



# Differential regulation of endobiotic-oxidizing cytochromes P450 in vitamin A-deficient male rat liver

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**1** The hepatic CYP4A-dependent  $\omega$ -hydroxylation of arachidonic acid and CYP2C11-dependent  $2\alpha$ -/ $16\alpha$ -hydroxylations of testosterone were decreased to 74 and 60% of respective control in microsomal fractions from vitamin A-deficient rats. Decreases in the rates of arachidonic acid  $\omega$ -1-hydroxylation and testosterone  $6\beta$ -,  $7\alpha$ - and  $17\alpha$ -hydroxylations were less pronounced.

**2** Corresponding decreases in microsomal CYP4A and CYP2C11 immunoreactive protein expression to 64 and 68% of respective control were observed in vitamin A-deficient rat liver. Expression of CYP3A proteins was unchanged from vitamin A-adequate control.

**3** Northern analysis revealed a selective decrease in CYP4A2 mRNA expression in vitamin A-deficient rat liver to ~5% of control; expression of the related CYP4A1/4A3 mRNAs was not decreased. CYP2C11 mRNA expression was also decreased in vitamin A-deficient male rat liver to 39% of control levels.

**4** Intake of the deficient diet containing all-*trans*-retinoic acid (ATRA) during the final week of the experiment restored CYP4A2 mRNA and CYP4A protein. Administration of exogenous androgen or episodic growth hormone was ineffective. In contrast, CYP2C11 expression was restored by ATRA and androgen, but not by growth hormone.

**5** From these studies it emerges that CYP4A2, a fatty acid  $\omega$ -hydroxylase in rat liver, is highly dependent on vitamin A for optimal expression, whereas CYP2C11 is indirectly down regulated by androgen deficiency resulting from vitamin A-deficiency. Altered CYP expression in vitamin A-deficiency provides insights into the relationship between dietary constituents and the intracellular formation of vasoactive eicosanoids as well as the clearance of androgenic steroids.

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**Keywords:** Cytochrome P450; vitamin A; all-*trans*-retinoic acid; hepatic microsomal fatty acid hydroxylation; hepatic microsomal steroid hydroxylation

**Abbreviations:** ATRA, all-*trans*-retinoic acid; CYP, cytochrome P450; GH, growth hormone; HETE, hydroxyeicosatetraenoic acid; IgG, immunoglobulin G; MT, methyltrienolone (17 $\beta$ -hydroxy-17-methylestra-4,9,11-trien-3-one); PP, peroxisome proliferator; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X (9-*cis*-retinoic acid) receptor

## Introduction

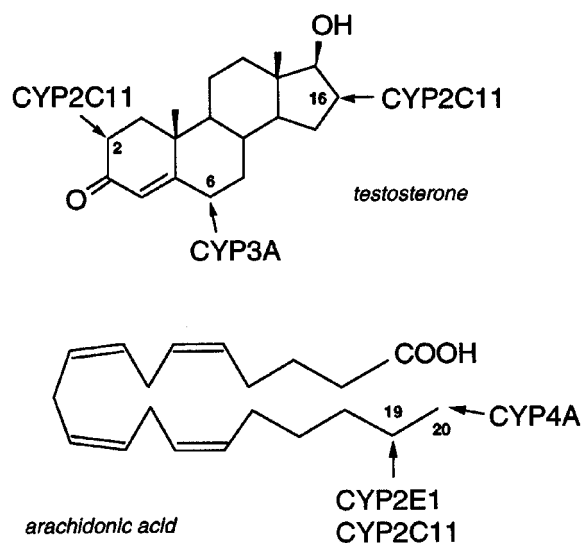
Male-specific cytochromes P450 (CYPs) in rat liver, including the steroid  $2\alpha$ -/ $16\alpha$ -hydroxylase CYP2C11 (Figure 1) and the fatty acid  $\omega$ -hydroxylase CYP4A2, are regulated by growth hormone (GH) acting in conjunction with androgen and other hormones (Gustafsson *et al.*, 1983; Morgan *et al.*, 1985; Skett, 1987; Sundseth & Waxman, 1992). Many CYP-mediated drug and endobiotic oxidations are deactivation processes and factors that influence hepatic CYP expression modify the duration of action of such agents. However, it is also apparent that CYP-mediated arachidonic acid oxidation generates terminal hydroxyeicosatetraenoic acids (HETEs; Figure 1) that modulate vascular reactivity in tissues (Fitzpatrick & Murphy, 1989; Harder *et al.*, 1997; Wang *et al.*, 1999).

Dietary factors also modulate hepatic CYP expression and function, but there is little information on the specific CYPs

involved or the mechanisms underlying these processes. Down regulation of the androgen-dependent CYP2C11 has been documented in vitamin A-deficient male rat liver, which coincided with a decline in circulating testosterone concentrations (Martini *et al.*, 1995). Replacement of dietary vitamin A esters with all-*trans*-retinoic acid (ATRA) prevented the loss of hepatic CYP2C11 expression and circulating androgen produced by vitamin A-deficiency (Martini *et al.*, 1995). Subsequently, administration of androgen to vitamin A-deficient male rats was found to restore hepatic CYP2C11 expression and function (Murray *et al.*, 1996).

Three members of the CYP4A subfamily have been identified in rat liver (Kimura *et al.*, 1989a, b; Tamburini *et al.*, 1984). On the basis of the limited information that is available, these proteins appear to possess similar functional properties and also appear to be regulated similarly (Aoyama *et al.*, 1990; Sundseth & Waxman, 1992; Wang *et al.*, 1999). Thus, the microsomal CYPs 4A are fatty acid hydroxylases that are inducible in rat liver by ligands of the peroxisome proliferator-activated receptors (PPARs) (Bell *et al.*, 1991;

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**Figure 1** Oxidation of testosterone and arachidonic acid by rat hepatic CYPs.

Sharma *et al.*, 1989; Sundseth & Waxman, 1992; Tamburini *et al.*, 1984), which are members of the steroid hormone receptor superfamily that act, in conjunction with the 9-*cis*-retinoic acid receptor (retinoid X receptor, RXR), as transcriptional activators of a number of target genes (Kliewer *et al.*, 1992). Previous studies have reported that vitamin A status modulates the response of hepatic genes such as CYPs 4A to peroxisome proliferators (PPs), like perfluorooctanoic acid (Sohlenius *et al.*, 1996), and the expression of nuclear hormone receptors, including those for vitamin A and thyroid hormone (Higueret *et al.*, 1992). The present study was undertaken because of the association between dietary vitamin A intake, which might modulate the intracellular availability of 9-*cis*-retinoic acid for binding to RXR, and the expression of PPAR-responsive genes such as CYPs 4A.

The major finding to emerge from the present study was that CYP4A2 expression is acutely sensitive to dietary vitamin A intake, more so than the related CYPs 4A1 and 4A3. Down regulation of CYP4A mRNAs in vitamin A-deficient male rat liver also decreased CYP4A immunoreactive protein and the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) from arachidonic acid. Inclusion of ATRA in diets that were otherwise deficient in vitamin A restored CYP4A2 expression and function to levels found in vitamin A-adequate control rat liver. Thus, the present findings establish a mechanistic link between dietary factors and the expression of CYP4A proteins that are modulators of vasoactivity in a range of tissues (Fitzpatrick & Murphy, 1989; Harder *et al.*, 1997; Wang *et al.*, 1999).

## Methods

### Chemicals

[ $\alpha$ - $^{32}$ P]-dCTP (specific activity 3000 Ci mmol $^{-1}$ ), Hyperfilm, ACS II, Hybond-N $^{+}$  filters, [4- $^{14}$ C]-testosterone (specific activity *ca.* 55 mCi mmol $^{-1}$ ) and reagents for enhanced chemiluminescence were obtained from Amersham Phar-

macia Biotech Australia (Castle Hill, NSW, Australia). All-*trans*-retinoic acid (ATRA), retinyl acetate and unlabelled steroids were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deoxynucleotidyl transferase and other biochemicals were obtained from Sigma or Boehringer-Mannheim (Castle Hill, NSW, Australia) and methyltrienolone (17 $\beta$ -hydroxy-17-methylestra-4,9,11-trien-3-one; MT) was purchased from DuPont-New England Nuclear (North Ryde, NSW, Australia). Human recombinant growth hormone (hGH) was provided generously by Aza Research (North Ryde, NSW, Australia). Reagents used in gel electrophoresis were obtained from Bio-Rad (Richmond, CA, U.S.A.). HPLC grade solvents were obtained from Rhone-Poulenc Chemicals (Baulkham Hills, Australia) and analytical grade reagents were from Ajax Chemicals (Sydney, Australia).

### Animal treatments

Studies were performed in accordance with the guidelines of the Australian National Health and Medical Research Council and were approved by the Institutional Animal Care and Ethics Committee. Male Wistar rats (*ca.* 3 weeks of age, *ca.* 60 g) were held under conditions of constant temperature and lighting (12 h light–dark cycle). Animals had free access to the vitamin A-deficient diet (ICN biochemicals, Seven Hills, NSW, Australia) over the 10 week experimental period; vitamin A-adequate control rats received the deficient diet that was supplemented with retinyl acetate (25 IU g $^{-1}$ ). After body weight had plateaued in the vitamin A-deficient animals (by 8–9 weeks of dietary conditioning), subsequent *in vivo* treatments were initiated. In one experiment animals received the deficient diet that was supplemented with 12  $\mu$ g ATRA g $^{-1}$  (Martini *et al.*, 1995) for a further 7 days. In another study MT was administered to vitamin A-deficient rats at a dose of 0.625 mgkg $^{-1}$  in propylene glycol by subcutaneous injection once daily for 5 days; treatment controls received solvent alone (Murray *et al.*, 1996). In a further experiment vitamin A-deficient rats were administered recombinant human growth hormone (hGH; Aza Research, West Ryde, NSW, Australia) in saline at a dose of 12.5  $\mu$ g 100 g $^{-1}$  body weight by subcutaneous injection at 12 h intervals for 7 days (Waxman *et al.*, 1991), during which animals were allowed free access to water and the appropriate diet.

Animals were killed by exsanguination under enflurane anaesthesia, livers were removed and perfused with ice cold saline, and part of the tissue ( $\sim$ 1 gram) was frozen in liquid nitrogen for the subsequent extraction of total RNA and determination of hepatic CYP mRNAs. The remainder was used in the preparation of hepatic microsomes by differential ultracentrifugation. Briefly, tissue was homogenized in 50 mM potassium phosphate buffer, (pH 7.4) containing 1 mM EDTA and 100 mM sucrose using a Potter-Elvehjem homogenizer and centrifuged at 10,000  $\times$  g for 10 min. The supernatant was ultracentrifuged at 105,000  $\times$  g for 60 min, followed by resuspension in buffer and resedimentation at 105,000  $\times$  g for 30 min. The final microsomal pellets obtained were resuspended in 50 mM potassium phosphate buffer, pH 7.4, that contained 20% glycerol and 1 mM EDTA, snap frozen in liquid nitrogen and stored at  $-70^{\circ}$ C until required in experiments (Murray *et al.*, 1999).

### *Microsomal arachidonic acid hydroxylation and analysis of HETE formation by gas chromatography-mass spectrometry*

The microsomal hydroxylation of arachidonic acid (30  $\mu\text{M}$ ) was determined in similar fashion to that described by Schwartzmann *et al.* (1990), except that unlabelled substrate was employed. Incubations (0.25 ml) contained 25  $\mu\text{g}$  microsomal protein, were conducted for 5 min at 37°C in 0.1 M phosphate buffer, pH 7.4) and were initiated with NADPH (1 mM). Reactions were terminated by the addition of glacial acetic acid (0.2 ml), placed in an ice bath, 20-HETE- $\text{d}_2$  (10 ng) was added (internal standard) and the mixture was extracted with ethyl acetate (1 ml). The extract was evaporated under a stream of nitrogen and the residue was treated with a mixture of pentafluorobenzyl bromide (40  $\mu\text{l}$ , 10% in acetonitrile) and DIPEA (20  $\mu\text{l}$ , 10% in acetonitrile) at room temperature for 30 min, followed by evaporation under nitrogen to yield the HETE pentafluorobenzyl esters. The sample was then treated with bis(trimethylsilyl)-trifluoroacetamide (20  $\mu\text{l}$ ) and anhydrous pyridine (20  $\mu\text{l}$ ) at 45°C for 20 min to yield the trimethylsilyl ethers. For analysis samples were run on a Hewlett-Packard 5890 Series II gas chromatograph coupled to a 5989B Mass Spectrometer. Samples were injected onto a HP-5MS column (30 m  $\times$  0.25 mm, 0.25  $\mu\text{m}$  film thickness) using helium as carrier gas. Samples were run using methane as the reagent gas for electron capture chemical ionization. The initial column temperature was 160°C programmed to 300°C at 20°C  $\text{min}^{-1}$ . The mass spectrometer was operated in selected ion monitoring mode and HETEs were detected by monitoring  $m/z$  391 (which corresponds to the loss of pentafluorobenzyl radical (M-181) from the molecular ion) and the corresponding  $m/z$  393 ion for 20-HETE- $\text{d}_2$  (internal standard). Peaks were identified by comparison with the retention times of authentic standards.

### *Microsomal steroid hydroxylation*

Microsomal testosterone hydroxylation activities were determined by methods described previously (Martini *et al.*, 1995). The  $^{14}\text{C}$ -labelled substrate (50  $\mu\text{M}$ , 0.18  $\mu\text{Ci}$ ) was incubated with 0.15 mg microsomal protein and NADPH (1 mM) at 37°C (0.1 M phosphate buffer, pH 7.4; 0.4 ml). Chloroform (5 ml) was added after 2.5 min, the tubes were transferred to an ice bath and shaken for 10 min. After centrifugation the chloroform phase was evaporated under  $\text{N}_2$ . The residue was applied to thin-layer chromatography (TLC) plates (Merck silica gel 60 F<sub>254</sub> type; Darmstadt, Germany) in a small volume of chloroform, run in dichloromethane:acetone (4:1) and then chloroform:ethyl acetate:ethanol (4:1:0.7) with air drying between (Waxman *et al.*, 1983). Radioactive metabolites were located on TLC plates by autoradiography (Hyperfilm-MP; Amersham) over 48–60 h and quantified by scintillation counting (ACS II; Amersham). To confirm the identity and homogeneity of the products of microsomal metabolism of  $^{14}\text{C}$ -labelled testosterone, radioactive regions corresponding to individual metabolites were scraped from the TLC plates and eluted from the silica with methanol. After application to new TLC plates along with the appropriate unlabelled standard hydroxytestosterone metabolite, the plates were resolved in dichloromethane:acetone (9:5) and subjected to autoradiogra-

phy. Thus, individual metabolites comigrated in two separate solvent systems and radioactivity was localized to one metabolite region. Intra-assay variation was estimated at 3% after analysis of three samples in triplicate. Inter-assay variation was 8% when microsomal fractions ( $n=3$ ) were analysed on three consecutive days. The linearity of product formation with protein concentration and time was confirmed for each metabolite; substrate conversion was  $15 \pm 2\%$  in microsomes from control rat liver.

### *Immunoquantitation of CYPs in rat liver microsomes*

The properties of the rabbit anti-rat CYP2C11 immunoglobulin G (IgG) used in this study has been described previously (Murray *et al.*, 1992). The goat anti-rat CYP4A1 and rabbit anti-human CYP2E1 IgGs were generously provided by Professor G.G. Gibson (School of Biological Sciences, University of Surrey, U.K.) and Professor M. Ingelman-Sundberg (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Sweden), respectively. The anti-rat CYP3A IgG was raised in female rabbits against CYP3A that was isolated from hepatic microsomes from dexamethasone-induced female rats according to established methods (Murray *et al.*, 1992). The purified CYP3A catalysed testosterone 2 $\beta$ - and 6 $\beta$ -hydroxylation in reconstituted enzyme systems and the IgG effectively inhibited testosterone 6 $\beta$ -hydroxylation in microsomal fractions from control rat liver.

Rat hepatic microsomes (5  $\mu\text{g}$  per lane; Lowry *et al.*, 1951) were incubated at 100°C for 5 min with 2% sodium dodecyl sulphate and 5% 2-mercaptoethanol and electrophoresed overnight at constant voltage (25 V) through 7.5% polyacrylamide gels (Laemmli, 1971) with minor modifications (Murray *et al.*, 1986). Microsomal proteins were then transferred electrophoretically (1.5 h at 70 V) to nitrocellulose sheets (Towbin *et al.*, 1979) and incubated with the anti-CYP IgG fractions (3.7  $\mu\text{g}$   $\text{ml}^{-1}$ ) for 120 min. Immunoreactive proteins were detected on nitrocellulose sheets (Protran, Medos Co., Lidcombe, NSW, Australia) by enhanced chemiluminescence (ECL RPN 2106 kit, Amersham Pharmacia Biotech Australia) and autoradiography on Hyperfilm-ECL (Amersham Pharmacia Biotech Australia) and the resultant signals were analysed by densitometry (Bio-Rad GS-700 Imaging Densitometer, Richmond, CA, U.S.A.). The linearity of the response under these conditions was confirmed for each CYP by loading several different quantities of microsomal protein over a 10 fold range and a 4 fold range of IgG concentrations.

### *Hepatic RNA extraction and analysis of CYP mRNAs*

Total RNA was extracted from male rat liver by the guanidinium thiocyanate/CsCl method and quantified spectrophotometrically (Sambrook *et al.*, 1989). Synthetic oligonucleotides were purchased from Bresatec (Adelaide, South Australia). Sequences were: CYP4A1 (5'-TAT-GGG-AAG-GGT-GCT-GGC-TT-3' (Sundseth & Waxman, 1992), corresponding to the complement of the 3'-untranslated region of the gene from nucleotides 1701–1720 (Kimura *et al.*, 1989a), CYP4A2 (5'-GCT-GGG-AAG-GTG-TCT-GGA-GT-3' (Sundseth & Waxman, 1992), corresponding to the complement of the genomic sequence (Kimura *et al.*,

1989a)), CYP4A3 (5'-ACT-GGG-ATG-GAG-TCT-GGA-GG-3' (Sundseth & Waxman, 1992), corresponding to the complement of the 3'-untranslated region of the gene from nucleotides 1691–1710 (Kimura *et al.*, 1989b)), CYP2C11 (5'-ATC-CAC-GTG-TTT-CAG-CAG-CAG-GAG-TCC-3' (Ram & Waxman, 1991), corresponding to the complement of the coding region of the gene from nucleotides 945–974 (Yoshioka *et al.*, 1987)), and 18S RNA (5'-CGG-CAT-GTA-TTA-GCT-CTA-GAA-TTA-CCA-CAG-3', corresponding to complement of the coding region of the gene from nucleotides 151–180 (Chan *et al.*, 1984)). Oligonucleotides were labelled with [ $\alpha$ - $^{32}$ P]-dCTP and deoxynucleotidyl transferase.

For Northern analysis, RNA (10  $\mu$ g lane $^{-1}$ ) was electrophoresed on 1% agarose in the presence of 2.2 M formaldehyde and then transferred to Hybond-N $^{+}$  nylon filters (0.45  $\mu$ m, Amersham). Hybridization and washing conditions were as described previously (Jiang *et al.*, 1994), and signals corresponding to CYP mRNAs were quantified after autoradiography (Hyperfilm MP, Amersham Pharmacia Biotech Australia) using a Fuji Phosphorimager. To demonstrate equivalence of RNA loading between samples, filters were stripped and rehybridized to the 18S ribosomal RNA  $\alpha$ - $^{32}$ P-labelled probe.

### Statistics

Data are presented throughout as means  $\pm$  s.e.mean. All measurements were made in samples from individual rats:  $n=6$  for catalytic measurements,  $n=4$  for protein data and  $n=3$  for mRNA data. Differences between means values from control and treatment groups were detected using one-way analysis of variance in conjunction with the Dunnett's  $q$ -test.

## Results

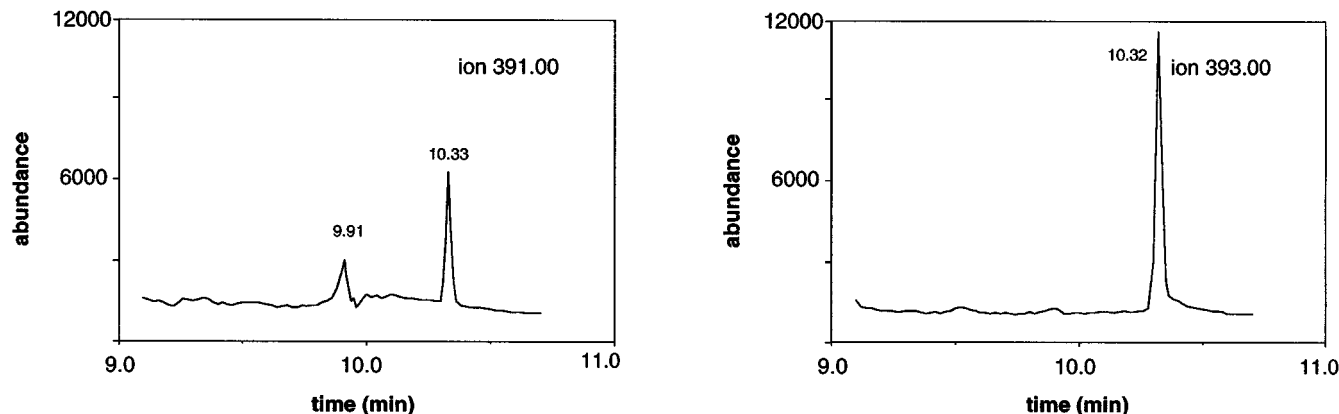
### CYP-dependent oxidation of arachidonic acid and testosterone in hepatic microsomes from vitamin A-deficient male rats

Intake of vitamin A-deficient diets by male rats over a 10 week period decreased hepatic and serum stores of vitamin A.

Thus, in control rats hepatic retinyl palmitate and serum retinol levels were  $150 \pm 20$  nmol g liver $^{-1}$  and  $1.2 \pm 0.1$  nmol ml $^{-1}$ , respectively, and, in vitamin A-deficient animals, were  $<0.3$  nmol g liver $^{-1}$  and  $<0.02$  nmol ml $^{-1}$ , respectively. Several CYP-dependent pathways of arachidonic acid and testosterone hydroxylation (Figure 1) were impaired in hepatic microsomes from vitamin A-deficient male rats. As indicated by the data in Table 1 CYP4A-dependent formation of 20-HETE from arachidonic acid, estimated by GC-MS (Figure 2), was decreased in vitamin A-deficient rat liver to 74% of control ( $1.34 \pm 0.08$  nmol mg protein $^{-1}$  min $^{-1}$  versus  $1.80 \pm 0.15$  in vitamin A-adequate control;  $P < 0.05$ ) and was restored by inclusion of ATRA in the experimental diet (Table 1). CYP2C11/CYP2E1-mediated  $\omega$ -1-hydroxylation of arachidonic acid (to 19-HETE) was decreased in vitamin A-deficient rat liver microsomes to 88% of control ( $P < 0.01$ ); this was also reversed by inclusion of ATRA in diets (Table 1). CYP2C11-mediated  $2\alpha$ - and  $16\alpha$ -hydroxylations of testosterone were decreased in microsomes from vitamin A-deficient rat liver (Figure 3) to 58 and 60% of the corresponding activities in vitamin A-adequate control liver ( $P < 0.01$  in both cases; Table 1). Inclusion of ATRA in the deficient diet during the final week of the study reversed this defect in CYP2C11 function (Table 1). Two other major pathways of testosterone hydroxylation were differentially affected by dietary vitamin A status. Thus, CYP3A-mediated testosterone  $6\beta$ -hydroxylation was decreased in hepatic microsomes from vitamin A-deficient rats to about 73% of control ( $P < 0.05$ ), whereas the apparent decrease in microsomal  $17\alpha$ -hydroxylation (to produce androstenedione) did not attain statistical significance. Testosterone was also converted to a number of minor products by microsomal CYPs and oxidoreductases; in total these products constituted approximately 20% of total testosterone biotransformation. Vitamin A-deficiency decreased the formation of these metabolites to 78% of control ( $P < 0.05$ ; Table 1).

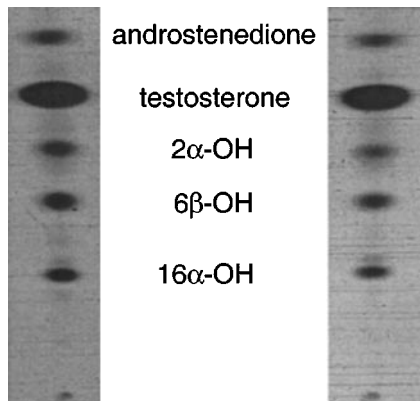
### CYP expression in microsomal fractions from control and vitamin A-deficient male rats

Immunoblotting experiments were undertaken with CYP-specific IgG fractions to corroborate the findings from arachidonic acid and testosterone hydroxylation measure-



**Figure 2** Representative ion chromatogram showing the elution and analysis of (left panel) 19-HETE (ion 391.00; retention time 9.91 min), 20-HETE (ion 391.00; retention time 10.33 min) and (right panel) 20-HETE-d $_2$  (internal standard; ion 393.00; retention time 10.32 min) during gas chromatography-mass spectrometry.

ments (Figure 4). In agreement with measurements of microsomal 20-HETE formation, expression of CYP4A immunoreactive protein was decreased to  $64 \pm 5\%$  of adequate control in vitamin A-deficient male rat liver ( $P < 0.01$ ; Figure 4); inclusion of ATRA in diets restored CYP4A expression ( $82 \pm 7\%$  of control). In contrast, CYP2E1 expression in vitamin A-deficient rat liver was unchanged from control but, consistent with earlier reports from this laboratory, CYP2C11 apoprotein content was decreased to  $68 \pm 9\%$  of control ( $P < 0.05$ ; Figure 4). Down



**Figure 3** Formation of oxidized testosterone metabolites in hepatic microsomes from (left panel) control and (right panel) vitamin A-deficient rats.

regulation of CYP2C11 in microsomes from vitamin A-deficient rat liver was reversed by inclusion of ATRA in the deficient diet during the final week of the study (week 10). Thus, impaired expression of CYP2C11 and not CYP2E1 appears more likely to be responsible for the decreased rates of arachidonic acid 19-hydroxylation as well as the decline in testosterone  $2\alpha$ - and  $16\alpha$ -hydroxylations. CYP3A apoprotein levels in vitamin A-deficient rat liver were decreased slightly to  $85 \pm 21\%$  of control, but this effect did not attain statistical significance. CYP4A and CYP2C11, but not CYP2E1 or CYP3A, are therefore important endobiotic-metabolizing CYPs that are down regulated in vitamin A-deficiency.

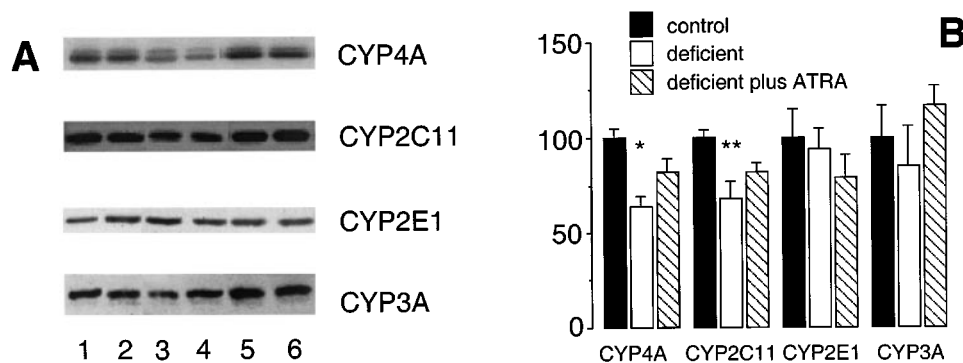
#### Expression of CYP mRNAs in vitamin A-deficient male rat liver

The effect of vitamin A-deficiency on CYP4A and CYP2C11 expression was explored further at the mRNA level. CYP4A2 mRNA was found to be highly dependent on vitamin A status, whereas the hepatic expression of the closely related CYP4A1 and CYP4A3 mRNAs was less dependent on the vitamin (Figure 5A). Inclusion of ATRA in the deficient diet over the final week of dietary manipulation reversed the down regulation of hepatic CYP4A2 expression produced by dietary vitamin A-deficiency ( $5 \pm 1\%$  of vitamin A-adequate control;  $P < 0.05$ ; Figure 5B). By comparison, effects of dietary vitamin A-deficiency on CYP4A1 mRNA ( $77 \pm 8\%$  of adequate control) and CYP4A3 mRNA ( $91 \pm 5\%$  of adequate

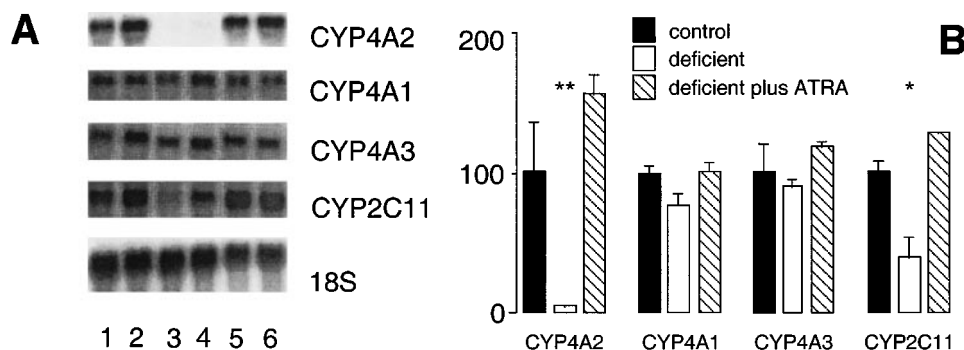
**Table 1** Arachidonic acid and testosterone hydroxylation in hepatic microsomes from rats that received vitamin A modified diets

Dietary vitamin A	Hydroxyarachidonic acid metabolite				Testosterone metabolite				
	19-	20-	2 $\alpha$ -OH	6 $\beta$ -OH	7 $\alpha$ -OH	16 $\alpha$ -OH	Androstenedione	5 $\alpha$ -dihydro	Other
	(nmol/mg protein/min)				(nmol/mg protein/min)				
Adequate (control)	0.66 $\pm$ 0.08	1.80 $\pm$ 0.15	1.63 $\pm$ 0.09	2.27 $\pm$ 0.19	0.36 $\pm$ 0.02	1.93 $\pm$ 0.09	1.47 $\pm$ 0.11	0.41 $\pm$ 0.10	1.02 $\pm$ 0.03
Deficient	0.58 $\pm$ 0.08*	1.34 $\pm$ 0.08**	0.94 $\pm$ 0.16*	1.66 $\pm$ 0.16**	0.23 $\pm$ 0.02*	1.16 $\pm$ 0.16*	1.11 $\pm$ 0.10	0.24 $\pm$ 0.05	0.80 $\pm$ 0.08**
Deficient Plus ATRA	0.66 $\pm$ 0.07	1.80 $\pm$ 0.16	1.47 $\pm$ 0.14	2.09 $\pm$ 0.06	0.24 $\pm$ 0.02*	1.76 $\pm$ 0.18	1.24 $\pm$ 0.07	0.26 $\pm$ 0.03	1.07 $\pm$ 0.05

Data are mean  $\pm$  s.e. mean of measurements made in microsomal fractions from individual rat livers ( $n = 6$ ). Different from control: \* $P < 0.01$ , \*\* $P < 0.05$ .



**Figure 4** Western analysis of CYP proteins in rat hepatic microsomal fractions after intake of vitamin A modified diets for 10 weeks. (A) Lanes 1,2 (vitamin A-adequate control), lanes 3,4 (vitamin A-deficient diet) and lanes 5,6 (vitamin A-deficient diet for 9 weeks followed by the deficient diet supplemented with ATRA  $12 \text{ mg kg}^{-1}$  for a further 7 days). (B) CYP protein expression after densitometric analysis (percentage of control). Different from control; \* $P < 0.01$ , \*\* $P < 0.05$ ; data are means  $\pm$  s.e. mean of estimates in four individual animals per group.



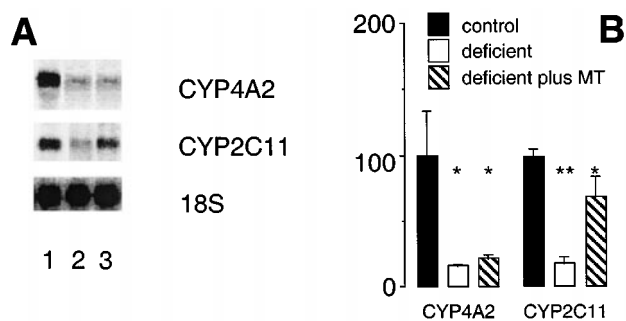
**Figure 5** Northern analysis of CYP mRNAs in liver of rats after intake of vitamin A modified diets for 10 weeks. (A) Lanes 1,2 (vitamin A-adequate control), lanes 3,4 (vitamin A-deficient diet) and lanes 5,6 (vitamin A-deficient diet for 9 weeks followed by the deficient diet supplemented with ATRA 12 mg kg<sup>-1</sup> for a further 7 days). The 18S rRNA signal was used to calibrate RNA loading between lanes. (B) Relative expression of CYP mRNAs in rat liver (percentage of control). Different from control; \**P*<0.01, \*\**P*<0.05; data are means ± s.e. mean of estimates in three individual animals per group.

control; Figure 5B) were not significant. Intake of the vitamin A-deficient diet for 10 weeks also decreased the expression of CYP2C11 mRNA (to 39 ± 15% of control; *P*<0.01; Figure 5B), an effect that was also reversed by intake of the ATRA-containing vitamin A-deficient diet, during the final week of the experiment.

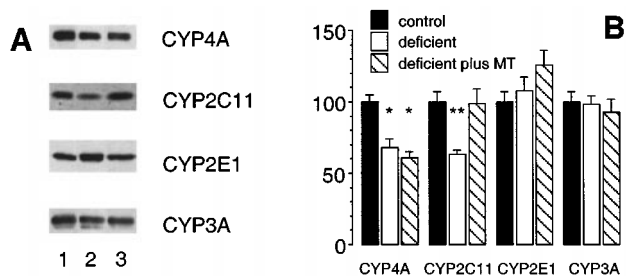
#### *In vivo modulation of CYP expression in liver of vitamin A-deficient rats by exogenous androgen and GH*

The expression of CYP4A2 and CYP2C11 in rat liver is male gender specific (Sundseth & Waxman, 1992) and dependent on androgen and episodic GH secretion by the pituitary. Since testicular androgen output is partially dependent on vitamin A (Appling & Chytil, 1981; Martini *et al.*, 1995), androgen (MT) was administered to vitamin A-deficient rats during the final week of dietary conditioning. The decrease in hepatic CYP4A2 mRNA content in vitamin A-deficiency was unaffected by exogenous androgen (remained at 22 ± 3% of control), whereas, consistent with earlier findings (Murray *et al.*, 1996), CYP2C11 mRNA expression was restored to control levels (Figure 6). These findings were complemented with data from immunoblotting experiments. CYP4A apoprotein was again decreased in vitamin A-deficient rat liver (to 68 ± 6% of control; *P*<0.01; Figure 7) but expression remained impaired after androgen administration (60 ± 4% of control; *P*<0.01). In contrast, the decrease in microsomal CYP2C11 protein elicited by dietary vitamin A-deficiency (to 63 ± 3% of control; Figure 7) was reversed by androgen (99 ± 10% of control). Consistent with earlier experiments, CYP2E1 and CYP3A apoprotein contents were not influenced significantly by dietary vitamin A intake and were similarly unaffected by MT administration (Figure 7).

GH also participates in the regulation of several CYPs in rat liver, including CYP4A2 (Sundseth & Waxman, 1992). In the present study GH was administered to vitamin A-adequate and vitamin A-deficient male rats to test whether GH deficiency may contribute to the impaired expression of CYP4A2 and CYP2C11 in vitamin A-deficient rat liver. A regimen involving the episodic administration of GH by subcutaneous injection, which has been documented previously to restore CYP2C11 expression in hypophysectomized male rats (Waxman *et al.*, 1991), was employed during the

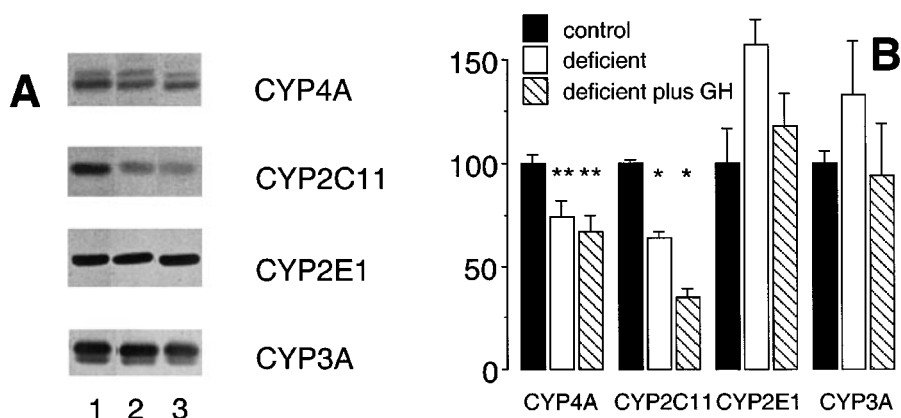


**Figure 6** Northern analysis of CYP mRNAs in liver of rats after intake of vitamin A modified diets for 10 weeks. (A) Lane 1 (vitamin A-adequate control), lane 2 (vitamin A-deficient diet) and lane 3 (vitamin A-deficient diet for 10 weeks and MT administration 0.625 mg kg<sup>-1</sup> s.c. daily during week 10 of the experiment). (B) Relative expression of CYP mRNAs in rat liver (percentage of control). Different from control; \**P*<0.01, \*\**P*<0.05; data are means ± s.e. mean of estimates in three individual animals per group.



**Figure 7** Western analysis of CYP proteins in rat hepatic microsomal fractions after intake of vitamin A modified diets for 10 weeks. (A) Lane 1 (vitamin A-adequate control), lane 2 (vitamin A-deficient diet) and lane 3 (vitamin A-deficient diet for 10 weeks and MT administration 0.625 mg kg<sup>-1</sup> s.c. daily during week 10 of the experiment). (B) CYP protein expression after densitometric analysis (percentage of control). Different from control; \**P*<0.01, \*\**P*<0.05; data are means ± s.e. mean of estimates in four individual animals per group.

final week of the experimental period. However, 12-hourly administration of GH in order to mimic the pulsatile release of GH by the male rat pituitary did not restore CYP4A or CYP2C11 immunoreactive protein (Figure 8), and was also without significant effect on the expression of either CYP2E1



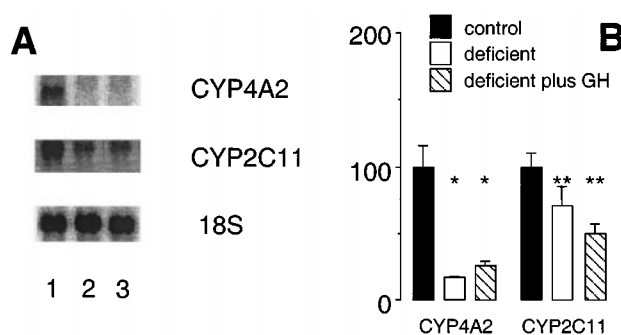
**Figure 8** Western analysis of CYP proteins in rat hepatic microsomal fractions after intake of vitamin A modified diets for 10 weeks. (A) Lane 1 (vitamin A-adequate control), lane 2 (vitamin A-deficient diet) and lane 3 (vitamin A-deficient diet) for 10 weeks and GH administration  $0.125 \text{ mg kg}^{-1}$  s.c. 12 hourly during week 10 of the experiment). (B) CYP protein expression after densitometric analysis (percentage of control). Different from control; \* $P < 0.01$ , \*\* $P < 0.05$ ; data are means  $\pm$  s.e. mean of estimates in four individual animals per group.

or CYP3A (Figure 8). Further, the decrease in CYP4A2 mRNA expression produced by vitamin A-deficiency (to  $17 \pm 1\%$  of adequate control;  $P < 0.01$ ; Figure 9) was not reversed by exogenous GH ( $26 \pm 3\%$  of adequate control;  $P < 0.01$ ; Figure 9). Similarly, exogenous GH did not reverse the decrease in CYP2C11 mRNA expression produced by vitamin A-deficiency (Figure 9B).

## Discussion

Dietary factors have been shown previously to modulate the activity of hepatic CYPs involved in endobiotic and xenobiotic oxidation (Walter-Sack & Klotz, 1996), but there is a deficiency of mechanistic information to account for such changes. From the present study the microsomal CYP-dependent oxidations of endobiotics, such as fatty acids and steroids that have important physiological roles in intracellular signalling pathways, are impaired in the liver of vitamin A-deficient rats. Thus, the microsomal CYP4A-mediated formation of 20-HETE from arachidonic acid and CYP2C11-dependent  $2\alpha$ -/16 $\alpha$ -hydroxylations of testosterone were decreased in rat liver after intake of the vitamin A-deficient diet. Consistent with these observations the microsomal expression of CYP4A and CYP2C11 proteins was also decreased in vitamin A-deficient male rat liver. Northern analysis indicated that the decrease in CYP4A immunoreactive protein was due to a selective impairment of CYP4A2 mRNA; the closely related CYPs 4A1 and 4A3 were essentially unchanged from control. As reported in earlier studies, CYP2C11 mRNA is decreased in vitamin A-deficient rat liver (Martini *et al.*, 1995). Thus, the dysregulation of endobiotic oxidizing CYPs in rat liver caused by dietary vitamin A-deficiency occurs at a pretranslational level.

The microsomal formation of 19-HETE from arachidonic acid was also decreased significantly in rat liver, although this was somewhat less pronounced than the decrease in 20-HETE formation. Arachidonic acid 19-hydroxylation activity has been attributed to several CYPs in rat liver, most notably CYP2E1 and CYP2C11 (Falck *et al.*, 1990;



**Figure 9** Northern analysis of CYP mRNAs in liver of rats after intake of vitamin A modified diets for 10 weeks. (A) Lane 1 (vitamin A-adequate control), lane 2 (vitamin A-deficient diet) and lane 3 (vitamin A-deficient diet for 10 weeks GH administration  $0.125 \text{ mg kg}^{-1}$  s.c. 12 hourly during week 10 of the experiment). (B) Relative expression of CYP mRNAs in rat liver (percentage of control). Different from control; \* $P < 0.01$ , \*\* $P < 0.05$ ; data are means  $\pm$  s.e. mean of estimates in three individual animals per group.

Laethem *et al.*, 1993). Since CYP2E1 expression was refractory to vitamin A-deficiency it appears that the decline in CYP2C11 expression is most likely to be responsible for the observed effects on rates of 19-HETE formation. This decrease in CYP2C11 expression is also responsible for the impaired capacity of microsomal androgen  $2\alpha$ -/16 $\alpha$ -hydroxylation in vitamin A-deficient rat liver. Apart from CYP2C11, the function of other CYP-dependent steroid hydroxylases was also decreased in vitamin A-deficient rat liver. Thus, intake of the deficient diet decreased the rate of CYP3A-mediated testosterone  $6\beta$ -hydroxylation, although this was less pronounced than the loss of CYP2C11-mediated oxidation pathways. CYP3A immunoreactive protein appeared to be slightly decreased in vitamin A-deficient rat liver but this did not attain statistical significance. This may be due to the multiplicity of the CYP3A subfamily. Thus, at least four members of this CYP subfamily have been identified in rat liver (Mahnke *et al.*, 1997; Miyata *et al.*, 1994; Nagata *et al.*, 1990; 1999), and it is possible that individual CYP3A proteins that are more active in steroid  $6\beta$ -hydroxylation

but that are expressed at relatively low levels in liver may be down regulated by dietary vitamin A-deficiency.

Factors that influence the expression of CYP4A protein may contribute to marked effects on hepatocellular homeostasis because of the established role of 20-HETE in vasoactivity (Wang *et al.*, 1999). The finding that hepatic CYP4A expression was impaired in vitamin A-deficiency may provide insight into the mechanisms by which diet influences the formation of vasoactive eicosanoids from arachidonic acid that elicit consequent effects on hepatic vascular tone. Similarly, CYP2C11 has been shown to be down regulated in models of chronic liver disease (Murray *et al.*, 1987a,b), portal bypass (Cantrill *et al.*, 1989) and inflammatory processes mediated by cytokines (Morgan, 1989; Wright & Morgan, 1990). Impaired hepatic androgen extraction because of decreased expression of CYP-hydroxylases increases the extrahepatic availability of substrate for oestrogen formation by aromatase activity in adipose tissue, thus contributing to hypogonadism and other hormonal imbalances that have been observed in liver disease (Gordon *et al.*, 1975). Decreased activity of CYP2C11 in vitamin A-deficient rat liver may contribute by a similar mechanism to the impaired testicular function and androgen production reported previously in these animals (Rich *et al.*, 1979; Martini *et al.*, 1995).

The mechanisms that are responsible for CYP down regulation in dietary vitamin A-deficiency were evaluated in a series of *in vivo* restitution experiments. ATRA, the biologically active form of vitamin A that serves as the endogenous ligand for retinoic acid receptors (Glass *et al.*, 1991), but which is normally absent from the diet, effectively substituted for dietary vitamin A esters to restore CYP4A2 and CYP2C11 in vitamin A-deficient rat liver. In these studies the deficient diet was supplemented with ATRA during the final (tenth) week of the experiment. This is complementary to previous studies in which supplementation of the deficient diet with ATRA over the entire period prevented the decline in CYP2C11 produced by vitamin A-deficiency (Martini *et al.*, 1995). Thus, both short- and long-term ATRA can substitute for dietary vitamin A in the maintenance of CYP expression in liver.

CYP4A2 exhibits a male-specific pattern of expression in rat liver and, like CYP2C11, its basal expression is regulated by hormonal factors (Sundseth & Waxman, 1992). In male rats androgen modulates the pulsatility of pituitary GH release mediated *via* the hypothalamo-pituitary-gonadal axis (Legraverend *et al.*, 1992; Waxman *et al.*, 1991; 1995). The present study provides evidence of a link between vitamin A intake and hormonal perturbations that lead to impaired expression of male-specific hepatic CYPs. Vitamin A status has been shown previously to influence androgen biosynthesis by testicular Leydig cells (Chaudhary *et al.*, 1989) and vitamin A-deficiency in male rats causes Leydig cell dysfunction (Rich *et al.*, 1979). The decrease in serum testosterone that has been reported in vitamin A-deficient male rats (Martini *et al.*, 1995) is consistent with the down regulation of male-specific CYPs. Indeed, administration of androgen to vitamin A-deficient male rats has been found to restore CYP2C11 without influencing vitamin A status: the animals remained vitamin A-deficient (Murray *et al.*, 1996). However, from the present study, regulation of CYP4A2 mRNA by vitamin A appears more complex and adminis-

tration of the exogenous androgen MT did not restore its expression in rat liver.

Pituitary GH gene transcription is regulated in part by ATRA (Bedo *et al.*, 1989), and circulating GH profiles are modulated by dietary vitamin A intake (Mallo *et al.*, 1992). The restoration of hepatic CYP2C11 in vitamin A-deficient rats by exogenous androgen clearly suggests that GH pulsatility was restituted adequately. However, attempts to restore CYP2C11 and CYP4A2 in vitamin A-deficient rat liver with exogenous GH according to a regimen that stimulated the expression of GH-regulated CYPs in liver of hypophysectomized male rats were unsuccessful. These findings are consistent with the assertion that vitamin A-deficiency is associated with perturbation, but not the abolition, of GH secretion by the rat pituitary. Thus, superimposition of exogenous GH pulses over circulating levels of endogenous GH was inadequate for the restoration of CYP expression in rat liver.

Considered together, several possibilities may contribute to the observations from restitution experiments. Androgen may regulate hepatic CYP4A2 mRNA expression more tightly than CYP2C11 mRNA. A number of studies have successfully restored CYP2C11 expression in liver of hypophysectomized male rats by several regimen even though the resultant plasma GH profiles were unphysiological (Waxman *et al.*, 1991); CYP4A2 expression may be more sensitive to circulating GH pulses. In this regard the studies of Sundseth & Waxman (1992) highlight several differences between the expression of CYP4A2 and other male-specific CYPs.

The basal regulation of hepatic CYPs 4A has not been explored. In contrast, a number of studies have established their inducibility by PP chemicals, such as clofibric acid (Gibson *et al.*, 1982; Tamburini *et al.*, 1984), which are ligands for PPAR $\alpha$ . PPARs are known to form heterodimers with the RXR, a common partner for many nuclear receptors, to activate target genes (Kliwer *et al.*, 1992). Heterodimers between PPAR $\alpha$  and RXR $\alpha$  are unusual in that the RXR $\alpha$  ligand 9-*cis*-retinoic acid enhances the peroxisome proliferator-dependent activation of PPAR $\alpha$ -responsive genes (Kliwer *et al.*, 1992). The possible association between dietary vitamin A intake, which might influence 9-*cis*-retinoic acid availability, and PPAR-mediated gene activation was investigated in the present study. Although CYPs 4A1-3 are all responsive to PPs (Sundseth & Waxman, 1992), the present study found that CYP4A2 was selectively impaired in vitamin A-deficiency. Moreover, studies in a PPAR $\alpha$ -null mouse line established that, although induction by PP chemicals was lost, the basal hepatic expression of CYPs 4A was unchanged from wild type control (Peters *et al.*, 1996). Thus, it appears clear that signalling pathways, distinct from PPAR $\alpha$ /RXR $\alpha$  which mediates CYP4A induction, are operative in the basal expression of these genes.

The JAK-STAT system is implicated in the regulation of GH-responsive genes, such as CYP2C11, in rat liver (Waxman *et al.*, 1995). Thus, GH pulses received at the hepatocyte activate JAK kinase *trans*-autophosphorylation, which in turn promotes the nuclear accumulation of tyrosine phosphorylated STAT5b (Darnell, 1997; Waxman *et al.*, 1991). The action of nuclear and/or cytosolic SH2-containing phosphatases returns the tyrosine phosphorylated STAT5b to a GH-responsive state following activation of target gene transcription (Ram & Waxman, 1997; Yu *et al.*, 2000).



Androgen plays a significant role in this process in that it maintains the episodic nature of pituitary GH release. In androgen deficiency continuous GH pulses would be expected to impair the responsiveness of the JAK-STAT system, leading to CYP2C11 down regulation. A similar process may contribute to the regulation of CYP4A2, which is also male gender-specific and GH responsive (Sundseth & Waxman, 1992).

The absorption of vitamin A *via* the oral route and the intracellular concentration of ATRA are tightly controlled (Blomhoff *et al.*, 1992). However, retinol and ATRA have been shown to upregulate the expression of certain CYPs in rat liver after *in vivo* injection, which bypasses intestinal regulation of absorption, (Wright *et al.*, 1997) or in primary hepatocytes cultured on matrigel (Westin *et al.*, 1993). From the present experiments the augmentation of vitamin A-adequate diets with ATRA and the intraperitoneal administration of ATRA did not increase hepatic CYP4A2 mRNA expression in rat liver (not shown). Thus, vitamin A does not appear to have a direct role in the maintenance of CYP4A2 expression in male rat liver.

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