Effects of Pb Exposure in Cell Culture on ATPase Activity in Rat C6 Glioma Cells

Scott Monk
University Undergraduate Fellow, 1993-1994
Texas A&M University
Department of Biochemistry

APPROVED

Fellows Advisor E. D. Harris

Honors Director

Abstract

The effects of low level lead (Pb) exposure in culture for 3 days on the non-specific ATPase activity of rat C6 glioma cells was studied. Cells were incubated in media containing Pb acetate at a concentration of 10^{-6}M, 10^{-5}M, or 5 x 10^{-5}M. Cells were harvested, the plasma membranes were isolated, and an ATPase assay was performed, with activity being determined at the end of incubation by the level of inorganic phosphate present in the assay solution. Significant changes were not seen in ATPase activity at any Pb concentration when the ATPase assays were performed with ATP at 3mM; the average difference between the activity of controls and that of 10^{-5}M Pb-treated cells for 4 experiments was only 5% of the average control ATPase activity. When ATPase assays were performed at a range of ATP concentrations with cells treated with 10^{-6}M and 10^{-5}M Pb, double reciprocal analysis showed that the kinetics of the ATPase activity appeared altered by the Pb treatment: the K_m and V_{max} for the enzyme were decreased in the Pb treated cells. Measured values were: Controls - K_m= 0.13mM, V_{max}= 2.85 μmol P_i/mg protein/hr; 1μM Pb - K_m= 0.10mM, V_{max}= 2.62; 10μM Pb - K_m= 0.08mM, V_{max}= 2.42 Thus, the measured effect of Pb treatment in culture on measured ATPase activity appears mixed and dependent on ATP concentration: the ATPase activity is inhibited at ATP concentrations above 0.2mM, but stimulated at ATP levels below that. A future, more thorough investigation of this mixed inhibition is warranted.

Introduction

Astroglial cells in rats have been shown to function as a protective "lead sink," sequestering Pb to apparently protect the surrounding neurons from damage caused by the metal (Holtzman, *et al.*, 1984). With the discovery that, even in adult rats, the blood brain barrier does not prevent the entry of Pb into the brain (Bradbury and Deane, 1993), the protective action of the astroglial cells is viewed as even more important since it serves as the last line of defense before Pb reaches the neurons in the brain. Great interest has been taken in the study of how astroglial cells, both *in vivo* and *in vitro*, are able to take up Pb to higher concentrations than other cells, and what biochemical alterations eventually result in Pb toxicity.

In one such study, Tiffany-Castiglioni, *et al.* (1987) showed that immature astroglial cells, cultured in 100μM Pb medium for three days, took up Pb to a level 1300 times that of the untreated cells, and accumulated copper to a level 4 fold higher than the controls. This latter effect is very interesting in light of a recent report of the cDNA sequence for the protein thought to be inactive in Menkes' disease (Vulpe, *et al.*, 1993). Menkes' disease is a copper transporting defect in which the mucosa cells of the intestine accumulate Cu to high concentrations and do not release it into the bloodstream. The cDNA for the inactive protein appears to code for a Cu transporting ATPase. Several studies, using various tissues from

several species, have shown that when Pb is added during the ATPase assay procedure, [Na^+ + K^+]ATPase activity (Nechay and Saunders, 1978; Chanez *et al.*, 1988) and [Ca^{2+},Mg^{2+}]ATPase activity (Mas-Oliva, 1989) are inhibited , so this lends weight to a hypothesis that Pb causes an accumulation of Cu inside astrocytes by inhibiting a Cu-transporting ATPase, as coded for by the Menkes' CDNA.

For this project, I studied the effect of Pb in culture medium on the ATPase activity of rat C6 glioma cells. The ATPase activity in C6 glioma cells is not nearly as well studied as that of primary astrocytes, but there have been several studies of [Na^++K^+]ATPase activity of C6 gliomas (Folbergrova, *et al.*, 1989; Sheedlo, *et al.*, 1987), and this cell line has been shown to be a reasonably good model for primary astrocytes in culture (Kimelberg, 1974). Cell lines offer the advantages of faster growth rates and lower costs than primary cells, which was an important consideration in this project. This study attempted to compare the ATPase activity in cells cultured in medium containing Pb to the activity seen in control cells. The effects of ATP concentration in the ATPase assay was also studied in Pb treated and control cells.

Materials and Methods

Materials

Rat C6 glioma cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's minimum essential medium,

Ham's F-12, and chemical reagents were purchased from Sigma (St. Louis, MO). Fetal bovine serum was obtained from Gibco (Grand Island, NY). Tissue culture flasks were obtained from Corning Inc. (Oneonta, NY).

Cell Culture

All experiments were conducted with C6 cells from passages ranging from 5 to 14. The glioma cells were cultured in DMEM/Ham's F-12 medium with 10% fetal bovine serum in $75cm^3$ plastic canted neck flasks in 95% air / 5% CO_2 humidified atmosphere at 36°C. Cells were passed every 1-2 weeks using Puck's solution, and the medium was changed every 2-3 days. For each experiment, the cells were allowed to grow for 5 days after the last passage before Pb treatment was begun, at which time the cells were confluent. For the 1 and 3 day exposures, the media was not changed. Pb dilutions were made in DMEM/F-10, 10% FBS medium from an aqueous stock of $10^{-3}M$ Pb acetate for $10^{-5}M$ and $5 \times 10^{-5}M$ Pb media; for $10^{-6}M$ Pb medium, the stock solution was diluted to $10^{-4}M$ before adding to the medium. Control cells were given medium which was $10^{-5}M$ Na acetate.

Plasma Membrane Preparation

Cells were harvested from flasks by adding 1-2ml 30mM Tris-HCl, pH 7.3 with 1% PMSF to each flask and scrapping with a plastic policeman. Cells from four flasks were combined to give enough cells for each sample. Samples were kept at 0-4°C through out the plasma membrane preparation. Cells were collected by centrifugation for 6 minutes at 850 x *g*. The pellet was

resuspended in 6ml 0.33M sucrose, 10mM Tris-HCl (pH 7.3) and sonicated for 15 sec at 50W. The broken cells were then centrifuged at 850 x *g* for 15 min. The supernatant was centrifuged at 30,000 x *g* for 30 min. The pellet was resuspended in approx. 2ml 0.25M sucrose, 10mM Tris-HCl (pH 7.3) and applied to an 80%, 40%, 17% sucrose gradient, with all layers being approximately equal volume. The samples were then centrifuged at 150,000 x *g* for 40 min in an SW 40 rotor. The resulting turbid interface bands were removed from the top of the 17% layer and at the 17%/40% interface. Sufficient 0.25M sucrose solution was added to this material to reach a total volume of approx. 6ml. The samples were then centrifuged at 150,000 x *g* for 40 min. The resulting pellet was suspended in 0.1-0.2ml of the 0.25M sucrose solution, and the membrane samples were then transferred to plastic Eppi tubes and stored frozen at -20°C; the membranes were usually thawed and assayed the next morning, but they were never stored frozen for longer than 3 days.

ATPase Assays

The ATPase activity was assayed in 1ml assay solution consisting of 0.01%Triton X-100, 30mM Tris-HCl (pH 7.0), 50μl of membrane sample (approx. 50-200μg protein), and Na_2-ATP and $MgCl_2$ at a ratio of 1:1; the Na_2-ATP was prepared daily to prevent excessive hydrolysis. Samples were incubated for 30 min at 37°C, and the incubation was stopped by adding trichloroacetic acid to a final concentration of 5%. The amount of inorganic phosphate produced was determined by the method of Fiske and Subbarow (1925), with solutions of K-PO_4 used as standards. Protein

concentration was determined with a Pierce BCA Protein Assay, using bovine serum albumin as the standard.

Results

The C6 glioma cells were cultured in the presence of Pb for 1 or 3 days, with 3 days being the standard period for this project. Pb concentrations used in the DMEM/F-12 medium were 1, 10 and 50μM Pb acetate. ATPase assays were performed with isolated plasma membranes, and activity was determined by measuring inorganic phosphate released.

ATPase assays at 3mM ATP

For the ATPase assays conducted at 3mM ATP, there was no significant difference ($p >> .05$) in ATPase activity between Pb treated cells and control cells. For 3 day assays, the average ($n = 4$) ATPase activity for controls was 4.10 ± 1.61 μmol P_i/mg protein/hr, for 1 μM Pb treated cells ($n = 3$) it was 4.66 ± 3.05, and the average value for 10 μM Pb treated cells ($n = 4$) was 3.87 ± 1.71. ("n" represents measurements from separate experiments, not replicates taken during the same assay). The ATPase activities varied substantially from one assay to the next (>250%), possibly because of slightly different culturing conditions from one group to the next. Despite this variance of measured ATPase activity between experiments, the average difference between controls and 10μM Pb treated cells was only 0.22 μmol P_i/mg protein/hr (5% of the average control value for ATPase activity). It is interesting to note that in one experiment in which the cells were frozen at -80°C for 11 days

ATPase Activity After 3 Day Exposure To Pb
Assayed With 3mM ATP
(μmol P_i/mg protein/hr)

	Controls	1 μM Pb	10 μM Pb	50 μM Pb
Mean	4.10 ± 1.61	4.66 ± 3.05	3.87 ± 1.71	3.42 (n=1)
Average Deviation From Controls	----------	+15.0%	-5.4%	-4.2%

Differences between Pb treated cells and controls (denoted as negative if control values were higher), were taken for each experiment, summed, and averaged for the number of values obtained.

before the membranes were isolated, the measured ATPase activity for the 10μM Pb treated cells was 50% greater than the activity measured for the controls (9.12 vs. 6.07 μmol P_i/mg protein/hr). This experiment was not included in the data analysis because the freezing of the cells differed from the protocol used with the other cells. It would be interesting to follow up this finding to determine if the freezing and thawing affects the ATPase activity of control and Pb treated cells differently. There was also no substantial effect seen even when cells were treated with medium containing 50μM Pb, either at 1 or 3 days (< 7% difference from controls), though this assay was performed only once.

ATPase assays conducted with varying ATP concentrations

Assays conducted over a range of ATP concentrations showed a slight mixed inhibition in which activity in cells exposed to Pb

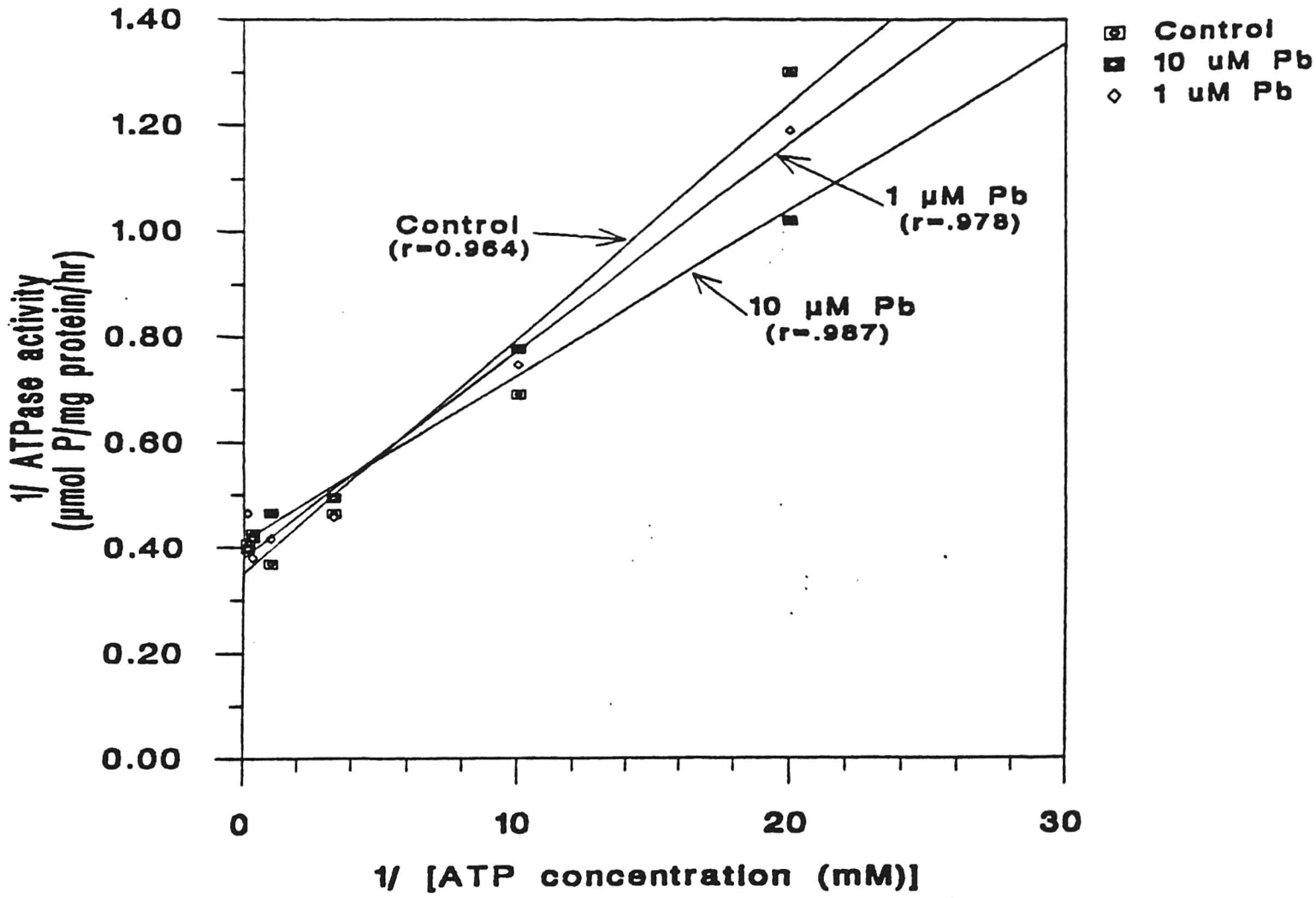

Fig. 1

Assays were performed with ATP at 6, 3, 1, .3, .1,and 0.05 mM. Assays were done in duplicate for each sample, and data points represent the averages of these duplicates.

n = 2	K_m (mM)	V_{max} (μmol P_i/mg protein/hr)
Controls	0.127	2.85
1 μM Pb	0.103	2.62
10 μM Pb	0.075	2.42

was lower than controls at ATP concentrations above 0.2mM, but greater than controls at ATP levels lower than 0.1mM (see double reciprocal plot). Double reciprocal analysis showed that the Pb treated cells had lower Km and lower Vmax than the controls. Since the assays were only done in duplicate, it is not possible to do valid statistical analysis of this data.

Discussion

My results indicate that 1 and 10μM Pb treatment of whole cell culture (C6) for 3 days has no significant effect on ATPase activity as measured in isolated plasma membranes when assayed at 3mM ATP. However, there does appear to be a change in the kinetics of the ATPase activity when the data are analyzed by double reciprocal over a range of ATP concentrations. This would mean that the effects of Pb exposure in culture on measured ATPase activity could be inhibiting or stimulating under the experimental conditions, depending on the level of ATP present. For the cells treated at 10μM Pb, the K_m value was 41% less than the that obtained for the controls. The V_{max} was also decreased, but less markedly. This suggests that the measured ATPases in the Pb treated cells were binding ATP with greater affinity than the control cells. The effect observed at the 1μM level is slight and possibly not significant, however, and this is the level at which many of the biochemical effects of Pb toxicity are observable. More study of the C6 ATPase activity under the conditions described needs to be conducted to determine if the effect is significant.

There are few studies with which to make close comparisons of these data. All published research that I found studied the effects of direct inhibition of ATPase activity by Pb (i.e. the Pb was added directly to the ATPase assay, rather than to cells in culture). The data obtained in this project were not a result of direct enzyme-inhibitor kinetics, but rather caused by the effects of Pb in a whole cell system carried over into assay by

isolated plasma membranes. Also, most published studies were investigating the effects of Pb on the [Na^++K^+]ATPase activity; the ATPase activity measured in this project did not include [Na^++K^+]ATPase activity, since neither Na^+ nor K^+ was added to the assay. Also, Dr. Yong Chang Qian of our lab has found that ouabain has no effect on the overall ATPase activity of the C6 glioma cells used in this experiment. Ouabain sensitivity is used to determine the amount of ATPase activity attributable to [Na^++K^+]ATPase (Kimelberg, 1974; Schimmel, *et al.*, 1973; Sheedlo, *et al.*, 1987). Therefore, we can be satisfied that the effects measured here were on ATPases other than [Na^++K^+]ATPase.

In their studies of [Na^++K^+]ATPase activity in tissues of several species, Nechay and Saunders (1978) found that the concentration for 50% inhibition (I_{50}) for Pb added to cell homogenate (with 3mM ATP) from rat brain cortex was 4×10^{-5}M. They also found that ouabain-resistant ATPase activity was 10-100 times more resistant than the [Na^++K^+]ATPase activity, indicating that significant inhibition of ATPase activity, other than [Na^++K^+] dependent, may require Pb concentrations well out of the physiologically-relevant range (less than 10^{-5}).

Further study of ATPase activity in C6's is needed to determine they effects of Pb over time. A time course study to determine the effects of Pb on ATPase activity over the first few hours of Pb exposure would give important knowledge of how quickly the ATPase activity recovers from an initial inhibition. Also, comparisons of inhibition seen in C6 glioma cells should be compared to similar experiments conducted with primary astrocytes

to determine validity of this cell line as a model for primary astrocytes in these experiments.

REFERENCES

Bradbury, M. W. B., and R. Deane, Permeability of the Blood-Brain Barrier to Lead, NeuroToxicology, 14 (1993) 131-136.

Chelly, J., Z. Tumer, T. Tonnesen, A. Petterson, Y. Ishikawa-Brush, N. Tommerup, N. Horn, and A. P. Monaco, Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein, Nature Genetics, 3 (1993) 14-15.

Engle, M. J., and J. J. Volpe, Glutamine synthetase activity of developing astrocytes is inhibited in vitro by very low concentrations of lead, Developmental Brain Research, 55 (1993) 283-287.

Fagan, M. J., and M. H. Saier, Jr., P-type ATPases of Eukaryotes and Bacteria: Sequence Analyses and Construction of Phylogenetic Trees, Journal of Molecular Genetics, 38 (1994) 57-99.

Folbergrova, J., V. Lisa, and V. Mares, Na^+, K^+ - ATPase Activity in Cultured C_6 Glioma Cells,

Glynn, I. M., and S. J. D. Karlish, Occluded Cation in Active Transport, Annual Review of Biochem., 59 (1990) 171-205.

Holtzman, D., J. E. Olson, C. DeVries, and K. Bensch, Lead Toxicity in Primary Cultured Cerebral Astrocytes and Cerebellar Granular Neurons, Toxicology and Applied Pharmacology, 89 (1987) 211-225.

Jencks, W. P., How Does a Calcium Pump Pump Calcium? Journal of Biological Chemistry, 264 (1989) 18855-18858.

Kimelberg, H. K., Active Potassium Transport and [Na^+ + K^+]ATPase Activity in Cultured Glioma and Neuroblastoma Cells, Journal of Neurochemistry, 22 (1974) 971-976.

Mas-Oliva, J., Effect of lead on the erythrocyte (Ca^{2+}, Mg^{2+})-ATPase activity, Molecular and Cellular Biochemistry, 89 (1989) 87-93.

Mercer, J. F. B., J. Livingston, B. Hall, J. A. Paynter, C. Begy, S. Chandrasekharappa, P. Lockhart, A. Grimes, M. Bhave, D. Siemieniak, and T. W. Glover, Isolation of a partial candidate gene for Menkes disease by positional cloning, Nature Genetics, 3 (1993) 20-22.

Nechay, B. R., and J. P. Saunders, Inhibitory characteristics of lead in sodium- and potassium- dependent adenosinetriphosphatase preparations derived from kidney, brain, and heart of several species, Journal of Toxicology and Environmental Health, 4 (1978) 147-159.

Schimmel, S. D., C. Kent, R. Bischoff, and P.R. Vagelos, Plasma Membranes from Cultured Muscle Cells: Isolation Procedure and Separation of Putative Plasma-Membrane Marker Enzymes, Proc. Nat. Acad. Sci. USA, 70 (1973) 3195-3199.

Sheedlo, H. J., S. Starosta-Rubinstein, T. J. Desmond, and G. J. Siegel, (Na^+ + K^+)-ATPase in C_6 glioma and rat cerebrum, Journal of Neuroimmunology, 15 (1987) 173-184.

Sierra, E. M., T. K. Rowles, J. Martin, G. R. Bratton, C. Womac,

and E. Tiffany-Castiglioni, Low level lead neurotoxicity in a pregnant guinea pigs model: neuroglial enzyme activities and brain trace metal concentrations, Toxicology, 59 (1989) 81-96.

Simons, T. J. B., Lead-Calcium Interactions in Cellular Lead Toxicity, NeuroToxicology, 14 (1993) 77-86.

Tiffany-Castiglioni, E., E. M. Sierra, J. Wu, and T. K. Rowles, Lead Toxicity in Neuroglia, NeuroToxicology, 10 (1989) 417-444.

Tiffany-Castiglioni, E., J. Zmudzki, J. Wu, and G. R. Bratton, Effects of Lead Treatment on Intracellular Iron and Copper Concentrations in Cultured Astroglia, Metabolic Brain Disease, 2 (1987) 61-78.

Vulpe, C., B. Levinson, S. Whitney, S. Packman, and J. Gitschier, Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase, Nature Genetics, 3 (1993) 7-10.