Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The role of antioxidants in maintaining the functional integrity of adrenocortical cells during in vitro exposure to endosulfan, an organochlorine pesticide, was investigated in rainbow trout (*Oncorhynchus mykiss*). Aminotriazole (ATA), an inhibitor of catalase (CAT), L-buthionine sulfoximine (L-BSO), an inhibitor of glutathione (GSH) synthesis, and *N*-acetyl cysteine (NAC), a glutathione precursor, were used to investigate the role of CAT and GSH redox cycle in protection against the adrenal toxicity of endosulfan, a pesticide that impairs cell viability (LC_{50} 366 μM) and cortisol secretion (EC_{50} 19 μM) in a concentration-related manner. Pretreatment with ATA and L-BSO enhanced the toxicity of endosulfan (LC_{50} and EC_{50}, respectively, 302 and 2.6 μM with ATA, 346 and 3.1 μM with L-BSO), while pretreatment with NAC had no significant effect on cell viability and increased the EC_{50} of endosulfan to 51 μM. CAT activity was significantly reduced following exposure to endosulfan when cells were pretreated with ATA. Pretreatment with L-BSO significantly decreased glutathione peroxidase (GPx) activity and reduced glutathione (GSH) levels in a concentration-related manner following exposure to endosulfan, while GSH levels were significantly higher in NAC pretreated cells compared to untreated cells. Finally, pretreatment with ATA and L-BSO increased, while pretreatment with NAC decreased, lipid hydroperoxides (LOOH) levels. CAT, GPx, and GSH were identified as important antioxidants in maintaining the function and integrity of rainbow trout adrenocortical cells and ATA, L-BSO, and NAC were identified as effective modulators of CAT and GSH redox cycle. Moreover, this study suggests that the glutathione redox cycle may be more efficient than catalase in protecting adrenocortical cells against endosulfan-induced oxidative stress.
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Keywords: Adrenocortical cells; Endosulfan; Cortisol; Viability; Oxidative stress; Antioxidant; Lipid peroxidation; Rainbow trout

Introduction

Oxidative stress is defined as a disruption of the prooxidant–antioxidant balance in favor of the former, leading to potential damage (Sies, 1991). It is a result of one of three factors: (1) an increase in reactive oxygen species (ROS), (2) an impairment of antioxidant defence systems, or (3) an incapacity to repair oxidative damage. The main damage induced by ROS results in alterations of cellular macromolecules such as membrane lipids (lipid peroxidation), DNA, and/or proteins. The resulting damage may alter cell func-

tion through changes in intracellular calcium or intracellular pH, eventually leading to cell death (Masaki et al., 1989; Kehrer et al., 1990; Swann et al., 1991). Recently, it has been reported that the endocrine-disrupting effects of the pesticide endosulfan in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) are mediated by oxidative stress (Dorval et al., 2003).

Enzymatic and nonenzymatic antioxidants such as reduced glutathione (GSH) and its precursor *N*-acetylcysteine (Bachowski et al., 1998) normally counteract damaging effects of intracellular ROS by either repairing the oxidative damage or directly scavenging oxygen radicals. The three most important specialized antioxidant enzymes are the superoxide dismutase (SOD) that converts O_2 into H_2O_2

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which is detoxified into H_2O and O_2 by either catalase (CAT) or peroxidases. The main peroxidase is glutathione peroxidase (GPx) which utilizes GSH as an electron donor (Ahmad, 1995). Although the antioxidant enzymes have been characterized in fish, the role of the antioxidant systems in maintaining the integrity of endocrine cells of any species, including teleost fish, have not yet been investigated.

A number of studies provided evidence for the capacity of organochlorine pesticides such as endrin, lindane, hexachlorocyclohexane (HCH), and others to induce oxidative stress in different organs of mammals (Bagchi and Stohs, 1993; Bagchi et al., 1995, Bachowski et al., 1998; Sahoo et al., 2000). Hincal et al. (1995) reported the oxidant stress-inducing effects of endosulfan, a chlorinated hydrocarbon insecticide, with an increase of lipid peroxidation and a significant alteration in glutathione redox cycle in cerebral and hepatic tissues of rats. In fish, modulation of antioxidant systems in liver by endosulfan, and the modulatory effect of preexposure to copper on the endosulfan-induced oxidative stress, have been reported by Pandey et al. (2001). Endosulfan belongs to the group of cyclodienes, chemicals that are potent inhibitors of Na^+/K^+ and Ca^{2+}/Mg^{2+} ATPase, essential for transport of ions across membranes, in mammals as well as in fish (Naqvi and Vaishnavi, 1993). The acute toxicity of endosulfan in fish is relatively high (LC_{50} 96 h = 1.5 μg/L in rainbow trout) (Ferrando et al., 1991), but it does not readily bioaccumulate in tissues (Naqvi and Vaishnavi, 1993). A previous study in our laboratory demonstrated that the endocrine disrupting effects of endosulfan, characterized as a concentration-dependent loss of capacity to secrete cortisol (Leblond et al., 2001; Bisson and Hontela, 2002), are mediated by oxidative stress in the adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) following acute in vitro exposure (Dorval et al., 2003). However, the relative importance of the various antioxidant systems mediating oxidative stress in endosulfan-induced adrenal toxicity has not been investigated thus far.

The objective of the present study was to investigate the role of the antioxidant defense systems in the adrenocortical cells of rainbow trout following acute in vitro exposure to endosulfan. To evaluate the role of the glutathione redox cycle, L-buthionine-[S,R]-sulfoximine (L-BSO) and *N*-acetylcysteine (NAC) were used. L-BSO, a specific and irreversible inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the rate-limiting step of glutathione synthesis (Griffith and Meister, 1979), was used to deplete intracellular glutathione levels. To increase glutathione levels, the antioxidant NAC was used as effective glutathione precursor. The role of catalase was determined by using the irreversible inhibitor aminotriazole (ATA). Cell viability, cortisol secretion, lipid peroxidation (LPO), antioxidant activities (catalase, glutathione peroxidase), and reduced glutathione (GSH) levels were evaluated to test the hypothesis that the antioxidant defense systems play an important role

in maintaining the function and integrity of the adrenocortical cells.

Materials and methods

Chemicals. Porcine adrenocorticotropin (ACTH[1–39]), minimum essential medium (MEM), bovine serum albumin (BSA), Hepes, reduced glutathione (GSH), reduced α-nicotinamide adenine dinucleotide phosphate (α-NADPH), glutathione reductase (GRd), propidium iodide (PI), cumene hydroperoxide, metaphosphoric acid (MPA), 3-amino-1,2,4-triazole (ATA), L-buthionine-[S,R]-sulfoximine (BSO), and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Collagenase/dispase mixture (collagenase from *Achromobacter iophagus* and dispase from *Bacillus polymixa*) was obtained from Boehringer Mannheim (Laval, Quebec, Canada). RIA kit for cortisol determination and 3-aminobenzoic acid ethyl ester (MS 222) for anesthesia were purchased from ICN Pharmaceuticals (Orangeburg, NY, USA). Hydrogen peroxide (H_2O_2), trypan blue, and culture plates (96-well) were obtained from Fisher Scientific (Nepean, Ontario, Canada). Endosulfan was purchased from Riedel de Haën (Diesenhofen, Germany), and GSH and LPO assay kits were obtained from Oxis Research Medicorp (Montreal, Quebec, Canada).

Experimental animals. Juvenile rainbow trout (*O. mykiss*) were obtained from a Pisciculture Laurentienne (body weight ∼ 100 g). Fish were maintained in a 600-L freshwater tank at 15 ± 1 °C, supplied with a constant flow rate of 3.8 L/min of filtered and oxygen-saturated water (hardness 70 mg/L $CaCO_3$). They were fed daily with commercial trout food at the manufacturer's recommended rate (10 g/kg of fish). Two weeks of acclimation were allowed before beginning the experiments.

Preparation of cell suspensions. Adrenocortical cell suspensions were prepared as described by Leblond and Hontela (1999). Fish were anesthetized with MS 222 and bled from the caudal vasculature to remove as much blood as possible. Head kidneys (also identified as interrenal or adrenal tissue, organs homologous to mammalian adrenal gland) were then dissected out and deposited into a tube containing MEM solution supplemented with 2.2 g/L $NaHCO_3$ and 5.0 g/L BSA (complete medium) at pH 7.4. The tissues were then cut into fragments and washed to remove a maximum of blood cells, resuspended in 2.5 mL of medium containing 2 mg/mL collagenase/dispase, and incubated for 60 min with slow agitation at room temperature. Following enzymatic digestion, the solution was filtered through a 30 μm mesh cloth and the filtrate, containing individualized cells, was transferred into a 15-mL polypropylene tube and centrifuged at 300*g* (1000 rpm) (IEC Centra-8R centrifuge) for 5 min. The supernatant was then removed and the pellet resuspended in 1.5 mL of MEM

solution. The cellular density was determined and adjusted to 75×10^6 cells/mL using a hemacytometer.

Exposure to pesticide. Cells were plated in a 96-well microplate at 150 μL of 75×10^6 cells/mL per well and preincubated for 2 h with 10 mM ATA, 1 mM BSO, 1 mM NAC, or both ATA/NAC (v/v, 1:1). The microplate was then centrifuged at 300g for 3 min and the supernatants were removed. Cellular pellets were resuspended in 150 μL of Ringer solution (13.0 g/L NaCl, 0.4 g/L CaCl$_2$, 0.4 g/L KCl, 0.4 g/L NaHCO$_3$, 2.0 g/L dextrose, and 11.9 g/L Hepes at pH 7.4) with or without toxicant for control wells, and incubated for 60 min at 15 °C. For the experiment, cells were exposed to endosulfan (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) in Ringer solution. Endosulfan was first dissolved in dimethyl sulfoxide (DMSO) at a final DMSO concentration of 5% v/v. Blanks with DMSO were used to demonstrate that 5% DMSO has no effect on cell viability or cortisol secretion (data not shown). Following exposure to endosulfan, the plates were centrifuged (300$g \times 3$ min) and cells were washed with 200 μL of Ringer solution and centrifuged again.

Stimulation of cortisol secretion and determination of viability. After the washing step, pellets were resuspended in 150 μL of MEM with 1 IU/mL ACTH, and incubated at 15 °C for 60 min. The optimal concentrations of ACTH and the incubation times have been determined previously (Leblond et al., 2001). The plate was then centrifuged at 300g for 3 min. Cortisol secretion was determined by radioimmunoassay (RIA) using the supernatants. Viability was assessed by flow cytometry using the exclusion dye propidium iodide (PI 1μg/mL). The cells were analyzed using a FAC-scan (Becton Dickinson, Rutherford, NJ) equipped with an argon laser emitting at 488 nm and 10,000 events were analyzed for each sample.

Enzyme assays. Enzyme activities and protein concentration were measured after lysis of cells in deionized water. Protein concentration was measured by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard.

CAT activity was measured by the decrease in absorbance at 240 nm ($\varepsilon = 0.04$/mM/cm) (Ultrospec 2000 Pharmacia Biotech) due to H$_2$O$_2$ consumption, according to Beers and Sizer (1952). The assay was performed using a solution of 12 mM H$_2$O$_2$ containing the cellular lysate in a final volume of 3 mL at 25 °C. One unit of CAT activity was defined as 1 μmol of H$_2$O$_2$ consumed/min/mg of protein.

GPx activities were estimated by the decrease in absorbance at 340 nm ($\varepsilon = 6.22$/mM/cm) due to NADPH oxidation. The measures were done in a coupled enzyme system where GSSG is reduced to GSH from excess glutathione reductase. The decrease in absorbance was followed by 50 mM Tris-phosphate buffer (pH 7.6) containing 0.1 mM EDTA, 2.4 mM GSH, 1 unit glutathione reductase, 0.16 mM NADPH, and 2.4 mM cumene hydroperoxide (adapted from Tappel, 1978). One unit of GPx activity was defined as 1 nmol of NADPH oxidized/min/mg of protein.

Reduced glutathione levels. GSH levels were measured using the GSH-400 method (Oxis Research Medicorp) based on a chemical reaction which proceeds in two steps, the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluoromethyl-quinolinium (reagent) and all mercaptans (RSH) which are present in the sample and a β-elimination reaction that specifically transforms the substitution product (thioethers) obtained with GSH into a chromophoric thione which has a maximal absorbance wavelength at 400 nm. The apparent molar extinction coefficient (ε) was determined using a GSH 0.5 mmol/L in 5% MPA (metaphosphoric acid) solution as standard.

Lipid peroxides determination. LPO levels were determined using the LPO-560 method (Oxis Research Medicorp) based on a direct colorimetric measurement of lipid hydroperoxides (LOOH). The assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides, the ferric ions binding the indicator dye, xylenol orange, to form a stable, colored complex that can be measured at 560 nm. To eliminate H$_2$O$_2$ interference from the LOOH measurement, the samples are pretreated with catalase and a sample blank is assayed to quantify and substract the non-LOOH produced 560 nm signal.

Cortisol secretion, as well as cell viability, enzyme activities, and GSH and LPO levels are expressed as a percentage of the control (cells incubated without endosulfan) from each individual fish. Control wells for each fish were included in each cell preparation because the variation in cortisol secretion and antioxidants levels between cells from different fish was less than 10% when expressed as a percentage of control, while the variation in the absolute values (ng cortisol/mL and IU) between fish was higher (up to 30%), due possibly to differences in the number of steroidogenic cells between fish (Leblond et al., in preparation).

Statistics. Data were transformed, when necessary, to respect normality. Statistical differences between responses of exposed cells and nonexposed controls were tested using a one-way analysis of variance (ANOVA) with a confidence range of $P < 0.05$. Differences in cortisol secretion, cell viability, enzymatic activities, reduced glutathione (GSH), and lipid hydroperoxide between pretreated and untreated cells were assessed using Student's t and Tukey-Kramer tests ($\alpha = 0.05$). The EC$_{50}$ (the concentration that inhibits cortisol secretion by 50%) and LC$_{50}$ (the concentration that kills 50% of the cells) were determined using the best fitting polynomial model with Jumpin software system.
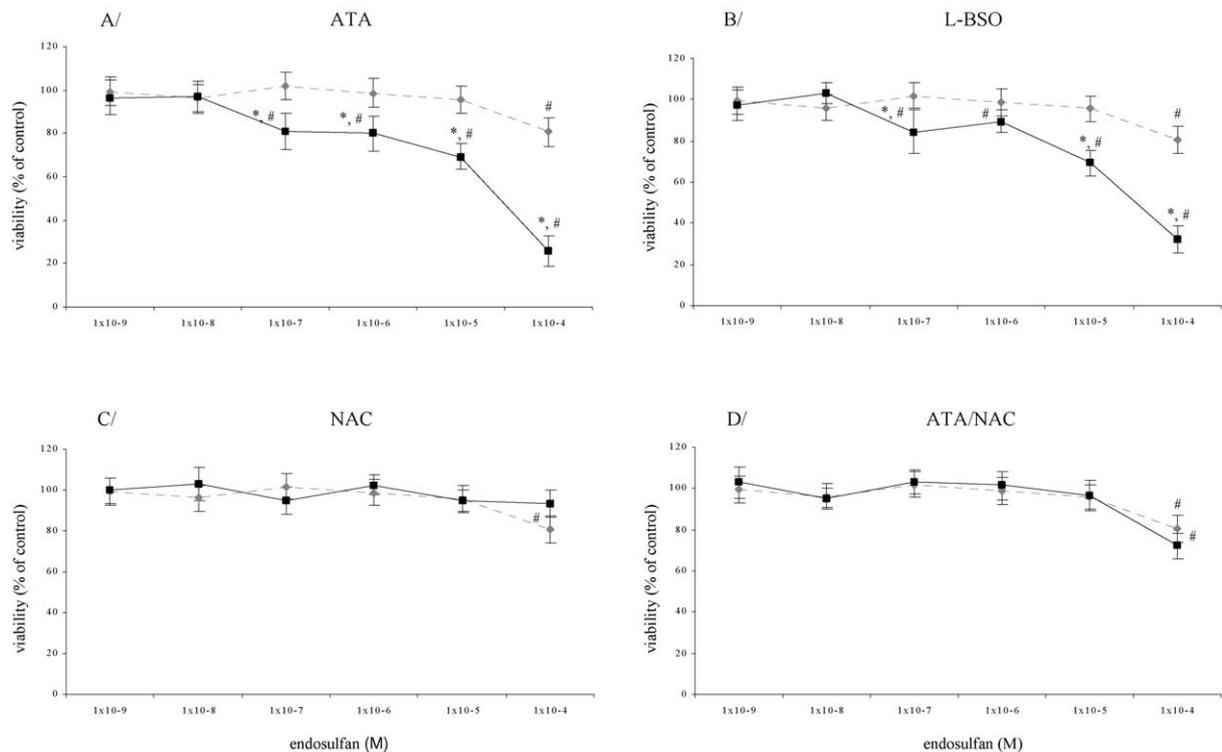
Fig. 1. Cell viability (mean $\pm$ SE, expressed as % of nonexposed control), assessed by flow cytometry using the exclusion dye propidium iodide (FACscan) method, following in vitro exposure to endosulfan for 60 min. Viability of cells pretreated for 120 min with 10 mM ATA, 1 mM L-BSO, 1 mM NAC, or ATA/NAC (1/1) is indicated by squares and by diamonds when untreated. * indicates significant differences between pretreated and untreated cells for each concentration of endosulfan and # indicates significant differences from nonexposed control (Student's t test and Tukey-Kramer test, $\alpha = 0.05$). Adrenocortical cells obtained from the adrenal tissue of one fish correspond to $n = 1$ and the number of fish for each concentration and experiment is 8.

Results

Cortisol secretion and cell viability

Responses of ACTH-stimulated adrenocortical cells to an in vitro exposure to endosulfan, with and without modulators, are shown in Figs. 1 and 2. The data are expressed as a percentage of respective controls not exposed to endosulfan (control taken as 100%). Concentrations of ATA (10 mM), L-BSO (1 mM), and NAC (1 mM) have been tested in a pilot study and selected because they had no apparent effects on viability and cortisol secretion in pretreated controls not exposed to endosulfan (Table 1). At these concentrations, ATA, L-BSO, and NAC were effective modulators of CAT activity and GSH levels (Table 2). Pretreatment with ATA had no effect on GPx activity.

Viability when cells were pretreated with ATA was impaired in a concentration-related pattern (Fig. 1A). At 10^{-7} M and higher concentrations of endosulfan, cytotoxicity was significantly greater in cells pretreated with ATA than without the inhibitor, with a median lethal concentration (LC_{50}, the concentration that kills 50% of the cells) of 302 μM of endosulfan compared to cells not pretreated with ATA (LC_{50} 366 μM, Table 1). Viability of L-BSO pretreated cells was also impaired in a concentration-related pattern (Fig. 1B). The cytotoxicity was significantly higher

following treatment with L-BSO (LC_{50} 346 μM) than without L-BSO (Table 1). Pretreatment with NAC had no significant effect on cell viability and no significant differences were observed between cells pretreated with NAC or not, up to 10^{-5} M endosulfan (Fig. 1C). Exposure up to 10^{-5} M endosulfan had no significant effect on viability of cells pretreated with both ATA/NAC (Fig. 1D). The general pattern of cell viability was similar between cells exposed to both ATA/NAC and cells without the modulators, and no significant differences were observed between cells that were pretreated or not.

Cortisol secretion following exposure to endosulfan and pretreatment with ATA, L-BSO, NAC, or both ATA/NAC are shown in Fig. 2. No significant differences were observed between the mean values of cortisol secretion in controls not exposed to endosulfan, with or without modulators, although cortisol secretion tended to be lower in ATA or L-BSO treated cells, as shown in Table 1. Cortisol secretion was significantly lower at 10^{-6} and 10^{-5} M endosulfan in cells pretreated with ATA (Fig. 2A) with a median effective concentration (EC_{50}, the concentration that inhibits cortisol secretion by 50%) of 2.6 μM with ATA and 19 μM without ATA. Cortisol secretion in L-BSO pretreated cells was significantly lower than without L-BSO at 10^{-8} to 10^{-5} M of endosulfan (Fig. 2B), with a EC_{50} of 3.1 μM. Cortisol secretion was significantly higher when cells were
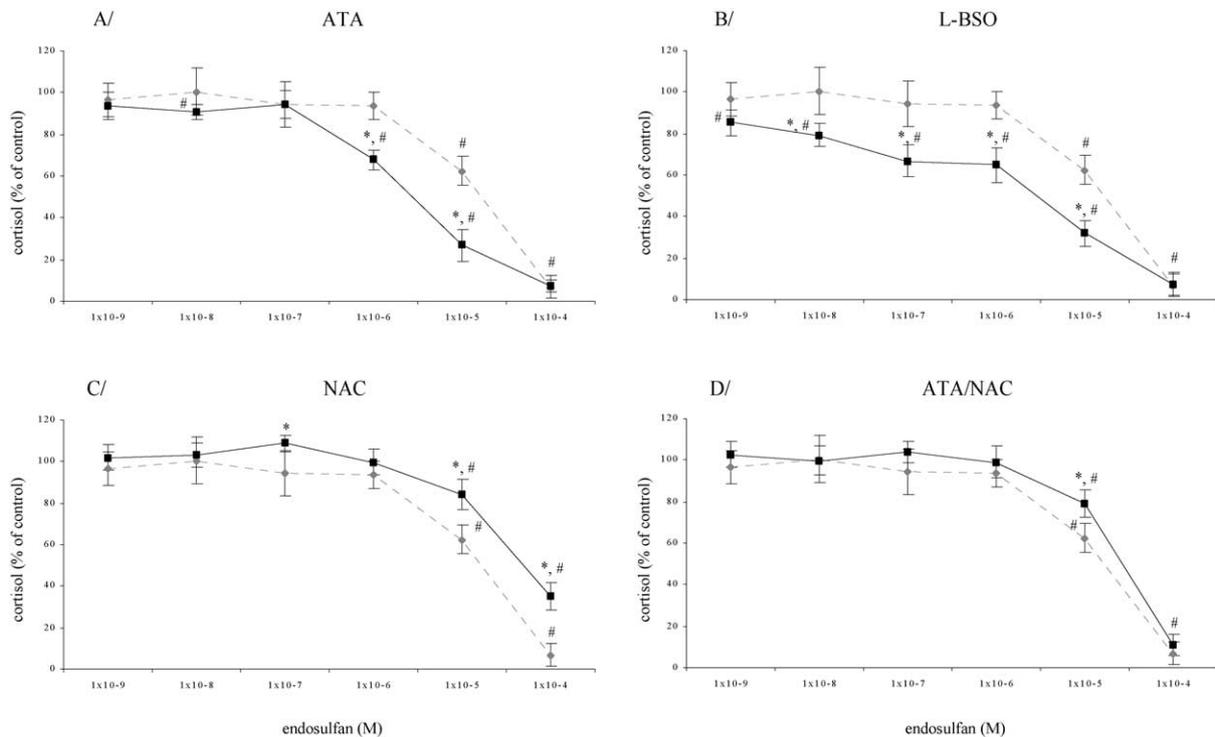
Fig. 2. Cortisol secretion by adrenocortical cells (mean ± SE, expressed as % of control) exposed in vitro to endosulfan for 60 min and subsequently stimulated with 1.0 IU/mL ACTH at 15 °C. Cortisol secretion by cells pretreated for 120 min with 10 mM ATA, 1 mM L-BSO, 1 mM NAC, or ATA/NAC (1/1) is indicated by squares, and diamonds were used for untreated cells. * indicates significant differences between pretreated and untreated cells for each concentration of endosulfan and # indicates significant differences from nonexposed control (Student's *t* test and Tukey-Kramer test, $\alpha = 0.05$). Adrenocortical cells obtained from the adrenal tissue of one fish correspond to *n* = 1 and the number of fish for each experiment is 9.

pretreated with NAC at 10^{-7}, 10^{-5}, and 10^{-4} M (Fig. 2C), with a EC_{50} of 51 μM. Finally, the general pattern of cortisol secretion in cells pretreated with both ATA/NAC was similar to the pattern in untreated cells and no signifi-

cant differences were observed between the two treatments except at 10^{-5} M where cortisol secretion was lower in untreated cells (Fig. 2D). The EC_{50} in cells exposed to both ATA/NAC was 29 μM.

Pretreatment with ATA and L-BSO increased the adrenotoxicity (ratio LC_{50}/EC_{50} that provides some information on the specificity of action of the chemicals tested in this

Table 1

Cortisol secretion (mean ± SEM) in rainbow trout (*Oncorhynchus mykiss*) adrenocortical cells in controls (no endosulfan) and the EC_{50} and LC_{50} in cells exposed to endosulfan and pretreated with aminotriazole (ATA), L-buthionine sulfoximine (L-BSO), *N*-acetyl cysteine (NAC), both ATA/NAC, or untreated

	Control cortisol secretion (ng/mL)	EC_{50} (μM)[a]	LC_{50} (μM)[b]	LC_{50}/EC_{50}
Untreated cells	17.31 ± 5.94	19	366	19.26
ATA	12.88 ± 4.52	2.6	302	116.16
L-BSO	13.55 ± 5.18	3.1	346	111.61
NAC	18.75 ± 6.17	51	N.D.	N.D.
ATA/NAC	16.51 ± 8.48	29	N.D.	N.D.

Note. Cells obtained from the adrenal tissue of one fish correspond to *n* = 1 and the number of replicates for each measure (cortisol secretion) is 8–9.

[a] EC_{50} (the concentration that inhibits cortisol secretion by 50%) and

[b] LC_{50} (the concentration that kills 50% of the cells) were determined using the best fitting polynomial model with Jumpin software system, and expressed in μM of endosulfan. Pretreatment with NAC and ATA/NAC did not induced 50% mortality in our system even at the highest concentration of endosulfan, thus LC_{50} could not be determined (N.D.).

Table 2

Effects of pretreatment with aminotriazole (ATA), L-buthionine sulfoximine (L-BSO) and *N*-acetyl cysteine (NAC) on catalase (CAT) and glutathione peroxidase (GPx) activities, reduced glutathione (GSH) and lipid hydroperoxides (LOOH) levels in adrenocortical cells (nonexposed to endosulfan), expressed as a % of increase ($\uparrow$) or decrease ($\downarrow$) compared to untreated controls

	CAT	GPx	GSH	LOOH
ATA	$\downarrow$ 40%[a]			$\uparrow$ 11%[a]
L-BSO		$\downarrow$ 12%[a]	$\downarrow$ 10%[a]	$\uparrow$ 11%[a]
NAC			$\uparrow$ 11%[a]	$\downarrow$ 9%[a]

[a] Significant differences between untreated and pretreated cells (Student's *t* test, $\alpha = 0.05$). Cells obtained from the adrenal tissue of one fish correspond to *n* = 1 and the number of replicates for each experiments is 8. Enzymatic activities of untreated controls after 120 min of incubation time were 19.54 ± 11.66 U for CAT and 44.14 ± 19.19 for GPx (one unit of enzyme activity defined as 1 μmol of product or substrate formed or consumed/min/mg of protein). GSH levels of untreated controls were 3.61 ± 1.46 U (one unit defined as 1 μM GSH/mg of protein).

system) of endosulfan (116.16 with ATA and 111.61 with L-BSO compared to 19.26 in untreated cells) (Table 1). Moreover, the EC_{50} in adrenocortical cells exposed to endosulfan and pretreated with ATA and L-BSO were six times lower compared to the EC_{50} in cells not pretreated. Thus, pretreatment with ATA and L-BSO increased the endocrine disrupting effects of endosulfan.

Antioxidative enzymes activities

CAT and GPx activities after a 60 min exposure in vitro to endosulfan with or without modulators are shown on Fig. 3. The data are expressed as a percentage of controls not exposed to endosulfan, without modulators. Parameters of each enzyme kinetic (volume assay, time, and number of measures) have been previously determined to obtain the optimal enzymatic reaction (Dorval et al., 2003). An incubation period of 60 min, necessary to reach maximal cortisol secretion with the optimal dose of ACTH (Leblond et al., 2001), was sufficient to induce optimal enzymatic activities and was therefore selected for the experiments with endosulfan so the results could be interpreted in relation with cortisol secretion and viability.

ATA, L-BSO, and NAC were efficient modulators of CAT and GPx and significant differences were observed in enzymatic activities in cells incubated with or without modulators (Table 2). ATA and L-BSO produced, respectively, a 40% decrease of CAT activity and 12% decrease of GPx activity in controls (cells not exposed to endosulfan) compared to control without ATA or L-BSO.

CAT activity in cells pretreated with ATA (Fig. 3A) was significantly reduced at 10^{-6}, 10^{-5}, and 10^{-4} M of endosulfan. Moreover, CAT activity was significantly lower when cells were pretreated with ATA at 10^{-9} to 10^{-4} M of endosulfan, than without ATA. GPx activity in cells pretreated with L-BSO was significantly reduced in a concentration-related manner as shown in Fig. 3B and was significantly lower at 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-4} M of endosulfan, compared to GPx activity in untreated cells. Although GPx activity was significantly reduced at 10^{-6} to 10^{-4} M of endosulfan in cells pretreated with NAC, a significantly greater reduction of GPx activity was observed without pretreatment with NAC (Fig. 3C).

Reduced glutathione (GSH) levels

GSH levels, measured in adrenocortical cells after 60 min exposure to endosulfan with or without modulators, are shown in Fig. 4. GSH levels in cells pretreated with L-BSO and in untreated cells decreased in a concentration-related pattern (Fig. 4A) and at 10^{-4} M of endosulfan, GSH levels were undetectable. Moreover, GSH levels were lower in cells pretreated with L-BSO compared to untreated cells at 10^{-9}, 10^{-8}, and 10^{-5} M endosulfan. Intracellular GSH in control following treatment with L-BSO was reduced by 10% compared to untreated control (Table 2). GSH levels
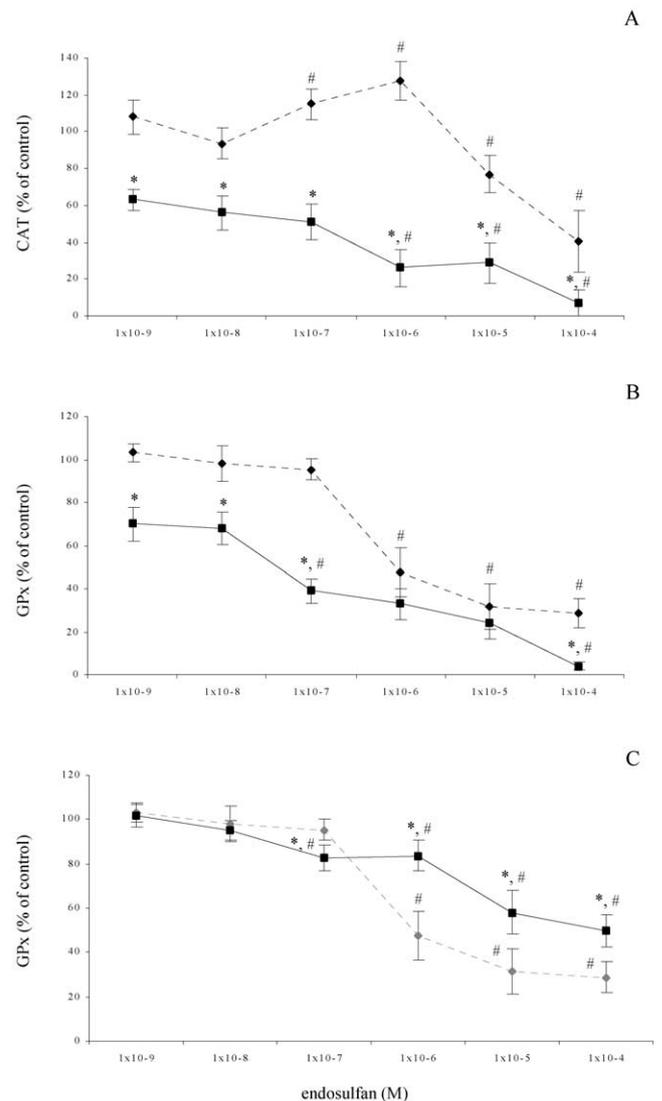
Fig. 3. CAT and GPx activities (mean ± SE, expressed as % of untreated control) in adrenocortical cells exposed in vitro to endosulfan for 60 min. Enzymes activities are represented by squares when cells were pretreated with 10 mM ATA (A), 1 mM L-BSO (B), or 1 mM NAC (C) and by diamonds when untreated. * indicates significant differences between pretreated and untreated cells for each concentration of endosulfan and # indicates significant differences from control (Student's *t* test and Tukey-Kramer test, $\alpha = 0.05$). The number of fish for each experiment is 8. Each point corresponds to the average of at least three replicates by fish and by concentration of endosulfan.

also decreased in a concentration-related pattern when cells were pretreated with NAC (Fig. 4B), but were, however, significantly higher at 10^{-7} to 10^{-4} M of endosulfan when NAC was used, compared to untreated cells. Moreover, NAC increased significantly the intracellular GSH levels in control compared to control without NAC by 11% (Table 2).

Lipid hydroperoxides

LOOH levels were measured in adrenocortical cells exposed to endosulfan, with or without ATA, L-BSO, and
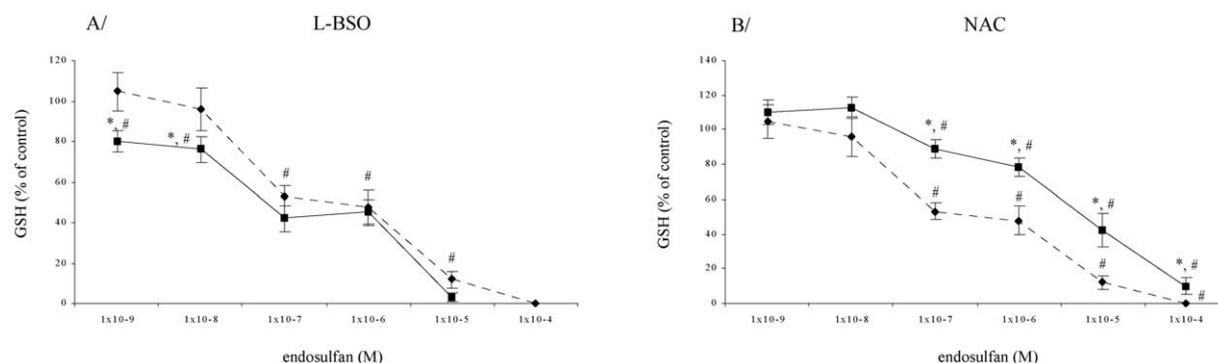
Fig. 4. Reduced glutathione (GSH) levels (mean ± SE, expressed as % of untreated control) in adrenocortical cells after 60 min exposure to endosulfan. GSH levels are represented by squares when cells were pretreated for 120 min with 10 mM ATA, 1 mM L-BSO, or 1 mM NAC and by diamonds when untreated. * indicates significant differences between pretreated and untreated cells for each concentration of endosulfan and # indicates differences from control (Student's *t* and Tukey-Kramer tests, $\alpha = 0.05$). Adrenocortical cells of one fish correspond to *n* = 1 and the number of fish for each experiment is 8.

NAC (Fig. 5). LOOH levels were significantly increased at 10^{-9} to 10^{-4} M of endosulfan in cells pretreated with ATA and significantly higher at 10^{-9}, 10^{-8}, 10^{-6}, 10^{-5}, and 10^{-4} M endosulfan compared to untreated cells (Fig. 5A). ATA increased significantly the LOOH levels by 11% in control compared to control without ATA (Table 2). LOOH levels were significantly increased in cells pretreated with L-BSO at 10^{-9} to 10^{-4} M of endosulfan (Fig. 5B). L-BSO increased LOOH levels in control by 11% compared to untreated control (Table 2). LOOH levels were significantly reduced in NAC pretreated cells at 10^{-9} to 10^{-4} M of endosulfan and significantly lower at all exposures to endosulfan compared to cells not pretreated with NAC (Fig. 5C).

Discussion

The present study demonstrated, for the first time, the protective role of the GSH redox cycle and catalase in adrenocortical cells of rainbow trout during acute in vitro exposure to endosulfan, and the important role of antioxidants in maintaining the function and integrity of these cells. A previous study by Dorval et al. (2003) provided evidence that endosulfan interferes with cortisol synthesis in adrenocortical cells at concentrations that are not cytotoxic. Endosulfan was also identified as a chemical inducing alterations in the activity of enzymes involved in oxidative stress and lipid peroxidation. The involvement of the two main cellular antioxidants, catalase and the GSH redox cycle, in protection of cells against oxidative stress induced by endosulfan, was evaluated in the present study in enzymatically dispersed adrenocortical cells, including steroidogenic cells. The role of catalase was studied using the catalase inhibitor, aminotriazole (ATA). GSH was depleted with L-buthionine-[S,R]- sulfoximine (L-BSO), a specific and irreversible inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyses the rate-limiting step of GSH synthesis (Griffith and Meister, 1979). The antioxidant *N*-acetyl cysteine (NAC) was used to increase GSH levels. NAC, a cysteine analogue, can act as an antioxidant by directly reducing free radicals and oxidants such as OH˙ and H_2O_2 and is a chemoprotective agent against the toxic effects of many compounds (McLellan et al., 1995; Gillissen et al., 1997). Recent studies reported that treatment with NAC could reverse oxidative stress in lead-exposed rats and hamsters (Ercal et al., 1996; Gurer et al., 1998).

The head kidney cell suspension is a heterogenous preparation composed mainly of lymphoid cells, but also steroidogenic cells, melanomacrophages and chromaffin cells (Wendelaar Bonga, 1997). However, the measure of cortisol secretion is a unique and highly specific response of steroidogenic adrenocortical cells, and is used, in this and previous studies (Dorval et al., 2003), as an indicator of functional integrity of these cells. The correlation between changes in cortisol secretion and changes in enzyme activities, GSH and hydroperoxide levels following various experimental treatments aimed at modifying the oxidative status of the cells, clearly indicates that the signal generated by the steroidogenic cells is detectable in this preparation. Thus the importance of antioxidants in adrenocortical cells can be tested, even if CAT and GSH levels may be modulated in all the cell types in the adrenal tissue.

A significant decrease of viability in cells pretreated with ATA was observed at concentrations of endosulfan that had no marked effect in cells incubated without the modulator. Moreover, a significant loss of cortisol secretion and a decrease of CAT activity were also observed in cells pretreated with ATA at concentrations of endosulfan that previously had no marked effect. Lipid hydroperoxides (LOOH) levels, used as an indicator of lipid peroxidation, a well-known mechanism of cellular injury and of oxidative stress in cells and tissues, were increased in cells pretreated with ATA, as well as in untreated cells, but no change was observed at the lowest concentrations of endosulfan with ATA. These results suggest that catalase plays an important role in maintaining the function and viability in adrenocortical cells of fish and is important to maintain the capacity of
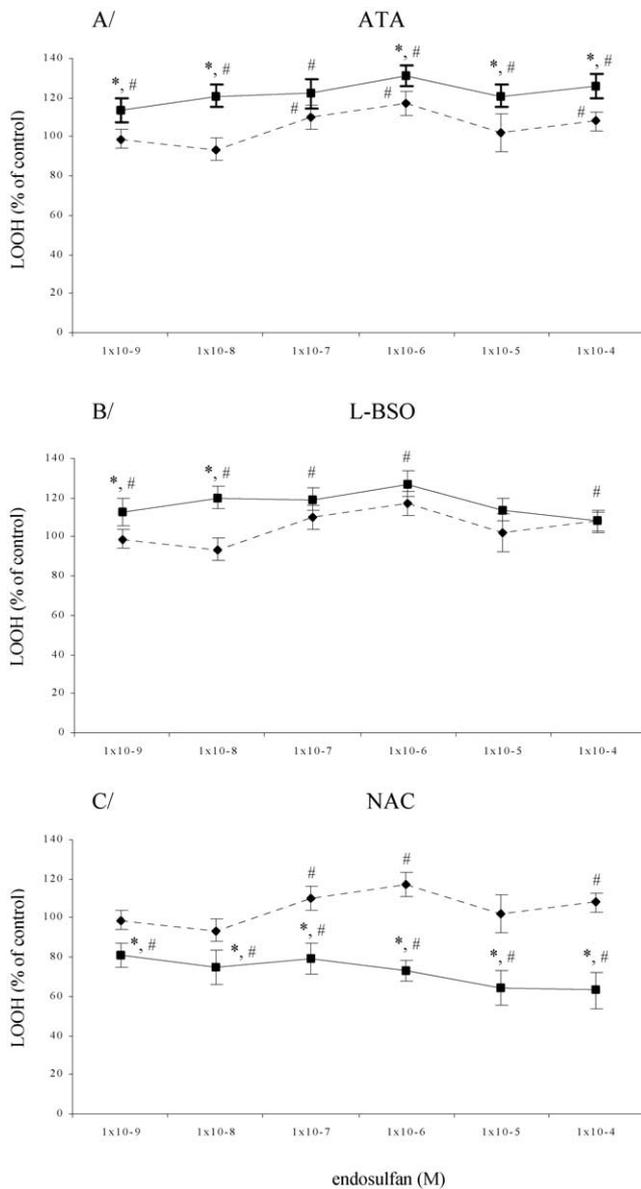
Fig. 5. Lipid hydroperoxide (LOOH) levels (mean ± SE, expressed as % of untreated control) in adrenocortical cells after 60 min exposure to endosulfan. LOOH levels in pretreated cells for 120 min with 10 mM ATA, 1 mM L-BSO, or 1 mM NAC are represented by squares and LOOH levels in untreated cells are indicated by diamonds. * indicates significant differences between treated and untreated cells for each concentration of endosulfan and # indicates significant differences from control (Student's *t* and Tukey-Kramer test, $\alpha = 0.05$). Adrenocortical cells obtained from the adrenal tissue of one fish correspond to $n = 1$ and the number for each experiment is 8.

cells to protect themselves against oxidants. CAT, associated with other enzymatic antioxidants (peroxidases, superoxide dismutase) is capable of removing, neutralizing, or scavenging oxy-intermediates and is, with the GSH redox cycle, the primary cellular enzymatic defense system against hydrogen peroxide (H_2O_2), that it converts to H_2O and O_2. Results from Spitz et al. (1992) on hamster fibroblast cell line provided evidence for the role of catalase as

a major component of cellular resistance to H_2O_2 toxicity. Moreover, reduced catalase activity following ATA treatment was shown to induce a reduction in resistance to H_2O_2-mediated toxicity and levels of catalase activity affected cellular cytotoxic responses to H_2O_2 in Chinese hamster ovary cells (Lord-Fontaine and Averill, 1999). Intracellular catalase was also shown to be important in prevention of oxidative DNA damage as well as deletions and GC–AT transitions upon cadmium exposure in hamster ovary-K1 cells (Chao and Yang, 2001).

The importance of the GSH redox cycle was also evaluated to define the role of GSH and associated enzymes in protecting adrenocortical cells against oxidative stress. Pretreatment with L-BSO decreased cell viability, cortisol secretion, and GPx activities at concentrations of endosulfan that previously produced no marked effects. L-BSO is a specific inhibitor of γ-glutamylcysteine synthetase and induces an irreversible depletion of intracellular GSH levels. Moreover, pretreatment with L-BSO induced lipid peroxidation, similar to pretreatment with ATA. Exposure up to 10^{-5} M endosulfan had no significant effect on viability, with or without pretreatment with NAC. However, pretreatment with NAC reduced the endosulfan-induced loss of cortisol secretion, the decrease of GPx activity, and the depletion of the intracellular GSH levels although not to the levels of controls. Lipid hydroperoxide levels were also significantly reduced when cells were pretreated with NAC. These results suggest that the GSH redox cycle plays an important role in maintaining the functional integrity and the capacity to secrete cortisol by adrenocortical cells following a chemical stress such as the exposure to endosulfan.

GSH redox cycle appears to be important in protecting adrenocortical cells against oxidative stress. Several recent studies documented the importance of intracellular GSH, via glutathione peroxidase and the GSH redox cycle, in protecting cells from oxidative stress caused by oxygen-derived species (Hayes and McLellan, 1999; Woods et al., 1999; O'Brien et al., 2001; Tsukamoto et al., 2002). Reduced (L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant cellular nonprotein thiol and plays a central role in maintaining cellular redox status. Thus, GSH is an essential antioxidant (Shen et al., 1997; Sies, 1999), that is also required for the antioxidant enzyme activities of glutathione peroxidase (GPx), reductase (GR), and transferase (GST) (Hayes and McLellan, 1999). The GPx and GST reactions either consume GSH or produce oxidized GSH (GSSG) that can be reduced to GSH through the action of GR and the reducing equivalents provided by NADPH. Depletion of GSH results in oxidative stress and increased cytotoxicity, whereas elevation of intracellular GSH levels is recognized as an adaptive response to oxidative stress (Woods et al., 1992; Ochi, 1995; Tian et al., 1997; Sagara et al., 1998). The cellular levels of GSH are controlled by multiple enzyme systems such as γ-glutamyltranspeptidase (γ-GT), amino acid transporters, and GR. Moreover, GSH biosynthesis is dependent on the activity of the rate-limiting en-

zyme, γ-glutamylcysteine synthetase (γ-GCS) (Ishikawa et al., 1997; Wild and Mulcahy, 2000), that makes regulation of γ-GCS expression and activity critical for GSH homeostasis (Griffith, 1999). It is also dependent on the availability of the amino acid precursors glutamate, glycine, and cysteine. An action of endosulfan on amino acid transport can thus not be excluded. An action on the enzymatic systems involved in GSH synthesis or GSSG reduction (GR, γ-GT, or γ-GCS) cannot be excluded either in our system. Indeed, an increase of γ-GT activity following GSH depletion and a correlation between exposure to compounds generating oxidative stress and a decrease in γ-GT activity were reported by van Klaveren et al. (1997).

Exposure up to 10^{-5} M endosulfan had no marked effect on viability of cells, pretreated or not with both ATA and NAC (inhibition of catalase and increase of GSH), while a significant decrease compared to control was observed at the higher concentration of endosulfan. These results suggest that the GSH redox cycle, rather than endogenous catalase, plays a critical role in intracellular antioxidant defence. The GSH redox cycle may effectively detoxify H_2O_2 when catalase is compromised. The importance of the GSH redox cycle in protection against oxidative stress was also demonstrated in rat gastric mucous cells exposed to H_2O_2 (Hiraishi et al., 1991), in cultured pulmonary artery endothelial cells following hyperoxia (Suttorp et al., 1991), and in cultured rabbit epithelial cells exposed to reactive oxygen metabolites generated by hypoxanthine and xanthine oxidase (Hata et al., 1997).

In conclusion, this in vitro study suggests that catalase and the GSH redox cycle are important in maintaining the secretory function and the integrity of adrenocortical cells exposed to endosulfan. Moreover, the GSH redox cycle appears to be more efficient than CAT in protecting the cells against oxidative stress. Oxidative stress can be prevented by the action of enzymatic antioxidant defenses (CAT, GPx) and by nonenzymatic antioxidants such as GSH. Under oxidative stress, ROOH are reduced by GSH with concomitant formation of GSSG. GSH also acts in the enzymatic first line antioxidant defense, as a cofactor in GPx mediated reduction of peroxides. Further investigation is required to understand the processes through which intracellular GSH levels are decreased following acute exposure of adrenocortical cells to endosulfan with possible effects on GR, γ-GT, or γ-GCS.

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