Bull. Environ. Contam. Toxicol. (1999) 63:582-589

Bulletin of Environmental Contamination and Toxicology

Effects of Chlorothalonil on Glutathione and Glutathione-Dependent Enzyme Activities in Syrian Hamster Embryo Cells

H. Bessi,[1] C. Cossu-Leguille,[2] A. Zaïd,[3] P. Vasseur[2]

[1] Faculty of Sciences and Technics, Laboratory of Ecotoxicology, B.P. 146, Mohammadia, Morocco
[2] Centre of Environmental Sciences, B.P. 94025, 57040 Metz Cédex 1, France
[3] U.F.R. Environment and Safety, Faculty of Sciences, Meknès, Morocco

Received: 6 May 1999/Accepted: 19 August 1999

Glutathione (GSH), the tripeptide glutamylcysteinylglycine, is the most abundant thiol of low molecular weight, and it plays a critical role in a variety of intracellular functions and metabolic regulation (Meister and Anderson 1983; Larsson et al. 1983; Deneke and Fanburg 1989). In mammalian cells, the intracellular concentration of GSH ranges between 0.5 and 10 mM depending on cell type (Kosower and Kosower 1978). GSH is usually present in its reduced form, less than 5 % comprising oxidized glutathione disulfide (GSSG). The thiol redox status is maintained by intracellular enzymes, glutathione peroxidases (GPOX), glutathione reductase (GR), and NADPH. GPOX neutralizes oxidative stress by reducing hydrogen peroxide and organic peroxides to the corresponding alcohols. This involves the concomitant oxidation of GSH, resulting in GSSG, which may be reduced by GR using NADPH as a reducing equivalent.

Numerous studies have demonstrated the important role of GSH in the protection of cells against reactive oxygen species and free radicals, as well as in detoxification of foreign electrophilic compounds (Sies et al. 1980; Yu 1994). GSH diverts electrophilic agents away from cellular targets and protects living cells either directly as a reducing agent or as a co-substrate for GPOX and glutathione-S-transferase (GST). The latter catalyzes the conjugation of electrophilic xenobiotic compounds with GSH (Meister and Anderson 1983).

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile), is a fungicide widely used in both agricultural and non-agricultural applications, including the aerial spraying of potatoes and other crops and wood treatment (IARC 1983). Owing to its four electrophilic chlorine atoms, chlorothalonil is highly reactive towards intracellular thiols such as GSH in living cells (Vincent and Sisler 1968; Rosanoff and Siegel 1981; NCI 1991). The fungitoxicity of chlorothalonil was due to its ability to inhibit glucose oxidation following depletion of GSH (Vincent and Sisler 1968; Rosanoff and Siegel 1981). This pesticide received particular attention due to its carcinogenic effects on the kidney and stomach of laboratory animals (NCI 1978; EEC 1993). Several *in vitro* studies have revealed that chlorothalonil is not genotoxic (Kawachi

Correspondence to: H. Bessi

et al. 1980; De Bertoldi et al. 1980). A previous study in our laboratory supported these observations and demonstrated that chlorothalonil was not an initiator but was a promoter of tumors in a multistage morphological transformation assay conducted on Syrian hamster embryo (SHE) cells (Bessi et al. 1994). Chlorothalonil was classified as a non-genotoxic carcinogen. Its carcinogenic effects in some fish species were reported to be in relation with the low antioxidant capacities of the species (Hasspieler et al. 1994).

Accordingly, the purpose of the present study, as a complement to our previous work (Bessi et al. 1994), was to investigate the effects of chlorothalonil on GSH pathways in SHE cells. Antioxidant status was evaluated by measuring the reduced and oxidized forms of glutathione and the activities of GR and GPOX.

MATERIALS AND METHODS

Analytical-grade chlorothalonil, also named 2,4,5,6-tetrachloroisophtalonitrile or 1,3-dicyanotetrachlorobenzene, or 2,4,5,6-tetrachloro-3-cyanobenzonitrile (97% purity) was provided by Interchim. Chem. Service (Montlucon, France). Impurities included tetrachlorophthalonitrile, chlorinated dicyanobenzenes, and pentachlorobenzonitrile. Chlorothalonil was dissolved in acetone and diluted with culture medium to the desired concentration just before use. Acetone was used in a final concentration of 0.2 %, which had no effect on cell response. The other chemicals were of the highest grade of purity, and were obtained from Sigma Chemical Co. (Saint Quentin Fallavier, France).

SHE cells were obtained from hamster embryos and prepared as previously described (Bessi et al. 1994). Cell suspensions resulting from embryo digestion were divided into vials which were stored in liquid nitrogen until the beginning of experiments. One vial was used for each series of experiment. Cells were thawed and then grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum and 2.2 g/L sodium bicarbonate, at 37°C and pH 7 in a humidified atmosphere of 10% CO_2.

After a preculture period of 24 h, cells were plated in culture dishes (10 mm diameter) and treated at 60-70 % confluency with chlorothalonil at concentrations ranging from 0.25 μg/mL to 0.75 μg/mL. The inoculum in culture dishes was adjusted so that cells would reach the same density at the end of the treatment time. The concentration of 1 μg/mL was not tested as it induced a high toxicity in the cell cultures. At the end of the treatment period, the test medium was removed and the cells were washed twice with cold PBS. They were immediately deproteinized with 10% HClO4 to which was added 2 m*M* EDTA (5 ml/dish). The samples were centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant was used for GSH and GSSG analyses, and the pellet was dissolved in 200 μL 1.0 *M* NaOH for protein quantitation according to the Bradford technique (1976).

GSH concentrations were quantified by means of HPLC analysis according to the method of Leroy et al. (1993). The GSH content in a given cell extract was expressed in μg/mg protein. In all experiments, GSH levels were determined using cultures of the same density to avoid the intracellular variation of GSH content during the cell's cycle. Indeed, Shaw and Chou (1986) reported that confluent and quiescent fibroblasts contained a lower level of GSH than their proliferating counterparts. GSSG was evaluated using the same method. After protein precipitation with 10% $HCLO_4$ mixed with 2m*M* Na_2EDTA, the reduced form was blocked with 20 m*M* N-ethyl maleimide at 0°C for 5 minutes. The oxidized form was then reduced with 15 mM dithiothreitol for 1 hr in the dark. The GSSG content was expressed as μg/mg protein.

At the end of the treatment period, the tested media were removed and the cell monolayers were washed twice with cold PBS. The cells were scraped off each dish and were solubilized with 300 μL of 1% Triton X-100. After centrifugation at 12,000 *g* for 30 min at 4°C, the supernatant fractions were used for enzymatic analyses and protein quantitation. Total GPOX activity was measured as described by Paglia and Valentine (1967) and modified by Lawrence and Burk (1976). GR activity was measured by the procedure of Carlberg and Mannervik (1985). The results were expressed in μM NADPH oxidized/min/mg protein. The percentage of changes induced by the treatment were expressed by the ratio of the difference between enzyme activities in control and treated cells over control activity.

Two series of independent experiments were conducted in order to test the effects of chlorothalonil on GSH and antioxidant enzymes. In each series of assays, two replicates were involved at each chlorothalonil concentration and each time point. Mean values of raw data are reported with S.D. in figures.

GSH concentrations were submitted to a standard multifactorial analysis of variance. GSH results were log-transformed in order to normalize the data and to achieve homogeneity of variance. Homogeneity of variance was checked by using the Cochran test. ANOVA was followed by multiple range test comparisons with Dunnett's t test for difference of means at a 5 % level of significance. The sample design was a three-factor factorial experiment, with two replicates per dose-exposure group (time of exposure with 8 fixed levels, chlorothalonil concentration with 4 fixed levels, the series of experiment with 2 random levels). The F-tests in the ANOVA table allowed us to identify the significant factors and the significant interactions amongst the studied factors. Statistical analyses were performed with STATGRAPHICS Plus Software (Manugistic, Version 2.0, Rockville, MD, USA).

RESULTS AND DISCUSSION

GSH content in control cells averaged 2.17±0..18 μg GSH/mg protein throughout the experiment. Chlorothalonil caused significant concentration and time-dependent

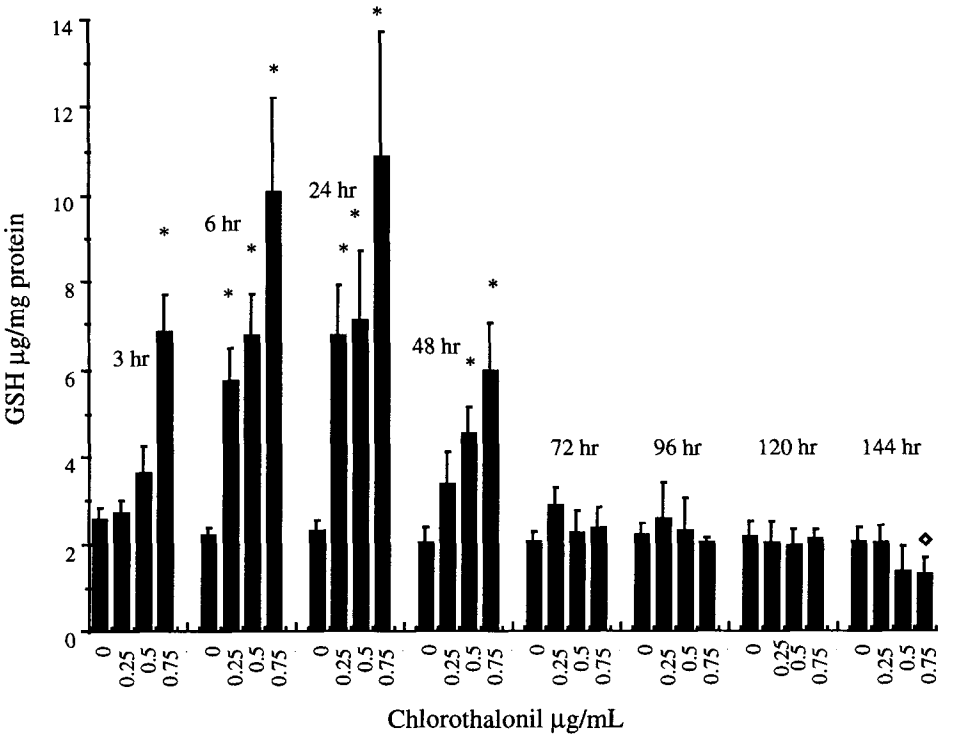

Figure 1. GSH content of SHE cells treated with 0.25, 0.5 and 0.75 μg/mL chlorothalonil during different lengths of exposure. Significant increase (*) or decrease (0) compared to controls (Dunnett's t test; $p<0.05$).

changes in intracellular GSH levels in SHE cells (Fig. 1). The outcome of the analysis of variance is presented in Table 1. An increase in reduced GSH levels was observed in cells treated with chlorothalonil during the first 48 hr of treatment. The

Table 1. Analysis of variance of log-transformed GSH concentrations (two independent experiments with replicates per dose-exposure group).

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F ratio	*P*
MAIN EFFECTS					
A : time	4.86194	7	0.69456	77.47	0.0000[a]
B : concentration	1.06093	3	0.35364	39.44	0.0000[a]
C : experience	0.00561	1	0.00561	0.63	0.4308[b]
D : duplicate	0.00886	1	0.00886	0.99	0.3227[b]
INTERACTION AB	1.993	21	0.0949	10.59	0.0000[a]
RESIDUAL	0.8428	94	0.0089		
TOTAL	8.7698	127			

[a] Significant at the 0.5% level ; [b] Not significant at the 0.5% level

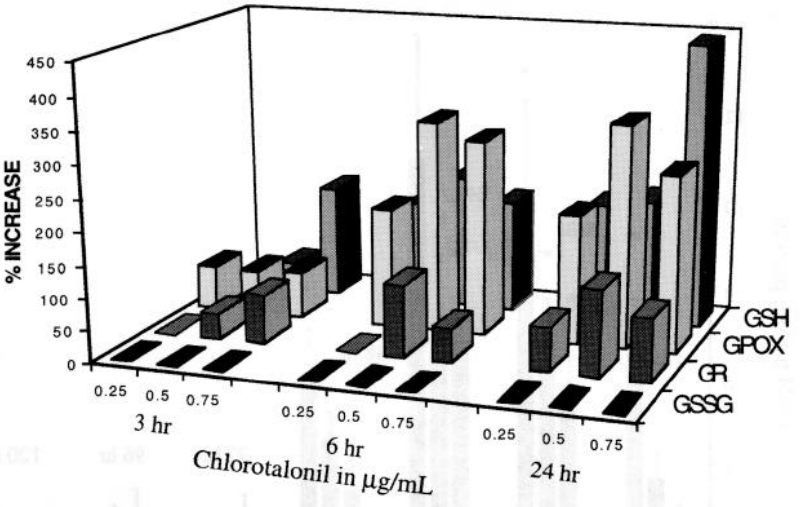

Figure 2. Percentage increase of antioxidant parameters compared to controls in SHE cells exposed to 0.25, 0.5, and 0.75 μg/mL chlorothalonil for 3, 6, and 24 hr.

ANOVA identified a statistically significant interaction between chlorothalonil concentration and time of exposure from 3 to 48 hr. The increase in GSH was already significant after 3 hr of treatment with 0.75 μg/mL chlorothalonil. Reduced GSH reached a maximum after 24 hr when increases of 183%, 195% and 440% in concentrations as compared to controls were noted in cells exposed to 0.25, 0.5, and 0.75 μg/mL chlorothalonil, respectively. Between 72 and 120 hr of treatment, GSH concentrations returned to control levels. After 144 hr of exposure, GSH dropped to 1.3 μg GSH/mg protein in cells treated with 0.5 and 0.75 μg/mL chlorothalonil. No change in GSSG levels was observed during the first 96 hr of treatment; beyond this time, a significant increase in GSSG was observed at 0.5 and 0.75 μg/mL chlorothalonil. GSSG increased by 15 and 22% after 120 and 144 hr of treatment at 0.75 μg/mL. The increase was less pronounced at 0.5 μg/mL (12 and 15% after 120 and 144 hr, respectively).

The activities of GPOX and GR were investigated during the first 24 hr of treatment. In control SHE cells, the GPOX mean activity was 15±4 μM NADPH/min/mg protein and GR activity averaged 59±8 μM NADPH/min/mg protein. Chlorothalonil treatment enhanced these activities. After 3 hr, the GPOX activities had increased by 70, 69, and 72% with 0.25, 0.5, and 0.75 μglmL chlorothalonil, respectively. After 6 hr, the increase in activity was more dramatic, reaching 186, 328, and 303% at the above concentrations. Thereafter, the activities seemed to stabilize. After 24 hr, the increase was 200, 343, and 273 % in cells treated with 0.25, 0.5, and 0.75 μg/mL chlorothalonil respectively, as compared to the controls (Fig. 2). GR activity increased in parallel, but to a lesser extent than GPOX activity. No change could be noted at 0.25 μg/mL chlorothalonil before 24 hr. The increase was significant after 3 and 6 hr of treatment with 0.5 and 0.75

μg/mL chlorothalonil only. After 3hr, the activities were respectively 38 and 76% higher than the controls at these concentrations. After 6 hr the increase was more pronounced. After 24 hr, the three concentrations, 0.25, 0.5, and 0.75 μg/mL of the fungicide, induced a significant increase in GR activity, respectively by 68%, 132%, and 96% of controls (Fig. 2).

Collectively, these results indicate that there was an alteration of GSH metabolic pathways in SHE cells exposed to chlorothalonil. This study demonstrated that GSH is rapidly synthetized in response to chlorothalonil injury. GSH levels in SHE cells, which averaged 2.2 μg or 7 n*M* per mg protein, are of the same order as that found in hamster cell lines, such as V79 with 3 n*M*/mg protein (Ochi 1993) and CHO with 17 n*M*/mg protein (Keiser et al. 1988). It is close to the GSH levels in hepatocytes, which range from 5 to 17 n*M*/mg protein in cell lines (Liu et al. 1993) and 8.5 n*M*/mg protein in rat hepatocytes (Vreugdenhil et al. 1991). The concentrations found in renal cells are higher, in the range of 20 - 30 n*M*/mg protein (Andreoli et al. 1992; Kang and Enger 1991). A comparison of the antioxidant enzyme activities of SHE cells with other mammalian cells is more difficult due to the different units used by authors to express activities.

After treatment of SHE cells with chlorothalonil, GPOX activity was the first antioxidant parameter to increase. This increase could be observed after only 3 hr of exposure to the lowest concentration tested, and before GSH levels were enhanced (Fig. 2). This increased activity seemed to result from the conjugation of chlorothalonil metabolites to reduced GSH. Indeed, selenium-independent GPOX not only reduces peroxides but also exhibits a GST activity. This explanation is supported by the fact that GR activity increased after GPOX (after 24 hr with 0.25 μg/mL), indicating that reduction of peroxides occurred during a second stage. The activation of GR as a consequence of an oxidation of GSH indicates that the metabolism of chlorothalonil induces the generation of organic peroxides. These peroxides are likely to produce cell damage if they are not rapidly neutralized by GPOX. It appears that GSH homeostasis and the thiol/dissulfide balance are maintained during the first 120 hr of treatment. Beyond this time, GSSG increases slightly, which indicates a deficiency in redox balance in SHE cells. The deficiency in the antioxidant capacities of SHE cells is critical since a drop in GSH levels is registered at the same time. As a consequence, the GSH/GSSG ratio decreases markedly from 10 in the controls to 5.6 and 3.5 at 0.5 and 0.75 μg/mL, respectively, after 144 hr of treatment. The inability to maintain the normally high GSH/GSSG ratio may increase susceptibility to oxidants (Tribble et al. 1987).

The results obtained in SHE cells are consistent with previous *in vivo* investigations that have shown that chlorothalonil is metabolized in rats via pathways which involve conjugation with glutathione (NCI 1991). In fish, chlorothalonil is also reported to be detoxified by GST catalyzed GSH conjugation in the liver and gills of channel catfish (Gallagher et al. 1991). The present study showed that a failure in redox

status may occur during prolonged periods of treatment, which is in agreement with the proposed mechanism for the fungitoxicity of chlorothalonil.

To summarize, the present paper has demonstrated that the fungicide chlorothalonil was able to induce a modulation of GSH content in SHE cells and of related enzyme activities involved in the homeostasis of the redox cycle. These alterations may initiate the deterioration of cellular defence. The involvement of the GSH pathway in the toxicological effects of chlorothalonil, outlined in this study, may partly explain the tumour promoting effects of chlorothalonil in SHE cells. The results suggest that the transforming effects of SHE cells after exposure to chlorothalonil may be related to GSH-depletion and a generation of organic peroxides. It would be useful to examine whether supplementation or correction of GSH depletion prevents the carcinogenic effects of the pesticide.

Acknowledgments. We warmly thank Mrs. Tracy Carmona for her assistance in correcting this paper, and Professor Armand Maul and Mr. Gilles Martignon for their collaboration in statistical analyses.

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