Liver microsomal membrane fluidity and lipid characteristics in vitamin A-deficient rats

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Rat liver microsomes (microsomal fraction) were isolated from vitamin A-deficient and -sufficient rats and analysed for membrane lipid characteristics. Membrane fluidity was found to be significantly decreased in microsomes from the vitamin A-deficient rats, but not in liposomes prepared from lipid extracts. Microsomes from vitamin A-deficient animals showed a significant decrease in $C_{18:2,\omega6}$ and an increase in $C_{22:5,\omega6}$ fatty acids.

INTRODUCTION

The metabolic or biochemical function of vitamin A, outside of the process of vision, is still incompletely understood. A great deal of evidence points to a function in the synthesis of some, though not all, glycoproteins (reviewed in [1]). Vitamin A deficiency certainly causes a decrease in the synthesis of certain glycoproteins, in particular a defect in glycosylation. This defect could be the result of changes in level or activity of glycosyl-transferases or of the acceptor protein of the oligosaccharide chain. Recent work from our laboratory (V. Chan & G. Wolf, unpublished work) has shown that microsomal vesicles from livers of vitamin A-deficient rats transferred the oligosaccharide chain of lipid-linked oligosaccharides to protein at about one-half the rate compared with vesicles from normal pair-fed control rats. Spiro & Spiro [2] have shown that the membrane fluidity of the membrane vesicles has a strong influence on the transfer of the oligosaccharide chain from oligosaccharide-lipid (OLL) to protein in the formation of glycoprotein; endoplasmic reticulum of thyroid glands showed decreased OLL and glycoprotein synthesis with increased membrane fluidity, presumably because of disruption of the juxtaposition of oligosaccharide donor or acceptor and enzyme. It was therefore of interest to determine whether the observed defect in glycoprotein synthesis in liver vesicles of vitamin A-deficient rats could be correlated with changes in membrane fluidity.

EXPERIMENTAL

Preparation of vitamin A-deficient and pair-fed control rats

Vitamin A deficiency was induced in 20-day-old male weanling Holtzman rats (30–40 g) by the method of Wolf *et al.* [3]. Animals were housed individually in suspended wire cages, with water available at all times and 12 h day/12 h night cycle. Animals were fed a vitamin A-deficient diet *ad libitum* for 6 weeks. At this time, control rats having approximately the same body weight were ranked and paired with the deficient rats. Deficient

rats were continuously fed the vitamin A-deficient diet *ad libitum*, while each control rat was fed the same amount of diet eaten by its paired deficient rat on the previous day. Control rats were given a weekly supplement of 2000 i.u. of vitamin A orally in the form of retinyl acetate dissolved in corn oil. Body weight and amount of food eaten were determined daily. The onset of vitamin A deficiency was taken to be the time when the deficient rats had lost 10–15% of their body weight and showed other symptoms of vitamin A deficiency (eye lesions, oily fur and impairment of walking pattern). Only animals without any signs of infection were sampled for experimentation.

Preparation of live microsomal membranes

The rats were fasted overnight and killed by decapitation. The livers were removed, weighed, minced, and suspended in 50 mM-Tris/HCl buffer, pH 6.9, with 0.25 M-sucrose (4 ml of buffer/g of liver). The suspension was homogenized by eight strokes of a Teflon pestle with a motor-driven homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min, the supernatant fraction at 10000 *g* for 15 min and the resulting supernatant fraction at 48000 *g* for 60 min. The 48000 *g* pellet representing the microsomal membrane fraction was resuspended in 50 mM-Tris/HCl buffer, pH 6.9. All procedures were carried out at 4 °C. The membrane fraction was used as an enzyme source and was prepared fresh for every experiment. The pellet obtained at 48000 *g* had higher specific enzyme activity (30% higher) than the 100000 *g* pellet (results not shown). Microsomal protein concentration was determined by the method of Lowry *et al.* [4], with bovine serum albumin as standard.

Lipid analysis

Total membrane lipids were extracted with chloroform/methanol (2:1, v/v) containing 0.005% butylated hydroxytoluene by the method of Folch *et al.* [5]. Fatty acids were then methylated with BF_3 as described by Morrison & Smith [6]. Fatty acid methyl esters were quantified by capillary g.l.c. with a SP-2330 30 m capillary column (Hewlett-Packard 5490 gas chroma-

tograph and 3392 integrator). Fatty acid methyl esters were identified by comparison of retention times to known standards. In addition, $C_{22:5,\omega6}$ fatty acid was identified by retention time and mass spectroscopy. Total membrane cholesterol and phospholipid were determined spectrophotometrically by the methods of Chiamori & Henry [7] and Bartlett [8] respectively.

Liposome preparation

Liposomes were prepared as described by Huang & Thompson [9], with slight modification. A portion of lipid extract (80–100 mg) was dried thoroughly and 4.5 ml of TBS buffer (5 mM-Tris/HCl/146 mM-NaCl/4 mM-KCl, pH 7.4) added. Samples were sonicated on ice with a Branson microsonifier (Branson Sonic Power Co., Danbury, CT, U.S.A.) for 45 min under N_2 . Liposomes were centrifuged for 45 min (105000 g, 4 °C) to remove multilamellar vesicles. Single unilamellar vesicles were then utilized for fluorescence-polarization studies.

Fluorescence polarization

Determination of membrane lipid fluidity by fluorescence polarization utilized the fluorophore 1,6-diphenylhexa-1,3,5-triene (DPH) (Kodak Laboratory Chemicals, Rochester, NY, U.S.A.). Purified membranes (0.25 mg of membrane protein) or liposomes (approx. 2 mg of lipid) were incubated for 45 min at 37 °C in a total volume of 1 ml containing 10 μ l of 1.5 mM-DPH. After incubation, membranes were washed by centrifugation in 75 mM-Tris, pH 7.4, for 30 min (180000 g, 4 °C) and resuspended with a Potter-Elvehjem homogenizer in 1 ml of Tris buffer. Steady-state fluorescence-polarization measurements were obtained in a SLM 4800 fluorescence spectrophotometer (SLM Instruments, Champaign, IL, U.S.A.) at an excitation wavelength of 360 nm. Emission readings were recorded at full spectrum with cut-off filters (3–75 filters; Corning Glass Works, Corning, NY, U.S.A.). Results were calculated as the fluorescence-anisotropy parameter $[(r_0/r) - 1]^{-1}$ [10], where r_0 , the maximal limiting anisotropy, was taken as 0.365 [11]. The anisotropy, r , is equal to $(I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp})$, where $I_{||}$ and $I_{\perp}$ are the intensities of light emitted parallel with, and perpendicular to, the plane of polarization of the excited light respectively. The anisotropy parameter is inversely related to the membrane fluidity. Background fluorescence and light-scattering were corrected for in each sample.

Statistics

All data were analysed by Student's t test utilizing the Statsoft statistical package (Statsoft, Tulsa, OK, U.S.A.) with an IBM-PC microcomputer.

RESULTS

Table 1 illustrates the data obtained for the fluorescence polarization of DPH in microsomal membranes (seven samples) and liposomes prepared from lipid extracts (two samples). The anisotropy parameter is directly related to membrane microviscosity and, hence, inversely related to the membrane fluidity. The liver microsomes from vitamin A-deficient animals had a significantly greater ($P < 0.01$) anisotropy parameter compared with their paired controls. However, liposomes

Table 1. Microsomal fluorescence polarization and cholesterol phospholipid content

All data (except liposome fluorescence polarization) are means $\pm$ S.E.M. for the number of samples indicated in parentheses. Liposome fluorescence polarization is the mean of two determinations. *Indicates deficient and sufficient are significantly different at $P < 0.01$.

Parameter	Vitamin A-sufficient	Vitamin A-deficient
Anisotropy parameter		
Membranes	0.840 $\pm$ 0.042 (7)	1.143 $\pm$ 0.080 (7)*
Liposomes	0.423 (2)	0.355 (2)
Cholesterol/phospholipid content		
μ mol of cholesterol/ mg of protein	0.213 $\pm$ 0.053 (7)	0.182 $\pm$ 0.036 (6)
μ mol of phospholipid/ mg of protein	0.468 $\pm$ 0.125 (7)	0.413 $\pm$ 0.115 (6)
μ mol of cholesterol/ μ mol of phospholipid	0.464 $\pm$ 0.064 (7)	0.474 $\pm$ 0.035 (6)

Table 2. Liver microsomal fatty acid profile

All data are means $\pm$ S.E.M. for seven animals in each group. *Indicates sufficient and deficient are significantly different at $P < 0.001$.

Fatty acid	Content (% , w/w)	
	Vitamin A-sufficient	Vitamin A-deficient
$C_{16:0}$	23.7 $\pm$ 1.6	23.2 $\pm$ 0.8
$C_{16:1}$	1.8 $\pm$ 0.2	0.7 $\pm$ 0.1
$C_{18:0}$	23.3 $\pm$ 1.4	22.8 $\pm$ 0.5
$C_{18:1,\omega9}$	11.1 $\pm$ 0.9	10.2 $\pm$ 0.7
$C_{18:2,\omega6}$	13.2 $\pm$ 0.6	9.2 $\pm$ 0.5*
$C_{20:4,\omega6}$	22.2 $\pm$ 2.5	20.5 $\pm$ 1.4
$C_{22:5,\omega6}/C_{22:4}$	0.9 $\pm$ 0.2	10.5 $\pm$ 0.6*
$C_{22:6,\omega3}$	4.3 $\pm$ 0.8	2.9 $\pm$ 0.3

prepared from the lipid extracts of several of these animals did not demonstrate the same alteration. That is, liposomes from vitamin A-deficient animals exhibited, if anything, a lower anisotropy parameter than those prepared from vitamin A-sufficient animals.

The cholesterol and phospholipid contents of liver microsomes are also shown in Table 1. No differences were observed in the cholesterol content, phospholipid content or cholesterol/phospholipid molar ratio. Table 2 presents the data concerning the fatty acid profile. There was a significant decrease ($P < 0.001$) of 30% in microsomes from the vitamin A-deficient animals of linoleic acid ($C_{18:2,\omega6}$) and a small, but not statistically different, decrease in arachidonic acid ($C_{20:4,\omega6}$). Furthermore, a peak identified as a combination of approximately 70% $C_{22:5,\omega6}$ and 30% $C_{22:4}$ fatty acid (positional isomer unknown) by g.c.-m.s. (results not shown) increased 10-fold in vitamin A-deficient animals ($P < 0.001$). This peak was shown by retention times of known standards not to consist of $C_{22:5,\omega3}$ fatty acid, and by m.s. to consist of $C_{22:5}$ and $C_{22:4}$ fatty acid in the

indicated ratios. The positional isomer of $C_{22:4}$ fatty acid is uncertain and could conceivably be either $\omega-3$ or $\omega-9$.

DISCUSSION

There are two aspects to the presently obtained data that should prove of great interest. The first is the alteration of membrane fluidity in response to a deficiency of vitamin A. Microsomes from deficient animals exhibited a marked decrease in membrane fluidity, as indicated by a 36% increase in the anisotropy parameter. Up until now there have been no reports of fluidity changes in vitamin A deficiency. A report by Jetten *et al.* [12] described an increased microviscosity in an embryonic carcinoma-cell line when incubated with retinoic acid and allowed to differentiate. However, this was done with a differentiating cell line, and its relationship to a non-differentiating animal tissue model is unclear. Most dietary studies relating to membrane lipid changes have been performed via an alteration of dietary lipid (see, e.g. [13–15]). In these cases the most typical changes involve alteration of fatty acid profile and/or cholesterol content. Changes in fluidity are most closely linked to changes in cholesterol content when the predominant fatty acid change is a shift in the ratio of various unsaturated fatty acids [13,14]. Storch & Schachter [16] demonstrated a relationship between membrane fluidity and Δ^9 -desaturase activity with a desaturase-inducing dietary protocol.

In our study, the relationship between membrane fluidity and membrane compositional change does not appear to be related to any observable lipid changes *per se*. This because none of the measured lipid parameters were altered in a direction consistent with the observed anisotropy changes. Furthermore, liposome preparations from the lipid extracts of several samples demonstrated an elimination of the effect of deficiency on the anisotropy parameter. If this were due to lipid modifications, the difference should persist in the liposome samples. Rather, these differences are more likely due to alterations in the protein component of the membrane.

Nothing is known at present about changes in membrane proteins as a result of vitamin A deficiency. Certainly, some proteins are underglycosylated or some glycoproteins are present at a lower concentration. However, whether these changes in glycoproteins are the cause or, as was suggested in the Introduction above, the consequence, of an altered membrane fluidity, remains to be determined.

The second aspect of these results that should prove of interest concerns the large increase of $C_{22:5,\omega6}$ fatty acid in the microsomes from vitamin A-deficient animals. Normally, this is present in very small amounts in liver tissues, as is the case in the microsomes from sufficient animals in the present study (0.9%, w/w). In the deficient animals there was a 10-fold increase. Two fatty acids were shown to co-migrate on the gas chromatograph: $C_{22:5,\omega6}$ and $C_{22:4}$. The $C_{22:4}$ fatty acid is potentially either an $\omega-3$ or an $\omega-9$ isomer. This could not be resolved with the available methodology. However, it is most likely an $\omega-9$ fatty acid, since both $C_{22:25,\omega6}$ and $C_{22:4,\omega9}$ fatty acids require the Δ^4 -desaturase as their final enzyme.

There is no previous report in the literature concerning

the potential effects of vitamin A on Δ^4 -desaturase. Alam & Alam [17] reported an increased activity of Δ^9 -desaturase in liver microsomes from vitamin A-deficient rats with no change in Δ^6 -desaturase. In their study they also indicated that there were not parallel changes in the levels of $C_{16:21}$ and $C_{18:1}$ fatty acids in these tissues. There have been two other reports in the literature [18,19] concerning fatty acid composition in liver membrane fractions from vitamin A-deficient rats. Krause *et al.* [18] only analysed mitochondrial membranes. Vaughan-Mitchell *et al.* [19] reported similar decreases to those seen here in $C_{18:2}$ and $C_{20:4}$ fatty acids. In neither case did the analysis extend to longer-chain-length or more unsaturated fatty acids. Hence the data presented here may indicate either an increased activity of the Δ^4 -desaturase or an altered flux through the overall pathway. Furthermore, the hypothesis of an increased flux or activity of a Δ^4 -desaturase is strengthened by the fact that the sum of the decrease (–5.7%) of $C_{18:2,\omega6}$ (–4.0%) and $C_{20:24,\omega6}$ fatty acids (–1.7%), which are the two identified precursors of $C_{22:5,\omega6}$ fatty acid, is very close to the increase of $C_{22:5,\omega6}$ fatty acid (approx. 6.6%). This takes into account the fact that 70% of the g.c. peak was identified as this fatty acid. Also, the analysis was done on a percentage-by-weight basis, with $C_{22:5}$ fatty acid being about 20% heavier than C_{18} and 10% heavier than C_{20} . Hence, virtually all of the increase in $C_{22:5,\omega6}$ fatty acid on a molar basis may be accounted for by the decrease in the two precursors.

In summary, we have demonstrated an altered membrane fluidity in liver microsomes from vitamin A-deficient animals that was not related to the lipid composition *per se*, as well as a marked increase in the level of a particular fatty acid, namely $C_{22:5,\omega6}$, the final metabolic step of which requires Δ^4 -desaturase.

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