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EFFECTS OF RP 52028 AND PHENOBARBITAL ON mRNA LEVELS OF INDUCIBLE AND CONSTITUTIVE SEX-SPECIFIC CYTOCHROME P450 ISOZYMES IN RAT LIVER*

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Abstract—Sex-related differences in basal levels of mRNA coding for various cytochrome P450 isozymes and their inducibility by 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (RP 52028) in comparison to phenobarbital (PB) were investigated in Sprague–Dawley rats. We observed that the inducible isozymes, namely cytochromes P450IIB1/2 and P450IIIA1/2 were barely detectable in non-induced animal livers. On the contrary, mRNAs coding for two constitutive forms of cytochrome, P450IIC7 and IIC11, were expressed at a high level in untreated rats in a sex-dependent manner. Cytochrome P450IIC11 mRNA was present in male rats only whereas P450IIC7 was expressed in both sexes but at a higher level in female rats. RP 52028 had a dose-dependent inducing effect on the P450IIB1/2 and IIIA1/2 isoforms in both sexes. After administration of a high dose (500 mg/kg), this molecule exhibited a pattern of induction similar to that of PB. Increases in the accumulation of these IIB1/2 and IIIA1/2 messengers were correlated with protein data, suggesting that RP 52028, like PB, induces these isozymes mainly through a pretranslational regulatory mechanism. On the other hand, PB and RP 52028 caused only a slight increase, less pronounced than in Wistar rats, in the mRNA level of the constitutive female-predominant P450IIC7, indicating a strain-related difference in inducibility of this isozyme. RP 52028 had no effect on P450IIC11 mRNA level in male rat liver, in contrast to the decreasing effect obtained with PB. Furthermore, the non-correlated changes in P450IIC11 mRNA level and microsomal testosterone 2α-hydroxylase activity after treatment with RP suggests that this molecule modulates the expression of P450IIC11 at a posttranscriptional level only.

Cytochromes P450 (P450§) are a super family of hepatic enzymes involved in a wide variety of monooxygenation reactions on endogenous compounds, as well as xenobiotics including numerous drugs [1]. Regulation studies have shown the induction of groups of individual isoforms of P450 by specific agents. This regulation by exogenous inducers allowed an initial classification of the inducible isozymes. However, protein sequencing data have resulted in a new classification based on the comparison of amino acid sequences. P450s have been classified into 13 gene families each one being subdivided into one or more subfamilies of which the major three are: IA, PAH-inducible P450s; IIB, PB-inducible P450s and IIIA, steroid-inducible P450s [2]. Besides these inducible isozymes, recent studies have focused on constitutive forms which metabolize endogenous substrates such as steroid hormones or vitamin D [3, 4]. These P450s are sex-specific isozymes responsible for the sex-related differences in drug metabolizing activities observed in the liver microsomes of several species, especially the rat [5]. The expression of constitutive P450s is regulated developmentally and controlled by the hormonal status of the animals [6–8]. These isozymes are either moderately responsive to, or repressed by the inducers previously evoked [9–12].

In a previous paper [13], we investigated the effects of 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (RP 52028), a new cytochrome P450 inducer, on liver microsomal monooxygenase activities. This xenobiotic is an antagonist of the peripheral benzodiazepine binding sites [14]. While used in clinical trials as an anticonvulsant and tranquilizer drug, it appears to be a potential inducer of the drug metabolizing enzymes. After repeated administration of RP 52028 in humans, an increase in its own metabolism rate (A. Uzan, personal communication), as well as in the urinary excretion of 6-β-hydroxycortisol [15], has been observed.

We reported that RP 52028 reproduces some of the hepatic effects of PB such as an important hepatomegaly, as well as a significant increase in liver weight and in microsomal protein and P450 content. RP 52028 was also demonstrated to be as potent as PB in inducing numerous P450 isozymes as shown by significant increases in related enzyme activities and by Western blot analysis [13].

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§ Abbreviations: P450, cytochrome P450; PB, phenobarbital; RP 52028, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl–0.015 M sodium citrate.

This compound also exhibited some sex-related differences in the induction of P450 enzymatic activities [13]. Therefore, RP 52028 could be considered to be a phenobarbital-like inducer and we may suggest that both molecules act through a similar induction mechanism. Although the mechanism by which phenobarbital exerts its inductive effect is still unknown, it is now well established that this induction process involves mainly an increased rate of gene transcription [16–18].

To confirm the PB-like inducing effect of RP 52028 and to investigate further the molecular events responsible for the induction of some particular forms of P450s, the steady state levels of the mRNAs coding for these isoforms were determined. Quantification studies were carried out by hybridization of liver total mRNA with the corresponding cloned cDNAs in both male and female rats. The effects of treatment with two different doses of RP 52028 on inducible isoforms, namely P450IIB1/IIB2 and IIIA1/IIIA2; and on sex-related isozymes such as P450IIC11, a male-specific form, and P450IIC7 which is expressed predominantly in the female; were studied in Sprague–Dawley rats of both sexes. The variations in mRNA levels were compared to those caused by phenobarbital treatment.

MATERIALS AND METHODS

Materials and reagents. 1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoleine carboxamide (RP 52028) was supplied by Rhône-Poulenc Santé (Centre de Recherches de Gennevilliers, France). Phenobarbital (PB) was purchased from Fluka (Buchs, Switzerland). [α-^{32}P]dCTP (3000 Ci/mmol) and Hybond-N nylon membrane (for Northern blot) were from Amersham International (Amersham, U.K.). Nitrocellulose used for slot blot was from Schleicher and Schüll (Dassel, F.R.G.). Autoradiography was carried out on X-Omat AR Kodak films. All other chemicals were of the finest grade available.

Animals and treatments. Male and female Sprague–Dawley rats (180–200 g) were obtained from Iffa-Credo (St Germain sur l'Arbresle, France). RP 52028 in sucrose solution was administered by gastric intubation at a daily dose of 110 mg/kg body weight (RB group) and 500 mg/kg body weight (RP group) for 5 days. PB, dissolved in saline, was administered intraperitoneally to rats at a daily dose of 80 mg/kg body weight for 5 days. Animals were killed on the sixth day following 18 hr of starvation. Livers were excised as quickly as possible, frozen immediately in liquid nitrogen, and kept at −80° until used for RNA extraction or preparation of microsomes.

RNA isolation. The frozen livers were pulverized and the total RNA was extracted using the following protocol: 1 g of tissue was homogenized using an Ultraturax in 20 mL of 10 mM sodium acetate, pH 5.0, buffer containing 6 M urea, 3 M lithium chloride, 200 μg/mL heparin and 0.1% SDS (w/v). After one night at 4°, the RNA was pelleted by centrifugation at 10,000 *g* for 20 min and washed three times in an 8 M urea–4 M lithium chloride solution. The pellet was further dissolved in 10 mM sodium acetate (pH 5.0)–0.1% SDS and extracted with phenol once at 65°, then twice at room temperature. RNA was recovered by ethanol precipitation. Poly(A)$^+$RNA was isolated using the mRNA Purification Kit from Pharmacia (France). All RNA preparations were quantitated by spectrophotometry as described by Maniatis *et al.* [19].

Slot blot. Serial dilutions of RNA in water were denatured in an equal volume of formaldehyde/20 × SSC (2v/3v) and applied to nitrocellulose membrane through the Biorad slot blot apparatus. The membrane was vacuum-dried at 80° for 2 hr.

Northern blot. Total RNA was denatured and electrophoresed through formaldehyde 1.2% (w/v) agarose gels as described by Lehrach *et al.* [20]. The RNA was transferred further by capillary blotting to Hybond nylon membrane in 20 × SSC overnight, as described by the manufacturer. The membranes were then fixed by exposure to UV (312 nm) for 5 min. Molecular size markers purchased from BRL (0.24–9.5 kb RNA Ladder) were run concurrently on gels, transferred and visualized by staining the filter with methylene blue, as described by the manufacturer.

Probes. Probes used in this study were the pB7 insert (a 1.7 kb near-full length P450IIB1 cDNA) [21], the pDex 12 insert (a 1.0 kb 3′ end of a P450 IIIA1 cDNA) [22], the p7C12 insert (a 1.2 kb fragment of the P450IIC7 cDNA) [7] and the p(M-1)-3 insert (a 1.6 kb near-full length P450IIC11 cDNA) [23]. All these cDNA probes were from the rat. cDNA inserts were ^{32}P-radiolabelled using Pharmacia random primer Oligolabelling Kit to a specific activity of 5×10^8–5×10^9 dpm/μg of DNA. The probes were purified further from unincorporated labelled nucleotides by gel filtration chromatography on a Nick column (Pharmacia-LKB).

Prehybridization, hybridization and washing conditions. Nylon sheets from Northern blots or nitrocellulose slot blots were prehybridized in a solution of 50% formamide, 5 × SSC, 0.1% SDS and 5 × Denhardt's reagent containing 200 μg/mL herring sperm DNA at 42° for 4 hr. The radioactive probes were denatured by heating at 100° for 5 min, chilled on ice and added to a fresh hybridization solution. Hybridization was performed at 42° overnight. The filters were washed twice in 2 × SSC–0.1% SDS, at room temperature for 5 min, followed by two washes in a 0.1 × SSC solution at a temperature varying between 25 and 65°, depending on the cDNA probes.

Autoradiography was carried out at −80° with Kodak X-Omat AR films in the presence of Dupont Cronex II intensifier screens. The same blots were hybridized subsequently with different cDNA probes. For removal of the probes, nylon membranes were washed with a boiling solution of 0.1% SDS and nitrocellulose sheets were washed in Tris–HCl 5 mM, pH 8.0 containing 0.1 × Denhardt's reagent, 0.05% sodium pyrophosphate and 0.2 mM EDTA, for 1 hr at 65°. Filters were monitored for background radiation by autoradiography.

Quantitative and statistical analysis. Autoradiograms were scanned on a computerized image-processing system (Histopericolor, Numelec,

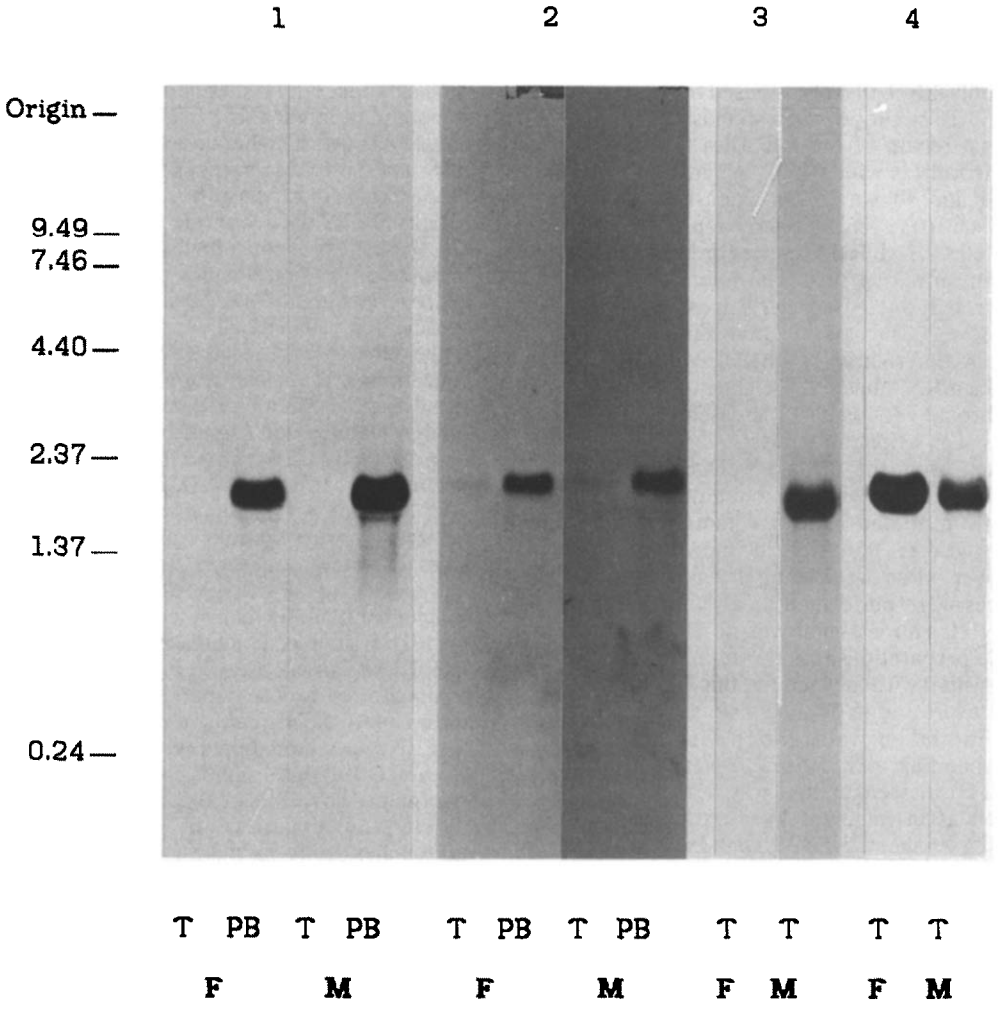

Fig. 1. Assessment of signal hybridization specificity. Total liver RNA (30 μg) extracted from untreated (T) and PB-treated (PB) male (M) and female (F) rats was analysed by Northern blot hybridized with ^{32}P-labelled probes; (1) pB7, (2) pDex12, (3) p(M-1)-3 and (4) p7C12 specific for P450IIB1/2, P-450 IIIA1/2, P450IIC11 and P450IIC7, respectively. Molecular weight markers in kb are indicated on the left. After washing, filters were autoradiographed overnight at −80°.

Biocom). For each probe, male and female rat liver mRNAs were quantitated on an autoradiogram obtained from the same X-ray film exposure. Depending on the time exposure, the dynamic range of the X-ray film was limited at either end of the plot of the loaded RNA amount versus densitometric scan value. Therefore, only two out of the three dilution values that were in the middle region of the hybridization calibration curve were used for calculation. Results are reported as arbitrary units of signal intensity and were tested for significance by the Mann–Whitney U Test.

Testosterone 2α-hydroxylation assay. Liver microsomes were prepared according to the method described previously [13]. Testosterone 2α-hydroxylation was assayed in 0.05 M Hepes buffer (pH 7.6), 15 mM $MgCl_2$, 0.1 mM EDTA, 5 mM glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, 0.5 mM NADP, 60 μM testosterone and 0.5 mg microsomal proteins, in a final volume of 2 mL. After incubation at 37° for 5 min, the reaction mixture was placed on ice for 5 min and the metabolites were extracted using a Sep-Pak C-18 cartridge. Separation of metabolites was performed by HPLC on a Superspher 100 RP-18 column by different isocratic systems described elsewhere [24].

RESULTS AND DISCUSSION

Specificity of the hybridization signals

Prior to quantification experiments, Northern blot analysis of mRNA preparations was performed to assess the specificity of the hybridization signals (Fig. 1). The pB7 cDNA probe strongly detected a 2.0 kb species in RNA preparations from PB-treated male and female rats which corresponded to both cytochrome P450IIB1 and P450IIB2 mRNAs. A slight band was detected in untreated rat livers which

may represent P450IIB2 only, whose gene is constitutively expressed in untreated rats, although at a very low level [25]. To examine the P450IIIA subfamily mRNA levels, we used the pDex 12 cDNA probe. This probe corresponds to the 3′ OH terminal region of the P450IIIA1 mRNA but also crosshybridizes with P450IIIA2 mRNA, which was cloned and shown to be extensively homologous with P450IIIA1 [26]. As shown in Fig. 1, the probe revealed a 2.1 kb RNA species present at a low basal level in untreated rats. The intensity of the signal was strongly increased in PB-treated animals from both sexes, which is in good agreement with the findings of Wrighton *et al.* [22], who demonstrated that the mRNA level of the glucocorticoid-inducible cytochrome P450 is also increased by non-steroidal agents such as phenobarbital.

When using the p(M-1)-3 cDNA probe, specific for P450IIC11, a band of about 1.9 kb was detected in both untreated male and female mRNAs, with much weaker intensity in the female mRNAs. However, when the washing stringency was increased (washes were performed at a temperature of 65°), the hybridization signal was no longer observed in female preparations, as shown in Fig. 1. This result is consistent with the fact that this P450IIC11 mRNA is not expressed in adult female rat liver [23].

Hybridization with the p7C12 cDNA probe, corresponding to P450IIC7, revealed the presence of a 2 kb messenger that was detectable in female as well as in male rat liver. After increasing the washing stringency, the 2 kb band was still detectable in both sexes. Thus, this isozyme is constitutively expressed in female and male rats, and cannot, therefore, be considered as a strictly sex-specific isoform. However, a clear difference could be observed between the sexes in the basal level of this P450 mRNA. Indeed, the autoradiogram revealed a higher concentration of this messenger in female than in male rat liver mRNA preparations. These findings confirm previous works showing that P450IIC7 mRNA is female-predominant [7, 27, 28].

No additional band and no shift in molecular size were detected in RNA preparations isolated from RP 52028-treated rat liver, whichever probe was used (data not shown).

Pattern of induction by RP 52028

The inductive effects of two doses of RP 52028 on the P450IIB1/2, IIIA1/2, IIC11 and IIC7 mRNAs were studied in male and female rat livers and compared to those of PB. The relative changes in the levels of each one of these four messengers were measured by slot blot hybridization.

Effects of RP 52028 *on inducible P*450 *mRNA levels*

In an experiment using the pB7 cDNA (Fig. 2A), an equally faint signal was observed in untreated male and female rats. It represented the amount of P450IIB2 messenger that is present at a very low level in liver from untreated animals, while P450IIB1 mRNA is detectable only after induction [25]. The level of P450IIB1/2 mRNA was increased in both sexes by RP 52028 in a dose-dependent manner, as higher levels were observed in animals treated with a high dose compared to those treated with a low dose of this molecule. The low dose of RP 52028 (i.e. RB-treated animals), molecularly equivalent to that of PB, produced a lesser inductive effect than this classical inducer. This suggests that RP 52028 is not as potent an inducer as PB. Treatment of male and female rats with a higher dose of RP 52028 resulted in a 38- and 24-fold increase, respectively, which was similar to the increasing effect of PB (Fig. 2A).

Our mRNA data confirmed the induction pattern of these two anticonvulsant drugs, obtained previously by enzymatic measurements of 7-pentoxyresorufin *O*-dealkylase activity which is specific for P450IIB1/2 isozymes [13]. This may suggest that RP 52028, like PB, acts mainly through gene transcription activation when inducing these P450IIB1/2 isoforms, although the hypothesis of mRNA stabilization cannot be excluded.

In a second experiment, we examined the P450IIIA1/2 mRNA levels. As shown in Fig. 2B, low amounts of these messenger species were detected in preparations isolated from untreated animals, with a noticeable difference between male and female rats. P450IIIA1/2 mRNA level was 2.6-fold higher in males than in females. After treatment by PB, a significant increase in the expression of P450IIIA1/2 was observed in both sexes. RP 52028 again showed a dose-dependent inducing effect on these isoforms. RP treatment resulted in a P450IIIA1/2 mRNA accumulation higher than that resulting from RB but not significantly different from PB treatment. For each of the three treatments a sex difference was observed, mRNA levels being lower in females than in males, as for the untreated animals. Male to female ratios were 2.6, 2.2, 2.1 and 1.3, respectively for control, PB-, RB- and RP-treated groups.

P450IIIA1 and IIIA2 have been shown to be regulated differentially [26]. P450IIIA1 mRNA was found to be absent in untreated male and female rat liver but was induced by dexamethasone and PB. On the other hand, P450IIIA2 mRNA was present in adult rats of both sexes, although at a higher level in males than in females. Moreover P450IIIA2 was not induced significantly by PCN or dexamethasone but was clearly increased after PB treatment. It is clear from these data that the messenger that was present in untreated animals of both sexes, although at a higher level in males than in females, resulted from P450IIIA2 gene transcription only. On the other hand, the increase of mRNA level observed after treatment with PB reflected the induction of both isoform P450IIIA1 and P450IIIA2. The consistent sex-related difference obtained after any treatment may be accounted for by induction of the P450IIIA2 isoform. This should be confirmed by use of specific oligonucleotide probes. Although we cannot explain yet why the sex difference was reduced after treatment with the higher dose of RP 52028 (ratio of 1.3), it is likely that this molecule, like PB, induced both cytochromes. It might also act mainly by increasing the transcription rate or stabilizing the mRNAs, as the changes in P450 gene expression reported here are in agreement with the immunochemical variations observed previously [13].

Effects of RP 52028 *on constitutive sex-specific P*450 *mRNA levels*

Constitutively expressed P450s have been demonstrated to be resistant to induction by a large number

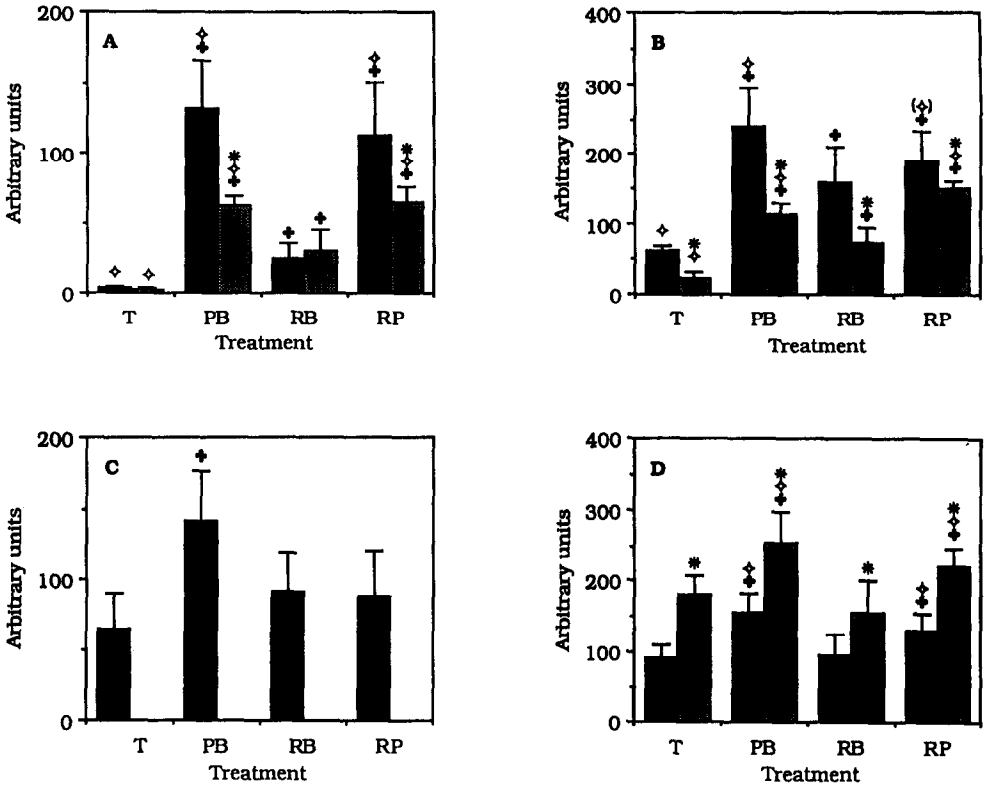

Fig. 2. Slot blot analysis of cytochrome mRNAs in liver from control and PB-, RB- and RP-treated male and female rats. (A) P450IIB1/2, (B) P450IIIA1/2, (C) P450IIC11, (D) P450IIC7. Graphic representation (mean ± SD) of densitometric quantitation of slot blot hybridization analysis (N = 8 from 4 animals). (■) Males, (▨) females. Significant differences in mRNA levels are marked by a sign in brackets and a simple sign for $P < 0.05$ and $P < 0.01$, respectively. ✣ Indicates results significantly different from control values, ◊ indicates results significantly different from RB values, * indicates results significantly different from male values.

of classical inducers [8]. However, on the basis of enzymatic activity data, RP 52028 was suspected as having a sex-differential inducing effect on some of these isozymes [13]. Therefore, we investigated the changes in gene expression of two sex-specific forms of P450, namely P450IIC11 and IIC7 mRNAs, after treatment of rats with the drug.

As shown in Fig. 2C, no hybridization signal could be detected in either control or treated female rat liver preparations with the p(M-1)-3 probe specific for P450IIC11. This is consistent with the works of other groups who observed independently either by immunochemistry [10] or by molecular hybridization to the same cDNA probe [23] that P450IIC11 elicited a strictly male-specific expression in the rat liver.

Treatment of male rats with phenobarbital resulted in a 2.2-fold increase in this P450 mRNA accumulation. Conversely, RP 52028 administration did not result in any significant change in P450IIC11 gene expression. These data are in contrast with our previous enzymatic results on propoxycoumarin *O*-depropylase activity [13]. This activity measurement was developed by Kamataki *et al.* [29] who demonstrated that the O-dealkylation reaction was catalysed by this male-specific P450IIC11 isozyme. However, livers from female rats surprisingly exhibited a low propoxycoumarin *O*-depropylase activity, which was increased 5.5-fold after RP treatment [13]. It is most likely that this activity is catalysed in female rats by another sex-specific isoform, possibly P450IIC6 which is known to have a high degree of sequence homology with P450IIC11 [30].

To ascertain the effect of RP 52028 on the synthesis of the male-specific P450 isoform, we measured the microsomal 2α-regioselective hydroxylation of testosterone which is known to be catalysed by P450IIC11 [31]. Figure 3 shows the rate of formation of 2α-hydroxytestosterone. It was depressed in PB-treated male rats to 26% of the value obtained in control microsomes. RP treatment also depressed the rate of testosterone 2α-hydroxylase activity to 53% of the control value. Liver microsomes from control or treated female rats did not exhibit any 2α-hydroxylase activity. When we compared 2α-hydroxylation of testosterone in male rat liver microsomes with the corresponding mRNA levels (Fig. 3), no correlation was observed. Such non-correlated changes in the level of P450IIC11 protein and mRNA in PB-treated rats have already been reported by Shimada *et al.* [11]. They suggest that this P450 isozyme is regulated at both transcriptional

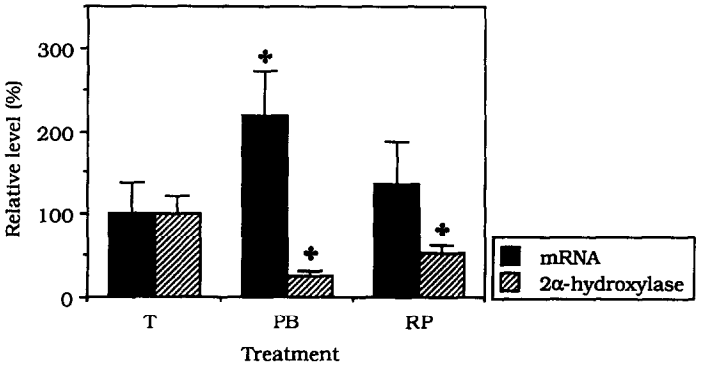

Fig. 3. Relative changes in testosterone 2α-hydroxylation activity and P450IIC11 mRNA level in PB- and RP-treated male rats. Results are expressed as mean ± SD. (N = 8 from 4 animals). Testosterone 2α-hydroxylation activity is expressed in nmol/mg protein/min (100% = 2.598 ± 0.568 nmol/mg protein/min). ✢ indicates results significantly different from control values ($P < 0.01$).

and post-transcriptional levels. In contrast to PB, the mRNA level was not increased in RP treated male rats and, moreover, 2α-hydroxylase activity was decreased less. This would reflect a one-step regulation mechanism, at the post-transcriptional level only.

In contrast to P450IIC11, P450IIC7 was expressed in both sexes in untreated animals, but at a higher level in female rats (Fig. 2D). This sex difference was also present after treatment with either PB or RP 52028. The ratios of female to male values were similar in control, PB-, RB- and RP-treated rats (1.92, 1.66, 1.62 and 1.70, respectively).

Administration of the low dose of RP 52028 did not result in any change in P450IIC7 mRNA level in either sex. Following treatment of rats with a higher dose of RP 52028, the mRNA level was slightly elevated in males and females and this was statistically significant. The low inducing effect of RP was quite similar to that observed after PB administration which did not cause a strong increase in P450IIC7 mRNA level. However, this last data was not consistent with the results published by Barroso *et al.* [7], who reported a strong induction of this P450IIC7 mRNA content after PB treatment in male and female Wistar adult rat livers.

Strain differences in cytochrome P450 have already been observed for PB-inducible isoforms IIB1 and IIB2 [32], as well as for a sex-specific isoform, P-450IIC13 [33]. Moreover, the results of Barroso *et al.* [7] were inconsistent with the work published by Bandiera *et al.* [8], who reported only a slight increase in this protein in PB-treated rats. To further investigate whether the use of Sprague–Dawley rats in our study could account for this discrepancy, we examined P450IIC7 mRNA levels in control and PB-treated Wistar rat livers (total RNA fractions were prepared in Prof. Lechner's laboratory at the Gulbenkian Institute using the method described in Materials and Methods). As shown in Fig. 4, the strong induction of this messenger after treatment with PB is consistent with the increase related by Barroso *et al.* [7] in this rat strain. Thus, some polymorphism in phenobarbiral inducibility of this P450 gene might explain these discrepancies.

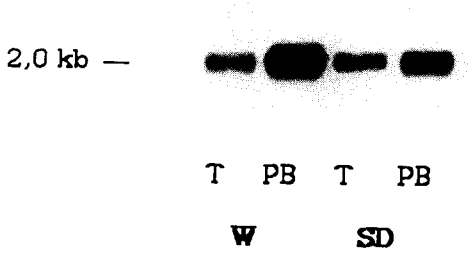

Fig. 4. Difference in PB-mediated induction of P450IIC7 between Sprague–Dawley and Wistar rats. Liver poly $(A)^+$ RNA (5 μg) extracted from untreated (T) and PB-treated (PB) male animals of both strains was analysed by Northern blot probed with [^{32}P]p7C12 cDNA. **SD** and **W** indicate Sprague–Dawley and Wistar rats, respectively. After washing, filter was autoradiographed overnight at −80°.

In conclusion, we have determined the patterns of induction of four P450 isoforms by RP 52028 and compared them to those obtained with the classical inducer phenobarbital. This study confirms our previous finding that RP 52028 is a new PB-like inducer. Our data show that besides causing a PB-type pleiotropic response in liver, RP 52028 treatment resulted in a similar induction profile of inducible P450 mRNAs. Indeed, as for PB, both cytochrome P450IIB1/2 and IIIA1/2 mRNA levels are increased by RP 52028 in a dose-dependent manner in both sexes. This pretranslational effect is mainly restricted to inducible isoforms, although a weak induction of the constitutive female-predominant P450IIC7 is also observed in liver of both sexes. However, in contrast to PB, RP 52028 does not appear to regulate the male-specific P450IIC11 at a transcriptional level, suggesting that these two inducers interfere with the hormonal regulation of this mRNA via different mechanisms.

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