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EFFECT OF ROCIVERINE ON P450-DEPENDENT MONOOXYGENASES AND ITS N-DEETHYLATION METABOLISM IN RAT LIVER MICROSOMES

SILVIA MENICAGLI, ANNALISA LIPPI,* MARCO CRISCUOLI* and PIER GIOVANNI GERVASI†

Laboratory of Genetics and Biochemical Toxicology, Istituto di Mutagenesi e Differenziamento, Consiglio Nazionale delle Ricerche, via Svezia, 10, 56124 Pisa, and *Laboratorio Guidotti, S.p.A., Company related with "A. Menarini- Industrie Farmaceutiche Riunite s.r.l.-" 56122 S. Piero a Grado, Pisa, Italy

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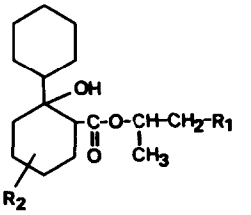
Abstract—Rociverine [2-(diethylamino)-1-methylethyl *cis*-1-hydroxy [bicyclohexyl]-2-carboxylate] citrate (ROC) is an antispasmodic agent therapeutically active in humans at doses of 0.5–1 mg/kg. This study investigated the effect of acute administration of the drug on hepatic microsomal cytochrome P450 (P450)-catalysed drug metabolism. Only high doses (≥100 mg/kg) of ROC were able to induce in rats the hepatic microsomal pentoxyresorufin *O*-depenthylase (PROD) and 16β-testosterone hydroxylase activities both associated with P4502B1/2 and the erythromycin *N*-dimethylase (ErD) and 2β-testosterone hydroxylase activities both dependent on P4503A1/2. However, at 100 and 200 mg/kg of ROC, the 16β-testosterone hydroxylase and PROD were the most induced activities, suggesting that P4502B1/2 are the isoforms most sensitive to ROC induction. Accordingly, ROC treatment enhanced, in a dose-dependent manner, the amount of P4502B1/2 and 3A1/2 in microsomes as assayed by western blotting. The northern blot analysis of ROC-treated rat liver showed that the P4502B1/2 induction appears to be regulated at the mRNA level as in the induction by phenobarbital (PB). The oxidative metabolism of ROC with hepatic microsomes from control or PB- and ROC-induced rats resulted in a *N*-deethyl ROC derivative (major metabolite) and an unknown minor ROC derivative. The kinetic parameters for the N-deethylation of ROC were studied with purified P4502B1 and with microsomes from control or rats treated with various inducers (phenobarbital, ethanol, β-naphthoflavone, dexamethasone and rociverine). It was found that phenobarbital-, dexamethasone- and rociverine-induced microsomes deethylated ROC with a V_{max} about five times higher than that (0.9 nmol/min/mg protein) of control microsomes, although with a similar affinity ($K_m \approx 0.3$ mM). In a reconstituted system, the purified P4502B1 metabolized ROC with a high deethylation rate (22 nmol/min/nmol P450). Moreover, the ROC deethylation was inhibited by compounds such as hexobarbital, metyrapone and triacetyloleandomicin, selective inhibitors for P4502B and/or P4503A enzymes. On the other hand ROC, when added *in vitro*, inhibited the 16β- and 2β-testosterone hydroxylases and the PROD and ErD activities. Taken together, these results indicate that the ROC-inducible P4502B and P4503A are involved in ROC deethylation. In conclusion, it has been demonstrated that ROC is a weak phenobarbital-like inducer of P450, probably able at high and reiterated doses to alter its own metabolism, at least in the rat liver.

Rociverine [2-(diethylamino)-1-methylethyl *cis*-1-hydroxy [bicyclohexyl]-2-carboxylate] citrate (ROC‡, Fig. 1), is an antispasmodic agent, therapeutically active at a single dose of 0.5–1 mg/kg in humans. The reported mechanisms of rociverine are a weak antimuscarinic activity and a direct muscle-relaxant activity, apparently due to inhibition of trans membrane fluxes of Ca^{2+}, which appear to be selective for smooth muscle [1, 2]. Unpublished metabolic studies have shown that orally administered ROC is extensively metabolized in rats. Three major metabolites of ROC (Fig. 1) have been detected by



Compound	ROC	R_1	R_2
Metabolite 1	(*N*-deethyl ROC)	$R_1 = NH—C_2H_5$	$R_2 = H$
Metabolite 2	(hydroxy *N*-deethyl ROC)	$R_1 = NH-C_2H_5$	$R_2 = OH$
Metabolite 3	(hydroxy ROC)	$R_1 = N-(C_2H_5)_2$	$R_2 = OH$

Fig. 1. Chemical structure of ROC and its major metabolites.

† Corresponding author: Dr P. G. Gervasi, Istituto di Mutagenesi e Differenziamento, CNR, Via Svezia, 10, 56124 Pisa, Italy.

‡ Abbreviations: ROC, rociverine citrate; PB, phenobarbital; DEX, dexamethasone; β-NF, β-naphthoflavone; 17-OT, 4-andosten-3,17-dione; 2α-OH, 2β-OH, 6α-OH, 6β-OH, 7α-OH, 16α-OH, 16β-OH correspond to 2α-, 2β-, 6α-, 6β-, 7α-, 16α, 16β-hydroxytestosterone, respectively; AnH, aniline hydroxylase; APD, aminopyrine demethylase; ErD, erythromycin demethylase; BzD, benzphetamine demethylase; pNPH, *p*-nitrophenol hydroxylase; ECOD, 7-ethoxycoumarin-*O*-deethylase; EROD, ethoxyresorufin-*O*-deethylase; PROD, pentoxyresorufin *O*-depentylase; DLPC, dilauroylphosphatidylcholine; P450, cytochrome P450; SSC, standard saline citrate.

GLC-MS of rat urine. Two of these metabolites appear to be N-monodeethylated ROC derivatives, indicating that oxidation of the diethylamino moiety is a primary metabolic pathway. Two urinary ROC metabolites, including the N-deethylated one, were found hydroxylated at the as yet unidentified 4 or 5 position of the substituted cyclohexane ring. When given at high doses but not after repeated administrations, ROC prolongs hexobarbital-induced sleeping time in mice or rats, a behaviour indicating a complex interaction of ROC with the enzymes involved in barbituate metabolism (unpublished data). This observation has prompted us to consider that ROC may affect drug-metabolizing enzymes particularly the cytochrome P450 (P450)-dependent enzyme system.

The microsomal P450, present in almost all tissues, consists of a superfamily of closely related isozymes which catalyse the oxidative metabolism of a wide variety of endogenous and foreign substrates [3]. One or more P450 forms exhibiting generally broad and overlapping substrate specificities can be induced by drugs or environmental compounds [4]. Consequently, the spectrum of specific isoforms in microsomes and their relative activities in microsomal oxidation can influence the metabolism of endogenous substrates and/or the metabolism and toxicity of coadministrated drugs or other xenobiotics. The present study was undertaken to analyse and characterize the induction or inhibition of ROC on the P450-dependent monooxygenases of rat liver. In addition, the *in vitro* deethylation of ROC to acetaldehyde by purified P4502B1 and by hepatic microsomes from rats treated with inducers of selected P450 isozymes has been studied.

MATERIALS AND METHODS

Chemicals. ROC was from the Guidotti Laboratories (Pisa, Italy); resorufin from Fluka (Buchs, Switzerland); 7-ethoxycoumarin from EGA-Chemie (Steinheim, F.R.G.). Benzphetamine was supplied by Upjohn Co. (Kalamazoo, MI, U.S.A.); enzymes, coenzymes and random priming kit were from Boehringer (Mannheim, F.R.G.) and [^{32}P]dCTP (3000 Ci/mmol) from New England Nuclear (Dreieich, F.R.G.). Ethoxyresorufin and pentoxyresorufin were synthesized from resorufin by ethylation with ethyl iodide and by pentylation by pentyl iodide, respectively [5]. Testosterone, 4-androsten-3,17-dione (17-OT), 16β-hydroxytestosterone (16β-OH), triacetyloleandomycin and corticosterone were supplied by the Sigma Chemical Co. (St Louis, MO, U.S.A.), 2α- 2β-, 6α-, 6β-, 7α- and 16α-OH were obtained from the Steroids Reference Collection (D. N. Kirk, Department of Chemistry, Queen Mary College, London). Rabbit anti-rat P4502B1 and P4503A1 polyclonal antibodies were purchased from Oxygene (Dallas, TX, U.S.A.). Goat anti-rabbit immunoglobulin G was purchased from Dako (Copenhagen, Denmark). Nitrocellulose filters (0.45 μm), goat serum, 4-chloro-1-naphthol and erythromycin were obtained from the Sigma Chemical Co. All other chemicals and solvents were of analytical grade and were obtained from common commercial sources.

Animal treatment and preparation of microsomes. Male Sprague–Dawley rats (5–6 weeks old, Charles River, F.R.G.) were injected i.p. with a phosphate-buffered solution (pH $\approx$ 6) of ROC at doses of 25, 50, 100 and 200 mg/kg for 4 days. Other inducers administered i.p. were: phenobarbital (PB) at 80 mg/kg for 3 days; β-naphthoflavone (β-NF) 40 mg/kg for 3 days; dexamethasone (DEX) 50 mg/kg for 4 days. Ethanol was administered at 15% (v/v) in the drinking water for 10 days. Animals were killed by CO_2 asphyxia; the livers were collected, the 100,000 *g* supernatants and microsomes were prepared as described previously [6]. The washed microsomal pellets were resuspended in 100 mM phosphate buffer, 1 mM EDTA, pH 7.4 and stored at −80°. Protein content was determined according to Lowry *et al.* [7] using bovine serum albumin as standard.

Enzymic assays. Hepatic cytochrome P450 and cytochrome b_5 content were measured by the method of Omura and Sato [8]. Microsomal aniline hydroxylase (AnH) was determined by measuring the formation of *p*-aminophenol as described by Ko *et al.* [9]. The aminopyrine (APD), erythromycin (ErD), benzphetamine (BzD) demethylase activities were assayed by measuring the formation of formaldehyde [10]. The *p*-nitrophenol hydroxylase (pNPH) was determined according to Reinke and Moyer [11]. The microsomal 7-ethoxycoumarin-*O*-deethylase (ECOD) activity was assayed by the fluorimetric determination of 7-hydroxycoumarin [12]. Ethoxyresorufin-*O*-deethylase (EROD) and pentoxyresorufin-*O*-depentylase (PROD) activities were determined by measuring the formation of the corresponding hydroxy product in a Perkin–Elmer spectrofluorimeter [13]. Microsomal epoxide hydrolase, glutathione *S*-transferase and UDP-glucuronyl transferase activities were quantitated as previously reported [14] using as substrate, safrole oxide, 1-chloro-2,4-dinitrobenzene and 1-naphthol, respectively. Testosterone hydroxylase was assayed as reported earlier [15] according to a HPLC method described by Platt *et al.* [16]. Difference spectra were determined according to Shenkman *et al.* [17]. Rociverine deethylation was assayed by measuring the formation of acetaldehyde by HPLC of the 2,4-dinitrophenylhydrazone derivative as reported previously [6] with the addition of semicarbazide to trap acetaldehyde as described by Yoo *et al.* [18].

Reconstituted system. Cytochrome P4502B1 and NADPH-cytochrome P450-reductase were purified from microsomes of male Sprague–Dawley rats treated with PB as described previously [19]. The reconstituted system contained in 1 mL of 0.1 mM phosphate buffer pH 7.4, 0.1 nmol of P4502B1, 0.3 nmol of P450 reductase, 30 μg of dilauroylphosphatidylcholine (DLPC) and substrate (1 mM). DLPC was prepared in water and sonicated immediately before use. After 30 min preincubation at room temperature, the reaction was carried out at 37° after the addition of 1 mM of NADPH; the incubation time was 15 min. Acetaldehyde was quantitated by HPLC [18].

Gel electrophoresis and immunoblotting. SDS–gel electrophoresis was performed using the discontinuous system of Laemmli [20], with a 1.5 mm thick gel and 3% and 7.5% acrylamide in the stacking

Table 1. Monooxygenase activities and other parameters in liver microsomes from rats treated with various ROC doses

Parameters	ROC doses (mg/kg) 0	25	50	100	200
Protein	18 ± 3	21 ± 2	17 ± 3	19 ± 3	20 ± 3
Cytochrome P450	0.68 ± 0.13	0.63 ± 0.12	0.70 ± 0.11	0.72 ± 0.14	0.81 ± 0.16
Cytochrome b_5	0.25 ± 0.07	0.22 ± 0.05	0.27 ± 0.05	0.30 ± 0.05	0.25 ± 0.07
Benzphetamine *N*-demethylase	4.1 ± 0.8	4.7 ± 0.4	6.1 ± 1.8	5.5 ± 0.5	6.4 ± 0.9†
Erythromycin *N*-demethylase	1.1 ± 0.3	1.3 ± 0.2	0.8 ± 0.3	1.8 ± 0.3†	4.2 ± 1.1*
Pentoxyresorufin *O*-depentylase	0.020 ± 0.004	0.025 ± 0.006	0.026 ± 0.004	0.040 ± 0.007†	0.12 ± 0.01*

Results are the means ± SD of three experiments. Each experiment used microsomes pooled from 3–4 animals.

Enzymic activities are expressed as nmol/min/mg of protein, P450 and b_5 content as nmol/mg of protein and protein content as mg/g of liver.

* Significantly different from control microsomes by Student's *t*-test ($P < 0.01$).

† $P < 0.05$.

and separation gel, respectively. Proteins were transferred from the slab gel to the nitrocellulose filters following the method of Towbin *et al.* [21]. Immunodetections were performed using rabbit anti-rat P4502B1 and P4503A1 polyclonal antibodies. The former antibody is able to crossreact with P4502B1 and 2B2, the latter with 3A1 and 3A2. The bands on the nitrocellulose membranes were quantified by a laser densitometer (Ultroscan 2202 LKB).

Isolation of RNA. Livers from untreated rats or rats treated i.p. for 4 days with 200 mg/kg ROC were dissected and cut into small pieces which were immediately frozen in liquid nitrogen and subsequently ground to a fine powder under nitrogen, in an Atomix blender (MSE Ltd, Manor Royal, Crawley, U.K.). Total hepatic RNA was prepared from frozen tissues by the method of Chomczynski and Sacchi [22] and quantified by measuring the absorbance at 260 nm. Poly(A)$^+$ RNA was isolated by two cycles of oligo (dT)-cellulose chromatography [23] and stored in 70% ethanol at −80° until use.

cDNA probe. A pGEM-4 vector containing a cDNA insert complementary to P4502B1/2 mRNA was kindly provided by Dr M. Ingelman-Sundberg (Dept of Physiological Chemistry, Karolinska Institute, Stockholm, Sweden). The 1.6 kb cDNA insert was excised from the vector by PTS1 digestion, separated by gel electrophoresis, recovered from the gel and ^{32}P-labeled by random priming kit using [^{32}P]dCTP.

RNA hybridization. Poly(A)$^+$ RNA was electrophoretically separated on 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon Hybond membrane for hybridization with labeled cDNA probe followed by autoradiography [23]. The nylon filter was exposed to UV light for 4 min. Prehybridization was carried out overnight at 42° in 5× Denhart's solution, 5× SSC, 0.5% SDS, 0.1 mg/mL of denatured salmon sperm DNA and 50% formamide.

The filter was hybridized in the same solution with the ^{32}P-labeled probe. Following overnight hybridization at 42°, the filter was washed with 2× SSC for 30 min at 42°, 2×SSC–0.05% SDS for 30 min at 42°, 1× SSC–0.05% SDS for 30 min at 42° and finally with 0.3× SSC–0.5% SDS for 15 min at room temperature. The filter was then exposed for varying lengths of time at −80° to Kodak X-Omat AR films using intensifying screens.

P450 nomenclature. The new nomenclature system for cytochrome P450 recommended by Nebert *et al.* [24] has been used in the text. Cytochromes P450 2A1, 2B1, 1A1, 1A2, 2B2, 2C13, 2C11, 2E1, 2C6, 3A1 are also known as cytochromes P450 *a, b, c, d, e, g, h, j, k, p*, respectively [3].

RESULTS

Effects of rociverine on hepatic drug-metabolizing enzymes

The effect of ROC administration on hepatic microsomal hemo-proteins and some P450-dependent monooxygenase activities is shown in Table 1. Treatment of rats with a buffered solution of ROC at doses of 25, 50, 100 and 200 mg/kg for 4 days had no significant effect on microsomal protein content, cytochrome b_5 and P450 content. The highest ROC dose (200 mg/kg) caused, compared to control, a slight but significant increase of the BzD activity (1.5-fold) and a marked enhancement of the P4503A1/2-associated ErD (3.5-fold) and P4502B1/2-linked PROD activities (6-fold). Significant increases (about twice control values) of these latter two activities were also observed after a ROC dose of 100 mg/kg corresponding to 0.193 mmol/kg. An equimolar dose of PB, a known strong inducer of P4502B1/2 [25], injected i.p. in rats for 4 days, elevated the hepatic PROD activity from 20 ± 0.4 (see Table 1) to 213 ± 27 pmol/min/mg of protein. Under these conditions the relative PROD induction by PB was about 5-fold greater than that of ROC. Other monooxygenase activities such as APD, EROD, pNPH, AnH, ECOD were also assayed (the control values of these enzymatic activities were 5.8 ± 1.2, 0.17 ± 0.03, 0.8 ± 0.2, 1.1 ± 0.2, 0.9 ± 0.2 nmol/min/mg of protein, respectively) but

Table 2. Hydroxylation of testosterone by liver microsomes of control and ROC-treated rats

Metabolites	Rociverine doses (mg/kg) 0	25	50	100	200
6α-OH	0.09 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.02	0.15 ± 0.04
7α-OH	0.24 ± 0.05	0.29 ± 0.04	0.19 ± 0.03	0.29 ± 0.04	0.30 ± 0.05
6β-OH	0.66 ± 0.18	0.79 ± 0.24	1.07 ± 0.31	1.14 ± 0.23†	1.31 ± 0.33†
16α-OH	0.93 ± 0.27	1.32 ± 0.17	1.33 ± 0.31	0.72 ± 0.22	0.95 ± 0.28
16β-OH	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.13 ± 0.04†	0.31 ± 0.06*
2α-OH	0.65 ± 0.13	0.85 ± 0.16	0.87 ± 0.25	0.41 ± 0.12	0.36 ± 0.11†
2β-OH	0.08 ± 0.03	0.12 ± 0.03	0.17 ± 0.04†	0.29 ± 0.07*	0.50 ± 0.14*
17-OT	1.14 ± 0.26	1.14 ± 0.22	1.32 ± 0.34	0.70 ± 0.21	1.12 ± 0.27
Total	3.83 ± 0.95	4.63 ± 0.88	5.08 ± 1.31	3.83 ± 0.95	5.16 ± 1.32

Results of testosterone hydroxylase activities expressed as nmol/min per mg of protein are the means ± SD of three experiments. Each experiment used microsomes pooled from 3–4 animals. Incubation were carried out at 37° for 15 min with 1 mg/mL of microsomal protein.

* Significantly different from control at $P < 0.01$.

† Significantly different from control at $P < 0.05$.

were not affected by any of the ROC treatments (results not shown). When the metabolism of an endogenous substrate, testosterone, was investigated, it was found that ROC at doses of 100 and 200 mg/kg is able to increase, compared to control microsomes, the 2β-OH and the 16β-OH hydroxylations, respectively associated with the P4503A1/2 and P4502B1/2 (Table 2). At the highest dose, the enhancement in the formation rate of these two metabolites was about 6-fold. In addition to the 2β-OH and 16β-OH hydroxylase induction, a ROC dose-dependent increase of 6β-OH hydroxylation was observed, but this oxidation is also linked to the P4503A1/2 along with other P450(s) [16]. Another effect of the highest doses of ROC was to depress (to about 60% of control value) the 2α-OH hydroxylation which is linked to the male specific P4502C11 [16], the major P450 form constitutively present in rat liver [26]. No other testosterone hydroxylations were significantly altered by ROC treatment nor were the microsomal epoxide hydrolase activity or other non-oxidative enzyme activities such as glutathione *S*-transferase and UDP-glucuronyl transferase significantly changed (data not shown).

SDS–PAGE and western blot analysis

Liver microsomes of control and ROC-treated rats were subjected to SDS–PAGE. In the electrophoretic pattern of liver microsomal proteins, ROC pretreatment resulted in a dose-dependent increase in intensity of at least two protein bands in

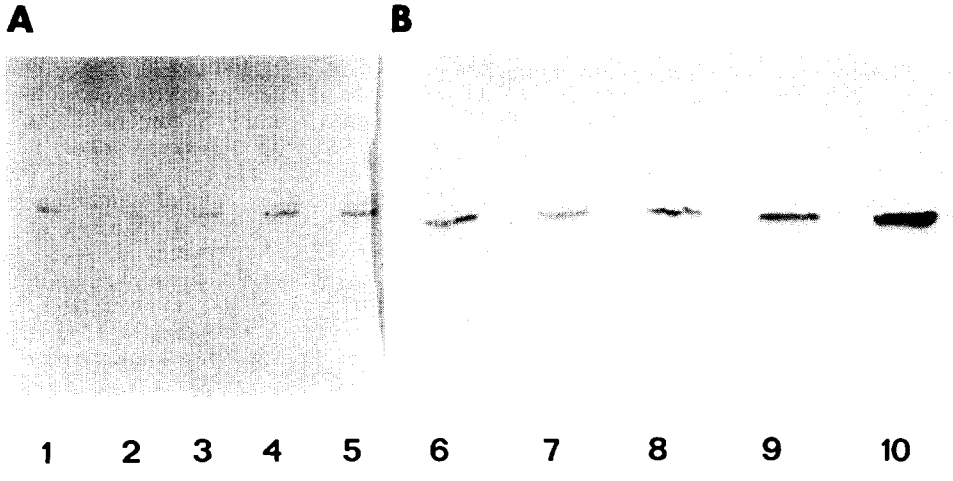


Fig. 2. Western blot analysis of liver microsomes from control and ROC-treated rats. Microsomes (12 μg protein in each lane) were subjected to SDS–PAGE followed by immunoblot analysis using polyclonal anti P4502B1/2 immunoglobulin G (panel A) or anti P4503A1 IgG (panel B). Lane 1 contained purified P4502B1 (1 pmol). Lane 2 contained control microsomes and lanes 3, 4, 5 microsomes from 50, 100, 200 mg/kg ROC-treated rats, respectively. Lane 6 contained control microsomes and lanes 7–10 microsomes from 25, 50, 100, 200 mg/kg ROC-treated rats, respectively.

the molecular mass range of 51–52 kDa. Western blots of liver microsomal proteins from ROC-treated rats, stained with rat P4502B1/2 and 3A1/2 specific antibodies showed these bands to be P4502B1 and P4503A1 and/or 3A2. The discrimination between the 3A isozymes is not possible as they have the same molecular mass and are immunochemically indistinguishable [27]. In microsomes from ROC-treated rats, anti P4502B1 did recognize a band, increasing with ROC dose and corresponding in migration position to purified P4502B1 (Fig. 2A). In addition to this protein, another band with a higher molecular mass, just visible in control microsomes according to previous observations [28], also increased with ROC dose and probably corresponds to P4502B2 (Fig. 2A). The P4502B1/2 content, which is approximately 0.5–2% of whole microsomal P450 population in untreated rat liver [26], enhanced to about 5, 12, 17% in 50, 100, 200 mg/kg ROC-treated rat liver as determined by densitometry and using as reference a standard curve performed with various concentrations of purified P4502B1. In addition, the anti P4503A1 showed that the band corresponding to the hepatic P4503A1/2 was induced in the microsomes of rats treated with 100 and 200 mg/kg of ROC (Fig. 2B). Compared to the untreated rats, the P4503A1/2 content, as determined by densitometry, increased to 120, 180, 280% after 50, 100, 200 mg/kg of ROC treatment, respectively. The anti P4503A1 also revealed another faint band with lower molecular mass which was not induced by ROC treatments and which could be the 50 kDa protein described by Gemzik *et al.* [27].

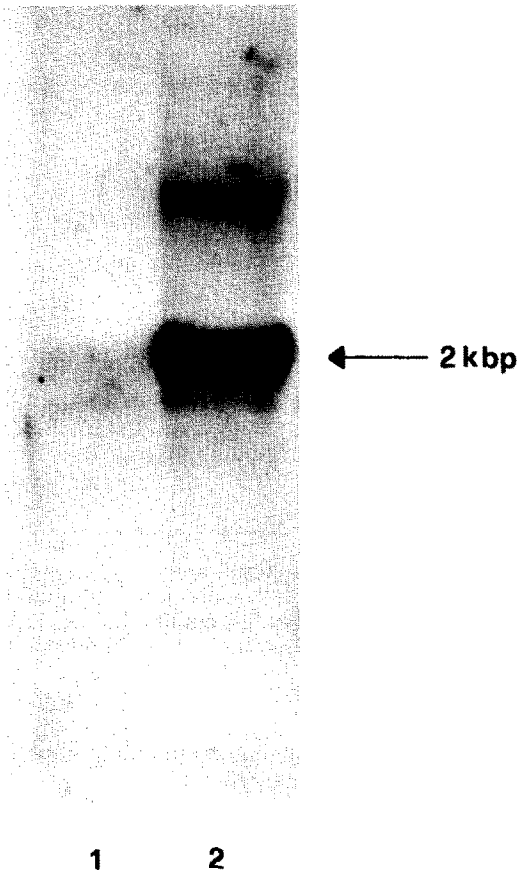


Fig. 3. Northern blot of rat liver mRNA complementary to P4502B1/2B2 cDNA probe. Poly(A^+) RNA (10 μg) from control rats (lane 1) or rats treated for 4 days with 200 mg/kg of ROC (lane 2) was fractionated by electrophoresis, transferred to nylon Hybond filter and hybridized with P4502B1/2 ^{32}P-labeled cDNA probe.

RNA hybridization analysis

The amount of hepatic mRNA coding for P4502B1/2 from control and ROC-treated rats was determined by northern blot analysis and autoradiography, by using a labeled cDNA probe which does not discriminate between the 2B1 and the 2B2 mRNA due to their extensive nucleotide sequence homology (>97%) [3]. The ^{32}P-labeled cDNA probe recognizes in the samples from ROC-treated rats, a single band of mRNA of approximately 2 kb (Fig. 3) which is the reported size of 2B1/2 mRNA [29]. In the samples of control rats a faint hybridization signal was just visible, in keeping with the very low constitutive expression of P4502B1/2 genes in untreated rats [26].

These results indicate that ROC treatment stimulates the accumulation of P4502B1/2 mRNA.

Inhibition by ROC of hepatic testosterone hydroxylases, PROD and ErD activities

In order to probe which P450s are involved in ROC oxidative metabolism, we determined the ability of ROC to inhibit the regioselective hydroxylation of testosterone in hepatic microsomes from ROC-pretreated rats, where P4502B1/2 and P4503A1/2 were induced. Figure 4 shows that ROC almost completely inhibited the P4502B1/2-mediated 16β-OH hydroxylase activity. In addition, the 2β-OH hydroxylase activity, dependent on P4503A1/2, was also inhibited (by about 85%). In contrast, the 2α-OH hydroxylase, dependent on the P4502C11, appeared refractory to inhibition.

Experiments with untreated microsomes (not shown), in which P4502C11 was not repressed, demonstrated that the P4502C11-linked 2α-OH and 17-OT hydroxylations were not inhibited by 1 mM of ROC. The strong inhibition (about 85% of control value) of 6β-OH hydroxylase which is almost completely dependent in untreated microsomes on P4502C13, -2C11, -3A1/2 [16], suggests that P4502C13 beside P4503A1/2, since P4502C11 is not involved, could be inhibited by ROC.

The susceptibility of 16β-OH and 2β-OH hydroxylase activities to inhibition by ROC, suggested that the P4502B1/2 and the P4503A1/2 may play a role in the oxidative metabolism of ROC. This susceptibility of P4502B1/2 and P4503A1/2 to the inhibition by ROC was confirmed when the ROC effect on the P4502B1/2-mediated PROD [30] and P4503A1/2-linked ErD [31] activities was assayed in hepatic microsomes from PB- and DEX-treated rats. ROC inhibited both the PROD and ErD

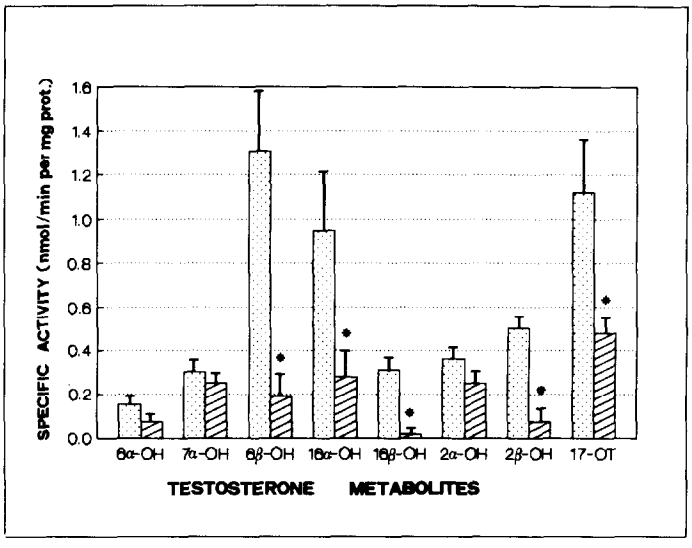


Fig. 4. ROC inhibition of testosterone hydroxylase activities by microsomes from 200 mg/kg ROC-treated rats. (▩) Without ROC; (▨) with 1 mM ROC. Testosterone was 1 mM in the incubation mixture. Data are the means ± SD (bar) of three experiments. Each experiment used microsomes pooled from 3–4 animals. * $P < 0.05$ compared to testosterone hydroxylase activities without rociverine.

activities in a competitive manner with an apparent K_i of 63 and 37 μM, respectively.

Deethylation of ROC by liver microsomes

The oxidative metabolism of ROC with hepatic microsomes from PB- and ROC-treated rats resulted in the *N*-deethyl ROC derivative (metabolite 1) and a minor unidentified metabolite which accounted for about 80% and 20%, respectively, of the total ROC metabolites as judged by GLC analysis (not shown). With microsomes from untreated rats, the minor ROC metabolite was still present but the metabolite 1 accounted for about 90% of the total ROC metabolism. Although the identity of the minor ROC microsomal metabolite remains to be established, it might be the hydroxy-ROC (metabolite 3) as suggested by the metabolites found in urine of ROC-treated rats. The deethylation of ROC gave rise to acetaldehyde that was quantitated as dinitrophenylhydrazone derivative by HPLC. This reaction was catalysed by P450 as it had an absolute requirement for molecular oxygen and NADPH; NADH was ineffective as a source of electrons. CO, bubbled in the incubation mixture, inhibited the reaction by 80%. Furthermore ROC, as typical of P450 substrates [17], bound to the active site of P450 in control microsomes resulting in a type I difference spectrum, with a peak at 390 nm and a trough at 420 nm. The double reciprocal plot (not shown) of the absorbance change versus ROC concentration allowed the determination of an apparent dissociation constant K_s of 17 μM and a maximal absorbance change ΔA_{max} of 0.016 absorbance units/nmol P450 in control microsomes.

Kinetic parameters

In order to investigate P450 isozyme selectivity in the deethylation of ROC to acetaldehyde, incubations were performed using liver microsomes from control, ROC-treated (200 mg/kg) rats and rats treated with various classical inducers of the P450 superfamily (β-NF, PB, ethanol, DEX). In all cases, the oxidative dealkylation of ROC followed a simple Michaelis–Menten kinetic and was linear up to 30 min and 1.5 mg/mL of microsomal protein. With a higher concentration of proteins from the various induced microsomes, the ROC deethylation was linear for shorter times. From the double reciprocal plots of the data of these deethylations, we obtained the apparent kinetic constants shown in Table 3. The apparent K_m for control microsomes was very similar to those of PB-, DEX- and ROC-treated microsomes. Ethanol-treated microsomes showed an approx. 4-fold higher K_m than that of control microsomes. Regarding the V_{max}, ethanol- and β-NF-induced microsomes catalysed the ROC deethylation at a rate equivalent to that of uninduced microsomes. In contrast, PB, DEX or ROC pretreatments increased V_{max} by 4–5-fold. When the rates were normalized for the different P450 levels (i.e. per nmol P450), the production of acetaldehyde was still 3–4-fold higher for PB-, DEX- and ROC-treated microsomes than that for uninduced microsomes, suggesting a specific role in the deethylation of ROC of the P450 forms, P4502B1/2 and -3A1/2, primarily induced by these compounds. The apparent involvement of P4502B1 in the ROC dealkylation is supported by the finding that this isozyme, in a reconstituted system, is able to catalyse the dealkylation at a higher rate than control and induced microsomes (Table 3). When hexobarbital, whose metabolism is dependent on P4502B1/2 and probably 3A1/2 [32, 33], was used as an inhibitor, the ROC deethylation was inhibited competitively with an apparent K_i of 250 μM. A further

Table 3. Values of apparent kinetic constants for the microsomal and P4502B1-mediated deethylation of ROC

Microsomes	K_m (mM)	V_{max} (nmol/min/mg protein)	V_{max} (nmol/min/nmol P450)
Control	0.29 ± 0.10	0.9 ± 0.3	1.3 ± 0.4
Ethanol	1.40 ± 0.40*	1.3 ± 0.3	1.7 ± 0.4
PB	0.48 ± 0.15	5.9 ± 1.2*	4.9 ± 1.0*
DEX	0.37 ± 0.11	4.6 ± 0.9*	4.4 ± 0.8*
β-NF	0.22 ± 0.08	1.6 ± 0.4	1.4 ± 0.3
ROC (200 mg/kg)	0.35 ± 0.11	4.7 ± 0.8*	5.8 ± 1.0*
Purified P4502B1	3.60 ± 0.80*	—	22 ± 3*

Values are reported as means ± SD for three experiments performed with different preparations of hepatic microsomes. Protein concentration was 1 mg/mL and incubation time was 30 min. The incubation of the reconstituted system containing P4502B1 was performed as described in Materials and Methods.

* Significantly different from control microsomes by Student's *t*-test, $P < 0.01$.

indication that P4502B1/2 and P4503A1/2 are chiefly responsible for the deethylation of ROC was obtained using P450 inhibitors such as metyrapone and triacetyloleandomycin selective for P4502B1/2 [34] and P4503A1/2 [35], respectively.

In liver microsomes from 200 mg/kg ROC-treated rats, the *in vitro* ROC deethylation at a substrate concentration of 1 mM was inhibited by 70–80% with either inhibitor at a concentration of 1 mM. It is likely that the ROC deethylation resistant to these inhibitors can be carried out by other microsomal P450 isoforms.

DISCUSSION

In this study we present evidence that ROC, when acutely administered to rats at high concentrations, affects some P450 dependent monooxygenases but not phase II enzymes. The drug at concentrations around 100 mg/kg or higher is capable of depressing the constitutive P4502C11 and inducing P4502B1/2 and P4503A1/2. The induction of these P450s was demonstrated by a significant increase in the pentoxyresorufin O-dealkylation and 16β-OH hydroxylation dependent on the former isozymes and in the erythromycin demethylation and 2β-OH hydroxylation dependent on the latter. The lower amount of P4502C11 in ROC-treated microsomes probably compensated for the induction of P4502B1/2 and P4503A1/2, so that the total microsomal P450 content did not change significantly in ROC-treated microsomes (see Table 1). Consistent with the enhancement of these monooxygenase activities, the amounts of immunodetectable P4502B1/2 and P4503A1/2 proteins were elevated, in a dose-related manner, in the microsomes from rats treated with ROC. It is of interest to note that in the liver from 200 mg/kg ROC-treated rats compared to control rats, both the immunodetectable P4502B1/2 level and the P4502B1/2-linked PROD and 16β-OH hydroxylase activities were enhanced in a similar manner (about 6–8-fold); a lower concordance was found between the enhancements of immunodetectable P4503A1/2 level (about 3-fold) and the P4503A1/2-associated ErD and 2β-OH hydroxylase activities (about 4–6-fold). The administration of ROC, however, did not induce either the P4502E1-mediated AnH, the pNPH activities [36] or the EROD activity linked to P450IA1 that is often involved in the bioactivation of procarcinogen and cellular toxicity [37]. Thus, ROC appeared to be an inducer like PB which is known to depress, in rat liver, the microsomal levels of P4502C11 and elevate the levels of P4502B1/2 and P4503A1/2 and, to a lesser extent, those of P4502A1 and P4502C6 [38]. However, it should be noted that the PB induction is not limited to the P450 genes, but is accompanied by enhanced production of glutathione *S*-transferase, epoxide hydrolase, UDP-glucuronyl transferase and other drug-metabolizing enzymes [25], whereas ROC does not affect these latter activities.

A comparison of P4502B1/2 proteins and the corresponding mRNA levels indicates that the increase of 2B1/2 proteins and their linked-monooxygenase activities parallels the increase of mRNA. Northern blot analyses provide evidence that ROC treatment in rat liver strongly induces the P4502B1/2 mRNA indicating that the drug acts at a pretranslational step in gene expression, as reported for PB induction [25]. Inasmuch as the level of P4502B1/2 is very low in untreated rat, being weakly detectable or undetectable by northern blot analysis [39, 40], the ROC potential for transcriptional activation of P4502B1/2 genes is significant. However, although transcriptional activation is the probable molecular mechanism for ROC induction of P4502B1 activity, further studies are needed to elucidate the role of transcription and the other regulatory mechanisms (RNA processing, mRNA stabilization, mRNA translation and protein stabilization).

It is well documented that many compounds augment the rate of their own metabolism by elevating the activity of certain P450 isozymes. The data presented here demonstrate that the P4502B1 and the DEX-inducible P4503A1 have a consistant if not major contribution to the deethylation of ROC. Thus, multiple ROC administrations, through the induction of these P450(s), could potentiate its own oxidative metabolism. In light of the probable

role of P4502B1 and P4503A1 in the ROC metabolism and the inhibition by ROC of PROD and ErD activities linked to the same isozymes, it can be explained why a single, but not a multiple, administration of ROC doses in rats *in vivo*, results in a substantial increase in hexobarbital sleeping times. After a single high ROC dose, the concentrations of drug found in hepatocytes appear sufficient to inhibit the metabolism of hexobarbital which is dependent on P4502B1/2 and probably P4503A1/2 [32, 33]. In fact, hexobarbital was found to be a competitive inhibitor (K_i = 250 μM) of ROC deethylation suggesting that ROC may inhibit the hexobarbital metabolism. After the repeated ROC doses, P4502B1/2 and P4503A1/2 are induced causing a higher biotransformation rate and clearance of the drug, weakening the inhibitory process and restoring the normal hexobarbital sleeping time. It should be noted that the apparent K_m(s) for ROC deethylation (0.22–0.48 mM) with various microsomes and the K_s (17 μM) and also the K_i(s) for the competitive inhibition by ROC of PROD and ErD activities (63 and 37 μM, respectively) are in disagreement in about one order of magnitude. Such a result is not surprising since both K_s and K_i reflect or can reflect the formation of enzyme–substrate complex in the absence of reducing equivalents, whereas K_m is obtained from the active monooxygenase system [41]. In addition, the secondary microsomal oxidation of ROC, although it accounts for only about 20% of total microsomal ROC metabolism, may play a role. It could be carried out by P4502B and 3A enzymes with a higher affinity than ROC deethylation. Further experiments are needed to establish whether P4502B, 3A or other P450 isoforms are involved in the oxidative metabolic pathway of this drug.

In conclusion, the results of this study suggest that ROC is a weak PB-type inducer of P450 in rat (on a molar basis, about five times less potent than PB in inducing P4502B), potentially able to alter its own metabolism if the drug is administered in a consistent and repeated manner.

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