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Suppression of the constitutive microsomal cytochrome P450 2C11 in male rat liver during dietary vitamin A deficiency

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Abstract—The effect of dietary vitamin A deficiency on hepatic microsomal cytochrome P450 (P450) and associated oxidase activities was examined in the male rat. Intake of a deficient diet by weanling rats over 10 weeks led to a pronounced decline in hepatic and serum vitamin A concentrations to levels that were beneath the limits of detection. These changes occurred concurrently with a decrease in total microsomal P450 to 77% of vitamin A adequate control. Measurement of microsomal androstenedione metabolism revealed respective decreases in 16α- and 6β-hydroxylation pathways to 61 and 71% of adequate control; 7α-hydroxylation was not significantly decreased. Immunoquantitation of the principal catalyst of steroid 16α-hydroxylation, the androgen-dependent P450 2C11, indicated a significant decrease in the microsomal content of the enzyme to 78% of control (13.7 ± 0.9 ng/μg protein in deficient rat liver versus 17.5 ± 0.5 in adequate control; $P < 0.005$). Serum testosterone appeared lower in vitamin A deficient male rats, but did not attain statistical significance. Administration of a diet containing excess vitamin A (500 IU/g) to rats for 10 weeks produced marked increases in hepatic vitamin A stores, but did not increase P450 2C11 activities. Thus, the expression and function of P450 2C11 is not related directly to hepatic vitamin A levels. The trend toward lower circulating androgen levels in male rats maintained on the deficient diet for 10 weeks may have a role in P450 2C11 down regulation, but other regulatory factors may also be disrupted in these animals.

Key words: retinoids; microsomal steroid hydroxylation; hepatic oxidoreductases; micronutrient regulation

The essential nutrient, vitamin A (retinol) is normally present in food in the form of retinyl esters but can also be derived from the *in vivo* cleavage of its precursor β-carotene [1]. Once absorbed, retinyl esters are hydrolysed to retinol prior to re-esterification (principally to the palmitate) and are transported to the hepatic perisinusoidal stellate cells [2]. When vitamin A dependent tissues require retinol, it is released from the liver bound to its transport protein, serum retinol binding protein, and transthyretin.

P450s* catalyse the oxidation of many lipophilic xenobiotics and endogenous compounds. Multiple genes code for distinct P450 proteins that are present in mammalian liver. Many factors regulate the hepatic expression of individual P450s, including sex steroids, growth hormone and other peptide hormones, and exposure to xenobiotics [3–5]. It has also been established that disease states [6–8] and dietary factors [9–11] play a role in the regulation of hepatic P450s. However, whereas the regulation of P450 expression by hormones and xenobiotics has been studied in some detail, the relationships between diet and specific P450 enzymes has been investigated less intensively. There have been a number of reports relating dietary vitamin A deficiency to impaired hepatic drug metabolizing capacity [12–14], but little information is available on the specific microsomal P450(s) subject to altered regulation by vitamin A deficiency in the rat.

In this study, the effects of dietary vitamin A manipulation on the function of several constitutive P450s were monitored using P450-specific androstenedione oxidations. The principal finding to emerge was that the activities of the male-specific P450s 2C11 and 3A2 (reflected by steroid 16α- and 6β-hydroxylation, respectively) were decreased in hepatic microsomes from vitamin A deficient male rats. The decrease in P450 2C11 activity was attributed to decreased expression of P450 2C11 protein content in vitamin A deficient rat liver.

* Abbreviations: androstenedione, androst-4-ene,3,17-dione; IgG, immunoglobulin G; P450, cytochrome P450; TBS, Tris-buffered saline; BSA, bovine serum albumin.

Materials and Methods

Chemicals. [^{14}C] Androstenedione (54.5 mCi/mmol) was obtained from Amersham Australia (NSW, Australia). Metabolite standards were obtained from either the Sigma Chemical Co. (St Louis, MO, U.S.A.) or the MRC Steroid Reference Collection (Queen Mary's College, London, U.K.). Biochemicals were from either Sigma or Boehringer-Mannheim (Sydney, Australia). HPLC solvents were from Rhône Poulenc Chemicals (Baulkham Hills, NSW, Australia) and miscellaneous reagents were from Ajax Chemicals (Sydney, Australia).

Animals. Weanling male Wistar rats (3 weeks of age) were obtained from the Department of Animal Care at Westmead Hospital, housed in wire cages without access to faeces and received a vitamin A deficient diet (ICN biochemicals, Seven Hills, Australia) for 10 weeks; vitamin A adequate controls received the same diet supplemented with 25 IU retinyl acetate/g. During this period, serum was collected by tail vein puncture for the estimation of serum hormone levels. In a separate experiment, rats were placed on the basal diet containing 500 IU retinyl acetate/g over 10 weeks; controls received the vitamin A adequate diet.

After the period of dietary manipulation, rats were fasted overnight and killed under ether anaesthesia. Blood was obtained from the abdominal aorta and the livers were removed, perfused with ice-cold saline and frozen in liquid nitrogen. Washed microsomes were prepared by standard methods [15].

Assay of microsomal androstenedione hydroxylation. Incubations contained [^{14}C]androstenedione (50 μM, 0.18 μCi/0.4 mL reaction), 0.15 mg microsomal protein and 1 mM NADPH and were run for 2.5 min at 37° (0.1 M phosphate buffer, pH 7.4) [16]. Reactions were terminated on ice and by the addition of 5 mL of chloroform. The organic phase was evaporated under nitrogen and each sample was applied in chloroform to TLC plates (silica gel

Table 1. Hepatic and serum vitamin A concentrations and hepatic microsomal P450 content in male rats after dietary manipulation of vitamin A intake

Dietary vitamin A group	Liver retinol (μg/g liver)	Liver retinyl palmitate (μg/g liver)	Serum retinol (ng/mL)	P450 (nmol/mg protein)
Adequate control (8)	0.66 ± 0.06	80 ± 10	330 ± 20	1.11 ± 0.04
Deficient (8)	ND*	ND	ND	0.86 ± 0.05‡
Adequate control (6)	0.25 ± 0.04	35 ± 5	399 ± 13	1.01 ± 0.07
Excess (6)	32.6 ± 3.0†	5920 ± 483†	378 ± 39	0.96 ± 0.05

Data are means ± SEM of determinations from (N) individual animals.
* ND, beneath limit of detection of the assay.
Significant difference from corresponding control, †$P < 0.001$, ‡$P < 0.002$.

60 including F_{254} indicator; Merck, Darmstadt, Germany). Plates were developed using standard solvent systems for the separation of metabolites of androstenedione [17]. Radioactive metabolites were located by autoradiography (Hyperfilm-MP, Amersham; exposed for 48–60 hr) and rates of formation were determined by scintillation counting (ACS II, Amersham).

Quantitation of serum retinol and hepatic vitamin A. Serum retinol, hepatic retinol and hepatic retinyl palmitate were extracted according to Azais *et al.* [18]. Retinoid quantitation was from standard curves prepared by extracting blank tissues that had been spiked with known quantities of the retinoids. The HPLC system used for the resolution of vitamin A derivatives was an Ultrasphere ODS column (5 μm, 25 cm × 4.6 mm i.d., Beckman Instruments Inc., San Ramon, CA, U.S.A.) attached to a Waters Associates HPLC system. Limits of detection were 1.0 and 0.5 ng for retinol and retinyl palmitate, respectively.

Immunodetection of P450 2C11 protein in hepatic microsomes. Rat hepatic microsomes (33 μg per lane) were incubated with 2% sodium dodecyl sulphate and 5% 2-mercaptoethanol at 100° for 5 min and then loaded onto polyacrylamide gels for overnight electrophoresis [19]. After electrophoresis, proteins were transferred to nitrocellulose sheets [20]. The sheets were washed sequentially in TBS (50 mM Tris and 200 mM sodium chloride, pH 7.4) for 30 min, TBS containing 3% BSA for 60 min and then incubated for 120 min with rabbit anti-P450 2C11 IgG (37 μg/mL; raised against P450 2C11 that had been isolated as before [7]). The male specificity of the IgG and its other properties have also been documented [7]). Several washes in TBS (5 × 5 min), and then in TBS containing 3% BSA (5 × 10 min), were followed by incubation with the combination of ^{125}I- and peroxidase-labelled donkey anti-rabbit IgG in TBS containing 3% BSA. After further washing in TBS (5 × 5 min), immunoreactive protein was visualized with 4-chloro-1-naphthol and hydrogen peroxide. Autoradiographs of the immunoblots obtained (Hyperfilm-MP) were analysed by densitometry (LKB Ultroscan XL, Bromma, Sweden).

Other assays. Microsomal protein was estimated by the method of Lowry *et al.*, with BSA as the standard [21]. Total P450 was measured by the spectrophotometric method of Omura and Sato [22]. Serum testosterone and oestradiol concentrations were estimated using commercial radioimmunoassay kits (CIS, Gif Sur Yvette, France).

Statistics. Data are presented as means ± SEM throughout the text. Differences between group means were detected with the Student's *t*-test.

Results and Discussion

The effect of dietary vitamin A manipulation on body parameters and vitamin A status in male rats. After 10 weeks of intake of a vitamin A deficient diet, male rats reached a plateau in their growth. This is consistent with an end point of vitamin A deficiency described in the literature [12, 23]; subsequent analysis of vitamin A levels in liver and serum confirmed the effectiveness of the approach. In general, similar time frames to that employed in this study have been found to produce vitamin A deficiency in the rat, although Becking reportedly achieved a vitamin A deficient state after as little as 20–25 days [24].

The data in Table 1 indicate the suitability of the model for the production of vitamin A deficiency. Thus, 10 weeks of dietary vitamin A deficiency resulted in striking decreases in hepatic retinol and retinyl palmitate (from 0.66 ± 0.06 and 80 ± 10 μg/g liver, respectively, to below the limit of detection of the assays, i.e. <1.0 and <0.5 ng, respectively). In contrast, in a separate experiment, dietary excess led to

Table 2. Microsomal androstenedione hydroxylation in vitamin A-deficient and adequate control male rat liver

Dietary treatment	Hydroxyandrostenedione metabolite 16α (nmol/min/mg protein)	6β (nmol/min/mg protein)	7α (nmol/min/mg protein)
Adequate control	2.98 ± 0.21	1.98 ± 0.18	0.24 ± 0.02
Deficient	1.82 ± 0.16†	1.41 ± 0.13*	0.26 ± 0.02
Percentage of control	61	71	108

Data are means ± SEM of estimates from eight individual microsomal fractions per group.
Significant difference from the corresponding control group, *$P < 0.05$, †$P < 0.001$.

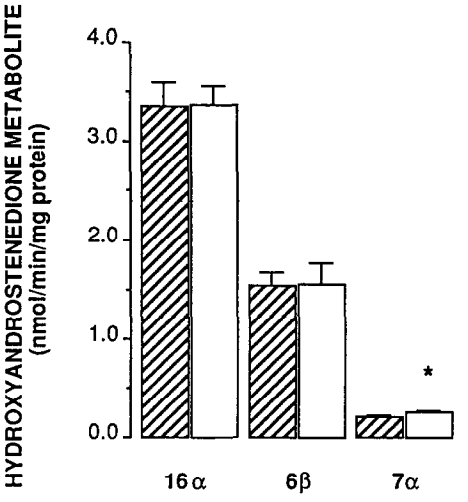

Fig. 1. Microsomal androstenedione hydroxylation in rat liver after intake of diets containing adequate (hatched bars) or excess (open bars) vitamin A for 10 weeks. Significant difference from control: *$P < 0.05$, $N = 6$ individual microsomal fractions per group.

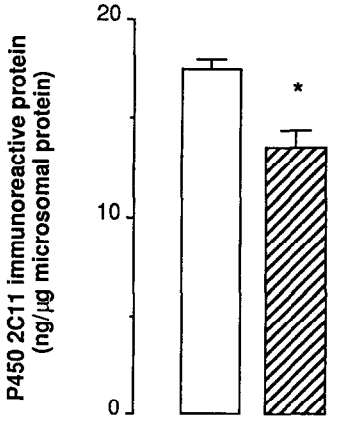

Fig. 2. Immunoquantitation of P450 2C11 in hepatic microsomes from weanling male rats that received either a vitamin A-adequate (open bars) or vitamin A-deficient (hatched bars) diet for 10 weeks. Significant difference from control: *$P < 0.005$, $N = 5$ individual microsomal fractions per group.

pronounced increases in hepatic retinol and retinyl palmitate (130- and 169-fold of control) but serum retinol was normal; this indicates that normal serum vitamin A homeostasis was maintained during the dietary regimen.

Dietary vitamin A modulation and hepatic microsomal drug metabolism in the male rat. The primary emphasis of this study was to investigate the effect of dietary vitamin A deficiency on the catalytic efficiency of individual P450 enzymes in male rat liver. In the vitamin A deficient group, hepatic P450 content was decreased to 77% of vitamin A adequate control (0.86 ± 0.05 nmol/mg protein versus 1.11 ± 0.04 in control; $P < 0.002$; Table 1).

The formation of individual hydroxysteroid metabolites has been ascribed essentially to distinct P450s [3, 17, 25]. Decreases in the rates of microsomal P450 2C11-mediated androstenedione 16α-hydroxylation in vitamin A deficient male rats to 61% of control was noted ($P < 0.001$; Table 2). There was also a significant decrease in androstenedione 6β-hydroxylation mediated by P450 3A2, to 71% of adequate control but P450 2A1-dependent androstenedione 7α-hydroxylation was not decreased. Dietary vitamin A excess did not influence P450 content (Table 1) or androstenedione hydroxylation activities associated with P450s 2C11 or 3A2 (Fig. 1). However, the apparent increase in 7α-hydroxylation activity attained statistical significance ($P < 0.05$).

It was found that immunoreactive P450 2C11 protein was significantly lower in hepatic microsomes from vitamin A deficient rats (13.7 ± 0.9 ng/μg protein versus 17.5 ± 0.5 in control; $P < 0.005$; Fig. 2). Earlier studies reported decreases in microsomal activities such as aminopyrine N-demethylation that are catalysed partially by P450 2C11 [12, 26]. To our knowledge the present study is the first to demonstrate down regulation of a specific P450 in dietary vitamin A deficiency.

Androgens are considered to maintain the high level of P450 2C11 expression in adult male rat liver [3, 4]. Decreases in testosterone production have been reported in rats fed a vitamin A deficient diet and this effect may be related to the requirement of the testis for retinol (or all-*trans*-retinoic acid) in androgen production [27]. Accordingly, serum was obtained from the tail vein during the study and when killed was assayed for serum testosterone and oestradiol. Circulating testosterone appeared to be decreased in male rats after 6 weeks of dietary vitamin A deficiency to 49% of adequate control (3.2 ± 1.2 compared with 6.5 ± 1.4 ng/mL, $N = 16$; $0.10 > P > 0.05$). After 8 and 10 weeks of intake of the deficient diet serum testosterone was 58 and 75%, respectively, of adequate control. However, these apparent decreases did not attain significance at any of the time points due to the wide inter-individual variation observed within groups (mean coefficient of variation of these data was 79%). Serum oestradiol concentrations were unaltered between the groups at each of the time points examined (data not shown). It is possible that the trend toward lower levels of testosterone in serum may contribute to the decrease in P450 2C11 content and activity. It is also possible that intracellular testosterone concentrations may be more sensitive indicators of the effects of vitamin A on androgen-dependent processes in the male rat.

General discussion. Several reports have related dietary vitamin A deficiency to impaired hepatic drug metabolizing capacity [12–14]. The present study indicates that microsomal P450 2C11-mediated androstenedione 16α-hydroxylation activity was decreased in vitamin A deficient male rat liver due to decreased expression of P450 2C11 protein. There have been reports that dietary vitamin A deficiency or supplementation at non-toxic doses modifies hepatic drug metabolism [10, 28].

In a previous study, we found an approximate 50% increase in P450 3A immunoreactive protein in rats that had received a vitamin A-supplemented diet for 15 weeks prior to sacrifice [10]. In the present study, microsomal steroid 6β-hydroxylase activities were not altered in vitamin A excess relative to the vitamin A adequate group. The reason for this discrepancy is unclear but the diets varied somewhat in composition. The change from the original diet was necessitated by the finding that vitamin A was readily detectable in the basal diet even when not supplemented [10]; thus, animals could not be made completely vitamin A deficient. The present studies using the commercial diet were undertaken to provide a standardized basis for future studies of vitamin A deficiency. Apart from the differential effect of the two diets on P450 3A2, it is noteworthy that the two diets produced quite different effects on hepatic vitamin A levels. As reported previously, the original supplemented diet (vitamin A adequate, to which only 25 IU/g had been added) produced

hepatic retinyl palmitate levels of 2.47×10^5 IU/g liver after 15 weeks of intake [10]. The corresponding levels in the present study, after 10 weeks of intake of the commercial diet (to which 500 IU vitamin A had been added), were 5920 μg/g liver (1.08×10^4 IU/g, based on the relationship that 1 IU vitamin A is equivalent to 0.55 μg retinyl palmitate [18]). Thus, although administered over a shorter experimental period, high level supplementation of the commercial vitamin A deficient diet produced hepatic retinyl palmitate levels that were about 20-fold lower than those produced by the original diet. From these points it now appears appropriate to consider nutrient interactions in the interpretation of dietary effects on P450s.

From the present findings it appears that dietary vitamin A deficiency results in the down-regulation of P450 2C11. However, it is unlikely that the decline in total holo-P450 can be attributed completely to the loss of P450 2C11. The measurement of further P450-specific substrate oxidations should provide information on the altered regulation of other P450s by dietary vitamin A.

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