Lindane, a gap junction blocker, suppresses FSH and transforming growth factor β1-induced connexin43 gap junction formation and steroidogenesis in rat granulosa cells

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Abstract

The present study was designed to explore the role of gap junctions in follicle-stimulating hormone (FSH) and transforming growth factor β1 (TGFβ1)-stimulated steroidogenesis in ovarian granulosa cells of gonadotropin-primed immature rats. There were three specific aims. First, we investigated the effect of FSH and TGFβ1 as well as lindane (a general gap junction blocker) on the level of connexin43 (Cx43), the major gap junction constituent in granulosa cells, and on gap junction function. The second aim was to determine the effect of lindane on FSH and TGFβ1-stimulated progesterone production and the levels of two critical players, cytochrome P450 side-chain cleavage (P450scc) enzyme and steroidogenic acute regulatory (StAR) protein. The third aim was to further investigate the specific involvement of Cx43 gap junctions in FSH and TGFβ1-stimulated steroidogenesis using a Cx43 mimetic peptide blocker. Immunoblotting analysis showed that FSH plus TGFβ1 dramatically increased the levels of phosphorylated Cx43 without significantly influencing the level of nonphosphorylated Cx43, and this stimulatory effect was completely suppressed by lindane. Also, immunofluorescence analysis showed that Cx43 immunoreactivity increased in the FSH plus TGFβ1-treated group and predominantly appeared in a punctate pattern at cell–cell contact sites, and lindane reduced such cell periphery immunostaining. Furthermore, TGFβ1 enhanced the FSH-induced gap junction intercellular communication and lindane completely suppressed this effect. In addition, lindane suppressed the FSH and TGFβ1-stimulated increases in progesterone production and the levels of P450scc enzyme and StAR protein. This study demonstrates a clear temporal association between the Cx43 protein level/gap junction communication and progesterone production in rat ovarian granulosa cells in response to FSH and TGFβ1 as well as lindane. Furthermore, a specific Cx43 gap junction blocker suppressed FSH plus TGFβ1-stimulated progesterone production. In conclusion, this study suggests that Cx43 gap junctions may play a critical role in FSH plus TGFβ1-stimulated progesterone production in rat ovarian granulosa cells.

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Introduction

Gap junctions are intercellular plasma membrane channels that allow direct intercytoplasmic movement of small molecules (<1 kDa) such as nutrients, ions and second messengers between neighboring cells (Kumar & Gilula 1996). They are formed by the docking of connexons from two adjacent cells, and each connexon is thought to be a hexamer of connexin proteins (Kumar & Gilula 1996). Gap junctions are believed to play essential roles in organogenesis and the control of cell proliferation and differentiation (Kumar & Gilula 1996, Yamasaki & Naus 1996). Such communication among ovarian cells via gap junctions may also be involved in the control of follicular development, oocyte meiotic maturation, and luteal growth and regression (Grazul-Bilska *et al.* 1997, Ackert *et al.* 2001, Kidder & Mhawi 2002). Gap junctions between ovarian granulosa cells contain predominantly connexin43 (Cx43) which is present at all stages of follicle development with large antral follicles having the strongest

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immunostaining intensity (Mayerhofer & Garfield 1995, Granot & Dekel 1997, Kidder & Mhawi 2002). Recent studies reported that in Cx43-deficient female mice, ovarian follicles were arrested in the early preantral stages, and that cellular communication between granulosa cells was disrupted (Juneja *et al.* 1999, Ackert *et al.* 2001, Kidder & Mhawi 2002, Gittens *et al.* 2003), suggesting that Cx43 gap junction communication is critical for ovarian folliculogenesis.

At present, our understanding of the hormonal control of Cx43 gap junction in ovarian cells is limited. Follicle-stimulating hormone (FSH) is the major regulator of growth and development of antral follicles in the ovary (Hirshfield 1991, Richards 2001). It was reported that FSH stimulated gap junction formation and turnover in rat ovarian granulosa cells (Burghardt & Matheson 1982). The expression of Cx43 was increased with follicular growth and decreased after the ovulatory luteinizing hormone surge and during follicular atresia (Schreiber *et al.* 1993, Wiesen & Midgley 1993, 1994, Mayerhofer & Garfield 1995, Okuma *et al.* 1996, Granot & Dekel 1997). Recently, FSH was demonstrated to increase intercellular communication as well as the levels of Cx43 mRNA in a rat ovarian granulosa cell line (Sommersberg *et al.* 2000). In the male, FSH also increased gap junction communication in primary testicular Sertoli cells, the equivalent to granulosa cells in the female (Pluciennik *et al.* 1994). Apart from external hormonal regulation, ovarian cells function in response to local factors including cytokines such as transforming growth factor β1 (TGFβ1) (Benahmed *et al.* 1993). TGFβ1 plays important autocrine/paracrine roles in modulating ovarian cell functions including the facilitation of gonadotropin-induced proliferation and differentiation (induction of luteinizing hormone receptors, progesterone production and aromatase activity) and the ovulatory process (Dorrington *et al.* 1993, Inoue *et al.* 2002). TGFβ1 was reported to increase the mRNA and protein levels of Cx43 in endothelial cells (Larson *et al.* 1997, 2001). In contrast, TGFβ1 inhibited gap junction intercellular communication and decreased the phosphorylation of Cx43 in osteoblasts (Wyatt *et al.* 2001) and glioma cells (Robe *et al.* 2000). It remains elusive as to whether TGFβ1 modulates gap junction function in ovarian granulosa cells where gap junctions are well developed.

Therefore, the objective of the present study was to investigate the role of gap junctions in FSH and TGFβ1-stimulated progesterone production (a marker of differentiation) in rat ovarian granulosa cells. There were three specific aims to achieve this goal; The first was to determine the effect of FSH and TGFβ1 as well as a general gap junction blocker, lindane (γ-hexachlorocyclohexane), on the level of gap junction protein Cx43 in rat granulosa cells. Lindane was reported to rapidly inhibit gap junction communication and with longer treatment it caused a loss of gap junctions and phosphorylated Cx43 proteins in liver epithelial cells (Guan *et al.* 1995) and Sertoli cells (Defamine *et al.* 2001). In addition, lindane has been widely used as a pesticide in agriculture and has been reported to accumulate in the ovary and testis (Szymczynski & Waliszewski 1983, Lindenau *et al.* 1994, Dalsenter *et al.* 1996). Thus, the second aim was to investigate the effects of lindane on FSH and TGFβ1-stimulated progesterone production as well as on two key players in steroidogenesis, steroidogenic acute regulatory (StAR) protein and cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme. StAR protein is responsible for mediating the rate-limiting step in steroidogenesis, the transport of cholesterol from the outer to the inner mitochondrial membrane (Christenson & Strauss 2001, Stocco 2001) where P450scc enzyme resides and catalyzes the initial step of steroid hormone biosynthesis, the production of pregnenolone. The third aim was to determine the specific involvement of Cx43 gap junction on FSH andTGFβ1-stimulated progesterone production by employing a Cx43 mimetic peptide blocker. Cx43 peptide blockers have been reported to interfere with gap junction communication in airway cells (Boitano & Evans 2000) and aortic smooth muscle cells transfected with Cx43 cDNA (Kwak & Jongsma 1999).

Materials and Methods

Materials

Ovine FSH (oFSH-19-SIAFP) and equine chorionic gonadotropin were purchased from the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr A F Parlow (Harbor-UCLA Medical Center, Torrance, CA, USA). Recombinant human TGFβ1 was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Penicillin and streptomycin were from GIBCO Invitrogen Corporation (Carlsbad, CA, USA). Mouse monoclonal antibody against Cx43 was obtained from BD Transduction Laboratories (Lexington, KY, USA). Antiserum against StAR protein was produced and characterized as previously described (Clark *et al.* 1994). Antiserum against P450scc enzyme (Hu *et al.* 1991) was kindly provided by Dr Bon-Chu Chung (Academia Sinica, Taipei, Taiwan). Mouse monoclonal antibody against β-actin, fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibody, bovine alkaline phosphatase, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and Lucifer Yellow were from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were purchased from Sigma Chemical Co. unless otherwise stated.

Animals

Immature Sprague–Dawley rats (23–25 days) were obtained from the Animal Center at National Yang-Ming

University (Taipei, Taiwan). Rats were maintained under controlled temperature (20–23 ° C) and light conditions (14 h light:10 h darkness). Food (Lab Diet from PMI Feeds Inc., St Louis, MO, USA) and water were available *ad libitum*. This study was conducted in accordance with both the United States National Research Council's Guide for the Care and Use of Laboratory Animals and institutional guidelines.

Cell culture and treatment

Isolation of ovarian granulosa cells from equine chorionic gonadotropin-treated immature rats was performed as previously described (Hwang *et al.* 1996). Briefly, immature rats were injected once subcutaneously with 15 IU pregnant mare serum gonadotropin for 48 h to induce the development of multiple follicles to antral follicle stage. Ovarian granulosa cells of mid- to large-sized antral follicles were then isolated and plated into 24-well plates coated with matrigel (derived from Engelbreth-Holm-Swarm sarcoma tumors; Sigma Chemical Co.) at approximately 5×10^5 viable cells per well in 500 μl of growth medium (Dulbeco's Modified Eagle's Medium (DMEM)/F-12, 1:1 medium containing 2 μg/ml bovine insulin, 0·1% fatty acid-free BSA, 100 U/ml penicillin and 100 μg/ml streptomycin) and allowed to attach for 24 h at 37 ° C, 5% CO_2-95% air. Cultured cells were then washed and incubated in 500 μl of incubation medium (DMEM/F12, 1:1 medium containing 0·1% lactalbumin hydrolysate, 100 U/ml penicillin and 100 μg/ml streptomycin) for 24 h before the beginning of treatment. Cells were treated once with FSH and/or TGFβ1 for 24 or 48 h. To study the effect of lindane on steroidogenesis, cells were pretreated with dimethylsulfoxide (DMSO) vehicle or various doses of lindane for 24 h, and then treated with vehicle, 10 ng/ml FSH and/or 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of various doses of lindane for an additional 24 or 48 h. To study the effect of Cx43 mimetic peptides on steroidogenesis, cells were cultured in serum-free media as described above except that antibiotics were omitted to prevent possible interaction with the oligopeptides (Kwak & Jongsma 1999). Cells were treated with Cx43 peptide blocker (containing the rat Cx43 extracellular domain sequence, amino acid residues 180–195) or control peptide (containing intracellular domain sequence, amino acid residues 201–211) during plating and allowed to attach for 24 h. Cells were then pretreated with Cx43 mimetic peptides for another 24 h and then treated with vehicle or 10 ng/ml FSH plus 5 ng/ml TGFβ1 in the absence or presence of the Cx43 peptides for an additional 48 h. In every experiment each treatment group was performed in triplicate. At the end of incubation, conditioned media were collected, cleared by centrifugation, and stored at –70 ° C until assayed for progesterone content by enzyme immunoassay. Cell number was determined using the crystal violet assay as previously described (Gillies *et al.* 1986).

Enzyme immunoassay of steroids

Progesterone levels in conditioned media were measured using enzyme immunoassay as previously described (Ke *et al.* 2004). Progesterone antibody was produced and characterized as previously described (Lu *et al.* 1996). The sensitivity of the assay was 5 pg per well, and the intra- and inter-assay coefficients of variation were 4·0% ($n=3$) and 7·8% ($n=3$) respectively.

Immunoblot analysis of Cx43, StAR protein and P450scc enzyme

Granulosa cells (approximate 5 to 6×10^6) were cultured in matrigel-coated 60 mm culture dishes, pretreated with DMSO vehicle or 40 μM lindane for 24 h, and then treated with control vehicle, 10 ng/ml FSH and/or 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 24 or 48 h. The cells were washed with ice-cold PBS and then extracted with lysis buffer (radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail and 1 mM of the phosphatase inhibitors Na orthovanadate and NaF). Cell lysates were analyzed for the presence of Cx43, StAR protein and P450scc enzyme with β-actin used as an internal control. Cell lysates (30 μg protein each) were analyzed by 10% SDS-polyacrylamide gel electrophoresis and electroblotting as previously described (Ke *et al.* 2004). Specific signals were detected using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, England) according to the manufacturer's protocol. Relative quantification of ECL signals on x-ray film was analyzed using a two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA). To identify the phosphorylated forms of Cx43, aliquots of the cell lysate (40 μg protein each) were digested with or without 60 units of bovine alkaline phosphatase at 37 ° C for 2 h in the absence or presence of alkaline phosphatase inhibitors (3 mM Na orthovanadate and NaF) prior to immunoblotting.

Immunofluorescence analysis of Cx43

Granulosa cells (approximate 2·5 x 10^6) were cultured in matrigel-coated 35 mm culture dishes, pretreated with DMSO vehicle or 40 μM lindane for 24 h, and then treated with control vehicle, 10 ng/ml FSH or FSH plus 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 48 h. Cells were rinsed with PBS twice, fixed in 4% paraformaldehyde at room temperature for 30 min, washed with PBS and

permeabilized with 0·05% Triton X-100 for 2 min. Cells were washed, blocked in 3% BSA plus 3% normal goat serum for 1 h and then sequentially incubated for 1 h with Cx43 monoclonal antibody (1:250 dilution) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibody (1:100). Nuclei of cells were also stained using 2 μg/ml DAPI for 1 h. Photographs were taken using a fluorescence microscope (Olympus BX50, Shinjuku-Ku, Tokyo, Japan) at x 100 magnification and SPOT image capture system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Gap junction communication: scrape-loading dye transfer assay

Granulosa cells (approximate 3 x 10^6) were cultured in matrigel-coated 35 mm culture dishes, pretreated with DMSO vehicle or 40 μM lindane for 24 h, and then treated with control vehicle, 10 ng/ml FSH or FSH plus 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 24 or 48 h. The gap junction function was assessed as previously described (El-Fouly *et al.* 1987, El-Sabban *et al.* 2003, Yeh & Hu 2003) with modifications. At the end of culture, the confluent monolayer of cells were scraped with a sharp blade to create two fine linear wounds, quickly rinsed with PBS and loaded with 1 ml of Lucifer Yellow (LY, 1 mg/ml in incubation medium). The dye solution was removed 5 seconds later and the culture was quickly rinsed four times with PBS. The amount of LY dye transferred from the scraped edge to the neighboring cells was examined under fluorescent microscope at x 100 magnification and SPOT image capture system (Diagnostic Instruments). Two representative images were taken per dish. The relative extent of cell coupling was determined by the following two methods; first, the relative number of LY positive cells was calculated as the ratio of total number of LY-labeled cells to the cell number on the scraped edge (determined by the corresponding phase contrast image) relative to that of the control group (Yeh & Hu 2003). Secondly, the relative fluorescent intensity was calculated as the ratio of total fluorescent intensity of LY (determined using ImageQuant analysis system, Molecular Dynamics) to the cell number on the scraped edge relative to that of the control group (El-Sabban *et al.* 2003).

Statistics

Data are presented as the mean ± S.E. and were analyzed by ANOVA and Duncan's multiple range test at a significance level of 0·05 using the general linear model of the SAS program (SAS Institute Inc., Cary, NC, USA). Differences between two treatment groups were analyzed using the Student's *t*-test at a significance level of 0·05.

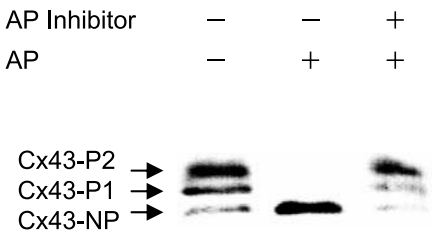


Figure 1. Characterization of Cx43 in rat ovarian granulosa cells. Cells were treated with 10 ng/ml FSH plus 5 ng/ml TGFβ1 for 48 h. Cell lysates were prepared, and aliquots of 40 μg protein each were digested with or without alkaline phosphatase (AP; 60 units, 37 ° C for 2 h) and in the absence or presence of AP inhibitors (Na orthovanadate and NaF), and then analyzed by immunoblotting using an antibody that recognizes nonphosphorylated (Cx43-NP) and phosphorylated forms of Cx43 (Cx43-P1, Cx43-P2).

Results

Effect of FSH, TGFβ1 and lindane (a nonselective gap junction blocker) on connexin43 protein levels and gap junction communication

Gap junctions, predominantly composed of Cx43, are well developed in ovarian granulosa cells. To investigate the potential role of gap junctions in ovarian steroidogenesis, we first determined the regulatory role of FSH and TGFβ1 as well as lindane on the protein levels of Cx43 in rat granulosa cells using immunoblotting and immunofluorescence techniques. Cx43 predominantly existed in two phosphorylated forms, designated Cx43-P1 and Cx43-P2, and both could be converted to the nonphosphorylated form (Cx43-NP) by alkaline phosphatase (Figure 1). FSH moderately increased the level of Cx43-P2 (but not Cx43-P1) on day 1 of culture and the increment subsided on day 2 of culture (Figure 2). TGFβ1 together with FSH markedly increased the levels of both Cx43-P1 and Cx43-P2 from day1 to day 2 of culture, while TGFβ1 alone had no significant effect (Figure 2). Lindane, on the other hand, completely suppressed FSH and TGFβ1-increased Cx43-P levels to control values in day 1 and day 2 cultures (Figure 2). Also, lindane moderately reduced Cx43-P levels in FSH-treated or TGFβ1-treated groups on day 2 of culture (Figure 2). The level of Cx43-NP remained scarce in all the treatment groups. We then determined the cellular localization of Cx43 using immunofluorescence. Immunostaining of Cx43 appeared predominantly as a punctate pattern at cell–cell contact sites, with the highest intensity in the FSH plus TGFβ1-treated group as compared with the FSH-treated and the control groups (Figure 3). In addition, lindane reduced the FSH and TGFβ1-stimulated increase in Cx43 cell periphery immunostaining (Figure 3). Furthermore, gap junction communication was examined using the scrape loading–dye transfer assay. The extent of cell coupling on day 1 and day 2 culture was increased in FSH- and FSH plus TGFβ1-treated groups as compared

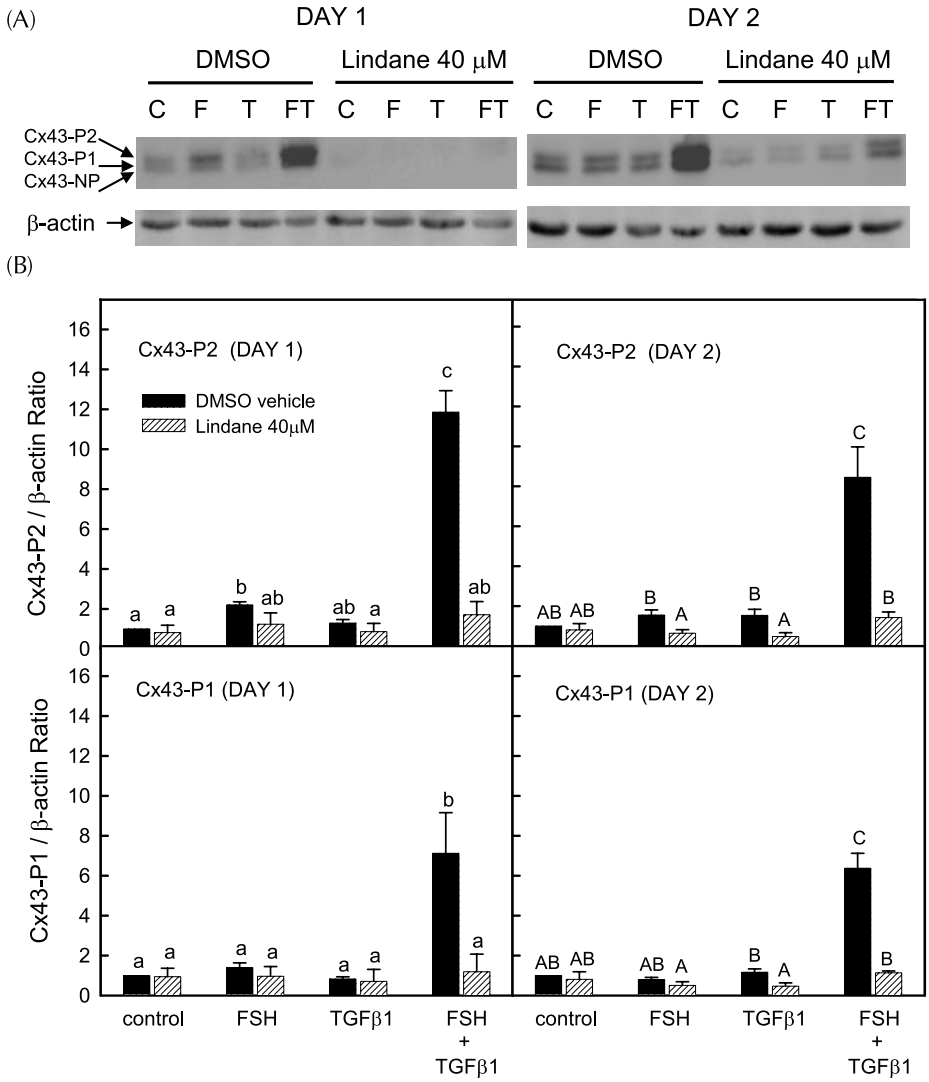


Figure 2. Effects of FSH, TGFβ1 and lindane on the Cx43 level in rat ovarian granulosa cells. Cells were plated and allowed to attach for 24 h, pretreated with DMSO vehicle or 40 μM lindane for 24 h, and then treated with vehicle, 10 ng/ml FSH and/or 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 24 h (DAY 1) or 48 h (DAY 2). (A) Cell lysates were prepared for immunoblotting analysis of Cx43, with β-actin used as an internal control, and quantification was performed using scanning densitometry. (B) Data are expressed as the mean (± S.E.) density ratio of Cx43:β-actin relative to that of the respective control value from three independent experiments. Different lower-case (or upper-case) letters indicate significant differences among treatment groups ($P<0{\cdot}05$). Cx43-P1, Cx43-P2: phosphorylated forms of Cx43. C: control; F: FSH; T: TGFβ1; FT: FSH+ TGFβ1. The levels of Cx43-NP were scarce in all treatment groups.

with the control group, with lindane suppressing this effect of FSH plus TGFβ1 (FSH plus TGFβ1>FSH>control ≈FSH plus TGFβ1 plus lindane) (Figure 4).

Effects of gap junction blockers on FSH and TGF β1-stimulated steroidogenesis

Since lindane suppressed the FSH plus TGFβ1-stimulated increases in the phosphorylated Cx43 levels and gap junction communication in rat granulosa cells, we then determined the effect of lindane on FSH and TGFβ1-stimulated progesterone production (a marker of differentiation) as well as on the levels of StAR protein and P450scc enzyme (two critical players in progesterone production). Lindane dose-dependently (10 to 40 μM) suppressed FSH plus TGFβ1-stimulated progesterone production during days 1 to 2 of culture (Figure 5), but had no significant effect on basal and FSH-stimulated

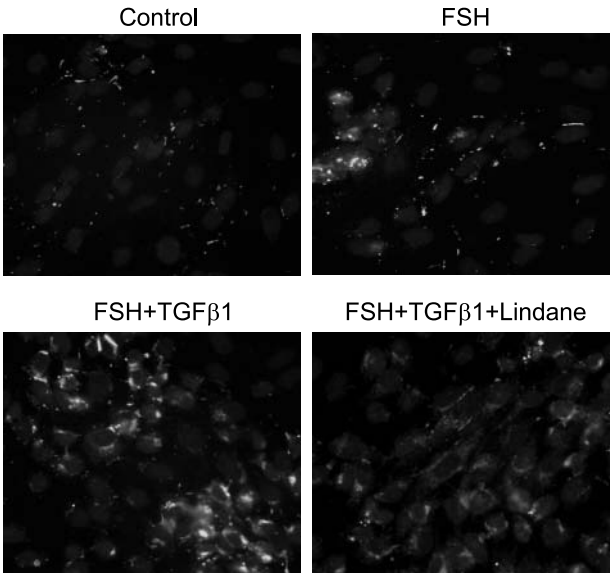
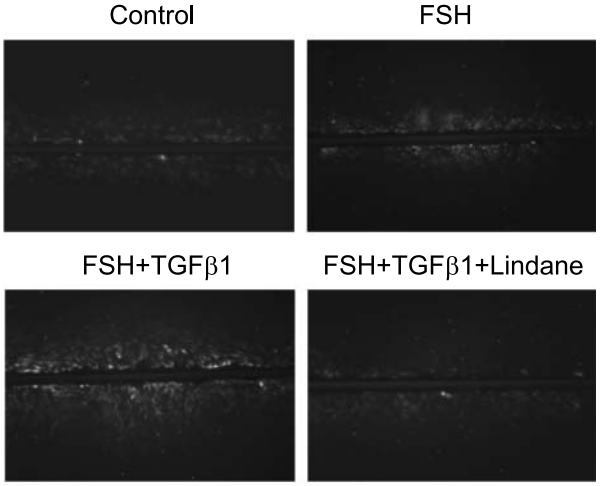


Figure 3. Effects of FSH, TGFβ1 and lindane on the cellular localization of Cx43 in rat ovarian granulosa cells. Cells were plated and allowed to attach for 24 h, pretreated with DMSO vehicle or 40 μM lindane for 24 h, and then treated with vehicle, 10 ng/ml FSH, or FSH plus 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 48 h. Cells were then fixed, permeabilized and analyzed for the localization of Cx43 using immunofluorescence. Nuclei of cells were also stained using DAPI. Representative photographs from one experiment are presented. Similar results were observed in three independent experiments. Note that lindane suppressed the FSH plus TGFβ1-induced increase in the Cx43 immunostaining intensity predominantly at the cell–cell contact sites.

steroidogenesis (Figure 5). Also, TGFβ1 alone or in the presence of lindane did not affect progesterone production (data not shown).

We further demonstrated that FSH and TGFβ1 as well as lindane exerted a differential regulation of StAR protein and P450scc enzyme. Consistent with our most recent study (Ke *et al.* 2004), FSH increased the levels of StAR protein (but not P450scc enzyme) in rat granulosa cells (Figures 6 & 7 respectively). FSH together withTGFβ1 dramatically increased the levels of both StAR protein and P450scc enzyme, while TGFβ1 alone had no effect on the levels of both proteins (Figures 6 & 7 respectively). Interestingly, this study demonstrates for the first time that lindane significantly reduced the FSH plus TGFβ1-



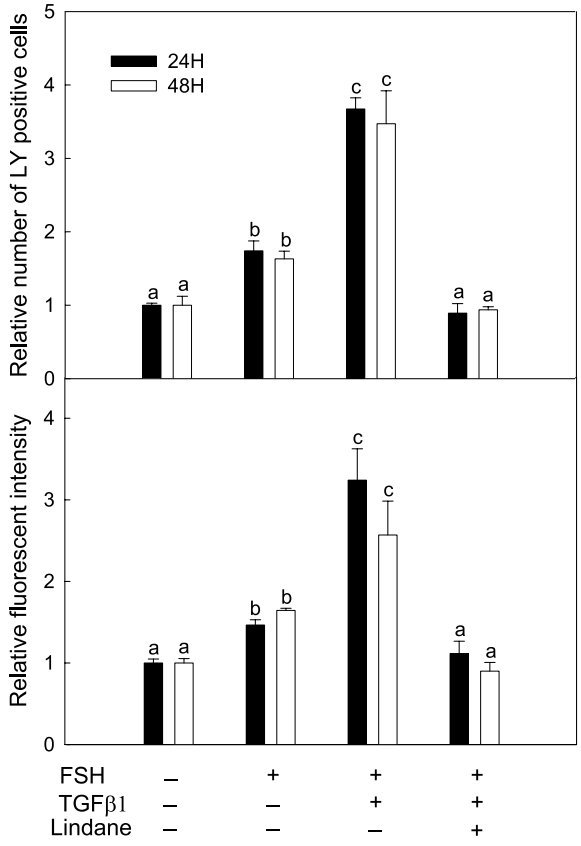


Figure 4. Effects of FSH, TGFβ1 and lindane on gap junction communication in rat ovarian granulosa cells. Cells were plated and allowed to attach for 24 h, pretreated with DMSO vehicle or 40 μM lindane for 24 h, and then treated with vehicle, 10 ng/ml FSH, or FSH plus 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 24 or 48 h. Scrape loading–dye transfer assay using Lucifer Yellow (LY) and the relative quantitative analysis were conducted as described in the Materials and Methods. Representative photographs of 48-h treatment groups from one experiment are presented. The extent of cell coupling was determined by the number of LY positive cells (the total number of dye positive cells / the cell number on the scraped edge) and the fluorescent intensity (total fluorescent intensity / the cell number on the scraped edge). Data are expressed as the mean (± S.E.) number of LY positive cells (or fluorescent intensity) relative to that of the respective control value from three independent experiments. Different lower-case letters indicate significant differences among treatment groups ($P<0{\cdot}05$). Note that both quantitative analyses show similar results.

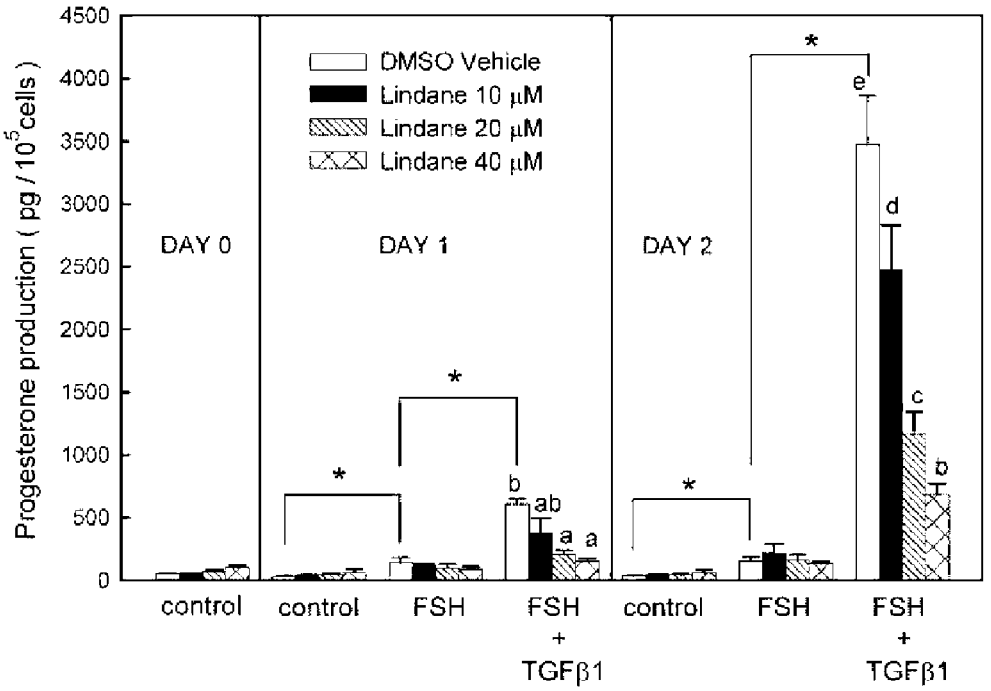


Figure 5. Dose-dependent effects of lindane (a nonselective gap junction blocker) on FSH and TGFβ1-regulated progesterone production in rat ovarian granulosa cells. Cells were plated and allowed to attach for 24 h, pretreated with DMSO vehicle or 40 μM lindane for 24 h (DAY 0), and then treated with vehicle, 10 ng/ml FSH and/or 5 ng/ml TGFβ1 in the absence (DMSO vehicle) or presence of lindane for an additional 24 h (DAY 1) or 48 h (DAY 2). Progesterone levels in conditioned media were analyzed using enzyme immunoassay. Data are expressed as the mean (± S.E.) progesterone level of triplicate samples from three independent experiments. Different lower-case letters indicate significant differences among the FSH plus TGFβ1-treated groups ($P<0{\cdot}05$). There were no significant differences among the control or the FSH-treated groups. An asterisk indicates a significant difference between the two groups ($P<0{\cdot}05$).

induced increases in the StAR protein and P450scc enzyme levels, in which a greater suppression was observed in the P450scc enzyme level (Figures 6 & 7). In addition, lindane increased the basal level of StAR protein but not P450scc enzyme (Figures 6 & 7).

To further determine the specific involvement of Cx43 gap junctions in the FSH and TGFβ1-stimulated progesterone production, a Cx43 peptide blocker was used. Cx43 peptide blockers have been reported to interfere with gap junction communication in airway cells and aortic smooth muscle cells (Kwak & Jongsma 1999, Boitano & Evans 2000). A Cx43 peptide blocker inhibited the FSH and TGFβ1-stimulated progesterone production in rat ovarian granulosa cells in a dose-dependent manner, while a Cx43 control peptide had no effect (Figure 8).

Discussion

Gap junctions containing predominantly Cx43 are well developed in granulosa cells (Mayerhofer & Garfield 1995, Granot & Dekel 1997, Kidder & Mhawi 2002), and they play critical roles in ovarian functions including folliculogenesis and oocyte meiotic maturation (Grazul-Bilska *et al.* 1997, Kidder & Mhawi 2002, Gittens *et al.* 2003). In the present study, we demonstrate for the first time that TGFβ1 enhanced FSH-stimulated increases in the levels of the phosphorylated forms of Cx43 (Cx43-P1 and Cx43-P2) and gap junction communication in rat ovarian granulosa cells, and lindane (a general blocker of gap junction) suppressed such increases. This study further demonstrates that disruption of gap junctions by lindane and by a specific Cx43 mimetic peptide blocker suppressed FSH plus TGFβ1-stimulated progesterone production in rat ovarian granulosa cells. Interestingly, there is a clear temporal association between the Cx43 protein level/gap junction communication and progesterone production in ovarian granulosa cells in response to FSH, TGFβ1 and lindane. In addition, this study reveals that lindane reduced the FSH plus TGFβ1-stimulated increases in P450scc enzyme and StAR protein. Together, these results indicate that TGFβ1 enhancement of FSH-facilitated progesterone production (a granulosa cell differentiation marker) in rat ovarian granulosa cells involves the regulation of gap junction function, and that lindane may impair ovarian cell steroidogenic function.

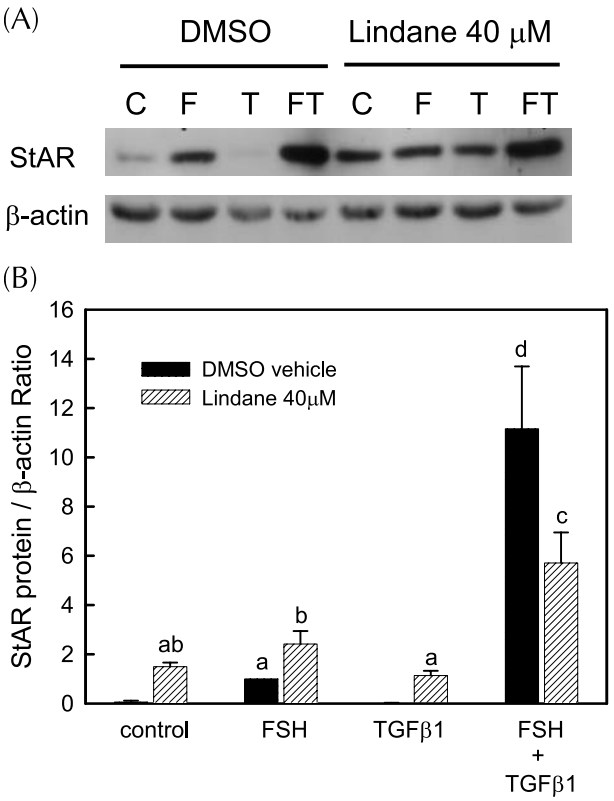


Figure 6. Effects of lindane on FSH and TGFβ1-induced StAR protein levels in rat ovarian granulosa cells. Cells were treated as described in figure 3. Cell lysates were prepared for immunoblotting analysis of StAR protein, β-actin was used as internal control and quantification was performed using scanning densitometry. Data are expressed as the mean (± S.E.) density ratio of StAR:β-actin relative to that of the FSH-treated group value from four independent experiments. Different lower-case letters indicate significant differences among treatment groups ($P<0{\cdot}05$).

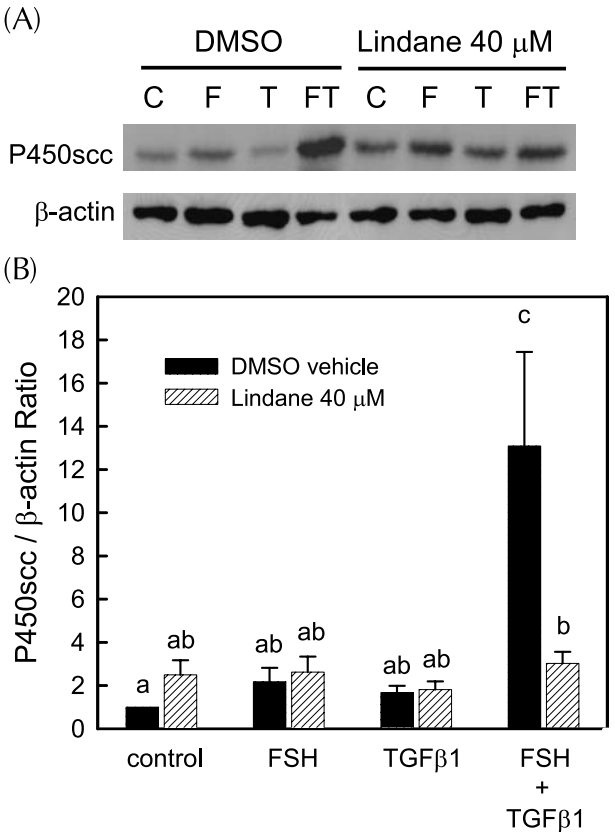


Figure 7. Effects of lindane on FSH and TGFβ1-induced P450scc enzyme levels in rat ovarian granulosa cells. Cells were treated as described in figure 3. Cell lysates were prepared for immunoblotting analysis of P450scc enzyme, β-actin was used as internal control and quantification was performed using scanning densitometry. Data are expressed as the mean (± S.E.) density ratio of P450scc:β-actin relative to that of the control group value from four independent experiments. Different lower-case letters indicate significant differences among treatment groups ($P<0{\cdot}05$).

At present, the understanding of the hormonal control of Cx43 gap junction in ovarian cells is limited. FSH may stimulate the expression of Cx43 as suggested by the following three lines of evidence; first, we showed that FSH moderately increased the level of phosphorylated Cx43 (Cx43-P2) in primary rat granulosa cells, while the levels of non-phosphorylated Cx43 (Cx43-NP) remained relatively low as in the control group. Secondly, a recent study reports that FSH increased the level of Cx43 mRNA in a rat ovarian granulosa cell line (Sommersberg *et al.* 2000). Thirdly, the expression of Cx43 was increased during gonadotropin-induced follicular growth and decreased after the ovulatory LH surge and during follicular atresia (Schreiber *et al.* 1993, Wiesen & Midgley 1993, 1994, Mayerhofer & Garfield 1995, Okuma *et al.* 1996, Granot & Dekel 1997). FSH regulation of the Cx43 level or gap junction function may be mediated partly through the cAMP pathway because cAMP up-regulated the permeability of gap junctions in human granulosa cells that also contain predominantly Cx43 (Furger *et al.*1996). In addition, an earlier study reported that cAMP-induced rapid increases in gap junction permeability may be partly attributed to the increase in trafficking and/or assembly of Cx43 in plasma membrane gap junctional plaques, a phenomenon seen in many cell types including rat granulosa cells, hepatocytes and myometrial cells (Burghardt *et al.* 1995). On the other hand, TGFβ1 was reported to increase Cx43 mRNA and protein levels in endothelial cells (Larson *et al.* 1997), and to inhibit gap junction intercellular communication and decrease the phosphorylation of Cx43 in osteoblasts (Wyatt *et al.* 2001) and brain cells (Robe *et al.* 2000). However, we did not observe any significant effect of TGFβ1 alone on the protein level of Cx43 in rat ovarian granulosa cells, yet TGFβ1 greatly augmented the FSH-stimulated increase in Cx43 protein level and its localization to plasma membrane plaques. We

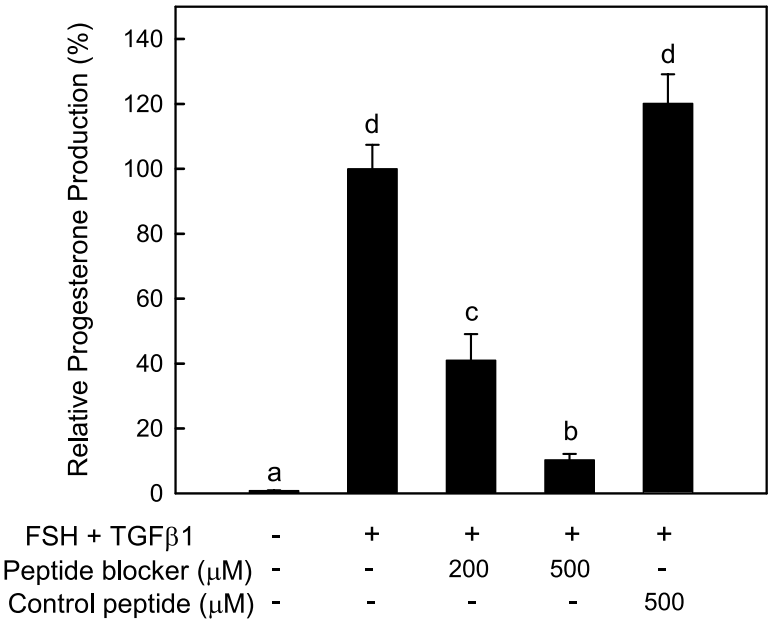


Figure 8. Effect of Cx43 mimetic peptide (a selective gap junction blocker) on FSH and TGFβ1-stimulated progesterone production in rat ovarian granulosa cells. Cells were given Cx43 peptide blocker or control peptide during plating and allowed to attach for 24 h, pretreated with peptides for another 24 h, and then treated with vehicle or 10 ng/ml FSH plus 5 ng/ml TGFβ1 in the absence or presence of the Cx43 peptides for an additional 48 h. Progesterone levels in conditioned media were analyzed using enzyme immunoassay. Relative progesterone production was calculated using the mean progesterone production (pg/1 × 10^5 cells) of FSH plus TGFβ1-treated group as 100%. Data are expressed as the mean (± S.E.) percentage of progesterone level of triplicate samples from three independent experiments. Different lower-case letters indicate significant differences among treatment groups ($P<0{\cdot}05$).

cannot, at the present time, rule out the possibility that FSH and TGFβ1 may affect the turnover of Cx43.

The gap junction protein Cx43 is present in multiple phosphorylated forms in the plasma membrane of ovarian follicular cells (Godwin *et al.* 1993, Granot & Dekel 1994), a finding that is also true in our present study. *In vivo* studies demonstrated that gonadotropin or estrogen induction of follicular growth was accompanied by an increase in the levels of Cx43 protein and mRNA with concurrent induction of the phosphorylation of Cx43 protein, while LH down-regulated the Cx43 gene concomitantly with its stimulation of ovulation and corpus luteum formation (Schreiber *et al.* 1993, Granot & Dekel 1997). The current *in vitro* study also demonstrated that FSH increased the level of phosphorylated Cx43 and gap junction communication in rat ovarian granulosa cells, and that the effect of FSH was enhanced by TGFβ1. The phosphorylation of Cx43 may occur through protein kinase A (PKA)- and protein kinase C (PKC)-dependent pathways (Godwin *et al.* 1993, Granot & Dekel 1994). FSH or PKA catalytic subunit could reverse the PKA inhibitor-induced reduction of cell–cell communication in primary granulosa cells (Godwin *et al.* 1993). In addition, cell–cell communication stopped when cells were injected with alkaline phosphatase but returned either spontaneously within 20 min or within 2–3 min after injection with PKA catalytic subunit or PKC (Godwin *et al.* 1993). Together, these studies suggest that phosphorylation of Cx43 in granulosa cells is essential for cell–cell communication, and that FSH may act partly through PKA in promoting the phosphorylation of Cx43.

The present study employed a general chemical blocker of gap junctions, lindane, to explore the potential role of gap junctions in steroidogenesis, and showed that lindane suppressed FSH plus TGFβ1-stimulated progesterone production in rat ovarian granulosa cells. It was of interest to attempt to determine the mechanism(s) whereby lindane inhibits hormone-induced steroidogenesis. Lindane has been shown to be a nonselective inhibitor of inositol metabolism as it moderately inhibits the activity of phosphatidylinositol synthase (Parries & Hokin-Neaverson 1985). Recently, lindane was reported to impair gap junction intercellular communication by promoting the intracellular localization of Cx43 within the Rab5 positive endosomes in a Sertoli cellline (Mograbi *et al.* 2003). This effect of lindane requires Cx43 phosphorylation and acti-

vation of the extracellular signal-regulated kinases (ERK) but not c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (Mograbi *et al.* 2003). Our present study also shows that lindane reduced the levels of phosphorylated Cx43 and the cell surface immunostaining intensity, as well as gap junction communication in FSH and TGFβ1-stimulated ovarian granulosa cells. In addition, lindane was reported to inhibit gap junction communication in rat uterine myocytes through an arachidonic acid-sensitive and cAMP-independent mechanism though lindane increased the levels of intracellular cAMP (Criswell & Loch-Caruso 1995). Also, lindane inhibited myometrial gap junctions and spontaneous oscillatory contraction by a phospholipase C-mediated pathway (Wang & Loch-Caruso 2002). Together, these studies indicate that lindane may act through phospholipase C/inositol phosphate, ERK and arachidonic acid pathways, but not through the cAMP pathway, to inhibit gap junction communication. Whether this is true for the inhibitory effect of lindane on FSH and TGFβ1-induced increases of Cx43 protein level, gap junction communication and steroidogenic activity in ovarian granulosa cells awaits future study. In addition, this study demonstrates that lindane suppression of the FSH and TGFβ1-induced increases of Cx43-P2 and Cx43-P1 levels was not due to dephosphorylation since no increase in the nonphosphorylated (Cx43-NP) form was evident. This is consistent with an earlier study in rat liver epithelial cells (Guan & Ruch 1996). These results suggested that lindane may affect the synthesis and/or degradation of Cx43 but not its phosphorylation.

Consistent with our most recent study (Ke *et al.* 2004), this study demonstrates that FSH increased the levels of StAR protein in rat ovarian granulosa cells and TGFβ1 augmented the FSH effect. Also, FSH together with TGFβ1 increased the level of the P450scc enzyme. Interestingly, this study demonstrates for the first time that lindane exhibits differential regulation of the levels of StAR protein and P450scc enzyme in rat ovarian granulosa cells. Lindane (40 μM) suppressed the FSH plus TGFβ1-induced increase in the P450scc enzyme level without affecting its basal level. In addition, lindane moderately reduced the FSH plus TGFβ1-induced, but not the FSH-induced, increase in StAR protein level. Also, lindane increased the basal level of StAR protein. This indicates that lindane inhibition of FSH and TGFβ1-stimulated steroidogenesis may partly attribute to its suppression of the levels of P450scc enzyme and StAR protein. In addition, a recent report demonstrated that lindane inhibited dibutyryl cAMP-stimulated progesterone production in the mouse MA-10 Leydig tumor cell line, and in contrast to our study, lindane reduced the StAR protein level but not the P450scc enzyme level (Walsh & Stocco 2000). At this point, we can only assume the difference may be due to the differences in the cell types utilized, as well as in the crosstalk between lindane and different stimulants (cAMP or FSH plus TGFβ1). Although we observed that lindane increased the basal level of StAR protein but not P450scc enzyme in rat granulosa cells, lindane had no significant effect on basal progesterone production. We further examined the specific role of Cx43 gap junctions in the FSH and TGFβ1-regulated steroidogenesis in rat granulosa cells by employing a specific Cx43 mimetic peptide blocker; the result shows that the Cx43 peptide blocker dose-dependently reduced the FSH plus TGFβ1-stimulated progesterone production.

Overall, the present study suggests that Cx43 gap junction formation may play a critical role in FSH plus TGFβ1-promoted steroidogenesis in rat ovarian granulosa cells. Lindane may repress female reproductive function partly through negative regulation of ovarian steroidogenic activity.

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