated to an increase of HNF3β expression, while the decline of CYP2C11 expression is unlikely due to a decrease of STAT5 activation

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ABSTRACT

Ageing affects drugs metabolism influencing the therapeutic efficacy and safety of drugs. By using the experimental model of aged male rats, we investigated the influence of ageing on some CYP2C isoforms, the most important CYP450 sub-family in rats. The activity of the male specific CYP2C11 is decreased by 55% in senescent male rats. This correlates with a significant reduction of both protein content (80%) and mRNA (60%) indicating a demasculinization process. The expression of CYP2C12, a female specific isoform, is induced in senescent male rats indicating a feminization process. Neither the activity nor the expression of CYP2C6, a female predominant isoform, is modified in senescent male rats. Thereafter, certain putative GH mediators like some liver enriched transcription factors (LETFs) or STAT5b were investigated. The amount of HNF3β mRNA, a transcription factor involved in the up-regulation of CYP2C12, has been shown to increase by about three-fold in senescent male rats. With regard to STAT5b, which has been reported to be involved in the male specific regulation of CYP2C11, large amounts of phosphorylated STAT5 were observed in the liver of senescent male rats. These results indicate that while the induction of CYP2C12 during ageing could be due, at least partially, to the enhanced HNF3β expression, the decline of CYP2C11 is unlikely related to a decrease of STAT5 activation.

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1. Introduction

Elderly people are an increasing part of the population [1]. Elderly are the most medicated segment of society and they are presumed to be more vulnerable to drug toxicity and side-effects due to various change in their pharmacodynamic and pharmacokinetics profiles. Indeed, 10% of all elderly admissions is related to adverse drug reactions and toxicities [2,3]. This is the consequence, at least in part, of a decline in drug-metabolizing enzymes [4] and should require an adjustment of therapeutic doses in older patients [5]. In rats as well as in other species, the clearance of many but not all drugs is impaired during ageing [6–12]. Indeed, ageing affects P450 enzymes in a specific manner as shown by a decrease in the protein content of some but not all P450 isoforms in senescent male rats [8,12,13]. In addition to age, gender can also

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Abbreviations: BSA, bovine serum albumin; CYP, cytochrome P450; GH, growth hormone; LETF, liver enriched transcription factor; HNF, hepatocyte nuclear factor; JAK, Janus tyrosine kinase; STAT, signal transducer and activator of transcription

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influence the expression and the activities of cytochrome P450 enzymes. In this regard, rat liver contains at least a dozen sex-dependent isoforms of cytochrome P450 [14–16]. Some of them are sex-specific, i.e. they are exclusively expressed either in male (for example, CYP2C11, CYP3A2, and CYP2A2) or in female (CYP2C12) rats. Others P450s are sex-predominant isoforms (CYP3A18, CYP32A23, CYP3A9, CYP2C6, etc.), i.e. they are expressed in both sexes but to a higher extent in males than in females or vice versa. For example, CYP2C6 is a female-predominant isoform for which male rat liver expresses ~60% of the levels found in female liver [17,18].

The effect of both age and gender on the expression of P450s is mediated by growth hormone (GH) and more specifically by its secretion profile. Indeed, in rats as well as other species, GH is secreted in a sex dependent manner [14]. In adult male rats, GH is secreted in episodic bursts (200–300 ng/ml of plasma) about every 3.5 h. Between the peaks, growth hormone levels are undetectable (≤2 ng/ml). In adult females the hormone pulses are more frequent and irregular and are of lower magnitude than those in males, whereas the interpulse concentrations of growth hormone are always measurable (40 ng/ml). These sexually differentiated pituitary GH secretion profiles are responsible for establishing and maintaining the sex-dependent patterns of cytochrome P450 genes expression [14]. During ageing, the GH secretion profile of the male rats changes and becomes closer to the one occurring in females. In fact, in senescent male rats, GH secretion becomes more continuous leading to a loss of the typical male GH devoid interpulse period [13].

The GH mediated intracellular mechanisms involved in the regulation of cytochrome P450 expression are still unclear. Liver enriched transcription factors (LETFs) such as certain hepatocyte nuclear factors (HNFs) and C/EBP have been proposed as potential candidates in these regulatory pathways [19]. In addition, a role for the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway in mediating the actions of GH has been reported. Indeed, one of the STAT proteins, STAT5b, is highly responsive to the male pulsatile GH pattern and is proposed to be a key mediator of the sexually dimorphic response of liver CYPs to GH [16,20,21].

In the present study, we investigated the influence of ageing on the activity and expression of the male specific CYP2C11, the female specific CYP2C12 (only mRNA level) and the female predominant CYP2C6 isoforms in both adult (9 months) and senescent (24 months) male rats. CYP2C12 gene has been reported to be regulated by HNF6 [22], HNF4α [23,24], HNF3β [25] and by C/EBPα [26]. A change in LETFs expression during ageing may have consequences on CYP2C12 expression. Indeed, we have previously shown that HNF1α is increased by about two- to three-fold in senescent male rats with putative consequence on CYP2E1 expression [27]. Therefore, we determined the mRNA levels of, HNF3β, HNF6 and C/EBPα in the liver of adult and senescent males rats. Subsequently, we analysed the influence of ageing on STAT5b because this factor has been proposed to be involved in the up-regulation of CYP2C11 [28]. Therefore, we hypothesised that the continuous secretion of GH in senescent male rats leads to a desensitization of the JAK2/STAT5 pathway explaining the age-related changes of cytochrome P450 enzymes expression. Accordingly, we analyzed the influence of ageing on STAT5b basal expression and activation (phosphorylation) in adult and senescent male rats. We compare these results with those obtained in male and female rats of the same age (3 months).

Finally, a morphological study was also performed and liver histological sections of both adult and senescent rats were analysed by haematoxylin/eosin, periodic acid-Schiff (PAS) and methylgreen pyronine staining under light microscopy.

2. Materials and methods

2.1. Animals

Male Wistar rats of 9 and 24 months were purchased from Harlan (The Netherlands). Male and female Wistar rats of 3 months old were bought from the University central animal breeding facilities. Rats were 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 1 week of acclimation, rats were weighed and killed under pentobarbital anesthaesia (60 mg/kg i.p.). The liver was removed. A part was used to prepare liver microsomes, another to prepare nuclear extract and the rest of the liver was frozen at −80 °C.

2.2. Chemicals

Testosterone and nortestosterone were purchased from Roche. 2-α-Hydroxy testosterone was kindly provided by Prof. Guillouzo. Diclofenac was from Sigma. 4-OH-diclofenac, rabbit polyclonal antibodies against CYP2C11 and goat polyclonal antibodies against CYP2C6 were from Gentest. Mouse polyclonal antibodies against STAT5b were from Zymed. Rabbit polyclonal antibodies against phosphorylated-STAT5 were from Cell Signaling. Primers for RT-PCR were synthesized by Eurogentech. Solvents used were of HPLC grade and all other chemicals were of the purest quality available.

2.3. Morphology of liver sections

Histological sections obtained from liver specimens were fixed in Carnoy's fixative and embedded. The general morphology was observed after haematoxilin/eosin coloration. Glycogen stores were visualised after periodic acid Schiff (PAS) staining and nucleic acid were colored by methylgreen pyronin reagent.

2.4. Preparation of liver microsomes

Hepatic microsomal fractions were prepared by differential centrifugation as described previously [29]. The amount of protein was determined by the method of Lowry using BSA as standard [30].

2.5. Liver nuclear extract preparation

Liver nuclear extracts were prepared from 100 mg of tissue using a Nuclear extract kit (Active Motif) according to the manufacturer's instructions.

2.6. CYP2C11 activity

The rate of testosterone hydroxylation by rat liver microsomes was quantified by measuring the formation of 2-α-hydroxytestosterone [31]. Briefly, 0.4 mg of liver microsomal protein, 0.1 M phosphate buffer pH 7.4, 0.1 M KCl, 1 mM EDTA, 6 mM $MgCl_2 \cdot 6H_2O$, 8 U of glucose-6-phosphate dehydrogenase, 6.6 mM glucose-6-phosphate were pre-incubated at 37 °C for 5 min with various concentrations of testosterone ranging from 0 to 1000 μM in a total volume of 500 μl. The incubation was started by the addition of a solution of 1.2 mM NADPH. After 12 min, the reaction was stopped by the addition of 200 μl of acetonitrile–acetic acid [95:5] and tubes were placed on ice. Nortestosterone was added as internal standard. After centrifugation for 10 min at 10,000 × *g*, testosterone and its metabolites were extracted from 600 μl of supernatant with 3.0 ml of dichloromethane. After mixing and centrifugation for 10 min at 500 × *g*, the organic layer was evaporated and conserved at −20 °C. The residue was dissolved in 60 μl of mobile phase and analysed by HPLC. The stationary phase consisted of a C18 column and the mobile phase (water/methanol/tetrahydrofurane; pH 4) [62:29.6:8.4] was delivered to the column at a flow rate of 1.5 ml/min. The results were expressed as micrograms metabolites/min mg protein.

2.7. CYP2C6 activity

The rate of diclofenac hydroxylation by rat liver microsomes was quantified by measuring the 4-OH-diclofenac formation [32]. About 0.2 mg of liver microsomal protein, 0.1 M phosphate buffer pH 7.4, 0.1 M KCl, 1 mM EDTA, 6 mM $MgCl_2 \cdot 6H_2O$, 8 U of glucose-6-phosphate dehydrogenase, 6.6 mM glucose-6-phosphate were pre-incubated at 37 °C for 5 min with various concentrations of diclofenac ranging from 0 to 110 μM in a total volume of 1000 μl. The incubation was started by the addition of a solution of 1.2 mM NADPH. After 20 min, the reaction was stopped by the addition of 200 μl of acetonitrile–acetic acid [95:5] and tubes were placed on ice. Diflunisal was added as internal standard. After centrifugation for 10 min at 10,000 × *g*, samples were analyzed by HPLC. The stationary phase was C18 column and the mobile phase (50 mM phosphate buffer pH 7/acetonitrile) [75:25] was delivered to the column at a flow rate of 1.2 ml/min. The results are expressed as nmol metabolites/min mg protein.

2.8. Western immunoblotting

Samples loading were as follow: 1 μg of liver microsomal proteins for CYP2C11 and CYP2C6 detection, 24 μg of liver nuclear extract for P-STAT5 detection and 40 μg of liver homogenate for STAT5b detection were subjected to (SDS)-polyacrylamide gel electrophoresis (12% separating gel) followed by electroblot to a 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. Next, they were probed with polyclonal rabbit anti-rat CYP2C11 antibody (1/5000), CYP2C6 antibody (1/1000) for 2 h at 25 °C or P-STAT5 (1/1000), STAT5b (1/350) antibodies overnight at 4 °C in a fresh solution of BSA (5%, w/v) in TBS buffer–Tween 0.1%. The membranes were washed and reprobed with goat anti-rabbit, goat anti-mouse or mouse anti-goat antibody coupled to horseradish peroxidase (1/10,000) at room temperature. Immunodetection was performed using the ECL^{TM} detection kit (Amersham, UK). The film was scanned and the density of the bands was calculated using the program Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands). The amount of protein loading in each lane was assessed by staining the blots with Ponceau S. The specificity of each antibody was previously checked and prior to the measurements of samples, linear conditions between protein concentrations and optical density was established. The results are expressed as arbitrary optical density units.

STAT5b antibody is specific for the STAT5b protein and does not react with STAT5a. P-STAT5 antibody detects STAT5a and STAT5b only when phosphorylated at tyrosine 694. This antibody does not react with other STAT proteins.

2.9. RT-PCR

Total RNA was extracted from approximately 30 mg of rat liver using a SV total RNA isolation kit (Promega) according to manufacturer's instructions. The cDNA was synthesized in a total volume of 20 μl of reaction mixture containing 1 μg of RNA or hnRNA in the RT reaction buffer (Invitrogen), 10 nmol/l dithiothreitol, 25 pg of oligo(dT) primer, 0.5 mmol/l each deoxyribonucleoside triphosphate (dNTP), 200 units of superscript II Rnase H$^-$ Reverse Transcriptase (Invitrogen). Reaction mixtures were incubated for 1 cycle at

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

	Primers		PCR conditions	
	Forward	Reverse	Number of cycles	Annealing temperature (°C)
CYP2C11	TGCCCCCTTTTTACGAGGCT	GGAACAGATGACTCTGAATTCT	26	64
CYP2C12	TATAAACTCAATACGTTCTGAG	TTTTACATTAACTTCAGAAACTG	32	54
HNF3β	ACCCCACGAATCTCAGCTGCA	CAAGGTAGCGCCATAAGGAGA	33	58
HNF6	CAGCACCTCACGCCCACCTC	CTTCCCATGTTCTTGCTCTTTCC	34	54
C/EBPα	CCCGTGCCCAGCCCTCAT	CACCTTCTGCTGCGTCTCCAC	34	57
STAT5b	TGATTATAGCGGCGAGATCC	ACCAGCTCATTTCCACCAAC	34	54
IGF-1	CACATCTCTTCTACCTGGCACTC	GGATGGAACGAGCTGACTTTGTA	25	53
β-Actin	CTGACCGAGCGTGGCTACAG	GGTGCTAGGAGCCAGGGCAG	23	62

42 °C for 2 min, at 42 °C for 50 min and were terminated by heating at 70 °C for 15 min and then chilled to 4 °C. PCR reaction mixture 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, 2 μl of cDNA, and 1 U of 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 72 °C for 1 min 30 s. Subsequently, the extension step was followed for 10 min at 72 °C. The β-actin mRNA was used as housekeeping gene to normalize the CYP mRNA content. The levels of mRNA were 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.

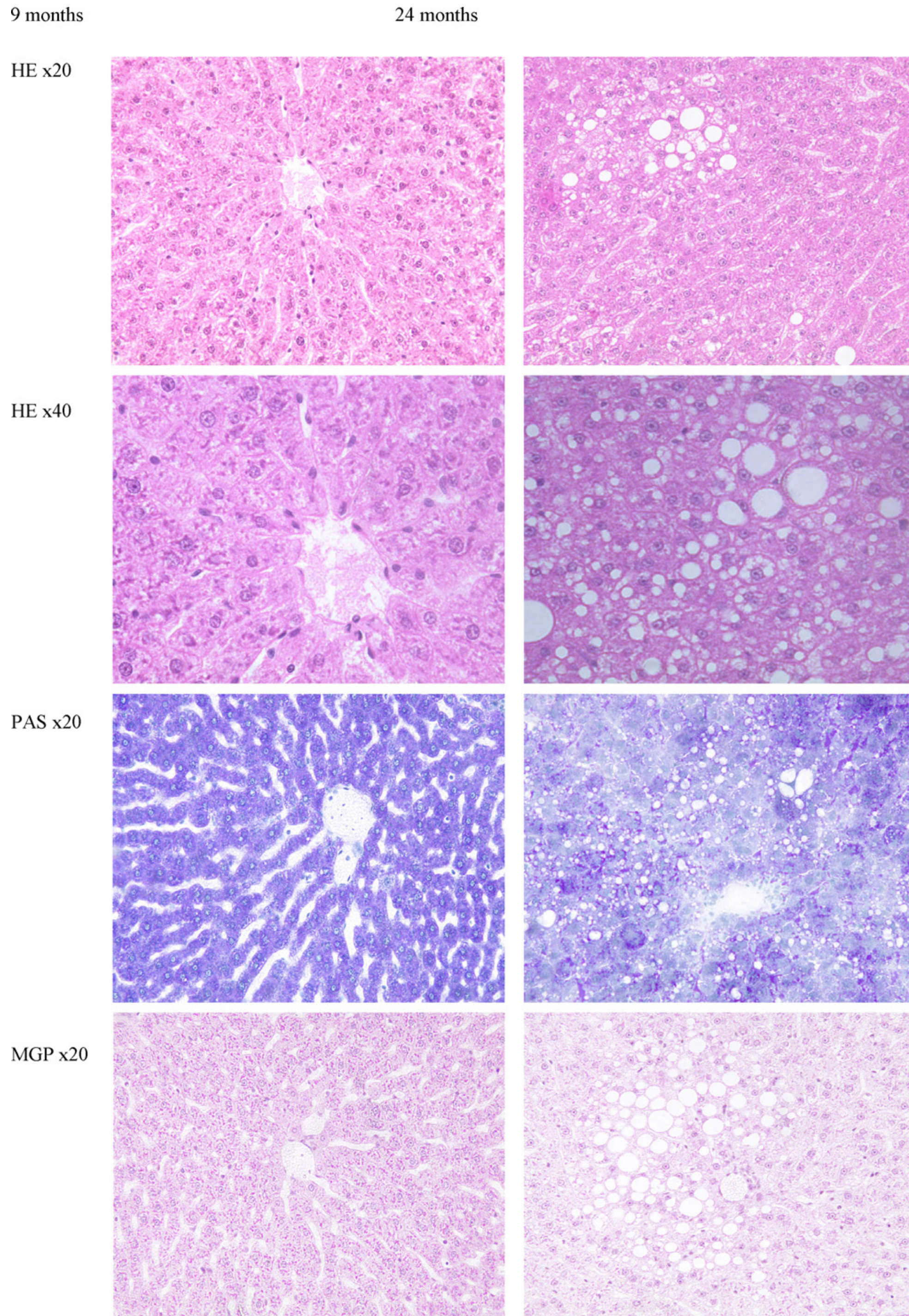

Fig. 1 – Histological pictures of the rat liver at 9 and 24 months. Histological sections were fixed in Carnoy's fixative, embedded in paraffin and stained with hematoxylin–eosin H/E (20×; 40×), PAS colouration (20×), and methylgreen pyronin (MGP) (20×).

2.10. Analysis of kinetic data

Kinetic data (Vmax and Km) were determined by the non-linear regression program WinNonlin (SCI, Software, Lexington, KY, USA) assuming single enzyme Michaelis–Menten kinetics.

2.11. Statistical analysis

Each group was composed of six rats. The results presented in the tables correspond to the mean ± S.E.M. Data were analyzed using *t*-test to determine the statistical significance among the two groups. The level of significance was set at $p < 0.05$.

3. Results

3.1. General histology

To characterize the experimental ageing rat model, a general morphological analysis of the livers from adult (9 months) and senescent (24 months) male Wistar rats was carried out. The hepatic cells have been reported to be increased in volume and variability in size in senescent rats. The general morphology was assessed by staining liver sections with hematoxylin/eosin (Fig. 1). In adult rats, we observed that the liver structure was well preserved while the histological sections from livers of senescent rats showed cells with a big number of

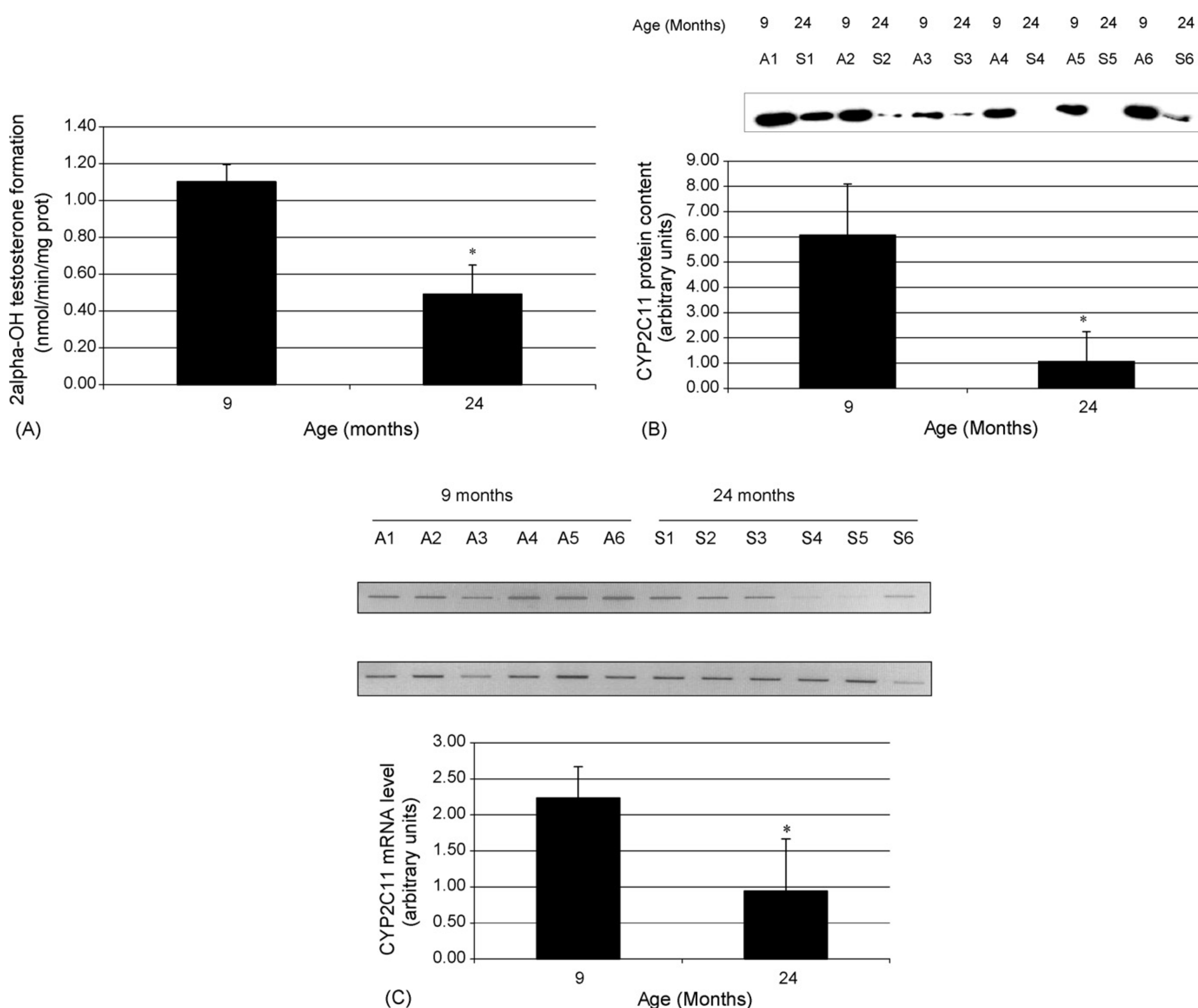

Fig. 2 – Effect of ageing on CYP2C11-mediated-2α-testosterone hydroxylation (a), amount of CYP2C11 proteins (b) and CYP2C11 mRNA levels (c). (a) Testosterone hydroxylation was quantified by recording (HPLC) the formation of 2α-OH-testosterone after 12 min incubation of rat liver microsomes at 37 °C. The values are expressed as Vmax (nmol metabolite produced/min mg protein). (b) The content of CYP2C11 protein was assessed by immunoblotting of CYP2C11 in rats of 9 and 24 months. Representative Western Blot showing CYP2C11 detection with polyclonal rabbit anti-rat CYP2C11 antibody. About 1.0 μg of liver microsomal protein was loaded per lane of adult (lanes 1, 3, 5, 7, 9, and 11) and senescent (lanes 2, 4, 6, 8, 10, and 12) rats. Values are expressed as arbitrary units obtained after densitometry of the respective electrophoretic bands. (c) The levels of CYP2C11 mRNA were evaluated by RT-PCR and values are expressed as arbitrary units obtained by densitometry after normalization to β-actin mRNA expression levels. (*) $p < 0.05$ as compared to animals 9 months old.

cytoplasmic lipid vacuoles (steatosis), a few number of pycnosis, and the presence of inflammatory cells. Sinusoid spaces appeared dilated and some necrotic zones around the periportal spaces were observed. Moreover, we found a weak increase in the number of binucleate hepatic cells with advancing age. A strong decrease of glycogen (as shown by PAS staining) was also observed in liver of senescent rats. Finally, both cytoplasmic and nuclear acidic material appears hypochromatic indicating a strong loss of RNA in senescent rats (methylgreen pyronin staining). These results confirm liver degeneration in senescent rats.

3.2. *Influence of age on the male specific CYP2C11 activity, protein and mRNA levels*

The CYP2C11 activity was measured by recording the oxidation of testosterone to 2-α-hydroxy-testosterone in liver microsomes prepared from adult (9 months) and senescent male rats (24 months). Fig. 2(a). shows that, in senescent rats, the rate of formation of 2-α-hydroxy-testosterone is significantly decreased by 55% ($p < 0.05$). No significant change in the value of Km for 2-α-hydroxytestosterone was observed indicating that the affinity of the enzyme for the substrate is unaffected by ageing (data not shown). Regarding the expression of CYP2C11, Fig. 2(b) and (c) Show that the age-related decrease of testosterone oxidation is well correlated to a significant decrease ($p < 0.05$) of 80% and 60% of CYP2C11 protein content (2B) and mRNA level (2C), respectively, in the liver of the male senescent rats.

3.3. *Influence of age on the female specific CYP2C12 mRNA levels*

Since no specific substrate reflecting the enzyme activity of CYP2C12 in male rats has been reported until now, we measured CYP2C12 mRNA levels in the liver of adult and senescent male rats. Fig. 3 shows that, in senescent male rats, an induction by about 10-fold, as compared to adult male rats, can be observed indicating that a feminization process is occurring.

3.4. *Influence of age on the female predominant CYP2C6 activity and protein levels*

After the experiments performed on both male and female specific isoforms, we were interested to know if the sex predominant CYP2C6 respond in a similar manner to ageing than the sex specific CYP2C11 and CYP2C12. To answer this question, we determined the activity and the protein of CYP2C6, a constitutive-predominant isoform expressed at an about 40% higher concentration in female compared to male liver [17,18]. The CYP2C6 activity was measured by recording the oxidation of diclofenac to 4-hydroxy-diclofenac in liver microsomes prepared from adult and senescent male rats. Both the rate of formation of 4-OH-diclofenac as well as the CYP2C6 protein content remained constant between 9 and 24 months (Table 2).

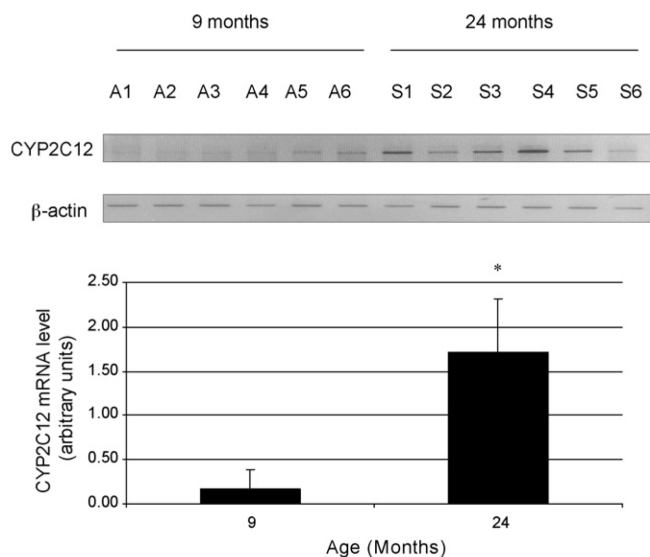

Fig. 3 – Effect of age on CYP2C12 mRNA levels. The levels of CYP2C12 mRNA were evaluated by RT-PCR and values are expressed as arbitrary units obtained by densitometry after normalization to β-actin mRNA expression levels. (*) $p < 0.05$ as compared to animals 9 months old.

3.5. *Influence of age on LETFs mRNA levels*

As previously reported, both CYP2C11 and CYP2C12 are regulated by various LETFs. Consequently, a change in their expression during ageing may have consequences on the expression of these CYPs. Accordingly, we measured the mRNA levels of HNF3β, HNF6 and C/EBPα in the liver of adult and senescent male rats (Fig. 4). Although the mRNA levels of HNF6 and C/EBPα remain relatively constant from 9 to 24 months, the mRNA levels of HNF3β are significantly increased by about 1.8-fold ($p < 0.05$) in livers of senescent rats.

Table 2 – CYP2C6 activity and protein content in liver of adult and senescent rats

	Adult rats	Senescent rats
Diclofenac hydroxylation activity (nmol/min mg protein)	0.69 ± 0.04	0.64 ± 0.15
CYP2C6 protein content (optical density units)	25.2 ± 4.3	25.4 ± 8.1

Diclofenac hydroxylation was quantified by recording (HPLC) the formation of 4-OH-diclofenac after 20 min incubation of rat liver microsomes at 37 °C. The values are expressed as Vmax (nmol metabolite produced/min mg protein). The content of CYP2C11 protein was assessed by immunoblotting of CYP2C6 in rats of 9 and 24 months. The CYP2C6 protein band was detected by using a polyclonal goat anti-rat CYP2C6 antibody. About 1.0 μg of liver microsomal protein was loaded per lane of adult and senescent rats. Values are expressed as arbitrary units obtained after densitometry of the respective electrophoretic bands.

3.6. *Influence of age and sex on basal expression and on activation of STAT5b*

Since STAT5b has been demonstrated to be a key mediator of the sexually dimorphic response of liver CYPs to GH, the hypothesis that a continuous secretion of GH in senescent male rats leads to a desensitization of the JAK2/STAT5 pathway during ageing was tested. To validate first our experimental approach, we carried out immunoblottings against STAT5b and phosphorylated STAT5 in female rats and compared the results to those obtained in male rats of the same age (3 months). Indeed, it has been reported that in females, where the GH is secreted in a continuous manner, the JAK2/STAT5 pathway is desensitised [20]. Fig. 5(A) shows that although STAT5b mRNA levels are significantly lower (by about 30%) in female compared to male rats (left panel), the liver homogenate STAT5b protein contents are similar in both male and female rats (right panel). As expected, a lower level of activated STAT5 was observed in nuclear extracts prepared from females in comparison to males (right panel). On the other hand, Fig. 5(B) shows that in adult and senescent male rats, similar amounts of STAT5b mRNA and protein content are observed. Regarding the activation of STAT5, large amounts of phosphorylated STAT5 are detected in some adult and senescent male rats (right panel), showing that the JAK/STAT pathway is not desensitized in the liver of senescent male rats. It should be noted that STAT5 becomes phosphorylated only if the rat was in a pulse of GH at the time of the rat was killed [21].

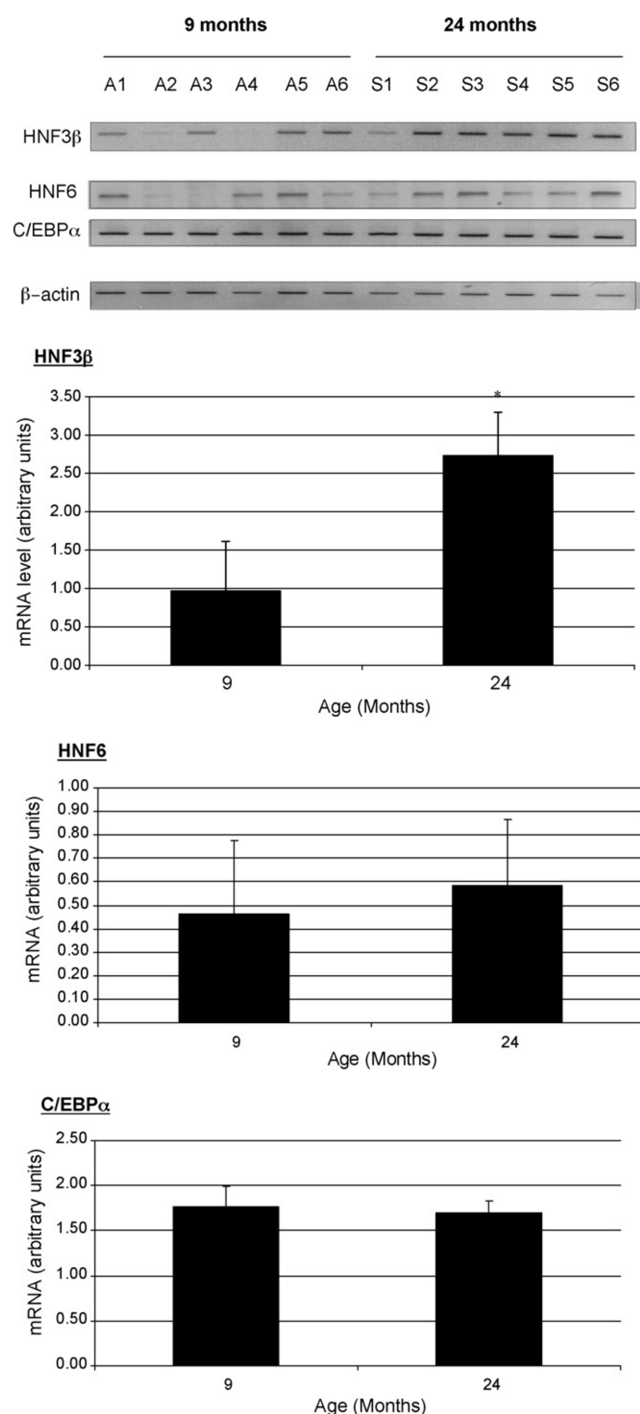

Fig. 4 – Effect of age on HNF3β, HNF6 and C/EBP mRNA levels in liver of adult and senescent male rats. The mRNA levels of HNF3β, HNF6 and C/EBP were evaluated by RT-PCR and values are expressed as arbitrary units obtained by densitometry after normalization to β-actin mRNA expression levels. (*) $p < 0.05$ as compared to animals 9 months old.

3.7. *Influence of age on IGF-1 mRNA level*

Since IGF-1 has been shown to be directly regulated by STAT5b, we measured the mRNA level of IGF-1 in adult and senescent male rats. As shown in Fig. 6, no change in the amount of IGF-1 mRNA was observed with ageing (1.5 ± 0.3 arbitrary units for adult rats versus 1.5 ± 0.4 arbitary units for senescent rats).

4. Discussion

To analyse the influence of ageing on drug metabolism, the experimental model of ageing rat was first characterized. In agreement with previous reports [33,34], histological sections of senescent male rats showed hepatocytes with typical senile morphology.

In the present study we showed a demasculinization of hepatic CYP enzymes. Indeed, we observed a drastic decrease in both the activity and the expression of the male specific CYP2C11. In addition, we also observed a feminization of the hepatic CYP enzymes. Indeed, we showed that CYP2C12, a gene which is only expressed in female rats, is also expressed in the liver of senescent male rats. These results are in agreement with previous findings reporting that hepatic isoforms of CYPs are demasculinized (suppression of male specific isoforms expression) and feminized (induction of female specific isoforms expression) in senescent male rats [8,9,13,35].

The demasculinization and feminization of CYP have been attributed to changes in the typical male GH secretion profile. Interestingly, although the lower pulse amplitude is the most dramatic change in the senescent GH profiles, it has been reported to have little relevance to the ageing-induced changes in CYP expression observed in male rats [13]. Indeed, even a 95% reduction of the pulse height in the episodic male

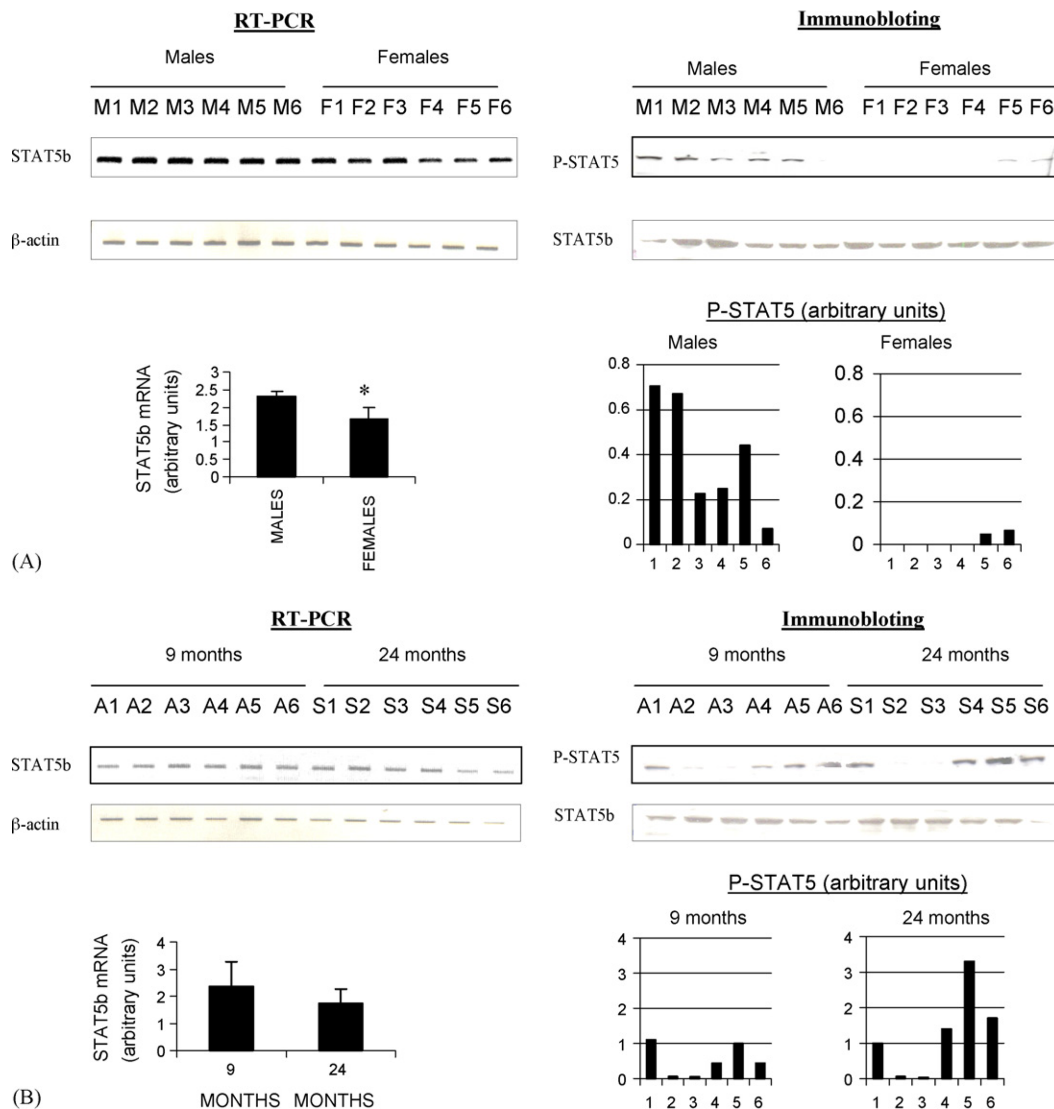

Fig. 5 – Effect of sex (A) and age (B) on STAT5b mRNA level, STAT5b protein content and STAT5 activation. (a) RT-PCR of STAT5b (left panel) and immunoblots of homogenate STAT5b and nuclear phosphorylated STAT5 (P-STAT5) (right panel) in liver of female and male rats of 3 months. Densitometric analyses are shown for both STAT5b mRNA and phosphorylated STAT5 electrophoretic bands. The STAT5b mRNA levels are lower in female compared to male rats (left panel), but the contents of STAT5b protein are similar in both male and female rats (right panel). (*) $p < 0.05$ as compared to males. (b) RT-PCR of STAT5b (left panel) and immunoblots of homogenate STAT5b and nuclear phosphorylated STAT5 (P-STAT5) (right panel) in liver of adult and senescent male rats. Densitometric analyses are shown for both STAT5b mRNA and phosphorylated STAT5 electrophoretic bands. Similar amounts of STAT5b mRNA and protein content are observed in adult and senescent male rats.

GH profile has been shown not to suppress normal levels of male specific CYP isoforms [18,36]. In addition, a similar or even greater decrease in the GH pulse amplitudes found in the senescent male rats have no inductive effects of female dependent CYP isoforms [18]. In contrast, Dhir and Shapiro [13] have shown that the changes of CYP expression are due to the loss of the GH-devoid interpulse due to a continuous secretion of just nominal levels of the hormone. In adult rats, normal male-like expression levels of CYP2C11, CYP3A2 and CYP2A2 are absolutely dependent on the free GH interpulse period whereas the expression of the female specific CYP2C12 is dependent on a continuous GH secretion. Accordingly, when GH is administered continuously in young male rats, the expression of the male specific isoforms is completely suppressed while the female specific CYP2C12 is expressed [17,37]. Moreover, it has been demonstrated that male specific CYPs are not only responsive to the free-GH period but also to its duration. Indeed, when the interpulse length approaches 2 h or less, male-specific CYP expression starts to decline [18]. These two major modifications in the GH secretion profile in senescent male rats may therefore contribute to the age-related changes in the expression of the CYP expression.

Regarding CYP2C6, a female predominant isoform [18], its expression is influenced but it is not dependent (like female

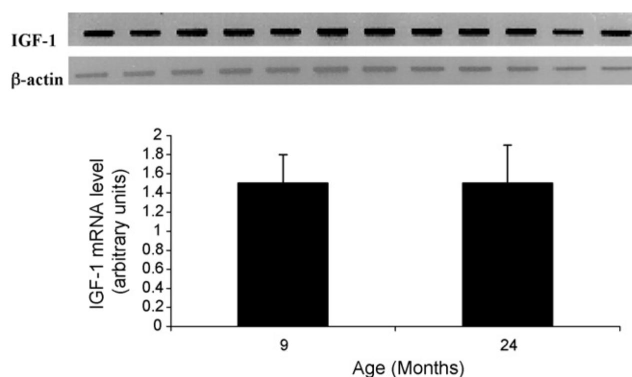

Fig. 6 – Effect of age on IGF-1 mRNA in liver of adult and senescent male rats. The levels of IGF-1 were evaluated by RT-PCR and values are expressed as arbitrary units obtained by densitometry after normalization to β-actin mRNA expression levels.

specific isoforms) on a continuous GH secretion. Therefore, its expression is more favourable to a more continuous secretion of GH even if pulses are of lower amplitudes. Accordingly, the expression of CYP2C6 should be increased in senescent rats. However, in agreement with a previous report demonstrating no age-associated change in the basal level of CYP2C6 mRNA [38], our results show that both the activity and the expression of CYP2C6 remained constant from 9 to 24 months. This suggests that CYP2C6 is less sensitive to changes in the GH secretion profile than sex-specific CYP isoforms.

Although the GH-related intracellular mechanisms involved in the regulation of the CYP genes remain unclear, they likely reflect the integrated actions of an array of liver transcription factors involving LETFs and STAT5 [19].Previous studies have shown that some LEFTs could play a role in the sex-dependent regulation of liver genes expression. For instance, a critical role of HNF4α has been reported in a HNF4α-deficient mouse liver [39]. Moreover, it has been shown that some HNFs, like HNF6 [23] or HNF3β [19,39], themselves regulated by GH, are also subject to a sexual dimorphism. In consequence, these HNFs could collaborate with STAT5b to mediate the GH-dependent activation of the expression of sexually dimorphic CYPs. For instance, GH-activated STAT5b has been shown to inhibit the synergistic action of HNF3β and HNF6 on the CYP2C12 promoter activation in vitro [26].

CYP2C12 expression has been reported to be up-regulate by several LETFs including HNF3β, HNF4α, HNF6 and C/EBPα [22–27]. We reported previously a drastic decrease of HNF4α expression with ageing [35]. The mRNA levels of both HNF6 and C/EBPα did not change in senescent rats but a strong increase in the level of HNF3β mRNA was observed. Since HNF3β has been shown to be regulated by GH [40] and, to be more expressed in female than in male rats [19], the feminization of the HNF3β expression in senescent male rats could be explained by the change of the GH secretion profile (more continuous secretion of GH like in females). Accordingly, the induction of CYP2C12 expression in the livers of senescent male rats may be, at least partially, due to the enhanced expression of HNF3β. However, the increase of HNF3β has only been observed at the level of mRNA, and the increase of the protein contents as well as of the binding activity to the CYP2C12 promoter remains to be proven.

Previous studies have reported a key role of the JAK2/STAT5 pathway in the GH regulation of CYP enzymes. Indeed, the importance of STAT5b for sex-specific expression of CYPs has been established by the loss of sexually dimorphic CYP gene expression seen in STAT5b-null mice [41–43]. Moreover, previous studies reported a desensitization of the JAK2/STAT5 pathway by the female pattern of continuous GH stimulation. Indeed, a high level of phosphorylated STAT5b during plasma GH pulse in adult male rats but not in female rats has been reported [16,20]. In fact, a continuous secretion of GH down-regulates the STAT5b pathway by enhancing the dephosphorylation of both STAT5b and the GHR-JAK kinase signaling complex [44].

Accordingly, we hypothesized that the continuous secretion of GH in senescent male rats may also desensitize the JAK2/STAT5 pathway and consequently bring a typical female P450 expression in the liver of senescent male rats. First, to validate our experimental approach, we carried out immunoblottings against STAT5b and phosphorylated STAT5 in female rats and compared the results to those obtained in male rats of the same age (3 months). We observed that although the female livers contain lower amounts of STAT5b mRNA than males, the protein contents are similar. In accordance with previous studies [16,20], we reported much lower activation of STAT5 in females compared to males, confirming the desensitization of the JAK2/STAT5 pathway.

After the validation of our method, we carried out the measurements in adult and senescent male rats. In senescent male rats, no significant changes in both STAT5b mRNA and protein contents were observed. Conversely to females, in senescent male rats the activation of STAT5 is still occurring. Indeed, large amounts of phosphorylated STAT5 have been observed in senescent male rats. The possibility that the phosphorylated STAT5 observed in nuclear extract prepared from senescent male rats corresponds to phosphorylated STAT5a (which could remain activated in senescent rats) and not to STAT5b (which would not be activated anymore) seems unlikely. Indeed, if the STAT5 protein bands observed in senescent rats correspond to the activated STAT5a, then we should also observe bands with the same intensity in females than those observed in senescent male rats. In addition, STAT5a is less expressed than STAT5b in the liver. Therefore, if STAT5b is not (or less) activated in senescent rats, and then the protein band detected in these animals correspond only to activated STAT5a, the intensity of these protein bands should be lower. In addition, the fact that the levels of IGF-1 mRNA, a gene directly regulated by STAT5b [45], do not change with ageing suggests also that STAT5b is always activated in senescent male rats.

According to this last finding, the decline of CYP2C11 cannot be attributed to a commensurate decline in STAT5 activation and nuclear translocation. Although the reason of such a discrepancy is not understood to date, some explanations can be proposed: (i) the decline of CYP2C11 expression could be due to a decrease of STAT5b binding activity and not to a decrease of its activation during ageing; (ii) STAT5b acts via an indirect mechanism to regulate a sex-specific liver gene.

Recent studies have suggested that STAT5b may regulate CYPs expression indirectly via the trans-activation of early response genes encoding transcriptional activators and repressors [46,47]; (iii) STAT5b is not involved in the male specific expression of CYP2C11 suggesting therefore involvement of another GH-dependent pathway. A recent study reported an inadequacy of the JAK2/STAT5 pathway to mediate episodic GH-dependent regulation of hepatic CYP2C11 [48]. These authors suggest a possible involvement of mitogen-activated protein kinase in episodic growth hormone regulation of CYP2C11 [48].

Further complementary studies on the role of the JAK2/STAT5b signalling pathway in the age-related decline of CYP2C11 are necessary to better understand the cellular mechanism of the demasculinization of cytochrome P450 enzymes in senescent rats.

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