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Evaluation of malondialdehyde as an index of lead damage in rat brain homogenates

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Lipid peroxidation *in vitro* homogenates of brain was examined as sequela of lead toxicity. The levels of malondialdehyde (MDA) in homogenates of rat brain (1 ml, 5% w/v) treated with lead (50 μg) alone or in combination with ascorbic acid (100 μg), alphatocopherol (100 μg) or hydroquinone (100 μg) were evaluated. The levels of MDA were consistently evoked by lead in a dose-related manner. The toxicity of lead was further advanced by the action of the pro-oxidant drug ascorbic acid on the brain. However, the anti-oxidant drugs alphatocopherol and hydroquinone decreased the toxic effect of lead on the brain. These results clearly show that the enhanced lipid peroxidation may provide a basis of lead-induced neurotoxicity.

Keywords: lead, lipid peroxidation, pro-oxidant, anti-oxidant, rat brain

Introduction

The role of lipid peroxidation in living tissues has received considerable attention as a potential health hazard of exposure to certain metals. Lipid peroxidation, which is an exceedingly damaging process, has been known to occur via peroxidation of unsaturated fatty acids in all aerobic biological systems. Free radical damage to membrane phospholipids is an important factor in the development of neuropathologic and neurotoxicologic conditions. The ions of certain inorganic compounds, such as iron and copper, are powerful promotors of free radicals (Barber & Bernheim 1967). Also, other ions like cadmium, cobalt, mercury, nickel, tin and vanadium, including lead, are known to generate lipid peroxidation in target tissues of rodents (see Sunderman 1986). Increased lipid peroxidation and deformability of erythrocytes have been implicated with lead poisoning in rats (Levander *et al.* 1977). Lipid peroxidation and degradation of phospholipids have been observed in different areas of the brain of rats exposed to lead (Shafiq-ur-Rehman 1984). However, the molecular mechanisms whereby the trace metals initiate and/or propagate lipid peroxidation *in vivo* are conjectural. In the present study, an attempt has been made to examine the role of lead in the promotion of lipid peroxidation in the homogenates of rat brain subjected to pro-oxidant and anti-oxidant activity.

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Materials and methods

Lipid peroxidation assay

As previously described (Shafiq-ur-Rehman 1984), young adult rats, weighing 100–150 g, were killed by decapitation. Brain (retaining the cervical part) was rapidly removed and placed in a Petri dish on an ice bath. After carefully removing the meninges, the brain was weighed on a single pan electrical balance and immediately deep frozen until use.

The quantitative determination of malondialdehyde (MDA), an index of lipid peroxidation, was performed by the 2-thiobarbituric acid (TBA) reaction method (Shafiq-ur-Rehman 1984) as follows. Brain was homogenized in chilled 150 mM KCl using a teflon pestle to give a 5% w/v homogenate. One milliliter of brain homogenate was aerobically incubated at 37 °C in a water bath-cum-metabolic shaker (180 strokes min^{-1} of 2 cm amplitude) for 2 h. The solution was immediately cooled with tap water and the formation of lipid peroxidation was stopped by the addition of 1 ml of cold 10% w/v trichloroacetic acid. For zero-time samples, brain homogenate was taken with 1 ml of 10% w/v trichloroacetic acid in another test tube. The solution was

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thoroughly mixed and centrifuged at 2000 r.p.m. for 10 min. In another test tube, 1 ml of supernatant was taken and allowed to react with an equal volume of 0.67% w/v TBA (Sigma, St Louis, MO) for 10 min in a boiling water bath. This mixture was cooled with tap water and diluted with 1 ml of double-distilled water. The absorbance was recorded at 535 nm in a Beckman Model UV/VIS-DU-6 spectrophotometer. The results were expressed as nmol of MDA formed 30 min^{-1} (the molecular extension coefficient of MDA expressed as $E_{535} = 1.56 \times 10^5$; Utley *et al.* 1967).

Study of incubation time

The effect of aerobic incubation time on the formation of lipid peroxides was observed at 37°C in the control and lead (50 μg) treated brain homogenates. The brain homogenates were incubated with 50 μg lead at intervals of 10 min up to 4 h and assayed for MDA as described above.

Study of increasing concentration of lead

The formation of MDA was examined in the brain homogenates treated with different dose schedules of lead from 2 to 60 μg. A corresponding control set of experiments was also run and assayed for MDA.

Study of the effect of lead on lipid peroxidation under pro-oxidant and anti-oxidant systems

The toxic ability of lead as the promotor of lipid peroxidation in the brain was tested in pro-oxidant and anti-oxidant environments. The brain homogenates were incubated in the presence of the pro-oxidant, ascorbic acid (100 μg), as well as the anti-oxidants, alphatocopherol (100 μg) or hydroquinone (100 μg), in separate sets. For the interaction studies, the brain homogenates bearing the pro-oxidant or anti-oxidant system (concentration as above) were allowed to react with lead (50 μg). The concentration of 50 μg of lead was used since the maximum lipid peroxidation was observed with this dose. The production of lipid peroxidation was determined as described in the assay procedure; other conditions were kept same.

Statistical analysis

The statistical analysis of data was performed by Student's *t*-test. $P < 0.05$ was considered as significant.

Results

Effect of incubation time on lipid peroxidation

The molecular formation of MDA (i.e. lipid peroxidation) in homogenates of brain attained a linear relationship with aerobic incubation time until 90 min. Subsequently, the lipid peroxidation culminated to an equilibrium state when further aerobic incubation did not produce extra MDA in the brain homogenates until the end of the experiment, i.e.

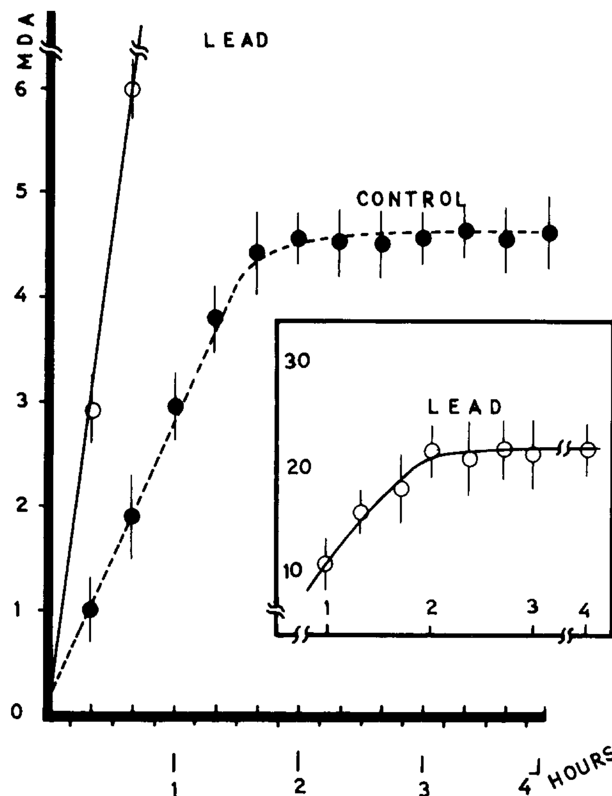

Figure 1. Effect of varying incubation periods on MDA formation (nmol 30 min^{-1}) without (●) and with 50 μg lead (○) in brain homogenates. The insert graph is an extended plot of lead-treated experiments. The experimental conditions were as described in the text. Each point represents the mean ± SE ($n = 3, 4$).

4 h. A similar pattern of the formation of lipid peroxidation has been shown in lead-treated brain homogenates. However, lead-induced production of lipid peroxidation was consistently much higher than the corresponding untreated controls (Figure 1).

Effect of increasing concentration of lead on lipid peroxidation

Lipid peroxidation was consistently evoked in homogenates of rat brain by lead in a dose-related manner up to the concentration of 45 μg. Subsequently, a plateau of lipid peroxidation (up to 20 nmol MDA) was obtained until the end of the experiment (i.e. 60 μg lead) (Figure 2).

Effect of lead on lipid peroxidation under the pro-oxidant system

Ascorbic acid elevated the levels of lipid peroxidation by 55% ($P < 0.01$) in homogenates of brain as compared with control. Lead damage was much higher as the levels of lipid peroxidation in brain homogenates were increased by 350% ($P < 0.001$) as compared with control. Addition of ascorbic acid to the brain homogenates containing lead enhanced lipid peroxidation by 25% ($P < 0.05$) as compared with the corresponding lead-treated brain homogenates (Figure 3).

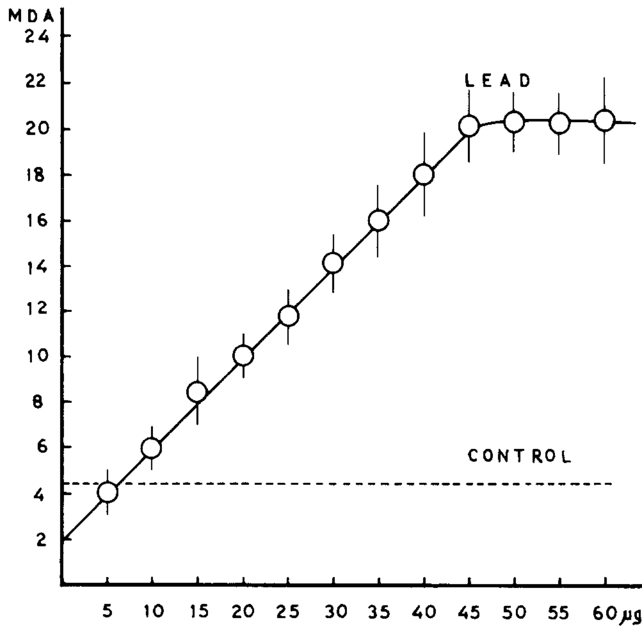

Figure 2. Effect of various concentrations of lead on the formation of MDA (nmol 30 min^{-1}) in brain homogenates. Rat brain homogenates were preincubated without (---) and with different concentrations of lead (○) for 2 h at 37°C. The experimental conditions were as described in the text. The bar represents standard error ($n = 3, 4$).

Effect of lead on lipid peroxidation under the anti-oxidation system

Alphatocopherol suppressed the formation of lipid peroxides in brain homogenates by 82% ($P < 0.001$) as compared with control. Hydroquinone also reduced the levels of lipid peroxidation in brain homogenates by 59% ($P < 0.001$) as compared with control. The toxic effect of lead is decreased in brain homogenates by alphatocopherol to 39% ($P < 0.01$) and by hydroquinone to 69% ($P < 0.001$) (Figure 3).

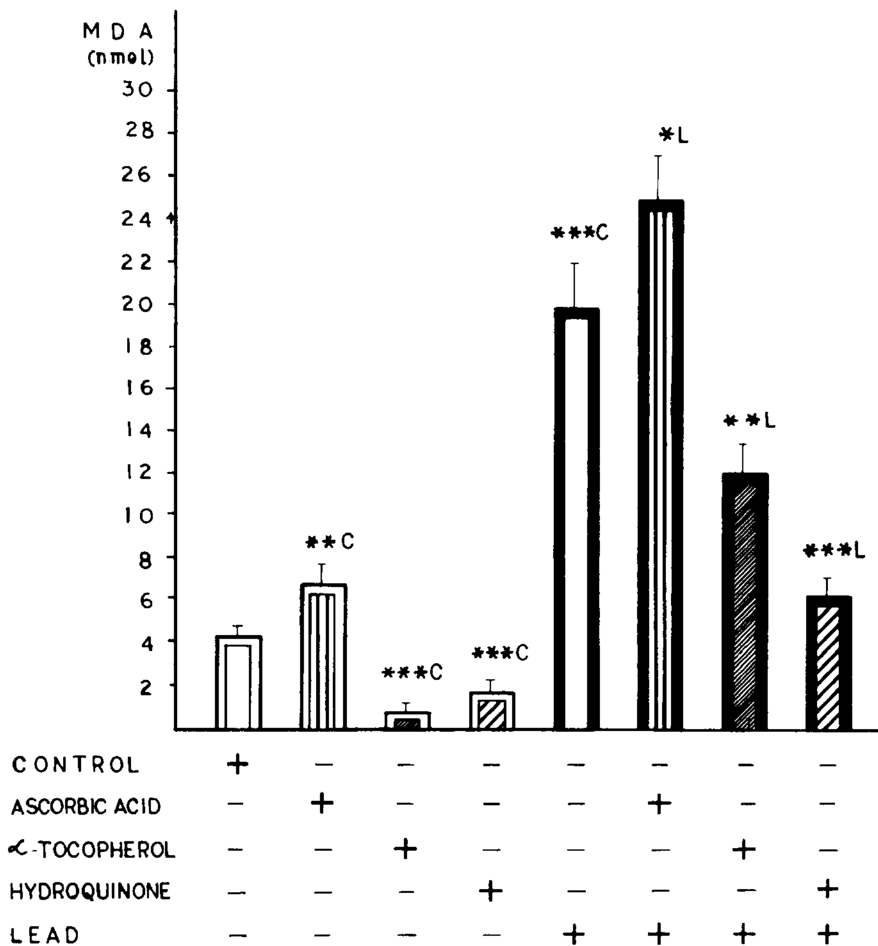

Figure 3. Effect of lead (50 µg), pro-oxidant (100 µg ascorbic acid) and anti-oxidants (100 µg alphatocopherol or hydroquinone) on the formation of MDA (nmol 30 min^{-1}) in brain homogenates. For detail see text. Each bar represents the mean ± SE of six separate experiments. Asterisks denote significant differences: C, from untreated control groups; L, from lead-treated groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) using Student's t-test.

Discussion

The evidence for tissue injury caused by certain toxins has been associated with enhanced fragility of various cell membranes. Phospholipids are the principal components of the membrane structure and function, and constitute the bulk of oxidative lipids. It has been shown recently that a mechanism by which free radicals produce cell damage operates through their oxidative effects on membrane phospholipids (Freeman & Crapo 1982). The double bonds in the skeleton of unsaturated fatty acids are oxidized during lipid peroxidation producing aldehydes (e.g. MDA, a TBA reactant). In a large number of tissues, it is now generally accepted that lipid peroxides play an important role in the pathogenetic process, particularly strong evidence having been generated by their involvement in liver (Poli *et al.* 1982), kidney (Cojocel *et al.* 1989) and brain (Uysal *et al.* 1989) toxicity. In the present findings, the formation of lipid peroxidation is enhanced in brain homogenates by lead exposure. In an earlier study, Shafiq-ur-Rehman (1984) also found that lead exposure enhanced the production of lipid peroxidation in certain brain areas. The degradation of phospholipids was also apparent in those areas of the brain which had elevated levels of lipid peroxidation and lead accumulation (Shafiq-ur-Rehman 1984).

Brain is particularly susceptible to peroxidation because of the presence of large amount of phospholipids containing polyunsaturated fatty acids. It is known that polyunsaturated fatty acids, especially arachidonic acid, are very sensitive to free radical attack (Bus & Gibson 1979). A close association among phospholipid methylation, arachidonic acid release from phospholipid and increased prostaglandin was found (Crew *et al.* 1980). Metabolically more active phosphatidylcholine, a major methylated phospholipid, is synthesized by the transmethylation pathway (Hirata & Axelrod 1980). The phosphatidylcholine is cleaved by Ca^{2+}-activated phospholipase A_2 to lyso-phosphatidylcholine and arachidonic acid. It has been shown that phosphatidylcholine contained most of the arachidonic acid (80%) incorporated into the phospholipids (Crew *et al.* 1980). A recent report suggested that lead exposure inhibited the process of phospholipid methylation in human erythrocyte membranes (Shafiq-ur-Rehman & Abdulla 1993). Also, the composition of phospholipids of the erythrocyte membranes is influenced by lead exposure, indicating a decrease in the levels of phosphatidylcholine (Shafiq-ur-Rehman & Abdulla 1993). It seems that the reduced composition of phosphatidylcholine might be due to inhibition by lead of the transmethylation pathway enzyme, methyltransferase, which in turn limited the synthesis of phosphatidylcholine from phosphatidylethanolamine. Further, the degradation of phosphatidylcholine into arachidonic acid and the metabolite prostaglandin by lead activation of phospholipase A_2 would then be facilitated. Since lead is known to inhibit sulfhydryl containing proteins and directly substitute for processes which are normally activated by calcium (Audesirk 1985), this raises the possibility that lead could activate phospholipase A_2-dependent processes. Consistent with the interpretation is the observation that the enhanced lipid peroxidation is associated with stimulation of arachidonic acid metabolism and prostaglandin formation in NaCl supplemented gastric mucosa (Takahashi *et al.* 1991).

It may be argued that the brain is a system of regional heterogeneity where morphological, functional and chemical differences play a vital role. There is evidence for the regional anomalies in the brain of lipid peroxidation, phospholipid composition and lead absorption (Shafiq-ur-Rehman 1984). However, little is known about the relationship between endogenous factors present in the brain tissues and lipid peroxidation. Ascorbic acid plays a significant role in the maintenance of the redox potential of brain, and perhaps in membrane repair and remodeling. Evidence obtained from the present study and elsewhere (Sharma 1977) suggests that ascorbic acid is concerned with the acceleration of non-enzymic lipid peroxidation in rat brain. Further, lead-induced increase in lipid peroxidation is aggravated by the addition of ascorbic acid to the brain homogenates. It suggests the role of pro-oxidant factors in the brain, which can advance the neurotoxic effect of lead.

Further examination of the toxic ability of lead as the promotor of lipid peroxidation in the brain and its protection by anti-oxidants, alphatocopherol and hydroquinone, was performed. Hydroquinone is known to inhibit the mitochondrial formation of lipid peroxidation in rat brain (Sharma & Krishna Murthi 1980). In this study, lipid peroxidation is inhibited by hydroquinone in brain homogenates. Furthermore, treatment with hydroquinone suppressed the toxic impact of lead on the brain. Lipid peroxidation initiation and propagation reactions can be prevented or terminated by the action of free radical scavengers or peroxidation quenching mechanisms. Every cell in the biological system has an extremely effective network of defense mechanisms against oxidative destruction. The most abundant powerful anti-oxidant in biological systems is alphatocopherol. Neuropathologic investigations support the role of alphatocopherol as an essential factor for maintenance of the integrity and stability of biological membranes by protecting the structural and functional phospholipids from oxidative deterioration (Tappel & Zalkin 1959). Alphatocopherol presumably reacts with free radical intermediates of lipid peroxidation and with peroxides, producing anti-oxidant properties. The present finding shows the alphatocopherol as a powerful inhibitor of lipid peroxidation in rat brain homogenates. Also, the toxic effect of lead is markedly decreased in the brain by alphatocopherol.

Other defense systems, however, which act against the formation of toxic lipid peroxides by removing or scavenging the initiating free radicals, such as $O_2^{-\bullet}$ and H_2O_2, are superoxide dismutase and catalase. These enzymes quench the production of secondary, more damaging free radicals, such as $^{\bullet}OH$. Arai *et al.* (1987) have shown less involvement of H_2O_2 and probably also $^{\bullet}OH$ as they observed a 7.5% inhibition of lipid peroxidation in brain by catalase under hyperoxia. McCay *et al.* (1976) reported that when microsomes were incubated in a peroxidizing medium, there was loss of unsaturated fatty acids and accumulation of

MDA, a product of chain cleavage. Again working with enzyme systems, the workers (McCay *et al.* 1976) concluded that selenium-dependent glutathione peroxidase could not reduce lipid peroxides when they were acylated, as in the case of biological membranes, and therefore the enzyme must act at the stage of reducing H_2O_2 and preventing initiation of lipid peroxidation. Grossman & Wendel (1984) supported the above contention that esterified fatty acids could not be the substrates for selenium-dependent glutathione peroxidase. This enzyme is found in the cytosol and the mitochondrial matrix of rat liver (Flohé & Schlegel 1971, Green & O'Brien 1970), and presumably the compartmentation is the same in other tissues and species. This would support the hypothesis of McCay *et al.* (1976) that the enzyme reduces H_2O_2 rather than lipid peroxides as the lipid peroxides would be situated in the hydrophobic region of the membrane and would not be accessible to selenium-dependent glutathione peroxidase activity. Valenzuela *et al.* (1989), however, found increases of catalase activity in cerebellum and cerebrum and selenium-glutathione peroxidase in cerebellum by lead in suckling rats. Inconsistently with the observations of Valenzuela *et al.* (1989), it has been reported that lead altered the defense properties of the normal cells as the activities of anti-oxidant enzymes catalase and glutathione peroxidase were found to be diminished in rat brain (Sandhir *et al.* 1994). This was accompanied with altered glutathione status, i.e. reduction of reduced-glutathione and accumulation of oxidized-glutathione levels.

Judging by the experimental observation obtained herein, it is evident that lead exerts its toxic impact on the brain by strongly inducing the formation of lipid peroxidation. The toxic effect of lead on the brain is decreased by alphatocopherol. Tissues of the central nervous system containing elevated levels of ascorbic acid and/or phospholipids as well as low levels of alphatocopherol are particularly susceptible to lipid peroxidation by lead exposure. As such, enhanced lipid peroxidation may provide a basis for lead induced brain toxicity.

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