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STIMULATION OF PEROXIDATION IN RAT LIVER MICROSOMES BY (COPPER, ZINC)-METALLOTHIONEINS

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The abilities of pig liver (copper, zinc) metallothionein I and rat liver zinc metallothionein II to modify lipid peroxidation in incubations of liver microsomes have been compared with the activities of reduced glutathione, mannitol, quinacrine, EDTA, dimethyl-pyrroline-N-oxide and phenyl-butyl-nitrone. Lipid peroxidation was determined by assay of thiobarbituric acid reactive substance formation in incubations of microsomes with iron/ADP or a mixture of xanthine and xanthine oxidase. Zinc metallothionein II had no effect on the extent of peroxidation in either system but (copper, zinc) metallothionein I caused a stimulation of peroxidation initiated by xanthine and xanthine oxidase, all other compounds tested were inhibitory. Gel exclusion chromatography of incubations of (copper, zinc) metallothionein I with xanthine and xanthine oxidase revealed aggregation of the metalloprotein. This may have exposed copper in a form capable of initiating peroxidation.

KEY WORDS: Metallothioneins, lipid peroxidation, glutathione, liver microsomes.

INTRODUCTION

Damage to polyunsaturated fatty acids in cell membranes may be caused by generation of radicals often derived from metabolism involving oxygen.[1] Many systems in the cell may prevent this damage, including superoxide dismutases, catalase, peroxidases, glutathione (GSH) and vitamin E.[2] GSH can act as an antioxidant in several ways; as a substrate for peroxidases, by interaction with a microsomal protein and by direct reaction with radicals.[3,4] It has recently been suggested that the protein metallothionein (MT) also functions in the cell as an antioxidant, since prior induction of MT synthesis by injection of zinc reduces the hepatotoxic effects of carbon tetrachloride in rats and since cells with increased MT levels are less susceptible to the damaging effects of X-irradiation.[5,6] Carbon tetrachloride and X-radiation can stimulate lipid peroxidation in cells. Moreover MT can react *in vitro* with hydroxyl and superoxide radicals,[7] both of which initiate lipid peroxidation. In this paper we examine the effects of (copper, zinc-) or zinc-MTs on peroxidation in incubations of liver microsomes from rats.

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MATERIALS & METHODS

Livers were obtained from rats which had consumed a semi-synthetic diet based on torula yeast[8] for 8 weeks from weaning. The diet was supplemented with 100 mg tocopherol acetate and 0.1 mg Se as Na_2SeO_3/kg. Liver microsomal fractions were prepared using a Ca^{2+} precipitation technique.[9] The microsomes were washed once with 0.15 M KCl and resuspended by gentle homogenisation in 0.15 M KCl to a final protein concentration of 20 mg/ml, determined by the Biuret method.[10]

Rats and pigs were injected on 3 successive days with 10 mg Zn (as $ZnSO_4$)/kg i.p. and 1.5 mg Cu (as diethylamine copper oxyquinoline sulphonate; 'Cujec', ICI Tasman Ltd, Upper Hutt, New Zealand)/kg s.c. respectively. Animals were killed 24 hours after the last injection. Rat zinc MT-II and pig (copper, zinc) MT-I were prepared as described previously.[11,12] The copper:zinc ratio in the pig MT was 2.5:1.

Incubations were carried out in 50 mM Tris/HCl pH 7.4 containing 0.15 M KCl at a microsomal protein concentration of 1 mg/ml. Peroxidation in incubations was initiated using (a) 6 μM $FeCl_2$, 2 mM ADP and 0.5 mM ascorbic acid or (b) 0.18 mM xanthine, 0.2 units/ml xanthine oxidase and 0.5 mM ascorbic acid. All inhibitors of peroxidation were dissolved in 50 mM Tris/HCl containing 0.15 M KCl, pH 7.4; final concentrations are given in Table I Incubations, open to the atmosphere, were maintained at 37 °C in a shaking water bath (120 cycles/min). Reactions were stopped by addition of 0.5 ml samples to 2.5 ml 15% trichloroacetic acid, whereupon thiobarbituric acid reactive substance (TBARS) was determined.[13] Data are presented as nmoles malonaldehyde (MDA) formed/mg protein, using E_{530} mM for MDA of 1.56×10^5.

Aliquots (2 ml) of incubations of MTs with xanthine and xanthine oxidase were chromatographed on a Sephadex G.75 column (900 mm length × 14 mm diameter) equilibrated with 0.01 M Tris:Acetate pH 7.4. 3.5 ml fractions were collected and copper and zinc determined by atomic absorption spectrophotometry.[12] Recovery of copper and zinc from the column was 95 to 105%.

TABLE I

Thiobarbituric acid reactive substance (TBARS as nmoles MDA mg/protein) in microsomes from rats (Fe/ADP incubations, 10 min xanthine/xanthine oxidase incubations, 15 min)[a]

Additions		TBARS	
		Fe/ADP	xanthine/ xanthine oxidase
Nil		2.38 ± 0.13	4.07 ± 0.14
ZnMT II	0.1 mM	2.29 ± 0.08	4.74 ± 0.69
CuMT I	0.1 mM	2.77 ± 0.40	8.76 ± 0.67[b]
GSH	1.0 mM	0.57 ± 0.05[b]	0.83 ± 0.07[b]
GSH	0.5 mM	0.58 ± 0.08[b]	1.03 ± 0.28[b]
GSH	0.1 mM	0.77 ± 0.19[b]	1.61 ± 0.11[b]
EDTA	0.1 mM	0.76 ± 0.28[b]	1.11 ± 0.08[b]
Quinacrine	1.1 mM	0.08 ± 0.01[b]	0.11 ± 0.02[b]
Mannitol	30.0 mM	1.08 ± 0.11[b]	1.19 ± 0.04[b]
PBN	12.5 mM	1.05 ± 0.13[b]	1.19 ± 0.14[b]
DMPO	12.5 mM	1.02 ± 0.12[b]	0.97 ± 0.08[b]

[a] Incubations were carried out as a described in methods, results are mean ± SEM of 4 incubations.
[b] Significantly different from Nil additions $p < 0.001$.

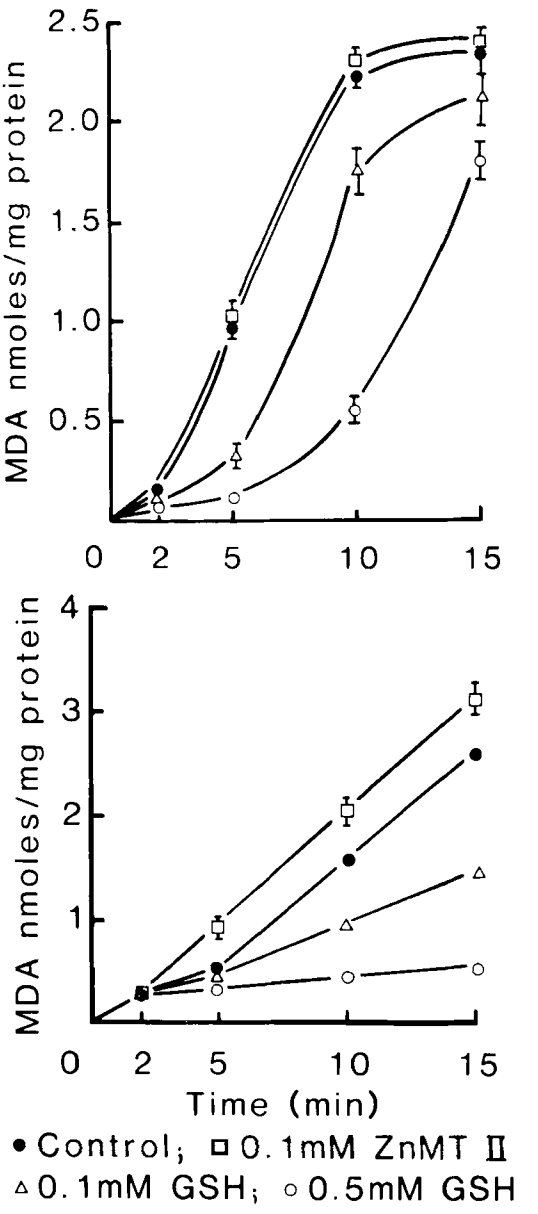

FIGURE 1 (a) Iron/ADP and (b) xanthine/xanthine oxidase induced peroxidation in liver microsomes. Incubations were carried out as described in methods. results are means of 4 incubations ± SEM (vertical bars), in some cases SEMs are smaller than the symbols on the graphs.

Tris, ascorbic acid, ADP, xanthine, xanthine oxidase (type III), GSH, EDTA, quinacrine, phenyl-butyl-nitrone (PBN), dimethyl-pyrroline-N-oxide (DMPO) and thiobarbituric acid were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were obtained from BDH, Poole, Dorset, U.K. and were of 'Analar' grade or better.

RESULTS

Figure 1 shows TBARS formation in microsomes incubated in the presence and absence of GSH or ZnMT II using either iron/ADP or xanthine/ xanthine oxidase to initiate peroxidation. Whereas 0.1 or 0.5 mM GSH delayed and decreased the extent of peroxidation in both systems, ZnMT II was without effect in the iron/ADP system and caused a small increase in the xanthine/xanthine oxidase system. (Copper, zinc) MT I had no effect in the iron/ADP system and increased TBARS formation when xanthine oxidase was used to initiate lipid peroxidation (Table I). All the other compounds tested inhibited TBARS formation (Table I). With the exception of quinacrine which caused 95% inhibition, none of the compounds tested for antioxidant activity affected xanthine oxidase activity, as assessed by uric acid formation (not shown).

Incubation of (copper, zinc) MT I with xanthine/xanthine oxidase caused zinc to be lost from the protein and some aggregation to a higher molecular weight form (Figure 2a). Incubation of (copper, zinc) MT I alone (Figure 2b) or with xanthine or xanthine oxidase (not shown) caused no change in the copper and zinc in the protein. Xanthine/xanthine oxidase treatment did not, however, cause loss of zinc from zinc MT II (Figure 2c).

DISCUSSION

The stimulation of TBARS formation, by (copper, zinc) MT I in incubations using xanthine/xanthine oxidase as initiator of peroxidation may be due to changes in the state of copper in the protein. Hydrogen peroxide generated by the xanthine/xanthine oxidase reaction causes oxidation of Cu (I) to Cu (II) in hepatic (copper, zinc) MT and in yeast copper–thionein as demonstrated by its conversion to an EPR detectable form.[14] This is associated with the movement of the copper in incubations to a fraction of higher molecular weight than MT and loss of zinc from the metalloprotein (Figure 2a). Copper can be a potent initiator of lipid peroxidation[15] and the effect of the xanthine/xanthine oxidase on (copper, zinc) MT I may be to facilitate this reaction. Peroxidation was stimulated despite the release of zinc which can prevent the formation of free radicals in liver microsomes.[16] The failure of xanthine/xanthine oxidase to influence the zinc binding to zinc MT II is consistent with the lack of effect of this MT on peroxidation under the conditions investigated.

The inhibition of TBARS formation by GSH in incubations of microsomes with iron/ADP is in accord with previous observations.[3] Since GSH did not inhibit xanthine metabolism by xanthine oxidase, it is unlikely that the inhibition of peroxidation in these incubations was due to effects on superoxide and hydrogen peroxide generation by the enzyme. Rather inhibition (in both radical-producing systems) probably occured by direct interaction of GSH with a microsomal protein[3]

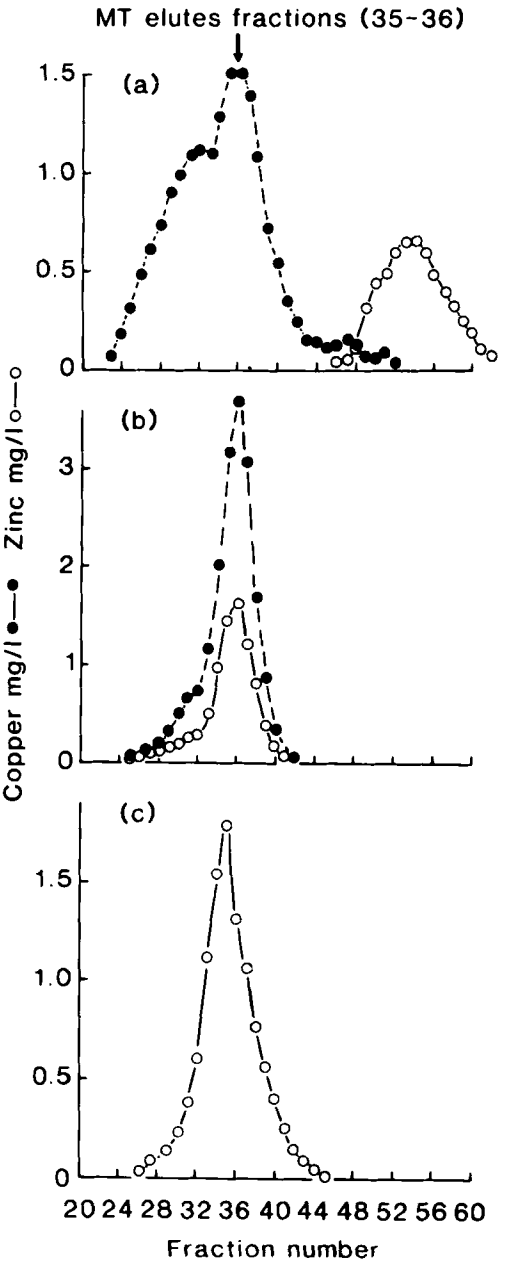

FIGURE 2 Copper and zinc in fractions obtained from the separation of metallothioneins on a Sephadex G.75 column. (a) (Copper, zinc) MT I incubated for 20 min with xanthine/xanthine oxidase as described in the text. (b) (Copper, zinc) MT I incubated for 20 min without xanthine/xanthine oxidase. (c) ZnMT II incubated as (a).

or by direct scavenging of radicals.[4] The concentrations of GSH needed to inhibit peroxidation (0.1–1 mM) were far below those normally present in cells (< 10 mM).[17]

MT contains 20 cysteine residues/molecule, and consequently the effective thiol concentrations in incubations containing 0.1 mM MT was 2.0 mM cysteine equivalents. Even at this high SH concentration no inhibition of peroxidation was detected. When it is considered that MT concentrations in the liver of normal rats are < 0.01 mM and are only 0.04 mM in stressed rats[18] it seems likely that any contribution of MT to inhibition of lipid peroxidation must be relatively minor *in vivo*, especially when GSH is present at normal levels.

When iron/ADP is added to incubations, small molecules such as GSH would be expected to have ready access to the sites on the microsomes where peroxidation is initiated. Since MT has a molecular weight of 6500 daltons, it could be less accessible to these sites and consequently it could be argued that these incubations are not an adequate test of the protein's potency as an inhibitor of peroxidation. However, xanthine/xanthine oxidase is thought to initiate peroxiation by generation of O_2^- and H_2O_2, with subsequent formation of the hydroxyl radical. Despite the fact that MT can react *in vitro* with O_2^- and hydroxyl radicals generated by a xanthine/xanthine oxidase system,[7] this apparently does not prevent the initiation of lipid peroxidation, in contrast to the other inhibitors tested.

Any involvement of MT in the prevention of lipid peroxidation may therefore be indirect and depend on the binding of potentially injurious copper ions (and possibly other metal ions) in an unreactive form. Under conditions where copper in (copper, zinc) MT can be oxidised this may facilitate peroxidation reactions in the cell.

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