Divergent Roles for Glutathione in Lindane-Induced Acute and Delayed-Onset Inhibition of Rat Myometrial Gap Junctions

Rita Loch-Caruso,*† Brad L. Upham,‡ Craig Harris,* and James E. Trosko‡

*Toxicology Program, Department of Environmental Health, University of Michigan, Ann Arbor, MI 48109–2029; and ‡National Food Safety & Toxicology Center, Department of Pediatrics and Human Development, Michigan State University, East Lansing, MI 48824–1392

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INTRODUCTION

Gap junctions are comprised of transmembrane hexameric connexon hemi-channels that are joined from adjacent cells to form intercellular channels. The proteins that make up the connexon hemi-channel belong to the connexin family (Evans and Martin, 2002). Gap junctions are necessary for normal development and homeostatic function, evidenced by disorders of the skin, heart, nervous system, ear, and eye that occur in animals or humans with mutated or deleted connexin genes (Evans and Martin, 2002). Gap junctions are also necessary for parturition, with increased expression of connxin43 and increased abundance and size of gap junctions in uterine smooth muscle immediately preceding parturition (Garfield et al., 1977; Risek et al., 1990). Gap junction intercellular communication is the direct cell-to-cell transfer of low molecular weight ions and molecules through gap junction channels. Toxicant-induced inhibition of gap junction communication is associated with pathophysiologic responses, most notably tumorigenesis (Trosko and Ruch, 1998).

The insecticide lindane (γ-hexachlorocyclohexane) was initially reported to inhibit gap junction intercellular communication in the Chinese hamster V79 cell line (Tsushimoto et al., 1983). Subsequently, lindane was shown to inhibit gap junction intercellular communication in a variety of cell types, including rodent hepatocytes (Ruch and Klaunig, 1986), the rat liver WB-F344 cell line (Guan et al., 1995), and rat myometrial cells (Criswell et al., 1995; Loch-Caruso et al., 2003). Although lindane’s insecticidal activity is attributed to neuroexcitation via inhibition of GABA A chloride channels (Joy et al., 1995), lindane’s ability to act as a liver tumor promoter and to inhibit uterine contractions have been linked to inhibition of gap junctions (Krieger and Loch-Caruso, 2001; Loch-Caruso et al., 2003; Ruch and Klaunig, 1986; Tsushimoto et al., 1983).

Until recently, lindane was used widely as an insecticide in agricultural, forestry, and household applications (ATSDR, 2003). However, lindane’s use now is restricted in the United States to certain seed treatments (U.S. Environmental Protection Agency, 2002), and the European Union has banned its use in agriculture (Weinhold, 2001). Lindane continues to be available as a pharmacological treatment of scabies and lice, with up to 1 million lindane prescriptions written annually in the United States (U.S. Food and Drug Administration Center for Drug Evaluation and Research, 2003). Because of its
A brief (1-h) exposure to lindane induces a biphasic inhibition of myometrial gap junctions as measured by Lucifer yellow dye transfer (Krieger and Loch-Caruso, 2001). The biphasic pattern is characterized by an initial acute inhibition in the presence of lindane, followed by rapid reversal after termination of lindane exposure and subsequent redevelopment of a secondary, delayed-onset inhibition of gap junction intercellular communication without further exposure. Additionally, oxidative stress is evident in myometrial cells after a brief (45 or 60 min) exposure to lindane (Krieger and Loch-Caruso, 2001). Because antioxidants prevent both the acute phase and delayed-onset phase of lindane-induced inhibition of Lucifer yellow dye transfer, a mechanistic role for oxidative stress in lindane-induced inhibition of myometrial gap junctions has been proposed (Krieger and Loch-Caruso, 2001). Recent reports suggest that glutathione (GSH) oxidation may be involved in regulation of gap junctions under conditions of oxidative stress (Upham et al., 1997). Increased generation of reactive oxygen species such as superoxide can lead to oxidation and depletion of GSH under conditions of oxidative stress (Reed, 1990). A role for GSH in lindane’s biphasic inhibition of myometrial gap junctions has not been previously investigated. The present study investigates the hypothesis that depletion of cellular GSH is a mechanistic link between lindane-induced oxidative stress and inhibition of myometrial gap junction communication. Superoxide formation was measured in response to lindane, and modulators of cellular GSH were used to probe the role of GSH in lindane-induced inhibition of myometrial gap junctions. Because lindane inhibits myometrial gap junctions in a biphasic manner (Krieger and Loch-Caruso, 2001), the effects of modulators were investigated immediately after a 1-h exposure (acute inhibition) and again 24 h after termination of lindane exposure (delayed-onset inhibition). Our results show that pharmacological modulation of GSH produced opposite effects on the acute and delayed-onset phases of the lindane-induced inhibition of myometrial gap junction intercellular communication.

METHODS

Chemicals. Lindane (γ-hexachlorocyclohexane, 99% purity) was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of 50 mM lindane was made in dimethyl sulfoxide (DMSO). Lucifer yellow and propidium iodide dyes were obtained from Molecular Probes (Eugene, OR). Roswell Park Memorial Institute (RPMI) cell culture medium was purchased from Gibco (Grand Island, NY) and was supplemented with iron-enriched bovine calf serum (BCS; HyClone, Logan, UT). L-buthionine-[S,R]-sulfoximine (BSO), 2-oxothiazolidine-4-carboxylate (OTC), GSH monoethyl ester, diamide, and all other chemicals used were obtained from Sigma Chemical Co.

Cell isolation and culture. Myometrial smooth muscle cells were isolated from gestation day 10 (GD10) Sprague-Dawley rats as previously described (Caruso et al., 1990). Briefly, rats were anesthetized with ether and euthanized by exsanguination or cardiac puncture. Uteri were removed to ice-cold physiologic saline solution (PSS) containing 135 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 1 mM CaCl2, 1 mM Hepes, and 5 mM glucose, adjusted to pH 7.4 with 1 N NaOH. Each uterus was dissected free of embryos, cervix, ovaries, and adipose tissue, then diced and digested in an enzyme solution containing 300 μg/ml type II collagenase, 300 μg/ml trypsin, and 200 μg/ml deoxyribonuclease I. The digest was filtered to remove large tissue clumps, and the filtrate was centrifuged to pellet the cells. After repeated washing of the cells with calcium/magnesium-free phosphate-buffered saline (CMF-PBS; 2.68 mM KCl, 1.5 mM K2PO4 (monobasic), 136.9 mM NaCl, 8.1 mM Na2PO4 (dibasic heptahydrate) at pH 7.2), cells were seeded into flasks containing RPMI medium supplemented with 10% BCS. Cells were incubated at 37°C with 5% CO2 atmospheric conditions. The medium was changed every 2 days, and cells were subcultured every 6–7 days, just before confluence. Isolated cells were characterized as smooth muscle cells by indirect immunofluorescence labeling with mouse anti-α-smooth muscle–specific actin monoclonal antibody as described elsewhere (Caruso et al., 1990). Alpha-actin labeling indicated that cultures were at least 99% smooth muscle cells. Unless otherwise indicated, all experiments used myometrial cells at passage two, and all incubations were at 37°C in a 5% CO2/95% air atmosphere in RPMI medium supplemented with 10% BCS.

Lucifer yellow dye transfer after lindane exposure. To assess gap junction intercellular communication, myometrial cells in culture were injected with a mixed dye solution of 0.8% Lucifer yellow and 0.02% propidium iodide in CMF-PBS following procedures previously described (Criswell et al., 1995). Lucifer yellow is a low molecular weight hydrophilic fluorescent dye that diffuses between cells through connexin43 gap junctions but does not cross the non-gap junction plasma membrane of healthy cells. Propidium iodide is a fluorescent dye that rapidly binds to nucleic acids and was included with Lucifer yellow in the injected solution to label injected cells. Cells were injected with dye at the end of a 1-h exposure to lindane while kept in the lindane-containing medium or, after rinsing, were injected in lindane-free medium. An injection pressure of 6.5 psi for 200 ms was used for each injection. Gap junction communication was determined by visual examination 5–10 min after injection with an epifluorescence microscope (Nikon Diaphot) for Lucifer yellow dye fluorescence in cells adjacent to and in direct contact with the injected cell. Dye transfer was quantified as follows:

\[
\% \text{Dye transfer} = \frac{\text{number of adjoining cells with Lucifer yellow fluorescence}}{\text{total # of adjoining cells}} \times 100
\]

Individual culture dishes were used for one time-point only, subsequent to random assignment to treatment groups. There were five dishes per group and 6–10 injections per dish.

Superoxide production by cultured cells. Quantification of superoxide production by cultured myometrial cells was based on the method of Boota et al. (1996). Briefly, cells were removed from flasks after a 5-min incubation with 0.25% crude trypsin in CMF-PBS at 37°C, then plated at 5500 cells/cm2 and incubated 24 h at 37°C to allow for attachment and growth. After rinsing the cells with PBS (0.9 mM CaCl2, 2.68 mM KCl, 1.47 mM K2PO4 (monobasic), 0.5 mM MgCl2·6H2O, 8 mM Na2PO4·7H2O at pH 7.2), 1 ml of reaction buffer (2 mM glucose, 1 mM CaCl2, 1.3 mM MgCl2, 4 mM KCl, 100 mM NaCl, 10 mM NADH, 0.25 mM NADP+, 10 ng/ml Cu,Zn-superoxide dismutase) was added to the dishes. Ferricytochrome c was added at a final concentration of 70 μM, and Cu,Zn-superoxide dismutase was simultaneously added to half of the plates at 40 μg/ml final concentration. The reaction was stimulated by adding lindane or solvent (controls). After 1, 15, 30, or 60 min, the reaction was stopped by transferring aliquots of supernatants to microcentrifuge tubes containing 25 μl of 1 mM N-ethylmaleimide per 200 μl sample supernatant. Absorbance was then read at 550 nm, and superoxide production was determined based upon the difference in cytochrome c reduction in the absence or presence of superoxide dismutase. An extinction coefficient of 0.038 absorbance units/mM at 550 nm was used.
21.1 mM$^{-1}$ cm$^{-1}$ was used for calculations. The results were expressed as nmoles superoxide released/1 × 10^5 cells.

**GSH depletion experiments.** Buthionine sulfoximine was used to deplete myometrial cells of GSH. To verify depletion, myometrial cell cultures were exposed to 1 mM BSO or distilled deionized water (solvent controls) for 4 h, and then processed for high-performance liquid chromatography (HPLC) analysis of GSH. To evaluate effects of GSH depletion on lindane-induced inhibition of gap junction intercellular communication, myometrial cell cultures were incubated with 1 mM BSO for 4 h, followed by a 1-h incubation with 100 μM lindane in the continued presence of BSO. As solvent controls for BSO, cell cultures were exposed to 0.1% distilled deionized water for 4 h followed by a 1-h exposure to 100 μM lindane. An additional solvent control group consisted of cell cultures exposed for 4 h to 0.1% distilled deionized water followed by a 1-h exposure to 0.2% DMSO, the solvent used to deliver lindane. A third control group consisted of cultures exposed to 1 mM BSO without lindane for the total 5-h incubation. At the end of the treatment period, cells were either immediately processed for microinjection with Lucifer yellow dye or were rinsed and cultured an additional 24 h in fresh, lindane-free medium before microinjection with Lucifer yellow dye.

**GSH augmentation experiments.** Glutathione monoethyl ester and oxothiazolidine carboxylate (OTC) were used to increase intracellular GSH. Augmentation of cellular GSH was verified by HPLC in myometrial cells exposed to 3 mM GSH monoethyl ester for 1 h or to 5 mM OTC for 65 min. Cell cultures exposed to distilled deionized water for 1 h or 0.1% DMSO for 65 min served as solvent controls for the GSH monoethyl ester and OTC treatments, respectively. To evaluate effects of GSH depletion on lindane-induced inhibition of gap junction intercellular communication, myometrial cell cultures were pretreated with 1 mM or 4 mM GSH monoethyl ester for 15 min or OTC for 5 min prior to a 1-h incubation with 100 μM lindane in the continued presence of the GSH augmenters. As solvent controls, cell cultures were treated with 0.2% DMSO for 1 h. Additional cultures were exposed to 100 μM lindane alone for 1 h or to the GSH augmenters alone for the duration of the combined pretreatment and lindane-exposure periods. At the end of the treatment period, cells were either immediately processed for microinjection with Lucifer yellow dye or were rinsed and incubated an additional 24 h in fresh, lindane-free medium before microinjection with Lucifer yellow dye.

**Measurement of cellular thiol.** High performance liquid chromatography was used to assay cellular GSH and cysteine in myometrial cells exposed to lindane or GSH modulators according to procedures previously described (Fahey and Newton, 1987) and modified (Harris, 1993). To prepare samples for HPLC analysis, the incubation medium was decanted from myometrial cell cultures, and the attached cells were quickly rinsed three times with prewarmed CMF-PBS before cell lysis in 200 mM methanesulfonic acid. The lysate was collected with a cell scraper and transferred to microfuge tubes, flash frozen in liquid nitrogen, and stored at −70°C. Thawed samples underwent ultrasonic cell disruption while on ice. Protein was precipitated by the addition of 4 M sodium methanesulfonate, and samples were centrifuged for 5 min at 150 × g and 5°C. Supernatants were transferred to new microfuge tubes containing HEPPS buffer (1 M HEPPS, 5 mM diethylene triamine, pH 8.5), and pellets were set aside for protein analysis. Monobromobimane solution (1.5 mg dissolved in 1 ml acetonitrile) was added to derivitize the supernatant samples, which were maintained in the dark at room temperature for 20 min. To stop the reaction, 400 mM methanesulfonic acid was added to samples in the dark. Derivitized samples were shielded from light and stored at −70°C until assay by HPLC, as previously described, using fluorescence detection (Harris, 1993). The HPLC results were then expressed on a per milligram protein basis. Protein was measured by the Bradford method (Bradford, 1976) as modified for use with a microtiter plate spectrophotometer.

**Statistical analysis.** Unless otherwise indicated, data were reported as a ± standard error of the mean (s.e.m.). Data analysis was conducted with SigmaStat (Jandel Scientific Software, San Rafael, CA). Superoxide production data were arcsine transformed to normalize the data, then analyzed by two-way analysis of variance (ANOVA). Other data were analyzed by one-way ANOVA or, if tests of normality failed, by the Kruskal-Wallis test. If a significant effect was detected, post hoc comparisons of means were conducted by the Student-Newman-Keuls pairwise multiple comparison test. A p value ≤ 0.05 was considered statistically significant.

**RESULTS**

**Superoxide Production**

Because the heme group of cytochrome c contains iron that can be reduced from Fe$^{3+}$ to Fe$^{2+}$, reduction of cytochrome c can be used to detect reactive oxygen species, and comparison of cytochrome c reduction in the presence and absence of superoxide dismutase allows specific detection of superoxide. As measured by superoxide dismutase–inhibitable reduction of cytochrome c, superoxide production increased in lindane-exposed cultured myometrial cells in a time-dependent manner (ANOVA, p < 0.0001). Exposure to 100 μM lindane increased superoxide production compared with time-matched solvent controls to 1.02, 0.863, 0.732, and 0.773 nmoles/1 × 10^5 cells after 1, 15, 30, and 60 min, respectively (Fig. 1, p ≤ 0.05). Likewise, exposure for 1, 15, 30, and 60 min to 200 μM lindane increased superoxide production to 1.05, 1.23, 1.49, and 1.99 nmoles/1 × 10^5 cells, respectively (Fig. 1, p ≤ 0.05). Increases observed with exposure to 200 μM lindane were not statistically significant compared with 100 μM lindane at each time-point. These results identified superoxide as a reactive oxygen species generated in myometrial cells in response to lindane.

**Effects of GSH Modulators on the Acute Phase of Lindane-Induced Inhibition of Gap Junction Communication**

Our previous study indicated that lindane inhibited myometrial gap junction communication in a biphasic manner, characterized by an acute phase after 1 h of exposure to lindane,
recovery after removal of lindane exposure medium, and then redevelopment of a secondary, sustained inhibition beginning 2 h after termination of exposure and continuing for at least 24 h (Krieger and Loch-Caruso, 2001). Using Lucifer yellow dye transfer to assess inhibition of gap junction communication, the present study compared the effects of GSH modulators on the acute versus the secondary, delayed-onset, sustained phase of lindane-induced inhibition of myometrial gap junction communication.

The effect of depletion of GSH on the acute phase of lindane-induced inhibition of gap junction communication was investigated by treating myometrial cells with the GSH depleter BSO before and during a 1-h exposure to 100 μM lindane. Treatment with 1 mM BSO blocked the acute phase of inhibition of dye transfer in myometrial cells (Fig. 2; Kruskal-Wallis, \( p < 0.004 \)). Cells exposed to lindane alone transferred dye to 3.1% of adjoining cells, compared with 99% dye transfer in the control cultures (Fig. 2; \( p < 0.05 \)). In contrast, dye-transfer levels in cell cultures exposed to BSO alone or BSO plus lindane exceeded 96% and were not significantly different from levels observed in the control group exposed to neither lindane nor BSO (Fig. 2). Because GSH depletion with BSO protected myometrial cells from lindane-induced inhibition of Lucifer yellow dye transfer, this experiment suggested that GSH had a facilitative, rather than protective, role in mediating acute actions of lindane on myometrial gap junctions.

To evaluate the acute effects of GSH augmentation on lindane-induced inhibition of gap junction intercellular communication, myometrial cells were treated with GSH monoethyl ester or OTC before and during exposure to lindane.

Glutathione monoethyl ester is rapidly cleaved intracellularly to release GSH and thereby elevate cellular GSH (Anderson et al., 1985), whereas OTC increases the availability of intracellular cysteine for GSH synthesis (Williamson and Meister, 1981). An acute exposure (1 h) to 100 μM lindane significantly reduced dye transfer regardless of treatment with GSH monoethyl ester (Fig. 3A; ANOVA, \( p < 0.0001 \)). Cells exposed to lindane alone transferred dye to 3.8% of adjoining cells, significantly decreased compared with cells exposed to GSH monoethyl ester (4 mM) alone or with control cells lacking exposure to GSH monoethyl ester and lindane (Fig. 3A; \( p < 0.05 \)). Although 1 mM GSH monoethyl ester had no
significant effect on lindane-induced inhibition of dye transfer, 4 mM GSH monoethyl ester further depressed dye transfer to 0.40% in lindane-exposed cells compared with cells exposed to lindane and other treatments (Fig. 3A; \( p \leq 0.05 \)). Because GSH monoethyl ester releases GSH intracellularly to elevate cellular GSH, the intensification of lindane-induced acute inhibition of dye transfer by GSH monoethyl ester (at 4 mM) provides further support that GSH has a facilitative role in lindane’s acute actions on myometrial gap junctions.

Similar to the results with 1 mM GSH monoethyl ester, an acute exposure (1 h) to 100 \( \mu \)M lindane significantly reduced dye transfer, regardless of treatment with 5 mM OTC prior to and during lindane exposure (Fig. 3B; Kruskal-Wallis, \( p < 0.002 \)). Dye transfer was depressed to similar extents in cell cultures treated with lindane alone or lindane plus OTC, and this effect was significant compared with that observed in cell cultures exposed to OTC alone and control cell cultures exposed to neither lindane nor OTC (Fig. 3B; \( p \leq 0.05 \)). Treatment with OTC alone for the duration of the exposure period had no effect on lindane-induced inhibition of dye transfer. This experiment shows that increasing the availability of intracellular cysteine for GSH synthesis by treatment with OTC failed to protect myometrial cells from acute lindane-induced inhibition of gap junction intercellular communication.

**Effects of GSH Modulators on the Secondary, Delayed-Onset Phase of Lindane-Induced Inhibition of Gap Junction Communication**

The potential role of GSH in the secondary, delayed-onset, sustained inhibition of myometrial gap junctions that develops 2–24 h after termination of exposure to lindane (Krieger and Loch-Caruso, 2001) was evaluated by assessing the effects of GSH modulators on Lucifer yellow dye transfer 24 h after termination of exposures. Selective inhibition of \( \gamma \)-glutamyl synthase, the rate limiting enzymatic step of GSH synthesis, by BSO (1 mM) before and during a 1-h exposure to 100 \( \mu \)M lindane did not prevent inhibition of dye transfer 24 h after termination of lindane exposure (Fig. 4; Kruskal-Wallis, \( p < 0.004 \)). Instead, BSO further decreased dye transfer during the secondary, delayed-onset phase of lindane-induced inhibition of dye transfer compared with cultures treated with lindane alone (Fig. 4; \( p < 0.05 \)). The transfer of dye to adjoining cells was significantly decreased in cell cultures exposed to lindane alone or to lindane and BSO, as compared with control cultures exposed to neither lindane nor BSO and cultures exposed to BSO alone (Fig. 4; \( p \leq 0.05 \)). Because depletion of cellular GSH with BSO magnified the secondary, delayed-onset inhibition of Lucifer yellow dye transfer by lindane, these data suggest that GSH may provide some protection from lindane’s sustained actions on myometrial gap junctions.

Experiments were performed in a similar manner to evaluate the effects of GSH augmentation on the secondary, delayed-onset, sustained inhibition of myometrial gap junctions by lindane. To promote increased intracellular GSH concentrations, cells were exposed to GSH monoethyl ester (3 mM) or OTC (5 mM) prior to and during a 1-h exposure to 100 \( \mu \)M lindane, and then evaluated for effects on Lucifer yellow dye transfer 24 h after termination of exposure. Cells treated with GSH monoethyl ester plus lindane exhibited high levels of dye transfer 24 h after termination of exposures, indistinguishable from control cells (exposed to neither lindane nor GSH monoethyl ester) and cells exposed to GSH monoethyl ester only (Fig. 5A). Consistent with previous experiments, lindane depressed dye transfer to 52% in cell cultures 24 h after termination of exposure, significantly decreased compared with all other treatments (Fig. 5A; Kruskal-Wallis, \( p < 0.01 \); Student-Newman-Keuls, \( p \leq 0.05 \)). These data show that GSH monoethyl ester protected myometrial cells from the secondary, delayed-onset inhibition of Lucifer yellow dye transfer by lindane, in contrast to the apparent facilitative role of GSH monoethyl ester in lindane’s acute actions on myometrial gap junctions.

Likewise, increasing the availability of intracellular cysteine for GSH synthesis by treatment with OTC protected cells from lindane-induced sustained inhibition of gap junction communication. Twenty-four hours after termination of exposure, high levels of dye transfer were observed in cells treated with lindane plus OTC, similar to levels of dye transfer observed in controls exposed to neither lindane nor OTC and cells exposed to OTC alone (Fig. 5B). Consistent with previous experiments, lindane depressed dye transfer to 56% in cell cultures 24 h after termination of exposure, significantly decreased compared with all other treatments (Fig. 5B; Kruskal-Wallis, \( p < 0.003 \); Student-Newman-Keuls, \( p \leq 0.05 \)). These data show that OTC, like GSH monoethyl ester, protected myometrial cells from the secondary, delayed-onset, sustained inhibition of Lucifer
yellow dye transfer by lindane. As such, these data further indicate that GSH may have divergent roles in lindane’s acute versus delayed-onset actions on myometrial gap junctions.

**Pharmacologically Induced Changes in Cellular Thiol Concentrations**

Buthionine sulfoximine, GSH monoethyl ester, and OTC effectively altered intracellular GSH levels under exposure conditions similar to those used in the dye-transfer experiments (Table 1, ANOVA, \( p < 0.0001 \)). Buthionine sulfoximine (1 mM for 4 h) reduced intracellular GSH to at least 45% of control amounts (Table 1, \( p \leq 0.05 \)). Conversely, GSH monoethyl ester (3 mM for 1 h) and OTC (5 mM for 65 min) each increased intracellular GSH levels compared with corresponding controls (Table 1, \( p \leq 0.05 \)). Intracellular cysteine was not significantly altered in cells treated with OTC compared with solvent control cells (0.741 ± 0.11 \( \mu \)g/mg protein and 0.608 ± 0.13 \( \mu \)g/mg protein, respectively.

**DISCUSSION**

Gap junction intercellular communication is regulated by availability of connexin, by insertion of the connexon hemi-channel in the membrane, and by gating mechanisms involving phosphorylation, calcium, pH, and membrane potential (Evans and Martin, 2002). Additionally, a recent report found that hydrogen peroxide–induced inhibition of gap junction communication was GSH-dependent (Upham et al., 1997). The present study is the first report investigating a role for GSH in myometrial gap junction regulation.

Previously, it was shown that lindane increases myometrial lipid peroxidation and nitro blue tetrazolium reduction to formazan, relatively nonspecific indices of oxidative stress. The present study identified superoxide as a reactive oxygen species produced by myometrial cells exposed to lindane, consistent with previous reports that lindane increases superoxide production in neutrophils (English et al., 1986), rat liver (Junqueira et al., 1986), and mouse placenta and fetal liver (Hassoun and Stohs, 1996). Because excessive production of superoxide depletes cellular GSH (Ishikawa and Sies, 1989), the increased production of superoxide is consistent with reports that lindane decreases GSH and induces oxidative stress in myometrial cells (Krieger and Loch-Caruso, 2001). Lindane-induced acute production of superoxide is linked to biphasic inhibition of myometrial gap junctions by our previous finding that cotreatment with superoxide dismutase during a 1-h exposure to lindane prevents both the acute and delayed-onset phases of lindane’s inhibition of gap junction

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glutathione (GSH) (( \mu )g/mg protein)</th>
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<tbody>
<tr>
<td>Solvent control (DDW)</td>
<td>5.09 ± 0.10(^a)</td>
</tr>
<tr>
<td>Buthionine sulfoximine (1 mM for 4 h)</td>
<td>2.29 ± 0.45(^b)</td>
</tr>
<tr>
<td>Solvent control (DDW)</td>
<td>4.95 ± 0.31</td>
</tr>
<tr>
<td>Glutathione monoethyl ester (3 mM for 1 h)</td>
<td>10.24 ± 0.21(^b)</td>
</tr>
<tr>
<td>Solvent control (0.1% DMSO)</td>
<td>4.21 ± 0.77</td>
</tr>
<tr>
<td>Oxothiazolidine carboxylate (5 mM for 65 min)</td>
<td>6.26 ± 0.015(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Data are expressed as the mean amount of thiol (\( \mu \)g) per mg protein ± S.E.M., measured by high performance liquid chromatography (HPLC) at the end of the incubation times indicated (\( n = 3 \)).

\(^b\)Significantly different from corresponding control (\( p \leq 0.05 \)).
communication (Krieger and Loch-Caruso, 2001). Superoxide likely initiates formation of other reactive species also, because the hydroxyl radical scavenger D-mannitol and the lipid peroxidation interrupter \( \alpha \)-tocopherol reverse lindane-induced inhibition of myometrial gap junction communication (Krieger and Loch-Caruso, 2001). Taken together, the current and previously published report (Krieger and Loch-Caruso, 2001) support a reactive oxygen species–dependent mechanism for lindane-induced inhibition of myometrial gap junctions.

Glutathione can protect cells against damage from reactive oxygen species and free radicals that arise during conditions of oxidative stress (Anderson et al., 1985; Reed, 1990). In the present study, pharmacological treatments were used to modify intracellular GSH concentration during lindane exposure. Pretreatment with BSO reduced cellular GSH by more than half, as expected based on its ability to inhibit the rate-limiting enzyme in GSH synthesis, \( \gamma \)-glutamylcysteine synthetase (Meister and Anderson, 1983). Treatment with OTC significantly elevated myometrial GSH, consistent with responses in other cells (Harris et al., 1987) and its ability to increase GSH synthesis by increasing availability of intracellular cysteine (Williamson and Meister, 1981). Treatment with the cell-permeable GSH monoethyl ester, which is de-esterified intracellularly to release GSH (Anderson et al., 1985), doubled myometrial GSH.

Because depletion of cellular GSH with BSO prevented inhibition of Lucifer yellow dye transfer in the presence of lindane, GSH may be necessary for the acute phase of lindane-induced inhibition of myometrial gap junctions. Moreover, a sufficiently high concentration of GSH methyl ester intensified lindane’s acute inhibition of Lucifer yellow dye transfer, supporting a mechanistic role for GSH in the acute phase of lindane-induced inhibition of myometrial gap junctions. A mechanistic role for GSH in the acute phase of lindane-induced inhibition of myometrial gap junctions is in agreement with a report that BSO-induced depletion of GSH prevents acute inhibition of gap junction intercellular communication by the pro-oxidant hydrogen peroxide in the rat liver WB-F344 cell line (Upham et al., 1997).

Previously, we reported that oxidative stress occurs in myometrial cells exposed to lindane under exposure conditions identical to those used in the present study (Krieger and Loch-Caruso, 2001). During cellular oxidative stress, GSH oxidation to GSSG is promoted, and our recent study indicates that cellular GSH/GSSG ratios decrease markedly after a brief (1-h) lindane exposure (Caruso et al., submitted). Altered cell redox status, as reflected by depressed GSH/GSSG ratios, increases oxidation of cell proteins and S-glutathionylation (Poole et al., 2004). A brief (1-h) exposure to lindane stimulates S-glutathionylation in myometrial cells (Caruso et al., submitted), supporting a possible role for S-glutathionylation in lindane-induced modulation of myometrial gap junctions. S-Glutathionylation would be reversible by thioredoxin, glutaredoxin, or GSH (Poole et al., 2004), consistent with the initial reversal of Lucifer yellow dye transfer that follows termination of lindane exposure in the myometrial cells (Krieger and Loch-Caruso, 2001). Consequently, a possible mechanism for acute inhibition and initial recovery of myometrial gap junction inhibition could involve reversible S-glutathionylation of protein cysteines under oxidative stress conditions induced by lindane.

In contrast to the acute phase of lindane-induced inhibition of myometrial gap junctions, depletion of cellular GSH with BSO enhanced the delayed-onset inhibition of gap junction intercellular communication by lindane, and augmentation of cellular GSH with OTC or GSH methyl ester blocked the delayed-onset phase of lindane-induced inhibition of gap junction intercellular communication. The BSO-induced exacerbation of the delayed-onset phase of lindane-induced gap junction communication agrees with previous reports that depletion of cellular GSH renders cells more vulnerable to an oxidative stress–inducing compound (Mitchell et al., 1983; Nishida et al., 1997).

Because the delayed-onset phase of inhibition of gap junction intercellular communication is prevented by cellular GSH (this study) as well as other antioxidants (Krieger and Loch-Caruso, 2001), the mechanism underlying the delayed-onset inhibition may involve an oxidative stress-mediated inhibition of proteins necessary for gap junction function. Additionally, GSH could facilitate reduction of S-glutathionylated proteins (Poole et al., 2004), restoring connexin function and preventing further irreversible oxidation of protein cysteines.

The delayed-onset inhibition of myometrial gap junction communication reaches maximum inhibition 4 h after cessation of lindane exposure (Krieger and Loch-Caruso, 2001) and is sustained for at least 96 h (Caruso et al., submitted). Although connexins have relatively short half-lives (Evans and Martin, 2002), our recent study found no loss of connixin43 after 4 h of exposure to lindane, suggesting that the mechanism likely does not involve inhibition of expression of connixin43 (Loch-Caruso et al., 2004).

Phosphorylation of the gap junction protein connixin43 by protein kinase C is a mechanism by which some agents, particularly phorbol esters, inhibit connixin43 gap junctions (Lampe and Lau, 2000). Although lindane activates protein kinase C in myometrial cells (Criswell et al., 1995), lindane exposure of myometrial cells does not induce phosphorylation at connixin43 ser368, a known protein kinase C phosphorylation target. This finding suggests that the mechanism of lindane-induced inhibition of myometrial gap junctions is independent of protein kinase C phosphorylation of connixin43 (Loch-Caruso et al., 2004). Regulation of myometrial gap junctions by GSH, as implicated in the present study, provides an alternative to protein kinase C for a mechanism by which some toxicants, such as lindane, may inhibit gap junctions.

The concentration of lindane used in the present study is about 100-fold higher than serum concentrations observed in...
occupationally exposed human populations (Baumann et al., 1980) and is significantly higher than concentrations reported in human blood samples of populations lacking known exposure to lindane (Kutz et al., 1991). Nonetheless, because lindane induces a sustained inhibition of myometrial gap junction communication at nanomolar concentrations (Loch-Caruso et al., 2003), an improved understanding of lindane effects on gap junctions may have relevance for understanding potential health risks. It is beyond the scope of the present investigation, however, to resolve whether lindane poses a reproductive risk to pregnant women by interfering with myometrial gap junctions.

Although exact mechanisms have yet to be elucidated, the present study suggests that GSH has distinct but contrasting roles in each stage of the biphasic lindane-induced inhibition of myometrial gap junction communication. The initial acute response of myometrial gap junctions to lindane appears to be GSH-dependent because depletion of cellular GSH prevented acute inhibition. In contrast, depletion of cellular GSH enhanced the delayed-onset phase of lindane-induced inhibition of myometrial gap junctions. Because gap junctions and GSH are each important to cellular and tissue homeostasis (Meister and Anderson, 1983; Trosko and Ruch, 1998), further studies are warranted to investigate the potential pathways that may utilize GSH to signal gap junction inhibition in myometrial and other cells.

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