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Changes in the Glutathione-Redox Balance Induced by the Pesticides Heptachlor, Chlordane, and Toxaphene in CHO-K1 Cells

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Organochlorine insecticides owe their environmental significance to their strong persistence throughout and ability to biomagnify in the trophic chain. Although most of them were banned in the eighties, residues of these compounds may still be present in food, representing a risk for human health. The acute and chronic toxic effects of such compounds in non-target animals are not completely clear, however. The pathogenic effects observed in experimental animals exposed to organochlorine insecticides include, among others, hypothyroidism, porphyria, hypertension, myocarditis, neurotoxicity, estrogenic effects and cancer (Stevenson *et al.*, 1999), but the principal molecular mechanism involved in these processes remains unclear. Bagchi *et al.* (1992) proved that endrin, dieldrin and chlordane, among the most toxic and persistent cyclodiene insecticides, activate rat neutrophils, a process involving superoxide anion (a reactive oxygen species: ROS) production. ROS generation is the first step in many toxic processes, such as lipid peroxidation, single strand-DNA damage and nitric oxide induction in peritoneal mice macrophages (Bagchi *et al.*, 1993). The ROS species induced by organochlorine insecticides can be neutralised by the cellular antioxidant system, comprising both mitocondrial Mn-containing and cytosolic Cu,Zn-containing superoxide dismutases (SODs) as well as glutathione peroxidase and reductase, which are instrumental in the balance between reduced and oxidised forms of glutathione (Meister and Anderson, 1983). Although superoxide anion radicals can be rapidly eliminated, the hydroxyl free radical ($OH^{\cdot}$), formed by the homolytic fission of hydrogen peroxide in the presence of transition metals, is not effectively scavenged by the antioxidant system and may be responsible for toxicity.

Although a wealth of information has been accumulated on the oxidative stress induced by cyclodiene insecticides (Bagchi *et al.*, 1992; Bagchi *et al.*, 1993) little is known about the cytotoxic effects of other organochlorine insecticides such as toxaphene (a chlorinated camphene widely used in the eighties) on the sulphur-redox balance. This study

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compares the dose-responsive effects of toxaphene as well as the two cyclodiene insecticides chlordane and heptachlor on glutathione content and interconversion in CHO-K1 cells, to ascertain whether oxidative stress is involved in the toxicity of these pesticides in mammalian cells.

MATERIALS AND METHODS

Hams F12 culture medium, gentamicine sulphate (10 mg/ml), neutral red dye (NR), reduced and oxidised glutathione (GSSG) and glutathione reductase, were from Sigma Chemical Co. (St. Louis Mo. USA). 1,2,4,5,6,7,8,8a-octachloro-4,7-methane-3a,4,7,7a-tetrahydroindane (chlordane), 1,4,5,6,7,8,8-heptachloro-3a,4,7a-tetrahydro-4,7-endo-5,8-methaneindene (heptachlor), and toxaphene (chlorinated camphene containing 67-69 % in chlorine) were acquired from Alltech Associates (Deerfield, Illinois, U.S.A.). Foetal calf serum was purchased from Boehringer Mannheim GmbH (Germany). All other reagents were of standard laboratory grade.
CHO-K1 cells were obtained from the American Type Culture Collection (ATCC CCL 61). The cells ($10^4/cm^2$) were plated in monolayer in 22.1 cm^2 polystyrene tissue culture dishes with Hams-F12 supplemented with 25 mM Hepes buffer (pH 7.4), 10 % heat inactivated foetal calf serum and gentamicin (30 μg/ml). Cells were routinely subcultured twice a week with as few subpassages as possible to maintain genetic homogeneity. Cells were counted in an improved Neubauer haemocytometer and viability was determined by the exclusion intake of Trypan Blue dye. Cells were grown to 65 % confluence (day 3, mid-log phase) and then pulsed with different concentrations of organochlorine insecticides dissolved in 1 % DMSO. Control wells were pulsed with a similar volume of DMSO solution. After 24 h cells were washed with PBS and the medium was renewed. Cells were harvested in 0.25 M sucrose and 1 mM EDTA and disrupted by sonication at 4° C. The disrupted cells were centrifuged at 12,000 x g for 5 min at 4 ° C, and the supernatant was used to determine sulphur-redox species and the enzymes involved in glutathione metabolism.

Insecticide cytotoxicity was estimated in CHO-K1 cells using the neutral red (NR) uptake assay described by Borenfreund and Puerner (1985). The values in this vital staining procedure represent the percentages of dead cells. Cells were grown to 65 % confluence and then pulsed with different concentrations of organochlorine insecticides (ranging from 0.005 to 0.3 mM). The cells were washed after 24 h and placed on fresh medium containing 40 mg/ml NR dye. After 3 h cells were washed with PBS and fixed with a solution containing 0.5 % formaldehyde and 1 % $CaCl_2$ in distilled water. Colour was developed with 0.2 ml of a solution containing 1 % acetic acid, 59 % ethanol and water. The absorbance of each plate at 540 nm was determined in an automatic ELISA reader and

Sigma Plot (version 2.1, Microsoft®) was used to fit curves in non-linear regression. NR values were obtained from the respective probit/log curves.

A final volume of the glutathione reductase mixture contained 500 μl potassium phosphate buffer, 0.2 M, pH 7.0 with 2 mM EDTA, 50 μl of a 2 mM NADPH solution dissolved in 10 mM HCl-Tris, pH 7.0, 50 μl of 20 mM GSSG, 300 μl distilled water and 100 μl of freshly isolated cellular extracts. The absorbance rate of the reaction mixture at 340 nm was determinated after incubation at 30 °C for 1 min. One unit of glutathione reductase activity is defined as the amount of enzyme able to reduce 1 μmol of GSSG per min per mg of soluble protein.

The total glutathione peroxidase assay was performed as described by Wendel (1981). For this, 100 μl of cell extracts were assayed in 1 ml final volume of a solution containing 500 μl of potassium phosphate buffer 0.25 M, pH 7.0, 2.5 mM EDTA, 2.5 mM sodium azide, 100 μl of 10 mM GSH, 100 μl of 2.5 mM NADPH in 0.1 % $NaHCO_3$ solution and 100 μl of 2.4 units/ml freshly prepared glutathione reductase in PBS. Following a 1 min preincubation at 37 ° C, the reaction was started by adding 100 μl of 12 mM tert-butyl peroxide (Sigma) to the reaction mixture. The rate of absorbance change at 366 nm was monitored for 1 min at 37 °C. One unit of glutathione peroxidase is defined as the amount of enzyme that oxidised 1 μmol of GSH per min per mg of soluble protein. The Bradford (1976) method was used to assay the protein.

The glutathione-S-transferase assay was performed as described by Habig et al. (1974). For this, 100 μl of cell extracts were assayed in 1 ml final volume of a solution containing 800 μl of sodium phosphate buffer 0.2 M, pH 6.5, 50 μl of 20 mM 1-chloro-2,4-dinitrobenzoic acid (Sigma) in 95% ethanol, 50 μl of 20 mM GSH. The rate of absorbance change at 340 nm was monitored for 1 min at 25 °C. One unit of glutathione-S-transferase is defined as the amount of enzyme that conjugate 1.0 μmol of 1-chloro-2,4-dinitrobenzene with reduced glutathione per min per mg of soluble protein.

Total glutathione levels were determined spectrophotometrically using the method described by Akerboom and Sies (1981). The monolayers of CHO-K1 cells harvested from the plastic surface of the 22.1 cm^2 polystyrene petri dishes, using a trypsin/EDTA solution, were washed twice with PBS pH 7.4 and disrupted by sonication with 500 μl of 1 M cold perchloric acid (PCA) containing 2 mM EDTA. An aliquot (100 μl) of the extracts were neutralised with 70 μl of 1 M KOH and 70 μl of 0.3 M MOPS, prior to total glutathione determination. The protocol for total glutathione was as follows: 730 μl potassium phosphate buffer, 0.1 M, pH 7.0, containing 1 mM EDTA, 200 μl of neutralised cell extracts, 50 μl of 4

mg/ml NADPH dissolved in 0.5 % $NaHCO_3$ and 20 µl of 1.5 mg/ml 5,5'-ditiobis(2-nitrobenzoic acid) (Sigma) in 0.5 % $NaHCO_3$ were combined to reach a final volume of 1 ml. The reaction was started with 6 units of glutathione reductase (Sigma) and measured at 412 nm for 1 min at 25 °C. The slopes for each determination were interpoled on a previously constructed calibration curve, with total glutathione content expressed as nmol per mg of total protein.

Oxidised glutathione (GSSG) was assayed in freshly acidified cell extracts using the method described by Anderson (1985). CHO-K1 cultures were harvested and disrupted as above. PCA-treated extracts were then centrifuged and neutralised with 2 µl of 2-vynilpyridine (Fluka) and 824 µl of 25 % trietanolamine. One ml of final volume of the reaction mixture contained 650 µl sodium phosphate buffer, 0.1 M pH 7.5, that included 6.3 mM EDTA, 50 µl of 4 mg/ml NADPH, 100 µl of 6 mM DTNB, 200 µl of cell extracts, and 1 unit of glutathione reductase. Like total glutathione, GSSG was estimated by interpolating the slopes of each assay on a standard curve and expressing GSSG content as nmol per mg of total protein.

RESULTS AND DISCUSSION

Organochlorine pesticide cytotoxicity was determined using the NR incorporation assay, which monitors lysosomal cell function. Chlordane, heptachlor and toxaphene were added to CHO-K1 cultures in the mid-log phase (day 3); and incubation for 24 h, the NR technique was used to analyse cell viability. The probit/log curves yielded NR_{50} values of 22.16, 23.90 and 106.73 µM, for chlordane, hepatchlor and toxaphene, respectively. Fractions of NR_{50} ($NR_{6.25}$, $NR_{12.5}$ and NR_{25}) value of each pesticide were used to determine the biochemical parameters assayed in the present report.
The metabolic effects of cyclodiene insecticides on the sulphur-redox cycle were measured after 24 h of cellular exposure to the agents, in the presence of foetal calf serum in the culture medium. After incubation, cells were extensively washed with PBS and then assayed for glutathione content. Table 1 summarises total and GSSG content, determined in mid/late log phase after exposure to sublethal concentrations of chordane, toxaphene and heptachlor. Significant ($P < 0.01$) depletion of total glutathione was found at heptachlor concentrations equivalent to the pesticide's $NR_{6.25}$ and RN_{25} values. No changes, however, were found in chlordane treated cells. In cells exposed to toxaphene, in turn, the total glutathione content more than doubled at all the concentrations studied. Unlike total glutathione, GSSG levels only rose significantly at concentrations equivalent to the NR_{25} for the pesticides studied.

Table 1. Intracellular glutathione (total and oxidised) content in 24 h-polyhalogenated -treated CHO-K1 cells.

		Glutathione content (nmol/mg protein)		
Insecticide	glutathione	$NR_{6.25}$	$NR_{12.5}$	NR_{25}
heptachlor	total	14.5 ± 0.8 ***	22.77 ± 0.18	18.60 ± 0.10**
	GSSG	0.24 ± 0.06	0.25 ± 0.04*	0.49 ± 0.02***
chlordane	total	21.36 ± 1.53	27.71 ± 3.04	19.41 ± 1.17*
	GSSG	0.14 ± 0.01***	0.24 ± 0.02	0.45 ± 0.02***
toxaphene	total	62.02 ± 3.39**	85.86 ± 7.8***	51.05 ± 3.66**
	GSSG	0.13 ± 0.01***	0.29 ± 0.01	0.37 ± 0.01**

(**) $P \leq 0.01$; (***) $P \leq 0.05$; values in the control samples were 23.48 ± 0.67 nmol/mg protein for total glutathione and 0.32 ± 0.05 for GSSG, respectively obtained in control cultures. CHO-K1 cultures were grown up to 65 % confluence (day 3) and then were incubated for an extent of 24 h with fresh medium that contained concentrations of chlordane, heptachlor and toxaphene equivalent to their $NR_{6.25}$, $NR_{12.5}$ and NR_{25}. Each value is the average ± SE of three different samples.

The figures in Table 2 illustrate the significant increase in glutathione reductase activity associated with exposure to chlordane ($P< 0.005$) and toxaphene ($P< 0.001$). At chlordane and toxaphene concentrations equivalent to their $RN_{6.25}$, values, glutathione reductase activity was almost two fold higher than recorded for the control culture, declining at higher pesticide levels. Heptachlor, however, induced no change at any of the concentrations studied. Attention is drawn to the inhibitory effect of toxaphene on glutathione reductase at higher concentrations.

Table 2. Glutathione reductase activity in CHO-K1 cells exposed to fractions of the mid-lethal concentration of polyhalogenated insecticides.

	Enzymatic activity (nmol/min/mg protein)		
Insecticide	$NR_{6.25}$	$NR_{12.5}$	NR_{25}
heptachlor	39.83 ± 2.22	40.30 ± 2.65	33.26 ± 0.84
chlordane	64.26 ± 3.41**	45.26 ± 1.85*	28.50 ± 0.61**
toxaphene	61.20 ± 0.69***	32.80 ± 3.44	11.07 ± 0.73***

(***)$P \leq 0.001$; (**)$P \leq 0.005$; (*) $P \leq 0.01$; values in the control samples were 34.19 ± 1.33 nmol/min/mg protein. After exposition cells were harvested and the enzymatic activity was determined under standard assay conditions. Each value is the average ± SE of triplicate samples.

Table 3 shows that chlordane induced a significant increase in total glutathione peroxidase activity at concentration equivalent to the

pesticides's $NR_{6.25}$ value, whereas heptachlor and toxaphene did not. Induction of glutathione peroxidase is associated with overexpression of SOD, the enzyme that controls superoxide anion disruption and cell homeostasis (Warner *et al.*, 1993).

Table 3. Glutathione peroxidase activity in CHO-K1 cells exposed to fractions of the mid-lethal concentration of polyhalogenated insecticides.

	Enzymatic activity (nmol/min/mg protein)		
	$NR_{6.25}$	$NR_{12.5}$	NR_{25}
heptachlor	159.83 ± 11.49	164.47 ± 5.79**	142.40 ± 2.52*
chlordane	205.70 ± 0.28***	151.46 ± 1.57**	134.10 ± 0.73**
toxaphene	115.70 ± 6.26	62.62 ± 7.95**	60.06 ± 2.84***

(***)P ≤ 0.001; (**)P ≤ 0.005; (*)P ≤ 0.01; values in the control samples were 129.19 ± 9.68 nmol/min/mg protein. Cells were grown until mid-log phase and then equivalent concentrations to their $NR_{6.25}$, $NR_{12.5}$ and NR_{25} were added for 24 h to the cultures. After exposition cells were harvested and the enzymatic activitiy was determined under standard assay conditions. Each value is the average ± SE of triplicate samples.

A similar pattern of behaviour was observed in glutathione-S-transferase, one of the enzymes involved in phase II detoxification in mammals (Fukami, 1980). Toxaphene produced a dose-response inhibition of this enzyme, which fell to barely one-third of the control activity at the concentration equivalent to the NR_{25} value for this pesticide. No significant changes, however, were found in the presence of the two cyclodiene insecticides (Table 4).

Table 4. Glutathione-S-transferase activity in CHO-K1 cells exposed to different concentrations of organochlorine insecticides.

	Enzymatic activity (nmol/min/mg protein)		
Insecticide	$NR_{6.25}$	$NR_{12.5}$	NR_{25}
heptachlor	**44.53 ± 1.62**	**44.33 ± 0.33***	**41.92 ± 1.08**
chlordane	**47.13 ± 0.55***	**45.33 ± 0.45***	**42.50 ± 1.88**
toxaphene	**31.46 ± 1.25****	**17.80 ± 0.39*****	**15.80 ± 1.11*****

(***)P ≤ 0.005, (**)P ≤ 0.01; (*) P ≤ 0.05, values in the control samples were 42.05 ± 1.81 nmol/min/mg protein. After exposition cells were harvested and the enzymatic activity was determined under standard assay conditions. Each value is the average ± SE of triplicate samples.

Significant induction of glutathione reductase activity would explain the increases observed in total glutathione content with no accompanying

change in GSSG after exposure to the lowest concentrations of chlordane and toxaphene. The high total glutathione content at higher concentratioins of toxaphene could be due to the decline in glutathione peroxidase activity under such conditions.

Several authors have reported that toxiciti of a number of organochlorine pesticides involves oxidative stress processes (Bachowski *et al.*, 1998; Hassoun *et al.*, 1993). Hassoun et al. (1993) showed that oxidative stress was the primary cause of lipid peroxidation and the rupture of single-strand DNA induced by the cyclodiene agents endrin and chlordane, the chlorinated ethane DDT and the gamma isomer of hexachlorocyclohexane, lindane. Recent studies have concluded that cancer in mice induced by aldrin and the aldrin metabolite dieldrin develops due to promoting-like mechanisms that may involve the inhibition of intercellular communication (Trosko et al., 1987), or the massive production of ROS (Stevenson et al., 1999). Other alterations of cellular homeostasis, after exposure to polyhalogenated insecticides such as expression of heat shock proteins (Bagchi et al., 1996) or apoptosis induction (Rought et al., 2000) have been also described.

In conclusion, cyclodiene pesticides induced increases in total glutathione peroxidase activity in CHO-K1 cultures after exposure for 24 h. Whereas total glutahtione content remained unchanged after exposure to chlordane due to a significant increase in glutathione reductase levels, the tripeptide content was low after exposure to heptachlor. Toxaphene, however, caused a significant accumulation of glutathione content due to increased glutathione reductase activity at low concentrations and the declining in glutathione peroxidase at higher levels.

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