SELENIUM INDUCED GLUTATHIONE PEROXIDASE ACTIVITY IN MOUSE NEUROBLASTOMA CELLS

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Summary

Mouse neuroblastoma cells grown in medium containing 10 percent fetal bovine serum have negligible amounts of glutathione peroxidase activity. Introduction of selenite to the medium to produce a concentration of 600 nM resulted in a 30-fold increase in the enzyme activity. This increase is directly proportional to the concentration below 60 nM and levels off at concentrations above this value. Selenate produces no increase in enzyme activity when present alone nor does it inhibit induction when present with selenite. Tellurite produces no increase in enzyme activity when present alone but does inhibit induction when present with selenite.

Selenium has been shown to be an important nutrient for cells in tissue culture. McKeehan et al. (1) demonstrated an increase in the colony size of WI-38 cells in tissue cultures with added Se and Guilbert et al. (2) found that some of the serum requirement for colony formation by haemopoietic cells from bone marrow could be replaced by Se. Both groups speculate that the growth-promoting properties of Se may result from its incorporation in the selenoenzyme glutathione (GHS) peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) (3). As part of an investigation of the antioxidant capacity of mouse neuroblastoma cells, we assayed their GSH peroxidase activity and found that the initially low enzyme activity was very sensitive to the concentration of Se in the medium.

Materials and Methods

Mouse neuroblastoma cells (clone N18) were grown in Dulbecco's modified Eagle's medium (4) without antibiotics or antimycotics (KB Biological, Inc.) supplemented with 10% fetal bovine serum in a humidified atmosphere of 10% CO_2-90% air at 37°C in Falcon 75 cm^2 flasks. Supplementation of the media was achieved by adding appropriate aliquots of 150x stock solutions of neutralized selenious acid ("specpure" grade from Johnson Matthey Chemicals Ltd.), sodium selenate (Pfaltz & Bauer Inc., Stamford, CT) and sodium tellurite (Pfaltz & Bauer Inc., Stamford, CT) to each selected flask. Control

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Table I Effect of Selenite, Selenate and Tellurite on Glutathione Peroxidase Activity

Days in Culture	Treatment	Specific Activity Units/mg[a]
2	Serum (A)[b]	N.D.[c]
	Serum (A) + 30 nM Se	0.97
4	Serum (A)	0.21
	Serum (A) + 30 nM Se	9.1
6	Serum (A)	1.15 ± 0.58[d]
	Dialyzed Serum (A)	0.61
	Serum (A) + 15 nM Se	6.6
	Serum (A) + 30 nM Se	13.8 ± 1.6
	Serum (A) + 60 nM Se	26.5 ± 7.2
	Serum (A) + 300 nM Se	33.6
	Serum (A) + 600 nM Se	34.6
	Serum (A) + 6 μM Se	29.4
	Serum (A) + 60 μM Se	29.3
	No Serum[e]	N.D.
	No Serum + 30 nM Se	14.0
	Serum (B)	1.4
	Serum (B) + 30 nM Se	15.1
	Serum (C)	2.8
	Serum (C) + 30 nM Se	20.0
	Serum (A) + 30 nM Se VI[f]	1.46
	Serum (A) + 60 nM Se VI	1.72
	Serum (A) + 30 nM Se + 30 nM Se VI	13.7
	Serum (A) + 30 nM Te[f]	0.70
	Serum (A) + 30 nM Se + 30 nM Te	5.8
	Serum (A) + 30 nM Se + 60 nM Te	2.4

[a]Enzyme units are expressed as nanomoles of NADPH oxidized per minute at 24°C. The non-enzymatic rate has been subtracted. The average non-enzymatic rate is 0.52 nanomoles of NADPH oxidized per minute.

[b]Growth medium consists of 10% v/v fetal bovine serum plus 90% v/v Delbecco's modified Eagle's medium. The letter in parentheses indicates the particular serum lot.

[c]Not detectably different from corresponding non-enzymatic rate.

[d]Standard deviations are only given for those enzyme activities that were determined by three or more experiments each on a different passage. The values with no standard deviations are the average of two experiments except those of serums B and C which are the averages of three determinations done on one experiment.

[e]The cells in this experiment were fed serum-supplemented medium for four days and serum-free medium for the final two days.

[f]Se VI represents represents selenium introduced as the selenate ion. Te represents tellurium introduced as the tellurite ion.

flasks received equivalent volumes of the diluent. Each treatment or control group always contained four flasks and was considered a single experiment. Flasks were fed every other day at which time either selenite, selenate, tellurite or diluent was also replenished. Cytodifferentiation of neuroblastoma cells was initiated by removal of serum from the culture medium (5) and these cells were then fed every day until they were harvested.

The cells were harvested by discarding the growth medium and removing the cells with an EDTA solution pH 7.2 (6). The harvested cells from four flasks were pooled, centrifuged and the supernatant discarded. The pellet was resuspended and homogenized in 25 mM potassium phosphate buffer pH 7.0, containing 100 mM KCl and 1 mM EDTA. Homogenization was carried out using a polytron (Brinkmann Instruments) operated at an intermediate speed for two thirty second periods at 0°C. The homogenate was immediately centrifuged at 112,000 x g for 60 minutes. A sample of the supernatant was removed for a protein determination (7) and the remaining supernatant was placed in a plastic pro-vial (Costar) and frozen in liquid nitrogen. Within three days the vials were thawed and assayed by a modification of the method of Paglia and Valentine (8) using cumene hydroperoxide dissolved in 95% ethanol as the substrate (9, 10).

Supernatants stored in liquid nitrogen did not lose their activities after fifteen days when compared to the same supernatants assayed fresh, immediately after isolation.

Cumene hydroperoxide (Pfaltz & Bauer Inc., Stamford, CT) was assayed for purity (11) and found to have a 81.5% peroxide content.

The final concentrations of the reagents in each cuvette were as follows: 91 mM potassium phosphate pH 7.0, 3.6 mM EDTA, 2 mM GSH, 0.1 mM NADPH, 0.16 mM cumene hydroperoxide, 1 unit of glutathione reductase (Sigma) and a 0.1 ml sample of the supernatant or in blank cuvettes 0.1 ml of homogenization buffer.

A 100 ml sample of serum lot A was dialyzed for 24 hours against 2 liters of 0.13 M sodium chloride. After 24 hours the saline was changed and the dialysis was continued for 26 hours. The dialyzed serum was then sterilized by filtration through a type HA Millipore filter.

Results and Discussion

GSH peroxidase activity of cells cultured in media unsupplemented with Se was uniformly low. Addition of 30 nM Se resulted in a dramatic increase in GSH peroxidase activity. This particular Se concentration was chosen because it produced optimal growth in the experiments of McKeehan *et al.* (1). With both Se supplemented and unsupplemented media there was an increase in the specific activity of GSH peroxidase the longer the cells were in culture (Table I).

The anomalously low enzyme activity values obtained for Se supplemented and unsupplemented two-day cells resulted from the low number of cells harvested. The low enzyme content of the resulting high speed supernatants was only slightly greater than the corresponding non-enzymatic blank. Since both the blank and the enzyme activities vary independently within each assay, negative enzyme activities can occur. The resulting average is therefore much lower for two-day cells. Some of the individual enzyme activities included in these averages were of the same magnitude as those of four-day

cells. When cultured six days in the presence of 30 nM Se, differentiated and undifferentiated cells had the same GSH peroxidase specific activity.

Neuroblastoma GSH peroxidase activity is directly proportional to the concentration of Se introduced as the selenite ion below a concentration of 60 nM. Concentrations above this value produced a small increase but specific activity was essentially constant for all higher concentrations. The low levels of GSH peroxidase exhibited by cells grown without Se supplementation are due to traces of Se in the serum for when serum was removed from control cultures to achieve differentiation and no Se was added, no detectable GSH peroxidase activity remained after two days. Cultures grown on dialyzed serum produced only about one-half the normal enzyme activity for control cultures. This indicates that while some selenium compound in serum is removable there remains a selenium compound which is active but no readily extracted. This has been noted by others (1).

None of the experiments described here exceed 6 days, during which time cell number, viability and protein content did not differ among selenite, selenate or tellurite supplemented media and unsupplemented media until a concentration of 6 μM selenite was equalled or exceeded. At this concentration cell number dropped by one-half and for the two higher concentrations was reduced to one-fifth and one-tenth of the control number. Viability also decreased drastically and the cell pellets from the cultures containing 30 μM and 60 μM selenite were colored red by the presence of elemental selenium produced by the reduction of selenite. This red selenium remained with the pellet after the 112,000 x g centrifugation. The inference of selenite at a concentration of 10 μM with mitosis has been noted (12).

Selenate at concentrations of 30 nM and 60 nM had no effect on GSH peroxidase activity. Apparently the cell has no mechanism to reduce this form to selenite. Selenate also does not inhibit GSH peroxidase activity induced by simultaneously present selenite.

Similarly, tellurite produces no increase in GSH peroxidase activity when

present alone. Oppositely, it does proportionally decrease the expected GSH peroxidase activity known to be produced by 30 nM selenite present alone. Non-enzymatic glutathione oxidation is catalyzed by selenite and is also inhibited by simultaneously present tellurite (13).

At what point in the induction of GSH peroxidase tellurite exerts its inhibiting influence is unknown. Tellurite may in some form be incorporated into the enzyme resulting in hybrid forms which have no or different activities depending on the relative number of selenium and tellurium atoms in each enzyme molecule.

In any case the data from the selenate and tellurite experiments support the idea that the oxidation state of the metal is of great importance.

Such a system of neuroblastoma cells and a Se depleted serum would serve as an excellent model to determine whether GSH peroxidase is synthesized *de novo* or pre-existing molecules are activated by the presence of Se. Also the susceptibility of neuroblastoma cells to lipid peroxidation at various Se levels could be determined. Pragmatically, the system could be used as a bioassay for Se, particularly the amounts available in serum samples. All of these possibilities are being explored.

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