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Original Contribution

ANTIOXIDANT ROLE OF α-LIPOIC ACID IN LEAD TOXICITY

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Abstract—The assumption of oxidative stress as a mechanism in lead toxicity suggests that antioxidants might play a role in the treatment of lead poisoning. The present study was designed to investigate the efficacy of lipoic acid (LA) in rebalancing the increased prooxidant/antioxidant ratio in lead-exposed Chinese hamster ovary (CHO) cells and Fischer 344 rats. Furthermore, LA's ability to decrease lead levels in the blood and tissues of lead-treated rats was examined. LA administration resulted in a significant improvement in the thiol capacity of cells via increasing glutathione levels and reducing malondialdehyde levels in the lead-exposed cells and animals, indicating a strong antioxidant shift on lead-induced oxidative stress. Furthermore, administration of LA after lead treatment significantly decreased catalase and red blood cell glucose-6-phosphate dehydrogenase activity. In vitro administration of LA to cultures of CHO cells significantly increased cell survival, that was inhibited by lead treatment in a concentration-dependent manner. Administration of LA was not effective in decreasing blood or tissue lead levels compared to a well-known chelator, succimer, that was able to reduce them to control levels. Hence, LA seems to be a good candidate for therapeutic intervention of lead poisoning, in combination with a chelator, rather than as a sole agent. © 1999 Elsevier Science Inc.

Keywords—Lipoic acid, Lead poisoning, Oxidative stress, Antioxidants, Free radicals

INTRODUCTION

Lead poisoning has been among the most studied health problems over the years. Despite the tremendous amount of data accumulated, the known mechanisms of lead toxicity are incapable of explaining some of the toxic effects of lead [1,2]. One current theory as to how lead exerts its toxic effects suggests that lead-induced oxidative stress may be a possible contributor to the pathogenesis of lead poisoning. Some in vitro studies pointed to elevated production of reactive oxygen species (ROS) by lead treatment [3–5]. These findings were further supported by in vivo studies in lead-exposed animals [6,7] and workers [8–10] where increased lipid peroxidation and altered antioxidant defense systems were found. Therefore, we believe that antioxidants should be considered as a component of an effective treatment for lead poisoning.

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Alpha-Lipoic acid (LA) is a naturally occurring compound that was shown to be synthesized by animals and humans [11]. It functions as a cofactor in several mitochondrial multienzyme complexes involved in energy production. In these reactions LA is reduced to dihydrolipoic acid (DHLA), that is reoxidized by lipoamide dehydrogenase accompanied with NADH formation [12]. Both LA and DHLA were shown to have antioxidant potential in in vitro and in vivo systems [13–15]. This new view of an old cofactor, LA, led scientists to explore the antioxidant properties and therapeutic implications of the compound in diseases associated with oxidative stress.

In the present study the main goal was to investigate the beneficial effects of LA on altered oxidative stress parameters after lead treatment in in vitro and in vivo systems. To achieve this goal, malondialdehyde (MDA) levels were measured as an indicator of lipid peroxidation. Also determined were glutathione (GSH) levels, that represent the thiol status of the cells; catalase (CAT) activity, as an antioxidant enzyme; and glucose-6-phosphate dehydrogenase (G6PD) activity, as an important

reducing equivalent provider for red blood cells (RBCs). Furthermore, the effectiveness of LA in protecting cells against the cytotoxic effect of lead was examined in an in vitro model where Chinese hamster ovary (CHO) cells were used. Another aim of the study was to investigate whether LA, that is known to chelate some of the heavy metals such as Cu^{2+}, Zn^{2+}, Mn^{2+}, is capable of decreasing lead levels in the blood and tissues of lead-treated Fischer 344 rats.

MATERIALS AND METHODS

Materials

The *N*-(1-pyrenyl)-maleimide, 1,1,3,3-tetramethoxypropane, and 2-vinyl pyridine were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA). Reagents used in GSH and MDA analysis were HPLC grade.

Cell culture studies

Cells were propagated in Ham's F12 culture media supplemented with 10% fetal calf serum and 0.5% glutamine. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Colony formation assays

Exponentially growing cells were collected after trypsinization and centrifuged at $1000 \times g$ for 5 min. The resulting cell pellets were resuspended in fresh media and counted on a hemocytometer. Between 100–2000 cells were plated into small (60 mm) petri dishes and incubated for 4 h to allow cell attachment to the surface. Cells were then treated with a respective concentration of lead as lead acetate for an additional 6 h. The cells were then washed and covered with 5 mL of fresh medium containing either media only or 50 μM LA enriched media and incubated for 7–10 d. After this incubation period, resulting cell colonies were stained with methylene blue and counted. Results reported from colony formation assays represent at least five separate experiments performed in triplicate. The colony efficiency (CE) was calculated as (Eqn. 1):

$$\mathrm{CE} = \frac{\text{Colonies counted}}{\text{Cells seeded}} \times 100 \qquad (1)$$

A survival curve was constructed by plotting the surviving fraction (number of colonies counted divided by the number of cells seeded times the colony efficiency of the control) from the lead only and LA + lead groups versus lead concentration.

Oxidative stress studies

Cells (50×10^4 cells/flask) were plated into flasks and incubated for 4 h to facilitate attachment. Cells were then treated with lead, LA and superoxide dismutase (SOD) + CAT as indicated below: The control group was incubated in the basic media for 24 h and the Pb only group was incubated with a media containing 500 μM lead as lead acetate for 20 h. At the end of that period, the media was changed and cells were incubated for an extra 4 h in a plain media. Cells in the Pb + SOD + CAT group were incubated with 500 μM lead for 20 h and the media was then removed and the cells were incubated in a fresh media including 200 U SOD + 200 U CAT for 4 more h. Cells from the Pb + LA group were treated with 50 μM LA containing the media for 4 h after incubation with 500 μM lead for 20 h. All solutions were always prepared immediately before use. At the end of the incubation time, the cells were trypsinized and resuspended in fresh media, homogenized, and analyzed for oxidative stress parameters.

Animal studies

All experiments were performed with Fischer 344 male rats weighing 75–100 g. The animals were housed in stainless steel cages in a temperature-controlled room (22°C) with a 12-h light:dark cycle. They were fed with standard rat chow (Purina rat chow). The animals were randomized into 4 groups. Group I ($n = 10$) served as the control and was given only standard rat chow and water for 6 weeks. Group II ($n = 10$) received 2000 ppm lead acetate in its drinking water for 5 weeks and, during the sixth week, this group received plain water. Group III ($n = 7$) received 2000 ppm lead acetate in its drinking water for 5 weeks and, during the sixth week, these animals received plain water and 25 mg/kg/d LA was administered by ip injection. LA was dissolved in ethanol:saline (1:1). Another group of three animals was fed with standard rat chow and plain water for the first 5 week and the vehicle (ethanol:saline) for LA was injected during the sixth week. Because the results from the vehicle only group did not show any significant difference from the control group, data from vehicle-injected animals is not included in the tables. Group IV ($n = 5$) received 2000 ppm lead for 5 weeks and was treated with 90 mg/kg/d succimer, a well-known chelator, during the sixth week. Lead levels in the blood and tissue of this group were used to evaluate the chelating effect of LA by comparing the results. At the end of the

sixth week, after overnight fasting, the animals were anesthetized with metofane and blood samples were collected via intracardiac puncture using heparin as an anticoagulant. Plasma and the buffy coat were removed by centrifugation for 10 min at 3000 rpm. The RBCs were washed three times with an equal volume of cold saline. The RBC samples were maintained at −70°C for MDA assays (not longer than 7 d) and at 4°C for CAT assay (not longer than 4 d) [16]. The tissue samples were also collected after sacrificing and kept at −70°C until analyzed.

Gutathione determinations were performed using the HPLC methodology developed by Winters et al. [17]. HPLC determination of MDA content was utilized as a marker of lipid peroxidation, according to Draper et al. method, [18] with minor modifications [19]. Catalase activity was determined spectrophotometrically [16] by using H_2O_2 as a substrate. Determination of G6PD activity was performed by using a spectrophotometer, as detailed in Tietz [20] where glucose-6-phospate and $NADP^+$ were used as substrates. Protein levels were estimated by the method of Bradford [21] using concentrated Coomassie Blue (Bio-Rad). Hemoglobin contents of the RBC samples were measured spectrophotometrically, as detailed by Tietz [20]. Lead levels in tissue and blood were assayed by atomic absorption spectroscopy (Varian SpectrAA) by the CDC-certified analytical laboratory at the Springfield-Greene County Department of Public Health, Springfield, MO, USA.

Statistical analysis

The nonparametric Mann-Whitney U-test was used to analyze the significance of the differences between the control and experimental groups.

RESULTS

Cell culture studies

Colony formation. Figure 1 represents the survival curve, generated by plotting the survival fractions of cells treated with lead in the presence or absence of LA against increasing concentrations of lead. Incubation of CHO cells with lead did inhibit colony formation in a concentration-dependent manner. Seventy percent inhibition of colony formation was accomplished with 500 μM lead acetate. Administration of 50 μM LA after lead treatment opposed the inhibitor effect of lead on survival fractions.

Glutathione levels. Table 1 displays the results of the GSH measurements of cells, after treatment to lead, in the presence or absence of LA, "SOD + CAT." Lead-

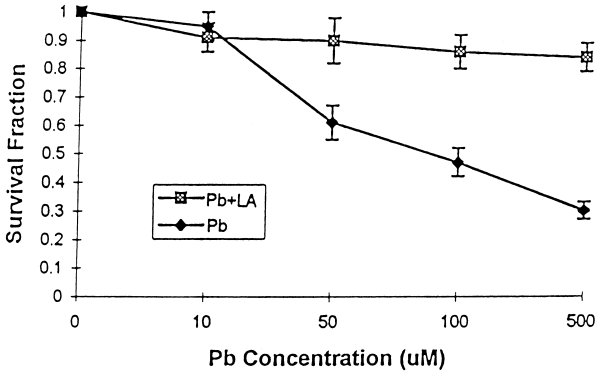

Fig. 1. Survival curve of CHO cells exposed to Pb in the presence or absence of 50 μM LA.

treatment diminished GSH levels that, then, notably increased to the control levels with "SOD + CAT" incubation. Lipoic acid treatment, after lead treatment, greatly increased GSH levels (207% of the lead-treated group).

Malondialdehyde levels. Malondialdehyde levels of cells from the control and treated groups are shown in Table 1. MDA levels of lead-exposed cells were 45.5 ± 5.8 nmol/100 mg protein that represents 205% of the levels in the control group. Incubation with "SOD+CAT," and LA was efficient in diminishing increased MDA levels due to lead treatment.

Catalase activity. Table 1 shows the CAT activity of cells from the control and treated groups. Lead treatment induced a statistically significant increase in CAT activity ($p < .05$). Cells treated with LA, after lead treatment, exhibited 19.0 U/mg protein CAT activity that was significantly lower than that of lead-treated cells ($p < .05$).

Table 1. Selective Oxidative Stress Parameters of Lead-Exposed CHO Cells in the Presence and Absence of LA or SOD + CAT

	GSH (nmol/mg protein)	MDA (nmol/100 mg protein)	Catalase Activity (U/mg protein)
Control	41.4 ± 3.2	22.6 ± 3.0	16.5 ± 5.3
Pb only	31.5 ± 1.5*	45.5 ± 5.8[§]	26.2 ± 3.3[‖]
Pb + SOD + CAT	42.0 ± 6.2[†]	22.7 ± 5.5[†]	—
Pb + LA	66.2 ± 2.3[‡]	18.7 ± 5.0[†]	19.0 ± 3.5[†]

All values represent mean ± SD of three through five experiments.
* $p < .005$, compared to the corresponding value of control group.
[†] $p < .05$, compared to the corresponding value of lead group.
[‡] $p < .01$, compared to the corresponding value of lead group.
[§] $p < .01$, compared to the corresponding value of control group.
[¶] $p < .05$, compared to the corresponding value of control group.

Table 2. Selective Oxidative Stress Parameters of Red Blood Cells From Lead-Treated Fischer 344 Rats

	Control	Pb	Pb + LA
GSH (nmol/g Hb)	24.0 ± 1.0	17.4 ± 1*	20.8 ± 2.5†
Catalase (U/g Hb)	98 ± 33	167 ± 14*	121 ± 30†
MDA (nmol/g Hb)	39 ± 7	61 ± 20*	33 ± 5†
G6PD (U/g Hb)	21.5 ± 1.1	26.2 ± 1.5*	23.0 ± 2.4†

All values represent mean ± SD for 5–10 samples.
* $p < .01$, compared to the corresponding value of control group.
† $p < .05$, compared to the corresponding value of lead group.

Table 4. Blood and Tissue Lead Levels From Fischer 344 Rats

	Control	Pb only	Pb + LA	Pb + Succimer
Blood lead levels (μg/dL)	0.2 ± 0.5	36.4 ± 4.4*	28.7 ± 4.1	2.0 ± 1.0†
Brain lead levels (ppb)	0.15 ± 0.07	1.4 ± 0.5*	1.1 ± 0	0.3 ± 0.1†
Kidney lead levels (ppb)	0.45 ± 0.4	10.5 ± 1.6*	9.5 ± 0.6	2.0 ± 0.6†

All values represent mean ± SD for 5–10 samples.
* $p < .001$, compared to the corresponding value of control group.
† $p < .005$, compared to the corresponding value of lead group.

Animal Studies

GSH levels. Lead treatment caused significant decreases both in RBCs ($p < .01$) and brain ($p < .05$) GSH levels. Administration of LA, after lead treatment, was effective in increasing the diminished GSH levels both in the RBCs (Table 2) and brain (Table 3). On the other hand, the increase in kidney GSH levels, that was observed in lead-treated animals ($p < .005$), was still high after LA treatment (Table 3).

Malondialdehyde levels. Malondialdehyde levels were significantly elevated in the RBCs (Table 2, $p < .01$), brains (Table 3, $p < .05$) and kidneys (Table 3, $p < .05$) of lead-treated rats. Administration of LA significantly decreased MDA levels in all three tissues.

Catalase activity. Pb treatment induced increases in CAT activity both in RBCs (Table 2, $p < .01$) and kidneys (Table 3, $p < .05$). The enzyme activity reverted to the control levels in both RBCs (Table 2) and kidneys of LA-treated animals, after lead-treatment (Table 3). Brain CAT activity was undetectable.

G6PD activity. Activity of G6PD was measured in the RBCs of the control, lead-treated and LA-treated rats. Table 2 shows the results. Enzyme activity increased considerably with lead treatment ($p < .01$). Furthermore, LA administration to lead-treated rats decreased the G6PD activity ($p < .05$).

Blood and tissue lead levels. Table 4 contains the lead levels in the blood and tissues of the control, lead-treated, LA-treated and succimer-treated rats. Blood, brain and kidney lead levels were profoundly elevated with lead treatment ($p < .001$). Succimer, a well-known chelating agent, significantly removed lead from the blood and tissues mentioned above ($p < .005$). No significant decreases in blood and tissue lead levels were obtained by LA administration.

DISCUSSION

Lead is known to have toxic effects on several biologic systems with the central nervous system, kidneys, and the hematologic and reproductive systems as the main targets. The mechanisms by which lead causes its deleterious effects have yet to be determined. In recent studies, however, some toxic effects of lead have been attributed to lead-induced oxidative stress [5,7,10]. Results from the present study testify to enhanced MDA content in lead-treated CHO cells and rats. The peroxidation of membrane phospholipids eventually leads to loss of membrane integrity and,

Table 3. Effect of LA on Brain and Kidney Oxidative Stress Parameters of Lead-Treated Fischer 344 Rats

	Brain		Kidney		
	GSH (nmol/mg protein)	MDA (nmol/100 mg protein)	GSH (nmol/mg protein)	MDA (nmol/100 mg protein)	CAT (U/mg protein)
Control	18.4 ± 1.4	15.0 ± 1.8	0.07 ± 0.04	7.2 ± 1.4	0.14 ± 0.04
Pb only	16.1 ± 1.5*	22.0 ± 0.5*	0.52 ± 0.20†	10.0 ± 0.8*	0.26 ± 0.01*
Pb + LA	20.6 ± 1.1‡	19.4 ± 1.5§	0.61 ± 0.10	8.4 ± 1.5§	0.19 ± 0.01§

All values represent mean ± SD for 5–10 samples.
* $p < .05$, compared to the corresponding value of control group.
† $p < .005$, compared to the corresponding value of control group.
‡ $p < .001$, compared to the corresponding value of lead group.
§ $p < .05$, compared to the corresponding value of lead group.

finally, to cell death. Our results from studies of CHO cells indicate that lead treatment inhibits cell survival and increases lipid peroxidation. Treatment of SOD + CAT increased the number of cells and reversed the effects of lead on oxidative stress parameters suggesting ROS as a possible contributor to the cell damage that occurred. The increases in lipid peroxidation in lead-treated CHO cells and rats were accompanied by alterations in their antioxidant defense systems, including decreased GSH levels and increased CAT and G6PD activity.

The assumption of the possible role of disrupted prooxidant/antioxidant balance in lead toxicity prompted us to explore whether antioxidants abate this toxic effect of lead. We previously suggested thiol supplementation to restore the redox status of cells after treatment to lead. *N*-acetylcysteine was proposed for this purpose; its effects on lead-induced oxidative damage were shown to be beneficial [22]. On the other hand, LA and DHLA seem to be an ideal antioxidant couple because of their several properties. They: (i) have the ability to scavenge some reactive species; (ii) can regenerate other antioxidants such as vitamins E and C, and GSH from their radical or inactive forms; and (iii) both have metal chelating activity [12,23]. It is also suggested that LA has a considerable advantage over *N*-acetylcysteine in opposing GSH loss, because LA is effective in a micromolar range while, millimolar *N*-acetylcysteine is needed for a similar effect [24]. The capability of LA to cross the blood-brain barrier [25] is also an advantage because the brain is an important target for lead poisoning. In light of this, the present study was undertaken to determine the beneficial effects of LA on lead-induced oxidative stress.

The current data display diminished lipid peroxidation in LA-treated CHO cells and rats that have been treated with lead. Lipid peroxidation was determined by examining the levels of MDA, a byproduct of the peroxidation process. Several studies have shown that both LA and DHLA scavenge ROS and chelate transition metals [15, 26,27]. Therefore, it is plausible to assume that they inhibit the lipid peroxidation process that is known to be triggered by ROS and transition metals. Furthermore, incubating lead-treated CHO cells with LA resulted in considerably increased cell survival along with attenuated lipid peroxidation.

Further evidence of the efficacy of LA in relieving lead-induced oxidative stress includes the increase in GSH content and decreases in both CAT and G6PD activity in lead-treated cells or animals after LA administration. Previous studies reported elevated GSH levels in murine neuroblastoma and melanoma cells [28] and in human T-lymphocyte Jurkat cells [24] after incubation with LA. The beneficial effects of LA on animals subjected to oxidative stress by radiation were documented by Busse et al. [28] and on those subjected to cerebral ischemia by Panigrahi et al. [25]. Data from the current study indicates elevated GSH levels after LA administration to CHO cells and Fischer 344 rats challenged by oxidative stress via lead treatment. DHLA is known to reduce glutathione disulfide (GSSG) to GSH; however, elevations in GSH cannot be solely explained by reduction of GSSG, because GSSG is normally present at less than 10% of GSH concentrations [29]. LA could either mitigate GSH consumption by acting as an alternate ROS scavenger or increase GSH levels by stimulating its biosynthesis with an unknown mechanism. A recent hypothesis to explain how LA stimulates GSH biosynthesis came from Packer et al. [30]. They suggested that LA administration can induce increases in GSH levels by facilitating transport of cystine, the limiting factor in GSH synthesis, into the cells. Once LA is taken up by the cell it is immediately reduced to DHLA that is then released. The released DHLA induces a chemical reduction of extracellular cystine to cysteine. Cysteine can be taken up rapidly (10 times more) by the cells than cystine and can then be used in the biosynthesis of GSH.

G6PD is the first enzyme in the pentose phosphate pathway that provides most of the extramitochondrial nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) to cells. The pathway is more important for RBCs because RBCs lack mitochondria. Within the RBCs, NADPH-reducing equivalents are necessary for keeping GSH in its reduced form through the enzyme glutathione reductase. NADPH is also important for catalase activity, albeit as a co-factor and not as a substrate [31]. The most important output of the pathways regulation has been suggested to be the NADPH/$NADP^+$ ratio [32]. The turnover of the pathway is shown to increase under oxidative stress conditions where demand for NADPH increases [33]. Under oxidative stress conditions, formation of GSSG would be expected to increase during consumption of hydrogen peroxide via glutathione peroxidase. Glutathione disulfide will then be reduced to GSH by glutathione reductase using NADPH as a substrate. In the present study the increase in CAT activity in lead-exposed cells and animals may indicate further consumption of NADPH. Therefore, stimulation of G6PD activity in lead toxicity to compensate for increased utilization of NADPH seems plausible. Lipoic acid administration is further supported by this scenario. Lipoic acid treatment of animals receiving lead for 5 weeks returned G6PD activity to control levels, that can be explained by the decreased need for NADPH. LA may achieve this by acting as an alternative sulfhydryl nucleophile to GSH, thereby preventing its oxidation to GSSG in detoxification reactions against ROS. Likewise,

increased CAT activity, noted in lead-treated cells and animals, was found to be reversed by a 1-week treatment to LA, that might result in decreased NADPH utilization.

Despite its antioxidant effect, LA was also shown to form stable complexes with Mn^{+2}, Cu^{+2}, Zn^{+2} [34], as well as chelating cadmium and iron [15,27]. DHLA, rapidly formed by the reduction of LA in cells, has two sulphydryl groups that suggest a promising chelating effect for lead. Therefore, we investigated whether LA is capable of removing lead from the bloodstream and target organs. Although 25 mg/kg/d LA injections for 7 d, after treatment to lead (2000 ppm) for 5 weeks, resulted in a minor abatement in blood lead levels, no significant decreases were detected in lead levels in the brains or kidneys. A well-known chelator, succimer (that also possesses two sulfhydryl groups) returned lead levels in both the blood and tissue back to the control values.

The present study was not designed to elucidate whether LA or DHLA is responsible for the observed antioxidant effects, but to conclude that LA administration resulted in decreased lipid peroxidation and reversed alterations in antioxidant defense system components. Because oxidative stress is known to cause cell death due to the imbalance in prooxidant/antioxidant ratio, the cell survival would alter after lead treatment and LA treatment. This cause-effect relation is evidenced in LA-treated CHO cells that were oxidatively challenged by the lead treatment. Therefore it can be concluded that the increased cell survival in LA-treated cells along with improved oxidative stress parameters reflect the antioxidant action of LA in lead-treated cells. Results from the study of lead levels in blood, brains and kidneys suggest that the beneficial effects of LA on oxidative stress parameters are not related to its ability to remove lead from target cells but are associated with LA's potential for bolstering thiol antioxidant capacity. The promising antioxidant effects of LA make it a good candidate for therapeutic intervention of lead poisoning in combination with a chelator rather than as a sole agent. Further studies need to be made to determine the optimum dosages and duration for LA administration to obtain the greatest medical benefits in treating lead poisoning.

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ABBREVIATION LIST

CAT—catalase
CHO—Chinese hamster ovary
DHLA—dihydrolipoic acid
GSH—glutathione
G6PD—glucose-6-phosphate dehydrogenase
LA—α-lipoic acid
MDA—malondialdehyde
$NADP^+$—β-nicotinamide adenine dinucleotide phosphate
NADPH—β-nicotinamide adenine dinucleotide phosphate, reduced form
RBC—red blood cells
ROS—reactive oxygen species
SOD—superoxide dismutase