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Zinc Suppression of Free Radicals Induced in Cultures of Rat Hepatocytes by Iron, t-Butyl Hydroperoxide, and 3-Methylindole[1] (42786)

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Abstract. The effect of zinc on lipid peroxidation initiated by either ferric-nitrilotriacetate, t-butyl hydroperoxide, or 3-methylindole was studied using primary monolayer cultures of rat liver parenchymal cells. The malondialdehyde content of the cells and culture medium was used to estimate the extent of lipid peroxidation. As the zinc concentration of the culture medium was increased from 1 to 48 μM, peroxidation was diminished. Cellular zinc and metallothionein levels were proportionally increased by supplemental zinc. Zinc supplementation of the medium inhibited NADPH-cytochrome *c* reductase activity and stimulated glutathione peroxidase activity. The uptake of iron into the hepatocytes was significantly reduced as the level of zinc was raised, suggesting that zinc antagonizes uptake of chelated iron into isolated hepatocytes and in this way blocks iron-induced peroxidation. Furthermore, induction of metallothionein synthesis by zinc may contribute to the reduction in free radicals. Spectra from electron spin resonance studies, using phenylbutylnitrone as a spin-trapping reagent, demonstrated that free radical production was inversely related to the zinc concentration of the culture medium. Spin trap data suggest that metallothionein added to lysed cells *in vitro* decreases free radical production. Studies using the spin trap, 3,3,5,5-tetramethylpyrroline-*N*-oxide indicated that cumulatively the predominant radical present in the cultures was a phenyl radical with hydroperoxide or methylindole. Collectively, our data demonstrate that zinc inhibits free radical production and lipid peroxidation in cultured hepatocytes. The mode of action of zinc could occur via free radical scavenging by zinc-induced metallothionein and/or by processes related to cytochrome *P*-450 and glutathione peroxidase, since these were also found to be sensitive to zinc supplementation levels of the culture medium.

There is an increasing body of literature which suggests that oxidative damage to cells has a role in many pathological conditions including carcinogenesis and aging. Prooxidant conditions caused by reactions related to active oxygen and free radical formation may lead ultimately to chromosomal damage and neoplastic growth (1). Specifically, activation of dioxygen to superoxide, H_2O_2, and hydroxyl radical ($\cdot OH$) leads to a variety of subsequent reactions. These have been implicated in numerous deleterious processes including lipid peroxidation and oxidative damage to DNA and chromosomal proteins. For example, iron reacts with hydrogen peroxide to produce hydroxyl radicals ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$). The extremely reactive $\cdot OH$ then participates in a cascade of deleterious events (2). Since iron is a nutrient with important physiological functions, it would be expected that its metabolism is highly regulated in order to restrict its participation in intracellular reaction (3).

A diversity of compounds and conditions are known to cause lipid peroxidation including high energy irradiation (4), ozone (5), carbon tetrachloride (6, 7), adriamycin (8), paraquat (9), iron (2, 10), 3-methylindole (11), and t-butyl hydroperoxide (12). These compounds generate reactive species within cells which could include the hydroxyl radical (13, 14). Cellular functions are subsequently compromised as a result of extensive peroxidative damage and ultimately longevity may be diminished (2, 15).

Peroxidation of tissue lipids is a common finding in animals of marginal or deficient zinc intake (16, 17). With time this could

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lead to cellular damage associated with aging, cancer, and altered regulatory processes. The effect of zinc may be related to an inhibition of NADPH-dependent lipid peroxidation (18, 19). Furthermore, the generalized effect of zinc on membrane stabilization could be explained on the basis of control of peroxidation (20). In addition, high levels of dietary zinc fed to rats or the addition of zinc to liver homogenates has also been shown to inhibit lipid peroxidation (17, 21).

Liver cells in primary culture provide a particularly useful tool to characterize cellular lipid peroxidation and free radical production. The purpose of the experiments described here was to evaluate the effect of zinc in the response of liver cells to a variety of initiators of free radical formation and peroxidation. Evidence is presented which suggests that free radicals can be induced in monolayer cultures of liver parenchymal cells by agents that may have different modes of action. Our data suggest that zinc plays a role in suppression of free radical formation and lipid peroxidation in rat hepatocytes by mechanisms which may involve metallothionein synthesis, exclusion of extracellular iron, and/or changes in the activities of cytosolic or microsomal enzymes.

Materials and Methods. *Materials.* Waymouth's MB 752/1 medium and fetal bovine serum were obtained from GIBCO (Grant Island, NY). Collogenase (type III) was from CooperBiomedical (Malvern, PA). Hepes, Tes, *N*-t-butyl-α-phenylnitrone (PBN), 3,3,5,5-tetramethylpyrroline-*N*-oxide (TMPO), nitrilotriacetic acid (trisodium salt), t-butyl hydroperoxide (t-BHP) and 3-methylindole (3MI), calf skin collagen, fatty acid-free bovine serum albumin, rabbit metallothionein, and insulin were purchased from Sigma Chemical Co. (St. Louis, MO). ^{59}Fe and [^{3}H]leucine were purchased from DuPont NEN Products (Boston, MA).

Hepatocyte monolayer cultures. Hepatocytes were from 150 to 200 g male, Sprague–Dawley strain rats (obtained from the University of Florida colony). They were fed a commercial diet (Purina Rat Chow; Ralston Purina Co., St. Louis, MO) and tap water *ad libitum.* Preparation of the cells was as described previously (22) with some modifications. Briefly, the freshly isolated hepatocytes were washed four times and suspended in Waymouth's 752/1 medium (pH 7.4), supplemented with 15 m*M* Hepes, 10 m*M* Tes, 30 m*M* $NaHCO_3$, 0.5 m*M* pyruvate, streptomycin sulfate (100 μg/ml), penicillin G (100 units/ml), gentamycin sulfate (50 μg/ml), alanine (0.4 μM), serine (0.5 μM), fetal bovine serum (10% w/v), and insulin (1 μg/ml). Cell viability, as determined by trypan blue exclusion, was greater than 85%. Aliquots containing 1×10^6 cells were placed on 60-mm culture dishes which had been previously coated with 20–40 μg of calf skin collagen. After 2 hr, viable parenchymal cells remained attached to the dish and formed a monolayer, while nonattached cells were removed with the medium by aspiration.

Treatment of cultures. Following the attachment period, the medium was removed and fresh Waymouth's medium was added, supplemented as before except that it contained bovine serum albumin (2 mg/ml) in place of fetal bovine serum and insulin was omitted. The zinc concentration of the medium was normally 1 μM. Zinc sulfate was added to the medium such that final zinc concentrations were 1, 16, 24, 32, or 48 μM and in one study 4 and 8 μM. These were verified by atomic absorption spectrophotometry (AAS).

After incubation for 18 hr, the cells were washed as described previously (22) and fresh medium containing ferric nitrilotriacetic acid (FeNTA 10 μM) and ascorbic acid (10 μM) was added (10). The FeNTA was prepared as described by White and Jacobs (23). Alternatively, fresh medium containing either t-BHP (2 m*M*) or 3MI (8 m*M*) was added (24) that had been supplemented with zinc. Aliquots of the medium were removed and cells harvested at various time intervals for 2 hr. For some experiments, the cells were lysed by homogenization (Polytron with P10 generator). Alternatively, a solution of 0.2% SDS in 0.2 *M* NaOH was added for 1 hr to hydrolyze cellular components (22). For iron uptake studies, the medium was supplemented with ^{59}Fe (20 μ/Ci plate). The cells were washed with EDTA-containing buffer (22), harvested, and ^{59}Fe uptake measured with a gamma spectrometer. For leucine incorporation studies, [^{3}H]leucine was added to the medium (1.5 microcurie per dish) for 1

hr. The cells were then washed and protein was precipitated by 10% trichloroacetic acid to measure incorporation into leucine (25).

Electron spin resonance studies. Spin trapping experiments were started after the cells had been in culture for 18 hr. PBN was added to serum-free mdium at 0.1 *M* and the cells were incubated for an additional 1 hr (11). This spin trap was extracted from the cultures with *n*-hexane (1:1, v/v). The hexane extract was purged and concentrated to 2 ml with N_2 and transferred to a quartz tube under anaerobic conditions. For *in vitro* studies, cells were harvested and lysed by homogenization (as described above) before TMPO (0.4 *M*) was added. In some cases, rabbit liver metallothionein was also added at a concentration of 0.25 μM. The TMPO-containing homogenates were incubated in capped tubes at 37°C for 30 min. Spectra were obtained under aerobic conditions using a quartz flat cell.

Spectral intensity and hyperfine splitting characteristics of spin trap adducts were determined at room temperature with a Bruker ER 200D-SRC electron magnetic resonance spectrometer operated under the following conditions: microwave frequency, 9.4 GHz; incident power, 20 mW; modulation frequency, 100 kHz; field intensity, 1 G; and a time constant of 500 msec.

Analytical procedures. Malondialdehyde (MDA) concentrations of the medium were measured as thiobarbituric acid (TBA)-reactive substances (26). Absorbance was read at two wavelengths 520 and 532 nm (27) using a MDA-TBA complex as a standard (28). Zinc content of the cells were measured by AAS after the cells were digested with 0.2 *M* NaOH (containing 0.2% SDS). Metallothionein was measured by the ^{109}Cd binding method (29). Total cell protein concentrations (30) and activities of cytochrome *c* reductase (31), glutathione peroxidase (32), and lactate dehydrogenase (33) were measured spectrophotometrically. Glutathione peroxidase activity was calculated as the decrease in glutathione concentration expressed as units per milligram protein (one unit = 1% substrate disappearance/min) (34). Differences between treatment means were evaluated using analysis of variance and Duncan's multiple range test (35).

Results. The production of malondialdehyde in the cell cultures over a 2 hr period was taken to be an index of lipid peroxidation. The concentrations of zinc added to the medium were chosen because previous experiments (22) have shown that zinc in the micromolar range produced changes in cellular zinc exchange and total zinc content of cultured rat hepatocytes. At concentrations of 32 and 48 μM Zn, the production of malondialdehyde (MDA; TBA reactive-products) in the presence of FeNTA and ascorbic acid and significantly reduced as compared to lower zinc concentrations (Table I).

Uptake of ^{59}Fe in the presence of FeNTA and ascorbic acid was significantly reduced at zinc concentrations of 16 μM or above (Table II). Iron uptake at 32 and 48 μM zinc was lower than the other zinc supplemental levels. Since ^{59}Fe exchanges rapidly with iron in the NTA complex, ^{59}Fe uptake can be equated with an increase in total cellular iron. Incorporation of [^{3}H]leucine into TCA-precipitable proteins over a 2 hr time period was not affected by the zinc concentration of the medium (Table II). Therefore, it is unlikely that the differences in MDA production could be attributed to a toxic effect of zinc. Lactate dehydrogenase activity of the medium after incubation with iron and zinc averaged 0.25 units/mg protein (data not shown). No differences in activity

TABLE I. SUPPRESSION BY ZINC OF MALONDIALDEHYDE FORMATION INDUCED IN HEPATOCYTES BY IRON

$[Zn^{2+}]$ (μM)	Malondialdehyde (ng/mg protein) Minutes			
	30	60	90	120
1	12 ± 10[a]	20 ± 10[a]	28 ± 5[a]	34 ± 8[a]
16	16 ± 10[a]	24 ± 6[a]	28 ± 6[a]	30 ± 12[a]
24	10 ± 6[a,b]	16 ± 8[a]	23 ± 12[a]	26 ± 6[a]
32	3 ± 5[b]	3 ± 3[b]	4 ± 2[b]	7 ± 5[b]
48	nd	nd	3 ± 2[b]	11 ± 12[b]

Note. Monolayer cultures of hepatocytes were incubated for 18 hr with serum-free medium containing varying concentrations of zinc. Ferric nitrilotriacetic acid and ascorbic acid (both at 10 μM) were added for an additional 2 hr of incubation. Means ± SD are shown (n = 6). Means with a different superscript letter are significantly different ($P < 0.05$). Also, nd = not detectable.

TABLE II. INCORPORATION OF [^{3}H]LEUCINE INTO PROTEIN AND ^{59}Fe UPTAKE BY HEPATOCYTE CULTURES IN THE PRESENCE OF ZINC

[Zn^{2+}] (μM)	Leucine incorporation (ng/mg protein)	Fe uptake (ng/mg protein)
1	456 ± 101	108 ± 7[a]
16	465 ± 62	37 ± 3[b]
24	387 ± 58	31 ± 2[c]
32	385 ± 113	22 ± 4[d]
48	453 ± 109	22 ± 2[d]

Note. Monolayer cultures of hepatocytes were incubated for 18 hr with serum-free medium containing varying concentrations of zinc prior to Fe uptake or leucine incorporation experiments. Ferric nitrilotriacetic acid and ascorbic acid (both at 10 μM) alone with ^{59}Fe (1 μCi/plate) were added for a 2 hr uptake period. [^{3}H]leucine incorporation into cell proteins precipitable in 10% TCA were measured as a 2 hr pulse labeling. Means ± SD are shown (n = 6). Means with a different superscript letter are significantly different ($P < 0.05$).

were found among cells incubated with varying concentrations of zinc. Therefore, it is likely that the plasma membrane was intact for the duration of the experiments.

Zinc is known to be a primary inductor of metallothionein. As the zinc concentration in the medium was raised from 1 to 48 μM, so the metallothionein concentrations rose from 0.14 to 1.01 mM, an increase of 7.5-fold at 48 μM Zn, suggesting an increase in expression of the gene (Table III). The cellular zinc concentration increased as the zinc concentration of the medium was raised (Table III). Metallothionein and cellular zinc levels are directly related but the magnitude of metallothionein induction is greater. These increases occur at lower zinc concentrations than needed to produce a significant reduction in malondialdehyde content of the hepatocyte cultures.

Electron spin resonance (ESR) spectra of the hexane extracts of cells incubated with zinc, FeNTA, and ascorbic acid for 1 hr in the presence of the spin-trap PBN showed that cultures with higher levels of supplemental zinc had proportionally less free radicals trapped (Fig. 1). This is based on reduction in signal amplitude. The reduction in PBN adducts was reduced to control (no iron) levels at 48 μM Zn. Other spin traps when added to the hepatocyte cultures gave similar evidence of free radical suppression.

A typical ESR spectrum from the spin trap TMPO indicating free radical production is shown in Fig. 2a. TMPO was added to cells cultured in the presence of FeNTA and ascorbate showed evidence of free radical formation based on pronounced signal intensity. In order to examine the effect of metallothionein added *in vitro,* cells were first cultured for 18 hr with 1 or 32 μM Zn. It was observed that the addition of rabbit metallothionein to lysed cells, which had been previously incubated with 1 μM zinc, decreased the amount of the signal generated by addition of iron (Fig. 2b vs 2d). This was observed under conditions when bovine serum albumin (2 mg/ml) was also present in abundance. However, when the cells had been previously incubated with 32 μM Zn, the addition of this small amount of metallothionein had no effect on the amplitude of the spectra (Fig. 2c vs 2e). Similar results were obtained with unpure rat metallothionein.

In order to examine the specificity of the effect on peroxidation, organic inducers of peroxidation were examined as well. As shown in Tables IV and V, the concentration

TABLE III. METALLOTHIONEIN CONCENTRATION OF HEPATOCYTES FOLLOWING INCUBATION WITH ZINC

[Zn^{2+}] (μM)	Metallothionein (mM)	Cellular Zn (mM)
1	0.14 ± 0.04[a]	1.08 ± 0.04[a]
4	0.14 ± 0.01[a]	0.84 ± 0.04[a]
8	0.13 ± 0.00[a]	0.96 ± 0.04[a]
16	0.32 ± 0.05[b]	1.56 ± 0.08[b]
24	0.32 ± 0.07[b]	1.88 ± 0.04[c]
32	0.54 ± 0.07[c]	2.00 ± 0.04[c]
48	1.01 ± 0.12[d]	2.60 ± 0.12[d]

Note. Monolayer cultures of hepatocytes were incubated for 18 hr with serum-free medium containing varying concentrations of zinc. Metallothionein and zinc was measured by the Cd(II) binding method and atomic absorption spectrophotometry, respectively, after 18 hr in culture. Molar concentration of metallothionein was based on cell H_2O content of 2.5 μl/mg cell protein. Cells from two plates were pooled for each individual measurement. Means ± SD are shown (n = 4 for MT and 3 for Zn). Means with a different superscript letter are significantly different ($P < 0.05$).

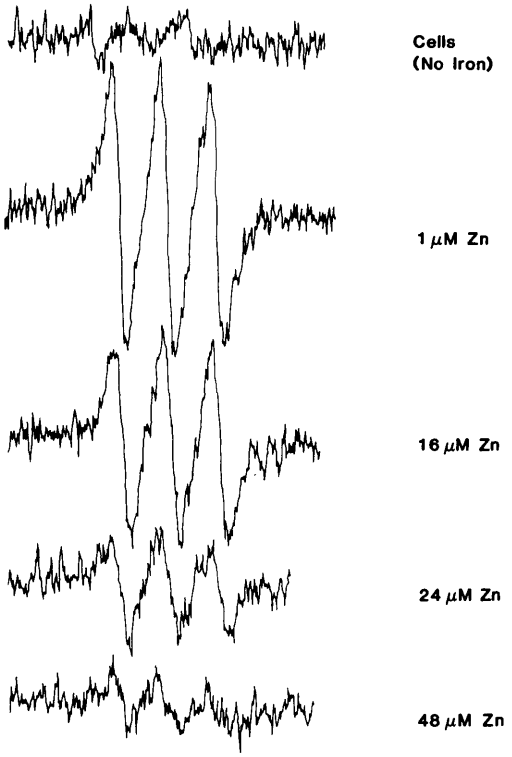

FIG. 1. ESR spectra from hepatocyte cultures which had been incubated for 18 hr with 1, 16, 24, or 48 μM Zn. The cells were then incubated with PBN and iron NTA for 1 hr.

of MDA in the presence of either 3MI or t-BHP also increased with time, but decreased as the zinc concentration was increased from 1 to 48 μM. After 60 min the concentration of MDA in the presence of t-BHP when zinc was 32 or 48 μM was significantly less than when the zinc concentration was 1, 16, or 24 μM (Table IV). After 2 hr, however, all cells cultured with supplemental zinc had significantly less MDA than the control (1 μM basal level). In the presence of 3MI, the production of MDA by 90 to 120 min is proportional to supplemental zinc levels (Table V). In the presence of 16 μM Zn, however, significant decreases in MDA were seen. Greater variation was encountered repeatedly in experiments with 3MI or FeNTA.

Zinc also decreased the numbers of free radicals trapped by the spin-trap PBN in the presence of either 3MI or t-BHP, as can be seen from the first derivative ESR spectra shown in Fig. 3. The amplitude of the peak of the nitroxide moiety of the PBN adduct generated in the presence of either t-BHP or 3MI decreased as the zinc concentration in the culture medium was increased. Since the protein concentration in culture plates did vary over the range of the zinc concentrations needed to detect suppression, the amplitude of the nitroxide peak was assumed to

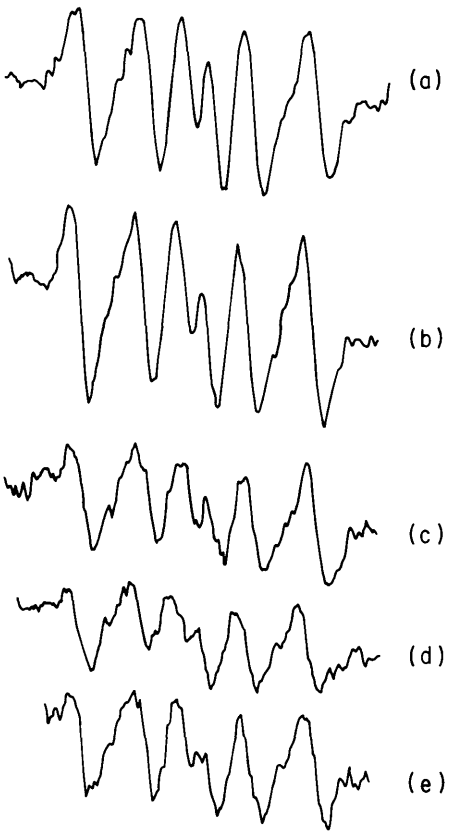

FIG. 2. ESR spectra from hepatocyte cultures which had been incubated for 18 hr with either 1 or 32 μM Zn and then were treated as indicated below. (a) Incubated with TMPO and iron NTA and ascorbate (each 10 μM) for an additional 30 min. (b and d) After incubation with 1 μM Zn, cells were lysed and cellular material including medium was incubated for 30 min at 37°C with 10 μM Fe and TMPO; (d) also contained 1 μM zinc-metallothionein for the 30 min incubation. (c and e) After incubation with 32 μM Zn, the cells were lysed and cellular material including medium was incubated for 30 min with 10 μM Fe and TMPO. (e) Also contained 1 μM zinc-metallothionein for the 30 min incubation.

TABLE IV. SUPPRESSION BY ZINC OF MALONDIALDEHYDE FORMATION INDUCED IN HEPATOCYTES INCUBATED WITH t-BUTYL HYDROPEROXIDE

$[Zn^{2+}]$ (μM)	Malondialdehyde (ng/mg protein) Incubation time (min)			
	30	60	90	120
1	87 ± 41^{a}	178 ± 75^{a}	197 ± 30^{a}	278 ± 119^{a}
16	$59 \pm 25^{a,b}$	$115 \pm 22^{b,c}$	$161 \pm 86^{a,b,c}$	191 ± 44^{b}
24	86 ± 40^{a}	$142 \pm 22^{a,b}$	$177 \pm 49^{a,b}$	168 ± 47^{b}
32	19 ± 15^{b}	82 ± 19^{c}	105 ± 22^{c}	152 ± 69^{b}
48	$44 \pm 52^{a,b}$	80 ± 19^{c}	$124 \pm 66^{b,c}$	136 ± 27^{b}

Note. Monolayer cultures of hepatocytes were incubated for 18 hr in serum free medium containing varying concentrations of zinc. t-Butyl hydroperoxide (1 mM) was added for an additional 2 hr of incubation. Means ± SD, are shown ($n = 6$). Means with a different superscript letter are significantly different ($P < 0.05$).

be proportional to the number of free radicals trapped.

No data concerning the type of radicals present in the cell cultures were possible using PBN as the spin trap. However, by using TMPO and through comparison with known ESR spectra, it was possible to identify that by 30 min after induction of peroxidation, the phenyl radical was the predominant TMPO adduct present in the cell cultures (Fig. 4). This was produced whether the free radicals were generated by t-BHP or 3MI.

Clearly the mechanism of free radical suppression induced by t-BHP or 3MI could be different. Free radical scavening by metallothionein could be a central factor contributing to the effect, however. As shown above, the addition of zinc to the culture medium resulted in proportional increase in metallothionein concentration up to 7.5-fold at 48 μM Zn. However, raising the zinc concentration above basal levels also significantly increased the activity of the cytosolic enzyme glutathione peroxide (GPx) while it significantly decreased the activity of the microsomal enzyme NADPH-cytochrome *c* reductase (Table VI). Thus, at 16 μM Zn and above, GPx activity was approximately twice that seen at 1 ppm Zn. In contrast, the activity of NADPH-glutathione c-reductase was decreased by 2.5-fold as the zinc level was raised from 1 to 16 μM and was almost 5-fold lower at 48 μM Zn. This suggests the suppressive effect of zinc could be operative at many cellular levels.

Discussion. Substances which reacted with TBA (malondialdehyde) are assumed to be proportional to the extent of lipid peroxidation. Although the TBA method is not totally specific, it is an accepted index of peroxidation (2, 36). Furthermore, the generation of free radicals produced under identical conditions in these experiments was similarly reduced, which suggests that the TBA-MDA method was a valid estimate of peroxidation.

Iron loading of liver cells has been shown to stimulate malondialdehyde production previously (10). This could involve Fenton's chemistry where Fe^{2+} and H_2O_2 generate free radicals which then react with polyunsaturated fatty acids. An end product of this reaction is malondialdehyde (2). The FeNTA complex was chosen to ensure availability of the iron at physiological pH. Interestingly, increased supplemental zinc decreased iron uptake into the cells, suggesting a common mechanism for chelated iron/zinc uptake. Whether the reduction of malondialdehyde produced by zinc was a direct effect, at least in part, of reduced cellular iron concentration is unknown.

It was also shown that as the supplemental zinc concentration was raised, the metallothionein concentration increased. Further-

TABLE V. SUPPRESSION BY ZINC OF MALONDIALDEHYDE FORMATION IN HEPATOCYTES INCUBATED WITH 3-METHYLINDOLE

$[Zn^{2+}]$ (μM)	Malondialdehyde (ng/mg protein) Incubation time (min)			
	30	60	90	120
1	13 ± 5^{a}	17 ± 4^{a}	17 ± 6^{a}	72 ± 8^{a}
16	7 ± 7^{a}	$10 \pm 7^{a,b}$	$12 \pm 7^{a,b}$	$69 \pm 10^{a,b}$
24	7 ± 5^{a}	13 ± 8^{a}	$12 \pm 6^{a,b}$	$55 \pm 21^{b,c}$
32	10 ± 11^{a}	13 ± 10^{a}	$9 \pm 10^{a,b}$	44 ± 11^{c}
48	nd	3 ± 2^{b}	5 ± 4^{b}	10 ± 11^{d}

Note. Monolayer cultures of hepatocytes were incubated for 18 hr in serum-free medium containing varying concentrations of zinc. 3-Methylindole (8 mM) was added for an additional 2 hr of incubation. Means ± SD are shown ($n = 6$). Means with a different superscript letter are significantly different ($P < 0.05$). Also, nd = not detectable.

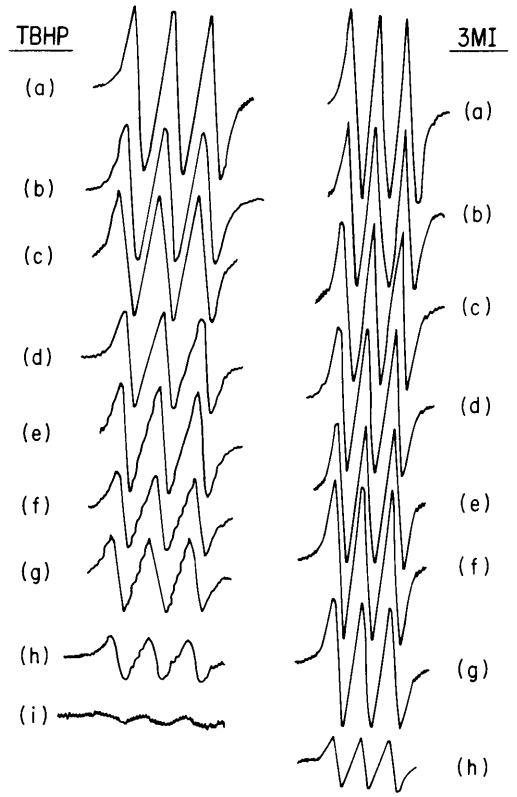

FIG. 3. ESR spectra of PBN from cell cultures which had been incubated for 18 hr with either 1, 4, 8, 16, 24, 32, or 48 μM zinc, (a) through (g), respectively. The cells were then incubated for 1 hr with PBN and either t-butyl hydroperoxide (TBHP) or 3-methylindole (3MI) as indicated. Control spectra of PBN are also shown: medium plus either t-butyl hydroperoxide (h) or 3-methylindole (h) and cells with medium containing PBN only (i) (center field 3320 G, scan width shown approximately 50 G).

more, metallothionein added to cultures containing a far greater concentration of albumin also reduced free radical formation. Therefore, it is tempting to speculate that the induced metallothionein levels also had a role in the scavenging free radicals generated. Recently, Thornalley and Vasak (37) showed that metallothionein has properties that suggest it could act as a free radical scavenger. Using a Fenton-type reaction (i.e., Fe^{2+} + H_2O_2) and the xanthine/xanthine oxidase reaction to generate oxygen radicals, they demonstrated that, based on comparison of rate constants, metallothionein was particularly effective in quenching hydroxyl radicals compared to superoxide ions. They found that these radicals caused metal loss from the protein and thiolate oxidation. It was proposed that glutathione would regenerate reduced sulfhydryl groups of metallothionein for subsequent rebinding of metals.

Girotti *et al.* (38) have observed with an *in vitro* system using erythrocyte membranes that Zn^{2+} inhibited peroxidation generated by the xanthine/xanthine oxidase system. They reasoned initially that Zn^{2+} interfered with redox cycling of iron by competing for membrane binding sites. It was subsequently shown that metallothionein added to this *in vitro* system reduced iron-mediated membrane peroxidation (39). Thus it seems likely that the antioxidant activity of metallothionein and reduced uptake of iron into hepatocytes may have contributed to the reduction in free radicals trapped and the reduction of malondialdehyde produced.

It has been reported that zinc inhibits microsomal NADPH oxidation and NADPH-dependent cytochrome *c* reductase activity (19, 40). Zinc binding to NADPH may explain zinc's mode of action in inhibiting NADPH-dependent cytochrome *c* reductase activity observed in the hepatocyte cultures in the present experiments. Kubow *et al.* (11) have proposed that 3MI is metabolized by the cytochrome *P*-450 complex which generates a 3MI radical. By inhibiting microsomal cytochrome *c* reductase, when the zinc concentration is 16 μM or above, 3MI radical formation would be reduced. Thus it seems likely in the case of 3MI, that zinc, by inhibiting the formation of the 3MI radical in the microsomes, would subsequently reduce subsequent lipid peroxidation.

t-Butyl hydroperoxide is a substrate for GPx (24). As the zinc concentration was increased, so the activity of this selenoenzyme was also increased and then by implication, the concentration of t-BHP would be more rapidly lowered. Thus, as was seen, at the higher zinc concentrations, the concentration of MDA, and the number of free radicals trapped was reduced in the presence of t-BHP, these reductions being due to the rapid removal of intracellular t-BHP at the

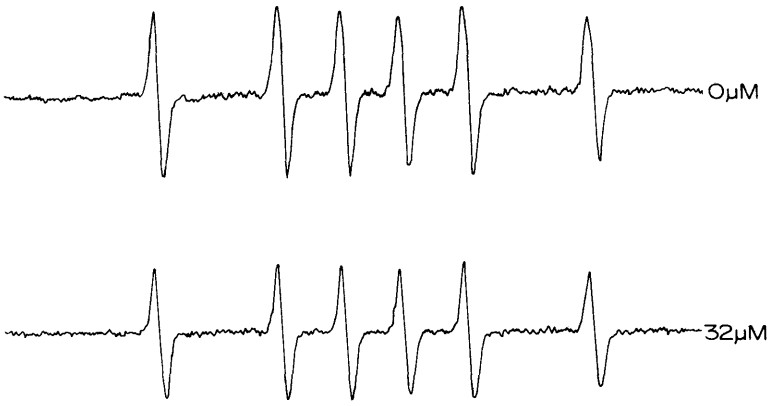

FIG. 4. ESR spectra obtained from cell cultures which had been incubated for 18 hr with either 1 or 32 μM zinc. t-Butyl hydroperoxide was added and the cells were then incubated for 30 min with the spin trap TMPO (center field 3450 G, scan width shown approximately 100 G).

highest zinc concentrations. To our knowledge, the increase in GPx activity by zinc is a novel finding. It is in contrast to zinc deficiency, which does not affect activity of the enzyme (41).

Collectively the data presented suggest that zinc suppresses lipid peroxidation by affecting many different cellular functions: decrease in iron uptake, concentration of metallothionein, and the activity of NADPH cytochrome *c* reductase and glutathione peroxidase. At present it is difficult to establish if one of these functions is dominant, however, it seems likely that depending on the initiator used, one of these mechanisms would be dominant, the other mechanisms playing a secondary one. In the present experiments, the phenyl radical may be the predominant radical after 30 min incubation of cells with 3MI or t-BHP. Phenylalanine and tyrosine residues of cell proteins or from the culture medium could be the source of these radicals. Further studies using other promoters of lipid peroxidation, shorter time courses, and other spin trapping models will be needed to shed more light on the mechanisms by which zinc, directly or indirectly, exerts antioxidant properties in cells.

TABLE VI. EFFECT OF SUPPLEMENTAL ZINC ON GLUTATHIONE PEROXIDASE AND NADPH-CYTOCHRONIC *c* REDUCTASE ACTIVITIES IN HEPATOCYTES

$[Zn^{2+}]$ (μM)	Glutathione peroxidase (units/mg protein/min)	NADPH-cytochrome *c* reductase (nmoles/mg protein/min)
1	$16 \pm 4^{a,b}$	85 ± 15^{a}
4	12 ± 2^{b}	$53 \pm 6^{b,c}$
8	$22 \pm 2^{a,c}$	$63 \pm 15^{a,b}$
16	$32 \pm 2^{d,e}$	$34 \pm 10^{c,d}$
24	$25 \pm 1^{c,e}$	$37 \pm 1^{c,d}$
32	$30 \pm 4^{d,e}$	$29 \pm 16^{c,d}$
48	32 ± 5^{d}	18 ± 17^{d}

Note. Monolayer cultures of hepatocytes were incubated for 20 hr in serum-free medium containing varying concentrations of zinc. Means ± SD are shown ($n = 6$). Means with a different superscript letter are significantly different ($P < 0.05$).

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