

Effect of choline-deficiency and methotrexate administration on peroxisomal β -oxidation, palmitoyl-CoA hydrolase activity and the glutathione content in rat liver

Rolf Kristian Berge¹, Niels Aarsaether, Asle Aarsland, Asbjørn Svoldal² and Per Magne Ueland²

Laboratory of Clinical Biochemistry, University of Bergen, Haukeland Sykehus, 5021 Bergen and ²Clinical Pharmacology Unit, Department of Pharmacology, University of Bergen, Haukeland Sykehus, 5027 Bergen, Norway

¹To whom reprint requests should be sent

Hepatic metabolism of long-chain fatty acids was studied in male rats fed a defined choline-deficient (CD) diet with and without choline and after methotrexate (MTX) administration. Peroxisomal β -oxidation was increased ~4-fold in the peroxisome-enriched fraction of CD-fed animals, whereas the catalase activity was increased 1.3-fold. The urate oxidase activity was marginally affected. The CD-fed rats also revealed elevated capacity for hydrolysis of palmitoyl-CoA in the cytosolic fraction (2.0-fold), whereas the microsomal palmitoyl-CoA hydrolase activity was decreased. Notably, the increased peroxisomal β -oxidation, the catalase activity and palmitoyl-CoA hydrolase activities (the membrane-bounded and cytosolic) were almost fully prevented by adding choline to the CD-diet. Thus, the change in these enzyme activities appears to be a consequence of a choline-deficiency provoked by the CD diet. MTX administration of normal fed rats (ND diet) had no effects on the peroxisomal β -oxidation, catalase activity and urate oxidase activity. MTX treatment of the ND-fed animals, however, increased the mitochondrial palmitoyl-CoA hydrolase activity and decreased the microsomal enzyme activity. As choline-deficiency and MTX increased the hepatic lipid level, the overall results suggest that fat accumulation is not an 'induction signal' for increased peroxisomal β -oxidation. The CD diet alone increased the reduced glutathione content in liver, whereas MTX did not significantly change this level. Whether the changes of H₂O₂-generating peroxisomal oxidation of long-chain fatty acids may be an important step in a chain of events, which eventually results in tumour formation by choline-deficiency, should be considered.

Introduction

The liver has a very active mechanism for the oxidation and phosphorylation of choline. In the oxidative pathway choline is converted to betaine which acts as methyl donor to homocysteine, yielding methionine. Methionine is then a precursor for S-adenosylmethionine (S-Ado-Met*). Thus, choline can have a sparing effect on methionine requirements.

The conversion of homocysteine to methionine and thereby to S-Ado-Met is also highly dependent on folate metabolism. Methotrexate (MTX) inhibits the enzyme dihydrofolate reductase, and

thereby blocks the regeneration of tetrahydrofolate from dihydrofolate. This in turn is expected to cause cellular depletion of reduced folates (1).

Long-term treatment of patients as well as experimental animals with MTX induces hepatic injury characterized by fatty infiltration and fibrosis (2,3). A chronic methyl-group deficiency can be produced in rats fed a diet deficient in choline and MTX (4-6). This in turn may cause fatty accumulation in the liver and reduce the amount of circulating phospholipids and apoproteins (5).

Recently, we have observed that both choline-deficiency and MTX treatment changed the activities of the fatty-acid-metabolizing enzymes (7). The data suggest that lipid accumulation in the liver of a choline-deficient (CD) diet may be related to an enhanced esterification of long-chain fatty acids concomitant with a reduction of their oxidation (7). Fat-accumulation in the liver during MTX treatment, however, could not be attributed simply to reduction of fatty acid oxidation as the carnitine palmitoyltransferase activity and the microsomal glycerophosphate acyltransferase activity were increased (7).

Rats fed diets devoid of choline developed liver cancer (8,9). The mechanism by which this diet induces cancer is not fully elucidated. It has been proposed that DNA alterations due to free radical generation (10,11) and/or by causing hypomethylation of specific genes (12) may be important biological responses in the initiation of liver cancer due to a CD diet.

Long-term treatment of experimental animals with hypolipidemic-peroxisome-proliferating drugs induces lipid peroxidation and liver tumours (13). The mechanism by which hypolipidemic-peroxisome-proliferating drugs exert their promoting effect (14) has been attributed to free radical damage of cell membranes and DNA following an excess production of H₂O₂ (13) through the enhanced H₂O₂-generating peroxisomal oxidation of fatty acids.

A number of changes have been reported in hepatic fatty acid metabolism and morphology (15-20), notably at the level of mitochondria, endoplasmic reticulum and peroxisomes on treatment with many hypolipidemic drugs (15-18), and feeding rats a diet with high doses of fish oils (19,20). At the peroxisomal level, a considerable proliferation of small peroxisomes with an increased capacity of β -oxidation of long-chain fatty acids and elevated capacity for hydrolysis of long-chain acyl-CoA has been observed (15-20). In addition, increased synthesis of triglycerides accompanied with an increase of lipid droplets (22) is an early change seen in rat hepatocytes treated with peroxisome proliferators. Thus, the overall results strongly suggest that an important regulation of hepatic fatty acid metabolism by peroxisome proliferators may be attributed to a cellular control both of substrate concentration (long-chain acyl-CoA) and of organelle biogenesis (18,20).

The aim of the present study was to examine in more detail the changes in specific activities and subcellular distribution of palmitoyl-CoA hydrolase, peroxisomal β -oxidation and peroxisomal-associated enzymes on administration to rats of a

*Abbreviations: S-Ado-Met, S-adenosylmethionine; MTX, methotrexate; CD, choline-deficient; ND, normal diet; GSH, glutathione; N, M, L, P and S, nuclear, mitochondrial, peroxisome-enriched, microsomal and cytosolic fractions respectively.

Table I. Liver biochemical parameters by choline deficiency and MTX administration

Parameter measured	ND diet	CD diet	CD diet + choline	ND diet + MTX	CD diet + MTX
△ Body weight (g)	42 ± 6	44 ± 6	43 ± 6	37 ± 3	29 ± 5
Liver weight (g)	10.3 ± 0.9	15.3 ± 1.2	9.8 ± 0.8	10.9 ± 0.6	12.6 ± 0.4
Liver weight/body weight (%)	4.0 ± 0.2	5.7 ± 0.2	4.1 ± 0.2	4.0 ± 0.1	4.5 ± 0.4
Triglycerides (μmol/g liver)	5.9 ± 0.4	35.2 ± 4.2	12.0 ± 3.2	7.3 ± 0.3	10.0 ± 2.8
Cholesterol (nmol/g liver)	9.3 ± 1.4	16.5 ± 1.3	10.5 ± 0.5	10.0 ± 0.5	9.2 ± 1.3
Palmitoyl-CoA synthetase	^b 3.6 ± 0.2 ^c 29 ± 2	4.2 ± 0.2 36 ± 3	3.7 ± 0.1 26 ± 4	4.1 ± 0.2 35 ± 1	3.5 ± 0.3 29 ± 2
Carnitine palmitoyltransferase	^b 1.3 ± 0.2 ^c 11.0 ± 2.6	0.9 ± 0.1 7.5 ± 0.2	1.4 ± 0.1 11.3 ± 0.4	1.0 ± 0.1 8.7 ± 0.6	0.5 ± 0.2 6.2 ± 0.7
Glycerophosphate acyltransferase	^a 143 ± 15 ^c 1.1 ± 0.2	120 ± 6 1.5 ± 0.2	152 ± 0.1 1.2 ± 0.1	290 ± 5 2.2 ± 0.2	137 ± 7 0.9 ± 0.2
Palmitoyl-CoA hydrolase	^b 3.7 ± 0.3 ^c 31 ± 2	2.9 ± 0.3 24 ± 4	3.1 ± 0.3 29 ± 3	3.2 ± 0.3 27 ± 4	3.3 ± 0.2 32 ± 4
Peroxisomal β-oxidation	^a 240 ± 20 ^c 1.7 ± 0.2	570 ± 30 3.7 ± 0.4	340 ± 20 2.7 ± 0.2	280 ± 30 1.5 ± 0.2	220 ± 10 1.2 ± 0.2
Catalase	^b 65 ± 2 ^c 548 ± 52	54 ± 7 493 ± 51	61 ± 3 520 ± 40	57 ± 6 496 ± 60	53 ± 9 505 ± 60
Urate oxidase	^a 760 ± 60 ^c 6.2 ± 0.4	703 ± 40 5.8 ± 0.3	715 ± 30 6.0 ± 0.2	642 ± 35 5.6 ± 0.2	448 ± 25 4.4 ± 0.3

The tabulated values represent means ± SD for four animals.

The total activity (nmol/min/mg liver)^a or (μmol/min/g liver)^b was measured in the combined nuclear + postnuclear fractions.

The specific activity (nmol/min/mg protein)^c was measured in the postnuclear fraction.

normal (ND) diet and a CD diet. Particular interest has been focused on the contribution of the choline-deficiency. It was also deemed of interest to consider the peroxisomal β-oxidation in relation to the cellular glutathione (GSH)-pool. This important thiol compound may be responsible for the defence against free radicals and H₂O₂ and, furthermore, be important in the regulation of cellular proliferation (23).

Materials and methods

Chemicals

[1-¹⁴C]Palmitoyl-CoA was purchased from New England Nuclear (Boston, MA, USA). Palmitoyl-CoA and reduced GSH were obtained from Sigma Chemical Co. (St Louis, MO, USA). MTX was purchased from Nycomed (Oslo, Norway). All other chemicals were obtained from common commercial sources, and were of reagent grade.

Animals and diet

Male Wistar rats weighing 180–220 g at the beginning of the study were randomly selected for MTX treatment, feeding a CD diet without and with choline (7) and a defined standard pellet diet (ND) (15). They were housed individually in metal wire cages in a room with 12-h light/dark cycles and a constant temperature of 20 ± 3°C. The animals were acclimatized to the facility at least 5 days before the start of the experiments.

Treatment of animals

The animals were divided into five groups each containing four animals and treated as follows.

(i) One group was fed the CD diet and received no drug. These animals are referred to as the CD group.

(ii) One group was fed the CD diet plus choline where the concentration of choline in the CD diet was similar to the standard pellet diet. These animals are referred to as the CD + choline group.

(iii) One group was fed the ND diet and received no MTX. These animals are the controls and designated ND group.

(iv) One group was fed the ND diet and were injected i.p. with MTX at a dose of 350 μg/kg once daily. These animals are referred to as the ND + MTX group.

(v) One group was fed CD diet and was injected with MTX (350 μg/kg/24 h). These animals are referred to as the CD + MTX group.

MTX was dissolved by adding sterile water to an ampoule containing suffi-

cient sodium chloride to make an isotonic solution. The control animals were injected with physiological saline.

The rats had free access to water and diets and the experiments lasted for 10 days.

Subcellular fractionation and enzyme assays

The rats were killed by decapitation and livers homogenized in ice-cold sucrose medium (0.25 M sucrose in 10 mM Hepes buffer pH 7.4) as described (16,17). The homogenates were fractionated into nuclear (N), mitochondrial (M), peroxisome-enriched (L), microsomal (P) and cytosolic (S) fractions (17,20).

The subcellular marker enzymes were determined as earlier described (15–20). Protein was assayed by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

Palmitoyl-CoA hydrolase was assayed by measuring the formation of [¹⁴C]-palmitate (15,17). The incubation medium contained 15 mM Hepes buffer pH 7.5, 2 mM EDTA, 0.01% (w/w) Brij-58, 60–100 μM palmitoyl-CoA and protein in the range 10–50 μg.

Palmitoyl-CoA-dependent dehydrogenases (usually termed peroxisomal β-oxidation) were measured by recommended procedure (17). The incubation medium for this assay consisted of 50 μM Hepes buffer pH 7.4, 4 mM dithiothreitol, 1 mM CoA, 0.2 mM NAD⁺, 0.01% Brij-58 (w/w), 40–50 μg protein and the palmitoyl-CoA concentration was in the range 40–60 μM (17).

Determination of GSH in tissues

The reduced GSH content was determined in an acid extract by a modification of Anderson (24). The GSH-bimane derivative was quantitated by HPLC on a 3 μM ODS Hypersil column, equilibrated and eluted with 14.2% methanol and 0.25% glacial acetic acid adjusted to pH 3.9 with NaOH. The retention time of the GSH-bimane derivative was 3.2 min.

Statistical methods

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

Results

Effect of diets

Hepatic pleiotropic response. Diet intakes of all animal groups were comparable. The liver weights, the relative liver size and the hepatic triglyceride and cholesterol contents were higher in rats receiving the CD diet, relative to those fed the ND diet (Table I). Additional supply of choline to the CD diet almost fully normalized this pleiotropic response to values found in the ND diet

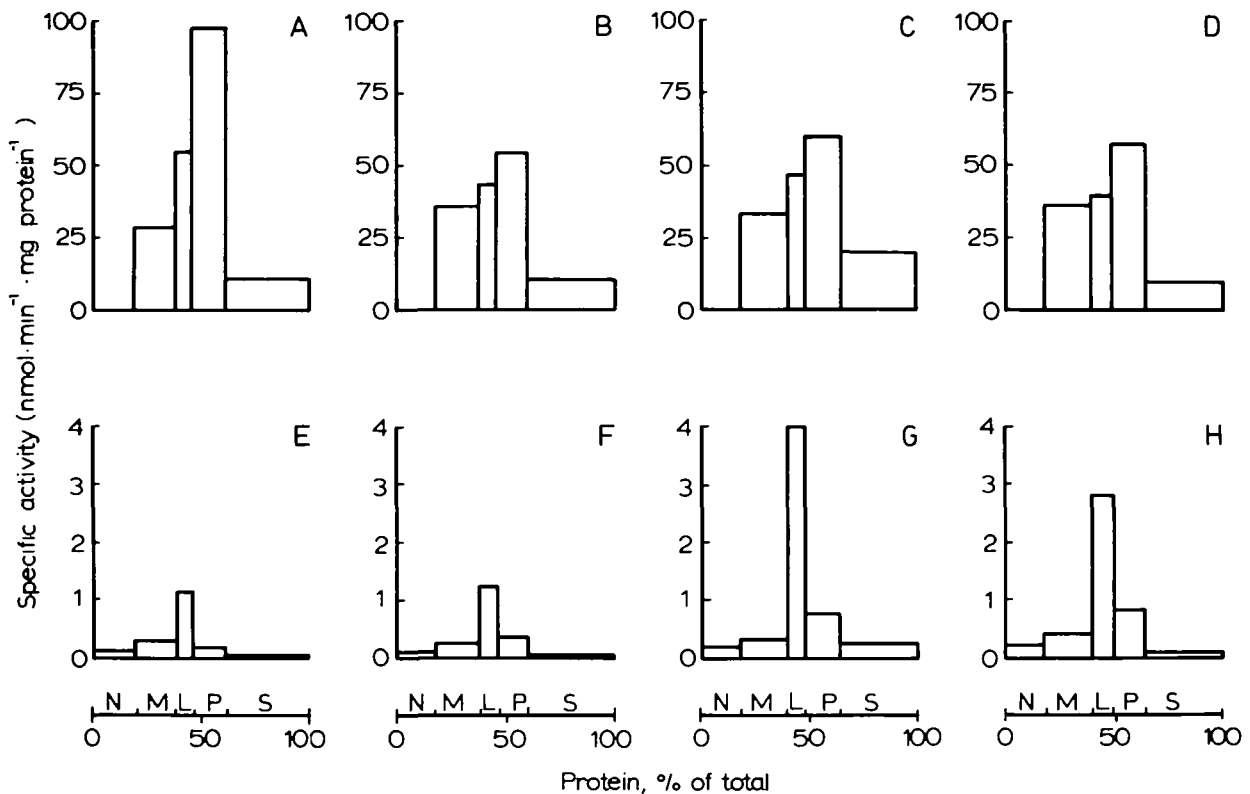


Fig. 1. Intracellular distribution of palmitoyl-CoA hydrolase (A–D) and peroxisomal β -oxidation (E–H) in the liver of ND-fed control (A,E), ND-fed + MTX (B,F), CD-diet (C,G) and CD-diet + MTX (D,H) animals. The MTX dose was $350 \mu\text{g/day/kg}$ body weight and the experiment tested for a 10-day period. N is the nuclear fraction; M, the mitochondrial fraction; P, the microsomal fraction and S, the soluble fraction. Three animals were tested separately and the tabulated values are the means. The measurements varied not more than $\pm 10\%$ from the mean value.

animal group, except for the triglyceride level (Table I). In accordance with previous findings (7) the CD diet increased the total palmitoyl-CoA synthetase activity, decreased the carnitine palmitoyltransferase activity and had no effect on the total glycerophosphate acyltransferase activity (Table I). On addition of choline to the CD diet the changed enzyme activities were almost completely normalized (Table I).

Peroxisomal enzymes. The total peroxisomal oxidation of long-chain fatty acids in the liver homogenates increased >2 -fold in rats fed the CD diet and additional supply of choline prevented this increase (Table I). The total activities of catalase and urate oxidase, well-known peroxisomal marker enzymes (13), and palmitoyl-CoA hydrolase, a multilocalized enzyme (10), were marginally affected by the CD diet and the CD diet plus choline.

Effects of MTX in rats fed a standard diet or a CD diet

Fatty acid metabolizing enzymes. MTX significantly increased the total palmitoyl-CoA synthetase activity in liver from rats given a normal diet, but decreased the enzyme activity of rats fed a CD diet (Table I). The total carnitine palmitoyltransferase activity and glycerophosphate acyltransferase activity of both diet groups were changed after MTX treatment in a similar way as earlier reported (7).

The total peroxisomal β -oxidation decreased after MTX exposure, irrespectively of diet intake (Table I). The total and specific activities of catalase in the whole liver homogenates of both animal diet groups seemed to decrease, but this could not be statistically verified because of unequal variance. MTX administration decreased the urate oxidase activity in both diet groups, especially in animals fed a CD diet (Table I). The total

palmitoyl-CoA hydrolase activity was marginally affected by MTX treatment.

Subcellular fractionation studies

Peroxisomal enzymes. The distribution of protein and marker enzymes for mitochondria, peroxisomes, microsomes and cytosolic fraction were for all groups of animals essentially similar to our previous findings for rat liver homogenates (17,20). The isolated cellular fractions appeared to be rather 'pure' based on marker enzyme activities (17,20,25).

MTX treatment of the animals fed the ND diet marginally affected the peroxisomal β -oxidation system in the L fraction (Figure 1). MTX decreased this enzyme activity in the animals fed the CD diet (Figure 1). The specific catalase activities in the L fraction of both diet groups were decreased by MTX treatment, whereas the enzyme activity in the S fraction was essentially unaffected (data not shown). MTX exposure of animals fed both ND and CD diets decreased the urate oxidase activity (data not shown).

On feeding the rats ND diet, MTX appeared to enhance the specific activity of the mitochondrial palmitoyl-CoA hydrolase while the hydrolase activity in the P fraction was decreased $\sim 45\%$ (Figure 1). In animals fed the CD diet, the multi-organelle activities of palmitoyl-CoA hydrolase were slightly changed with MTX (Figure 1).

On feeding the rats a CD diet alone, however, the specific activity of the peroxisomal oxidation of long-chain fatty acids was increased 4- and 2-fold in the L and P fractions respectively (Figure 1). Notably, this membrane-bound increased activity was almost fully prevented by addition of choline (Table II).

Table II. Effect of a defined CD diet and a CD diet + choline on some peroxisome-associated enzyme activities^{a,b} in a particulate membrane fraction^c (MLP) and a cytosolic fraction (S)

Parameters measured	ND diet		CD diet		CD diet + choline	
	MLP	S	MLP	S	MLP	S
Peroxisomal β -oxidation	136 \pm 27		308 \pm 25		201 \pm 27	
Catalase	38 \pm 0.8	15 \pm 1	51 \pm 1	16 \pm 2	44 \pm 0.6	15 \pm 1
Urate oxidase	630 \pm 20		590 \pm 18		600 \pm 20	
Palmitoyl-CoA hydrolase	5.4 \pm 0.3	1.8 \pm 0.2	4.3 \pm 0.2	3.5 \pm 0.5	4.6 \pm 0.3	2.7 \pm 0.2

The tabulated values represent means \pm SD for four animals.

The total activity (nmol/min/g liver^a or (μ mol/min/g liver)^b was measured in the isolated MLP fraction (membrane-bounded)^c and the cytosolic fraction.

In the CD-diet-fed animals, the activity of catalase in the particulate membrane (MLP) fraction was increased \sim 1.3-fold. This increase of activity was almost fully prevented by choline in the diet (Table II). The activity of urate oxidase was slightly decreased in the two CD-fed groups (Table II).

Next to peroxisomal β -oxidation, the most conspicuous alteration caused by diets was observed in the activity of palmitoyl-CoA hydrolase. On feeding the CD diet, the microsomal hydrolase activity was decreased with a concomitant increased cytosolic palmitoyl-CoA hydrolase activity (\sim 2-fold) (Figure 1C). The mitochondrial palmitoyl-CoA hydrolase activity was slightly affected in the CD-fed animals (Figure 1C). Addition of choline to the CD diet almost fully normalized the particulate membrane-bounded and cytosolic palmitoyl-CoA hydrolase activities to values found in the ND-diet-animal group (Table II).

Effect on reduced glutathione

Choline deficiency increased the amount of GSH \sim 1.6-fold ($P < 0.05$) in the liver, whereas MTX decreased the amount \sim 40% ($P < 0.01$) in rats fed the CD diet (Table III). Thus, it seems that MTX counteracts the increased amount of GSH induced by the CD diet.

Discussion

Hepatomegaly and accumulation of liver lipids were found to be caused by the choline deficiency, as the relative liver weight and increased hepatic lipids (especially the cholesterol content) were almost fully prevented by addition of choline to the CD diet (Table I).

The present study has further confirmed previous findings (7) that MTX exposure and especially choline deficiency change the activities of palmitoyl-CoA synthetase, glycerophosphate acyltransferase and carnitine palmitoyltransferase. Whether the increase in specific mitochondrial glycerophosphate acyltransferase activity, concomitant with decreased carnitine palmitoyltransferase activity, may affect the development of fatty metamorphosis in the liver, induced by choline deficiency (7), should be considered (Table I). Increased glycerophosphate acyltransferase activity in the microsomal fraction (7) may be an important factor for increased liver triglycerides by MTX administration (Table I).

Induction of peroxisomal palmitoyl-CoA oxidation, palmitoyl-CoA hydrolase, glycerophosphate acyltransferase and partly catalase is a feature shared with mammalian proliferation of peroxisomes. Peroxisomal β -oxidation and palmitoyl-CoA hydrolase can often be increased $>$ 10- and 5-fold respectively by different hypolipidemic drugs (i.e. clofibrate and tiadenol) (15–18) and somewhat less with certain high-fat diets (19,20).

The results of the present experiments clearly demonstrate that a CD diet exerts an effect on peroxisomal enzymes. In the

Table III. Effect of MTX on the GSH content in the liver of rats fed either a ND diet or CD diet. The animals were injected i.p. with MTX (350 mg/day/kg body weight) for 10 days

Treatment	GSH (μ mol/g liver)
ND diet	11.7 \pm 3.6
ND diet + MTX	9.8 \pm 0.7
CD diet	18.1 \pm 1.5*
CD diet + MTX	10.5 \pm 2.5**

The results are given as means of determination \pm SD.

* $P > 0.05$ compared with the CD diet group.

** $P < 0.01$ compared with the CD diet group.

peroxisome-enriched L fraction, the activity of the peroxisomal β -oxidation was enhanced by \sim 4-fold and to a slighter extent in the P fraction (Figure 1). The catalase specific activity in the particulate membrane fraction was increased 1.3-fold (Table II) whereas the activity in the S fraction was not altered (data not shown). The urate oxidase activity in the membrane-fraction was not affected by choline deficiency (Table II). The subcellular distribution of palmitoyl-CoA hydrolase activity revealed changes as a result of the dietary regimes. The cytosolic palmitoyl-CoA hydrolase activity was increased concomitant with a decreased microsomal located palmitoyl-CoA hydrolase activity (Figure 1). The mitochondrial palmitoyl-CoA hydrolase activity was slightly affected. Notably, these phenomena are very similar to those caused by treatment with various peroxisome proliferators, i.e. hypolipidemic drugs (15) and high-fat diets (20) or to modulation in responses to altered physiological status, e.g. starvation, diabetes (26).

It has been reported that peroxisome proliferators change the distribution profile of peroxisomal β -oxidation, catalase and especially the palmitoyl-CoA hydrolase among subcellular fractions in rat liver (15–17). This could be due to the peroxisomes' great sensitivity to damage (15), giving rise to leakage of peroxisomal matrix enzymes, and to organelle biogenesis forming heterogeneous populations of peroxisomes with different average sedimentation coefficients (S) (15,17). The cellular distribution of palmitoyl-CoA hydrolase in choline-deficient animals is very similar to the picture obtained with peroxisome proliferators. In addition the increased palmitoyl-CoA activity in the cytosolic fraction caused by choline deficiency (Figure 1) is a characteristic feature shared by peroxisome proliferation (16,18).

The increase in specific peroxisomal β -oxidation activity appears not to be a consequence of an essential fatty-acid deficiency (20,27). As peroxisomal β -oxidation, catalase activity and both the cytosolic- and membrane-bounded palmitoyl-CoA hydrolase activities were almost completely and selectively normalized by

choline added to the CD diet, the overall results strongly suggest that these enzyme changes appear to be a consequence of a choline deficiency provoked by the CD diet (Table II).

In contrast to choline deficiency, MTX administration of animals fed the ND diet did not stimulate the peroxisomal β -oxidation in either L and P fractions (Figure 1) and the catalase and urate oxidase activities were marginally affected (Table I). Regarding the total palmitoyl-CoA hydrolase activities, MTX treatment decreased the microsomal palmitoyl-CoA hydrolase activity but had no stimulating effect on the cytosolic enzyme activity (Figure 1). Thus, based on biochemical markers MTX has no peroxisome-proliferating effect.

Megamitochondria formation appears to be related to the development of an essential fatty-acid deficiency (27). Increased mitochondrial palmitoyl-CoA hydrolase activity is a biochemical parameter of this biological event (20). The increased palmitoyl-CoA hydrolase activity in the M fraction after MTX treatment (Figure 1) may be a consequence of megamitochondria formation as increased size of mitochondria has been reported by MTX exposure (2).

We have reported that clofibrate, tiadenol and niadenate, all hypolipidemic drugs, as well as the potent tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate, induced differentiation of the mouse embryo fibroblast line C3H/10T 1/2 Cl8 cells to adipocytes (14). The earliest change seen in rat hepatocytes treated with clofibrate (21,22) and high fat concentration (22) are an increased synthesis of triglyceride and increased peroxisomal β -oxidation. Accumulation of lipid may be a common 'trigger' for peroxisome proliferation accompanied by an increase of the peroxisomal β -oxidation enzyme system. MTX administration and choline deficiency both favour fat deposition (Table I), but only choline deficiency increased the peroxisomal β -oxidation. Thus, the results of the present experiments suggest that fat accumulation in the liver is not a stimulus for induction of peroxisomal β -oxidation confirming the conclusions based on experiments with chlorpromazine (28). Accordingly, it would appear more likely that the message for peroxisome proliferation is some intermediate of triglyceride synthesis, i.e. long-chain acyl-CoA (18).

A chronic methyl-group deficiency can be produced in rats fed a diet deficient in choline (6). Methyl-group deficiency alone has been shown to produce a significant incidence of liver tumours in rats (9–11). The disruption of methionine metabolism seems to be important in hepatocarcinogenesis. However, the mechanisms by which a CD diet exerts its promoting and/or carcinogenic effects are not known. It is of interest to note that increased lipid peroxidation has been observed in livers of rats fed a CD diet (11,29). One of the manifestations of membrane damage is lipid peroxidation, which is a process that is initiated by oxygen radicals.

Choline deficiency decreased the liver S-Ado-Met content (31) and present data have demonstrated that dietary deficiency can induce an increase in the synthesis of a H_2O_2 -generating peroxisomal β -oxidation enzyme system accompanied by a catalase activity that is marginally affected. It would seem possible that an early change in lipid metabolism may be the first step in a chain of events (i.e. peroxidation) which eventually results in tumour formation by choline deficiency. An excess production of H_2O_2 and genesis of free radicals due to enhanced H_2O_2 -generating peroxisomal β -oxidation and uncontrolled lipid peroxidation (11,29) may lead to early DNA damage (10). This effect may be an important event leading to initiation of carcinogenesis in the liver of rats fed a CD diet. It is also noteworthy

that the GSH level was increased by choline deficiency—a phenomena which is also observed after hypolipidemic peroxisome proliferators (13). This may result in a further compromise of the endogenous defence against excess H_2O_2 generation.

In conclusion, our studies have shown that the effects of choline deficiency were similar to those of short-term feeding of peroxisome proliferators: in increasing the activities of peroxisomal β -oxidation and palmitoyl-CoA, especially the cytosol located (15), causing a marginal effect on the activities of catalase and urate oxidase, and causing changes in the rate of esterification and oxidation of fatty acids (15,17). Although most of the agents which cause peroxisome proliferation *in vivo* cause accumulation of lipid, the results by MTX administration and choline deficiency suggest that the converse is not true. Hence, it is conceivable that lipid itself is not an induction signal for peroxisome proliferation accompanied by increased peroxisomal β -oxidation. Whether the excess production of H_2O_2 by the increased H_2O_2 -generating peroxisomal oxidation of fatty acids may be an important step in a chain of events, which eventually results in tumour formation and hepatocellular carcinomas by long-term choline deficiency, should be considered.

Acknowledgements

The authors are grateful to Mr Svein Krüger for excellent technical assistance. The work was supported by the Norwegian Research Council for Science and Humanities, the Norwegian Society for Fighting Cancer and the Norwegian Cancer Society.

References

- Jackson, R.C. (1984) Biological effects of folic acid antagonists with anti-neoplastic activity. *Pharmacol. Ther.*, **25**, 61–82.
- Nyfors, A. and Poulsen, H. (1977) Morphogenesis of fibrosis and cirrhosis in methotrexate-treated patients with psoriasis. *Am. J. Surg. Pathol.*, **21**, 235–243.
- Custer, R.P., Freeman-Narro, M. and Narro, S.A. (1977) Hepatotoxicity in Wistar rats following chronic methotrexate administration: a model of human reaction. *J. Natl. Cancer Inst.*, **58**, 1011–1013.
- Barate, A.J. and Beckenhauer, H.C. (1985) Sparing effect of vitamin B₁₂ on hepatic betaine in methotrexate-treated rats. *Biochem. Arch.*, **1**, 161–163.
- Vance, J.E. and Vance, D.E. (1986) Special pools of phospholipids are used for lipoprotein secretion by cultured hepatocytes. *J. Biol. Chem.*, **261**, 4486–4491.
- Copeland, D.H. and Salmon, W.D. (1946) The occurrence of neoplasms in the liver, lungs and other tissues of rats as a result of prolonged choline deficiency. *Am. J. Pathol.*, **22**, 1059–1079.
- Aarsaether, N., Berge, R.K., Aarsland, A., Svandal, A. and Ueland, P.M. (1988) Effect of methotrexate on long-chain fatty acid metabolism in liver of rats fed a standard or a defined, choline-deficient diet. *Biochim. Biophys. Acta*, in press.
- Choshal, A.K. and Farber, E. (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis*, **5**, 1367–1370.
- Poirier, L.A., Mikol, X.B., Hoover, K. and Creasia, D. (1983) Liver tumor formation in rats fed methyl-deficient, amino acid-defined diets with and without diethylnitrosamine initiation. *Proc. Am. Assoc. Cancer Res.*, **24**, 97.
- Rushmore, T.H. and Farber, E., Ghoshal, A.K., Parodi, S., Pala, M. and Taningher, M.C. (1986) A choline-devoid diet, carcinogenic in the rat, induces DNA damage and repair. *Carcinogenesis*, **7**, 1677–1680.
- Rushmore, T.H., Lim, Y.P., Farber, E. and Ghoshal, A.K. (1984) Rapid lipid peroxidation in the nuclear fraction of rat liver induced by a diet defined in choline and methionine. *Cancer Lett.*, **24**, 251–255.
- Locker, J., Reddy, T.V. and Lombardi, B. (1986) DNA methylation and hepatocarcinogenesis in rats fed a choline-devoid diet. *Carcinogenesis*, **7**, 1309–1312.
- Reddy, J.K. and Rao, M.S. (1986) Peroxisome proliferators and cancer: mechanisms and implications. *TIPS*, November, 438–440.
- Lillehaug, J.R., Aarsaether, N., Berge, R.K. and Male, R. (1986) Peroxisome proliferators show tumor-promoting but no direct transforming activity *in vitro*. *Int. J. Cancer*, **37**, 97–100.

15. Berge, R.K. and Bakke, O.M. (1981) Changes in lipid metabolizing enzymes of hepatic subcellular fractions from rats treated with tiadenol and clofibrate. *Biochem. Pharmacol.*, **30**, 2251–2256.
16. Berge, R.K., Aarstrand, A., Bakke, O.M. and Farstad, M. (1983) Hepatic enzymes, CoASH and long-chain acyl-CoA in subcellular fractions as affected by drugs inducing peroxisomes and smooth endoplasmic reticulum. *Int. J. Biochem.*, **15**, 191–204.
17. Berge, R.K., Flatmark, T. and Osmundsen, H. (1984) Enhancement of long-chain acyl-CoA hydrolase activity in peroxisomes and mitochondria of rat liver by peroxisomal proliferators. *Eur. J. Biochem.*, **141**, 637–644.
18. Berge, R.K. and Aarstrand, A. (1985) Correlation between the cellular level of long-chain acyl-CoA, peroxisomal β -oxidation, and palmitoyl-CoA hydrolase activity in rat liver. Are the two enzyme systems regulated by a substrate-induced mechanism? *Biochim. Biophys. Acta*, **837**, 141–151.
19. Berge, R.K. and Thomassen, M.S. (1985) Effects of high fat diets on the activity of palmitoyl-CoA hydrolase in rat liver. *Lipids*, **20**, 49–52.
20. Berge, R.K., Flatmark, T. and Christiansen, E.N. (1987) Effect of a high fat diet with partially hydrogenated fish oil on long-chain fatty acid metabolizing enzymes in subcellular fractions of rat liver. *Arch. Biochem. Biophys.*, **252**, 269–276.
21. Capuzzi, D.M., Intenzo, C.M., Lackman, R.D., Whereat, A.F. and Scott, D.M. (1983) Stimulation of fatty acid utilization by sodium clofibrate in rat and monkey hepatocytes. *Biochem. Pharmacol.*, **32**, 2195–2203.
22. Hertz, R., Arnon, J. and Bar-Tana, J. (1985) The effect of bezafibrate and long-chain fatty acids on peroxisomal activities in cultured rat hepatocytes. *Biochim. Biophys. Acta*, **836**, 192–200.
23. Shaw, J.P. and Chou, I.N. (1986) Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. *J. Cell. Physiol.*, **122**, 193–198.
24. Anderson, M. (1985) Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.*, **113**, 548–551.
25. Berge, R.K., Stensland, E., Aarstrand, A., Tsegai, G., Osmundsen, H., Aarsaether, N. and Gjellesvik, P.R. (1987) Induction of cytosolic clofibroyl-CoA hydrolase activity in liver of rats treated with clofibrate. *Biochim. Biophys. Acta*, **918**, 60–66.
26. Berge, R.K., Hosøy, L.H. and Farstad, M. (1984) Influence of dietary status on liver palmitoyl-CoA hydrolase, peroxisomal enzymes, CoASH and long-chain acyl-CoA in rats. *Int. J. Biochem.*, **16**, 403–410.
27. Christiansen, E.N., Flatmark, T. and Kryvi, H. (1981) Polydispersity of rat liver peroxisomes induced by the hypolipidemic and carcinogenic agent clofibrate. *Eur. J. Cell Biol.*, **24**, 62–69.
28. Price, S.C., Hall, D.E. and Hinton, R.H. (1985) Lipid accumulation in the livers of chlorpromazine-treated rats does not induce peroxisome proliferation. *Toxicol. Lett.*, **25**, 11–18.
29. Perera, M.I.R., Demetris, A.J., Katyal, S.L. and Shinozuka, H. (1985) Lipid peroxidation of liver microsome membranes induced by choline-deficient diets and its relationship to the induction of γ -glutamyl transpeptidase-positive foci. *Cancer Res.*, **45**, 2533–2538.
30. Lillehaug, J.R. and Berge, R.K. (1986) The tumor promoter 12-O-tetradecanoylphorbol-13-acetate increases the activities of some peroxisome-associated enzymes in *in vitro* cell culture. *Br. J. Cancer*, **53**, 121–127.
31. Svardal, A., Ueland, P.M., Berge, R.K., Aarstrand, A., Aarsaether, N., Lønning, P.E. and Refsum, H. (1987) Effect of methotrexate on homocysteine and other sulphur compounds in tissues of rats fed a normal or a defined, choline-deficient diet. *Cancer Chemother. Pharmacol.* in press.

Received on October 22, 1987; accepted on January 21, 1988