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INFLUENCE OF α-NAPHTHYLISOTHIOCYANATE (ANIT) ON MICROSOMAL CYTOCHROME P-450, PROTEIN AND PHOSPHOLIPID CONTENT IN RAT LIVER

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Abstract—In rats suffering from ANIT induced cholestasis plasma and microsomal phospholipid content was measured using lipid extraction, thin-layer chromatography and phosphorus determination. Microsomal protein and cytochrome P-450 content were also measured. Plasma phospholipids were found to be increased without similar changes within the liver. In ANIT-treated animals the hepatic microsomal protein content was increased whereas the microsomal phospholipid and cytochrome P-450 content remained unchanged.

THE ACTIVITY of several microsomal enzymes may depend on the presence of phospholipids in these membranes. Treatment of isolated microsomes with phospholipase C resulted in a decrease in the specific activity of the NADH: semidehydroascorbic acid oxido-reductase (EC 1.6.5.4). The enzyme system could be reactivated by addition of a mixture of LPC* and PC.[1] Comparable results have been obtained with other microsomal enzymes.[2] The activity of a recombined enzyme system containing cytochrome P-450 and NADPH-cytochrome *c* reductase also depends on the addition of PC.[3]

ANIT treatment of rats leads to a cholestatic jaundice which is associated with a distinct increase of plasma phospholipids presumedly due to increased phospholipid synthesis in the liver and possibly to diminished bile secretion. Under normal circumstances an increase in plasma phospholipid content is accompanied by an increase in liver microsomal phospholipid content since Le Kim *et al.* showed that these phospholipid pools are exchangeable.[4] On the other hand the drug metabolizing capacity of the liver *in vivo* is reduced under these experimental conditions,[5,6] which could be the result of altered microsomal phospholipid content or composition.

Microsomal phospholipids as well as microsomal cytochrome P-450 and protein content were measured in animals with ANIT induced cholestasis.

MATERIALS AND METHODS

Chemicals. 1-Naphthylisothiocyanate (ANIT) was purchased from EGA Chemie KG Steinheim, Germany. Reagents for the determination of plasma bilirubin, SGPT, alkaline phosphatase and cholesterol were obtained from Boehringer, Mannheim, Germany. Other reagents used were of analytical grade.

* Abbreviations: LPC = lysophosphatidylcholine; S = sphingomyelin; PC = phosphatidylcholine; PI + PS = phosphatidylinositol + phosphatidylserine; PE = phosphatidylethanolamine.

Animals. Male rats (FW 49, Thomae, Biberach, Germany, about 250 g body wt) received commercial diet (Altromin^{R}, Lage, Lippe, Germany) and water *ad lib.* Fifteen hours before sacrifice food was withdrawn to lower the liver glycogen content. Animals were kept under constant room temperature.

ANIT cholestasis. Forty eight hours before sacrifice the animals received 100 mg ANIT dissolved in 1 ml olive oil/100 g body wt by gastric intubation.

Microsomes were prepared using a modification of the method of Hogeboom and Schneider.[7,8] Animals were lightly anesthesized with ether and blood samples were taken by cardiac puncture. The liver was removed and homogenized (1 g/10 ml) in ice-cold SVT-buffer solution (0·25 M saccharose, 10^{-3} M Versen, 0·02 M Tris buffer pH 7·4). After sedimenting the mitochondria and nuclei by two centrifugations at 20,000 *g* for 15 min (Sorvall RC-2, rotor SS 34) the supernatant was centrifuged for 90 min at 105,000 *g* (Spinco L2-50 B, rotor 30, 30,000 rev/min). The supernatant was discarded and the microsomes were suspended in 1·18% potassium chloride and recentrifuged. Thereafter the microsomes were suspended in 0·05 M Tris–HCl buffer pH 7·5. Microsomal protein was determined by the method described by Bode *et al.*[9]

Cytochrome P-450 was measured using the method described by Omura and Sato[10] and modified by Schoene *et al.*[11] The recovery of microsomal protein, calculated from the difference in the cytochrome P-450 content in the original homogenate and the microsomal fraction as described by Greim *et al.*[12] was found to be 100 per cent. The cytochrome P-450 content was calculated using a millimolar extinction coefficient of 91 cm^{-1} (cytochrome P-450–490 nm).[13]

Extraction and separation of lipids. 1 ml microsomal suspension (containing microsomes from 1 g liver wet weight, 10–12 mg protein) was extracted three times (with 50, 25 and 15 ml, total 90 ml) with methanol–chloroform (30:60, v/v) at room temperature. The combined extracts were washed once with $MgCl_2$.[14] The organic phase was then evaporated and the phospholipid containing layer was dissolved in 2 ml chloroform–methanol (25:15, v/v). This extract was used for thin-layer chromatography.[15] 10–20 μl of the lipid phase was applied to silica gel H plates (30 g silica gel, (Stahl), particle size 10–40 μm, 58 ml double-distilled water, 0·2 mm thickness). Chromatograms were developed for about 2 hr in chloroform–methanol–acetic acid–H_2O (25:15:4:2, by vol.). Phospholipid containing spots were visualized by iodine vapour and these regions were scraped off into test tubes. Phosphorus content was determined by the method described by Bartlett.[16] Thus lysophosphatidylcholine (LPC), sphingomyelin (S), phosphatidylcholine (PC), phosphatidylinositol and phosphatidylserine (PI and PS) and phosphatidylethanolamine (PE) could be determined. The front contained neutral lipids and cardiolipin. Plasma phospholipids were determined in the same way after extraction of 1 ml plasma.

RESULTS

As shown in Table 1 ANIT treatment induced cholestatic hyperbilirubinemia. This was indicated by enhanced plasma bilirubin associated with an increase in alkaline phosphatase activity and plasma cholesterol level. The total phospholipid content of plasma was significantly increased. The most important increase was found in the PC-fraction. The LPC and S-fractions were enhanced to a smaller degree. PI + PS and PE were almost unchanged. These alterations in the plasma phospholipid con-

TABLE 1. LIVER FUNCTION TESTS AND PLASMA PHOSPHOLIPIDS IN RATS 48 hr AFTER TREATMENT WITH ANIT (100 mg/kg BODY WT)

	Control n = 6	ANIT n = 6	P
Bilirubin (mg/100 ml)	0·4 ± 0·1	7·4 ± 0·7	0·0005
SGPT (mU/ml)	40 ± 6	275 ± 86	0·0005
Alkaline phosphatase (mU/ml)	250 ± 35	1037 ± 177	0·0005
Cholesterol (mg/100 ml)	65 ± 9	453 ± 66	0·0005
Total phospholipids (μmoles P/ml)	3·2 ± 0·6	11·1 ± 1·5	0·0005
LPC (μmoles P/ml)	0·9 ± 0·2	2·2 ± 0·4	0·0005
S (μmoles P/ml)	0·4 ± 0·1	1·1 ± 0·2	0·0005
PC (μmoles P/ml)	1·5 ± 0·2	7·2 ± 1·0	0·005
PJ + PS (μmoles P/ml)	0·3 ± 0·1	0·5 ± 0·2	0·025
PE (μmoles P/ml)	0·2 ± 0·1	0·2 ± 0·1	NS

Values are expressed as mean ± S.D. NS = not significant.

tent were not accompanied by similar changes in the phospholipid content of the microsomal fraction of the liver.

Table 2 shows that the total phospholipid content of the liver per 100 g body wt remained unchanged and similar results were obtained in the isolated microsomal fraction. Cytochrome P-450 per 100 g body wt also was unchanged. ANIT treatment increased the liver wet weight and since the microsomal protein content per g liver was not reduced, there was a distinct increase in microsomal protein content of the liver per 100 g body wt (Tables 2 and 3). Microsomal cytochrome P-450 content and total phospholipid content, both based on microsomal protein, were reduced in ANIT-treated animals (Table 3). Therefore, in ANIT-treated animals there was an increase in microsomal protein without an increase in cytochrome P-450 and phospholipid content. No significant change was observed in the cytochrome P-450/phospholipid ratio in these animals (Fig. 1).

TABLE 2. LIVER WET WEIGHT, MICROSOMAL PROTEIN, CYTOCHROME P-450 CONTENTS AND PHOSPHOLIPID CONTENT AND COMPOSITION IN RATS 48 hr AFTER TREATMENT WITH ANIT (100 mg/kg BODY WT).

	Experimental conditions		
	Control n = 6	ANIT n = 6	P
Liver wet wt (g/100 g body wt)	3·25 ± 0·26	4·31 ± 0·36	0·0005
Microsomal protein (mg/g liver wet wt)	47·9 ± 8·2	52·1 ± 10·1†	NS
Cytochrome P-450 (nmoles/100 g body wt)	147 ± 20	155 ± 18*	NS
Total phospholipid content (μmoles P/100 g body wt)	164 ± 18	182 ± 31*	NS
S (μmoles P/100 g body wt)	5·5 ± 1·7	6·8 ± 2·8†	NS
PC (μmoles P/100 g body wt)	73·2 ± 9·1	81·0 ± 15·4†	NS
PJ + PS (μmoles P/100 g body wt)	18·0 ± 4·1	15·5 ± 3·0†	NS
PE (μmoles P/100 g body wt)	32·3 ± 6·6	26·5 ± 6·7†	NS

* Tested in liver homogenate.
† Determined in isolated microsomes and corrected for 100 per cent recovery (see Methods).
NS = not significant.

TABLE 3. CYTOCHROME P-450, PHOSPHOLIPID AND PROTEIN CONTENT OF RAT LIVER MICROSOMES 48 hr AFTER TREATMENT WITH ANIT (100 mg/kg BODY WT)

Condition	n	mg Microsomal protein 100 g body wt	nmoles Cytochrome P-450 mg microsomal protein	μmoles P mg microsomal protein
Control	6	155 ± 23	0·97 ± 0·21	0·8 ± 0·1
ANIT	6	224 ± 39	0·71 ± 0·14	0·6 ± 0·1
P		0·0025	0·025	0·0025

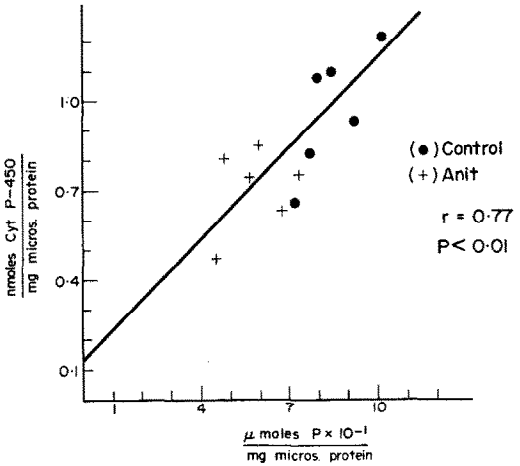

FIG. 1. Microsomal cytochrome P-450/phospholipid ratio in liver from control (●) and ANIT-treated rats (×).

DISCUSSION

Cholestatic hyperbilirubinemia induced in rats by ANIT treatment has been described previously by several authors.[17] Bile flow is zero under these experimental conditions.[18] It could be concluded from this that the increase in plasma cholesterol and phospholipid content is the consequence of reduced secretion of these substances into the bile and this would agree with the unchanged hepatic phospholipid content (Table 2). However, other factors such as shortened half-life, red blood cells with enhanced liberation of phospholipids and cholesterol[19] or enhanced phospholipid synthesis in the liver, as suggested by Kattermann,[20] must be taken into consideration. In human cholestasis the synthesis of a specific LDL lipoprotein (LPX) with a high phospholipid and cholesterol content has been found.[21]

Drug tolerance of the animals *in vivo* is diminished.[22] Since liver cytochrome P-450 content per 100 g body wt remained unchanged (Table 2) functional impairment of microsomal drug metabolizing activity must be considered. Greim *et al.*[12] presented strong evidence that increased bile acid concentration could be one of the factors leading to competitive inhibition of drug metabolizing capacity. On the other hand as it may be seen from the results in Table 3, the composition of the microsomal membranes in ANIT cholestasis is altered with regard to the protein/cytochrome P-450 and phospholipid ratio. *In vitro* this leads to a reduced drug metabolizing activity based on microsomal protein content.[23] Since no evidence has been found, that

there is any change in the cytochrome P-450 phospholipid ratio (Fig. 1) or in the phospholipid composition of the microsomal fraction of the liver (Table 2) the question must be raised if the increase of microsomal protein could be an additional factor influencing drug metabolizing activity of the liver *in vivo*.

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