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Toxicity of Acrylonitrile in a Human Neuroblastoma Cell Line and Its Effect on Glutathione and Glutathione-S-Transferase

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Acrylonitrile (ACN), used extensively in the manufacture of acrylic polymers, is a well known neurotoxic compound. Several cases of acrylonitrile intoxication resulting from exposure to fumes of synthetic rubber and polymerization, as well as accidental, acrylonitrile poisonings have been described. These reports emphasized the involvement of the central nervous system (CNS) and gastrointestinal tract (IARC 1979; O' Berg 1980).

The toxicity of acrylonitrile has been attributed to release of the cyanide (CN) ion *in vivo*. However, differences in symptoms and CN blood concentrations associated with poisoning by inorganic cyanides and acrylonitrile led to some questions about that mechanism (Paulet and Desnos, 1961). Its oxidative metabolism in the mammalian liver is well known. The epoxide, 2-cyanoethylene oxide, is formed via P-450 mediated reactions. The epoxide can then follow three possible pathways: alkylation of proteins and DNA, hydration of the epoxide by epoxide hydrolase, or nucleophilic attack of the epoxide by glutathione (GSH) (Guengerich et al., 1981). The unstable cyanohydrins, resulting from the hydration reaction, then spontaneously decompose to release cyanide (Fig. 1). The glutathione adduct is excreted as mercapturic acid, N-acetyl-S-(2-hydroxyethyl)-L-cysteine, and the cyanide is metabolized to thiocyanate prior to excretion in the urine. No information exists about biotransformation of these compounds in neurons (Walum and Peterson, 1984).

In order to further study the toxicity and the metabolism of acrylonitrile in neurons, we evaluated the cytotoxic effect of acrylonitrile and production of CN *in vitro* using the neuroblastoma SK-N-BE. This cell line is extensively used as an *in vitro* neuronal model in many laboratories (Spinedi et al., 1990) because it possesses neuronal voltage-operated calcium channels, neuronal nicotinic receptors and neuron specific enolase. Moreover, these cells can differentiate *in vitro* in response to retinoic acid extending long neurites. However, no adequate information exists on the metabolic activity of this culture.
Changes in GSH and glutathione-S-tranferase (GST) were investigated because GSH appears to act as an antioxidant in the protection of cells from chemical injury by ACN and/or its metabolites (Pilon et al., 1988, Szabo et al., 1977)

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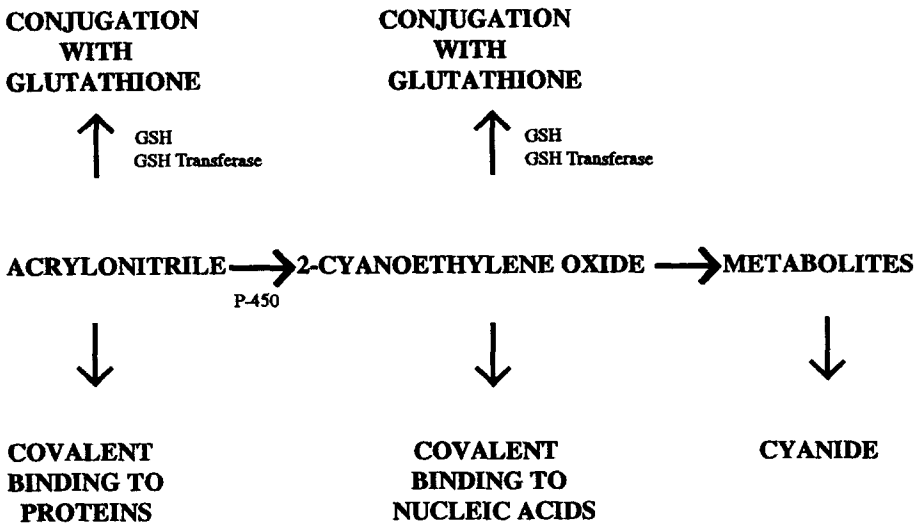

Figure 1 Proposed scheme for acrylonitrile metabolism in vivo

MATERIALS AND METHODS

Reduced and oxidized glutathione (GSH and GSSG) were obtained from Boehringer-Mannheim (Mannheim, Germany) N-Ethylmaleimide (NEM), o-Phthaldialdehyde (OPT) and 1-chloro-2,4 dinitrobenzene (CDNB) were obtained from Merck (Darmstadt, Germany). Acrylonitrile was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). The other compounds were readily available commercial products and were used without further purification.

The SK-N-BE cell line was obtained from American Type Culture Collection (Rockville, MD , USA). SK-N-BE cells were grown in Eagle's Minimum Essential Medium with Earle's salts, 10% heat inactivated fetal bovine serum, penicillin (5000 IU/ml) and streptomycin (5000 ug/ml). Acrylonitrile was dissolved in culture medium and added to Petri dishes 24 hours after plating. The acrylonitrile solution was freshly prepared in media at the beginning of each experiment. Cells were exposed to different concentrations of acrylonitrile (from 5 nM to 150 nM) for 24 hours. At the end of exposure the number of total cells and the number of viable and non-viable cells were determined. Cells which excluded trypan blue were considered to have an intact cell membrane and to be viable. The concentration required to kill 50% of the cells (LD_{50}) was derived from a graphic representation of the data.

GST was determined according to Habig et al. (1974) in a thermostated cell compartment at 30 °C using a Beckman spectrophotometer (Model 34) . Cells (10^6) were washed with a phosphate-buffered saline (PBS: 140 mM NaCl; 2.7 mM KCl; 6.5 mM Na_2HPO_4; 1.5 mM KH_2PO_4) and centrifuged at 1500 g for 10 min at 4°C. The pellet was suspended again in 3 ml of 22 mM potassium phosphate buffer (pH 7) and sonicated for 30 min at 0°C. The suspension was centrifuged at 4°C at $105x10^3$ g for 1 hour and used for the assay.

The assay of GSH and GSSG was performed according to Hissin and Hilf (1976) using a Perkin-Elmer fluorometer (Model LS-50). $20x10^6$ cells were washed with PBS and centrifuged at 1500 g at 4°C for 10 min. The pellet was suspended in 1 ml of 0.1 M sodium phosphate-0.005 M EDTA buffer (pH 8.0) and sonicated at 0°C for 10 min. 250 ul of 25% HPO_3 (meta-phosphoric acid) was added as protein precipitant. The total suspension was centrifuged at 4°C at $105x10^3$ g for 30 min to obtain the supernatant for the assays. For determination of GSH 100 ul of supernatant was added to 1.8 ml of phosphate-EDTA buffer and 100 ul of the OPT solution, containing 100 ug of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm. For determination of GSSG, a 0.5 ml portion of the supernatant was incubated at room temperature with 200 ul of 0.04 M NEM for 30 min to interact with GSH present in tissue. To this mixture, 4.3 ml of 0.1 N NaOH was added. An aliquot of the mixture was taken for measurement of GSSG, using a procedure similar to the one used for GSH determination, except that 0.1 N NaOH was employed as diluent rather than phosphate-EDTA buffer.

Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Cyanide concentration in the cultured media was measured at the end of acrylonitrile exposure. The specimens were treated with 0.2 M KOH (pH 9.75). The assay for cyanide was performed with an Amel polarographic analyzer (Model 433) equipped with a high-performance static mercury drop electrode. The linearity of the method has been verified for cyanide at a final concentration ranging from 1 to 500 ng; coefficient of variation (CV) of the analysis results to be 6.1 %.

RESULTS AND DISCUSSION

The cytotoxicity of ACN and KCN in the SK-N-BE neuroblastoma cell line was expressed in terms of LD_{50}. These values are very different for the two compounds, the cytotoxicity of KCN (2.5 $\pm$ 0.3 uM) being at least one order of magnitude lower than for ACN (72.5 $\pm$ 10 nM). The neuroblastoma is particularly sensitive to ACN. Since the CN was hypothesized to be responsible for the toxicity of ACN, we carried out experiments to determine whether CN was indeed produced from ACN in this cell line. We therefore performed a polarographic analysis of the culture medium of SK-N-BE neuroblastoma cells in presence of 60 nM ACN (Fig 2). This analysis gave a spectrum with a waveform and potential values which were identical to those of an authentic standard of cyanide at the final concentration of 10 nM.

The lack of stoichiometry between cyanide release and the concentration of ACN may be due to metabolic enzyme deficiencies in the human neuroblastoma cells. Nevertheless, this finding is consistent with the presence of metabolic systems (e.g. P450's) capable of converting ACN to unstable cyanohydrins that spontaneously decompose to release cyanide.

To evaluate the toxicity of KCN on this neuroblastoma cell line, the LD_{50} of this compound was determined. Since we found an LD_{50} value of 2.5 ± 0.3 uM, comparatively much higher with respect to ACN (72.5 $\pm$ 10 nM) the hypothesis that the toxicity observed at high concentration of ACN in SK-N-BE cells could due to CN production seems to be excluded. This statement together with the

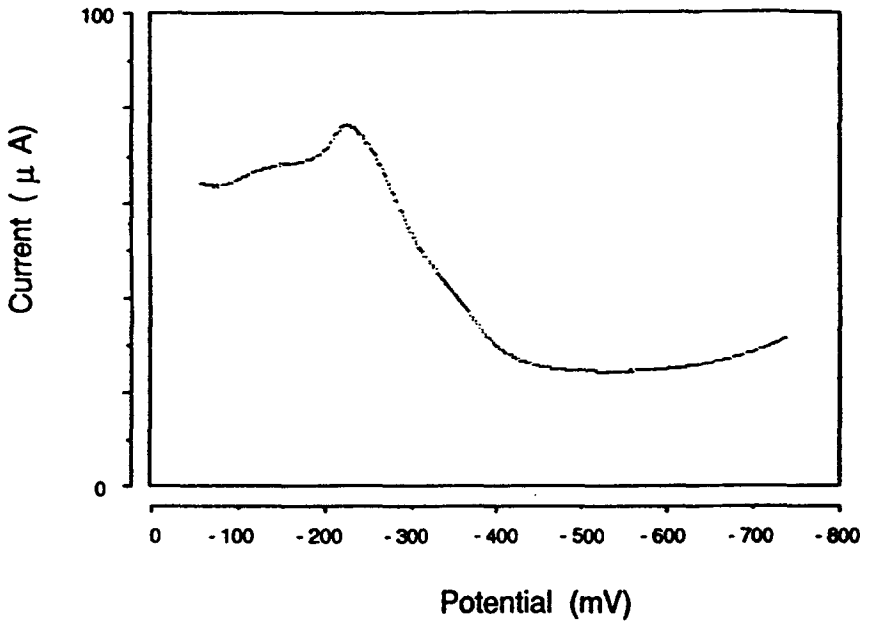

Figure 2 Polarographic analysis of culture medium of neuroblastoma SK-N-BE cells, in the presence of acrylonitrile (60 nM). Output signal at full scale = 100 uA

Table 1 Effects of acrylonitrile on the concentrations of GSH and GSSG, the ratio of GSH to GSSG and GSH-S-transferases activity.

Treatment with Acrylonitrile (nM)	GSH[a] (nmol/mg protein)	% vs Control	GSSG[a] (nmol/mg protein)	% vs Control	GSH/GSSG[a] (nmol/mg protein)	% vs Control	GSH-S-Tranferase[b] (umol CDNB/ min*mg)	% vs Control
Control	15.87±4.43		3.45±0.72		4.60		0.46±0.05	
5	15.28±3.36	-3.72	3.92±0.99	13.62	3.90	-15.21	0.45±0.09	2.22
15	14.60±1.99	-8.00	3.25±0.73	-5.80	4.49	- 2.39	0.43±0.07	-4.44
30	18.26±2.61	15.06	4.85±1.39	40.88	3.76	-18.26	0.42±0.05	-6.67
60	14.66±2.33	-7.62	4.01±1.01	16.23	3.66	-20.43	0.42±0.03	-6.67

[a] Data expressed as mean ± standard error; n=6

[b] Data expressed as mean ± standard error; n=5

very limited metabolic production of CN that we have observed led us to postulate the formation of a more reactive intermediate. The activity of GST with respect to ACN doses (Table 1) seems to confirm this hypothesis. In fact, the

GST activity in the neuroblastoma is considerably lower than that detected in human tissues such as liver and brain (146 umol and 40 umol * mg^{-1} protein * min^{-1} respectively, Theodore et al., 1985). In any case, the exposure of the cells to several concentrations of ACN did not produce any significant change in this enzymatic activity with respect to control activity. This hypothetical substance would accumulate in the neuroblastoma cell cultures due to one or several enzyme deficiencies such as GST. Moreover, the glutathione levels (in the oxidized and reduced forms) in the exposed cells are substantially unchanged. However, exposure to 30 nM ACN induces a rise (statistically non significant) of the oxidized glutathione which led to a decrease in the GSH/GSSG ratio.

The explanation of these data is not easy.
We would expect lower levels of glutathione in exposed cells because the tripeptide is used for conjugation with reactive intermediates during the metabolism of ACN. However, the low GST activity of this cell line could explain the incongruity of our results. On the other hand, it is well know that in cells the glutathione is a redox buffer system and only a marked oxidative stress or cellular aging are able to change the GSH/GSSG ratio substantially (Gilbert, 1989). Although this cell line appears to possess certain metabolizing systems, these seem to not be present in sufficient quantities to mimic neuron behaviour *in vivo* in terms of a xenobiotic compound which becomes neurotoxic after metabolism. To prevent this, the cells should be exposed to a metabolic activation system derived from the postmitochondral fraction (S-9) of livers from rats pretreated with Arochlor 1254 (Parkinson et al., 1983). Because the differences with neurons *in vivo* could be due to the undifferentiated state of this cell line, it also would be worth comparing exposure of the cell line both in an undifferentiated and an experimentally differentiated (e. g. dibutyryl AMP - or 5'-bromo-2'deoxy-uridine - treated) state.

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