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THE INFLUENCE OF AGEING ON THE INDUCTION OF THE mRNAs OF RAT LIVER CYTOCHROMES P450IIB1 AND P450IIB2

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Abstract—To investigate the influence of age on the regulation of the cytochromes P450IIB1 and P450IIB2 the levels of the messenger RNAs for these two cytochromes were determined in liver cytoplasmic RNA of rats of various ages after maximal induction with either phenobarbital or isosafrole and in untreated rats. The levels of these mRNAs were determined by solution hybridization with a RNA-probe (riboprobe system) complementary to both mRNAs. This study showed a marked decrease in the maximal induction levels of these mRNAs between the ages of 12 and 36 months irrespective of the type of inducer used. To assess whether this age-related decrease could be found for both individual mRNAs also solution hybridization experiments were performed with deoxyoligonucleotide probes of a defined sequence. The data presented in this paper show that ageing influences the levels of both the cytochrome P450IIB1 and P450IIB2 mRNA in a similar way. After induction the amount of mRNA for P450IIB1 was in all age groups measured four- to five-fold higher than that of P450IIB2. These data indicate that previously observed age-related changes in the cytochrome P450 system could be related to a lower accumulation of its mRNAs.

The function of the hepatic microsomal monooxygenase system includes the oxidation of exogenous compounds such as drugs and carcinogens. This process of enzymatic oxidation is generally considered as one of the most important steps in the biotransformation pathway [1, 2]. Cytochrome P450 serves as the terminal oxidase within the monooxygenase system and consists of a number of enzymes with partly overlapping substrate specificities. Age-related changes in microsomal oxidative enzyme activity, especially with lipophilic substrates, are observed. There is general agreement that this reflects changes in the cytochrome P450 system. The influence of ageing on the cytochrome P450 system in rats has been extensively studied (for reviews see Refs 3–5). Most reports show a general age-related decline in the cytochrome P450 content in rat liver microsomes and in enzymatic activities, although also age-related increases in cytochrome P450 activities have been reported [5, 6]. Age-related decreases in P450 activities are much more pronounced in male rats than in female ones, due to changes in gender-specific, constitutive forms of cytochrome P450, which in male rats are dependent on testosterone levels [7]. Up till now it is still not clear how these changes take place, but obviously they have enormous implications for treatment of the elderly patient [8].

As far as the inducible types of the cytochrome P450 system are concerned not many data are available with regard to the influence of ageing. Furthermore, to our knowledge only one study has been performed with respect to the influence of age on the messenger RNA levels of the cytochrome P450 system in rats [9].

In this paper, data are presented with regard to the influence of ageing on the levels of messenger RNA for cytochrome P450IIB1 and P450IIB2 (nomenclature according to Nebert *et al.* [10]) in male rats either untreated or maximally induced with either phenobarbital or isosafrole.

MATERIALS AND METHODS

Materials. Riboprobe Gemini vector pGEM4, RNase inhibitor (RNasin) and RNase-free DNase were obtained from Promega Biotec (Madison, WI). SP6-polymerase, S1-Nuclease, T4 polynucleotide kinase and Pvu II were obtained from Bethesda Research Laboratories (Gaithersburg, MD). RNase A and RNase T1 were obtained from Boehringer Mannheim (F.R.G.). [5-^{3}H]CTP (25 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from the Radiochemical Center (Amersham, U.K.).

Animals and treatment. Male BN/BiRij rats were maintained under "clean conventional" conditions as described by Hollander [11]. The average weight of the 3-, 12-, 24- and 36-month-old animals were 263 ± 12, 317 ± 23, 362 ± 21, and 376 ± 29 g, respectively (means ± SD). The rats were treated either with phenobarbital (60 mg/kg, administered intraperitoneally), isosafrole (150 mg/kg, administered intraperitoneally) once daily for 3 consecutive days, the last dose being given 16 hr prior to killing. Control rats were treated with corn oil. All rats were fasted overnight before killing.

RNA preparation. Total cytoplasmic RNA was prepared according to Shore and Tata [12]. For most experiments, the total cytoplasmic RNA preparation

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† Abbreviations: EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

was used without further purification. Poly(A)$^+$-RNA was isolated by two rounds of chromatography through oligo(dT)-cellulose.

cDNA probes and synthetic deoxyoligonucleotides. A cytochrome P450IIB1/IIB2 cDNA probe (R17) was obtained as a generous gift from Dr M. Adesnik and a mouse cytochrome P450IA2 cDNA probe (P_3-450) was obtained as a generous gift from Dr D. W. Nebert. The 1035 bp insert of the R17-clone [13] and the 1470 bp insert of the P_3-450 clone [14] were isolated and subcloned in a Riboprobe Gemini vector, pGEM-4 and pGEM-3, respectively, containing both a SP6-promotor and a T76-promotor.

Two specific deoxyoligonucleotides were synthesized and characterized as described by Omiecinski *et al.* [15]. The deoxyoligonucleotides used in this study had the following base sequences: 3'-d(AGTGTGGCCGATGGTTGG)-5' for P450IIB1 and 3'-d(AGAGTGTCCGGTGGTAGG-5' for P450IIB2 (differences in the sequence are underlined). Each deoxyoligonucleotide has identical A-T and G-C content.

Preparation of [^{3}H]-labeled complementary RNA probes. The Riboprobe pGem-4 vector containing the R17-insert was linearized with Pvu II and used as templates for *in vitro* run-off transcription reactions using the SP6-promotor; the pGem-3 vector containing the P_3-450-insert was linearized with HindIII and transcriptions were done using the T7-promotor. Transcription was performed in 50 μL volumes containing 40 mM Tris–HCl, pH 7.5/ 7.5 mM $MgCl_2$/ 2.5 mM ATP/ 2.5 mM GTP/ 2.5 mM UTP/ 50 units RNasin/ 10 mM dithiothreitol/ 1 μg linearized T7-polymerase plasmid template DNA/ 10 units SP6-polymerase/ and 30 μM [^{3}H]CTP ($\pm$25 μCi). The incubation was done for 1 hr at 37°. Following the RNA synthesis the DNA template was removed by adding RNase-free DNase to a final concentration of 1 unit/μg DNA. Incubation was performed for 15 min at 37°. The mixture was extracted with an equal volume of phenol:chloroform (1:1, v:v) and with an equal volume of chloroform. The RNA was recovered by addition of 1/10 volume of 3 M sodium acetate, pH 5.5 and 2.5 volumes of absolute ethanol (−20°). Using this protocol [^{3}H]cRNA was routinely obtained with a specific activity of 0.05–0.1 $\times$ 10^6 cpm/μg.

Solution hybridization experiments using [^{3}H]cRNA probes. Solution hybridization experiments were carried out in 5 μL sealed capillary tubes containing 80% formamide/ 40 mM Pipes, pH 6.7/ 0.4 M NaCl/ 1 mM EDTA/ 3000 cpm [^{3}H]cRNA and varying amounts of the RNA under study (for RNA from induced rats hybridizations were done for 2 hr with 0.01–5 μg RNA; for RNA from control rats hybridizations lasted 24 hr with 5–50 μg RNA). The reaction mixture was heated at 85° for 5 min and hybridized at 45°. After incubation, samples were diluted 100-fold in 10 mM Tris–HCl, pH 7.5/ 5 mM EDTA/ 300 mM NaCl/ 40 μg/mL RNase A/ 2 μg/ mL RNase T1 and incubated for 1 hr at 37°. tRNA (20 μg) was added as a carrier and hybrid formation was monitored by determining the percent of input [^{3}H]cRNA which was insoluble in 10% trichloroacetic acid. Trichloroacetic acid-precipitable material was collected on nitrocellulose filter and counted by liquid scintillation spectroscopy.

5'-End labeling of deoxyoligonucleotide probes. Synthetic deoxyoligonucleotides were 5'-end labeled with T4 polynucleotide kinase as described by Richardson [16] with minor modifications. Final reaction volumes of 15 μL contained 25 pmol of oligomer/ 66 mM Tris–HCl, pH 7.6/ 10 mM $MgCl_2$/ 1.7 mM Spermidine/ 5 mM dithiothreitol/ 125 μCi [γ-^{32}P]-ATP and 3 units T4 polynucleotide kinase. Incubations were performed for 1 hr at 37°, after which another 3 units of the enzyme were added for a further 60 min incubation. Reactions were stopped by addition of EDTA to 20 mM. Labeled oligonucleotides were separated from unincorporated label by chromatography os Sephadex G25. The specific radioactivities obtained this way was about 6 $\times$ 10^6 cpm/pmol and were identical for both the P450IIB1 and P450IIB2 probes.

Solution hybridization experiments using deoxyoligonucleotides. Solution hybridization experiments were carried with poly(A)$^+$-RNA in 5 μL sealed capillary tubes containing 0.75 M NaCl/ 0.2% SDS/ 4 mM EDTA/ 20 mM Tris–HCl, pH 7.5/ 2000 cpm [^{32}P]oligonucleotide/ and 0.01–20 μg of the RNA under study. Hybridizations were performed for 4 hr at 52°. After hybridization the samples were treated with S1-Nuclease (8 units/mL) in a buffer containing 0.75 M NaCl/ 2.8 M $ZnSO_4$/ 70 mM sodium acetate, pH 4.5/ 10 μg/mL single stranded salmon sperm DNA/ 5 μg/mL double stranded salmon sperm DNA for 1 hr at 37°. Hybrid formation was monitored by determining the percent of input [^{32}P]oligonucleotide which was insoluble in 10% trichloroacetic acid. Trichloroacetic acid-precipitable material was collected on nitrocellulose filter and counted by liquid scintillation spectroscopy.

Statistical evaluation. Statistical calculations were performed using Student's *t*-test with Bonferroni corrections. Values were considered to be significantly different if $P < 0.05$.

RESULTS

Cytochrome P450IIB1/IIB2 mRNA sequence content

The combined sequence of the cytochrome P450IIB1/IIB2 mRNAs were determined with solution hybridizations using a single-stranded [^{3}H]-labeled complementary RNA probe. Figure 1 shows the kinetics of the solution hybridizations using this probe. Log Rot-values, where 50% of the cRNA was hybridized were used to compare the specific mRNA content within different RNA fractions. The results of the relative content of the cytochrome P450IIB1/ IIB2 mRNAs with age are given in Table 1. In untreated rats, no influence of age on the levels of these mRNAs were observed. After treatment with either phenobarbital or isosafrole the levels of the cytochrome P450IIB1/IIB2 mRNAs were greatly elevated. The induction factor for these two inducers in 3-month-old rats was $\approx$500 and $\approx$250, respectively. With age the maximal inducibility of the cytochrome P450IIB1/IIB2 mRNAs by both phenobarbital and isosafrole was greatly reduced.

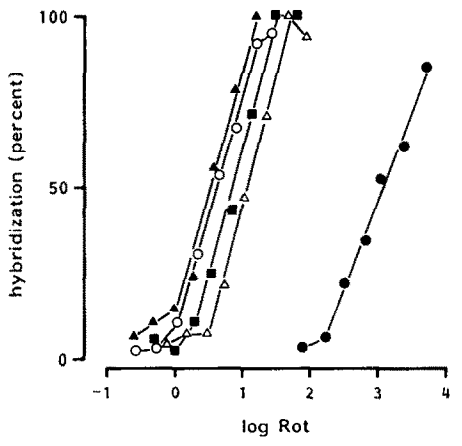

Fig. 1. Hybridization kinetics of different RNA fractions prepared from rat liver using [^{3}H]RNA complementary to cytochrome P450IIB1/IIB2 mRNAs. Solution hybridization experiments were carried out as described in Materials and Methods with varying amounts of hepatic total RNA. Expressed is the percentage of [^{3}H]cRNA probe hybridized versus the logarithm of the product of the initial RNA-concentration and the time of hybridization. The complementary RNA was hybridized to total cytoplasmic RNA from untreated 3-month-old rats (●), and to total cytoplasmic RNA from phenobarbital induced 3- (▲), 12- (○), 24- (■) and 36-month-old (△) rats.

Table 1. The combined sequence content of the cytochromes P450IIB1 and P450IIB2 mRNAs in rat liver with age

Age (months)	Control	Phenobarbital	Isosafrole
3	1	489 ± 91	247 ± 36
12	0.89 ± 0.24	472 ± 54	254 ± 32
24	0.93 ± 0.19	263 ± 60*	132 ± 20*
36	0.78 ± 0.26	147 ± 24†	69 ± 24†

Solution hybridization experiments were performed in 5 μL capillaries containing varying amounts of RNA and 3000 cpm [^{3}H]cRNA. All hybridizations were conducted for 2 hr except for the control RNA which was hybridized for 24 hr. Data were derived from the log Rot-curves as shown in Fig. 1 and were expressed relative to the 3-month-old control value. Values are expressed as means ± SD for six animals.

* Value differs significantly ($P < 0.05$) from 3- and 12-month values.

† Value differs significantly ($P < 0.05$) from 3-, 12- and 24-month values.

Starting from 12 months of age a gradual decline in the amount of these mRNAs ws observed up to 36 months of age with the levels of the cytochrome P450IIB1/IIB2 mRNAs in 3- and 12-month-old rats about three- to four-fold higher as compared to the 36-month-old rats.

Table 2 shows the influence of age on the levels of the cytochrome P450IA2 mRNA, which is not inducible by phenobarbital, in control and phenobarbital-treated rats. It can be seen that no age-related changes in the levels of this mRNA occur.

Table 2. The relative amount of cytochrome P450IA2 mRNA in phenobarbital-treated and untreated rat liver with age

Age (months)	Control	Phenobarbital
3	1	0.91 ± 0.21
12	0.85 ± 0.20	0.97 ± 0.13
24	1.03 ± 0.12	1.07 ± 0.32
36	0.96 ± 0.10	1.04 ± 0.24

Solution hybridization experiments were performed in 5 μL capillaries containing varying amounts of RNA and 3000 cpm [^{3}H]cRNA. All hybridizations were conducted for 24 hr. Data were derived from the log Rot-curves as shown in Fig. 1 and were expressed relative to the 3-month-old control value. Values are expressed as means ± SD for six animals.

Synthetic deoxyoligonucleotide probes

Specific synthetic deoxyoligonucleotide probes were used to discriminate between the cytochrome P450IIB1 and P450IIB2 mRNAs. These oligomeric 18-mer probes were tested in the solution hybridization conditions used in this paper to exclude cross-hybridization. The results of this test are shown in Fig. 2. Poly(A)$^+$-RNA from 3-month-old phenobarbital-treated rats was hybridized with radiolabeled P450IIB1 probe (Fig. 2; Panel A). No significant difference was observed when a 100-fold molar excess of unlabeled P450IIB2 oligomer was added, whereas the hybridization was enormously reduced with a 100-fold molar excess of unlabeled P450IIB1 oligomer. This experiment was repeated but with radiolabeled P450IIB2 as the oligomer probe and only a 100-fold molar excess of the unlabeled P450IIB2 oligomer caused a decrease in the hybridization signal (Fig. 2; panel B).

Relative sequence content of the individual cytochrome P450IIB1 and P450IIB2 mRNAs

Levels of the individual cytochrome P450IIB1 and P450IIB2 mRNA were determined by means of solution hybridization to the specific synthetic deoxyoligonucleotide probes. Using this method we were not able to detect cytochrome P450IIB1 or P450IIB2 mRNA sequences in untreated animals (data not shown). Table 3 shows the results obtained in induced animals. It was apparent that the mRNAs for both enzymes in all age groups examined are two-fold higher in phenobarbital-treated rats as compared with isosafrole treated animals. In addition, in all age groups after treatment with either of the two inducers the relative levels of the cytochrome P450IIB1 mRNA are four- to five-fold higher than those of the P450IIB2 mRNA.

DISCUSSION

Cytochrome P450 is an enzyme system which is thought to play an important role in the age-related changes in drug metabolism. A lot of literature data is available on the relationship between ageing and cytochrome P450 in rats and these data mainly con-

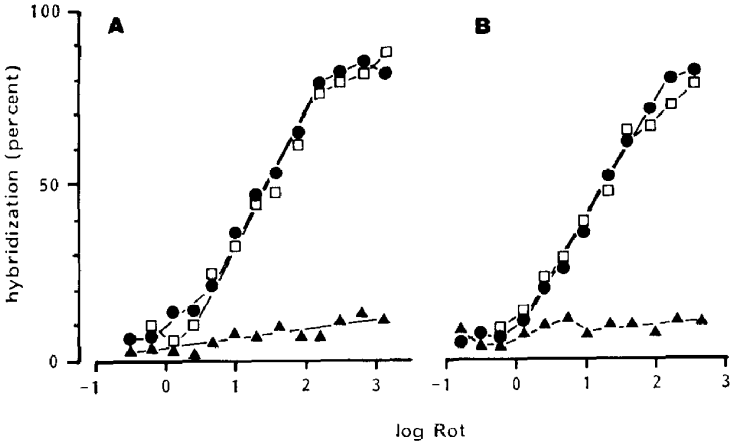

Fig. 2. Solution hybridization analyses with cytochrome P450IIB1 and P450IIB2 synthetic deoxyoligonucleotide probes. Solution hybridization with the deoxyoligonucleotide probes were carried out as described in Materials and Methods with varying amounts of poly(A)$^+$-RNA of phenobarbital-treated 3-month-old rats. Expressed is the percentage of [^{32}P]deoxyoligonucleotide probe hybridized versus the logarithm of the product of the initial RNA-concentration and the time of hybridization. Panel A, the poly(A)$^+$-RNA was hybridized to [^{32}P]P450IIB1 oligomer alone (●), to [^{32}P]P450IIB1 oligomer plus a 100-fold molar excess of unlabeled P450IIB2 oligomer (□), and to [^{32}P]P450IIB1 oligomer plus a 100-fold molar excess of unlabeled P450IIB1 oligomer (▲). Panel B, the poly(A)$^+$-RNA was hybridized to [^{32}P]P450IIB2 oligomer alone (●), to [^{32}P]P450IIB2 oligomer plus a 100-fold molar excess of unlabeled P450IIB1 oligomer (□), and to [^{32}P]P450IIB2 oligomer plus a 100-fold molar excess of unlabeled P450IIB2 oligomer (▲).

Table 3. The relative amount of the individual mRNAs for the cytochromes P450IIB1 and P450IIB2 in rat liver with age

Age (months)	Cytochrome P450IIB1		Cytochrome P450IIB2	
	Phenobarbital	Isosafrole	Phenobarbital	Isosafrole
3	100	47 ± 10	26.1 ± 3.2	12.3 ± 2.6
12	103 ± 11	51.0 ± 6.3	24.0 ± 5.3	13.1 ± 1.9
24	56.3 ± 7.2*	26.1 ± 4.7*	12.6 ± 2.4*	6.5 ± 1.3*
36	23.1 ± 6.4†	12.0 ± 5.0†	5.7 ± 1.1†	3.2 ± 1.0†

Solution hybridizations were performed in 5 μL capillaries containing varying amounts of poly(A)$^+$-RNA and 2000 cpm of the specific ^{32}P-labeled deoxyoligonucleotide probe. All hybridizations were conducted for 8 hrs as described in Materials and Methods. The level of the cytochrome P450IIB1 mRNA in 3-month-old phenobarbital-treated rats is arbitrarily set to 100. All other values are expressed relative to this value. Values are expressed as means ± SD for six animals.

* Value differs significantly ($P < 0.05$) from 3- and 12-month values.

† Value differs significantly ($P < 0.05$) from 3-, 12- and 24-month values.

cern the activity of the cytochrome P450 [3–5]. The majority of these studies report a decrease in the P450 activity with age, although in some cases no changes and even an increase in P450 activity were described.

Although a lot of research effort has been put in cytochrome P450 and ageing, very few data are available on the molecular regulation of cytochrome P450 with age. In this paper, the influence of age is described on two inducible cytochrome P450 mRNAs, P450IIB1 and P450IIB2 using fluid-hybridizations with a specific complementary RNA probe. This cRNA probe could not discriminate between the two mRNAs, due to their extensive nucleotide sequence homology (>97%) [17, 18]. No change with age was observed in the amount of the cytochrome P450IIB1/IIB2 mRNAs in untreated rats. Both phenobarbital and isosafrole were able to induce these mRNAs, but the level of induction decreased dramatically between 12 and 36 months of age. The amount of cytochrome P450IA2, a non-phenobarbital-inducible mRNA, did not change with age in phenobarbital-treated rats and in untreated animals, indicating that the observed age-related changes in the cytochrome P450IIB1/IIB2 mRNAs are a specific phenomenon. An age-related decrease in the amount of cytochrome P450IIB1/IIB2 mRNAs in phenobarbital treated rats was also observed by Richardson *et al.* [9], who observed a 50% decrease between 6 and 29 months of age.

Dilella *et al.* [19] used *in vitro* translations to determine the levels of these mRNAs in phenobarbital-treated rabbits and observed a decrease with age as well.

So far, no studies were performed to investigate whether the observed age-related decrease in P450IIB1/IIB2 mRNA content is specific for one or both mRNAs. To address to this question two synthetic deoxyoligonucleotides were used, that are able to discriminate between the P450IIB1 and P450IIB2 mRNAs, as described by Omiecinski *et al.* [15]. It could be seen that in the livers of rats of all age groups examined and after induction with either phenobarbital or isosafrole, the level of the P450IIB1 mRNA was always four- to five-fold higher than that of the P450IIB2 mRNA. From these data it can be concluded that ageing exerts a similar influence on both the cytochrome P450IIB1 and the P450IIB2 mRNA.

In conclusion, the levels of both cytochrome P450IIB1 and P450IIB2 mRNA do not change with age in untreated rats. The inducibility of these mRNAs, however, decreases between 12 and 36 months of age. Further investigations have to show whether these changes are a result of a changed transcription rate of the cytochrome P450IIB1 and IIB2 genes and/or of a changed turnover rate of these mRNAs.

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