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Biochimica et Biophysica Acta 1291 (1996) 60–66

BBA Biochimica et Biophysica Acta

Differential effects of neuroleptic agents on hepatic cytochrome *P*-450 isozymes in the male rat

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Received 4 January 1996; revised 23 April 1996; accepted 25 April 1996

Abstract

We report the effects of various dopamine receptor-blocking drugs on gene and protein expression, as well as the activity of several hepatic cytochrome *P*-450 (CYP) enzymes in the male Sprague-Dawley rat. At equipotent doses (with respect to receptor blockade and behavioural tests), the dopamine D_2-receptor selective sulpiride and remoxipride gave a conspicuous down-regulation of CYP2C11 and its associated androstenedione 16α-hydroxylation activity as well as of the CYP2C11-specific mRNA. The average immunoidentified CYP2C11 levels correlated with the CYP2C11-specific mRNA levels in all treatment groups ($r = 0.994$), indicating a transcriptional mechanism. The CYP3A protein was also selectively down-regulated. In contrast, androstenedione 5α-reduction was significantly increased. Clozapine, a non-selective neuroleptic, gave the same effects on the steroid metabolism as sulpiride and remoxipride. In contrast, diverging effects were observed for clozapine, compared to sulpiride and remoxipride, on the immunoidentified CYP1A2, CYP2B1, and CYP3A. These proteins were elevated by clozapine, and down-regulated by sulpiride and remoxipride. Our results are of interest for the interpretation of preclinical dose ranging toxicity tests of neuroleptic agents in rats. They may also be relevant in relation to certain interactions and adverse reactions observed in the clinical use of these drugs. The down-regulation of certain CYP enzymes is most likely mediated by an interaction with the growth hormone secretion.

Keywords: Cytochrome *P*-450; Hepatic regulation; mRNA; Neuroleptic; (Rat liver)

1. Introduction

The members of the cytochrome *P*-450 (CYP) enzyme family catalyze the oxidation of numerous drugs and other xenobiotics, and also a variety of endogenous substrates such as steroids, fatty acids and prostaglandins [1]. The 'hypothalamo-pituitary-liver axis' and the role of growth hormone (GH) in the hepatic sex-specific CYP metabolism have been thoroughly investigated in the rat [2]. Endocrine manipulations modulating the physiological GH secretion have revealed a crucial role of GH in the regulation of the sex-specific hepatic metabolism of steroids [3,4] and drugs [5–7]. Pharmacological intervention studies in our laboratory have shown that certain hepatic drug and steroid metabolic reactions are selectively down-regulated by morphine [6,8–10], whereas other pathways are up-regulated [9]. This may be related to the effects of morphine on GH via the dopaminergic system [9,10]. These findings have raised questions about the effects on the hepatic CYP enzyme system of pharmacological monoaminergic intervention by the dopaminergic receptor antagonists. Such intervention therapy is the basis of many pharmacological treatments in patients with various psychiatric and neurological disorders. Neuroleptics clearly affect the secretion of GH [11,12], but the mechanisms of the effects on GH secretion by monoaminergic intervention are not well understood. The effects of GH on hepatic CYP enzymes are closely dependent on the sex-specific pattern of GH secretion in the rat. The male pulsatile secretion pattern is believed to initiate transcription of the male-specific CYP enzyme genes, possibly through interaction between GH-regulated nuclear factors and DNA regulatory sites [13].

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PII S0304-4165(96)00046-3

We have investigated the effects on the hepatic CYP system in the male rat of different neuroleptics with antipsychotic effects. Two representatives of the neuroleptic group with main affinity for the dopamine D_2-receptor (sulpiride and remoxipride) were studied together with the atypical neuroleptic agent clozapine. The drugs were administered to the rats via mini-osmotic pumps. All three agents caused marked effects on certain isozymes, but clozapine, a non-selective neuroleptic, caused an elevation of several forms of CYP not observed for sulpiride, or remoxipride.

2. Materials and methods

2.1. Animals and tissue handling

Male Sprague-Dawley rats initially weighing 170 g were used (ALAB, Sollentuna, Sweden). Body weights were recorded before the treatment was started and before sacrifice. All rats were kept under standard conditions. The animals were sacrificed on the morning of day 15. The livers were excised immediately after sacrifice and put on wet or dry ice for isolation of microsomes [9] or RNA [14], respectively. Protein determination was performed as previously described [15].

2.2. Drug treatments

Administration of the neuroleptic drugs was performed by continuous dosing through the use of mini-osmotic pumps (Alzet) for 14 days. The daily average doses of the different drugs (mg/kg/day and μmol/kg/day, respectively), were as follows: sulpiride 137 and 401; remoxipride 31.3 and 73.4; and clozapine 114 and 349. Control animals were sham operated and kept under similar conditions until sacrifice. Two rats that had lost their pumps were excluded from the study. These rats belonged to the group treated with sulpiride (2).

2.3. Metabolic assays

Incubations of liver microsomes, cofactors and substrates were performed under conditions of linearity with time and protein. The assays were performed at 37°C for 10 min and the reactions were stopped on dry ice. The frozen incubates were stored at −80°C until extraction and analysis. The steroid substrates were dissolved in methanol, 2 μl per incubate. Larger volumes of this solvent inhibited the reactions. The *N*-demethylation and *O*-dealkylation of ethylmorphine was assayed as described previously, at 1.01 mM substrate concentration and 0.1 mg protein per ml incubate. The incubation time was 5 min [16]. The 16α-hydroxylation and 5α-reduction of 4-androstene-3,17-dione was assayed at a concentration of 0.6 mM androstenedione and 1.0 mg microsomal protein per ml incubate. The incubation time was 10 min. Tritiated substrate was used, and metabolites were separated by thin-layer chromatography [5].

Dehydroepiandrosterone (DHA)-16α-hydroxylation was measured as follows. The DHA and protein concentration was 0.1 mM and 0.1 mg per ml, respectively. Incubation time was 15 min. The analysis of 16α-OH-DHA was based on the method by Taylor et al. [17] with small modifications. Briefly, 0.1 ml of incubate or standards in aqueous solution was diluted with 1.0 ml distilled water and extracted with 5 ml of dichloromethane by a slow shaking for 15 min. The samples were centrifuged at 2000 rpm for 5 min at 4°C. The aqueous phase was aspirated and 4 ml of the dichloromethane phase were transferred to new tubes. This solvent volume was evaporated under nitrogen at 35–40°C. The residues were kept dry at −20°C. For the analysis, the residue was dissolved in 200 μl of the eluent (65% methanol in water) and 50 μl were injected on the column. Chromatography was performed on a Nova-Pak Phenyl 4 μm Radial-Pak cartridge (Millipore) at an eluent flow rate of 0.7 ml/min. Detection was carried out at 205 nm (detection limit 5–10 ng/ml) and the retention time was about 5 min.

2.4. Immunoblotting experiments

Liver microsomes from individual rats were pooled on an equal protein basis, and 10 and 20 μg protein, respectively, were separated by SDS-PAGE (9%). After transfer to nitrocellulose membranes, the proteins were probed with polyclonal and/or monoclonal antibodies against the various *P*-450s, and the immunoidentified protein bands were visualized by [^{125}I]Protein A labeling and quantitated in an LKB laser densitometer [9]. Results were expressed as percentage of control samples. Antibodies against *P*-450s 1A1/2, 2B1, 2C11, 3A1, and 4A1 were raised as described [18]. The antibody raised to CYP1A2 crossreacts with CYP1A1, the antibody to CYP2B1 also reacts with CYP2B2, and the antibody to CYP4A1 reacts with CYP4A2 and CYP4A3.

2.5. Preparation of RNA

Total cellular RNA was isolated by homogenization of the tissue in 4 M guanidinium thiocyanate with subsequent extraction with phenol-chloroform [14]. RNA concentrations were determined from the optical density at 260 nm.

2.6. Solution hybridization of RNA

The expression of CYP2C11 and CYP2C12 was analyzed by hybridization of RNA to complementary cRNA probes in solution [19,20]. cRNA probes were synthesized in vitro using the Riboprobe Gemini system (Promega), and radiolabelled with [^{35}S]UTP (Amersham). A 50 bp oligonucleotide synthesized from the determined sequence

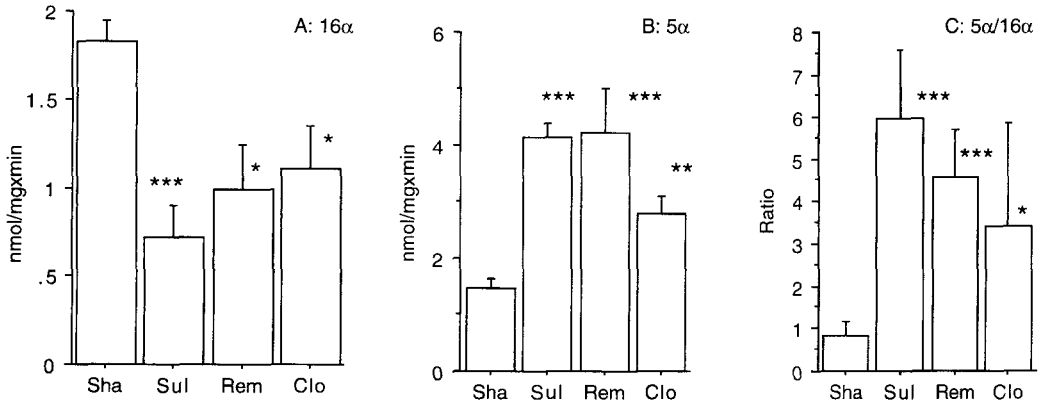

Fig. 1. Effects of neuroleptics on hepatic androstenedione 16α- hydroxylation and 5α-reduction. Liver microsomes from male rats treated with sulpiride (Sul), remoxipride (Rem), and clozapine (Clo) were used. Control rats were sham operated (Sha). (A) Androstenedione 16α-hydroxylation. The activity in control groups was 1.83 nmol/mg/min. (B) Androstenedione 5α-reduction. The activity in controls was 1.46 nmol/mg/min. (C) Ratio 5α/16α. Bars indicate the mean values of 6, 3, 6, and 5 individual rats in the respective treatment groups (left to right). Vertical lines are standard deviations and asterisks indicate the level of significance (* $P < 0.05$, * * $P < 0.01$, * * * $P < 0.001$).

of the CYP2C12 gene and a 190 bp fragment corresponding to the 3′ part of the CYP2C11 gene were used as templates for cRNA synthesis, as described previously [21]. Plasmids containing the CYP2C11- and CYP2C12-specific fragments were kindly provided by Dr. Inger Porsch Hällström (Karolinska Institute, Stockholm). ^{35}S-Labelled cRNA (20000 cpm/incubation) was hybridized at 65°C overnight in microcentrifuge vials containing 0.6 M NaCl, 20 mM Tris-HCl (pH 7.5), 4 mM EDTA, 0.1% SDS, 1 mM dithiothreitol, 25% formamide for 2C11, 20% formamide for 2C12, and 10–50 μg RNA per vial. After incubation, the samples were treated for 45 min with 1 ml of a solution containing 40 μg RNAse A, 2 μg RNAse T_1 (Boehringer) and 100 μg salmon sperm DNA. Radioactive RNA–RNA hybrids protected from RNAse digestion were precipitated by addition of 100 μl 6 M trichloroacetic acid, collected on a filter (Whatman GF/C) and quantitated by scintillation counting. Data represent the mean of 3–6 animals per point, mean of duplicate determinations. The hybridization method using RNA was validated against hybridization with TNA and found to correlate ($r = 0.86$).

All results are expressed as means ± S.D. for each group of rats based on data from five to eight individual liver microsomal preparations or liver specimens, except in the sulpiride group of Figs. 1 and 2 (see legends).

Statistical analysis was performed using the Student's *t*-test and the level of significance was either $P < 0.05$ (*), $P < 0.01$ (* *), or $P < 0.001$ (* * *).

3. Results

3.1. Steroid metabolic reactions in the liver

The rat male-specific 16α-hydroxylation of androstenedione, which is catalyzed predominantly by CYP2C11 [22], was significantly suppressed by treatment with all agents (Fig. 1A). Sulpiride and remoxipride suppressed the

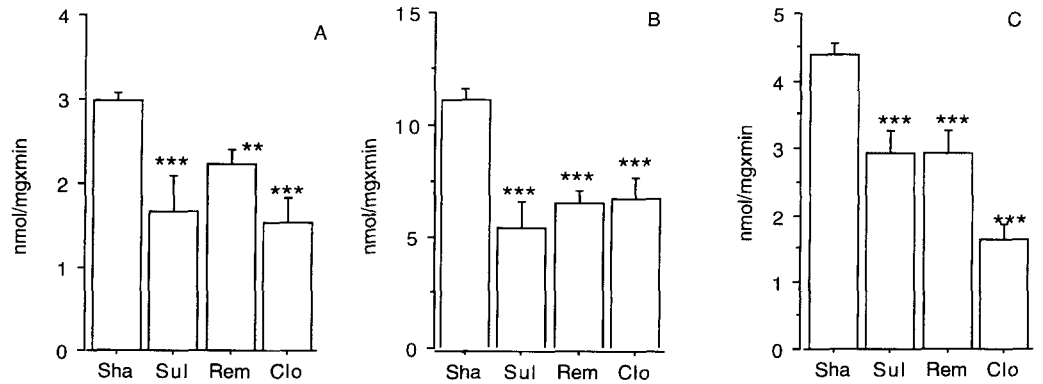

Fig. 2. Dehydroepiandrosterone (DHA) 16α-hydroxylation and ethylmorphine *N*- and *O*-dealkylation in liver microsomes from male rats treated with various neuroleptic drugs. (A) DHA 16α-hydroxylation. The enzymatic activity in the control group was 2.98 nmol/mg/min. (B) Ethylmorphine *N*-dealkylation. The average enzyme activity in control rats was 11.15 nmol/mg/min. (C) Ethylmorphine *O*-dealkylation. The average enzyme activity in control rats was 4.29 nmol/mg/min. Other details are given in the legend of Fig. 1.

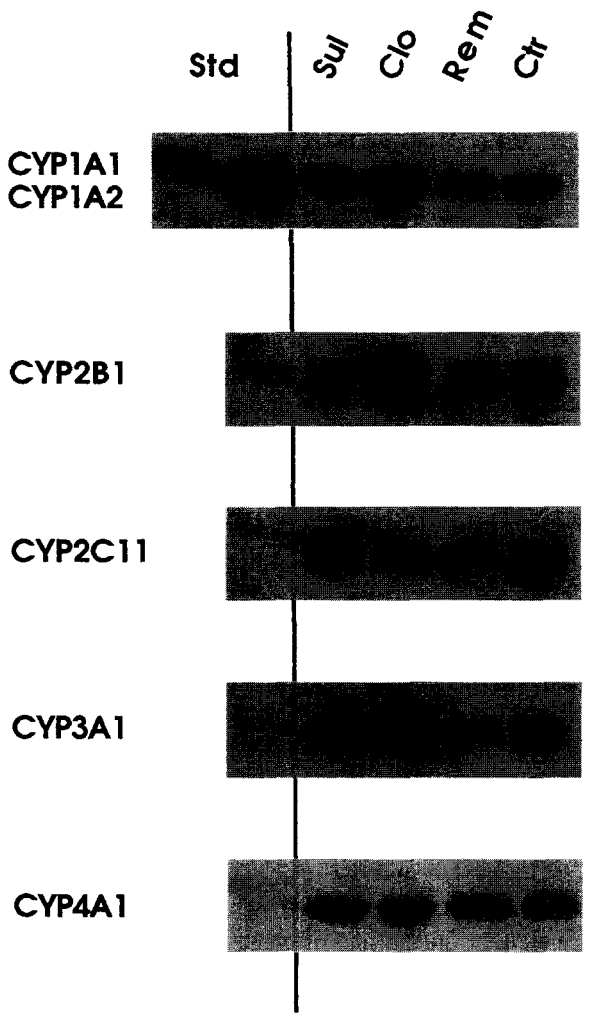

Fig. 3. Effects of neuroleptic drugs on hepatic cytochrome *P*-450 isozyme expression. Immunoblotting of pooled liver microsomal samples from male rats treated with various neuroleptic drugs was carried out as described in Fig. 1. Each blot (except that of CYP2C11) included a standard (Std) of the respective isozyme. Abbreviations are the same as in Fig. 1. 10 μg of protein was loaded per lane.

Table 1
Changes in immunoidentified hepatic cytochrome *P*-450 (CYP) isozymes in male rats after treatment with neuroleptic agent

CYP isozyme	Treatment with		
	sulpiride	clozapine	remoxipride
1A2	98	*179*	**71**
2B1	**66**	*187*	75
2C11	**64**	93	84
3A	**74**	*195*	**57**
4A1	117	116	95

The drugs were administered for 14 days via mini-osmotic pumps. The density of the immunoblotting bands shown in Fig. 3 was measured by laser scanning densitometry and expressed as percentage of control values (mean of two bands shown in Fig. 3). Visual decreases (bold type) and increases (italics) in Fig. 3 are marked.

reaction to 40% ($P < 0.001$) and 54% ($P < 0.05$) of control values, respectively. The corresponding value for clozapine was 60% ($P < 0.05$).

A corresponding significant increase of the rat female-specific androstenedione 5α-reductase activity was observed (Fig. 1B) after treatment with sulpiride (2.8-fold; $P < 0.001$), remoxipride (2.9-fold; $P < 0.01$), and clozapine (1.9-fold; $P < 0.01$). As a corollary of these changes, the 5α/16α-ratio (Fig. 1C) was raised for sulpiride (7.3-fold), remoxipride (5.6-fold), and clozapine (3.4-fold). This ratio is often used as an index of metabolic feminisation in the rat [3,23].

The dehydroepiandrosterone (DHA) 16α-hydroxylase was suppressed to 56%, 75%, and 51% of control in liver microsomes from rats treated with sulpiride, remoxipride, and clozapine, respectively (Fig. 2A).

3.2. Drug metabolic reactions in the liver

Treatment with sulpiride, remoxipride, and clozapine significantly suppressed the *N*-demethylation of ethylmorphine to 48%, 58%, and 61% of control, respectively (Fig.

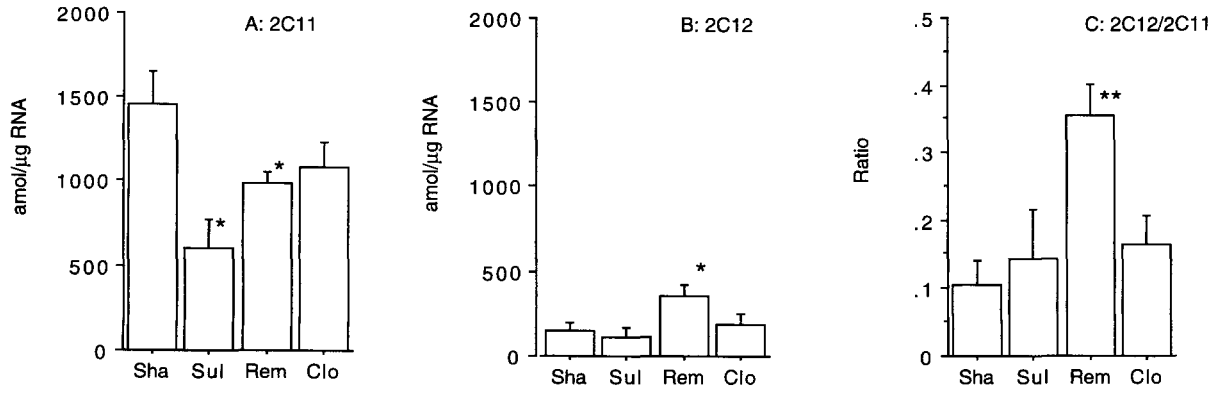

Fig. 4. Cytochrome *P*-4502C11- and 2C12-specific mRNA content in liver from male rats treated with different neuroleptic drugs. mRNA levels for (A) CYP2C11 and (B) CYP2C12 were monitored by solution hybridization (see Section 2) in total RNA prepared from groups of male rats treated with the various neuroleptic drugs and expressed as amol mRNA/μg total RNA. (C) Ratio 2C11/2C12. For abbreviations and statistical information, see legend of Fig. 1.

2B). The *O*-deethylation of this drug (Fig. 2C) was also significantly suppressed by pretreatment with these agents (to 67%, 67%, and 38% of control, respectively).

3.3. Immunoblotting of liver microsomal samples

Immunoblotting was performed on liver microsomes pooled from the animals of each treatment group so that the contribution of each individual animal was equal on a protein basis. Shown in Fig. 3 are immunoblots of microsomes from the different treatment groups probed with different antibodies, and the corresponding relative values of the band densities are given in Table 1. Treatment with sulpiride gave a decrease in the expression of CYP2B1, CYP2C11, and CYP3A. Decreased band densities for CYP3A were also observed in pooled microsomes from rats treated with remoxipride.

Interestingly, the effects of clozapine were different from those of the other neuroleptics. Clozapine markedly increased the band density of CYPs 1A2, 2B1, and 3A.

3.4. CYP2C11- and CYP2C12-specific mRNA levels

Analysis of the mRNA expression of CYP2C11 and 2C12 following treatment with the various neuroleptic drugs was carried out using a solution hybridization method. Treatment with sulpiride and remoxipride lead to a significant decrease in the expression of CYP2C11-specific mRNA (Fig. 4A). An increase in the mRNA expression of the female-specific CYP2C12 enzyme was seen in the rats treated with remoxipride (Fig. 4B). As a corollary, there was a significant increase of the 2C12/2C11 mRNA ratio in this treatment group (Fig. 4C).

The treatment effects on the CYP2C11 band density in Western blots were compared with the effects on the CYP2C11-specific mRNA for all treatment groups (Fig. 5). A high degree of correlation was found ($r = 0.994$).

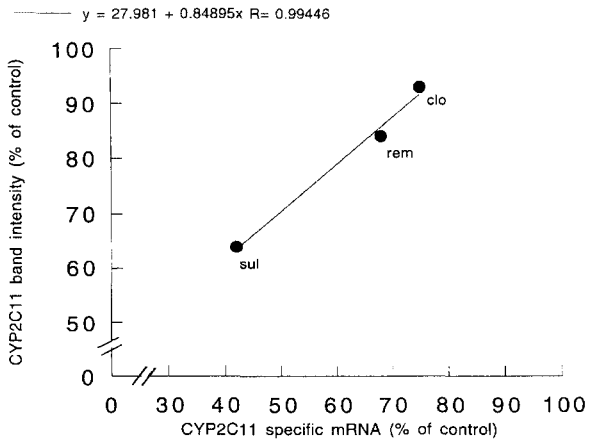

Fig. 5. Correlation between CYP2C11-specific mRNA and the corresponding protein levels in male rats treated with neuroleptic drugs. The data are also retrieved in Fig. 3 and Fig. 4, and the correlation coefficient (r) is 0.994. For abbreviations, see legend of Fig. 1.

4. Discussion

The specific perturbation of certain hepatic CYP enzymes and associated metabolic pathways described here for several neuroleptic agents may have implications for the evaluation of preclinical toxicity studies in rats. Treatment with these drugs may also have endocrine implications since several endogenous steroids are substrates of the affected enzymes.

The neuroleptics studied here have different chemical structures and affinities for neurotransmitter receptors and binding sites in the brain. Clozapine in particular has different effects on the brain metabolism and turnover of dopamine than sulpiride, consistent with its non-selective receptor binding profile [24]. The doses were chosen on the basis of equipotency in respect of the dopamine D_2 receptor occupancy in the caudate-putamen, as assessed in vivo with the selective D_2 receptor ligand [^{3}H]raclopride. With the doses used, about 80% of the dopamine D_2 receptors in the caudate-putamen, and probably also in other parts of the brain, should be constantly blocked during the experiment [25]. Equipotency was also prevailing on the basis of behavioral studies estimating the ED_{50} values for blockade of apomorphine-induced stereotypes [26].

All of the investigated neuroleptics caused a marked reduction in the male-specific 16α-hydroxylation of androstenedione in the rat. The drugs also increased the female-specific 5α-reduction of androstenedione, and hence, feminized the androstenedione metabolism, as assessed by the 5α-reduction/16α-hydroxylation ratio in the male rat. The down-regulation of the CYP2C11-associated 16α-hydroxylation [22] predicted a decrease of CYP2C11. Indeed, the density of the a-CYP2C11 antibody identified band (Table 1, Fig. 3) was markedly decreased by sulpiride but less so by remoxipride, consistent with the conspicuous effects on the 16α-hydroxylation. The compiled data from the treatment groups showed that the effects on CYP2C11 and its specific mRNA correlated well ($r = 0.994$; Fig. 5). This suggests the decreased enzyme expression to be mediated by a transcriptional mechanism.

Similar to the effects on 16α-hydroxylation of androstenedione, the 16α-hydroxylation of DHA and the dealkylations of ethylmorphine were consistently decreased by all drugs.

The maintenance of the male-dominant expression of the androstenedione 16α-hydroxylase (CYP2C11) is dependent on the intermittent secretion of GH characteristic of male rats. Administration of GH feminizes the metabolism of androstenedione in the male rat, leading to a decrease of the 16α-hydroxylase and an increase in the 5α-reductase activity [6,7,23,27]. It is possible that the neuroleptics studied feminize the male rat pattern of androstenedione metabolism by affecting the GH release [12]. The well-known effect on prolactin release has probably no part in the perturbation of the hepatic CYP enzyme

system [28]. The possibility that these effects are mediated through interaction with dopamine D_2-receptors in the CNS was not supported in our preliminary studies with low-dose neuroleptics such as haloperidol (unpublished). Theoretically, the suppression of the 16α-hydroxylation and the *N*- and *O*-dealkylation reactions may have been caused by direct hepatotoxicity. However, clozapine induced the expression of several other CYP isozymes, suggesting a regulatory rather than a general toxic effect on the hepatocytes. In addition, the CYP2C12-specific mRNA as well as the female-specific androstenedione 5α-reductase were increased in all treatment groups.

Both clozapine and sulpiride caused a decrease in ethylmorphine *N*-demethylase activity, a reaction believed to be catalyzed by CYP3A2 and CYP2C11, formerly denoted cytochrome *P*-450 PCN-E and UT-A, respectively [29]. Therefore, it is surprising that these drugs exerted a divergent effect on the immunoidentified CYP3A protein. The reason for this discrepancy is not clear.

There seems to be almost no information in the literature on the effect of neuroleptic agents on the CYP enzymes. However, rats treated with chlorpromazine, a less specific dopamine receptor-blocking agent, were down-regulated in their immunoidentified CYP2C11 protein and its associated metabolism of testosterone [30]. In contrast, the CYP2B1-associated *O*-depentylation of pentylresorufin was induced. This is at variance with our findings of a down-regulation of the CYP2B enzyme protein by sulpiride and remoxipride.

Other drugs were also reported to decrease the levels or activities of certain CYP isozymes. We recently reported a similar and conspicuous down-regulation of certain CYP isozymes by morphine treatment [9]. These effects could not be reproduced by treatment with pethidine, indicating that a non-opioid receptor mechanism is involved. Waxman et al. [31] found a marginal or moderate suppression of the male-specific *P*-450 2c/UT-A protein (CYP2C11) by some classical inducers including phenobarbitone and β-naphthoflavone. No effect on the female-specific *P*-450 2d/UT-I (CYP2C12) was noted. The CYP2C11-catalyzed 16α-hydroxylation of androstenedione was, however, not suppressed by phenobarbitone. This is at variance with our findings of a parallel reduction in both protein and 16α-hydroxylation. Certain macrolide antibiotics may also cause a decrease in 'male-specific *P*-450' and associated enzyme activity [32], possibly related to the formation of stable *P*-450–metabolite complexes [33]. Pretranslational suppression of CYP2C11 [34] as well as CYP2C12 [35] by interferon inducers was also described recently.

Our results bear interest and implications for the interpretation of acute and chronic toxicity studies with neuroleptic agents, since the dose-exposure proportionality may be deranged by metabolic perturbations, and may be different in male and female rats. Monoaminergic intervention is the basis of many pharmacological treatments in large patient groups with psychiatric, neurological or other disorders. Our data may have relevance for certain drug–drug interactions observed in the clinical use of neuroleptic drugs [36], such as inhibition of imipramine and nortriptyline metabolism by haloperidol and perphenazine, respectively. These interactions may be explained by effects on specific CYP enzymes similar to those described here in the rat. Some of the endocrine adverse drug reactions of neuroleptics may be caused by perturbation of steroid metabolism, e.g. gynaecomasty, which has been associated with a deranged metabolism of sex steroids [37]. Studies in man are warranted to see if our results in rats bear any relevance for the mechanism behind these and other significant adverse effects of neuroleptics.

Acknowledgements

We thank Mrs. Birgitta Ask for excellent assistance in the laboratory and Mrs. Elisabeth Agell for excellent secretarial help. We also thank Dr. S.-O. Ögren, Astra Arcus AB, for valuable comments on this paper. This study was supported by grants from the Swedish Medical Research Council (04X-04496) and the Swedish Cancer Society.

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