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# Physiological factors affecting the expression of FMO1 and FMO3 in the rat liver and kidney

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**Abstract**

FMO1 and FMO3, the main FMOs described in the rat, are highly expressed in the liver and the kidney. The age, from 3 to 11 weeks, and gender-dependent expression of FMO1 and FMO3 in the rat liver and kidney were investigated. Based on the enzyme activities, protein levels and mRNA levels, this study demonstrates an important increase in the expression of the FMO3 in the liver of male rats during a period that corresponds to the acquisition of the sexual maturity. Rat liver FMO1 remains unchanged during this period of observation. The evolutions of both isoforms in the kidney of the male rat are similar to those observed in the liver. On the contrary, the important decrease in the total flavin-containing monooxygenase (FMO) activity observed in the liver of female rat is linked to a considerable decrease in the FMO1-dependent activity, FMO1 protein and FMO1 mRNA levels as a function of age. The expression of the FMO3 in the liver does not seem to be affected by the age of the female rat. Inversely, the expression of FMO1 in the female rat kidneys does not seem to be modified as a function of age while the expression of FMO3 is strongly increased. © 2002 Published by Elsevier Science Inc.

*Keywords:* Flavin-containing monooxygenase; Rat; Development; Expression; Liver; Kidney

## 1. Introduction

The microsomal flavin-containing monooxygenases (FMO) catalyze the oxidation of nucleophilic nitrogen, sulfur, phosphorus and selenium atomic centers present in a number of drugs, pesticides and other environmental chemicals [1,2]. FMOs exist as a multigene family with one member per family (for a total number of five families) designated FMO1–FMO5. Orthologous sequences share at least 80% of amino acid identity whereas homologous FMOs are 52–57% identical [3]. The expression of the different FMO isoforms in the same organ is now well documented [4]. FMO1 and FMO3 are the predominant isoforms expressed in the liver and kidney of several animal species [5–7].

In animal models, it has been suggested that the FMOs are possibly induced by nutrient substrates [8,9]. Evidence exists for hormonal and developmental control of FMO expression. In the rabbit lung, FMO2 has been previously shown to be induced during late gestation in pregnant animals, presumably as a result of glucocorticoids or progesterone plasma levels increase [10]. FMO2 is also induced in the rabbit kidney at about the time of parturition. Results obtained after experimental administration of these steroids in male or non-pregnant female rabbits suggest the involvement of glucocorticoids or progesterone to induce FMO2 in the lung, but only cortisol in the kidney [11]. In human beings, FMO1 is found in the fetal liver only and FMO3 is predominant in the adult liver. But FMO3 does not appear sex-dependent in the adult human liver [12,13]. In the mouse liver, FMO3 expression has been shown to be sex-dependent and expressed only in females [14]. In males, the repression of FMO3 expression appears to be due to testosterone [15].

There is compelling evidence in the rat that gender-related differences exist in hepatic microsomal FMO, with males usually showing higher imipramine (IMI) *N*-oxidase and thiobenzamide *S*-oxidase activities than females [16].

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*Abbreviations:* FMO, flavin-containing monooxygenase; IMI, imipramine; TMA, trimethylamine; kb, kilobases; RT-PCR, reverse transcription polymerase chain reaction; kDa, kilo-dalton; SEM, standard error of the mean.

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The male rat has overall higher levels of FMO protein than the female [17]. Other studies using gonadectomy and hormone replacement indicate positive regulation of FMOs by testosterone and negative control by estradiol in the rat liver [18]. Despite the increasing amount of data implicating hormones as the major regulating FMO metabolism, the precise mechanisms remain unknown.

At the time of these studies [16–18] the rat FMO1 was the only isoform fully described in the rat. Since these studies, the rat FMO3 was described for the first time by Moroni *et al.* [19] and completely characterized by Lattard *et al.* [20] and the expression of FMO5 was suggested by Cherrington *et al.* [21]. As a consequence, the purpose of this study was to determine more definitively the relationship between gender and development in the expression of FMO1 and FMO3 in rat liver and kidney by examination of catalytic activity, protein and mRNA levels. The measurement of FMO1 or FMO3 mRNA levels was achieved by the use of a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) assay.

## 2. Materials and methods

### 2.1. Chemicals

DNA polymerases, cDNA synthesis components were purchased from Promega. Oligonucleotides were manufactured by OligoExpress. Sequencing were performed by GenomeExpress. All other chemicals were of the highest reagent or electrophoresis grade commercially available.

### 2.2. Animals

OFA-Sprague–Dawley rats were obtained from a commercial breeder (IFFA-CREDO) and acclimated for a minimal period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature (22°). Rat food and water were available *ad libitum* until sacrifice. In order to study the effect of age, 20 male rats and 20 female rats, were divided in five groups (four male or female rats/group; 20–24 days; 36–39 days; 44–47 days; 54–61 days and >75 days). The rats were killed by decapitation. Liver and kidney were removed quickly, frozen in liquid nitrogen and stored at −80° until analysis.

### 2.3. Enzyme assays

Microsomes were prepared from liver, kidney samples by differential centrifugation as previously described [19]. Proteins concentrations were determined by the method of Bradford [22] using serum bovine albumin as a standard. Methimazole oxidation was measured according to the method of Dixit and Roche [23] at 412 nm. Sample and reference cuvettes contained a 1 mL reaction mixture of 0.1 M tricine/KOH, pH 8.5, 0.1 mM EDTA, 0.06 mM 5,5′-dithio-bis(nitrobenzoic acid), 0.2 mM dithiothreitol, 0.1 mM NADPH, 0.1–0.2 mg microsomal protein and 1.0 mM methimazole (sample cuvette only).

In order to evaluate the FMO1 and FMO3 parts, the inhibiting effects on methimazole oxidation of FMOs specific substrates (trimethylamine, 0.1 mM and imipramine, 0.5 mM) were performed. The substrates were added to sample and the reference cuvettes subsequent to the addition of methimazole (i.e. methimazole oxidation was monitored for 2 min first).

### 2.4. Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting were performed as described by Laemmli [24] and Towbin *et al.* [25], respectively. Microsomal proteins (10, 30 or 50 μg) were resolved by electrophoresis on a 10% polyacrylamide gel under denaturing conditions, electrophoretically transferred to polyvinylidene difluoride membrane (Bio-Rad) and blocked for 1 hr with a PBS (phosphate-buffered saline) buffer containing 5% milk. The membrane was incubated for 4 hr with rabbit polyclonal anti-rat FMO1 (1:1000) or anti-rat FMO3 IgG (1:1000). Following a washing sequence in PBS, alkaline phosphatase-linked antibodies anti-rabbit IgG were used as secondary antibodies and blots were visualized with an alkaline phosphatase conjugate substrate kit (Bio-Rad). Quantification was performed by densitometry using a scanner employing NIH Image version 1.61 software (public domain, Wayne Rasband, National Institutes of Health, available from the Internet).

### 2.5. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA were extracted from rat liver and kidney samples by using SV total RNA isolation system (Promega). Total RNA concentrations were evaluated spectrophotometrically from absorbance at 260 nm. The 260/280 ratios were between 1.8 and 2.0. The first strand cDNA template was synthesized from total RNA (two concentrations: 0.5 and 1.5 μg were used) in the presence of an oligo$(dT)_{18}$ (500 pmol) in 20 μL of standard reverse transcription buffer (50 mM Tris–HCl pH 8.3, 3 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol and 200 μM of each deoxynucleotide triphosphate). After an initial denaturation step at 70° for 2 min, 200 units of Moloney murine leukemia virus reverse-transcriptase RNAs H minus (MMLV-RT) (Promega) were added and the reaction was incubated at 37° for 90 min and then at 70° for 5 min. Reverse transcription of the polyadenylated RNAs from each organ was performed simultaneously on all the animals of one experiment.

Two microliter of resulting cDNA were amplified by PCR using specific primers of rat FMO1 or FMO3 designed from FMO1 (Genbank accession number M84719) or FMO3 (Genbank accession number AF286595) nucleotide

sequence, respectively. The sequence of the sense primer FMO1-S1 and the antisense primer FMO1-AS1 were (5′-TGTCAAGGGAAGCAAAGC-3′) and (5′-CCTGAATCA-AAGACTCGGC-3′), respectively. The PCR was performed using FMO1-S1 and FMO1-AS1 primers (50 pmol), *Taq* DNA polymerase (1.5 units, Promega) in 50 μL of 1.5 mM $MgCl_2$ PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100 and 200 μM of each deoxynucleotide triphosphate). The amplification was performed at 94° for 90 s, 27 cycles of 94° for 30 s, 56° for 45 s and 72° for 60 s, followed by a final extension at 72° for 5 min. The size of the amplified fragment was 293 bp. To control for differences in initial RNA levels and tube-to-tube variation in RT-PCR efficiency, a fixed amount (120 fg) of a non-homologous DNA fragment (mimic 1/3) containing the primer templates for both rat FMO1 and FMO3 was included in each PCR and co-amplified with the target cDNA. Mimic product length using FMO1-S1 and FMO1-AS1 primers was 447 bp. The amplified products were easily separated according to their size on 2% agarose gel stained with ethidium bromide.

The same strategy was performed with specific FMO3 primers. The sequence of the sense primer FMO3-S1 and the antisense primer FMO3-AS1 were (5′-GTGTTTTCCA-GACTTCCC-3′) and (5′-ATACCACCAGTCAGAAAT-3′), respectively. The PCR was performed in similar conditions, with the same amount (120 fg) of the same non-homologous DNA fragment (mimic 1/3). The amplification was performed at 94° for 90 s, 27 cycles of 94° for 30 s, 58° for 45 s and 72° for 60 s, followed by a final extension at 72° for 5 min. The sizes of the amplified fragment and mimic product, using FMO3-S1 and FMO3-AS1 primers, were 568 and 410 bp, respectively. The PCR products were separated on an ethidium bromide-stained 2% agarose gel.

The reverse transcription efficiency are controlled by the PCR amplification of a β-actin corresponding fragment.

Numeric photographs of the gels were acquired using a high performance CCD Camera (COHU®) and the Perfect Image 5.3 Software (Clara Vision). The relative intensity of the bands was quantified by Gel Analyst Software (Clara Vision). The target cDNA-to-mimic ratio was used as a relative estimate of mRNA abundance.

### 2.6. Nucleotide sequence determination

To confirm the specificity of the PCR amplification, the amplified products were sequenced by GenomeExpress. The sequences found were compared with published sequences of the rat FMO1 and the FMO3.

### 2.7. Statistical analysis

Data are presented as the mean ± 2 SEM obtained from four individual rats. Statistical analysis was done by using the multiple comparison of Kruskal–Wallis test, using StatView 5.0 software. Comparison between two groups was analysed by using Mann–Whitney test. $P < 0.05$ was the accepted level of significance.

## 3. Results

### 3.1. Catalytic activities

The presented activities are the parts of the total activity inhibited by either the imipramine or the trimethylamine. The imipramine is a known substrate for FMO1. Up to a concentration of 1 mM for imipramine, the methimazole *S*-oxidase activity of the purified FMO3 [19] or recombinant FMO3 [20] is not modified while the methimazole *S*-oxidase activity of the purified rat FMO1 is inhibited (decrease of 65%) by 0.5 mM imipramine [19]. The part of methimazole *S*-oxidase activity which was inhibited by imipramine (methimazole *S*-oxidase activity total—methimazole *S*-oxidase activity residual after imipramine's addition) is used as a catalytic index for FMO1 activity even though it represents only a part, about 65%, of the total FMO1 activity.

In the same way, the trimethylamine being a substrate for FMO3, the part of methimazole *S*-oxidase activity which was inhibited by trimethylamine (100 μM) (methimazole *S*-oxidase activity total—methimazole *S*-oxidase activity residual after trimethylamine's addition) is used as a catalytic index for FMO3 activity. Indeed, up to a concentration of 100 μM for trimethylamine the methimazole *S*-oxidase activity of FMO1 is not inhibited while it provokes a 45% inhibition of the dependent methimazole *S*-oxidase activity of the purified FMO3 [19]. This catalytic index was used as an estimate of the FMO3 activity although it represents only a part, about 45%, of the total FMO3 activity.

### 3.2. Semi-quantitative analysis of FMO1 and FMO3 protein abundance by western-blotting

Immunoblot analysis of recombinant rat FMO1 using rabbit polyclonal anti-rat FMO1 revealed the presence of a single immunoreactive protein which migrated at ∼56 kDa. The microsomal enzyme was recognized at the same molecular weight. Rabbit polyclonal anti-rat FMO3 weakly recognized the purified rat FMO1 [19]. By immunoblot analysis, the recombinant rat FMO3 and the microsomal enzyme cross-reacted with rabbit polyclonal anti-rat FMO3, at the same molecular weight (∼56 kDa). Rabbit polyclonal anti-rat FMO1 did not recognize the recombinant rat FMO3. Quantification of the stained bands was performed by densitometry. Fig. 1 shows the relation between the amount of purified recombinant rat FMO3 (Fig. 1A) [20] or liver microsomal protein (Fig. 1C) and the relative intensity of the detected signal. The relation between the relative intensity of the detected signal and the logarithm of the FMO3 amount (Fig. 1B) was linear ($r = 0.991$). A similar relation between the amount of purified FMO1 protein and the relative intensity

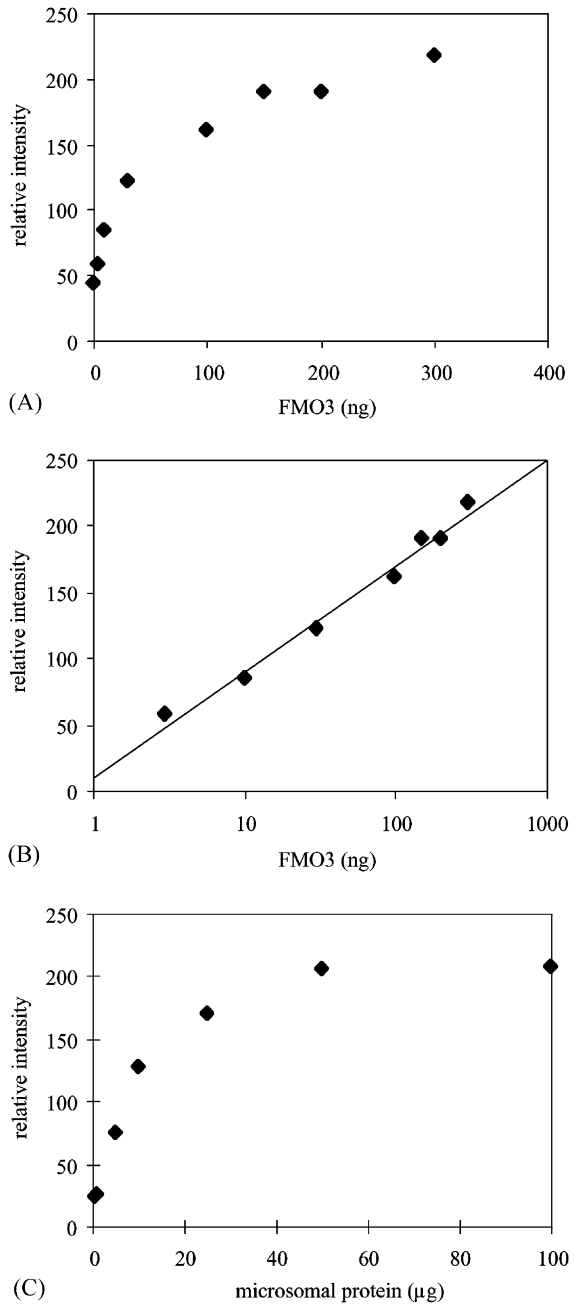


Fig. 1. Semi-quantitative analysis of FMO3 protein abundance by western blotting. (A) Relation between the amount of purified recombinant rat FMO3 and the relative intensity of the detected signal. (B) Log-linear relation between the amount of purified recombinant rat FMO3 and the relative intensity of the detected signal. (C) Relation between the amount of liver microsomal protein and the relative intensity of the detected signal.

of the detected signal have been observed with rabbit polyclonal anti-rat FMO1 (data not shown).

### *3.3. Semi-quantitative analysis of FMO1 and FMO3 mRNA abundance by RT-PCR*

The size of the FMO1 fragment amplified by PCR was 293 bp. Its nucleotide sequence was identical to the published rat FMO1 sequence [26]. To take into account

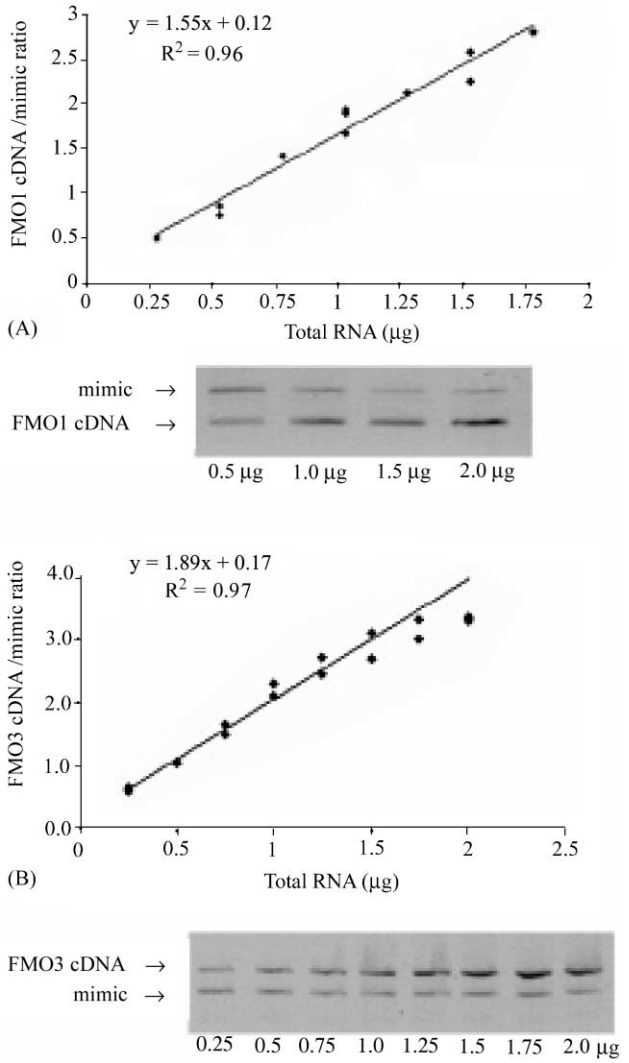


Fig. 2. Effects of (A) increasing amount of total RNA used in the reverse reaction on FMO1 cDNA-to-mimic ratio in the male rat liver and (B) increasing amount of total RNA used in the reverse reaction, on FMO3 cDNA-to-mimic ratio in the male rat liver.

tube-to-tube variation in PCR efficiency, and to allow comparison between FMO1 and FMO3, a unique mimic fragment (mimic 1/3) containing the primer templates for both rat FMO1 and FMO3 was co-amplified. Linear relations between the cDNA-to-mimic ratio and either the amount of FMO1 cDNA added in the PCR reaction (data not shown) or the amount of total RNA reversed (Fig. 2A) were observed. The reverse step did not introduce any marked bias in the assay. Furthermore the amplification of the mimic and the FMO1 fragment were, as a function of PCR cycles identical (data not shown). In spite of an observed competition between the mimic and the FMO1 fragment, these results clearly indicate that the FMO1 cDNA-to-mimic ratio may be used as a good estimate of the relative amount of FMO1 mRNA present in a tissue.

The same method was used to assess the relative FMO3 mRNA abundance. The size of the FMO3 fragment amplified by PCR was 568 bp. Its nucleotide sequence was identical to

the published rat FMO3 sequence [20]. The product size of the co-amplified mimic 1/3 was 410 bp. Linear relations between the FMO3 cDNA-to-mimic ratio and either the amount of FMO3 cDNA (result not shown) or the amount of total RNA reversed (Fig. 2B) were observed.

FMO1 and FMO3 mRNA are presented as the ratio cDNA-to-mimic. Reverse transcription were carried out for each RNA sample using 0.5 and 1.5 μg of total RNA. As a consequence two different cDNA-to-mimic ratios were obtained after PCR amplification. This was done in order to obtain a cDNA-to-mimic ratio in the linear range of the standard curve presented in Fig. 2B. Results are presented, and comparisons are realized, using the cDNA-to-mimic ratio obtained from a single quantity of total RNA reversed. The amplification of the mimic and the FMO3 fragment were, as a function of PCR cycles identical (data not shown).

### 3.4. Age-dependent expression of FMO1 and FMO3 in the liver and the kidney

Fig. 3 presents the evolution of total methimazole *S*-oxidase activity as a function of age in male and female livers and kidneys. In the male liver, total methimazole *S*-oxidase activity increased as a function of age (from $3.6 \pm 0.4$ nmol $min^{-1}$ $mg^{-1}$ to $4.6 \pm 0.4$ nmol $min^{-1}$ $mg^{-1}$). In the kidney of male rats total methimazole *S*-oxidase activity increased as a function of age ($2.7 \pm 0.7$ nmol $min^{-1}$ $mg^{-1}$ in the male rat aged 20–24 days) up to a maximum of $4.7 \pm 0.7$ nmol $min^{-1}$ $mg^{-1}$ in the male rat aged 44–47 days, and then it decreased again. In the female kidney, total methimazole *S*-oxidase activity progressively increased as a function of age (from $2.1 \pm 0.2$ nmol $min^{-1}$ $mg^{-1}$ to $4.4 \pm 0.5$ nmol $min^{-1}$ $mg^{-1}$). In the liver of females 20–24 days old, methimazole *S*-oxidase activity was very high ($9.3 \pm 0.88$ nmol $min^{-1}$ $mg^{-1}$). This activity decreased very strongly as a function of age (a decrease of 67%).

#### 3.4.1. Age-dependent expression of FMO1 and FMO3 in the male liver

Fig. 4 presents the evolution of FMO1 and FMO3 according to age in the livers of male rats. The catalytic index for FMO1 remained stable, while this for FMO3 increased significantly ($P = 0.04$, from 0.3 nmol $min^{-1}$ $mg^{-1}$ at the age of 20–24 days to 1.6 nmol $min^{-1}$ $mg^{-1}$ at the age of more than 75 days) (Fig. 4A). Western blotting

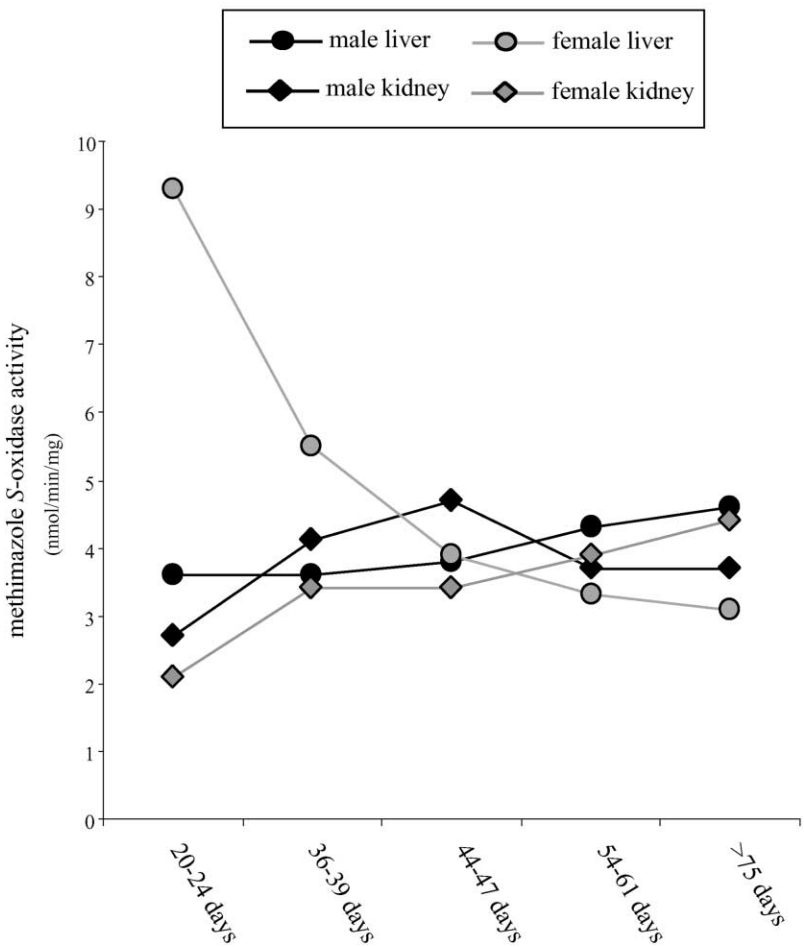


Fig. 3. Evolution of total methimazole *S*-oxidase activity as a function of age in male and female livers and kidneys. Each data point represents the mean for four animals.

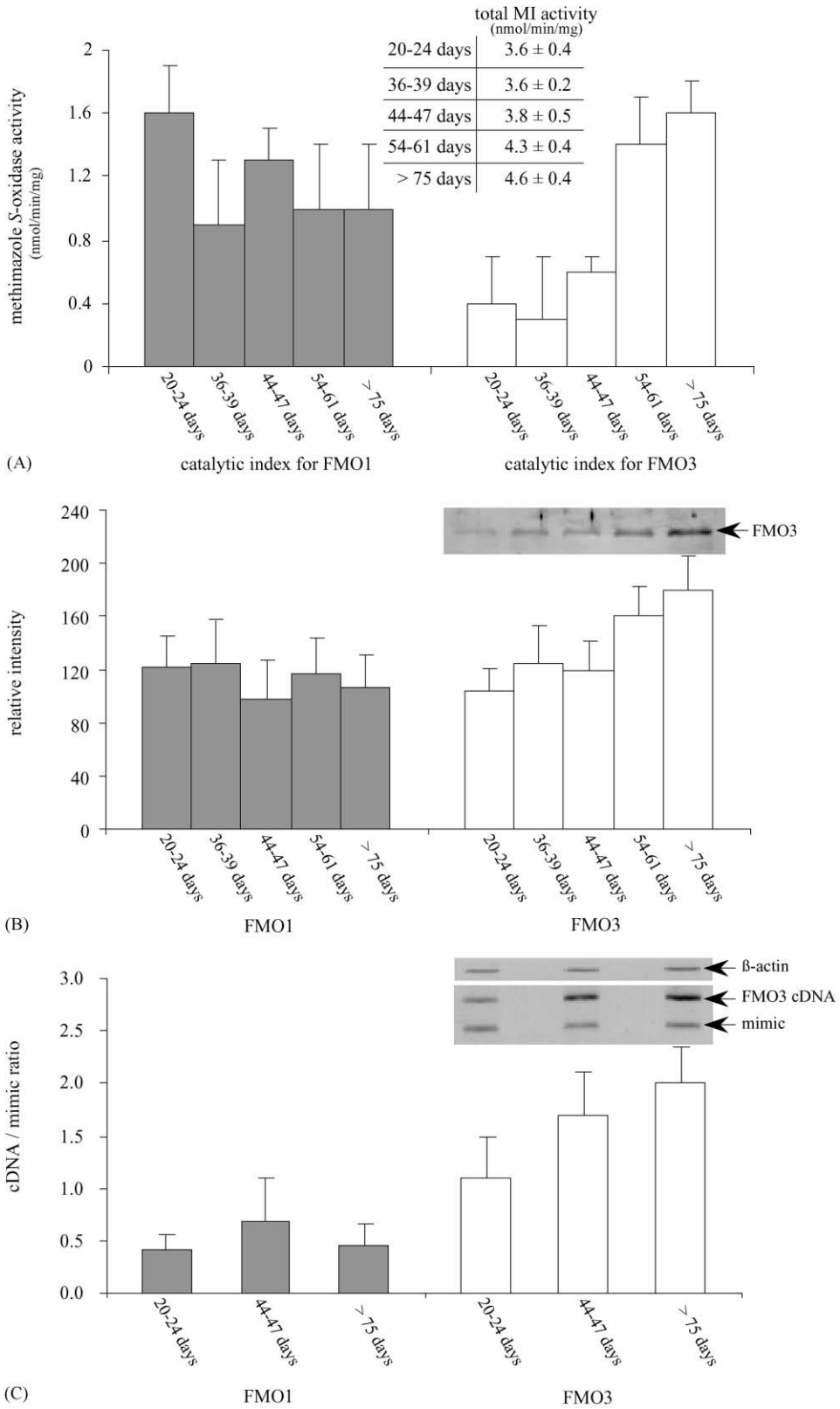


Fig. 4. Evolution of male rat liver FMO1 and FMO3 as a function of age. (A) FMO1 and FMO3 ($P < 0.05$) activity in male liver according to age. The presented activities are the parts of the total methimazole *S*-oxidase activity inhibited by either the IMI (catalytic index for FMO1) or the TMA (index for FMO3 activity). Each data point represents the mean ± 2 SEM for four animals. (B) FMO1 and FMO3 ($P < 0.05$) protein levels in male liver according to age. Protein levels were obtained using densitometry of immunoblots probed with antibodies raised against rat FMO1 or FMO3. Each data point represents the mean ± 2 SEM for four animals. (C) FMO1 and FMO3 ($P < 0.05$) mRNA levels in male liver as a function of age. FMO1 and FMO3 mRNA levels are estimated by the cDNA-to-mimic ratio, using densitometry of FMO1 or FMO3 amplified fragment and mimic product. The presented results are obtained from a reverse transcription of 1.5 μg of total RNA. Each data point represents the mean ± 2 SEM for four animals.

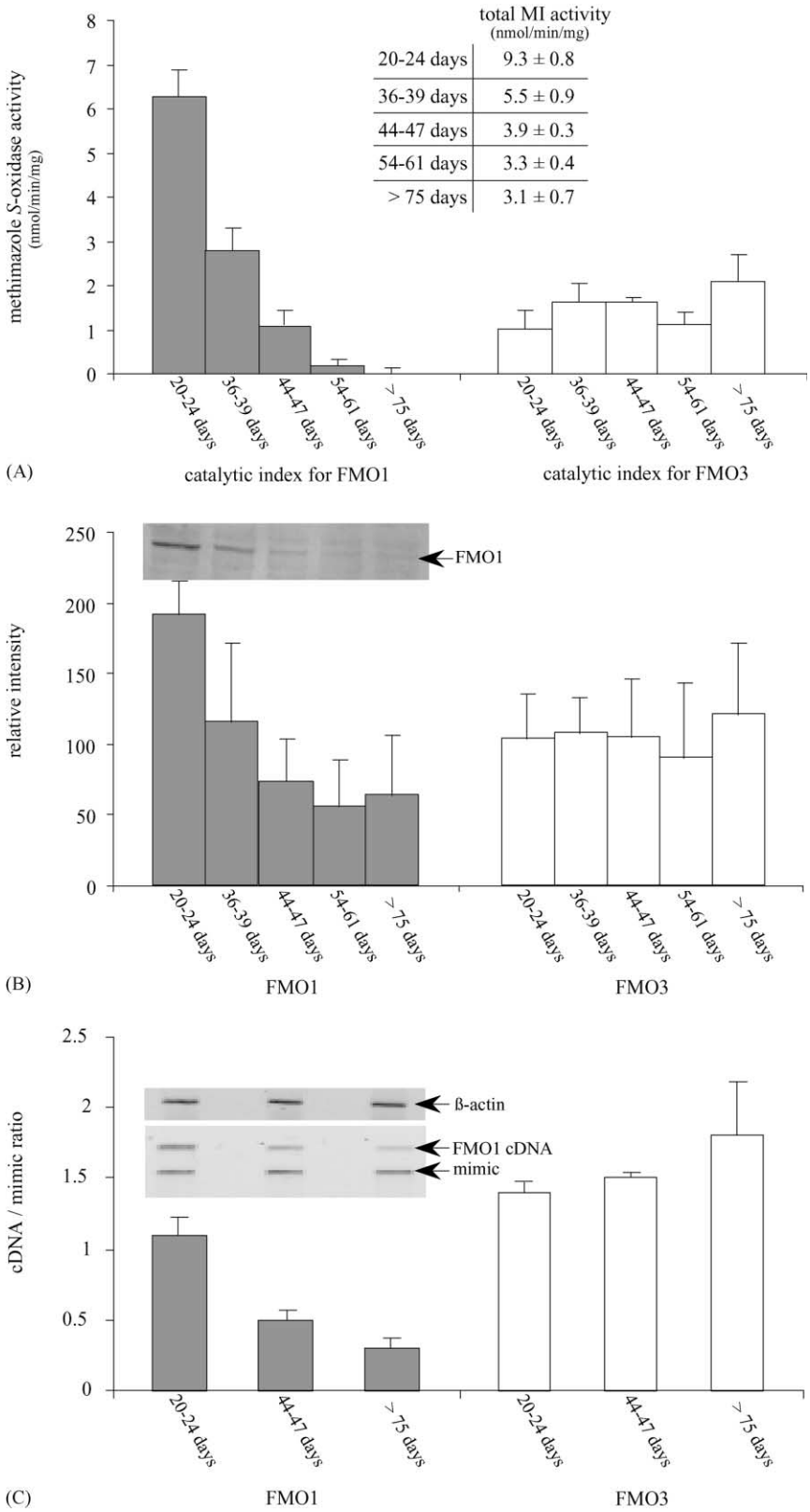


Fig. 5. Evolution of female rat liver FMO1 and FMO3 as a function of age. (A) FMO1 ($P < 0.05$) and FMO3 activity in female liver according to age. The presented activities are the parts of the total methimazole *S*-oxidase activity inhibited by either the IMI (catalytic index for FMO1) or the TMA (catalytic index for FMO3). Each data point represents the mean ± 2 SEM for four animals. (B) FMO1 ($P < 0.05$) and FMO3 protein levels in female liver according to age. Protein levels were obtained using densitometry of immunoblots probed with antibodies raised against rat FMO1 or FMO3. Each data point represents the mean ± 2 SEM for four animals. (C) FMO1 ($P < 0.05$) and FMO3 mRNA levels in female liver according to age. FMO1 and FMO3 mRNA levels are estimated by the cDNA-to-mimic ratio, using densitometry of FMO1 or FMO3 amplified fragment and mimic product. The presented results are obtained from a reverse transcription of 1.5 μg of total RNA. Each data point represents the mean ± 2 SEM for four animals.

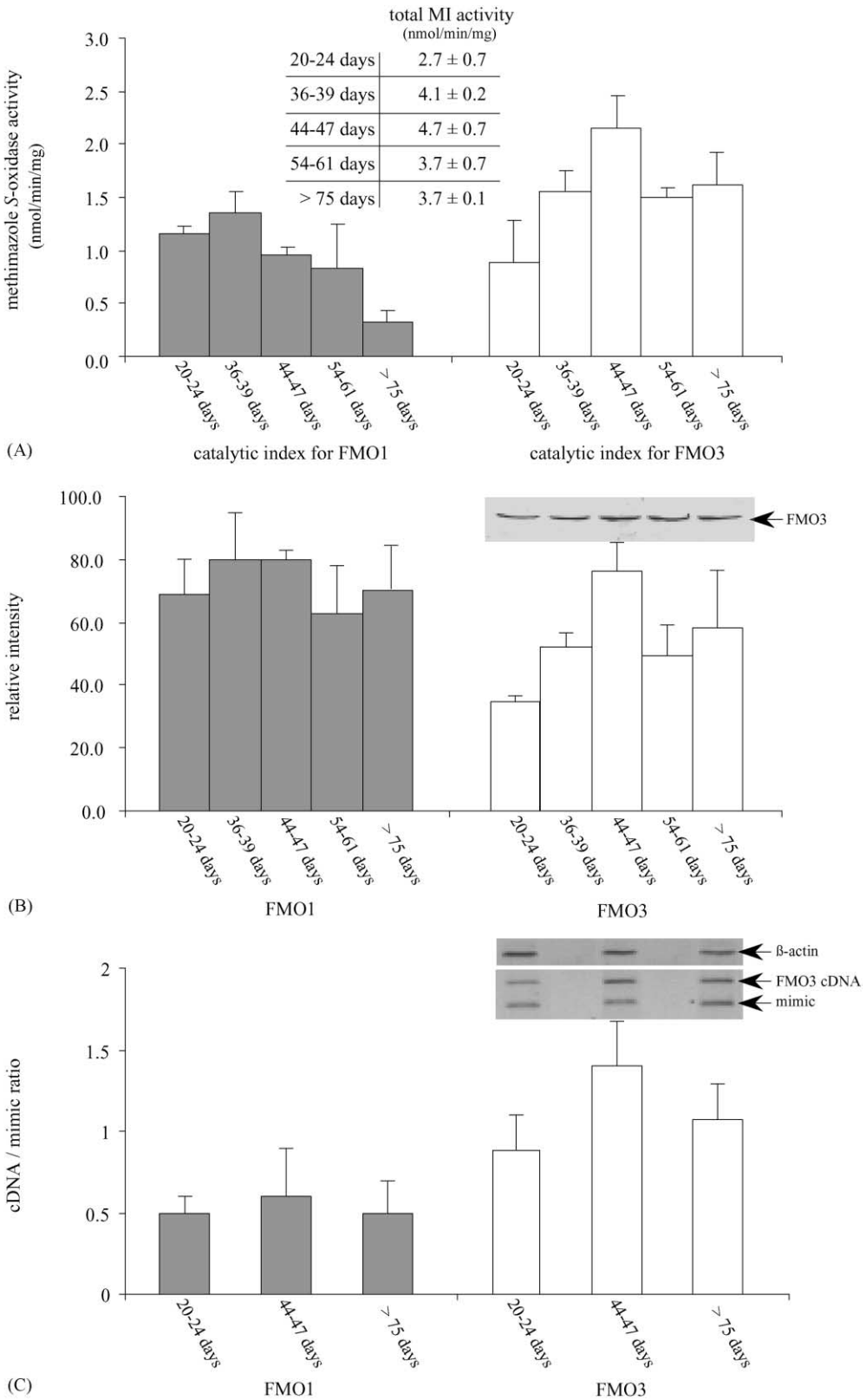


Fig. 6. Evolution of male rat kidney FMO1 and FMO3 as a function of age. (A) FMO1 and FMO3 ($P < 0.05$) activity in male kidney according to age. The presented activities are the parts of the total methimazole *S*-oxidase activity inhibited by either the IMI (catalytic index for FMO1) or the TMA (catalytic index for FMO3). Each data point represents the mean ± 2 SEM for four animals. (B) FMO1 and FMO3 ($P < 0.05$) protein levels in male kidney according to age. Protein levels were obtained using densitometry of immunoblots probed with antibodies raised against rat FMO1 or FMO3. Each data point represents the mean ± 2 SEM for four animals. (C) FMO1 and FMO3 ($P < 0.05$) mRNA levels in male kidney. FMO1 and FMO3 mRNA levels are estimated by the cDNA-to-mimic ratio, using densitometry of FMO1 or FMO3 amplified fragment and mimic product. The presented results are obtained from a reverse transcription of 0.5 μg of total RNA. Each data point represents the mean ± 2 SEM for four animals.

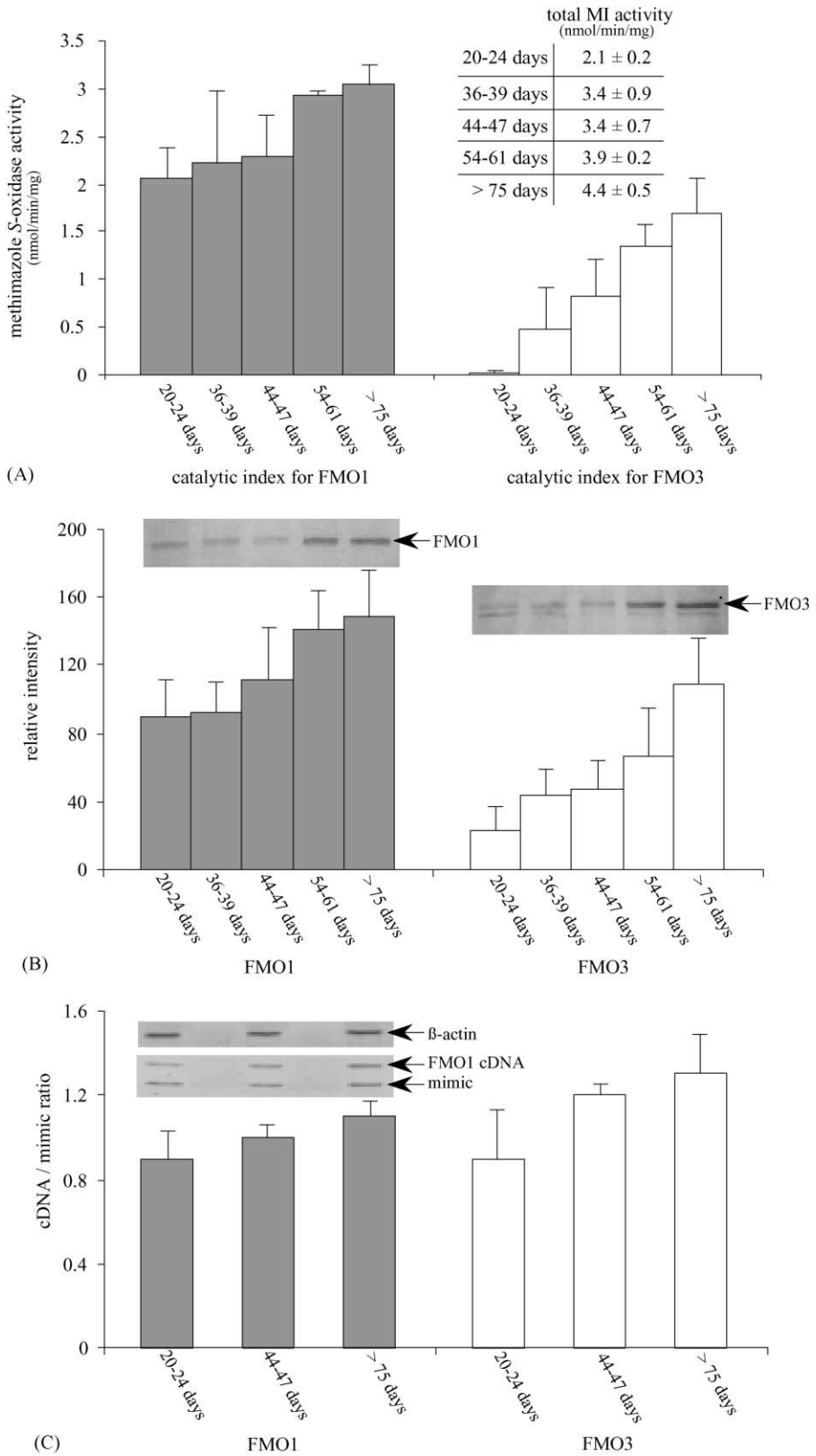


Fig. 7. Evolution of female rat kidney FMO1 and FMO3 as a function of age. (A) FMO1 and FMO3 ($P < 0.05$) activity in female kidney according to age. The presented activities are the parts of the total methimazole *S*-oxidase activity inhibited by either the IMI (catalytic index for FMO1) or the TMA (catalytic index for FMO3). Each data point represents the mean ± 2 SEM for four animals. (B) FMO1 and FMO3 ($P < 0.05$) protein levels in female kidney according to age. Protein levels were obtained using densitometry of immunoblots probed with antibodies raised against rat FMO1 or FMO3. Each data point represents the mean ± 2 SEM for four animals. (C) FMO1 and FMO3 ($P < 0.05$) mRNA levels in female kidney. FMO1 and FMO3 mRNA levels are estimated by the cDNA-to-mimic ratio, using densitometry of FMO1 or FMO3 amplified fragment and mimic product. The presented results are obtained from a reverse transcription of 0.5 μg of total RNA. Each data point represents the mean ± 2 SEM for four animals.

(Fig. 4B) and semi-quantitative RT-PCR (Fig. 4C) confirmed the increase of the FMO3 protein and the corresponding mRNA. Any significant difference in the evolution of the FMO1 protein and mRNA quantity was noted.

*3.4.2. Age-dependent expression of FMO1 and FMO3 in the female liver*

The catalytic index for FMO1 greatly decreased according to age while no evolution of the catalytic index for FMO3 was noted (Fig. 5A). Western blotting confirmed these evolutions (Fig. 5B) of FMO1 and FMO3. Furthermore, the FMO1 mRNA level strongly decreased (85% decrease) in the liver of the rats aged more than 75 days in comparison with the 20–24 days old rats. In contrast the level of FMO3 mRNA remained unchanged (Fig. 5C).

*3.4.3. Age-dependent expression of FMO1 and FMO3 in the male kidney*

The evolution of the FMO1 and FMO3 in the kidney of male rats according to age is presented in the Fig. 6. The catalytic index for FMO1 remained unchanged, while the corresponding index for FMO3 presented an apogee in male rats aged 44–47 days (Fig. 6A). Results obtained by western blotting confirmed these evolutions for FMO1 and FMO3 (Fig. 6B). Using RT-PCR (Fig. 6C), no significant variation was observed for the FMO1 mRNA level while FMO3 mRNA levels were maximal for 44–47 days old male rats. By using the calibration curve presented in Fig. 2B, a 100% increase was observed with 44–47 days old rats in comparison to 20–24 days old rats. The FMO3 mRNA level for the 75 days old rats is similar to the level observed with the 20–24 days old rats.

*3.4.4. Age-dependent expression of FMO1 and FMO3 in the female kidney*

The evolution of the FMO1 and FMO3 in the kidney of female rat according to age is presented in the Fig. 7. No significative evolution of the catalytic index for FMO1 according to the age was noted (Fig. 7A). Conversely, a very significant increase of the catalytic index for FMO3 was observed (from undetectable for 20–24 days old rats to 1.5 nmol $min^{-1}$ $mg^{-1}$ for rats aged more than 75 days). Using western blotting, a similar evolution was observed (Fig. 7B). The FMO1 mRNA level was not significantly modified while the FMO3 mRNA level increased significantly according to age (Fig. 7C).

## 4. Discussion

FMO1 was the first FMO isoform described in the rat and is usually considered as the main isoform in the rat liver. A second isoform, FMO3, which presented different catalytic properties, was described some years ago [19,27] and recently characterized [20]. A limited and sexually independent expression of FMO5 was recently evidenced in the rat liver by the use of an antiserum raised against a recombinant rabbit FMO5 [21]. In the fields of the pharmacology and the toxicology, 2 months old rats (200 g) which corresponds to the period of acquisition of sexual maturity are commonly used. Various authors show, in other animal species, that the expression of the FMOs is, dependent on the hormonal status. In order to estimate the expression of FMO1 and FMO3 in laboratory rats during the development, we set up a protocol taking into account a catalytic approach, an immunoquantification of these isoforms and a semi-quantitative method to assess FMO1 and FMO3 mRNA level.

The catalytic activity of the FMOs is measured using the method of Dixit and Roche [23] based on the oxidation of the methimazole, which is an excellent substrate for both the FMO1 and FMO3 ($K_m$ in the micromolar range) [19]. Taking into account that the expression of FMO5 seems to be low, and the methimazole a poor substrate for FMO5 ($K_m$ 3–10 mM) [28], the possible involvement of this isoform in the *S*-oxidation of methimazole should be very limited. The evaluation of the activity of each isoform is very difficult because of the absence of specific substrates or inhibitors. As a consequence we have used, in order to analyze the evolution of each isoform, a catalytic index which corresponds to the inhibition of the methimazole *S*-oxidase activity produced by selective inhibitors imipramine or trimethylamine in condition where no effect was observed towards the other isoform. Nevertheless, due to the absence of an accepted method for the specific measurement of the specific activity of FMO1 or FMO3, we have decided to analyze these indexes in the presence of the immunoquantification of the corresponding proteins and mRNAs only.

The immunoquantification of both isoforms is realized by two antisera, developed in the laboratory, against purified rat FMO1 and FMO3 [19]. The relative intensity of the signal is correlated to the log of the concentration of the purified FMOs or the quantity of microsomal proteins. This fact has to be kept in mind when the variations of the relative intensity of both isoforms are discussed. Furthermore, the important variations observed between various western blottings prevent direct comparisons between western blottings and even more so, comparisons between isoforms. Consequently, each graph is the result of either a single western blotting, or of a set of western blottings each containing a sample stemming from the various compared groups.

Validation of the RT-PCR assay was an important prerequisite of this study. It was carried out by first sequencing the amplified products (FMO1 and FMO3), which showed a perfect analogy with the published sequences for rat FMO1 and FMO3. The second validation was achieved by establishing the relation between the cDNA (FMO1 and FMO3) to mimic ratio on one hand with increasing amounts of liver or kidney cDNA and on other with increasing amounts of total RNA from liver and kidney. The linear relations between cDNA-to-mimic ratio and either the amounts of cDNA or the amounts of total RNA indicate that the

cDNA-to-mimic ratio may be used as a good estimate of the relative amount of FMO1 and FMO3 mRNA. The similar efficiency of the PCR amplification for the specific fragment and its corresponding mimic fragment associated with the use of a unique mimic containing the primer templates for both rat FMO1 and FMO3 allow the direct comparison of the concentration of the mRNA corresponding to these two FMOs. Errors due to the possible differences in the reverse transcription efficiency are controlled by the PCR amplification of a β-actin corresponding fragment.

The total activity of the FMOs in the liver of the male rat increases from 3 to 11 weeks. Das and Ziegler [29] also observed an increase of dimethylaniline *N*-oxidase activity in the liver of the male rat from 3 to 24 weeks. The FMO1-dependent activity and also the immunoquantification of the FMO1 and the quantification of the FMO1 mRNA remain unchanged during the period of observation. This is coherent with the study presented by Dannan and Guenguerich [18] which shows no evolution of the FMO1 immunoquantification between 4 and 14 weeks in the male rat. On the contrary, all the FMO3 dependent parameters studied, including the catalytic activity, immunoquantification and quantification of the mRNA, demonstrates an important increase in the expression of the FMO3 during the period of observation which corresponds to the puberty of the rat and to the beginning of the androgens secretion. This phenomenon is particularly surprising since the testosterone was found in mouse to be a powerful negative regulator for the expression of the FMO3 [30,15]. The FMO3 is, indeed, undetectable in the male mouse or in the castrated male mouse treated by testosterone while the expression levels observed in the female and in the untreated castrated male mouse are great [30,15]. The regulation of the FMO3 expression in the liver of these two rodents is thus totally different. From 2 months, the FMO3 activity is equivalent and further predominates over the FMO1 activity in the liver of the male rat.

The total FMO activity decreases in the liver of female rat according to the age. All the parameters studied, linked to the FMO1, decrease in a coherent manner. While the FMO1 activity is extremely important in the liver of the very young female (about 6 nmol $min^{-1}$ $mg^{-1}$), this activity becomes almost undetectable in females of more than 75 days. Dannan *et al.* [18] also observed a decrease, using immunoquantification, of the FMO1 (of about 50%) according to age. These authors showed a negative regulation of the FMO1 by the 17β-estradiol. They observed that a treatment with this hormone reduced the expression of the FMO1 in the female rat previously gonadectomized at birth. Interestingly, Coecke *et al.* [31] observed, using rat hepatocytes co-culture, a partial inhibiting effect of 17β-estradiol (56% of decrease) of the methimazole *S*-oxidase and a considerable decrease of the signal FMO1 in western blotting. Thus, *in vivo*, it seems coherent to think that the acquisition of sexual maturity, due to the secretion of estradiol, is responsible for a collapse of the expression of the FMO1. The important decrease in the FMO1 mRNA should be, at least in part, due to a transcriptional regulation of the FMO1 expression.

Inversely, the expression of the FMO3 does not seem to be affected by the age of the female rat. As a consequence, from the age of 45 days, the catalytic activity of the FMO3 becomes predominant. As a consequence, in both genders, FMO1 is the main catalyst in young animals and FMO3 becomes predominant after sexual maturity. In the female, this phenomenon is due to the collapse of the expression of FMO1 and in the male is linked to the increase of the expression of the FMO3. In the human such a situation is also described. FMO1 is expressed at high levels in the liver of the human fetus but is lacking in the adult liver [5] and FMO3, which is expressed at low levels in the fetal liver, is the predominant isoform in the adult human liver [6]. In the adult rat, as in the adult human but contrary to the mouse, there is no gender dependence for the expression of FMO3 in the liver.

Very few studies are dedicated to the renal FMOs even though the methimazole *S*-oxidase activities are equivalent to the hepatic activities. We had shown previously that FMO1 and FMO3 mRNA were present in the kidney of the rat [20]. No study evokes a regulation for the expression, in this organ, for one or other of these two isoforms. The evolutions of both isoforms in the kidney of the male rat are similar to those observed in the liver. The FMO1 remains unchanged and the level of expression for FMO3 increases but sooner than in the liver. By the 44th–47th day the activity and the mRNA are maximum. On the other hand, the expression of the FMO1 in the kidney of the female rat is not statistically modified according to age, while in the liver it collapses during the same period of observation. This demonstrates that the action of estrogens is totally different in these two organs as regards the expression of the FMO1. The regulation of the expression for FMO3 is also totally different because its expression is strongly increased in the kidney of the female rat as a function of age while in the liver no modification was observed.

In all the described situations we systematically observed a parallelism in the evolution of the mRNA, the protein and the corresponding activity. This was particularly observed in the liver for the FMO3 of the male rat and in the kidney for the FMO1 of the female. A transcriptional regulation, implying a positive effect of the testosterone for the FMO3 and a negative effect of the estradiol for the FMO1, is thus very likely. A post-transcriptional regulation is possible for example, for the expression of the FMO3 in the kidney, particularly in the female; where the variations, if they exist, of the mRNA are very limited with regard to the variations of the activity or the immunoquantification. For 20–24-day-old female rats, mRNA for the FMO3 is in great concentration while the protein or the corresponding activity are almost undetectable. In a similar way, the ratio FMO1 activity reported to the concentration of FMO1 mRNA is systematically more important in the

liver than in the kidney, and can also be explained by a more important instability of the protein in the kidney.

If we consider that FMOs are an important group of phase I xenobiotic-metabolizing enzymes and that the substrate specificities of FMO1 and FMO3 are different thus the variations in the expression of these enzymes should be of great pharmacological interest. Thus it becomes obvious that the metabolism and the pharmacokinetics of substrates of FMOs is gender-dependent and age-dependent in the rat.

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