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Comparative *In Vitro* Oxygen Radical Scavenging Ability of Zinc Methionine and Selected Zinc Salts and Antioxidants

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ABSTRACT. **1. The concentration-dependent scavenging abilities of zinc DL-methionine, zinc sulfate, zinc gluconate, zinc picolinate and selected free radical scavengers, including superoxide dismutase (SOD), catalase, mannitol, allopurinol and DL-methionine, were examined against biochemically generated superoxide anion, hydroxyl radical and hypochlorite radical plus hypochlorous acid, by chemiluminescence and cytochrome c reduction.**

2. Zinc methionine was the most effective of the zinc compounds that were tested. Following incubations with superoxide anion, hydroxyl radical, and hypochlorite radical-generating systems, in the presence of 50 μM zinc DL-methionine approximately 38%, 47% and 28% inhibition in reactive oxygen species generation was observed, respectively, compared to control groups.

3. The protective abilities of various zinc salts, as well as selected free radical scavengers and antioxidants were also assessed on phorbol ester (TPA)-induced lactate dehydrogenase (LDH) release from cultured PC-12 cells. Preincubation showed better protection than coincubation. Approximately 45% and 50% inhibition in TPA-induced LDH leakage was observed following preincubation with 50 μM zinc DL-methionine and 50 μM vitamin E succinate, respectively. Zinc DL-methionine exhibited better protection against LDH leakage than any other zinc salt tested.

4. The results indicate that zinc DL-methionine can attenuate the biochemical consequences of oxygen free radicals, and is comparable to other well-known antioxidants and free radical scavengers in the *in vitro* system that was employed. Copyright © 1997 Elsevier Science Inc. GEN PHARMAC 28;1:85–91, 1997.

KEY WORDS. Biochemically generated oxygen free radicals, cultured PC-12 cells, TPA, zinc salts, vitamin E, β-carotene, vitamin C, methionine, chemiluminescence, cytochrome c reduction, LDH leakage

INTRODUCTION

The roles of reactive oxygen species and free radicals in tissue injury and cell killing are well established (Halliwell *et al.*, 1992) . These highly reactive species act as cancer initiators and/or promoters, cause DNA damage, activate procarcinogens, and alter the cellular antioxidant defense system (Liochev and Fridovich, 1994; Comporti, 1989; Cerutti, 1991). Antioxidants function as inhibitors at both the initiation and promotion/progression/transformation stages of carcinogenesis, and protect cells from oxidative damage (Halliwell and Cross, 1991; Sies, 1991).

Zinc is a well-known membrane stabilizer, and functions in the maintenance of membrane structure and function (Bettger and O'Dell, 1981). Physiologically bioavailable zinc has been identified as a nutrient essential for normal growth, sexual development, wound healing, ability to fight infections, sense of taste, night vision, healthy epithelial tissue, cell-mediated immunity and other vital functions (King and Turnland, 1989). Zinc deficiency results in increased microsomal lipid peroxidation (Bettger and O'Dell, 1981; Sullivan *et al.*, 1980), enhanced production of carbon-centered radicals in lungs (Bray *et al.*, 1986), leakage of hydrogen peroxide from the NADPH-dependent cytochrome P-450 system (Hammermueller *et al.*, 1987) and DNA fragmentation (Xu and Bray, 1992; Cao and Chen, 1991). Evidence has indicated that zinc may protect sulfhydryl groups against oxidation (Bray and Bettger, 1990). However, little information is available regarding the radical scavenging ability of zinc and its ability to prevent the formation of reactive oxygen species.

Other naturally occurring antioxidants and free radical scavengers that have been extensively studied include vitamin E (α-tocopherol), vitamin C (ascorbic acid) and β-carotene. Vitamin E and other tocopherols are potent lipid-phase antioxidants and are reported to regulate signal transduction pathways involved with cellular proliferation (Burton *et al.*, 1983). Previous studies in our laboratories (Bagchi *et al.*, 1995) as well as by Ray and Fariss (1994) have demonstrated that excellent cytoprotection by vitamin E succinate occurs due to the subcellular disposition of vitamin E succinate and subsequent release of vitamin E at critical cellular sites. Humans with suboptimal vitamin E intake are thought to be at increased risk for many chronic aging-related diseases, such as cancer, cardiovascular and neurological disorders (Cooney *et al.*, 1995).

Bendich and Langseth (1995) have reviewed the health effects of vitamin C supplementation and high dietary intake. In general, long-term consumption of high levels of vitamin C reduces risks of cancer at several sites, cardiovascular disease and cataracts. Vitamin C supplementation decreases the duration and severity of symptoms associated with the common cold, but has little effect on the inci-

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dence of the common cold (Hemila, 1992). Dietary vitamin supplementation also decreases endogenous protein oxidative damage, production of malondialdehyde and lipid peroxidation, and maintains fatty acid unsaturation (Barja *et al.*, 1994).

Experimental and epidemiological evidence strongly indicates that dietary carotenoids play a preventive role in cancer and cardiovascular disease, and the function and action of carotenoids, especially β-carotene, have been the subject of extensive studies (Tsuchihashi *et al.*, 1995). Evidence indicates that the protective effects of β-carotene are due to its effectiveness as an antioxidant and free radical scavenger (Gottlieb *et al.*, 1993; Tsuchihashi *et al.*, 1995).

In this study, reactive oxygen species, including superoxide anion, hydroxyl radical and hypochlorite radical plus hypochlorous acid, were biochemically generated *in vitro*. The concentration-dependent scavenging abilities of various zinc salts, including zinc DL-methionine, zinc sulfate, zinc gluconate, zinc oxide, zinc citrate and zinc picolinate, were assessed by chemiluminescence and cytochrome *c* reduction, and were compared with selected oxygen free radical scavengers/antioxidants including superoxide dismutase, catalase, β-carotene, vitamin C, vitamin E and methionine.

Furthermore, following incubation of cultured neuroactive PC 12 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), a well known tumor promoter and an inducer of reactive oxygen species, enhanced release of lactate dehydrogenase (LDH) from the PC-12 cells was monitored as a measure of oxidative membrane damage and cytotoxicity (Bagchi *et al.*, 1995). LDH release was monitored following co- and preincubation with selected zinc salts, as well as oxygen free radical scavengers and antioxidants.

MATERIALS AND METHODS

Chemicals

Zinc oxide, zinc sulfate heptahydrate, β-carotene, DL-methionine, and 12-O-tetradecanoylphorbol-13-acetate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-gluconic acid, citric acid, picolinic acid, zinc carbonate hydroxide monohydrate, zinc hydroxide and zinc oxide were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The commercially available zinc DL-methionine, known as OptiZinc®, was obtained from InterHealth Company (Concord, CA, U.S.A.). All other chemicals used in this study were obtained from Sigma, and were of analytical grade or the highest grade available. RPMI 1640 medium (with L-glutamine), penicillin-streptomycin, gentamycin and trypsin was purchased from GIBCO Laboratories (Grand Island, NY, U.S.A.). Horse serum (heat inactivated) and fetal bovine serum were purchased from Hyclone (Logan, UT, U.S.A.).

Zinc citrate was prepared from zinc carbonate and citric acid as described by von Heldt (1943). Zinc gluconate and zinc picolinate were synthesized as described previously by Cai (1986) and Evans and Johnson (1980), respectively.

PC-12 cell line

The neuroactive PC-12 adrenal pheochromocytoma cell line (ATCC CRL 1721) was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). PC-12 cells were maintained and grown in culture flasks in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 0.2% gentamycin and 0.2% penicillin-streptomycin solution. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Trypsin solution was used to split cultures whenever they were grown to confluence. The number of cells was determined using a Coulter counter. Viability was checked by the Trypan Blue exclusion method.

Generation of reactive oxygen species

The oxygen free radicals were biochemically generated by the following procedures (Bagchi *et al.*, 1990). Superoxide anion radical was produced in a total volume of 2.0 ml by incubating xanthine (100 μM) in 5 mM Tris-HCl buffer with 8 mU/ml of xanthine oxidase. The incubation mixture to generate hydroxyl radical contained 5 mM Tris-HCl, 100 μM $FeCl_3$, 100 μM ethylene diamine tetracetic acid (EDTA), and 100 μM xanthine in a total volume of 2.0 ml. Xanthine oxidase (8 mU/ml) was added to initiate the production of hydroxyl radical. Hypochlorite radical plus hypochlorous acid were generated in 5 mM Tris-HCl buffer using 1 mM sodium hypochlorite in a total volume of 2.0 ml. Vitamin E [(±)-α-tocopherol], vitamin E succinate [(±)-α-tocopherol acid succinate] and β-carotene were dissolved in minimum volumes of ethanol that, when added to the incubation mixtures, did not exceed 1% of the total volume. This volume of ethanol had no effect on these experiments. Vitamin C and zinc salts were dissolved in water or cell culture media. All free radical scavengers, antioxidant and chemoprotectant solutions were freshly prepared prior to each experiment.

Chemiluminescence measurement

Chemiluminescence as an index of reactive oxygen species production was measured in a Chronolog Lumivette Luminometer (Chronolog Corp., Philadelphia, PA, U.S.A.) (Bagchi *et al.*, 1993). The assay was conducted in 3-ml glass minivials. The vials were incubated at 37°C prior to measurement and the background chemiluminescence of each vial was checked before use. Samples were preincubated at 37°C for 15 min and 4 μM luminol was added to enhance chemiluminescence. All additions to the vials, as well as chemiluminescence counting procedures, were performed under dim lighting conditions. Results were examined as counts/unit time minus background. Chemiluminescence was monitored for 6 min at continuous 30-sec intervals.

Measurement of lactate dehydrogenase (LDH) leakage

Leakage of LDH from cultured cells into the media was determined as described by Moss *et al.* (1986) by dilution of 10-μl aliquots of media to 2.6 ml with phosphate buffer (0.1 M, pH 7.4) and warming the mixtures to 37°C. The reactions were then initiated in a cuvette by adding 100 μl NADH (125 μM, final concentration) and 100 μl sodium pyruvate (300 μM, final concentration) to the diluted media. The changes in absorbance of the reaction solutions were measured at 340 nm for 3 min in a Perkin-Elmer Lambda 6 spectrophotometer (Norwalk, CT, U.S.A.). The concentrations of LDH in the media were determined by direct calculation based upon the decrease in absorbance.

Cytochrome c reduction

Superoxide anion production was measured by the cytochrome c reduction assay of Babior *et al.* (1973), and as described by Bagchi *et al.*(1993). The 1.0-ml reaction mixtures contained 0.05 mM cytochrome c, 100 μM xanthine and 8 mU xanthine oxidase, as well as the free radical scavengers and zinc salts listed in Table 1. The mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures on ice, and spectrophotometric determinations were carried out at 550 nm. Absorbance

TABLE 1. Generation of superoxide anion and inhibition by various agents

	Cytochrome c reduction (% inhibition) (nmol min^{-1} ml^{-1})	Chemiluminescence (inhibition)
Control	2.53 ± 0.16[a] (0)	8241 ± 926[a] (0)
SOD (200 μg/ml)	2.21 ± 0.14[b] (13)	N.D.
Catalase (200 μg/ml)	1.97 ± 0.18[b,g] (22)	N.D.
SOD (200 μg/ml) + Catalase (200 μg/ml)	0.62 ± 0.03[c] (76)	1484 ± 217[b] (82)
Zinc DL-methionine (10 μM)	2.37 ± 0.11[a] (6)	7579 ± 816[a] (8)
Zinc DL-methionine (25 μM)	2.08 ± 0.09[b] (18)	6594 ± 638[c] (20)
Zinc DL-methionine (50 μM)	1.53 ± 0.07[d] (40)	5111 ± 452[d] (38)
Zinc DL-methionine (100 μM)	1.22 ± 0.04[e] (52)	3871 ± 477[e] (53)
Zinc DL-methionine (200 μM)	1.05 ± 0.08[f] (58)	3626 ± 293[e] (56)
Zinc sulfate (50 μM)	2.46 ± 0.22[a] (3)	7828 ± 809[a] (5)
Zinc gluconate (50 μM)	2.02 ± 0.17[b] (20)	6922 ± 725[a,c] (16)
DL-methionine (30 μM)	1.82 ± 0.15[g] (28)	5192 ± 662[d] (37)
Zinc oxide (50 μM)	2.57 ± 0.19[a] (0)	8076 ± 740[a] (2)
Zinc citrate (50 μM)	2.58 ± 0.22[a] (0)	7912 ± 696[a] (4)
Zinc picolinate (50 μM)	2.22 ± 0.17[b] (12)	7333 ± 803[a] (11)

Xanthine (100 μM) in 5 mM Tris-HCl buffer was incubated with 8 mU/ml of xanthine oxidase to generate superoxide anion. Selected oxygen free radical scavengers, and zinc salts were individually added to the incubation mixtures and assayed for cytochrome c reduction and chemiluminescence. Data are expressed as the mean value of 6 experiments ± SD. N.D., not determined. Values with nonidentical superscripts are significantly different ($P < 0.05$).

values were converted to nmoles of cytochrome c reduced using the extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}/15 min, and the data were expressed as nmol cytochrome c reduced/min/ml.

Statistical analysis

Data for each group were subjected to analysis of variance (ANOVA). Scheffe's S method was used as the *post hoc* test. The data are expressed as the mean±standard deviation (SD). The level of statistical significance employed in all cases was $p<0.05$.

RESULTS

The abilities of various antioxidants/free radical scavengers and zinc salts to inhibit superoxide anion were determined and are presented in Table 1. Cytochrome c reduction and chemiluminescence assays were used to measure superoxide anion production. Superoxide anion produced from the reaction of xanthine and xanthine oxidase reduced cytochrome c at a rate of 2.53 nmol min^{-1} ml^{-1}. Individually, catalase and SOD had little effect on cytochrome c reduction. However, the reduction of cytochrome c was inhibited approximately 76% by using both SOD and catalase (Table 1). Concentration- dependent inhibition of superoxide anion as assessed by cytochrome c reduction was observed with zinc DL-methionine. Approximately 6%, 18%, 40%, 52% and 58% inhibition was observed following incubation with 10 μM, 25 μM, 50 μM, 100 μM and 200 μM concentrations of zinc DL-methionine (Table 1). At 50 μM concentrations of zinc sulfate, zinc gluconate and zinc picolinate, approximately 3%, 20% and 12% inhibition was observed, respectively, compared to control values for cytochrome c reduction (Table 1). A 28% inhibition in cytochrome c reduction was observed using 30 μM DL-methionine (Table 1). No inhibition was observed with zinc oxide or zinc citrate.

When chemiluminescence was used to assay superoxide anion, results similar to those obtained with the cytochrome c reduction assay were obtained (Table 1). The chemiluminescence response was inhibited by approximately 82% in the presence of catalase plus SOD. Approximately 8%, 20%, 38%, 53% and 56% inhibition in the chemiluminescence response was observed following incubation of the superoxide anion-generating system [xanthine (100 μM) + xanthine oxidase (8 mU/ml)] with 10 μM, 25 μM, 50 μM, 100 μM and 200 μM concentrations of zinc DL-methionine. Following incubation with 50 μM concentrations of zinc sulfate, zinc gluconate and zinc picolinate, inhibition of 5%, 16% and 11% was observed in the chemiluminescence response, respectively (Table 1). Approximately 37% chemiluminescence inhibition was observed following incubation of the superoxide anion-generating system with a 30 μM concentration of DL-methionine (Table 1). Zinc oxide and zinc citrate had little effect on superoxide anion production when assessed with the chemiluminescence assay.

The abilities of various zinc salts to inhibit hydroxyl radicals are presented in Table 2. Following incubation of the hydroxyl radical-generating system [xanthine (100 μM)+xanthine oxidase (8 mU/ml)+ferric chloride (100 μM)+EDTA (100 μM)] with 10 μM, 25 μM, 50 μM, 100 μM and 200 μM concentrations of zinc DL-methionine, approximately 16%, 22%, 47%, 52% and 55% inhibition in the chemiluminescence response was observed, respectively. Mannitol, a well-known scavenger of hydroxyl radical, inhibited chemiluminescence by approximately 85%. Following incubations with 50 μM concentrations of zinc sulfate, zinc gluconate and zinc picolinate, approximately 7%, 17% and 23% inhibition was observed in the chemiluminescence response, respectively, as compared to control values (Table 2). Approximately 46% inhibition was observed in the chemiluminescence response following incubation of the hydroxyl radical-generating system with a 30 μM concentration of DL-methionine. Zinc oxide and zinc citrate had little effect on hydroxyl radical production.

The abilities of various zinc salts to inhibit chemiluminescence associated with the hypochlorite radical are presented in Table 2. In the hypochlorite radical+hypochlorous acid-generating system, approximately 4%, 17%, 28%, 33% and 35% inhibition in chemiluminescence was observed following coincubation with 10 μM, 25 μM, 50 μM, 100 μM and 200 μM concentrations of zinc DL-methionine, respectively. Following incubation with 50 μM concentrations of zinc sulfate, zinc gluconate or zinc picolinate, approximately 10%,

TABLE 2. Generation of hydroxyl radical and inhibition by various agents

Addition	Chemiluminescence [% inhibition] (cpm/min)	
	Hypochloride radical + hypochlorous acid inhibition	Hydroxyl radical inhibition
Control	8617 ± 745[a] (0)	10,116 ± 953[a] (0)
Mannitol (1.25 μM)	N.D. (–)	1517 ± 86[b] (85)
Allopurinol (1.47 mM)	1810 ± 205[b] (79)	N.D. (–)
Zinc DL-methionine (10 μM)	8272 ± 384[a] (4)	8498 ± 691[c] (16)
Zinc DL-methionine (25 μM)	7152 ± 656[c] (17)	7893 ± 808[c] (22)
Zinc DL-methionine (50 μM)	6204 ± 633[c,d] (28)	5358 ± 477[d] (47)
Zinc DL-methionine (100 μM)	5775 ± 622[d] (33)	4849 ± 613[d] (52)
Zinc DL-methionine (200 μM)	5594 ± 616[d] (35)	4556 ± 521[d] (55)
DL-methionine (30 μM)	5613 ± 541[d] (35)	5470 ± 635[d] (46)
Zince sulfate (50 μM)	8359 ± 775[a] (3)	9407 ± 885[a] (7)
Zinc oxide (50 μM)	8014 ± 324[a] (7)	9612 ± 714[a] (5)
Zinc citrate (50 μM)	7755 ± 419[a] (10)	9715 ± 852[a] (4)
Zinc gluconate (50 μM)	6894 ± 718[c] (20)	8397 ± 874[c] (17)
Zinc picolinate (50 μM)	7410 ± 763[a] (14)	7789 ± 586[c] (23)

Hydroxyl radicals were generated *in vitro* using xanthine (100 μM), $FeCl_3$ (100 μM), EDTA (100 μM) and xanthine oxidase (8 mU/ml) in 5 mM Tris-HCl buffer. Hypochlorite radical plus hypochlorous acid were biochemically generated in 5 mM Tris-HCl buffer using 1 mM sodium hypochlorite. Allopurinol, mannitol, and zinc salts were individually added to the incubation mixtures and assayed for positive chemiluminescence response. Data are expressed as the mean value of 6 experiments ± SD. Values with nonidentical superscripts are significantly different ($P < 0.05$).

20% and 14% inhibition was observed in the chemiluminescence response, respectively, compared to control values (Table 2). Approximately 35% inhibition was observed following incubation of the hypochlorite radical plus hypochlorous acid-generating system with a 30 μM concentration of DL-methionine. As with the production of superoxide anion (Table 1) and hydroxyl radical (Table 2) production, zinc oxide and zinc citrate had little effect on hypochlorite radical production (Table 2). However, allopurinol produced a 79% inhibition of hypochlorite radical-induced chemiluminescence.

12-O-Tetradecanoylphorbol-13-acetate (TPA), a well-known tumor promoter, was used as an inducer of oxygen free radicals resulting in membrane damage with the subsequent leakage of LDH from the cells. A concentration of 100 ng TPA/ml was incubated with PC-12 cells in culture (Bagchi and Stohs, 1993), and an approximately 3.3-fold enhanced LDH leakage was observed at the 24-hr incubation time point. The concentration-dependent effects of selected zinc salts were assessed on the release of LDH into the medium following incubation of PC-12 cells with TPA, and compared with selected oxygen free radical scavengers/antioxidants. Both pre- and coincubation effects were assessed. Preincubations provided better protection in all cases than coincubation.

Table 3 shows the protective abilities of SOD, catalase, mannitol and allopurinol, singly and in combination, on the release of LDH by PC-12 cells in the presence of TPA (100 ng/ml) at the 24-hr incubation time-point. Oxygen free radical scavengers individually did not significantly attenuate the TPA-induced LDH leakage from PC-12 cells. However, following co- and preincubation of PC-12 cells with TPA (100 ng/ml) and the combination of SOD (200 μg/ml), catalase (200 μg/ml), mannitol (1.25 μM) and allopurinol (1.47 mM), decreases in LDH leakage of approximately 58% and 65%, respectively, were observed, compared to control values.

Following co- and preincubation of PC-12 cells with TPA in combination with β-carotene (10 μM), decreased LDH leakage of approximately 13% and 18%, respectively, was observed (Table 3). Co- and preincubation with vitamin C decreased the LDH leakage by 20% and 22%, respectively, compared to control values. When concentrations of vitamin C greater than 25 μM were added, increased LDH leakage occurred (data not shown). Approximately 45% and 50% inhibition in LDH leakage was observed following co- and preincubation with 50 μM vitamin E succinate, respectively, relative to control samples, and similar results were observed with vitamin E. Previous studies have shown that vitamin E succinate provides better protection than vitamin E against membrane damage and LDH leakage (Bagchi *et al.*, 1993). DL-Methionine was employed in concentrations of 10 μM, 30 μM and 50 μM. Approximately 16% and 21% inhibition of LDH leakage was observed at the 24-hr incubation time following co- and preincubation with 10 μM methionine, respectively, and 30 μM DL-methionine resulted in approximately 34% and 40% inhibition, respectively, relative to control values (Table 4). Approximately 44% and 47% inhibition of LDH leakage was observed at the 24-hr incubation time following co- and preincubation with 50 μM DL-methionine, respectively.

The ability of various zinc salts to prevent TPA-induced membrane damage and LDH leakage from PC-12 cells is presented in Table 4. Concentration-dependent radical scavenging abilities and protection from membrane damage were observed for zinc DL-methionine. Approximately 19%, 41% and 51% inhibition was observed following coincubation with 10 μM, 50 μM and 100 μM zinc DL-methionine, respectively, and preincubation with 10 μM, 50 μM and 100 μM zinc DL-merhionine resulted in approximately 23%, 45% and 54% inhibition, respectively, relative to control values. Approximately 31%, 11% and 13% inhibition of LDH leakage was observed following coincubation with 50 μM of zinc gluconate, zinc picolinate or zinc sulfate, respectively and, while following preincubation, approximately 32%, 23% and 18% inhibition was observed, respectively, relative to control values (Table 4). Zinc oxide and zinc citrate exhibited no scavenging effects.

DISCUSSION

The formation of reactive oxygen species was assessed by measuring production of chemiluminescence and the reduction of cytochrome *c*. Cytochrome *c* reduction is a relatively specific test for superoxide

TABLE 3. Effects of free radical scavengers and antioxidants on the release of lactate dehydrogenase (LDH) from TPA-induced cultured PC12 cells

TPA (100 ng/ml)	Oxygen radical scavengers/antioxidants	Units LDH/liter 24 hr
–	–	77.4 + 7.9[a]
–	–	256.8 ± 27.3[b]
+	SOD (200 μg/ml)	235.6 ± 30.4[b]
+	Catalase (200 μg/ml)	241.3 ± 21.8[b]
+	Mannitol (1.25 μM)	237.0 ± 20.7[b]
+	Allopurinol (1.47 mM)	247.6 ± 31.6[b]
+	SOD + catalase + mannitol + allopurinol (coincubated with TPA)	106.6 ± 12.3[c]
+	SOD + catalase + mannitol + allopurinol (preincubated 2 hr prior to the addition of TPA)	89.4 ± 10.1[a]
+	β-Carotene (10 μM) (coincubated with TPA)	223.4 ± 25.0[b]
+	β-Carotene (10 μM) (preincubated 2 hr prior to the addition of TPA)	210.2 ± 18.6[b]
+	Vitamin E succinate (50 μM) (coincubated with TPA)	142.4 ± 11.3[d]
+	Vitamin E succinate (50 μM) (preincubated 2 hr prior to the addition of TPA)	129.7 ± 14.4[e]
–	Succinic acid (50 μM)	242.5 ± 16.1[b]
+	Vitamin E (50 μM) (coincubated with TPA)	140.5 ± 16.6[d]
+	Vitamin E (50 μM) (preincubated 2 hr prior to addition of TPA)	127.4 ± 11.2[e]
+	Vitamin C (25 μM) (coincubated with TPA)	207.5 ± 20.5[b]
+	Vitamin C (25 μM) (preincubated 2 hr prior to the addition of TPA)	200.4 ± 11.7[d]
+	DL-methionine (10 μM) (coincubated with TPA)	219.7 ± 18.4[b]
+	DL-methionine (10 μM) (preincubated 2 hr prior to the addition of TPA)	203.2 ± 21.5[b]
+	DL-methionine (30 μM) (coincubated with TPA)	170.2 ± 19.7[d]
+	DL-methionine (30 μM) (preincubated 2 hr prior to the addition of TPA)	154.5 ± 14.3[d]
+	DL-methionine (50 μM) (coincubated with TPA)	142.4 ± 20.5[d]
+	DL-methionine (50 μM) (preincubated 2 hr prior to the addition of TPA)	136.2 ± 16.6[d]

PC-12 cells (25×10^4 cells/35 mm petri dish) in 2 ml of RPMI 1640 were incubated for at least 3 hr to allow cell adherence and TPA, and/or various concentrations of selected oxygen free radical scavengers, singly and in combination, and antioxidants were added to the cultures. The incubation was continued at 37°C in an atmosphere of 5% CO_2 for 24 hr. Media was collected from the cultures and assayed for lactate dehydrogenase activity. Data are expressed as the mean value of 6 experiments ± SD. Values with nonidentical superscripts are significantly different ($P < 0.05$).

anion production (Babior *et al.*, 1973; Ritchey *et al.*, 1981), but chemiluminescence is a more general assay for the production of reactive oxygen species (Fischer and Adams, 1985), and was used to measure formation of superoxide anion, hydroxyl radical and hypochlorite radical. TPA-induced enhanced release of lactate dehydrogenase (LDH) by cultured PC-12 cells was assessed as an index of cellular damage and cytotoxicity (Bagchi *et al.*, 1995; Hassoun *et al.*, 1995). These assays demonstrated the relative scavenging abilities of the selected free radical scavengers/antioxidants and various zinc salts.

The results in Tables 1 and 2 clearly demonstrate that zinc methionine served as a better chemoprotectant than other zinc salts that were tested. Due to its greater scavenging ability, concentration-dependent studies were conducted. Zinc DL-methionine significantly attenuated reactive oxygen species in a dose-dependent manner. Furthermore, zinc DL-methionine also attenuated TPA-induced LDH leakage from cultured PC-12 cells in a dose-dependent manner (Table 4), and is comparable to vitamin E and vitamin E succinate (Table 3).

Various investigators have proposed that a biochemical function of zinc is the maintenance of membrane structure and function (Bettger and O'Dell, 1981). Dietary zinc deficiency has been shown to increase the susceptibility of rat hepatic microsomes to lipid peroxidation both *in vitro* (Bettger and O'Dell, 1981) and *in vivo* (Sullivan *et al.*, 1980). The ability of dietary zinc deficiency to stimulate the production of carbon-centered free radicals in lung microsomes was reported by Bray *et al.* (1986). Hammermueller *et al.* (1987) have demonstrated that a zinc deficiency causes leakage of hydrogen peroxide from the NADPH-dependent cytochrome P-450 enzyme system. Xu and Bray (1992) have shown that dietary zinc deficiency in rats causes a functional and structural impairment of liver microsomal cytochrome P450 function *via* a free radical-mediated mechanism. The removal of zinc from thymocytes results in DNA fragmentation, which is believed to occur by one of several possible mechanisms (Xu and Bray, 1992; Cao and Chen, 1991). These results indicate that zinc acts as a membrane stabilizer and prevents the formation of reactive oxygen species. Zinc may exert these effects through a mechanism that may involve protection of sulfhydryl groups against oxidation (Bray and Bettger, 1990).

The oxygen radical scavenging ability of methionine is also well documented, and the results in Tables 1–4 confirm this effect. Under *in vitro* conditions involving formation of reactive oxygen species, rat liver mitochondria undergo swelling, lipid peroxidation and distinct disorganization of ultrastructure, and methionine provides excellent protection against these effects (Mehrotra *et al.*, 1991). Methionine has also been reported to be an effective quencher of

TABLE 4. Effects of zinc salts on the release of lactate dehydrogenase (LDH) from PC-12 cells in culture

Zinc-based antioxidants	Units LDH/liter (24 hr)				
	10 μM	25 μM	50 μM	100 μM	200 μM
None (TPA - 256.8 ± 27.3[a] U/L)	–	–	–	–	–
Zinc DL-methionine (coincubated with TPA)	209.4 ± 21.9[a,b]	181.3 ± 21.6[b]	151.5 ± 18.1[b,c]	127.2 ± 18.4[c,d]	120.3 ± 18.2[d]
Zinc DL-methionine (preincubated 2 hr prior to the addition of TPA)	197.5 ± 24.7[a,b]	170.2 ± 22.4[b]	140.7 ± 20.3[c]	119.2 ± 20.5[d]	120.5 ± 14.5[d]
Zinc gluconate (coincubated with TPA)	238.6 ± 15.7[a]	202.5 ± 19.3[a,b]	177.6 ± 24.5[b]	–	171.5 ± 22.5[b]
Zinc gluconate (preincubated 2 hr prior to the addition of TPA)	212.4 ± 24.5[a]	195.4 ± 18.5[a,b]	174.7 ± 23.3[b]	–	169.3 ± 24.7[b]
Zinc picolinate (coincubated with TPA)	247.6 ± 11.4[a]	–	228.5 ± 10.3[a]	–	220.7 ± 18.4[a]
Zinc picolinate (preincubated 2 hr prior to the addition of TPA)	227.3 ± 20.8[a]	–	198.4 ± 10.6[a,b]	–	192.4 ± 22.5[a,b]
Zinc citrate (coincubated with TPA)	252.3 ± 19.2[a]	–	254.6 ± 16.8[a]	–	261.5 ± 21.4[a]
Zinc citrate (preincubated 2 hr prior to the addition of TPA)	253.4 ± 26.4[a]	–	251.3 ± 18.7[a]	–	267.7 ± 30.3[a]
Zinc sulfate (coincubated with TPA)	252.6 ± 24.7[a]	–	224.6 ± 15.9[a]	–	197.6 ± 21.4[a,b]
Zinc sulfate (preincubated 2 hr prior to the addition of TPA)	236.5 ± 30.6[a]	–	210.7 ± 17.8[a,b]	–	191.5 ± 14.8[a,b]
Zinc oxide (coincubated with TPA)	–	–	247.6 ± 22.5[a]	–	–
Zinc oxide (preincubated 2 hr prior to the addition of TPA)	–	–	246.2 ± 18.4[a]	–	–

PC-12 cells (25×10^4 cells/35 mm petri dish) in 2 ml of RPMI 1640 were incubated for at least 3 hr to allow cell adherence and TPA, and/or various concentrations of zinc salts were added to the cultures. The incubation was continued at 37°C in an atmosphere of 5% CO_2 for 24 hr. Media was collected from the cultures and assayed for lactate dehydrogenase activity. Data are expressed as the mean value of 6 experiments ± SD. Incubation of PC-12 cells with TPA (100 ng/ml) resulted in 256.8 ± 27.3 Units of LDH leakage/liter at 24-hr incubation time. Values with nonidentical superscripts are significantly different ($P < 0.05$).

singlet molecular oxygen (Devasagayam *et al.*, 1991), an excellent hydroxyl radical scavenger and a potent cardioprotective agent (Blasig *et al.*, 1988). Methionine is oxidized to a sulfoxide by a reactive oxygen species that is dependent on hydrogen peroxide and heme for its production (Sagone *et al.*, 1984). Furthermore, methionine can also react with the same reactive oxygen species that degrade glutathione (Sagone *et al.*, 1984).

Methionine has been shown to modulate the antioxidant defense system in urolithic rats, and incorporation of methionine in the diet induced restoration of antioxidants in the liver of these animals (Selvam and Kurien, 1992). Methionine ameliorates perinatal hypoxic-ischemic brain damage (Thordstein *et al.*, 1993), and acts as a scavenger of the products of the myeloperoxidase system. Methionine may also be useful in inflammatory disorders, preventing tissue damage inflicted by this system (Borregaard *et al.*, 1987). The results clearly indicate that methionine scavenges superoxide anion (Table 1) and hydroxyl radicals (Table 2), as well as preventing oxidative membrane damage to PC-12 cells with the subsequent leakage of LDH (Table 3). Furthermore, the data suggest that the free radical scavenging ability of zinc DL-methionine may be due primarily to the methionine moiety. Zinc methionine is available as a zinc supplement, and has been shown to increase cell growth and enhance immune function in animals (Spears, 1989; Green *et al.*, 1988). Zinc has been shown to be highly bioavailable when given as zinc methionine (Wedekind *et al.*, 1992).

In summary, zinc methionine was found to be the most potent free radical scavenger compared to all other zinc salts that were tested. The results indicate that zinc DL-methionine provided excellent chemoprotection against oxygen free radicals and TPA-induced LDH leakage from cultured neuroactive PC-12 cells, and was comparable to vitamin E succinate with respect to these effects. The methionine moiety may be primarily responsible for these observed effects.

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