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HORMONAL PERTURBATION AS A POSSIBLE MECHANISM FOR THE ALTERATION OF CYTOCHROME P450 BY CYCLOPHOSPHAMIDE

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Abstract—The mechanism by which the cytotoxic agent cyclophosphamide (CP) alters cytochrome P450 and some associated enzymes in the male rat has been investigated. CP, administered as a high single dose, decreases the activity of the enzymes CYP2C6, CYP2C11, CYP3A2 and CYP2E1 and NADPH P450 reductase and increases the activity of steroid 5α-reductase. CP appears to exert its effect via an indirect mechanism that reaches its maximal effect 7 days after administration. The decreases in the activity of the enzymes CYP2C11, CYP2E1 and CYP3A2 are accompanied via a corresponding change in the amount of enzyme protein indicating that the alteration in expression of these enzymes occurs via changes in transcription and/or translation. Michaelis–Menten analysis confirmed this conclusion for the enzymes CYP2C11 and CYP3A2. The change in enzyme profile is accompanied by a reduction in the hormones, testosterone, TSH, T_4 and T_3. The reduction in hormone levels is also maximal 7 days after CP administration. To determine whether CP alters enzyme expression in the male rat via perturbation of hormonal regulation, daily replacement doses of hCG and/or T_3 were administered for 7 days after a single dose of CP and hepatic CYP and associated enzyme activities assessed. Results indicated that daily hormone replacement with either hCG and/or T_3 prevented the changes in expression of the hormone dependent enzymes NADPH P450 reductase, steroid 5α-reductase and CYP3A2 following a single dose of CP. In contrast for other enzymes including the male sex-specific enzyme CYP2C11, the female predominant enzyme CYP2E1 and the sex-independent enzyme CYP2C6, daily replacement of hCG and/or T_3 did not prevent the changes that occur 7 days following CP administration. As rats appeared anorexic and dehydrated and significant weight losses were recorded following CP treatment, blood was collected at the time of killing and subjected to biochemical analysis and a complete blood picture to identify any changes in such parameters that may have contributed to the changes in hormones and/or enzyme expression that occurred. However, significant variation in the treatment groups compared with controls for all parameters was not observed to occur except for an anticipated leukopoenia. We have concluded that CP alters the enzymes NADPH P450 reductase, steroid 5α-reductase and CYP3A2 via perturbation of the regulation of these enzymes by testosterone and/or thyroid hormones. However, while interference with regulation by testosterone and/or thyroid hormones may be part of the mechanism by which CP alters CYP2C11 and CYP2E1, other factors are contributing. Furthermore, the CP-mediated decrease in activity of CYP2C6, an enzyme shown to be independent of hormonal regulation, cannot be attributed to changes in hormone levels as expected. In addition we were able to eliminate the possibility that CP alters enzyme expression and/or hormone levels via alteration of biochemical parameters.

Key words: cyclophosphamide; cytochrome P450; testosterone; thyroid hormone; NADPH P450 reductase; steroid 5α-reductase

CP† is an alkylating agent commonly used in cancer chemotherapy and as an immunosuppressant [1]. CP requires metabolic activation by CYP enzymes to exert alkylating activity. The proposed mechanism is by an initial hydroxylation at C-4 [2]. The initial product 4-hydroxy CP undergoes ring-opening to form aldophosphamide. Aldophosphamide spontaneously decomposes to form acrolein, a metabolite previously implicated in causing the direct inactivation of CYP enzymes *in vivo* and *in vitro* [3–5], and phosphoramide mustard, the single biologically active alkylating metabolite. CYP2C6 and CYP2C11 have been shown to be the major catalysts responsible for the bioactivation of CP in uninduced rat liver microsomes [6].

Previously we have reported that 7 days following a single dose of CP (200 mg/kg) there is a decrease in CYP activity in male rats [7]. The enzymes decreased by CP include CYP2A1/2, CYP2B1, CYP2C11 and CYP3A2 as well as the associated enzyme NADPH P450 reductase. Co-administration of CP with NAC, an agent that provides free sulphydryl groups, did not prevent the alteration mediated by CP and so we concluded that CP is not

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† Abbreviations: CP, cyclophosphamide; NAC, *N*-acetylcysteine; AD (androstenedione), androst-4-ene-3,17-dione; testosterone, 17β-hydroxy-androst-4-ene-3-one; steroid 5α-reductase, NADPH:Δ^4-3-oxosteriod-5α-oxidoreductase; TSH, thyroid stimulating hormone; T_3, 3,3′,5-triiodo-L-thyronine; T_4, L-thyroxine; 4-MA, 7β-(*N*,*N*-diethylcarbamoyl) - 4 - aza - methyl - 5α - androstan - 3 - one; hCG, human chorionic gonadotrophin; GH, growth hormone; CYP, cytochrome P450.

acting directly to alter the activity of the various enzymes via its metabolite acrolein [7].

Recently, it has become evident that the rat liver is a sexually differentiated tissue and is subject to complex hormonal regulation [8–10]. CYP enzymes and other drug metabolizing enzymes are regulated by hormones such as the gonadal steroids, GH, the thyroid hormones and the glucocorticoids [8–10].

Le Blanc and Waxman [11] reported a selective alteration in the activity of hepatic CYP enzymes over a 9 day period following administration of CP 130 mg/kg i.p. It was postulated [11] that the feminized response, that is, the decrease in some male specific enzymes and the increase in the female specific enzyme steroid 5α-reductase following CP administration occurred via perturbation of the hormonal regulation of these enzymes as a concurrent decrease in testosterone was observed to occur. Le Blanc and Waxman [11] suggested that the CP metabolite acrolein may be acting at the hypothalamic pituitary axis to decrease testosterone levels. A more recent study by Chang and Waxman [12] concluded that the CP metabolite phosphoramide mustard appears to be responsible for the CP medicated changes in CYP2C11 and steroid 5α-reductase activity with acrolein potentiating the modulating action of the mustard.

In the present study, the effects of CP on CYP2E1 and steroid 5α-reductase have been examined. To examine the mechanism by which CP alters the quantitatively important CYP enzymes, CYP2C11 and CYP3A2, Michaelis–Menten studies have been performed. In addition, CYP2C11, CYP2E1, CYP3A2 and NADPH P450 reductase have been immunoquantitated.

We have postulated that CP (via phosphoramide mustard and/or acrolein) may be disturbing the plasma levels of hormones other than testosterone resulting in alterations in enzyme activity and expression. To examine this hypothesis, plasma levels of testosterone, TSH, T_4 and T_3 have been measured at various time intervals after CP administration. Results from hormone level studies indicated that decreases in all the hormones measured occurred following CP administration with maximum decreases occurring 7 days after CP administration.

To further examine the possibility that CP may be changing enzyme profiles via perturbation of hormonal regulation, daily replacement doses of hCG and/or T_3 were administered for 7 days after a single dose of CP and hepatic CYP and associated enzyme activities and, where possible, protein contents assessed.

It has previously been reported that states such as diabetes, fasting and uraemia can alter CYP expression via a change in hormone levels [13, 14]. As rats appeared anorexic, dehydrated and significant weight losses were recorded following CP treatment, blood was collected at the time of killing and subjected to biochemical analysis and a complete blood picture to identify any changes in such parameters that may have contributed to the changes in hormones and/or enzyme expression that occurred.

MATERIALS AND METHODS

Materials. 4-MA was a gift from Merck, Sharpe and Dohme. The NADPH P450 reductase and CYP3A antibodies were a gift from Prof. ME McManus, the CYP2E1 antibody was a gift from Dr ME Veronese and the CYP2C11 antibody was a gift from Dr M Murray. Nitrocellulose was purchased from Schleicher and Schuell (Dassel, Germany), horseradish peroxidase conjugated second antibodies from Silenus laboratories (Hawthorn, Australia) and all other reagents for Western blotting from Biorad (Sydney, Australia). T_3 was obtained from Glaxo (Liverpool, U.K.). hCG was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The testosterone assay kit was purchased from CIS Bio International (Gif-Sur-Yvette, Cedex, France). The rat TSH assay kit was purchased from Amersham Laboratories (Buckinghamshire, U.K.). Sources of other materials were as previously described [7, 15].

Animals. Adult male Hooded Wistar rats (180–220 g) were purchased from Gilles Plains Animal Resource Centre (Adelaide, Australia) and acclimatized under standard conditions. Animals were housed in metal cages and allowed free access to food and water.

Four *in vivo* experiments were conducted. In the first experiment, the animals were randomly assigned to six treatment groups (N = 6). One group was administered NaCl (0.9%) solution as a single i.p. dose and killed 7 days after administration. Three groups were administered CP (200 mg/kg) freshly prepared in NaCl (0.9%) as a single i.p. dose and killed on days 1, 4 and 7. Another group was coadministered CP and NAC where the NAC was given 0.5 hr before (200 mg/kg) and 0.5 hr after (200 mg/kg) CP (200 mg/kg), such that the total NAC dose was 400 mg/kg, and killed 7 days later. The final group received NAC (400 mg/kg) but no CP and were killed after 7 days. The same dose schedule of NAC was used where NAC was given before and after saline.

In the second experiment, rats were randomly assigned to six treatment groups (N = 6) and were injected i.p. with CP (200 mg/kg) freshly prepared in NaCl (0.9%), 1, 4, 7, 10 or 14 days prior to sacrifice. Control animals were injected i.p. with the same volume of NaCl (0.9%).

In the third experiment rats were randomly assigned to eight treatments (N = 5–6). One group were administered CP (200 mg/kg) freshly prepared in NaCl (0.9%) i.p. as a single dose 7 days prior to killing, to one group on day zero and then daily hormone replacement vehicle until the day of killing. The second group received CP on day zero plus daily T_3 5 μg/kg i.p. administered until the day of killing. The third group received CP on day zero plus daily hCG (500 IU/kg) s.c. administered until the day of killing. The fourth group received CP on day zero plus daily T_3 5 μg/kg i.p. and daily hCG (500 IU/kg) s.c. administered until the day of killing. Corresponding control groups were also administered with saline on day zero plus daily hormone or daily hormone vehicle.

In the fourth experiment, rats were randomly assigned to four treatment groups (N = 4) and were injected i.p. with CP (200 mg/kg) freshly prepared in NaCl (0.9%), 1, 4, or 7 days prior to sacrifice.

Control animals were injected i.p. with the same volume of NaCl (0.9%).

In the first three experiments, animals were killed by cervical dislocation and exsanguination, the livers removed and immediately placed in ice cold phosphate buffer (0.1 M, pH 7.4) with KCl (1.15%), prior to microsome preparation. All further steps were carried out at 4°. Microsomes were prepared by a method of differential centrifugation as previously described [16] and stored at −70° until used. Animals in the fourth experiment were anaesthetized with ether and blood was collected via cardiac puncture for biochemical and haematological analysis. Hepatic microsomes were also prepared as described above.

Assays. Protein concentration, P450 specific content, NADPH cytochrome P450 reductase and aminopyrine demethylase activities were determined as previously described [7, 15]. Steroid 5α-reductase activity was measured using AD as a substrate as described previously [15] and measuring the conversion of [^{14}C]AD to [^{14}C]5α-androstan-3,17-dione after the zone corresponding to this metabolite had been located by autoradiography. The [^{14}C]5α-androstan-3,17-dione metabolite was confirmed by Dr G Curry (Waite Agricultural Research Institute, Urrbrae, South Australia) using a F. Finnigan Mat TSQ 70 Mass Spectrometer. Aniline hydroxylase activity [17] and ethylmorphine demethylase activity [18] were measured as previously described.

Kinetic analysis. Microsomal protein (0.25 mg or 0.5 mg) obtained from rats (N = 3) in the first *in vivo* study was incubated in the presence of androstenedione (using 10–13 concentrations) over the range 7–350 μM to determine Michaelis–Menten parameters of CYP3A2 and CYP2C11. Androstenedione hydroxylase activity was assayed as described previously [15] except incubation mixtures also contained 1.0 μM 4-MA to inhibit 5α-reduction of the substrate catalysed by microsomal steroid 5α-reductase. Michaelis–Menten parameters for a single enzyme system were derived graphically from Eadie–Hofstee plots for individual rats. The values obtained were used as initial estimates for a non-linear least squares regression computer program (Multifit 2.0®, Day Computing, Cambridge, U.K.). Data were weighted according to the reciprocal of the observations squared.

Western blotting. SDS-PAGE was performed according to the method of Laemmli [19], using a 4.5% acrylamide stacking gel and a 7.5% separating gel in a Hoefer SE 500 gel apparatus. Samples (30 μg microsomal protein/lane) were solubilized with an equal volume of 'sample' buffer and heated at 100° for 10 min. Gels were stacked at 100 V (60 min) then run at 200 V (150 min) until the bromophenol blue marker reached the bottom of the gel.

For immunoblotting, samples were transferred from acrylamide gels to nitrocellulose membranes (25 mM Tris-HCl, 192 mM glycine, 20% methanol; 50 V overnight using a Hoefer TE series transfer electrophoresis unit). Membranes were blocked in 5% skim milk (1 hr, 37°). Immunoblot analysis was performed by incubation (3 hr, 37°) with dilutions of primary antibody (CYP2C11, 1:1,666; CYP2E1, 1:2000; CYP3A, 1:1000; NADPH P450 reductase 1:1000) followed by incubation (1 hr, room temp.), with horseradish peroxidase conjugated donkey anti-sheep (CYP2E1, CYP3A, NADPH P450 reductase) and sheep anti-rabbit (CYP2C11) and development with HRP colour development reagent.

Quantitation of the immunoblotted microsomal P450s was carried out by laser densitometry (LKB 2222-010 Ultro-Scan XL). Relative CYP levels were determined by direct comparison to a pooled hepatic microsomal sample which was always run in parallel. This pooled control sample was assigned a value of 1 and the enzyme protein values are expressed relative to this.

Plasma hormone assays. Plasma testosterone and TSH were measured by solid phase radio-immunoassay using commercially available kits. Plasma T_4, T_3 and some TSH levels were kindly analysed, using rat hormone specific assays, by the clinical chemistry department, The Queen Elizabeth Hospital (Adelaide, South Australia). Hormone levels were determined for N = 4–6 individual animals in each treatment group.

Clinical observations. Rats were observed daily for clinical signs in the fourth experiment, including appetite, appearance, excretory functions and discharges and mortality. In addition, individual body weight measurements and food and water consumption were monitored daily.

Clinical chemistry. Chemical analysis of blood from rats in the fourth experiment was carried out using a Cobas Mira analyser (Roche Diagnostics Systems, Hoffman La Roche, Basle, Switzerland). The following parameters were measured and/or calculated: sodium, potassium, chloride, osmolarity, glucose, urea, creatinine, creatinine clearance, phosphate, calcium, albumin, globulins, total protein, cholesterol, total bilirubin, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, creatinine kinase, alanine aminotransferase, lipase, sodium/potassium ratio and β-hydroxy butyrate.

Haematology. Haematological measurements of blood from rats in the fourth experiment were made using a Sysmex K-1000 analyser (Toa Medical Electronics Co. Ltd, Kobe, Japan). Haematological parameters measured included: erythrocyte count, haemoglobin, haematocrit, white blood count and platelets. Erythrocyte indices were also calculated (mean cell volume and mean corpuscular haemoglobin concentration).

Urinalysis. Animals in the fourth experiment were placed in metabolism cages and 24 hr urine samples were collected for urinalysis. The following parameters were measured: volume, appearance, colour, creatinine, pH, protein, glucose, ketones, bilirubin, occult blood and urobilinogens. The creatinine was measured using a Cobas Mira analyser (Roche Diagnostics Systems, Hoffman La Roche, Basle, Switzerland) and where appropriate, Ames multistix (Ames Division of Miles Laboratories, Elkhart, IN, U.S.A.) were used.

Statistical analysis. Statistical comparisons were by ANOVA and subsequently the Dunnet's multiple range test or the Bonferroni/Dunn multiple range test as appropriate. A P-value less than 0.05 (two-

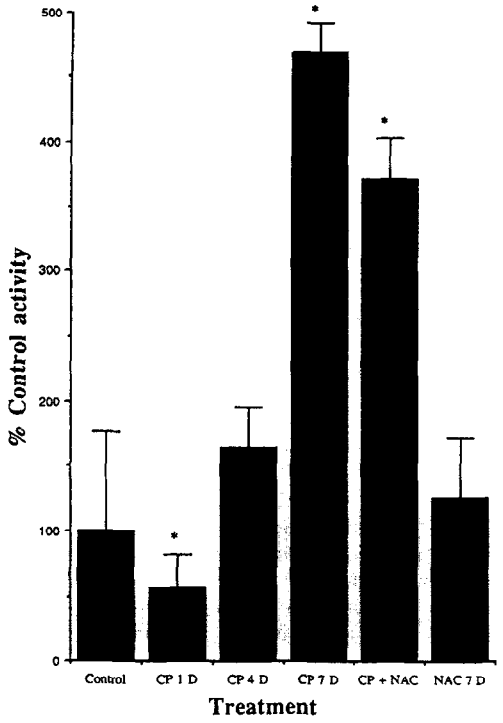

Fig. 1. Steroid 5α-reductase activity representing the mean ± SD for N = 6 individual rat hepatic microsome preparations relative to control values where 100 = 0.98 nmol/mg/min. *P < 0.05 versus saline control group.

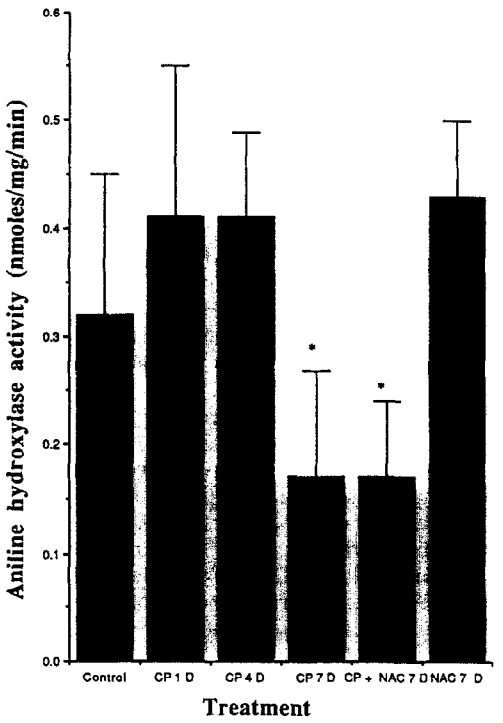

Fig. 2. Aniline hydroxylase activity representing the mean ± SD for N = 6 individual rat hepatic microsome preparations relative to control values. *P < 0.05 versus saline control group.

tailed) was considered to be significant unless otherwise stated.

RESULTS

Experiment 1

In Fig. 1 steroid 5α-reductase is clearly increased 7 days after CP administration and the increase observed is not prevented when CP is coadministered with NAC. In Fig. 2 it is evident that the activity of aniline hydroxylase activity is decreased 7 days after but not 1 or 4 days following CP administration and again was not counteracted by co-administration of CP with NAC. Mean apparent Michaelis–Menten parameters for AD 16α- and 6β-hydroxylases are shown for each treatment group in Table 1. The V_{max} values of both enzymes were not altered 1 or 4 days after CP administration but were reduced after 7 days while the K_m values remain unchanged for all groups.

Mean results from immunoquantitation analysis where antibodies to NADPH P450 reductase, CYP2C11 and CYP3A have been used are shown in Table 2. Statistical comparisons for Western blotting analysis show that NADPH P450 reductase, CYP2C11 and CYP3A protein contents were significantly decreased 7 days after CP administration and 7 days after co-administration of CP and NAC, consistent with the decrease in activity levels reported previously [7].

Experiment 2

In Fig. 3 it is evident that plasma levels of testosterone, TSH, T_3 and T_4 were reduced at all time intervals following CP administration. For testosterone the reduction was significant for all time intervals except after 4 days while TSH was significantly reduced after 7 days only. The decrease in T_4 was significant at all measured time points except after 1 day while T_3 was significantly reduced at all time points following CP administration. The nadir for all hormone levels occurred 7 days after CP administration (Fig. 3) while the maximal decrease in P450 content and aminopyrine demethylase activity measured in the hepatic microsomes prepared from the experiments occurred 10 days following CP administration (not shown).

Experiment 3

NADPH P450 reductase activity 7 days after CP administration was decreased with a corresponding decrease in the amount of enzyme (Tables 3 and 5). Administration of daily T_3 or daily T_3 plus hCG replacement prevented the decrease (Tables 3 and 5). Consistent with results from experiment 1 (Fig. 1), CP administration increased the activity of steroid 5α-reductase 7 days after its administration. Daily T_3 replacement did not prevent the increase in enzyme activity while daily hCG administration and coadministration of daily T_3 and hCG after CP dosing prevented the anticipated increase in steroid

Table 1. Michaelis–Menten parameters for AD 6β- and 16α-hydroxylase in hepatic microsomes from control, CP 1, 4 and 7 day, NAC and CP + NAC-treated rats

Treatment	AD 16α-hydroxylase		AD 6β-hydroxylase	
	K_m (μM)	V_{max} (nmols/mg/min)	K_m (μM)	V_{max} (nmols/mg/min)
Saline control	21.7 ± 9.2	3.2 ± 0.5	24.0 ± 4.2	1.8 ± 0.2
CP 1 day	14.7 ± 2.9	3.2 ± 0.4	21.6 ± 3.6	1.8 ± 0.2
CP 4 day	19.9 ± 2.6	3.1 ± 0.4	27.2 ± 5.9	2.4 ± 0.3
CP 7 day	18.2 ± 2.2	1.7 ± 0.5*	26.8 ± 3.8	1.1 ± 0.4*
NAC 7 day	18.3 ± 1.2	2.7 ± 0.1	23.5 ± 2.6	1.9 ± 0.1
CP + NAC 7 day	22.1 ± 9.9	1.0 ± 0.3*	25.0 ± 5.0	0.9 ± 0.5*

Activity values represent the mean ± SD for N = 3 individual rat liver microsome preparations.
* Significantly different to saline 7 day controls at $P < 0.05$.

Table 2. Immunoquantitation results for NADPH P450 reductase, CYP2C11 and CYP3A pretein levels in hepatic microsomes from control, CP 1, 4 and 7 day, NAC and CP + NAC-treated rats

Treatment	NADPH P450 reductase	CYP2C11	CYP3A
Saline control	1.3 ± 0.4	1.2 ± 0.3	1.1 ± 0.4
CP 1 day	1.5 ± 0.6	1.3 ± 0.3	1.3 ± 0.6
CP 4 day	1.3 ± 0.6	1.1 ± 0.5	1.0 ± 0.2
CP 7 day	0.3 ± 0.1*	0.3 ± 0.1*	0.2 ± 0.1*
NAC 7 day	1.6 ± 0.7	1.2 ± 0.2	1.4 ± 0.2
CP + NAC 7 day	0.4 ± 0.1*	0.3 ± 0.2*	0.5 ± 0.6**

Protein level values represent the mean ± SD for N = 6 individual rat liver microsome preparations.
* Significantly different to saline 7 day controls at $P < 0.05$.
** Significantly different to saline 7 day controls at $0.1 < P < 0.05$.

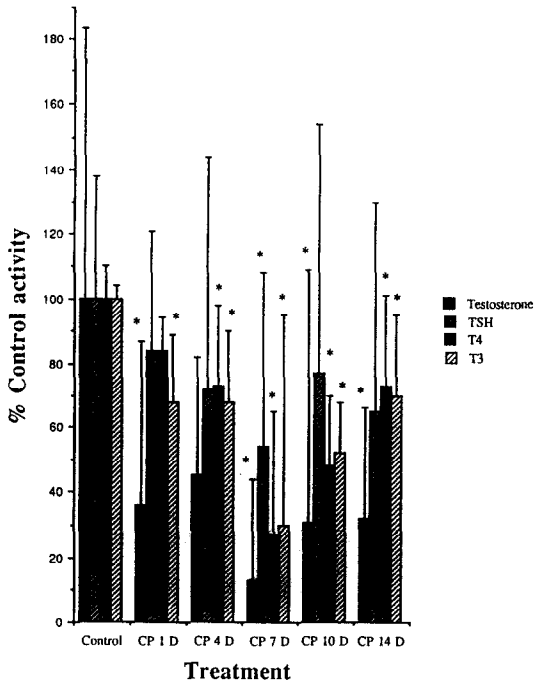

Fig. 3. Plasma hormone levels representing the mean ± SD for N = 4–6 individual rats relative to saline control values where 100 = 2.81 ng/mL for testosterone, 9.20 ng/mL for TSH, 43.42 ng/mL for T_4 and 1.02 ng/mL for T_3. *$P < 0.05$ versus saline control group.

5α-reductase (Table 3). The decrease in the activities of the enzymes erythromycin demethylase and androstenedione 6β-hydroxylase that occurred 7 days following CP administration were prevented when daily T_3, daily hCG and daily T_3 plus hCG were administered following CP dosing (Table 3).

In contrast to other enzymes including androstenedione 16α-hydroxylase, aniline hydroxylase and ethylmorphine hydroxylase, while activity is decreased 7 days following CP administration as expected, daily administration of T_3 and/or hCG did not prevent the decreases that occur following CP dosing (Table 4).

Immunoquantitation analysis of the enzymes NADPH P450 reductase, CYP2C11, CYP2E1 and CYP3A consistently demonstrated that when a decrease in the activity of a given enzyme occurred there was a decrease in the corresponding amount of protein. Similarly, when the decrease was prevented from occurring by daily hormone replacement after CP dosing, protein levels, as expected, remained unchanged as well as the activity of the enzyme relative to the appropriate controls (Table 5).

Experiment 4

The animals were observed to have reduced food consumption following CP treatment and to have lost weight. The average body weight increase for control rats 1, 4 and 7 days following saline administration was 3%, 12% and 17%, respectively. In comparison, 1, 4 and 7 days following CP administration, rats were recorded to have an

Table 3. Influence of CP administration 7 days before killing (± daily hormone replacement) on NADPH P450 reductase, steroid 5α-reductase, erythromycin demethylase and AD 6β-hydroxylase activities in hepatic microsomes from saline control, saline + T_3, saline + hCG, saline + T_3 + hCG, CP, CP + T_3, CP + hCG and CP + T_3 + hCG-treated rats

Treatment	NADPH P450 reductase activity (nmols/mg/min)	Steroid 5α-reductase activity (nmols/mg/min)	Erythromycin demethylase activity (nmols/mg/min)	AD 6β-hydroxylase activity (nmols/mg/min)
Saline control	134 ± 10	0.87 ± 0.28	0.70 ± 0.13	0.86 ± 0.12
Saline + T_3	129 ± 16	1.07 ± 0.33	0.69 ± 0.13	0.78 ± 0.07
Saline + hCG	170 ± 22	0.57 ± 0.06	0.70 ± 0.12	0.85 ± 0.14
Saline + T_3 + hCG	130 ± 12	0.66 ± 0.18	0.82 ± 0.07	0.82 ± 0.23
CP	80 ± 13*	2.37 ± 1.01*	0.30 ± 0.06*	0.19 ± 0.18*
CP + T_3	100 ± 24	2.93 ± 0.60*	0.61 ± 0.25	0.57 ± 0.41
CP + hCG	118 ± 18*	1.37 ± 0.25	0.59 ± 0.11	0.47 ± 0.15
CP + T_3 + hCG	102 ± 39	1.35 ± 0.32	0.77 ± 0.26	0.53 ± 0.37

Activity values represent the mean ± SD for N = 5–6 individual rat liver microsome preparations.
* Significantly different relative to corresponding saline (± daily hormone replacement) 7 day controls at $P < 0.05$.

Table 4. Influence of CP administration 7 days before killing (± daily hormone replacement) on AD 16α-hydroxylase, aniline hydroxylase and ethylmorphine demethylase activities in hepatic microsomes from saline control, saline + T_3, saline + hCG, saline + T_3 + hCG, CP, CP + T_3, CP + hCG and CP + T_3 + hCG-treated rats

Treatment	AD 6β-hydroxylase activity (nmols/mg/min)	Aniline hydroxylase activity (nmols/mg/min)	Ethylmorphine demethylase activity (nmols/mg/min)
Saline control	1.53 ± 0.49	0.38 ± 0.05	8.13 ± 1.31
Saline + T_3	1.31 ± 0.15	0.31 ± 0.04	6.41 ± 1.15
Saline + hCG	1.40 ± 0.29	0.30 ± 0.03	7.55 ± 0.67
Saline + T_3 + hCG	1.01 ± 0.08	0.26 ± 0.09	8.26 ± 0.59
CP	0.35 ± 0.17*	0.16 ± 0.04*	1.79 ± 1.52*
CP + T_3	0.43 ± 0.27*	0.11 ± 0.08*	2.96 ± 1.78*
CP + hCG	0.45 ± 0.18*	0.24 ± 0.09*	2.05 ± 0.68*
CP + T_3 + hCG	0.36 ± 0.27*	0.14 ± 0.07*	2.46 ± 1.24*

Activity values represent the mean ± SD for N = 5–6 individual rat liver microsome preparations.
* Significantly different relative to corresponding saline (± daily hormone replacement) 7 day controls at $P < 0.05$.

average weight loss of 6%, 7% and 13%, respectively. Results from biochemical and haematological analysis indicated there were no significant changes in the parameters measured at various time intervals after CP administration relative to controls except a decreased white blood cell count 1, 4 and 7 days following CP administration ($P < 0.05$, not shown).

DISCUSSION

Experiments conducted in this laboratory have indicated that 7 days after the administration of CP (200 mg/kg) to male hooded Wistar rats the activities of various CYP enzymes and associated enzymes are altered [7]. CP administered as a 200 mg/kg dose is double the LD_{50} for CP in rats [20]. If body surface area differences between the rat and the human are taken into account [21], CP administered as a 200 mg/kg dose to rats corresponds with the high doses typically administered to humans for cancer chemotherapy and immunosuppression. We have previously reported that CP significantly decreased the activity of the enzymes NADPH P450 reductase and androstenedione 6β- and 16α-hydroxylase, probes for CYP3A2 and CYP2C11, respectively [22], 7 days but not 1 or 4 days after its administration. In these experiments the co-administration of CP with NAC, an agent that provides free sulphydryl groups, did not alter the decrease in activity mediated by CP at 7 days [7]. If CP was acting indirectly via its metabolite acrolein at the hypothalamic–pituitary gonadal axis as initially suggested by Le Blanc and Waxman [11] then co-administration of CP with NAC could be expected to counteract these effects. However, this was not observed in our experiments and we concluded that CP, or a CP metabolite other than acrolein (possibly phosphoramide mustard), may be acting to perturb hormonal regulation or,

Table 5. Influence of CP administration 7 days before killing (± daily hormone replacement) on NADPH P450 reductase, CYP2C11, CYP2E1 and CYP3A protein levels in hepatic microsomes from saline control, saline + T_3, saline + hCG, saline + T_3 + hCG, CP, CP + T_3, CP + hCG and CP + T_3 + hCG-treated rats

Treatment	NADPH P450 reductase	CYP2C11	CYP2E1	CYP3A
Saline control	1.1 ± 0.4	0.7 ± 0.01	1.2 ± 0.1	0.9 ± 0.2
Saline + T_3	1.0 ± 0.2	1.2 ± 0.1	0.9 ± 0.2	1.1 ± 0.2
Saline + hCG	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.2
Saline + T_3 + hCG	1.2 ± 0.3	1.4 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
CP	0.6 ± 0.1*	0.5 ± 0.2*	0.2 ± 0.1*	0.4 ± 0.3*
CP + T_3	0.9 ± 0.3	0.7 ± 0.1*	0.3 ± 0.2*	0.7 ± 0.3
CP + hCG	1.02 ± 0.33	0.5 ± 0.1*	0.2 ± 0.1*	0.7 ± 0.1
CP + T_3 + hCG	0.9 ± 0.2	0.3 ± 0.1*	0.2 ± 0.2*	0.7 ± 0.2

Protein level values represent the mean ± SD for N = 5–6 individual rat liver microsome preparations.

* Significantly different relative to corresponding saline (± daily hormone replacement) 7 day controls at $P < 0.05$.

alternatively, acrolein may be acting at sites that are inaccessible to NAC [7]. Our conclusions are complementary to results obtained from a later study by Chang and Waxman [12] where phosphoramide mustard was identified as the CP metabolite that modulates the expression of some hepatic enzymes while acrolein appears to potentiate the effects of the mustard.

Results from experiments 1 and 3 indicated that 7 days after CP administration there was an increase in steroid 5α-reductase activity (experiment 1 (Fig. 1); experiment 3 (Table 3)) and a decrease in aniline hydroxylase activity (experiment 1 (Fig. 2); experiment 3 (Table 4)). The effects on both enzymes were not counteracted by NAC (experiment 1 (Figs 1 and 2)), essentially eliminating the possibility that acrolein changes the enzymes directly. Aniline hydroxylase is a probe for CYP2E1 [17]. Results from experiment 3 demonstrated that there was also a decrease in CYP2E1 enzyme expression corresponding with the decrease in activity of aniline hydroxylase 7 days after CP administration (Tables 4 and 5). These results are similar to results reported by Le Blanc and Waxman [11] except that where Le Blanc and Waxman [11] reported that CYP2E1 protein levels were elevated while aniline hydroxylase activity was decreased 3 days after CP administration, our results indicated that 4 days after CP administration both CYP2E1 protein levels (not shown) and aniline hydroxylase activity (Fig. 2) were not significantly different from controls.

The Michaelis–Menten parameters of AD 16α- and 6β-hydroxylase activity (probes for the enzymes CYP2C11 and CYP3A2) [22] in each treatment group were determined to investigate whether the observed decrease in activity 7 days after CP treatment caused a decrease in V_{max}, K_m or both V_{max} and K_m of the enzyme concerned and hence further characterize the mechanism by which CP alters P450 (Table 1). As the V_{max} values of the enzymes were not altered 1 or 4 days after CP administration but were reduced at 7 days and the K_m values remain unchanged for all groups, this provides definitive evidence that CP mediates a decrease in transcription and/or translation of these enzymes, that is, CP causes a reduction in the amount of enzyme rather than the activity of the enzyme or the affinity of the enzyme for the substrate. Co-administration of CP with NAC did not prevent the CP mediated decrease in transcription and/or translation at 7 days providing further evidence that acrolein is not acting directly to inactivate the enzymes (Table 1).

Consistent with the hypothesis that CP acts indirectly to decrease transcription and/or translation of the enzymes NADPH P450 reductase, CYP2C11 and CYP3A 7 days post CP treatment, results from Western blotting analysis indicated that protein levels (Table 2) were decreased 7 days after CP administration in conjuction with the measured decrease in activity previously reported [7]. Consistent with previous results NAC did not counteract the effects of CP (Table 2). This further indicates that CP requires up to 7 days to exert its effect, that is, CP and/or a metabolite(s) is acting indirectly as opposed to directly denaturing the cytochrome as previously suggested [3–5].

Le Blanc and Waxman [11] suggested that a CP metabolite, possibly acrolein, produced by CP may act at the hypothalamic–pituitary gonadal axis, decreasing testosterone, perturbing the hormonal regulation of CYP and consequently feminizing the enzyme profile. In experiment 2, in addition to decreased testosterone levels following CP treatment, we also observed a decrease in TSH, T_4 and T_3 (Fig. 3). All hormones reached their lowest levels 7 days after CP administration. As the decrease in TSH was accompanied by a corresponding decrease in T_3 and T_4 this suggests that CP mediates a decrease in TSH secretion and the decreases in T_3 and T_4 occur as a secondary effect. The maximal decrease in the levels of these hormones occurred 7 days following CP treatment which corresponded with the maximal decrease in CYP content (not shown) and aminopyrine demethylase activity (not shown). The thyroid hormones are also involved in the complex regulation

of hepatic enzymes including CYP [23–27]. In some instances the effects of the thyroid hormones are complementary to testosterone but can also be opposing [23–27].

The doses of T_3 [24] and hCG [11] used in the hormone replacement experiment were consistent with supplementation doses used by previous workers. Results from experiment 3 demonstrated that the decrease in NADPH P450 reductase activity and the corresponding decrease in the amount of protein that occurred 7 days following CP administration were prevented when T_3 or T_3 plus hCG were administered daily following CP dosing. In contrast, daily administration of hCG alone did not prevent the decrease in NADPH P450 reductase. NADPH P450 reductase is an enzyme that is positively regulated in the rat by the thyroid hormones [23, 28] and as it is apparent that supplementation with T_3 prevents the CP-induced changes in this enzyme we can conclude that CP alters NADPH P450 reductase via a reduction in the thyroid hormones.

Steroid 5α-reductase is a female sex-specific enzyme whose expression is stimulated by the continuous release of GH seen in female rats [29]. It is also positively regulated by the thyroid hormones [25]. CP increased the activity of steroid 5α-reductase in the male rat to the level seen in the female rat 7 days after its administration (Table 3). The T_3 replacement did not prevent the increase in enzyme activity as predicted as the thyroid hormones positively regulate the enzyme [25]. However, activity remained normal when daily hCG or T_3 plus hCG were administered following CP dosing. In conclusion, it appears that the decrease in testosterone that occurred following CP administration is responsible for the increase in steroid 5α-reductase that occurs as restoring testosterone levels prevented the feminization of the enzyme that occurs 7 days following CP administration. This conclusion is consistent with the data presented by Le Blanc and Waxman [11].

Erythromycin demethylase, which has been used as a probe for CYP3A2 [15], is an enzyme that is predominantly male [27]. It is suppressed by both male and female sex hormones via GH [27]. The characteristic continuous female GH secretion is somewhat more effective in suppressing the enzyme than the pulsatile male GH secretion and thus its expression is higher in the male [27]. In addition, the thyroid hormones have been reported to have a suppressive effect on the expression of CYP3A2 in both sexes [27]. It is therefore unexpected that 7 days after CP administration, when the hormones that suppress CYP3A2 are decreased, that CYP3A2 enzyme activity and content decreased [7] (Tables 2, 3 and 5). Results demonstrated however, that daily T_3 replacement, daily hCG administration and the daily administration of both T_3 and hCG in combination prevented the reduction in erythromycin demethylase (CYP3A2) activity that occurred 7 days following CP dosing. This suggests that CP alters CYP3A2 via hormonal perturbation.

The effects of CP on CYP3A2 were substantiated by the fact that the activity of androstenedione 6β-hydroxylase, which is also a probe for CYP3A2 [22] was prevented from decreasing when T_3, hCG and T_3 plus hCG were administered daily 7 days after CP administration. In conclusion, as the decrease in activity and content of CYP3A2 is prevented by hormone replacement, it is indicative that the mechanism of alteration of this enzyme is via hormonal perturbation.

In contrast, hormone replacement experiments also demonstrated that androstenedione 16α-hydroxylase activity, a probe for CYP2C11 [22] does not have either its activity or protein levels restored with hormone replacement with either T_3, hCG or the combination (Table 4). The fact that CYP2C11 is not maintained when CP dosing is followed by daily hCG administration is consistent with the results of Le Blanc and Waxman [11]. In addition we have also shown that CYP2C11 is not maintained when T_3 is administered following CP dosing. CYP2C11 is a male sex-specific enzyme that is regulated indirectly by both testosterone and the thyroid hormones via GH [30, 31]. As CYP2C11 activity and expression were not maintained by replacement of testosterone and/or T_3, it implies that CP does not alter the expression of this enzyme via hormonal perturbation. It could also be speculated that the release of GH that normally occurs in response to these hormones is impaired in the CP treated rat but studies where GH is measured and then possibly supplemented are required for clarification.

Aniline hydroxylase is a probe for CYP2E1 [17]. It is a predominantly female enzyme that is suppressed indirectly by both testosterone and oestrogen via their action on GH (male pulsatile GH secretion is more effective than female continuous GH secretion and thus its higher expression in females) [26]. The thyroid hormones appear to have no effect on CYP2E1 [26]. The decrease in CYP2E1 activity and expression in rats that occurred 7 days following CP administration (Tables 4 and 5) is, similarly to CYP3A2, not consistent with the hypothesis that CP alters CYP2E1 via hormonal perturbation. In contrast to CYP3A2, however, the decrease was not prevented by daily hCG administration or the combination of daily T_3 plus daily hCG (Tables 4 and 5).

Ethylmorphine demethylase has been used as a probe for CYP2C6 in the male rat [18]. It is a sex-independent enzyme [32] and its regulation is unaffected by thyroid hormones. CYP2C6 is one of the enzymes involved in CP activation in the uninduced male rat [6] and its activity was also decreased 7 days after CP administration (Table 4). Neither T_3 or hCG or the combination of T_3 and hCG prevented the decrease in CYP2C6 activity that occurred (Table 4). This is consistent with the assumption that as it is not a hormonally regulated enzyme, hormone replacement after CP administration will not prevent the changes that occur 7 days following CP administration.

In a number of experiments conducted, when the rats were observed daily for clinical signs of toxicity, CP treated rats were clearly anorexic and significant daily weight losses were recorded compared with controls. It has been reported that both diabetes and fasting can increase or decrease CYP enzyme

expression and activity in the rat [13]. In addition, it has been reported that uraemia can increase or decrease the protein levels and activity of certain CYP enzymes [14]. Both studies suggested that perturbation of hormonal regulation may be involved in the mechanism by which enzyme expression was altered in these states.

Given that the rats were anorexic after treatment with CP, it is possible that their blood glucose levels may have been altered and ketones elevated. In addition, the rats may have been uraemic as a result of impaired renal function. In experiment 4, we examined whether alteration of these biochemical parameters, or others, may be contributing to the changes in enzyme expression. Significant variation in the treatment groups compared with controls was not observed to occur except for a leucopoenia and hence we have essentially eliminated the possibility that CP alters enzyme expression via perturbation of such parameters. Myelosuppression consisting primarily of leucopoenia is the most significant of CP induced toxicities [33].

Clearly, endocrine regulation of enzymes is a complex area. Results from this series of experiments indicate that for the non-P450 enzymes, NADPH P450 reductase and steroid 5α-reductase and CYP3A2, daily hormone replacement of T_3 and/or testosterone prevents the changes in enzyme activity that occurs 7 days after CP administration. It is therefore apparent that the mechanism of the CP induced alteration of these enzymes appears to be via hormonal perturbation. However, while perturbation of hormonal regulation may be part of the mechanism by which CP alters the enzymes CYP2C11 and CYP2E1 other factors are also contributing. In contrast CP decreases CYP2C6 via a mechanism independent of hormones. In this series of experiments we have also been able to conclude that while alterations in biochemical parameters have previously been demonstrated to change the expression of various enzymes via a change in hormone levels [13, 14] this is not the mechanism by which CP disturbs hormone levels.

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REFERENCES

1. Kawabata TT, Chapman MY, Kim D-H, Stevens WD and Holsapple MP, Mechanisms of *in vitro* immunosuppression by hepatocyte-generated cyclophosphamide metabolites and 4-hydroxycyclophosphamide. *Biochem Pharmacol* **40**: 927–935, 1990.
2. Colvin M and Hilton J, Pharmacology of cyclophosphamide and metabolites. *Cancer Treat Rep* **65**: 89–95, 1981.
3. Berrigan MJ, Marinello AJ, Pavelic Z, Williams CJ, Struck RF and Gurtoo HL, Protective role of thiols in cyclophosphamide-induced urotoxicity and depression of hepatic drug metabolism. *Cancer Res* **42**: 3688–3695, 1982.
4. Marinello AJ, Bansal SK, Paul B, Koser PL, Love J, Struck RF and Gurtoo HL, Metabolism and binding of cyclophosphamide and its metabolite acrolein to rat hepatic microsomal cytochrome P-450. *Cancer Res* **44**: 4615–4621, 1984.
5. Gurtoo HL, Marinello AJ, Struck RF, Brajeswar P and Dahms RP, Studies on the mechanism of denaturation of cytochrome P-450 by cyclophosphamide and its metabolites. *J Biol Chem* **256**: 11691–11701, 1981.
6. Clarke L and Waxman DJ, Oxidative metabolism of cyclophosphamide: Identification of the hepatic monooxygenase catalysts of drug activation. *Cancer Res* **49**: 2344–2350, 1989.
7. McClure MT and Stupans I, Investigation of the mechanism by which cyclophosphamide alters cytochrome P450 in male rats. *Biochem Pharmacol* **43**: 2655–2658, 1992.
8. Lund J, Zaphiropoulos PG, Mode A, Warner M and Gustafsson J-A, Hormonal regulation of cytochrome P-450 gene expression. *Adv Pharmacol* **22**: 325–354, 1991.
9. Zaphiropoulos PG, Mode A, Norstedt G and Gustafsson J-A, Regulation of sexual differentiation in drug and steroid metabolism. *TIPS* **10**: 149–153, 1989.
10. Kobliakov D, Popvoa N and Rossi L, Regulation of the expression of the sex-specific isoforms of cytochrome P-450 in rat liver. *Eur J Biochem* **195**: 585–591, 1991.
11. Le Blanc GA and Waxman DJ, Mechanisms of cyclophosphamide action on hepatic P-450 expression. *Cancer Res* **50**: 5720–5726, 1990.
12. Chang TKH and Waxman DJ, Cyclophosphamide modulates rat hepatic cytochrome P450 2C11 and steroid 5α-reductase and messenger RNA levels through the combined action of acrolein and phosphoramide mustard. *Cancer Res* **53**: 2490–2497, 1993.
13. Ma Q, Dannan GA, Guengerich FP and Yang CS, Similarities and differences in the regulation of hepatic cytochrome P-450 enzymes by diabetes and fasting in male rats. *Biochem Pharmacol* **38**: 3179–3184, 1989.
14. Ikemoto S, Imaoka S, Hayahara N, Maekawa M and Funae Y, Expression of hepatic microsomal cytochrome P450s altered by uraemia. *Biochem Pharmacol* **43**: 2407–2412, 1992.
15. Stupans I and Sansom LN, The inhibition of drug oxidation by anhydroerythromycin, an acid degradation product of erythromycin. *Biochem Pharmacol* **42**: 2085–2090, 1991.
16. Lear L, Nation RL and Stupans I, Influence of morphine concentration on detergent activation of rat liver morphine-UDP-glucuronosyltransferase. *Biochem Pharmacol* **42**: S55–S60, 1991.
17. Ko I-Y, Park SS, Song BJ, Patten C, Tan Y, Hah YC, Yang CS and Gelboin HV, Monoclonal antibodies to ethanol-induced rat liver cytochrome P-450 that metabolises aniline and nitrosamines. *Cancer Res* **47**: 3101–3109, 1987.
18. Craig P, Murray M and Farrell G, Interferon suppresses the isoform-specific activities of hepatic cytochrome P450 in female rats. *Biochem Pharmacol* **43**: 908–910, 1992.
19. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680–685, 1970.
20. Wheeler AG, Dansby D, Hawkins HC, Payne HG and Weikel JH, A toxicologic and hematologic evaluation of cyclophosphamide (Cytoxan®) in experimental animals. *Toxicol Appl Pharmacol* **4**: 324–343, 1962.

21. Ings R, Interspecies scaling and comparisons in drug development and toxicokinetics. *Xenobiotica* **20**: 1201–1231, 1990.
22. Waxman DJ, Ko A and Walsh C, Regioselectivity and stereoselectivity of androgen hydroxylations catalysed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937–11947, 1983.
23. Ram PA and Waxman DJ, Thyroid hormone stimulation of NADPH P450 reductase expression in liver and extrahepatic tissues. Regulation by multiple mechanisms. *J Biol Chem* **267**: 3294–3301, 1992.
24. Yamazoe Y, Long X, Murayama N, Gong D, Nagata K and Kato R, Modulation of hepatic level of microsomal testosterone 7α-hydroxylase, P450a (P450IIA), by thyroid hormone and growth hormone in rat liver. *J Biochem* **108**: 599–603, 1990.
25. Ram PA and Waxman DJ, Pre-translational control by thyroid hormone of rat liver steroid 5α-reductase and comparison to the thyroid dependence of two growth hormone-regulated CYP2C mRNAs. *J Biol Chem* **265**: 19223–19229, 1990.
26. Waxman DJ, Morissey JJ and Le Blanc GA, Female-predominant rat hepatic P-450 forms j (IIE1) and 3 (IIA1) are under hormonal regulatory controls distinct from those sex-specific P-450 forms. *Endocrinology* **124**: 2954–2965, 1989.
27. Waxman DJ, Ram PA, Notani G, Le Blanc GA, Alberta JA, Morissey JJ and Sundseth SS, Pituitary regulation of the male-specific steroid 6β-hydroxylase P-450 2a (gene product IIIA2) in adult rat liver. Suppressive influence of growth hormone and thyroxine at a pre-translational level. *Mol Endocrinol* **4**: 447–454, 1990.
28. Waxman DJ, Morissey JJ and Le Blanc GA, Hypophysectomy differentially alters P-450 protein levels and enzyme activities in rat liver: Pituitary control of hepatic cytochrome P-450 reductase. *Mol Pharmacol* **35**: 519–525, 1989.
29. Mode A, Norstedt G, Simic B, Eneroth P and Gustafsson J-A, Continued infusion of growth hormone feminizes hepatic steroid metabolism in the rat. *Endocrinology* **108**: 2103–2108, 1981.
30. Morgan ET, MacGeoch C and Gustafsson J-A, Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. *J Biol Chem* **260**: 11895–11898, 1985.
31. Ram PA and Waxman DJ, Hepatic P450 expression in hypothyroid rats: Differential responsiveness of male-specific P450 forms 2a (IIIA2), 2c (IIC11), and RLM2 (IIA2) to thyroid hormone. *Mol Endocrinol* **5**: 13–20, 1991.
32. Westin S, Strom A, Gustafsson J-A and Zaphiropoulos P, Growth hormone regulation of the cytochrome P-450 IIC subfamily in the rat: inductive, repressive, and transcriptional effects of P450f (IIC7) and P-450PB1 (IIC6) gene expression. *Mol Pharmacol* **38**: 192–197, 1990.
33. Fraiser LH, Kanekal S and Kehrer JP, Cyclophosphamide toxicity—characterising and avoiding the problem. *Drugs* **42**: 781–795, 1991.