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Effects of *N*-acetylcysteine and 2,3-dimercaptosuccinic acid on lead induced oxidative stress in rat lenses

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Abstract

Lead (Pb) is known to disrupt the pro-oxidant/anti-oxidant balance of tissues which leads to biochemical and physiological dysfunction. The present study investigated the effects of exposure on the redox status of the lenses of Fisher 344 rats and examined whether antioxidant or chelator administration reversed these changes. Animals were given 5 weeks of 2000 ppm Pb exposure followed by 1 week of either antioxidant, chelator or distilled water administration. Glutathione (GSH) and cysteine (CYS) levels decreased in the Pb-exposed group. *N*-acetylcysteine or 2,3-dimercaptopsuccinic acid (Succimer) supplementation following Pb intoxication resulted in increases in the GSH and CYS levels. Protein bound glutathione (PSSG) and cysteine (PSSC) increased following Pb exposure. In the Succimer-treated animals, the PSSG decreased significantly. The glutathione disulfide (GSSG) levels remained unchanged. Malondialdehyde (MDA) levels, a major lipid peroxidation byproduct, increased following Pb exposure and decreased following Succimer treatment. Our results suggest that antioxidant supplementation, as well as chelation, following Pb exposure may enhance the reductive status of lenses.

Keywords: Lens; Lead poisoning; NAC; Succimer; Glutathione; Protein bound sulfhydryls

1. Introduction

Lead (Pb) poisoning negatively impacts various body systems, particularly the hematopoetic, central nervous, and lenticular systems (Grubb et al., 1986; Peters et al., 1994; Ercal et al., 1996b). Pb impairs the lenticular system through photoreceptor cell degeneration, rod cell functional alterations, and possibly inhibition of the retinal Na^+-K^+ ATPase following postnatal Pb exposure (Fox and Rubenstein, 1989; Fox et al., 1991;

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Fox and Katz, 1992). Pb poisoning has also been proven to decrease the glutathione (GSH), glutathione-S-transferase (GST) and essential trace metals in rat lenses (Dwivedi, 1996).

The changes in GSH levels, as well as antioxidant enzyme activities implicate oxidative stress in the toxicity of Pb. Disruption of the reducing status of tissues leads to the formation of reactive oxygen species (ROS) which damage essential biomolecules such as proteins, lipids and DNA (Halliwell and Gutteridge, 1990; Stohs and Bagchi, 1995). Previous studies have demonstrated that, following an oxidative insult, disulfide bonding between lens proteins and cellular thiols such as GSH and cysteine (CYS) occurs prior to cataract formation (Lou et al., 1990). In addition, lipid peroxidation has been shown to be closely associated with protein S-thiolation following cataractogenesis (Geller et al., 1990). Therefore, the effect of Pb exposure on protein disulfide bonding and lipid peroxidation should be investigated to determine the extent of lenticular oxidative modification.

Our lab has previously proposed thiol supplementation to restore tissue redox status following Pb exposure (Ercal et al., 1996a). *N*-acetyl cysteine (NAC), the treatment of choice for paracetamol poisoning, is rapidly deacetylated to cysteine and thus may increase GSH levels by providing the substrate for the rate limiting step in GSH synthesis (Corcoran et al., 1985). NAC may also directly scavenge free radicals through its free sufhydryl group (Aruoma et al., 1989). Thus, NAC administration may possibly modify the lenticular redox status.

Currently, chelation is the treatment of choice in Pb poisoning. 2,3-dimercaptosuccinic acid (Succimer), a dithiol-containing metal chelator, was the first orally administered chelator approved by the FDA for treatment of childhood Pb poisoning (Mann and Tavers, 1991). It has been used in China and Russia since the 1950s, and was classified by the FDA as an 'investigational new drug' in 1989. Succimer may be used to reduce blood and tissue Pb levels, thus removing the pro-oxidizing agent which leads to alterations in the lens reductive status.

While the molecular mechanism of Pb toxicity is currently being investigated, relatively little attention has been given to inhibiting the effects of Pb poisoning on the reductive status of the lenticular system. This study was designed to test the hypothesis that the decreased GSH observed in lens during Pb poisoning may be reversed by administration of a thiol-containing antioxidant or by chelation. In addition CYS, glutathione disulfide (GSSG), protein bound GSH and CYS (PSSG and PSSC), as well as malondialdehye (MDA), were measured to further quantify the oxidative status of the lenses.

2. Materials and methods

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 obtained from Sigma (St. Louis, MO, USA).

All experiments were performed with 20 adult male Fisher 344 rats weighing $\approx 100-150$ g each. The animals, randomized into four groups, were housed in stainless steel cages, in a temperature-controlled room (25°C) with a 12 h light:dark cycle. They were allowed distilled water and standard Purina rat chow ad libitum. The control group received distilled water for 6 weeks and the Pb group received 2000 ppm Pb acetate in distilled water for 5 weeks followed by 1 week of distilled water. The remaining animals were randomized into two groups, each of which received 2000 ppm of Pb for 5 weeks. During the sixth week, the NAC group received 800 mg/kg per day of NAC in distilled water, and the Succimer group received 90 mg/kg per day of Succimer in distilled water. Following week 6, the animals were anesthetized with metofane and blood samples were collected with Pb-free needles via intracardiac puncture. The animals were sacrificed and the lenses were collected. The samples were maintained at -70°C until assayed.

2.1. Blood and lens Pb determination

Blood and lens Pb levels were determined by graphite furnace atomic absorption spectroscopy in the CDC certified Analytical Laboratory of the Springfield Public Health Department

2.2. Glutathione and cysteine determinations by HPLC

A new method of GSH determination was developed in this laboratory to analyze γ-glutamyl cycle intermediates (Winters et al., 1995). Each lens was placed in 1 ml of serine–borate buffer (100 mM Tris–HCl, 10 mM borate, 5 mM serine, 1 mM diethylenetriaminepentacetic acid, pH 7.0). The tissue was homogenized on ice for 3 min with 5 s intervals of homogenization and rest, and derivatized with *N*-(1-pyrenyl)-maleimide (NPM). This compound reacts with free sulfhydryl groups to form fluorescent derivatives. After appropriate dilution, 250 μl a sample was added to NPM (750 μl, 1 mM in acetonitrile). The resulting solution was mixed and incubated at room temperature for 5 min. To stop the reaction 3 μl 2M HCl were added . After filtration through a 0.2 μm acrodisc, the derivatized sample was injected onto a 3 μm C_{18} column in a reverse phase HPLC system. CYS has been determined with GSH since it also forms fluorescent NPM derivatives.

2.3. Glutathione disulfide determination by HPLC

The determination of GSSG was accomplished by adding 44 μl of water to 40 μl of an appropriate dilution of the homogenate. To the sample mixture, 16 μl of 6.25% 2-vinylpyridine in absolute ethanol were added, and the mixture was allowed to incubate at room temperature for 60 min, after which time 95 μl of a 2 mg/ml solution of NADPH and 5 μl of a 2 units/ml solution of glutathione reductase were added. The solution was subsequently mixed and an aliquot of 100 μl was immediately withdrawn. To this aliquot, 150 μl of HPLC grade water and 750 μl of 1.0 mM NPM were immediately added to perform the GSH derivatization, as mentioned above.

2.4. Protein bound glutathione and protein bound cysteine determination

The new GSH method developed in this laboratory was modified as follows to measure PSSG and PSSC. The lens was homogenized in 0.5 ml of serine borate buffer and the proteins were precipitated by addition of an equal amount of ice cold 10% trichloroacetic acid (TCA). After 5 min on ice, the samples were centrifuged and the protein pellet was washed twice with ice cold TCA. The washed protein pellet was resuspended in 0.5 ml of serine borate buffer. 0.35 ml of this suspension was incubated at 37°C for 30 min with 0.145 ml of water and 0.005 ml of 2-mercaptoethanol (14.3 M). Following incubation, the samples were centrifuged and the resulting supernatant was derivatized with NPM (0.1 ml supernatant, 0.15 ml water, 0.75 ml NPM).

2.5. HPLC system

The HPLC system (Shimadzu) comprised a model LC-10A pump, a Rheodyne injection valve with a 20 μl injection filling loop, and a model RF535 fluorescence spectrophotometer operating at an excitation wavelength of 330 nm and an emission wavelength of 375 nm. The HPLC column was 100 × 4.6 mm and packed with 3 μm particles of C_{18} packing material. The mobile phase was 35% water and 65% acetonitrile containing 1 ml/l acetic acid and 1 ml/l *o*-phosphoric acid. The NPM derivatives were eluted from the column isocratically at a flow rate of 0.5 ml/min. Quantitation of the peaks from the HPLC system was performed with a Chromatopac, model C-R601 (Shimadzu).

2.6. Malondialdehyde determinations by HPLC

To 0.350 ml of lens homogenate, 0.550 ml of 5% trichloroacetic acid (TCA) and 0.100 ml of 500 ppm butylated hydroxytoluene (BHT) in methanol were added. The sample was then heated in a boiling water bath for 30 min. After cooling on ice, the samples were centrifuged at 1000 × *g* for 10 min. The supernatant was mixed 1:1 with saturated thiobarbituric acid (TBA). The

sample was once again heated in a boiling water bath for 30 min. After cooling on ice, 0.50 ml of the sample was extracted with 1.00 ml of *n*-butanol and centrifuged to facilitate the separation of the two phases (Draper et al., 1993). The resulting organic layer was first filtered through a 0.45 μm acrodisc and then injected onto a reverse phase 250 × 4.6 mm 3 μm C_{18} column. The mobile phase for this system was composed of 30% acetonitrile and 0.6% tetrahydrofuran in 5 mM phosphate buffer (pH 7.0). The reaction complexes were eluted from the column isocratically at a flow rate of 0.70 ml/min. 1,1,3,3-Tetramethoxypropane was used as standard.

2.7. Protein determination

The Bradford method was used to determine the protein content of the tissue samples using concentrated Coomassie Blue (Bio-Rad) and optical density determinations at 595 nm (Bradford, 1976). A standard curve using bovine serum albumin was constructed. The homogenized tissues were subjected to appropriate dilutions before protein was determined.

2.8. Statistical analysis

Tabulated values represent means ± S.D.s of at least four data points. One-way analysis of variance (ANOVA) and the Student–Newman–Keuls multiple comparison test was used to analyze data from experimental and control groups. P values < 0.05 were considered significant.

3. Results

Pb treatment caused an increase in blood Pb levels (Table 1). NAC administration caused a significant decrease in blood Pb levels ($P < 0.05$). However, Succimer administration returned the blood Pb levels to near normal.

Pb exposure caused a significant decrease in the GSH and CYS levels of lens in the experimental groups as compared to the control group (Table 2). NAC or Succimer treatment following Pb exposure caused the GSH levels of lens to return to near control levels. Although the CYS levels increased following NAC or Succimer administration, the results were not statistically significant when compared to the control or the Pb-treated groups.

Table 1
Effect of NAC or Succimer on blood Pb levels[a]

	Blood Pb levels
Control	1.0 ± 0.0
Pb	34.8 ± 4.2[b]
Pb + NAC	25.3 ± 2.9[b,c]
Pb + Succimer	2.5 ± 1.0[c]

[a] *N*-acetylcysteine is abbreviated as NAC; units are μg/dl; $n = 4$–6 Fisher 344 rats per group.
[b] $P < 0.05$ as compared to control.
[c] $P < 0.05$ as compared to control.

The GSSG levels in all treatment groups were unchanged (Table 3). Pb exposure caused a significant increase in the PSSG and PSSC levels but NAC treatment did not result in any changes in the PSSG or PSSC levels. Succimer administration significantly decreased the PSSG levels while the PSSC levels remained unchanged.

Pb exposure resulted in a significant increase in the MDA levels in the lens (Fig. 1). NAC administration had no beneficial effect. However, a significant decrease in the MDA levels was observed following Succimer administration.

Table 2
Effect of NAC or Succimer on GSH and CYS of Pb-exposed lens[a]

	GSH	CYS
Control	9.84 ± 0.98[c]	1.04 ± 0.03[c]
Pb	4.61 ± 0.59[b]	0.69 ± 0.16[b]
Pb + NAC	7.40 ± 1.50[c]	0.85 ± 0.06
Pb + Succimer	8.29 ± 2.66[c]	0.88 ± 0.11

[a] Glutathione, cysteine, and *N*-acetylcysteine are abbreviated as GSH, CYS, and NAC, respectively; units are nmol/mg protein; $n = 4$–6 Fisher 344 rats per group.
[b] $P < 0.05$ as compared to control.
[c] $P < 0.05$ as compared to Pb.

Table 3
Effect of NAC or Succimer on GSSG, PSSG, and PSSC levels in Pb-exposed lens[a]

	GSSG	PSSG	PSSC
Control	2.69 ± 0.76	0.02 ± 0.16[c]	0.28 ± 0.16[c]
Pb	1.51 ± 0.95	0.18 ± 0.02[b]	0.57 ± 0.03[b]
Pb + NAC	2.46 ± 1.14	0.20 ± 0.06[b]	0.89 ± 0.44[b]
Pb + Succimer	1.50 ± 0.63	0.08 ± 0.01[b,c]	0.59 ± 0.12[b]

[a] Gluthathione disulfide, protein bound glutathione, protein bound cysteine, and *N*-acetylcysteine are abbreviated as GSSG, PSSG, PSSC, and NAC, respectively; units are nmol/mg protein; $n = 4$–6 Fisher 344 rats per group.
[b] $P < 0.05$ as compared to control.
[c] $P < 0.05$ as compared to Pb.

4. Discussion

Pb is a pervasive environmental pollutant whose mechanism of toxicity is currently being investigated. Pb inhibits the heart, brain (Nechay and Saunders, 1978), retinal and possibly the lenticular (Bender, 1994) Na^+–K^+ ATPase pump activity. In addition, Pb accumulates in the epithelial layer of the lens, possibly through anionic interaction at the plasma membrane (Grubb et al., 1986). Unlike transition metals, Pb does not readily undergo valence changes (Stohs and Bagchi, 1995). In cerebral synaptosomal suspensions, Pb acetate appeared to enhance the pro-oxidant properties of ferrous iron but not to initiate excess generation of ROS in the same system (Bondy and Guo, 1996).

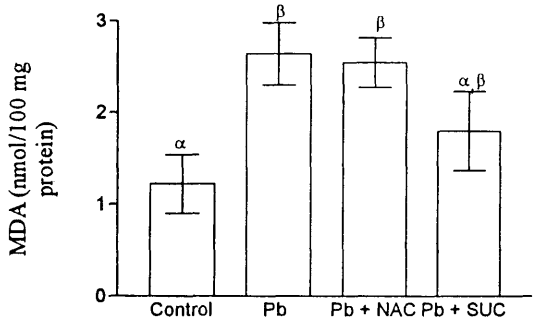

Fig. 1. $^{\alpha}P < 0.05$ as compared to Pb group; $^{\beta}P < 0.05$ as compared to control group; *N*-acetylcysteine and Succimer are abbreviated as NAC and SUCC.

While Pb may not directly generate ROS, evidence supports an indirect effect on antioxidant systems. Low level Pb exposure has resulted in decreased activity of GST (Dwivedi, 1996). In addition, Dwivedi (1987) has shown that low level Pb exposure decreases the GSH content of the rat lens by ≈50%. In the present study, the GSH levels in the lens were also decreased by 50% along with a 30% decrease in the CYS levels. The decline in GSH levels may be partially due to the observed decline in CYS availability following Pb exposure since CYS incorporation is a rate limiting step in GSH synthesis (Richmann and Meister, 1975). In addition, glutathione synthesis may also be inhibited due to enzyme active site modifications by Pb. However, oxidative conversion of the free sulfhydryl in GSH and CYS to a disulfide must account for part of the free thiol lost during an oxidative stress episode.

In tissues such as liver and brain, GSH is oxidized to GSSG in the presence of ROS resulting in a shift in the redox status of these tissues (Meister, 1981; Kaplowitz et al., 1985). However, previous studies have shown that GSSG does not accumulate in the lens during an oxidative stress episode (Lou and Dickerson, 1992; Willis and Schleich, 1996). Willis and Schleich (1996) proposed a mechanism for PSSG formation from GSSG that provides an alternative pathway for GSSG removal from the non-vascularized lens. Our study confirms that GSSG does not accumulate in the lens since no change in GSSG levels was observed following GSH depletion by Pb exposure.

PSSG and PSSC formation has been proposed as a possible protective mechanism guarding against protein–protein aggregation which occurs following oxidative stress prior to cataractogenesis (Willis and Schleich, 1996). H_2O_2-treated lenses demonstrated a loss in GSH, with an associated cortical haziness and swelling, as well as increases in PSSG and PSSC preceding protein–protein conjugation (Lou et al., 1995). Human age-related cataracts also have a significantly greater amount of protein–thiol mixed disulfides when compared to age-matched normal lenses (Lou et al., 1990). In the present study we have demonstrated that, following Pb exposure, the PSSG levels in lens

were dramatically increased and the PSSC levels were doubled. This indirectly confirms Dwivedi's hypothesis that Pb exposure may induce oxidative modification of the lens proteins which leads to functional damage in the form of cataractogenesis.

Alterations in the lens reductive status are also accompanied by increased lipid peroxidation during cataract formation (Babizhayev, 1996). In the membranes, lipid hydroperoxides may induce changes in permeability, order of lipid–protein environment and cause an uncoupling of the membrane bound Na^+–K^+ ATPase (Bender, 1994). The peroxidative damage to the lens cell membranes and biomolecules, induced by the lack of reductive detoxification of phospholipid hydroperoxides, has been proposed as a triggering mechanism of cataractogenesis (Babizhayev et al., 1992). MDA, a well-known lipid hydroperoxide decomposition product, increased in the lens following Pb exposure with concurrent declines in GSH and CYS. This further supports the hypothesis that Pb induces oxidative stress in the lens which may eventually lead to functional damage.

NAC, a thiol-containing antioxidant, is a poor scavenger of the superoxide anion and reacts slowly with H_2O_2. However, NAC is a powerful scavenger of HOCl and the hydroxyl radical (Aruoma et al., 1989). Thus NAC may act directly as a free radical scavenger or indirectly, following deacetylation, as a GSH precursor. In the present study, NAC administration following Pb exposure resulted in a significant increase in GSH levels in the lens. However, following NAC administration the CYS levels were intermediate between the control and Pb groups, resulting in no statistically significant changes in the CYS levels. It is possible that any additionally available CYS was rapidly converted to GSH. The PSSG, PSSC, and MDA levels were not significantly different from those in the Pb-exposed lenses. As noted previously, alterations in PSSG due to low level oxidative challenge may be reversed following oxidant removal (Lou et al., 1995). It is not surprising that NAC administration did not return these parameters to control levels since the pro-oxidant, Pb, is not completely chelated by NAC (Ercal et al., 1996a). This study demonstrated NAC's ability to return the GSH levels of Pb-exposed lenses to near control levels, further implicating oxidative stress as a possible factor in Pb toxicity.

Chelating agents are traditionally used to remove Pb from bones and soft tissues. Succimer is effective in lowering blood Pb levels in children with severe Pb poisoning (>45 μg/dl). Succimer also decreases the Pb burden of soft tissues such as blood, brain, and kidney (Mann and Tavers, 1991) and also removes Pb from bone (Pappas et al., 1995). In the present study, Pb chelation resulted in a partial restoration of the lens redox status as evidenced by increases in the GSH levels and decreases in the PSSG and MDA levels. Wang et al. (1997) proposed that PSSG was a better substrate than PSSC for thioltransferase, an oxidoreductase which reduces protein–thiol mixed disulfides following elimination of oxidative stress, since PSSG was more efficiently removed than PSSC following H_2O_2 exposure in cultured pig lenses. This possibly explains why, following Pb chelation, the PSSG levels decreased while the PSSC levels remained unchanged. We propose that chelation therapy at lower blood Pb levels (<45 μg/dl, as in this study) may have a beneficial effect upon the lens. However, following discontinuation of chelation therapy a subsequent increase in tissue and blood Pb burden associated with systemic Pb re-equilibration (Mann and Tavers, 1991) may result in a return to an oxidative imbalance in the lens.

The goal of this study was to examine the extent of oxidative stress in the rat lens following Pb exposure. We have demonstrated oxidative modification of protein sulfhydryl residues and lipids as well as confirming decreases in GSH levels following Pb administration. We have also shown that NAC or Succimer administration following Pb exposure can modify the observed decline in GSH levels of the lens. Since disturbance of the reductive status of the lens preceeds cataractogenesis, long term Pb exposure may be a risk factor for cataract development.

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