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LIPID PEROXIDATION IN CHOLINE-METHIONINE DEFICIENCY

K. HONG TAN†, DAVID J. MEYER and BRIAN KETTERER

Cancer Research Campaign Molecular Toxicology Group, Biochemistry Department, Middlesex Hospital Medical School, London W1P 6DP, UK.

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A deficiency of choline and methionine is hepatocarcinogenic and is associated with an apparent increase in lipid peroxidation. In this study the susceptibility of microsomes and nuclei to ferritin-dependent lipid peroxidation is examined together with the status of the peroxidation- protective systems. Choline-methionine deficiency caused an increase in Se-independent GSH peroxidases (GSH transferase subunit 2) and membrane vitamin E but a decrease in Se-dependent GSH peroxidase and microsomal GSH peroxidase activity. Choline-methionine deficient microsomes and nuclei were 4-fold more susceptible to lipid peroxidation induced *in vitro* by physiological concentrations of ferritin/ascorbate/ADP; and the peroxidation was less effectively inhibited by GSH and soluble GSH peroxidases than controls. The results indicate that a decreased level of Se-dependent and membrane GSH peroxidases is involved in the increase in lipid peroxidation observed in choline-methionine deficiency.

KEY WORDS: Choline-methionine deficiency, lipid peroxidation, nuclei, microsomes, GSH peroxidases.

INTRODUCTION

A choline-methionine (CM) deficient diet is hepatocarcinogenic either when administered alone[1–3] or as a co-carcinogen in a two stage carcinogenic regime in which diethylnitrosamine is the initiator[4]. As judged by the presence of conjugated dienes in membrane lipid, lipid peroxidation quickly develops in choline-methionine deficiency and the production of lipid peroxides has been associated with carcinogenic properties of these diets[5].

In the present study, enhanced susceptibility to ferritin-induced lipid peroxidation in microsomal and nuclear fractions from the livers of rats fed a CM deficient diet has been observed and the status of systems which protect against lipid peroxidation, namely GSH, GSH peroxidases and vitamin E have been examined.

METHODS AND MATERIALS

Materials

The following compounds were obtained from Sigma, Poole, Dorset, UK: ADP (grade III), ascorbate, bovine serum albumin, butylated hydroxy-toluene (2,6-di-tert-butyl-p-cresol), ferritin (equine spleen), GSH. GSH reductase and NADPH were

† Author for correspondence: K. Hong Tan, address as above.

from Boehringer, Lewes, East Sussex, UK. Sephadex G-100 was from Pharmacia, London W5, UK. All other reagents were of analytical grade.

Methods

Lipid peroxidation The buffer used throughout was based on physiological intracellular concentrations of K^+, Na^+, Mg^{2+}, Cl^- and phosphate ions[6]. Microsomal, nuclear and soluble supernatant fractions were prepared as follows: Male Wistar rats, inbred at the Courtauld Institute of Biochemistry and weighing 200–250 g were fed a standard diet (Diet 86; E. Dixon and Sons, Ware, Herts) or a CM deficient diet containing no more than 25 mg/kg choline and 0.25% methionine plus cystine (SDS, Witham, Essex) for up to 42 days. The microsomal and soluble supernatant fraction were prepared as previously described[6] whilst the nuclear fraction was prepared with a sucrose density gradient[7]. Both microsomal and nuclear fractions were washed thoroughly to remove soluble GSH transferases which may be loosely associated with the membranes[8].

Peroxidation in microsomal and nuclear fractions (3 mg/ml) was induced by incubation in a system resembling the intrahepatic environment and contained ferritin (0.7 μM) as the source of iron, ascorbate (1 mM), ADP (1 mM) and NADPH (0.3 mM) in buffer. Incubations were carried out at 37°C in air in a shaking water bath for 30 min. GSH was regenerated using NADPH and GSH reductase. Lipid peroxidation was measured as malonaldehyde release measured by a thiobarbituric acid method[6]. Vitamin E content of the membranes was determined by the disappearance of diphenylpicrylhydrazyl absorption at 516 nm.

Enzyme fractionation Se-dependent and Se-independent (GSH-transferase) GSH peroxidases in soluble supernatant were separated by applying a 5 ml portion of soluble supernatant to a Sephadex G-100 column (2.5 cm × 90 cm) and elution with buffer. Fractions of 4 ml were collected and GSH peroxidase and GSH transferase activities using cumene hydroperoxide and 1-chloro-2, 4-dinitrobenzene as substrates respectively were determined. Soluble hepatic GSH transferases were prepared by isoelectric focusing the combined GSH transferase-containing fractions from Sephadex G-100 chromatography. The GSH transferase subunit content of the fractions thus separated was determined by SDS polyacrylamide electrophoresis, HPLC according to the method of Ostlund-Farrants, A.K., Meyer, D.J., Coles, B. & Ketterer, B. (personal communication) and enzymically as follows: using cumene hydroperoxide which is utilized by subunits 1 and 2; 1,2-dichloro4-nitrobenzene which is utilized by subunit 3 and *trans*-4-phenyl-but-3-en-2-one which is utilized by subunit 4 respectively.

RESULTS

Effect of CM deficiency on GSH peroxidases

The soluble supernatant from livers of CM deficient and control rats were fractionated on Sephadex G-100 (Figure 1). The effect of CM deficiency was to reduce the Se-dependent GSH peroxidase activity by 55% while increasing the Se-independent

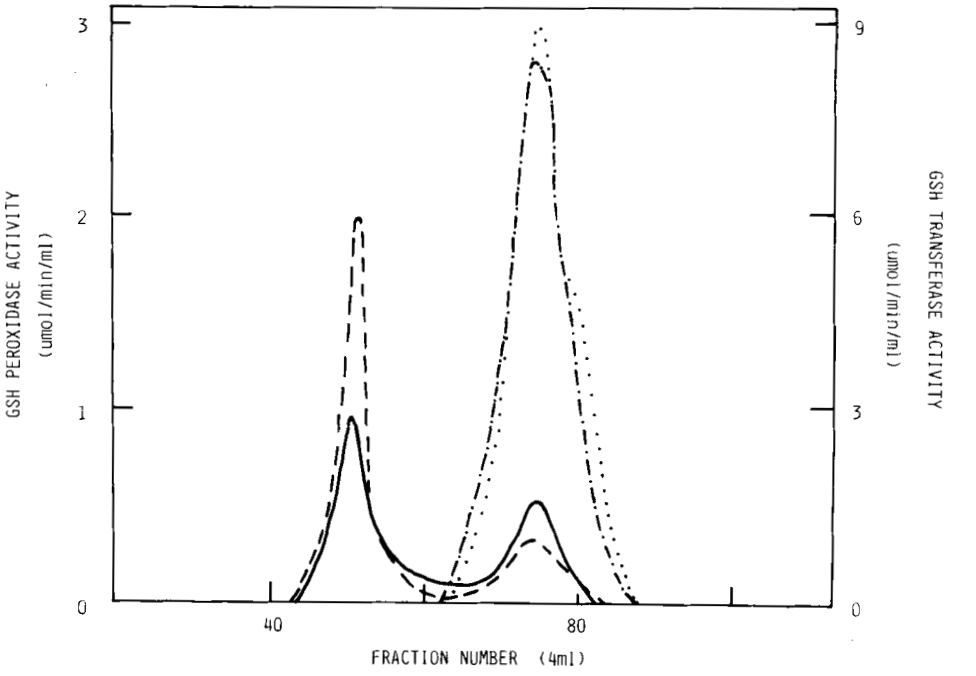

FIGURE 1 Separation of soluble supernatant from control and CM deficient rats. GSH peroxidase activity was measured with cumene hydroperoxide (– – –, control; ——, CM) and GSH transferase activity with 1-chlro-2,4-dinitrobenzene (· · · · · ·, control; · – · – ·, CM).

(GSH transferase) GSH peroxidase by 30%. The latter increase was due to differential effects of the diet on the content of the 4 main GSH transferase subunits of rat liver. Thus while the content of subunit 1 remained unchanged, subunits 2, 3 and 4 increased by 2-, 1.5- and 1.2-fold respectively (Table I). All these subunits are associated with GSH peroxidase activity towards both the model substrate cumene hydroperoxide and endogenous substrates linoleate hydroperoxide and thymine hydroperoxide, but subunit 2 has relatively high activity[9].

TABLE I
Subunit composition of the soluble hepatic GSH transferases

	GSH transferase subunit (mg/g liver)			
	1	2	3	4
Control	1.95	0.50	0.59	0.25
CM- diet	1.85	1.01	0.87	0.30

Note: GSH transferases from the soluble supernatant fraction of control and CM- diet rat livers were separated on Sephadex G-100, isoelectric focusing and analysed by pI, substrate specificity, SDS PAGE and HPLC.

Microsomal GSH peroxidases

The GSH peroxidase activity of liver microsomes from CM deficient rats was only 60% that of controls, being 0.4 μmol/min/mg protein compared with 0.7 μmol/min/mg protein for control rats indicating a loss of either intrinsic membrane GSH transferase activity or a soluble activity remaining strongly absorbed to the microsomes during the isolation procedure.

Effect of CM deficiency on microsomal vitamin E

Preliminary experiments indicate that CM deficient animals have increased levels of microsomal vitamin E. Control animals gave values of 83 μmol/g liver protein while CM deficient microsomes gave 260 μmol/g liver protein.

Lipid peroxidation

Microsomes and nuclei from livers of rats fed a CM deficient diet had an increased susceptibility to ferritin/ascorbate/ADP- induced lipid peroxidation (Table II). Rates were four-fold of controls after only 3 days on the diet. Microsomes reached a maximum at 11 days while nuclei showed slowly increasing susceptibility after as long as 42 days.

GSH-dependent inhibition of lipid peroxidation

a) with microsomes. We have previously shown that GSH alone inhibits microsomal lipid peroxidation induced with $FeCl_3$ and ADP/NADPH by 55%[6]. With ferritin as the iron source, GSH inhibited control microsomes from control liver by 80% whilst GSH plus soluble supernatant inhibited by 100% (Table III). In microsomes from CM deficient diet, GSH alone and GSH plus soluble supernatant were far less effective inhibitors of lipid peroxidation, inhibiting by only 55% and 85% respectively. This may be due to the combined effect of reduced soluble and microsomal GSH peroxidase activity, although it is noteworthy that microsomal GSH peroxidases have not been taken into account in previous studies of lipid peroxidation.

TABLE II
Ferritin-induced lipid peroxidation in isolated microsomes and nuclei

Additions	Malonaldehyde (nmol/min/ml)
Microsomes, control	16.8 ± 1.3
CM- diet	72.4 ± 1.8
Nuclei, control	0.3 ± 0.0
CM- diet	1.2 ± 0.1

The incubation mixtures contained microsomes or nuclei (3 mg protein/ml), 0.7 μM ferritin, 1 mM ascorbate, 1 mM ADP and 0.3 mM NADPH in physiological buffer. Incubations were at 37°C for 30 min. Peroxidation was recorded (mean ± S.D) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal and nuclear preparation; experiments with another microsomal and nuclear preparation gave similar results.

TABLE III
Effect of GSH and the soluble supernatant fraction on ferritin-induced lipid peroxidation in microsomes from control and CM- diet rats

Additions	Malonaldehyde (nmol/min/ml)
Control, none	11.9 ± 0.1
plus GSH	2.1 ± 0.3
plus GSH & soluble supernatant	0.0 ± 0.1
plus soluble supernatant	12.0 ± 0.9
CM- diet, none	57.6 ± 2.4
plus GSH	25.9 ± 1.2
plus GSH & soluble supernatant	19.1 ± 2.2
plus soluble supernatant	58.3 ± 0.5

Note: Incubations contained microsomes (3 mg protein/ml), 0.7 μM ferritin, 1 mM ascorbate, 1 mM ADP and 0.3 mM NADPH in physiological buffer and were performed at 37°C for 30 min. Where indicated, dialysed soluble supernatant (22 mg protein/ml) and 2.5 mM GSH were added to the assay mixture. Peroxidation was estimated (mean ± S.D) as nmol of malonaldehyde released/min per ml released in at least three simultaneous incubations.

TABLE IV
Effect of GSH and the soluble supernatant fraction on ferritin-induced lipid peroxidation in nuclei from control and CM- diet rats

Additions	Malonaldehyde (nmol/min/ml)
Control, none	0.50 ± 0.0
plus GSH	0.45 ± 0.0
plus GSH & soluble supernatant	0.01 ± 0.0
plus soluble supernatant	0.48 ± 0.1
CM- diet, none	1.70 ± 0.1
plus GSH	1.34 ± 0.1
plus GSH & soluble supernatant	0.31 ± 0.1
plus soluble supernatant	1.67 ± 0.2

Incubation mixtures contained nuclei (3 mg protein/ml), 0.7 μM ferritin, 1 mM ascorbate, 1 mM ADP and 0.3 mM NADPH in physiological buffer and were performed at 37°C for 30 min. Where indicated, soluble supernatant (22 mg protein/ml) and 2.5 mM GSH were added to the assay mixture. Peroxidation was recorded (mean ± S.D.) as nmol of malonaldehyde released/min per ml in at least three simultaneous incubations.

b) with nuclei. Whereas GSH alone had a substantial effect on microsomal peroxidation it had a much smaller effect on lipid peroxidation in nuclei, the inhibition in CM deficient nuclei being only 20% that of controls (Table IV). As with microsomes a large inhibition of GSH plus soluble supernatant was observed in control nuclei (98%) and a lesser effect was obtained with CM deficient nuclei by GSH plus CM deficient soluble supernatant (78%).

DISCUSSION

It is shown that CM deficient diets increase *in vitro* lipid peroxidation in microsomes and nuclei and decrease the ability of GSH dependent protective systems to prevent this peroxidation.

Reasons for the increase in lipid peroxidation in microsomes may be an increase in the content of polyunsaturated fatty acyl groups, a decrease in phospholipase activity, decreased microsomal GSH peroxidase or alterations in microsomal electron transport similar to those which have already been observed to be induced by N-acetyl-2-aminofluorene. The effect on nuclear lipid peroxidation may have a similar mechanism, but it is also possible that malonaldehyde released by peroxidizing nuclei may also originate from DNA and other macromolecules. With respect to the effect of diet on systems which protect against lipid peroxidation, it could appear that neither the increased level of vitamin E in the membranes nor the compensatory increase in Se-independent GSH transferases are sufficient to overcome the enhancement of lipid peroxidation in CM deficiency. The increase in GSH peroxidase activity can be calculated to increase the ability of the GSH transferase activity to reduce linoleate hydroperoxide 140% and thymine hydroperoxide 170% (Tan, K.H., Meyer, D.J. & Ketterer, B., personal communication). This may be sufficient to compensate for the loss of the combination of Se-dependent GSH peroxidases towards reduction of organic hydroperoxides; but does not compensate for the lost ability to reduce hydrogen peroxide and may result in a substantial increase in oxidizing free radicals in the system. Thus, from *in vitro* data it can be seen how the increased lipid peroxidation and its impaired inhibition could be involved in both initiation and promotion in carcinogenesis.

Acknowledgement

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