

Short Communication

MICROSOMAL LIPID PEROXIDATION AND OXIDATIVE METABOLISM
IN RAT LIVER: INFLUENCE OF VITAMIN A INTAKE

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Introduction

In several experimental animal systems, lack of vitamin A is associated with enhanced susceptibility to chemical carcinogenesis [1–3] and there are reports also of alterations in the activity of certain microsomal oxidation-reduction enzymes [4–6]. It seemed possible that both effects are related to loss of vitamin A protection against free radical-induced membrane-lipid peroxidation. We have accordingly undertaken parallel studies of peroxidation and various microsomal metabolic activities in both vitamin A-deficient and vitamin A-loaded rats, in comparison with normal controls.

Materials and Methods

Male weanling Sprague–Dawley rats initially weighing about 50 g were obtained from the Laboratory Animal Unit of the University of Hong Kong. There were divided into three groups and reared for 90 days on a basal semisynthetic diet [7] supplemented with different amounts of retinyl acetate (*ad libitum*), with free access to tap water. Supplementation was arranged so as to provide average retinyl acetate intake as follows: vitamin A-deficient group, 5 $\mu\text{g}/\text{day}$; vitamin A-excess group 500 $\mu\text{g}/\text{day}$; vitamin A-sufficient controls 90 $\mu\text{g}/\text{day}$.

After 90 days on special diet, the rats were fasted overnight for 16 h. In the morning, blood was collected from the orbital plexus by capillary puncture while the animal was under light ether anaesthesia. The blood

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clot was removed later by centrifugation and the serum was saved for vitamin A determination by the method of Hansen and Warwick [8]. After drawing blood, the anaesthetised rat was killed by cervical dislocation and its liver removed. A small portion of the sample was solubilised in 30% boiling KOH for 15 min and the tissue digest was used for vitamin A determination. The remaining tissue was stored at -80°C for a few days before assay.

To assess the effect of storage at -80°C , cytochrome *P*-450 levels were determined in microsomes isolated from fresh liver in comparison with levels from samples of liver stored frozen for 1 week. The latter averaged 10% lower. Other enzymes were not tested. It will be noted that the tissue samples were frozen in all experimental groups so that like is compared with like.

The frozen liver sample was thawed on ice, minced and homogenised in 0.1 M sodium phosphate buffer [9] (pH 7.4) in a Potter-Elvehjem homogenizer with Teflon pestle to give a 25% w/v homogenate. The microsomal fraction was prepared by differential centrifugation following the procedure of Tom and Montgomery [10] and protein concentration of the microsomal fraction was measured by the method of Lowry [11].

Microsomal aminopyrine *N*-demethylation activity was determined by the colorimetric assay of formaldehyde production with Nash's reagent [12]. Aniline hydroxylase activity was estimated by *p*-aminophenol production as described by Imai et al. [13]. NADPH-cytochrome *c* reductase activity was measured according to Williams and Kamin [14]. Microsomal generation of hydrogen peroxide was determined by the conversion of methanol to formaldehyde [15]. NADPH-dependent microsomal generation of superoxide anion radical was followed by the oxidation of adrenaline to adrenochrome at 20°C [16]. Microsomal lipid peroxidation was estimated by the formation of thiobarbituric acid-reactive products in the presence of NADPH and ADP-Fe(III) [10]. Assay of cytochromes *P*-450 and *b*₅ was carried out according to the method of Omura and Sato [17]. All chemicals used in this study were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. or E. Merck, Darmstadt, F.R.G.

The unpaired Student's *t*-test (2-tailed) was employed for evaluation of differences between groups.

Results

Signs of moderate vitamin A deficiency (anorexia, alopecia and slight skin lesions) were observed in rats which had been placed on a vitamin A-deficient diet for 90 days. Animals on excess vitamin A diet did not show any appreciable differences from controls in physical appearance or body weight (Table I).

Liver vitamin A was very low in the deficient group although the serum level remained at about 37% of control ($P < 0.001$). In the excess group, the liver vitamin A content was three times the control value and a 62% increase in serum level was observed ($P < 0.001$). A few determinations

TABLE I

EFFECT OF DIFFERENT LEVELS OF VITAMIN A INTAKE IN RATS ON LIVER AND SERUM LEVELS, LIVER MICROSOMAL ENZYME ACTIVITIES, LIPID PEROXIDATION AND THE GENERATION OF REACTIVE OXYGEN SPECIES

Results are quoted as mean \pm S.E.M. with number of animals in brackets. For methods, see text. Extent of statistical significance is indicated thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Unless otherwise indicated in the table, reaction rates and enzyme activities are expressed in terms of nmol of product/mg microsomal protein/h, the products being as follows: ^aformaldehyde, ^b*p*-aminophenol, ^cthiobarbituric acid-reactive products. ^dSuperoxide generation is expressed as increase in absorbance at 480 nm (absorbance units)/mg microsomal protein/h.

	Experimental groups		
	Vitamin A-sufficient	Vitamin A-deficient	Vitamin A-excess
Final body wt. (g)	262 \pm 2 (9)	193 \pm 7** (9)	247 \pm 2 (9)
Liver wet wt. (g)	11.9 \pm 0.6 (9)	9.2 \pm 0.5 (9)	9.7 \pm 0.5 (9)
Serum vitamin A level (μ g/100 ml)	31.9 \pm 0.7 (7)	11.9 \pm 0.5*** (7)	51.8 \pm 1.2*** (7)
Liver vitamin A content (μ g/g)	200 \pm 6 (4)	<0.5*** (4)	813 \pm 38*** (4)
Microsomal protein (mg/g liver)	32.6 \pm 1.5 (4)	26.7 \pm 2.0 (4)	27.7 \pm 3.9 (4)
Aminopyrine <i>N</i> -demethylase activity ^a	228 \pm 14 (6)	139 \pm 20** (6)	223 \pm 25 (6)
Aniline hydroxylase activity ^b	40.4 \pm 4.4 (6)	32.0 \pm 2.4 (6)	30.8 \pm 6.0 (6)
Cytochrome <i>c</i> reductase activity (nmol reduced/mg microsomal protein/min)	60.4 \pm 1.5 (6)	60.5 \pm 4.0 (6)	67.8 \pm 5.8 (6)
Cytochrome <i>P</i> -450 content (nmol/mg microsomal protein)	0.87 \pm 0.02 (4)	0.51 \pm 0.05** (4)	0.54 \pm 0.05** (4)
Cytochrome <i>b</i> ₅ content (nmol/mg microsomal protein)	0.72 \pm 0.03 (4)	0.17 \pm 0.03** (4)	0.26 \pm 0.04** (4)
Lipid peroxidation ^c	7.38 \pm 1.32 (4)	13.32 \pm 1.80* (4)	3.72 \pm 0.36* (4)
Superoxide generation ^d	4.18 \pm 0.54 (6)	2.69 \pm 0.25* (6)	3.75 \pm 0.27 (6)
Hydrogen peroxide generation ^a	534 \pm 24 (6)	369 \pm 28** (6)	535 \pm 28 (6)

were done subsequently on isolated microsomes: the results were similar to those for whole liver shown in Table I, allowing for a different basis of report (μg retinyl acetate per mg microsomal protein: normal animals 0.43, 0.95; vitamin A-excess, 2.83; deficient, 0.02, 0.04, 0.06).

Variations in vitamin A supply in the diet did not significantly alter liver wet weight or microsomal protein content (Table I). Cytochromes *P*-450 and *b*₅ contents were lowered in both experimental groups. The activity of cytochrome *P*-450-dependent aminopyrine *N*-demethylase was significantly diminished in the deficient group ($P < 0.01$), but no significant change in aniline hydroxylase activity (also cytochrome *P*-450-dependent) was detectable; and neither enzyme was affected in the vitamin A-excess group. There were also no alterations in microsomal NADPH-dependent cytochrome *c* reductase activities in either group.

Generation of activated oxygen species by microsomal enzymes in the presence of NADPH was also examined (Table I). The rate of both hydrogen peroxide and superoxide production was depressed in the deficient group, whereas no significant differences from control were observed in the excess group. The extent of microsomal lipid peroxidation was inversely related to the level of vitamin A in the liver. The deficient group exhibited greater lipid peroxidation, while the excess group was more resistant to this kind of damage than controls.

Discussion

Becking [18], Colby et al. [19] and Miranda et al. [6] have reported that vitamin A deficiency leads to impaired metabolism of both type I and II substances by the mixed function oxidase system, whereas on this point our observations agree with Hauswirth and Brizuela [4] and Siddik et al. [5], who found that only *N*-demethylation of type I substrates such as aminopyrine and ethylmorphine is affected, and not hydroxylation of type II substrates (e.g. aniline). Results in such experiments depend sensitively on experimental conditions but it seems safe to conclude that type I systems are more labile to vitamin A deficiency. That activities do not always parallel cytochrome *P*-450 levels may be related to independent variation in levels of the multiple forms of this protein.

It also seems possible that structural integrity of the microsomal membrane is important in relation to these vitamin A effects. Sufficient vitamin A is essential for maintenance of normal structure of the endoplasmic reticulum and other membranes [20]. Excess vitamin, however, also leads to increased membrane lability [21].

Highly reactive oxygen species such as superoxide radical ion and hydrogen peroxide can induce lipid peroxidation [22] and have been specifically implicated in oxidative destruction of membrane lipids [23]. We have observed, however, a lack of correlation between on the one hand the ability of liver microsomes to generate these reactive species and, on the other, the occurrence of peroxidation. It seems possible that: (i) the superoxide generating system, being membrane-bound, suffers from peroxidative

lipid damage at the same time as other microsomal membrane enzymes, (ii) the observed peroxidative damage is due to attack on membrane lipids by free radicals other than superoxide and (iii) hydrogen peroxide is not involved either. However, singlet oxygen remains among the likely candidates for attack on unsaturated membrane lipids [24]. Loss of superoxide and hydrogen peroxide generating capacity is apparently *not* directly related to a fall in cytochrome *P*-450 level, though again the existence of isoenzymes must be borne in mind here.

In our experiments, the effect of vitamin A on lipid peroxidation may be interpreted as perhaps a purely chemical quenching, especially since excess vitamin A depresses lipid peroxidation below the control level. But in other respects, it seems that excess vitamin A produces some deleterious effects on liver microsomes. We are apparently concerned with a vitamin whose effects are varied, both chemically and biologically speaking, and whose intake must be controlled, for health, between exceptionally narrow limits.

In a recent review [25] Ames has stressed the possibility that reactive oxygen species, not all of which are free radicals, may be largely responsible both for lipid peroxidation and for carcinogenesis. We have shown a distinct discrepancy between peroxidation and the generation of superoxide and hydrogen peroxide (relatively 'late' members in the series of active oxygen species). This lends force to the argument that if peroxidation does play a part in carcinogenesis it is most likely to be via an effect on membrane structure and hence the enzymes of xenobiotic metabolism and not, or not directly, by effects on DNA.

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