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Effect of methotrexate on long-chain fatty acid metabolism in liver of rats fed a standard or a defined, choline-deficient diet

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The effect of methotrexate on lipids in serum and liver and key enzymes involved in esterification and oxidation of long-chain fatty acids were investigated in rats fed a standard diet and a defined choline-deficient diet. Hepatic metabolism of long-chain fatty acids were also studied in rats fed the defined diet with or without choline. When methotrexate was administered to the rats fed the standard diet there was a slight increase in hepatic lipids and a moderate reduction in the serum level. The palmitoyl-CoA synthetase activity and the microsomal glycerophosphate acyltransferase activity in the liver of rats were increased by methotrexate. The data are consistent with those where the liver may fail to transfer the newly formed triacylglycerols into the plasma with a resultant increase in liver triacylglycerol content and a decrease in serum lipid levels. Fatty liver of methotrexate-exposed rats can not be attributed simply to a reduction of fatty acid oxidation as the carnitine palmitoyltransferase activity was increased. The methotrexate response in the rats fed the defined choline-deficient diet was different. There was a reduction in both serum and hepatic triacylglycerol and the glycerophosphate acyltransferase and palmitoyl-CoA synthetase activities. The carnitine palmitoyltransferase activity was unchanged. Hepatomegaly and increased hepatic fat content, but decreased serum triacylglycerol, total cholesterol and HDL cholesterol were found to be related to the development of choline deficiency as the pleiotropic responses were almost fully prevented by addition of choline to the choline-deficient diet. Addition of choline to the choline-deficient diet normalized the total palmitoyl-CoA synthetase and carnitine palmitoyltransferase activities. In contrast to methotrexate exposure, choline deficiency increased the mitochondrial glycerophosphate acyltransferase activity. The data are consistent with those of where fatty liver induction of choline deficiency may be related to an enhanced esterification of long-chain fatty acids concomitant with a reduction of their oxidation.

Introduction

Fatty metamorphosis of the liver is a side effect of long-term treatment of patients as well as in experimental rats with the anti-folate drug, methotrexate [1,2]. The lipotropic agents like methionine or choline may protect the liver against lipid infiltration [3–5].

There is some information in the literature on metabolic effects of methotrexate, which may be related to fat accumulation in the liver. Methotrexate causes cellular depletion of reduced

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folates [6,7]. This may lead to increased metabolic demand for betaine in the betaine homocysteine methyltransferase reaction, which serves as an alternative route of methionine synthesis in the liver [8]. Since choline is the immediate metabolic precursor of betaine, methotrexate may cause lack of intracellular choline [9].

Choline deficiency induces fatty accumulation in the liver and reduction in circulating phospholipids, triacylglycerol and apolipoproteins [10,11], but knowledge of the underlying biochemical processes is sparse. When choline levels are depleted there is a reduction of betaine content and choline utilization for biosynthesis of phosphatidylcholine [12,13], an important component of plasma membrane and serum lipoproteins [14].

It is conceivable that accumulation of fat in liver is associated with altered uptake or metabolic flux through pathways leading to synthesis or degradation of lipids. The free fatty acids taken up from the blood by the liver, and converted to the corresponding long-chain acyl-CoA through the action of different long-chain acyl-CoA synthetases (EC 6.2.1.3) [15], may either be oxidized or be involved in glycerol 3-phosphate esterification. This constitutes a branch-point connecting synthesis or degradation of lipids [15].

Glycerophosphate acyltransferase (EC 2.3.1.15) which is localized in both mitochondria and endoplasmic reticulum [16], is a key enzyme involved in glycero-lipid biosynthesis [15,16]. Diacylglycerols which are important intermediates of phosphoglycerols can be converted into triacylglycerols by reaction with long-chain fatty acyl-CoA.

Carnitine palmitoyltransferase (EC 2.3.1.21) appeared to be rate limiting in the transfer of activated fatty acids into the mitochondria [15]. Thus, this enzyme determined the flux through pathways oxidizing long-chain fatty acids to acetyl-CoA and thereby opposed the action of the anabolic enzymes like glycerophosphate acyltransferase.

Accumulation of fat in the liver may be due to increase in lipid biosynthesis, inhibition of lipid degradation and fatty acid oxidation or failure to transfer the newly synthesized triacylglycerols into plasma with a resultant increase in liver triacylglycerol content. We decided to evaluate the possibility of altered flux through opposing pathways during methotrexate exposure of rats fed a normal diet and a defined diet deficient in choline and vitamin B-12 by determining the palmitoyl-CoA synthetase, glycerophosphate acyltransferase, and carnitine palmitoyltransferase activities in the liver. Particular attention has been focused on (a) which enzyme system changes in specific activity and subcellular distribution and (b) the role of the choline deficiency.

Materials and Methods

[1-^{14}C]Glycerol 3-phosphate, purchased from New England Nuclear (Boston, MA, U.S.A.) was mixed with unlabelled glycero 3-phosphate (Sigma Chemical Co., MO, U.S.A.) to give a specific activity of 2000 cpm/nmol. Methotrexate was purchased from Nyco A/S (Oslo, Norway). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals

Male Wistar rats weighing 180–200 g were randomly selected for methotrexate treatment and were fed a defined choline-deficient diet without and with choline (600 mg/kg) and a defined standard pellet diet. They were housed individually in metal wire cages in a room maintained at 12 h light-dark cycles and constant temperature of $20 \pm 3°$ C. The animals were acclimatized to these conditions at least 5 days before the start of the experiments. The standards pellet contained 55% carbohydrate, 25% protein, 2.1% fat and all necessary minerals and vitamins including vitamin B-12 (24 μg/kg) and choline (600 mg/kg) [17]. The choline-deficient animals were fed with a semi-purified commercial choline-deficient diet containing 680 g sucrose, 200 g casein (British Drug Houses, Light White Soluble), 60 g soya oil, 40 g U.S.P. XIW salt mixture and a 20 g vitamin mixture which contained 25 mg thiamine hydrochloride, 22 mg riboflavin, 22 mg pyridoxine hydrochloride, 33 mg calcium pantothenate, 220 mg nicotiamide, 350 mg ascorbic acid, 5000 I.U. vitamin A, 600 I.U. vitamin D-2 and 51 mg tocopherol acetate in sufficient glucose to make 20 g.

Methotrexate was dissolved by adding sterile water to an ampule containing sufficient sodium

chloride to make an isotonic solution. The animals received increasing doses of this methotrexate solution corresponding to 160, 250 and 350 μg/day per kg body weight intraperitoneally once daily. The control animals were injected with physiological saline. The rats had free access to water and diets, and the experiments lasted for 10 or 16 days. After this time, the non-fasted rats were weighed, decapitated, exsanguinated to obtain blood samples and the livers were removed and immediately chilled on ice and weighed. Serum was prepared from the blood samples by centrifuging the clotted whole blood at $1000 \times g$ for 10 min.

Preparations of cell subfractions

The liver was homogenized in ice-cold sucrose medium (0.25 M sucrose in 10 mM Hepes buffer (pH 7.4) using a Potter-Elvehjem homogenizer at 720 rev/min and with two strokes of a loosely fitting Teflon pestle. Subfractions of the homogenates were prepared by a modified procedure [18] of De Duve et al. [19] using a Sorvall RC-5 refrigerated centrifuge and a HB-4 rotor (R_{min} = 4.8 cm and R_{max} = 14.6 cm). Cell debris, nuclear and partly whole cells (the nuclear fraction, N) were sedimented at a centrifugal effect of $rpm^2\ dt = 6.3 \cdot 10^7\ min^{-1}$ ($t = 10$ min). The pellet was washed twice and the collected supernatants (the cytosolic extract, E) were centrifuged at a centrifugal effect of $6.4 \cdot 10^8\ min^{-1}$ ($t = 10$ min). The sediment (the mitochondrial fraction, M) was washed twice. The combined supernatants resulting from the mitochondrial preparation, were centrifuged at a centrifugal effect of $4.3 \cdot 10^9\ min^{-1}$ ($t = 30$ min). The sediment was washed twice giving the light mitochondrial fraction (L) containing both peroxisomes, lysosomes and mitochondria. The combined supernatants resulting from the L fraction were centrifuged in a Beckman Ultracentrifuge (L-70) with the SW 41 rotor at 35000 rpm for 60 min (R_{min} = 6.63 cm, R_{max} = 15.23 cm). The combined supernatants resulting from preparation of the P fraction was termed the particle-free supernatant or cytosol (S). All procedures were performed at 0–4 °C. The different fractions were stored below −20 °C until analyzed.

Enzyme assays and other analytical methods

Glycerophosphate acyltransferase was assayed by a modification of the method of Daae and Bremer [20] and Nimmo and Nimmo [21]. The incubation comprised, in a total of 0.1 ml, 70 mM Hepes (pH 7.4) 7.5 mM NaF, 4 mM $MgCl_2$, 1 mM dithiothreitol, 2 mg/ml bovine serum albumin, 0.5 mM *sn*-[^{14}C]glycerol 3-phosphate, various amounts of palmitoyl-CoA and protein. After 6 min at 30 °C the incubation was stopped by the addition of 0.4 ml of water-saturated butanol and extraction of the glycerolipids was done as described [22].

Carnitine palmitoyltransferase was assayed as described by Bird et al. [22]. The reaction mixture contained 25 mm Tris-HCl (pH 7.4) in 150 mM sucrose, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1.3 mg/ml bovine serum albumin, 100 μM palmitoyl-CoA, 40–70 μg of protein in a final volume of 0.3 ml. After the preincubation for 2 min, the incubation was initiated by the addition of 0.7 mM L-[^{14}C]carnitine at 30 °C. After 3 min, the incubation was stopped by 1 ml 1 M HCl and 1 ml butanol and the extraction was done as described [23].

The enzymatic activity of palmitoyl-CoA synthetase was determined as described earlier [20].

The following marker enzymes were measured: succinate phenazine metosulfate oxidoreductase for mitochondria, acid phosphatase for lysosomes, catalase and urate oxidase for peroxisomes and rotenone-insensitive NADPH-cytochrome *c* reductase for microsomes [17,18].

Protein was determined employing the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) using and freeze-dried bovine immunoglobulin as the standard.

Statistical methods

A two-way analysis of variance and the Student's *t*-test was used for the comparison of values from the different groups. The *P* values were corrected using the Bonferroni correction.

Results

Body weight, liver weight and protein content

Diet intakes of all animal groups were comparable. The body weight of the rats decreased as

TABLE I

EFFECT OF METHOTREXATE ADMINISTRATION, A DEFINED, CHOLINE-DEFICIENT DIET AND A CHOLINE-DEFICIENT DIET + CHOLINE ON BODY WEIGHT, RELATIVE LIVER WEIGHT AND PROTEIN CONTENT

CP diet, a standard pelleted diet; CD diet, a defined, choline-deficient diet; CD diet + choline, a choline-deficient diet + choline. MTX, methotrexate. The lowest and highest values of individual rats in each treatment group are given. Calculated as mean ± S.D. of three animals, the significance is: * $P < 0.01$ compared to group 6. ** At least $P < 0.02$ compared to group 7. *** At least $P < 0.01$ compared to group 7. **** At least $P < 0.02$ compared to group 1. ***** $P < 0.02$ compared to group 7.

Group	Days			MTX, ip (μg/day per kg body)	Increased rat weight (g)	Liver weight (g)	Liver/body weight (%)
	CP diet	CD diet	CD diet + choline				
1	10			0	36– 48	9.2–11.4	3.8–4.1
2	10			160	40– 45	8.2– 9.4	4.0–4.3
3	10			350	26– 28 ****	10.4–11.5	3.9–4.1
4	16			0	102–113	10.2–11.4	3.8–4.0
5	16			250	91– 96	9.9–11.6	3.7–4.1
6			10	0	55– 60	9.5–10.2	3.9–4.2
7		10		0	38– 50	14.1–16.4 *	5.5–5.9 *
8		10		160	20– 33 *****	11.8–13.4 **	5.0–5.3 **
9		10		350	28– 34 *****	12.5–12.7 ***	4.7–4.9 ***

a function of methotrexate dose, irrespective of diet intake (Table I).

The liver weights and the relative liver size (liver weight expressed as percentage of body weight) were higher in rats receiving choline-deficient diet relative to those fed the same diet plus choline. Methotrexate administration did not essentially affect these parameters in rats given standard pellet diets, but decreased the relative liver size of choline-deficient rats (Table I).

The protein content per gram of liver was similar for all feeding groups (data not shown).

Serum lipids

Methotrexate caused a slight, but significant reduction of serum triacylglycerols and cholesterol (Table II).

The choline-deficient diet decreased the serum triacylglycerols. Additional supply of choline to the choline-deficient diet increased the amount of serum triacylglycerols (1.7-fold), cholesterol (1.3-fold) and HDL cholesterol (1.4-fold) to levels comparable those found in standard pellet diet rats.

TABLE II

EFFECT OF METHOTREXATE, CHOLINE-DEFICIENT DIET AND CHOLINE-DEFICIENT DIET + CHOLINE, ON SERUM LIPIDS OF RATS

* $P < 0.01$ compared to group 1. ** $P < 0.02$ compared to group 1. *** $P < 0.01$ compared to group 7. n.d., not determined.

Group	Days			MTX, ip (μg/day per kg)	Triacyl-glycerol (μmol/ml)	Cholesterol (μmol/ml)	HDL chole-sterol
	CP diet	CD diet	CD diet + choline				
1	10			0	2.7 ± 0.5	2.04 ± 0.31	n.d.
3	10			350	1.7 ± 0.1 **	1.47 ± 0.05 *	n.d.
6			10	0	2.2 ± 0.2 ***	2.5 ± 0.4 ***	2.3 ± 0.4 ***
7		10		0	1.3 ± 0.1	1.9 ± 0.5	1.6 ± 0.6

Hepatic lipids

Methotrexate administration to the standard pellet diet-fed animals caused a slight, but significant increase in liver cholesterol content, (Table III). The hepatic triacylglycerols in rats fed a choline-deficient diet were increased about 4-fold relative to standard pellet diet rats. Addition of choline to the deficient diet decreased the hepatic lipid contents relative to the choline-deficient-fed rats. However, the hepatic triacylglycerols and cholesterol were not fully normalized to values found in the standard pellet diet rats (Table III). Notably, when methotrexate was given to rats fed the choline-deficient diet there was a dose-dependent reduction of triacylglycerol content which approached levels obtained with the standard pellet diet (Table III).

Effects of methotrexate and choline deficiency on selected enzyme activities in total liver homogenates

The palmitoyl-CoA synthetase activity was increased in a dose-dependent manner following methotrexate exposure, whereas treatment decreased the specific palmitoyl-CoA synthetase activity of choline-deficient rats (Table IV). The specific activity of palmitoyl-CoA synthetase in whole liver homogenates of rats fed a choline-deficient diet was significantly increased from that of standard pellet diet rats (Table IV). Addition of choline decreased the specific activity of palmitoyl-CoA synthetase in whole homogenates of rats, which was comparable to the value obtained in the standard pellet-fed animals (Table IV).

Preliminary experiments were done to optimize the conditions used for preservation and assay of glycerophosphate acyltransferase activity. The optimal assay conditions for glycerophosphate acyltransferase in the microsomal and the mitochondrial fractions were the same. The enzyme activity in both fractions was linear with respect to protein up to 500 μg/ml and to time for 8 min (data not shown). Substrate saturation was observed at 80–120 μM palmitoyl-CoA. The microsomal glycerophosphate acyltransferase was decreased by about 30–50% following freezing and thawing.

Choline deficiency did not change the specific activity of glycerophosphate acyltransferase in whole liver homogenates of rats. Methotrexate administration increased the enzyme activity in a dose-dependent manner. Notably, when methotrexate was administered to rats fed the choline-deficient diet there was a significant decrease in the enzyme activity (Table IV).

Addition of choline to the choline-deficient diet increased the carnitine palmitoyltransferase activity to values obtained with the standard pellet-fed animals (Table IV).

Methotrexate administration had no significant effect on the carnitine palmitoyltransferase activity (Table IV).

TABLE III

HEPATIC LIPIDS IN RATS FED A CHOLINE-DEFICIENT DIET (CD DIET) A CHOLINE-DEFICIENT DIET + CHOLINE AND AN ORDINARY PELLETED DIET (CP DIET) WITH AND WITHOUT METHOTREXATE ADMINISTRATION

* $P < 0.01$ compared to group 7. ** $P < 0.02$ compared to group 7. *** $P < 0.02$ compared to group 1.

Group	Days			MTX, ip (μg/day/kg)	Triacylglycerol		Cholesterol	
	CP diet	CD diet	CD diet + choline		nmol/g protein	nmol/g liver	nmol/mg protein	nmol/g liver
1	10			0	52 ± 10	6.1 ± 0.6	72 ± 14	9.6 ± 0.5
2	10			160	58 ± 14	6.4 ± 0.5	68 ± 10	10.0 ± 1.1
3	10			350	64 ± 5	7.4 ± 0.4	85 ± 11	11.2 ± 0.4 ***
6			10	0	112 ± 20 *	12.7 ± 3.2	108 ± 10 *	10.8 ± 0.6 *
7		10		0	200 ± 20	30.4 ± 4.6	157 ± 8	17.5 ± 2.0
8		10		160	102 ± 16	15.6 ± 2.8	147 ± 8	16.6 ± 2.2
9		10		350	86 ± 12	9.1 ± 4.8 **	138 ± 12	15.4 ± 1.6

TABLE IV

EFFECT OF A CHOLINE DEFICIENCY AND METHOTREXATE EXPOSURE ON THE TOTAL SPECIFIC ACTIVITIES OF PALMITOYL-CoA SYNTHETASE, GLYCEROPHOSPHATE ACYLTRANSFERASE AND CARNITINE PALMITOYLTRANSFERASE IN WHOLE LIVER HOMOGENATES

The lowest and highest values of individual rats in each treatment group are given. Calculated as mean ± S.D. of three or four animals, the significance was: * $P < 0.01$ compared to group 1. ** $P < 0.02$ compared to group 4. *** $P < 0.01$ compared to group 6. **** $P < 0.02$ compared to group 6.

Group	Days			MTX, ip (μg/day per kg)	Palmitoyl-CoA synthetase		Glycerophosphate acyltransferase		Carnitine palmitoyltransferase	
	CP diet	CD diet	CD diet + choline		nmol/min per liver	nmol/min per mg protein	nmol/min per g liver	nmol/min per mg protein	μmol/min per g liver	nmol/min per mg protein
1	10			0	3650–3780	29–32	128–158	0.9–1.3	1.1–1.5	9.4–12.1
2	10			160	3750–3920	33–34	163–178	1.4–1.7	1.0–1.4	9.0–11.7
3	10			350	3950–4150 *	34–36 *	280–296 *	2.0–2.5 *	0.9–1.1	8.1– 9.5
4	16			0	n.d.	n.d.	147–151	1.0–1.5	n.d.	n.d.
5	16			250	n.d.	n.d.	219–261	1.7–2.2 **	n.d.	n.d.
6			10	0	3640–3720	21–31	148–170	1.4–1.7	1.3–1.5	10.5–12.0
7		10		0	3980–4250 ****	34–36 ****	163–175	1.4–1.7	0.7–0.9 ****	7.3– 7.7 ****
8		10		160	n.d.	n.d.	145–182	1.3–1.7	0.7–0.8	7.1– 7.6
9		10		350	3550–3650 ***	28–30 ***	126–140 ***	0.8–1.0 ***	0.6–0.8	6.9– 7.5

Subcellular fractionation studies

The dual localization of glycerophosphate acyltransferase in rat liver [16,24] led us to investigate whether methotrexate had a differential effect on the mitochondrial or microsomal enzyme.

The liver from four animals from each group were pooled and subcellular fractions were prepared as described in Materials and Methods. The distribution of protein and marker enzymes for mitochondria, lysosomes, peroxisomes and microsomes was essentially similar for all groups (Table V), and in accordance with published data [18,25]. The recovery of protein and enzyme activities was in the range of 90–110%. The nuclear fraction, N, was contaminated by cell debris and whole cells, but the other prepared cellular fractions appeared to be rather pure with low contamination based on marker enzymes (Table V). The amounts of succinate phenazine methosulfate oxidoreductase, urate oxidase and catalase suggest a 3–6% contamination of mitochondria and peroxisomes in the P fraction. However, about 20% of the acid phosphatase was found in the P fraction, confirming earlier results obtained by us [25]. The amount of rotenone-insensitive NADPH-cytochrome *c* reductase suggests a 8–10% contamination of microsomes in the mitochondrial fraction.

Glycerophosphate acyltransferase in the liver of control rats was recovered in the mitochondrial

TABLE V

SUBCELLULAR DISTRIBUTION OF MARKER ENZYMES IN THE LIVER HOMOGENATES OF NORMAL (CP), METHOTREXATE (MTX) AND CHOLINE-DEFICIENT RATS (CD)

The sum of the enzyme activities in the fractions are expressed as percent (corrected to 100% recovery) of the whole homogenates (i.e., postnuclear fraction + nuclear fraction, N). Three fractionations were carried out. The methotrexate dose was 350 μg/day per kg. The animals in the four groups were treated for 10 days.

Enzymes	Diets	Percentage distribution					Recovery (%)
		N	M	L	P	S	
Protein	CP	18.7	19.9	7.0	15.5	38.9	108
	CP + MTX	17.4	20.1	7.6	15.6	39.3	104
	CD	17.8	22.2	7.5	15.5	37.0	103
	CD + MTX	18.2	21.2	7.8	15.2	37.6	107
Succinate phenazine metosulfate oxidoreductase	CP	5.5	86.8	3.5	3.5	0.7	95
	CP + MTX	5.2	86.5	3.3	3.7	0.7	96
	CD	5.0	86.1	3.6	3.4	0.6	95
	CD + MTX	5.1	85.9	3.9	4.0	1.1	97
Acid phosphatase	CP	9.1	20.7	32.2	23.6	14.4	96
	CP + MTX	9.6	20.9	32.5	22.6	15.4	94
	CD	10.0	21.2	31.8	22.2	14.8	93
	CD + MTX	10.1	20.9	32.4	22.4	14.2	92
Catalase	CP	3.5	16.2	27.7	5.4	32.0	103
	CP + MTX	4.5	16.7	28.2	4.8	32.6	102
	CD	5.1	17.3	29.2	3.8	31.6	100
	CD + MTX	3.1	16.4	27.9	5.3	32.1	97
Urate oxidase	CP	5.4	17.8	73.8	2.8	0.2	92
	CP + MTX	5.0	17.4	72.8	3.8	0.2	96
	CD	5.0	16.8	75.2	2.7	0.3	102
	CD + MTX	4.9	16.9	74.2	3.7	0.3	101
Rotenone-insensitive NADPH-cytochrome *c* oxidoreductase	CP	15.0	9.6	10.8	56.4	8.2	91
	CP + MTX	14.0	8.7	10.7	56.3	8.3	94
	CD	13.8	8.2	11.8	58.1	8.1	96
	CD + MTX	14.5	9.8	10.5	56.7	7.9	104

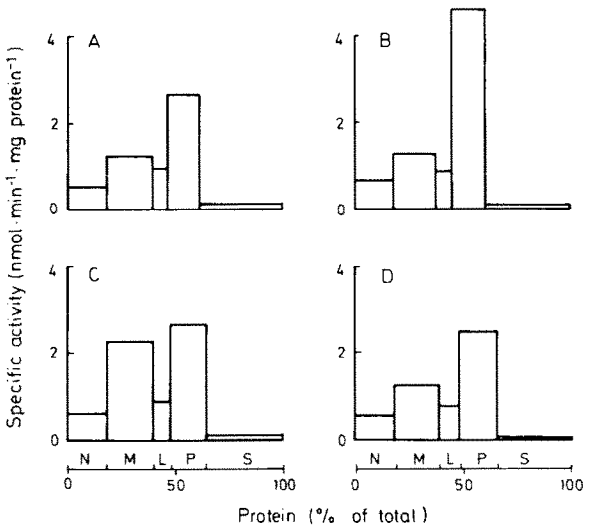

Fig. 1. Intracellular distribution of glycerophosphate acyltransferase in liver of pellet-fed – a defined choline-deficient diet – and methotrexate-treated rats. (A) Rats fed a standard pellet diet. (B) Rats fed a standard diet and methotrexate (350 μg/day per kg). (C) Rats fed a defined choline-deficient diet and (D) rats fed a choline-deficient diet plus methotrexate (350 μg/day per kg). Fraction N is the nuclear fraction; M, the mitochondrial fraction; L, the light mitochondrial fraction; P, the microsomal fraction and S, the soluble fraction. Four animals were tested separately and the tabulated values are the means. The measurements deviated by no more than 10% from the mean value.

(M) (27% of total activity) and microsomal (P) (46% of total activity) fractions. The specific activity of glycerophosphate acyltransferase (Fig. 1) was highest in the M and P fractions. This shows that the enzyme activity is enriched in these fractions, and confirms previous findings that glycerophosphate acyltransferase is located both in mitochondria and microsomes [16,24].

Administration of methotrexate at a dose of 350 μg/day per kg for 10 days increased the specific activity of the microsomal glycerophosphate acyltransferase about 2-fold, whereas the mitochondrial enzyme was not affected (Fig. 1B). Notably, methotrexate administration to rats fed a choline-deficient diet reduced both the mitochondrial and microsomal glycerophosphate acyltransferase as compared to choline-deficient diet alone (Fig. 1D).

Dose-response relationship of methotrexate

The specific activity of the microsomal glycerophosphate acyltransferase increased in a dose-dependent manner following administration of methotrexate, whereas the mitochondrial enzyme was essentially unaffected (Fig. 2A). In rats given the choline-deficient diet, methotrexate

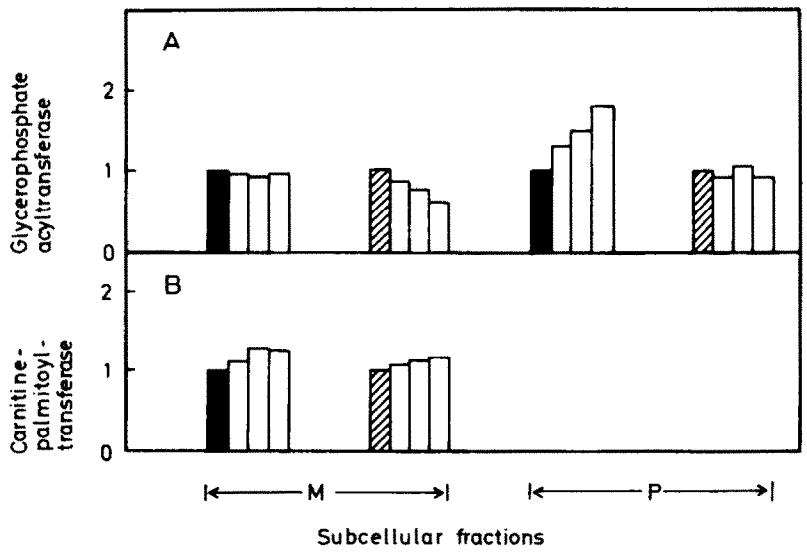

Fig. 2. The effect of the dose of methotrexate on the specific activities of mitochondrial and microsomal enzymes. (A) glycerophosphate acyltransferase (B) carnitine palmitoyltransferase. ■, rats fed a standard diet. ▨, rats fed a defined choline-deficient diet (lined columns). Increasing doses of methotrexate (from left to right: 160, 250 and 350 μg/day per kg body weight) (□) were administered. The specific activities are calculated relative to those of pellet-fed and choline-deficient diets (1.0). The isolated subcellular fractions are the mitochondrial fraction (M) and the microsomal fraction (P).

caused a dose-dependent reduction in the enzyme activity. The microsomal enzyme was unaffected by methotrexate (Fig. 2A).

Carnitine palmitoyltransferase was mainly (75–80%) recovered in the mitochondrial fraction. Fig. 2B shows that the enzyme activity was slightly increased in a dose-dependent manner up to 250 μg/day per kg following methotrexate administration to rats fed the standard diet.

Discussion

The standard diet is a commercially available pellet, containing all vitamins and lipotropes, and which has been fed to these animals during their whole life-span, and therefore supports their growth and normal function. Such a diet precludes that the methotrexate effects observed are limited to conditions of undefined nutritional deficiencies.

The choline-deficient diet is well defined and lacks vitamin B-12 and choline, and was given to these rats for 10 days. Vitamin B-12 was excluded from the diet because it may antagonize some metabolic effects of methotrexate [9]. Barak and Beckenhauer [9] have reported that a 10-day B-12-deficient regimen did not produce a true B-12 deficiency in the animals.

Animal studies have established that choline in the diet may modulate the hepatotoxic effects of methotrexate [3–5]. In order to elucidate that differences between diets were due to low choline in one diet the control diet was made by adding choline to the deficiency diet. Therefore, we decided to evaluate the effect of choline deficiency and methotrexate on the hepatic and serum lipids, and some key enzymes involved in lipid synthesis and degradation in liver of rats receiving both a standard pellet diet and a choline-deficient diet. Adaptive changes in the activities of these enzymes may reflect alterations in the metabolic flux through the corresponding pathways. Changes in enzyme activities are either caused by altered enzyme content in the liver or changes in enzyme activity induced by metabolites which serve as modulators of the catalytic activity. Our study does not distinguish between these two possibilities.

In agreement with earlier findings [26–28], removal of choline from the diet is associated with accumulation of hepatic triacylglycerols and cholesterol (Table III) and decreased serum lipids (Table II). Thus, the choline-deficient diet increases hepatic lipids but lipoprotein secretion is impaired [3,10,27]. Hepatomegaly and fatty liver were found to be related to lack of choline as the relative liver weight and hepatic triacylglycerols and cholesterol were fully prevented by addition of choline to the choline-deficient diet (Table I).

It has been demonstrated that removal of choline from the diet is associated with accumulation of hepatic triacylglycerols and increased activity of hepatic fatty acid synthetase [29]. Apart from this, most enzymological studies on choline deficiency have focused on phospholipid metabolism [11], and the only increase in enzyme activities observed was a moderate stimulation of phosphatidylethanolamine-*S*-adenosylmethionine methyltransferase activity [13].

In the present study, choline deficiency induced a moderate increase in palmitoyl-CoA synthetase and mitochondrial glycerophosphate acyltransferase activities and reduction of mitochondrial carnitine palmitoyltransferase activity. This response suggests that a greater part of activated fatty acids is directed from oxidation towards triacylglycerol esterification. The net result may be that excess diacylglycerol, generated by the increased mitochondrial glycerophosphate acyltransferase activity, is converted to triacylglycerols via acyl-CoA by palmitoyl-CoA synthetase, as depicted in Fig. 3. This adaptive response is in accordance with the observation that this dietary change caused a massive accumulation of hepatic triacylglycerol (Table III).

It is of interest to investigate how methotrexate interferes with lipid metabolism under diverse conditions. In rats fed a standard pellet, methotrexate induced a moderate increase in hepatic triacylglycerols and a reduction in serum triacylglycerols (Table II and III). This observation is in accordance with data published by Tuma et al. [3]. As observed for choline deficincy, the metabolic response is consistent with accumulation of hepatic triacylglycerols and impaired lipoprotein transport. Under this nutritional state, methotrexate induced a moderate increase in palmitoyl-CoA synthetase, carnitine palmitoyltransferase and microsomal glycerophosphate

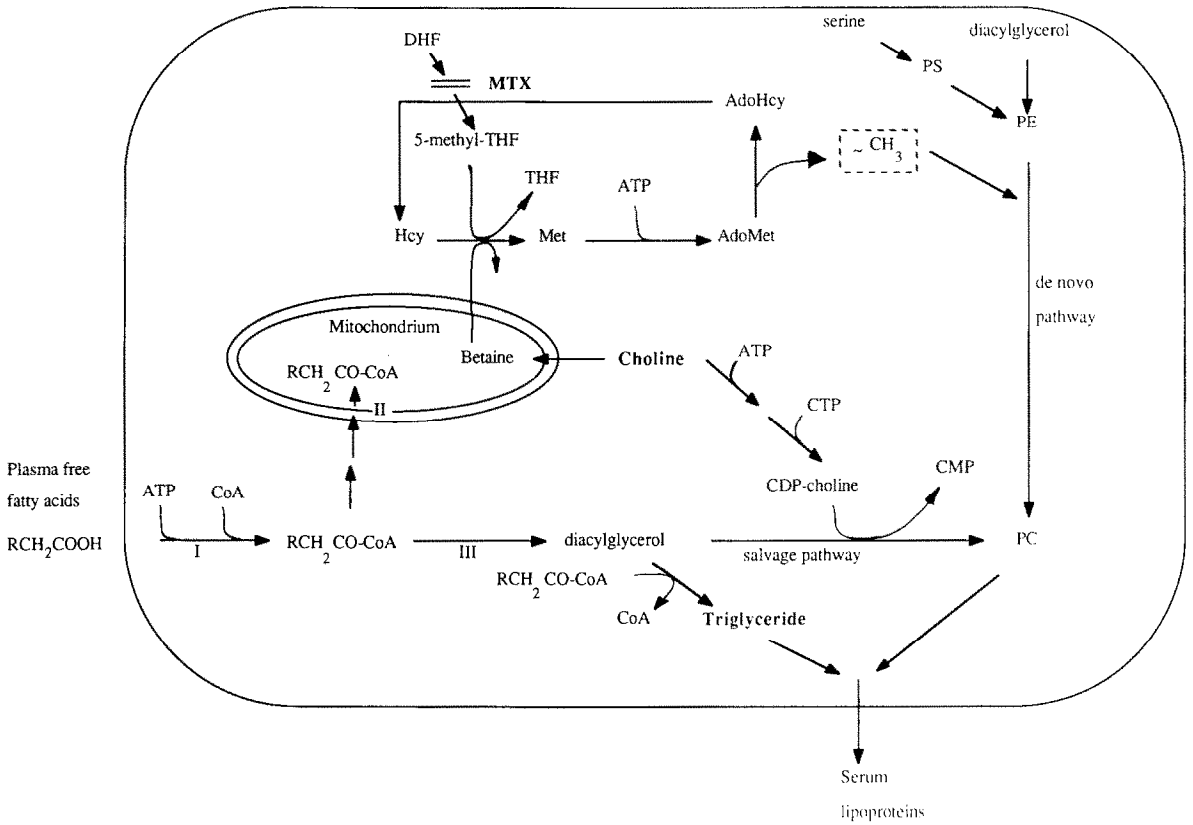

Fig. 3. Diagrammatic representation of a liver cell showing uptake of free fatty acids and their conversion to triacylglycerol esterification, mitochondrial oxidation and to phospholipid formation (de novo and salvage pathways) in relation to the metabolic pathways of choline and sulfur compounds. I, palmitoyl-CoA synthetase. II, carnitine palmitoyltransferase. III, glycerophosphate acyltransferase. PC, phosphatidylcholine, PE, phosphatidylethanolamine, PS, phosphatidylserine, Met, methionine, CH_3, active methyl group attached to AdoMet, *S*-adenosylmethionine MTX, methotrexate; DHF, dihydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; Hcy, homocysteine; AdoHcy, *S*-adenosylhomocysteine.

acyltransferase activities (Table IV, Fig. 2). The data suggest that fatty metamorphosis in the liver of methotrexate treatment may partly be related to an enhanced esterification on long-chain fatty acids (at the endoplasmatic levels), but can not be attributed simply to a reduction of their oxidation.

In rats fed the defined choline-deficient diet, the metabolic response to methotrexate is different. There is a marked reduction in hepatic triacylglycerol content (Table III) and the serum triacylglycerol was also reduced (data not shown), suggesting interference with lipoprotein secretion. Under this condition there is no increase in microsomal glycerophosphate acyltransferase activity and the activity of the mitochondrial enzyme is reduced (Table V). The mitochondrial fatty acid oxidation was marginally affected (Fig. 2B). The activity of the palmitoyl-CoA synthetase (Table IV) was also decreased in these animals. Thus, reduction in enzyme activities under these conditions may be, partly at least, related to an interference with the protein synthesis. Inhibition of protein synthesis and low adosyl-L-methionine concentration [30–33] may also decrease the formation of the protein and lipid moieties of lipoproteins necessary for lipoprotein secretion, and thereby contribute to accumulation of fat in the liver (Fig. 3).

In conclusion, choline deficiency seems to increase the flux of long-chain fatty acids through pathways providing them for triacylglycerols synthesis at the endoplasmatic reticulum (micro-

somes). This is in contrast to methotrexate where the activated long-chain fatty acids are esterified at the mitochondrial level. It is conceivable that hepatic accumulation of triacylglycerols, and low serum triacylglycerol levels may be related to impaired lipoprotein secretion both after methotrexate treatment and choline deficiency. Furthermore, the data suggest that methotrexate causes profound alteration in hepatic fatty acid metabolism, but the characteristics of this response are highly dependent on the status of the lipid metabolism at the time of exposure.

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