BIPHASIC LINDANE-INDUCED OXIDATION OF GLUTATHIONE AND INHIBITION OF GAP JUNCTIONS IN MYOMETRIAL CELLS

Rita Loch Caruso¹*

Brad L. Upham²

Craig Harris³

James E. Trosko⁴

¹Toxicology Program, Department of Environmental Health, University of Michigan, 1420 Washington Heights, Ann Arbor, MI 48109-2029. Email: rlc@umich.edu.

²National Food Safety & Toxicology Center and Department of Pediatrics and Human Development, Michigan State University, 243 Food Safety, East Lansing, MI 48824-1302. Email: upham@msu.edu.

³Toxicology Program, Department of Environmental Health, University of Michigan, 1420 Washington Heights, Ann Arbor, MI 48109-2029. Email: charris@umich.edu.

⁴National Food Safety & Toxicology Center and Department of Pediatrics and Human Development, Michigan State University, 246 Food Safety, East Lansing, MI 48824-1302. Email: trosko@msu.edu.

*Address correspondence to:
   Dr. Rita Loch-Caruso
   Toxicology Program
   Department of Environmental Health Sciences
   The University of Michigan
   1420 Washington Heights
   Ann Arbor, MI 48109-2029

Short Title: Glutathione Oxidation and Gap Junctions

Journal Section: Reproductive and Developmental Toxicology
ABSTRACT

The insecticide lindane (γ-hexachlorocyclohexane) inhibits gap junction intercellular communication in rat myometrial cells by a mechanism involving oxidative stress. We hypothesized that oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG) and subsequent S-glutathionylation provide a mechanistic link between lindane-induced oxidative stress and lindane’s inhibition of myometrial gap junction communication. Gap junction communication between cultured rat myometrial myocytes was assessed by Lucifer yellow dye transfer after microinjection. A biphasic pattern was confirmed, with dye transfer nearly abolished after 1 h of exposure to 100 µM lindane followed initially by recovery after lindane removal, and then the development 4 h after termination of lindane exposure of a delayed-onset, sustained inhibition that continued for 96 h. As measured by HPLC, cellular GSH varied over a 24-h period in a biphasic fashion that paralleled lindane-induced inhibition of dye transfer, whereas GSSG levels increased in a manner inversely related to GSH. In accordance, GSH/GSSG ratios were depressed at times when GSH and dye transfer were low. Lindane substantially increased S-glutathionylation in a concentration-dependent manner, measured biochemically by GSSG reductase-stimulated release of GSH from precipitated proteins. Furthermore, treatments that promoted accumulation of GSSG (50 µM diamide and 25 µM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)) inhibited Lucifer yellow dye transfer between myometrial cells. Findings that lindane induced GSH oxidation to GSSG with increased S-glutathionylation, together with the diamide and BCNU results, suggest that oxidation of GSH to GSSG is a component of the mechanism by which lindane inhibits myometrial gap junctions.
Key Words: glutathione, lindane, γ-hexachlorocyclohexane, gap junctions, myometrium, oxidative stress
Gap junction intercellular communication is the cell-to-cell diffusion of small molecules through gap junction channels that span the membranes of adjoining cells. Gap junction intercellular communication is important for normal cell function and homeostasis (Evans and Martin, 2002), and inhibition of gap junction communication has been linked to toxicological phenomena such as carcinogenesis (Trosko and Chang, 2000). Moreover, the formation of gap junctions between uterine smooth muscle (myometrial) cells is necessary during pregnancy for development of the coordinated uterine contractions required for parturition (Garfield et al., 1978; Garfield and Hayashi, 1981). Accordingly, inhibition of gap junction communication has been linked to inhibition of uterine contractions (Loch-Caruso, 1999).

Lindane, \(\gamma\)-hexachlorocyclohexane, is a pesticide that bioaccumulates in human adipose tissue (Robinson et al., 1990). Lindane-induced inhibition of gap junction communication was first observed in Chinese hamster fibroblasts (Tsushimoto et al., 1983) and more recently reported in myometrial myocytes (Criswell and Loch-Caruso, 1995; Criswell et al., 1995). Lindane inhibits gap junction intercellular communication between rat myometrial cells in a biphasic, temporal manner, characterized by an initial acute inhibition in the presence of lindane followed by rapid reversal after termination of lindane exposure, and then the subsequent redevelopment of a secondary, delayed-onset inhibition of gap junction intercellular communication without further exposure (Krieger and Loch-Caruso, 2001; Loch-Caruso et al., 2003).
Lindane inhibits gap junction intercellular communication by a mechanism that involves oxidative stress, as shown in hepatocytes (Ruch and Klaunig, 1986) and myometrial cells (Krieger and Loch-Caruso, 2001). Oxidative stress occurs when the redox state of the cell is perturbed with either an overproduction or underproduction of oxidants that can damage the macromolecular machinery of the cell or perturb cell signaling. Glutathione (L-$\gamma$-glutamyl-L-cysteinylglycine) is a predominant low molecular weight cellular thiol that is important for various cellular processes, including protection against damage from reactive oxygen species and free radicals that arise during conditions of oxidative stress (Meister and Anderson, 1983).

Glutathione is formed intracellularly in its reduced state. Reduced glutathione (GSH) is essential for the formation of DNA precursors, amino acid transport, and reduction of disulfide linkages, among other functions. The sulfhydryl moiety of GSH is particularly reactive and can serve as a nucleophile, reductant and free radical scavenger. Reactions involving GSH as a reductant normally lead to GSH oxidation to a disulfide form, GSSG (Mannervik et al., 1989), and decreased GSH/GSSG ratios that are associated with oxidative stress (Gardiner and Reed, 1994). Consistent with lindane’s pro-oxidant activity, lindane depressed cellular GSH in rat liver (Barros et al., 1988; Videla et al., 1990) and in rat embryos and visceral yolk sac (McNutt and Harris, 1994). Although earlier reports indicate that lindane induces oxidative stress in myometrial cells (Krieger and Loch-Caruso, 2001), effects of lindane on GSH and GSSG in myometrial cells have not been reported previously. The current study evaluates the hypothesis that oxidation of GSH to GSSG is a component of the mechanism by which lindane inhibits myometrial gap junction intercellular communication.
METHODS

**Chemicals.** Lindane (β-hexachlorocyclohexane, 99% purity), diamide and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) were purchased from Sigma Chemical Co. (St. Louis, MO). Lucifer yellow and propidium iodide dyes were obtained from Molecular Probes (Eugene, OR). RPMI cell culture medium was purchased from Gibco (Grand Island, NY) and was supplemented with iron-enriched bovine calf serum (BCS; HyClone, Logan, UT). All other chemicals used were obtained from Sigma Chemical Co.

**Cell isolation and culture.** Myometrial smooth muscle cells were isolated from midgestation (Day 10) Sprague-Dawley rats as previously described (Loch-Caruso et al., 1992). Myometrial cells established by this procedure express the gap junction protein connexin43 and regulate gap junctions in a manner consistent with in vivo responses (Caruso et al., 1990; Loch-Caruso et al., 1992; Loch-Caruso, 1999). 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 type III 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 K$_3$PO$_4$ (monobasic), 136.9 mM NaCl, 8.1 mM Na$_3$PO$_4$ (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% CO$_2$ 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 previously (Caruso et al., 1990). Alpha-actin labeling indicated 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% CO$_2$ /95% air atmosphere using RPMI medium supplemented with 10% BCS.

**Lindane exposure.** Cells were incubated for 1 h in RPMI medium containing 100 µM lindane derived from a stock solution of 50 mM lindane in dimethyl sulfoxide (DMSO). For the dye transfer experiments, cell cultures were either immediately processed for microinjection with Lucifer yellow dye or were rinsed three times with pre-warmed CMF-PBS after the 1-h lindane exposure, and then incubated with fresh medium without lindane for 0.25, 0.5, 1, 4, 24, 48 or 96 h before microinjection with Lucifer yellow dye. A minimum of 10 cells per culture dish were microinjected with Lucifer yellow dye and there were 6-7 dishes per treatment group. Myometrial cells were treated in a similar manner for HPLC evaluation of cellular GSH and GSSG immediately after a 1-h exposure to 100 µM lindane or 0.5, 1 or 24 h after 1-h exposure to 100 µM lindane (four culture dishes per treatment group). Solvent controls were incubated for 1 h in medium containing 0.2% DMSO and treated in a manner identical to the cultures exposed to lindane.
**Diamide exposure.** To promote oxidation of GSH to GSSG, myometrial cells were exposed to 50 µM diamide for 0.5 h. Solvent controls were exposed to 0.05% double distilled water (DDW). At the end of the exposure, the cells were microinjected and assessed for Lucifer yellow dye transfer in diamide-containing medium. The concentration and exposure duration for diamide were based on experiments in our laboratory that showed a maximal inhibitory effect on Lucifer yellow dye transfer under these conditions (data not shown). A minimum of six cells were microinjected in each of five dishes per treatment group.

**Exposure to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).** Cells were exposed to the GSSG reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) to prevent reduction of GSSG to GSH. Cells in culture were exposed for 4 h to 25 µM BCNU or to 0.05% DMSO (solvent control). At the end of the exposure time, the cells were microinjected and assessed for Lucifer yellow dye transfer in BCNU-containing medium. Exposure to 25 µM BCNU for 3 h produced virtually identical results as the exposure for 4 h (data not shown). A minimum of six cells were microinjected in each of five dishes per treatment group.

**Lucifer yellow dye transfer.** To assess gap junction intercellular communication, myometrial cells in culture were injected with mixed dye solution of 0.8% Lucifer yellow and 0.02 % propidium iodide in CMF-PBS, and then monitored for evidence of Lucifer yellow dye transfer to adjoining cells using procedures previously described (Criswell et al., 1995). Because of its low molecular weight and hydrophilicity, Lucifer yellow readily diffuses between cells through connexin43 gap junctions but does not cross the non-gap junction plasma membrane of healthy
cells. In contrast, propidium iodide rapidly binds to nucleic acids and serves as a marker to label injected cells. An injection pressure of 6.5 psi for 200 msec was used for dye 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:

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

Culture dishes were randomly assigned to treatment groups and utilized for one time-point only.

**Measurement of cellular thiols.** High performance liquid chromatography (HPLC) with electrochemical detection was used to assay cellular GSH and GSSG in myometrial cells exposed to lindane. 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 pre-warmed CMF-PBS before cell lysis in 0.05 N perchloric acid. After collecting the lysate using a cell scraper, a small aliquot was set aside for protein analysis and the remainder was filtered through a 0.22-μm Acrodisc. The lysate and filtrate samples were flash-frozen in liquid nitrogen and stored at -70°C until HPLC and protein analysis. After thawing, the filtrate samples were separated with a C18 5 μ MF-Plus HPLC column (Alltech Assoc., Deerfield, IL) using a mobile phase consisting of 50 mM sodium dihydrogen phosphate (monohydrate), 0.05 mM 1-octanesulfonic acid (sodium salt), and 2% acetonitrile, adjusted to pH 2.70 with phosphoric acid. A Coulochem II electrochemical detector (ESA, Inc., Chelmsford, Massachusetts) monitored
GSH and GSSG simultaneously in individual samples. Some samples were below the HPLC
detection limit of GSSG (3 ng), and these samples were assigned the mid-value between zero and
the detection limit value, i.e., 1.5 ng. The protein content of each HPLC sample was measured
using the Bradford method (Bradford, 1976) as modified for use with a microtiter plate
spectrophotometer, and cellular concentrations of GSH and GSSG were expressed as µg/mg
protein. For those samples with GSSG concentrations below the limit of detection, 1.5 ng (the
assigned GSSG value) was divided by the HPLC sample protein (expressed in mg).

**Quantification of S-glutathionylation.** Evaluation of S-glutathionylation was based upon the
method of Lou et al. (Lou et al., 1986) as modified by Hiranruengchok and Harris (1995).
Myometrial cell cultures were exposed to 50 or 100 µM lindane or to 0.1% DMSO (solvent
control) in RPMI medium containing 10% BCS for 1 h at 37 °C in a 5% CO2 atmosphere. The
cells were then rinsed with prewarmed CMF-PBS, drained, and quickly scraped in 200 mM
methanesulfonic acid. The cell suspensions were flash frozen using liquid nitrogen and stored at
-70 °C. Thawed cell and tissue samples underwent ultrasonic cell disruption while on ice, and
protein was precipitated by addition of 4 M sodium methanesulfonic acid. Samples were
centrifuged for 5 min at 150 x g at 4 °C. Protein pellets were washed with ice-cold 80% ethanol
and were suspended in sodium pyrophosphate (pH 8.4). An enzymatic reduction system (5 mM
EDTA, 1 mM dithiothreitol, 0.2 mM NADP+, 2.5 mM glucose-6-phosphate, 2 µg/ml glucose-6-
phosphate dehydrogenase (Leuconostoc mesenteroides) and 4 µg/ml GSSG reductase (from
Baker yeast)) was added to the protein suspension, and samples were incubated for 30 min at 37
°C to reduce disulfide bonds and release free GSH. Upon cooling to room temperature,
methanesulfonic acid was added at a final concentration of 200 mM to stop the reaction. Next,
protein was precipitated by adding an equal volume of 4 M sodium methanesulfonic acid. Samples were centrifuged and supernatants were transferred to tubes containing HEPPS buffer (1 M HEPPS and 5 mM diethylene triamine, pH 8.5). S-Glutathionylation was quantified as the amount of GSH released in the supernatant. The GSH amounts in each sample were evaluated using HPLC with fluorometric detection as described by Fahey and Newton (1987) and modified by Harris (1993). Samples were derivitized with monobromobimane solution at a final concentration of 0.2 mM and held in a dark environment at room temperature for 20 min. Methanesulfonic acid (400 mM) was then added to stop the reaction, and derivitized samples were stored at -70 °C away from light until assayed by HPLC. Thiol analysis was conducted as described by Harris (1993) using a scanning fluorescence detector. Following solubilization of the protein pellets set aside from each sample, protein values were determined using the Bradford assay (Bradford, 1976) modified for use with a microtiter plate spectrophotometer. The amount of S-glutathionylation was expressed as nmoles of resolved GSH per mg protein.

**Statistical Analysis.** Data are reported as the mean ± standard error of the mean (s.e.m.). Data analysis was conducted using SigmaStat (Jandel Scientific Software, San Rafael, CA) or Prism (GraphPad Software, Inc., San Diego, CA). Lindane-induced time-dependent changes in dye transfer were analyzed by two-way analysis of variance (ANOVA) with and without arcsine transformation of the data (to correct for non-normal distribution), and the results of the analyses were unaffected by the transformation. The HPLC measurements of GSH were analyzed by two-way ANOVA followed by post-hoc comparisons of means using the Student-Newman-Keuls test. The GSSG data set was not normally distributed because samples below the limit of GSSG detection were assigned the mid-value between zero and the detection limit value.
Consequently, the GSSG and GSH/GSSG ratio data were analyzed using the distribution-free Mann-Whitney Rank Sum Test to compare control and lindane-treated cells at each time-point. The S-glutathionylation data were analyzed by one-way ANOVA followed by post-hoc comparisons of means using Student-Newman-Keuls pairwise multiple comparison tests. The diamide and BCNU data were analyzed by the Mann-Whitney Rank Sum Test. A $p$-value less than or equal to 0.05 was considered statistically significant.

RESULTS

*Lindane-induced inhibition of gap junction intercellular communication*

Gap junction intercellular communication was assessed by injecting myometrial cells with a mixed dye solution of Lucifer yellow and propidium iodide, and subsequently monitoring the cell cultures for evidence of Lucifer yellow fluorescence in adjoining cells. Figures 1A and 1D show phase contrast images of cultured cells exposed for 1 h to solvent alone (controls) or 100 µM lindane immediately after microinjection of the dye solution. The propidium iodide rapidly bound to nucleic acids and served as a marker for the injected cell (arrows, Fig. 1B and 1E). In control cultures, Lucifer yellow fluorescence was observed in cells adjacent to the injected cell, indicating dye transfer (Fig. 1C), whereas Lucifer yellow transfer to adjacent cells was inhibited after a 1-h exposure to 100 µM lindane (Fig. 1F).

Lindane inhibited Lucifer yellow dye transfer between rat myometrial cells in a time-dependent manner, in contrast to control cells that did exhibit significant changes in dye transfer.
over time (Fig. 2; ANOVA time, treatment, and time x treatment interaction effects; p < 0.0001). A 1-h exposure to 100 µM lindane depressed dye transfer to 7.5% of adjoining cells compared with transfer to 97% of adjoining cells in solvent controls (Fig. 2, 0-h Recovery Time; p ≤ 0.05). Removal of the lindane-containing medium allowed rapid reversal of dye transfer inhibition, such that dye transfer recovered to 65% by 15 min after rinsing (significantly increased compared with 0-h Recovery Time; p ≤ 0.05) and 95% by 0.5 h after rinsing (Fig. 2). Dye transfer remained at control levels in the lindane-exposed cultures for an additional 30 min. However, a secondary, delayed-onset of inhibition developed by 4 h after rinsing, with dye transfer decreased to 66% (Fig. 2, p ≤ 0.05, compared with controls). This degree of inhibition of dye transfer was maintained for up to 96 h after termination of lindane exposure (Fig. 2, p ≤ 0.05, compared with controls). These data show a biphasic lindane-induced inhibition of gap junction intercellular communication between myometrial cells as measured by Lucifer yellow dye transfer. Following an acute phase of inhibition in the presence of lindane, dye transfer initially recovered but subsequently declined again as a secondary, delayed-onset inhibition developed that was sustained for at least 96 h.

Lindane-induced changes of cellular GSH and GSSG

Myometrial cells were assessed for cellular content of GSH and GSSG after exposure to lindane using HPLC with electrochemical detection. Similar to the biphasic effect of lindane-induced inhibition of dye transfer, lindane decreased GSH in a time-dependent biphasic manner (Fig. 3A; ANOVA time, treatment, and time x treatment interaction effects, p < 0.0001). Myometrial cells exposed to 100 µM lindane for 1 h had 0.74 µg GSH/mg protein, significantly
decreased compared with 5.2 µg GSH/mg protein in solvent controls (Fig. 3A; p ≤ 0.05).

Following removal of lindane-containing medium and rinsing, GSH increased after 0.5 h and recovered to control levels after 1 h (Fig. 3A; p ≤ 0.05, significantly increased compared with lindane-treated cells at 0 h). However, a secondary, delayed-onset depletion of GSH was evident 24 h after removal of lindane, with GSH levels at 1.7 µg/mg protein in lindane-treated cells compared with 5.3 µg GSH/mg protein in control cells (Fig. 3A; p ≤ 0.05). The results indicate that decreases in cellular GSH followed a biphasic pattern that paralleled lindane-induced inhibition of Lucifer yellow dye transfer.

Concentrations of GSSG and GSH were measured concurrently in the myometrial cells (Fig. 3B). Compared with control levels of 0.30 µg GSSG/mg protein, GSSG was elevated to 1.04 µg GSSG/mg protein immediately after the 1-h exposure to lindane (p < 0.03). After removal of lindane exposure media, GSSG concentrations declined to control levels at 0.5 h and 1 h. However, 24 h after cessation of exposure, GSSG increased to 0.98 µg/mg protein in lindane-exposed cells compared with 0.29 µg GSSG/mg protein in solvent controls (p ≤ 0.03). Consequently, GSSG increased in an inverse manner with respect to GSH and inhibition of gap junctions in myometrial cells after a 1-h exposure to lindane.

To more completely ascertain lindane modulation of cellular redox status, ratios of GSH/GSSG were determined by calculating the mean of individual sample GSH/GSSG ratios at each time-point from the data shown in Figures 3A and 3B. Immediately after a 1-h exposure to 100 µM lindane, the GSH/GSSG ratios decreased to an average of 0.98 compared with an average ratio of 17.5 in control cells (Fig. 3C, 0-h time-point; p ≤ 0.03). By 0.5 h after termination of lindane exposure, GSH/GSSG ratios partially recovered to 10.3 in lindane-exposed cells but still remained significantly depressed compared with 18.4 in control cells (Fig.
Complete recovery of GSH/GSSG ratios to control levels was observed 1 h after removal of the lindane exposure medium and rinsing. However, 24 h after removal of lindane, GSH/GSSG ratios decreased again to 1.88 in lindane-exposed cells compared with a ratio of 18.3 in control cells (Fig. 3C; p ≤ 0.03). The GSH/GSSG ratios changed a biphasic manner that paralleled the patterns of lindane-induced inhibition of Lucifer yellow dye transfer and depression of cellular GSH.

**Lindane-induced formation of S-glutathionylation**

Because lindane promoted oxidative cellular conditions immediately after a 1-h exposure, as indicated by the markedly reduced GSH/GSSG ratios at 0 h of recovery, the ability of lindane to promote S-glutathionylation under similar acute exposure conditions was determined. A 1-h exposure to 50 or 100 µM lindane stimulated S-glutathionylation in a concentration-dependent manner (Fig. 4; ANOVA treatment effect, p< 0.0001). S-Glutathionylation levels were low in control cells, as expected (averaging 0.20 nmoles released GSH/mg protein). However, lindane increased S-glutathionylation after a 1-h exposure to 50 or 100 µM lindane (1.6 and 3.4 nmoles released GSH/mg protein, respectively; significantly increased compared with controls, p≤0.05). Consequently, S-glutathionylation was induced immediately after an acute (1-h) exposure to lindane under experimental conditions in which the GSH/GSSG ratios were markedly depressed.
Diamide-induced inhibition of gap junction communication

Diamide directly and reversibly oxidizes GSH to GSSG without formation of radicals (Kosower and Kosower, 1976). Because lindane increased intracellular GSSG and depressed cellular GSH/GSSG ratios, cells were treated with diamide and evaluated using Lucifer yellow dye transfer in order to assess effects of GSH oxidation to GSSG on myometrial gap junction intercellular communication. A 30-min treatment with 50 µM diamide reduced the transfer of dye between myometrial cells to 14%, compared with 98.9% dye transfer in control cultures (Fig. 5; p < 0.01). These data show that diamide inhibited gap junction intercellular communication between myometrial cells as measured by Lucifer yellow dye transfer, indicating that myometrial gap junctions are modulated by cellular GSH oxidation.

Inhibition of gap junction communication by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)

To further investigate the role of GSSG in regulation of myometrial gap junctions, myometrial cells were treated with the GSSG reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) for 4 h, and then assessed for inhibition of gap junction communication by monitoring for Lucifer yellow dye intercellular transfer. Because GSSG reductase reduces GSSG to GSH, inhibition of GSSG reductase results in elevated cellular GSSG. After 4 h of treatment, Lucifer yellow dye transfer was depressed from 96.6% in controls to 2.0% in cells exposed to 25 µM BCNU (Fig. 5; p < 0.01). These results indicate that inhibition of GSSG reductase also inhibits myometrial intercellular gap junction communication, supporting the
conclusion that increased cellular GSSG leads to decreased gap junction communication in myometrial cells.

**DISCUSSION**

The direct cell-to-cell transfer of low molecular weight molecules and ions through gap junctions is a principal mechanism for intercellular signal transmission, coordination of tissue function, the exchange of nutrients and waste, and homeostasis (Nicholson, 2003). Gap junctions are regulated by mechanisms involving gene expression, phosphorylation, calcium, pH, and membrane potential (Evans and Martin, 2002). Recently, GSH was implicated in the regulation of gap junctions, also (Upham et al., 1997; Caruso et al., 2005). The present study examines oxidation of GSH to GSSG and S-glutathionylation as components of the mechanism by which lindane inhibits myometrial gap junction intercellular communication.

Exposure to the insecticide lindane (γ-hexachlorocyclohexane) inhibits gap junction intercellular communication between myometrial cells as assessed by Lucifer yellow dye transfer, consistent with previous reports in various cell types (Tsushimoto et al., 1983; Ruch and Klaunig, 1986; Criswell et al., 1995). Confirming our previous observations (Krieger and Loch-Caruso, 2001; Loch-Caruso et al., 2003), an initial rapid recovery followed termination of a brief exposure to lindane, with the subsequent development of a secondary, delayed-onset inhibition of gap junction communication. The present study extended previous reports by showing continued inhibition of myometrial gap junctions for 96 h after termination of lindane exposure. In contrast to myometrial cells, rat liver WB-F344 cells completely recover from inhibition of
gap junction communication 4 h after discontinuation of a 4-h exposure to lindane (Guan et al., 1995). Differences between myometrial cells and WB-F344 cells in recovery from lindane-induced inhibition of gap junctions may be related to differences in phosphorylation of connexin43 at the protein kinase C phosphorylation site of serine 368 (Loch-Caruso et al., 2004).

Cellular concentrations of GSH and GSSG in control myometrial cells were similar to uterine GSH and GSSG levels reported in literature (Suojanen et al., 1980), suggesting that the myometrial cell cultures maintained GSH/GSSG homeostasis in vitro similar to that observed in vivo. A brief (1-h) exposure to lindane acutely depleted GSH of cultured myometrial cells, consistent with reports that lindane reduces GSH in rat liver (Barros et al., 1988; Videla et al., 1990) and in rat embryos and visceral yolk sac (McNutt and Harris, 1994), and providing the first report of GSH depletion in myometrium in response to lindane. Furthermore, the present report provides the first demonstration of a lindane-induced biphasic effect on cellular GSH, characterized by recovery of cellular GSH to control levels within 1 h after termination of lindane exposure, and then the redevelopment of a secondary, delayed-onset decline of GSH observed 24 h after discontinuation of exposure to lindane. The lindane-induced changes in myometrial GSH followed a similar temporal pattern as inhibition of myometrial gap junction communication, suggesting that depletion of cellular GSH may be involved in lindane’s acute and delayed-onset inhibition of myometrial gap junctions. Decreased cellular GSH was correlated with diminished gap junction intercellular communication in rat-1 fibroblast variants (Masta et al., 1991) and in liver cell lines (Barhoumi et al., 1993), also.

After a brief (1-h) exposure to lindane, cellular GSSG varied in a biphasic manner that was inversely related to GSH, suggesting that GSH depletion was linked to oxidation of GSH to GSSG. Moreover, lindane depressed GSH/GSSG ratios in a manner that paralleled changes of
cellular GSH, providing additional support that lindane promoted GSH oxidation to GSSG. Because the GSH/GSSG pattern varied in a similar manner as inhibition of gap junctions, oxidation of GSH to GSSG may be related to lindane-induced inhibition of myometrial gap junctions.

The GSH/GSSG ratio is an excellent indicator of cell redox status (Schafer and Buettner, 2001). As such, the markedly depressed GSH/GSSG ratios observed immediately after a 1-h exposure to lindane is consistent with increased generation of superoxide and induction of oxidative stress previously observed in myometrial cells exposed to lindane under identical conditions (Krieger and Loch-Caruso, 2001; Caruso et al., 2005). Moreover, oxidative stress has been reported in liver cells exposed to lindane (Videla et al., 1990). Increased generation of superoxide, as was observed in myometrial cells under identical lindane exposure conditions (Caruso et al., 2005), would be expected to promote oxidation of cysteines of cellular proteins and subsequent S-glutathionylation. The present study provides the first report that lindane stimulates S-glutathionylation and is the first to associate S-glutathionylation with inhibition of gap junctions.

Because S-glutathionylation most likely occurs by direct reaction of GSH with an oxidized protein cysteine, the finding that lindane increased S-glutathionylation is consistent with cellular oxidative stress (Mannervik et al., 1989; Poole et al., 2004) and suggests a potential mechanism whereby lindane may acutely deplete cellular GSH. Moreover, S-glutathionylation may occur by direct reaction of GSSG with a protein thiol(ate), also (Poole et al., 2004). Consequently, the elevated GSSG and reduced GSH/GSSG ratios observed immediately after a brief (1-h) exposure to lindane could also contribute to the increased S-glutathionylation observed in myometrial cells under identical lindane exposure conditions.
Consistent with the reversibility of S-glutathionylation by thioredoxin, glutaredoxin or protein disulfide isomerase systems (Poole et al., 2004), GSH concentrations were restored to control levels 1 h after termination of lindane exposure in myometrial cells. Although initial recovery of GSH after discontinuation of lindane treatment theoretically could be the result of de novo synthesis of GSH, the rapid rate of recovery of GSH pools makes de novo synthesis unlikely. Moreover, lack of stoichiometry between GSSG and GSH levels suggests that regeneration of GSH from reduction of GSSG by NADPH-dependent GSSG reductase is an unlikely explanation for the initial recovery of GSH. Consequently, S-glutathionylation provides a plausible explanation for cellular GSH depletion during lindane exposure and, through its rapid reversal by thioredoxin, glutaredoxin or protein disulfide isomerase, S-glutathionylation may also explain the initial recovery of GSH observed 1 h after termination of lindane exposure.

Because the antioxidant and GSH modulation treatments that prevented the delayed-onset inhibition of gap junctions in previous studies were pre-/cotreatments with a 1-h lindane exposure, the lindane-induced delayed-onset inhibition of myometrial gap junctions likely is dependent on acute oxidative stress during the 1-h lindane exposure (Krieger and Loch-Caruso, 1997; Caruso et al., 2005). S-Glutathionylation was assessed in the present study and indices of oxidative stress were measured in previous studies (Krieger and Loch-Caruso, 1997; Caruso et al., 2005) immediately after lindane exposure because the available evidence suggested that the critical events were initiated during the 1-h lindane exposure. Nonetheless, it may be interesting to measure oxidative stress by means other than GSH and GSSG at various times after termination of lindane exposure, in order to better interpret the delayed-onset reductions of GSH and GSH/GSSG ratios. These studies are beyond the scope of the present investigation, however.
The development of secondary, delayed-onset decreases of GSH and GSH/GSSG ratios observed 24 h after cessation of a brief (1-h) lindane exposure suggest that a GSH-related mechanism is initiated during exposure to lindane that requires time to develop. A change of cellular redox status, as evidenced by depressed GSH/GSSG ratios immediately after a 1-h exposure to lindane, may modify protein expression of redox regulated genes (Allen and Tresini, 2000; Rahman et al., 2005) that, in turn, leads to the development of a secondary depletion of GSH and inhibition of gap junctions. A gene transcription-dependent process would be expected to require more than 1 h to develop, consistent with the delayed onset of the secondary inhibition of myometrial gap junctions. However, investigation of this mechanism requires further experimentation beyond the present study.

Alternatively, both the initial and delayed-onset inhibition of myometrial gap junctions by lindane may involve S-glutathionylation of connexin43, the gap junction protein abundantly expressed in the myometrium during parturition (Risek et al., 1990) and in myometrial cells in culture (Caruso et al., 1990). Because connexin43 has six extracellular cysteines that are critical for channel function (John and Revel, 1991) and three cysteines located on the cytoplasmic carboxy terminus, the connexin43 cysteines may become oxidized and glutathionylated under conditions of oxidative stress with consequential alteration of protein function, as observed with other proteins (Poole et al., 2004). The inhibition of myometrial gap junctions observed immediately after a 1-h exposure to lindane was shown to be dependent on cellular GSH (Caruso et al., 2005), consistent with a role for S-glutathionylation in development of acute inhibition of myometrial gap junctions. Although S-glutathionylation is readily reversible, the more oxidized forms of protein cysteines (e.g., sulenate, sulfinate, and sulfonate) are less so and may be irreversible (Poole et al., 2004). It is suggested that S-glutathionylation, which occurs under
acute lindane exposure conditions as shown by the present study, is mechanistically related to the acute inhibition of myometrial gap junctions, and that further, less readily reversible, oxidation of cell protein cysteines may develop over a longer period and possibly underlie the development of the secondary, delayed-onset inhibition of gap junctions. Although our data are consistent with this explanation, further study is required to examine long-term oxidative effects of lindane on cell proteins critical for gap junction regulation.

Treatment with diamide depressed myometrial gap junction intercellular communication, consistent with previous reports that diamide decreases gap junctional electrical coupling in crayfish giant axons (Campos de Carvalho et al., 1986) and augments phorbol ester-induced inhibition of gap junctions in WB-F344 rat liver cells (Hu and Cotgreave, 1995). Because diamide directly and reversibly oxidizes GSH to GSSG without formation of radicals (Kosower and Kosower, 1976), the rapid inhibition of myometrial gap junction communication by diamide implicates a role for GSH oxidation to GSSG in myometrial gap junction inhibition. Similar to diamide, the GSSG reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) inhibited myometrial gap junction communication. Because BCNU promotes cellular increases of GSSG by preventing reduction of GSSG to GSH, the ability of BCNU to inhibit myometrial gap junctions further suggests a role for GSSG in the regulation of myometrial gap junctions. To our knowledge, this is the first report that BCNU inhibits gap junctions and the first evidence supporting GSH oxidation as a more general regulatory mechanism of myometrial gap junctions.

A previous study in our laboratory suggested that lindane inhibits myometrial gap junctions and spontaneous oscillatory contractions by a phospholipase C-mediated mechanism (Wang and Loch-Caruso, 2002). Additional preliminary experiments further suggest that lindane's mechanism of inhibition of myometrial gap junctions is mediated by PKCε-dependent
activation of NADPH oxidase (Wang and Loch-Caruso, 1999). NADPH oxidase activation could be a mechanism by which lindane exposure leads to GSH oxidation in myometrial cells because lindane increases generation of superoxide (Caruso et al., 2005), NADPH oxidase activation increases superoxide production, and superoxide promotes GSH oxidation to GSSG.

Lindane greatly increases release of arachidonic acid from myometrial cells. Because arachidonic acid metabolism stimulates conversion of GSH to GSSG (Burch and Burch, 1990), release of arachidonic acid could initiate the lindane-induced GSH oxidation observed in the present study. However, an arachidonic acid metabolism-mediated mechanism does not appear to link lindane-induced oxidation of GSH to inhibition of gap junctions because the cyclooxygenase inhibitor indomethacin has only a marginal impact on lindane-induced inhibition of myometrial gap junctions (Criswell and Loch-Caruso, 1995). Lindane activates cAMP and calcium cell signaling pathways in myometrial cells, also, but a role for these signaling molecules in lindane-induced inhibition of myometrial gap junctions was not supported by experimental results (Criswell et al., 1994; Criswell and Loch-Caruso, 1995; Criswell et al., 1995; Criswell and Loch-Caruso, 1999).

Inhibition of gap junction intercellular communication has been linked to tumorigenesis, impaired uterine contraction, and other pathophysiologic phenomena (Loch-Caruso, 1999; Trosko and Chang, 2000). Furthermore, lindane-induced inhibition of myometrial gap junctions and spontaneous uterine contractions is prevented by antioxidant treatments (Krieger and Loch-Caruso, 2001), consistent with a critical role for oxidative stress-mediated inhibition of myometrial gap junctions in the dysregulation of uterine contractions. Although gap junctions serve to improve uterine muscle coordination and force generation at parturition (Garfield et al., 1978; Garfield and Hayashi, 1981), it is not known whether lindane presents reproductive risks
to women by inhibiting myometrial gap junctions and uterine contractions during pregnancy. The concentration of lindane used in the present study is about 100-fold higher than serum concentrations reported in humans after occupationally exposure (Baumann et al., 1980) or pharmaceutical use of lotion containing 1% lindane (Rauch et al., 1990). However, because lindane induces an inhibition of myometrial gap junctions that is sustained for at least 4 days after discontinuation of exposure (Loch-Caruso et al., 2003), further research on possible health impacts in pregnant women may be warranted. However, it is beyond the scope of the present investigation to resolve whether lindane poses a reproductive risk to pregnant women by interfering with myometrial gap junctions.

The parallel nature of lindane-induced inhibition of gap junction communication, GSH depletion and GSH/GSSG ratio decrease suggest that GSH oxidation to GSSG may be part of the mechanism by which lindane inhibits myometrial gap junction communication. Moreover, the data suggest a role for S-glutathionylation in lindane’s biphasic effects on myometrial GSH/GSSG and gap junction communication. Finally, inhibition of myometrial gap junctions by pharmacological treatments that elevate the ratio of GSH/GSSG suggest that the GSH/GSSG redox couple may serve as a more general regulatory mechanism of myometrial gap junctions. A mechanism by which S-glutathionylation could regulate gap junctions under conditions of GSH oxidation to GSSG is proposed, but elucidation of the exact mechanism remains beyond the scope of the present investigation.
ACKNOWLEDGMENTS

We thank Teresa Krieger-Burke for contributions to data collection and analysis, and Vincent Peterkin for laboratory assistance. We also thank Ellen Flatley for assistance with graphics. Support for R.L.C., B.L.U., C.H. and J.E.T. was from a National Institute of Environmental Health Sciences (NIEHS), NIH grant (P42 ES04911). This research was initiated with support from a grant (R01 ES06915) to R.L.C. from the NIEHS, NIH. Additional support was provided by the Laboratory Animal Core of the Center for the Study of Reproduction (Grant P30 HD18258 from NIH). Contents of the work are solely the responsibility of the authors and do not necessarily represent the official views of NIH.
REFERENCES


FIGURE LEGENDS

Figure 1. Transfer of Lucifer yellow dye between myometrial cells after a 1-h exposure to 100 μM lindane (bottom row) or to 0.2% DMSO alone (solvent controls, top row). A mixed dye solution of Lucifer yellow and propidium iodide was injected into a single cell (indicated by arrow) in a focus of cells, and the appearance of Lucifer yellow fluorescence in adjacent cells was scored as dye transfer. Panels A and D, phase contrast. Panels B and E, propidium iodide fluorescence. Panels C and F, Lucifer yellow fluorescence. In contrast to controls (Panel C), no Lucifer yellow dye transfer occurred between cells exposed to lindane (Panel F).

Figure 2. Time-dependent inhibition of Lucifer yellow dye transfer in myometrial cell cultures after a 1-h exposure to 100 μM lindane. The arrow indicates termination of lindane exposure followed by rinsing. Each bar represents the mean ± s.e.m. of 6-7 dishes with a minimum of 10 cells microinjected with dye per dish. Means that are significantly different (p ≤ 0.05) are labeled with different letters. Error bars not visible are too small to depict graphically.

Figure 3. Time-dependent effects on cellular GSH and GSSG in myometrial cells following a 1-h exposure 100 μM lindane. (A) GSH (μg/mg protein). (B) GSSG (μg/mg protein). (C) GSH/GSSG ratio. Arrows indicate termination of lindane exposure followed by rinsing. Each column represents the mean ± s.e.m. (n=4). Error bars not visible are too small to be depicted graphically. *, Significantly different from
time-matched controls (p ≤ 0.05). ⊕, Significantly different from all lindane-exposed groups (p ≤ 0.05). ⊖, Significantly different from lindane-exposed groups at 0 h and 1 h recovery time.

**Figure 4.** Concentration-dependent increase of S-glutathionylation in cultured myometrial cells exposed for 1 h to 50 or 100 µM lindane. Solvent control cells were exposed to 0.1% DMSO. Each bar represents the mean ± s.e.m. of nmol GSH released/mg protein (n=5 culture dishes). Different letters indicate significantly different mean values (p ≤ 0.05).

**Figure 5.** Inhibition of Lucifer yellow dye transfer between myometrial cells exposed in culture to modulators of cellular GSSG. In one experiment, cells were exposed to the GSSG reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or to 0.05% DMSO (solvent control) for 4 h. In a separate experiment, cells were exposed to 50 µM diamide or 0.05% double distilled water (solvent control) for 0.5 h. Each bar represents the mean ± s.e.m. of 5 dishes with a minimum of 6 cells injected with dye per dish. Asterisks indicate significantly different mean values from controls (p ≤ 0.05). Error bars not visible are too small to depict graphically.
A

![Graph A: GSH vs. Recovery Time](image)

B

![Graph B: GSSG vs. Recovery Time](image)

C

![Graph C: GSH/GSSG vs. Recovery Time](image)