Connexin 43 Gap Junction Protein Expression during Follicular Development in the Porcine Ovary

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ABSTRACT

Connexin 43, a member of the highly conserved connexin family of gap junction proteins, is expressed in the pig ovary. In other species, ovarian connexin 43 expression and phosphorylation are hormonally regulated. We characterized connexin 43 expression and phosphorylation in the ovaries of mature pigs during the estrous cycle and in pubertal gilts during follicular development induced by eCG (750 IU)/hCG (500 IU; 72 h later). Ovarian connexin 43 protein expression and phosphorylation were examined by immunoblot analysis. Connexin 43 was localized to specific follicular cell types during development by immunofluorescence. While no change in total connexin 43 protein expression was seen during the cycle, connexin 43 phosphorylation was significantly higher (p < 0.05) during the late follicular stage of the cycle than during the early luteal and early to mid-follicular stages. In ovaries of eCG/hCG-primed prepubertal gilts, connexin 43 protein levels remained steady, while phosphorylation of the protein increased significantly at 72 h and 84 h after eCG treatment (p < 0.05), then declined to pre-treatment levels by 96 h (24 h post-hCG administration). Immunoreactive connexin 43 was localized predominantly to granulosa cells of cyclic gilts and eCG/hCG-primed prepubertal gilts. Follicular connexin 43 was highest between 60 h and 84 h after eCG and declined after hCG administration. Connexin 43 was not detected in morphologically atretic follicles, stroma, or vascular tissue of the ovary. This is the first evidence that porcine ovarian connexin 43 phosphorylation is differentially regulated during follicular development. The results suggest that hormonally induced changes in connexin 43 phosphorylation may play a coordinating role in porcine follicular development.

INTRODUCTION

Gap junctions are specialized channels, composed of six identical membrane proteins or connexins, which connect the cytoplasmic compartments of neighboring cells and allow small molecules to pass from one cell to its neighbor. Morphological studies of the ovary in a variety of mammalian species have demonstrated the presence of gap junctions in granulosa cells of the follicle wall, in cumulus granulosa cells surrounding the oocyte, and between theca cells of the developing follicle [1–3]. These gap junctions form a metabolic syncytium between follicular cells, interconnecting the oocyte and surrounding granulosa cells. Connexin 43 belongs to this highly conserved, multi-gene gap junction protein family. In the rat, connexin 43 is expressed in granulosa cells of healthy developing follicles [4–6] and in the corpus luteum [7]. Immunoreactive connexin 43 has also been localized in the theca cell layer of the cyclic rat ovary and in ovarian stromal cells of the pregnant rat [4, 7]. Additionally, differential expression of four connexin genes, including connexin 43, has recently been described in the porcine follicle [8].

Gap junctions are thought to have a role both in maintaining tissue homeostasis and in the exchange of regulatory molecules involved in normal cellular growth and development. These associations are thought to be important in follicular development [9, 10]. Control of gap junction expression by gonadotropins and steroid hormones has been reported in the ovary of the rabbit and the rat. Gap junction formation in the rabbit ovary is first observed at the time of antrum formation [1], a time when the granulosa cell population enters a rapid phase of proliferation. This suggests that FSH, which is required for antrum formation in the rabbit, may also increase gap junction expression. In the prepubertal rat, administration of estradiol or diethylstilbestrol in the absence of endogenous gonadotropins stimulates gap junction expression between granulosa cells in preantral follicles as well as in healthy antral follicles [6, 11, 12]. Additionally, a decrease in connexin 43 expression following the LH surge and in follicles undergoing atresia has been reported in the rat during the cycle [6, 13]. This decrease in gap junction protein has also been observed in preovulatory follicles of the rat and rabbit after hCG treatment [14, 15]. Furthermore, connexin 43 mRNA expression is significantly down-regulated during proestrus in the rat [13] and in the functional stage of the porcine corpus luteum [8], supporting a role for increased concentrations of LH, due to the LH surge, as a negative regulator of connexin 43 expression [13, 16].

Phosphorylation can influence the biological activity of a protein. Phosphorylation/dephosphorylation of gap junction proteins has been shown to alter cell-to-cell communication [17], and second messengers, such as cAMP, which are associated with gonadotropin stimulation in the ovary, readily pass through these channels [9, 18–21]. In vitro studies have shown that connexin 43 is phosphorylated in several different tissues and cell lines, including the ovary [16, 22–24]. Moreover, studies suggest that ovarian connexin 43 phosphorylation is hormonally regulated. Incubation of follicles from eCG-primed rats with LH resulted in immediate, short-term phosphorylation of connexin 43 protein [16]. Additionally, in vitro exposure of porcine granulosa cells to FSH enhanced gap junctional conductance between adjacent cells, an effect that could be abolished by subsequent dephosphorylation of these junctions with alkaline phosphatase [23].

While ovarian gap junction expression during the cycle has been studied in other species [6, 14, 15], expression of porcine connexin 43 protein during cyclic follicular development has not been investigated. Whether connexin 43
protein expression and/or phosphorylation are differentially regulated during the porcine estrous cycle is unknown. In addition, since hormones alter ovarian gap junction expression in other species, we were interested in determining the effects of gonadotropins on porcine ovarian connexin 43 expression. Therefore, the objective of this study was to characterize connexin 43 expression and phosphorylation in the porcine ovary during the estrous cycle and in prepubertal gilts after in vivo gonadotropin stimulation. Additionally, we localized connexin 43 to specific ovarian cell types during follicular development.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and Gibco-BRL Life Technologies (Gaithersburg, MD), unless otherwise stated. Equine CG was obtained from Ayerst Veterinary Laboratories (Equiex, Guelph, ON, Canada) and Calbiochem Corporation (La Jolla, CA). Human CG (APL) was also from Ayerst Veterinary Labs. Alkaline phosphatase was purchased from Boehringer Mannheim (Indianapolis, IN). Gradient SDS-polyacrylamide gels (4–20%) were obtained from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence (ECL) kit and X-ray film for protein detection (Hyperfilm-ECL) were purchased from Amersham (Arlington Heights, IL). Monoclonal anti-rat connexin 43 antibody, polyclonal anti-rat connexin 43 antibody, purified normal mouse and rabbit immunoglobulins (IgG), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or goat anti-rabbit IgG, were obtained from Zymed Laboratories (South San Francisco, CA). Goat anti-mouse IgG-horseradish peroxidase-conjugated antibody was purchased from Transduction Laboratories (Lexington, KY).

Tissue Collection

Multiparous Duroc sows that exhibited at least two estrous cycles averaging 21 days were checked for estrus in the presence of a boar, and the first day of estrus was designated Day 0 of the cycle. Ovaries were collected from animals on Days 3, 13, and 19 of the cycle [25], times representing early, middle, and late stages of follicular development, respectively [26]. Ovaries were snap-frozen in liquid nitrogen and stored at -80°C until used. Tissue samples used for protein analysis were taken from portions of the ovary containing follicles and surrounding stroma. Care was taken to exclude luteal tissue from the analysis. In other experiments, prepubertal Landrace gilts (60–70 kg) housed at the Macdonald Campus Farm, McGill University, were treated i.m. with 750 IU eCG (Ayerst Veterinary Labs) and 72 h later with hCG to induce follicular growth and ovulation [27]. With this treatment, animals ovulated approximately 114 h post-eCG (42 h after hCG) [28]. Ovaries, devoid of corpora lutea, were collected via laparotomy at 48, 60, 72, 84, and 96 h after eCG administration or from untreated animals (0 h). Tissues were either frozen in liquid nitrogen for protein analysis or fixed in 4% formaldehyde and embedded in paraffin for immunohistochemistry. Initially, frozen tissues were used to localize connexin 43 in porcine ovaries during the cycle; however, only weak immunoreactivity was observed. In formaldehyde-fixed, paraffin-embedded ovaries from gonadotropin-primed pigs, there was better preservation of follicular architecture with less nonspecific fluorescence, which permitted specific cellular localization of connexin 43. Therefore, ovaries from mature pigs (68–90 kg), obtained from local abattoirs, were fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical studies. Only ovaries with evidence of fresh corpora lutea were used for immunohistochemistry, to ensure that they were representative of tissues from mature, cycling animals. Follicles were classified as small (1–2 mm), medium (3–5 mm), or large (≥ 6 mm).

Ovaries from immature rats (21 days), 48 h after priming with eCG (10 IU; Calbiochem Corp.), served as the positive control for connexin 43 detection [29] in both immunoblot analysis and immunohistochemical localization. Skeletal muscle, which is fused to form a functional syncitia and does not contain gap junctions [30], was used in immunoblot analysis as the negative control for connexin 43 detection. Immunoblot analysis and immunocytochemistry were repeated at least three times using ovaries from three or more animals per time point.

Protein Extraction

Protein was extracted from tissue samples by homogenization in 5 vol (1 g/5 ml) of boiling lysis buffer (10 mM Tris/HCl, 1% SDS; pH 7.4), followed by microwaving (Quasar, model #668W; Quasar Co., Elk Grove Village, IL) for 3 × 5-sec cycles at high power (~650 watts). Samples were then sonicated for 30 sec to reduce viscosity, and homogenates were centrifuged (12,000 g, 15°C) for 30 min to remove insoluble material. Protein concentration was determined using a detergent-compatible protein assay kit (DC Protein Assay; Bio-Rad).

Gel Electrophoresis and Immunoblotting

Protein samples (20 μg) were resolved by 4–20% gradient SDS-polyacrylamide gel electrophoresis under reducing conditions [31] and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking in 5% BSA in Tris-buffered saline (TBST; 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20), membranes were incubated with a mouse anti-rat connexin 43 antibody (0.5 μg/ml) in TBST/1% BSA overnight at 4°C. After being washed with TBST, blots were incubated with a peroxidase-conjugated mouse anti-IgG, diluted 1:5000 in TBST/5% nonfat dry milk, for 1 h at room temperature, and bound antibodies were detected by ECL. Multiple exposures (5–60 sec) to Hyperfilm-ECL were performed to maximize visualization of protein bands with minimal background. Results were similar in all experiments, and representative images are presented in the figures. Protein size was determined by including prestained protein molecular weight markers (Mr, 14.3–200 × 10^3) in the gels.

Dephosphorylation of Connexin 43

Phosphorylation of connexin 43 in ovarian samples was identified using methods described by Hossain et al. [24] with modifications. Briefly, alkaline phosphatase (15 U) was preincubated at 37°C for 30 min in 0.1 M diethanolamine (pH 10.0) and 1 mM MgCl₂ (final volume, 20 μl). Porcine ovarian homogenates (100 μg protein) were suspended in phosphatase reaction buffer (50 mM Tris/HCl, 10 mM MgCl₂, 150 mM NaCl, pH 8.0, with 1% SDS, 1% 2-mercaptoethanol, and 2 mM PMSF) [22] to a total volume of 35 μl. Dephosphorylation was initiated by the addition of 10 μl of ovarian homogenate in phosphatase reaction buffer to the alkaline phosphatase solution; a 3-h incubation at
Connexin 43 expression in the porcine ovary

37°C followed. Control reactions were carried out either in the absence of alkaline phosphatase or in the presence of both alkaline phosphatase and phosphatase inhibitors (150 mM sodium fluoride, 150 mM sodium pyrophosphate, 15 mM sodium orthovanadate) [16]. Samples (15 μl) were then mixed with an equal volume of loading buffer and processed for immunoblot analysis as described above.

**Densitometry and Statistical Analysis**

Connexin 43 protein was quantified in the immunoblots by scanning densitometry (Ultrascan X; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Immunoblot data are presented as the percentage of phosphorylated connexin 43 protein relative to total connexin 43 protein in both bands (% phosphorylated protein). To correct for variations in band intensity between blots, data from cyclic animals were standardized against the eCG-primed rat ovary control. Signal intensities of ovarian connexin 43 in the eCG/hCG-treated gilt model were corrected to those of the 0-h control group. All data are expressed as the mean ± SE for at least three independent experiments. All statistical analysis was performed with the StatView 4.02 application (Abacus Concepts, Berkeley, CA). Data were analyzed by analysis of variance and tested for differences by Fisher’s least-significant difference (LSD) for multiple comparisons. A value of \( p < 0.05 \) was accepted as significant.

**Immunohistochemistry**

Ovaries were serially sectioned (~10 μm) and mounted onto poly-L-lysine-coated slides. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and rinsed in tap water. Tissues were incubated in buffered trypsin (0.01% porcine pancreas type II trypsin, 0.1% CaCl₂, 20 mM Trizma base, pH 7.4) for 10 min at room temperature to unmask antigens altered by formalin fixation [32]. Sections were washed in tap water and then blocked with 0.1 M L-lysine in 0.015 M PBS containing 0.1% Triton X-100 for 1 h at room temperature. Tissues were incubated overnight at room temperature with either a monoclonal anti-rat connexin 43 antibody at a dilution of 1:20 in PBS, or a polyclonal anti-rat connexin 43 antibody at a dilution of 1:100 in PBS. Normal mouse or rabbit IgG served as negative controls. Sections were rinsed in PBS (2 × 5 min), then incubated with FITC-conjugated goat anti-mouse IgG or goat anti-rabbit IgG, diluted at 1:20 in PBS for 1 h at room temperature in a light-tight, humidified box. Slides were washed in PBS (2 × 5 min), then mounted using 5% n-propyl gallate. Adjacent sections were stained with hematoxylin and eosin, and follicles were classified as healthy or atretic in tissue sections on the basis of the morphological criteria of Hay et al. [33]. Atretic follicles were identified by the presence of pyknotic nuclei, fragmentation of the granulosa cell layer, and loss of basement membrane integrity [33-35].

**FIG. 1.** Immunoblot analysis of connexin 43 in the porcine ovary during the estrous cycle. a) Connexin 43 protein in ovarian samples from cyclic gilts on Days 3, 13, and 19 of the 21-day estrous cycle, separated by SDS-PAGE, was detected using a monoclonal mouse anti-rat connexin 43 antibody. Rat ovary (+) and porcine skeletal muscle (−) were used as controls. Numbers indicate approximate molecular masses of immunoreactive bands. d, Day. b) Changes in the relative amount (%) of phosphorylated connexin 43 protein were determined by densitometric analysis and calculated as described in Materials and Methods. Values with different letters are significantly different (\( p < 0.05 \)). c) Representative dephosphorylation of ovarian connexin 43. Day 19 porcine ovarian homogenates
RESULTS

Detection of Connexin 43 in the Porcine Ovary during the Cycle

Immunoblot analysis to detect connexin 43 in the porcine ovarian homogenates revealed the presence of two immunoreactive bands of 43 and 45 kDa at Days 3, 13, and 19 of the estrous cycle (Fig. 1a). A single immunoreactive band was detected in ovarian tissue from the eCG-treated rat control at 43 kDa, whereas no immunoreactivity was detected in skeletal muscle samples, even with longer exposure. To determine whether the 45-kDa immunoreactive band represented a phosphorylated form of connexin 43, samples were incubated with alkaline phosphatase. In the ovary at Day 19 of the cycle (Fig. 1c), alkaline phosphatase dephosphorylation increased the intensity of the lower-molecular-mass form of the protein (43 kDa) at the expense of the higher-molecular-mass form (45 kDa), which was almost completely abolished (Fig. 1c, lane 1). When ovarian samples were incubated in the presence of alkaline phosphatase plus phosphatase inhibitors (Fig. 1c, lane 2) or without alkaline phosphatase (Fig. 1c, lane 3) there was no loss in the intensity of the upper band. Similar dephosphorylation results were obtained when ovarian tissues from animals at Day 3 and Day 13 of the cycle were analyzed (data not shown). Total connexin 43 protein, expressed as the sum of the optical density units (OD units) of the phosphorylated and nonphosphorylated forms, was not significantly different at Day 3 (9.61 ± 3.49 OD units), Day 13 (5.78 ± 1.77 OD units) or Day 19 (7.81 ± 2.22 OD units) of the estrous cycle. Since the 45-kDa band was shown to be a result of phosphorylation, the relative intensity (percentage of total protein phosphorylated) of the 45-kDa phosphorylated form of connexin 43 in the porcine ovary was calculated (Fig. 1b). The relative amount of phosphorylated connexin 43 was not significantly different at Day 13 of the cycle (69.6% ± 0.7) from that at Day 3 (71.9% ± 1.0). However, by Day 19 the relative amount of phosphorylated connexin 43 had increased (76.7% ± 1.6) and was significantly higher (p < 0.05) than observed at either Day 3 or Day 13 of the cycle (Fig. 1b).

Effect of eCG/hCG Treatment on Expression and Phosphorylation of Connexin 43 Protein

The results show that connexin 43 protein exists in both a nonphosphorylated (43-kDa) and phosphorylated (45-kDa) form in the prepubertal gilt (Fig. 2a). Total connexin 43 protein in the ovary after eCG/hCG administration was not significantly different over the 96-h period, with OD values that ranged from 0.81 ± 0.07 (48 h) to 1.22 ± 0.09 (84 h). Equine CG treatment did not affect the relative intensity of the phosphorylated form of connexin 43 at 48 h (62.3% ± 4.8) and 60 h (61.4% ± 8.2) when compared to untreated (0 h) prepubertal controls (61.1% ± 5.8; Fig. 2b). However, at 72 h post-eCG, a significant increase (p < 0.05) in the 45-kDa phosphorylated form of connexin 43 was detected (80.7% ± 2.2) and was maintained through 84 h post-eCG (80.6% ± 2.3). By 96 h after eCG (24 h post-hCG administration), the relative amount of phosphorylated connexin 43 (64.7% ± 2.5) had returned to levels similar to those seen in untreated animals (Fig. 2b).

Immunohistochemistry

Punctate connexin 43 immunofluorescence was detected along the surface of granulosa cells of morphologically healthy, medium to large antral follicles from adult animals (Fig. 3a). No specific immunofluorescence was detected in follicles from prepubertal gilts before eCG/hCG treatment (Fig. 3b). Immunoreactive connexin 43 was first observed in granulosa cells of antral follicles at 48 h after eCG administration (Fig. 3c). Follicular connexin 43 immunoreactivity was sustained through 84 h post-eCG (Fig. 3, d and e). Identical results were obtained using either the monoclonal or polyclonal antibodies. No specific immunofluorescence was detected when tissues were incubated with the control normal mouse/rabbit immunoglobulin (Fig. 3f). Theca cells in ovaries from cyclic pigs, as well as in pre-
FIG. 3. Localization of connexin 43 gap junction protein in ovarian follicles from cyclic pigs and eCG/hCG-treated prepubertal gilts. Tissues were incubated with a) polyclonal rabbit anti-rat connexin 43 antibody (1:100) or b-f) monoclonal anti-rat connexin 43 antibody (1:20), and immunoreactive protein was visualized using an FITC detection system. G, granulosa cells; T, theca cells; arrows, punctate connexin 43 immunofluorescence; arrowheads, autofluorescent red blood cells. ×170 (reproduced at 96%). a) Connexin 43 in the granulosa cells of a medium-size (~4-mm) antral follicle of a cyclic gilt (CYC). b) An antral follicle from an immature gilt (I), before eCG/hCG treatment (0 H). c-e) Follicles from eCG-treated prepubertal gilts at 48 H (c), 60 H (d), and 84 H (e) after treatment. f) Control (CON), at 60 H post-eCG, incubated with normal mouse immunoglobulin control.
that connexin 43 exists in two forms in the porcine ovary, changes in connexin 43 phosphorylation during porcine follicular development. The present study provides new information concerning the nature of porcine ovarian connexin 43 expression both during the cycle and in response to exogenous gonadotropin stimulation. We report here that connexin 43 exists in two forms in the porcine ovary, a nonphosphorylated 43-kDa protein and a phosphorylated 45-kDa form. These results are in agreement with reports of multiphosphorylated forms of connexin 43 in the rat ovary [16, 36] and in nonreproductive tissues [22, 37–40]. Additionally, in the present study, we show by immunoblotting that the relative expression of the two forms of porcine ovarian connexin 43 changes during the cycle and in response to eCG/hCG treatment. Furthermore, the immunohistochemical studies reported here support and extend the immunoblotting data by localizing connexin 43 to specific cell types within the porcine ovary during development.

Gap junctions are thought to facilitate the exchange of regulatory molecules involved in normal cellular growth and development. In the ovary, these associations may help to coordinate cumulus cell/oocyte communication, oocyte maturation, and cyclic follicular development [9, 10]. There is evidence that differences in ovarian connexin 43 protein expression or phosphorylation could be associated in the hormonal environment of the follicle. During hormonally induced follicular development in the rat, connexin 43 expression and phosphorylation are reported to be sensitive to estrogen [5, 12], progesterone [12], and LH [15, 16]. Estradiol, alone or in conjunction with progesterone, has been shown to enhance follicular connexin 43 gene and protein expression in vivo [11, 12]. In addition, increased expression of connexin 43 protein in large antral follicles of proestrus rats has been reported [13]. Therefore, in the present study, we expected an increase in ovarian connexin 43 expression during the late follicular phase of the pig estrous cycle (Day 19), when estradiol is elevated both systemically [41] and locally [42] in preovulatory follicles. However, we found no change in total connexin 43 when compared with earlier time points during the cycle. This may have been due, in part, to rising levels of LH in the late follicular phase. LH is reported to inhibit connexin 43 expression in preovulatory follicles of the rat [5, 13, 15], and in vivo hCG administration down-regulates follicular connexin 43 expression [13, 36]. Thus, any estradiol-mediated increase in ovarian connexin 43 expression during the late follicular phase of the cycle may have been negated by the preovulatory increase in LH.

While total ovarian connexin 43 was similar during the pig cycle, we did observe a significant increase in phosphorylation of connexin 43 protein in the late follicular phase of the cycle. The relative amounts of connexin 43 protein phosphorylated during the early (Day 3) to middle (Day 13) stages of porcine follicular development were similar (71.9% and 69.9%, respectively), and are comparable to that reported in rat follicles isolated after eCG administration, but before LH treatment [16]. The moderate but significant increase in phosphorylated connexin 43 protein (76.7%) observed at Day 19 of the cycle may be attributed to the preovulatory peak in follicular estrogen levels, as estrogen has also been reported to stimulate connexin 43 phosphorylation [12]. Alternatively, phosphorylation may have been transiently enhanced by the onset of the LH surge or the transient periovulatory rise in plasma FSH concentration [42]. In isolated follicles from eCG-treated rats, LH induces a short-term increase in phosphorylation of connexin 43 [16]. Likewise, FSH stimulates protein kinase C-mediated connexin 43 phosphorylation in porcine granulosa cells in vitro [23].

Protein kinase-mediated phosphorylation of gap junction proteins has tissue- and cell-specific effects. In some tissues, phosphorylation can enhance cell-cell communication [43–47], while in other tissues, phosphorylation may attenuate communication between cells [46, 48, 49]. Using porcine granulosa cells, Godwin et al. [23] presented evidence to indicate that FSH-induced phosphorylation of gap junction proteins enhances gap junctional conductance in vitro. Furthermore, they postulated that in the ovary, cell-cell communication requires cycles of phosphorylation and dephosphorylation, and that protein kinase A and protein kinase C interact in regulating phosphorylation-mediated communication [23]. However, precisely how these protein kinases increase or decrease conductance between gap junctions within the ovary is unknown; it is likely that they are regulated by a number of factors. In porcine and rat granulosa cells, in which connexin 43 is one of two connexin subtypes present [8, 36], communication between cells is enhanced after protein kinase A stimulation, while protein kinase C had a positive effect on cell communication under basal conditions but reduced communication when the enzyme was maximally activated [16, 23].

FSH and LH, which stimulate follicular growth and maturation, appear to have opposing actions on ovarian gap junction expression and activity. In the rabbit, gap junctions between granulosa cells are first observed at the time of FSH-dependent antrum formation [1]. In the ovary of cyclic rats, granulosa cell connexin 43 protein expression increases with follicle development [7]. Furthermore, connexin 43 gap junction conductance is enhanced by FSH in cultured porcine granulosa cells [23]. Taken together, these observations suggest that FSH stimulates gap junction formation during follicular development. In contrast, LH acts as a negative regulator of connexin 43 expression and activity. LH is reported to have multiple effects on gap junction activity in the ovary, initially enhancing connexin 43 phosphorylation [16], then later inhibiting connexin 43 protein production and mRNA expression [5, 13, 16, 36]. In isolated rat follicles, connexin 43 protein expression and phosphorylation are attenuated in a time-dependent manner after LH treatment [13]. A decrease in ovarian connexin 43 mRNA expression is also observed in the rat after the LH surge [6, 13]. Additionally, in the pig, Itahana et al. [8] report that connexin 43 mRNA expression is significantly down-regulated in functional-stage corpora lutea.

We were interested in further investigating the role of FSH- and LH-like stimulation on porcine ovarian connexin 43. Therefore, in addition to investigating ovarian connexin 43 during the cycle, this study also focused on porcine ovarian connexin 43 expression in prepubertal gilts during follicular development induced by exogenous gonadotro-
pins. We monitored porcine ovarian connexin 43 protein during follicular growth after eCG treatment (0–72 h) and maturation of the follicle after the administration of hCG (84–96 h). In response to eCG, which has predominantly FSH-like activity, we found that the ratio of phosphorylated to nonphosphorylated connexin 43 protein was significantly enhanced without any change in total ovarian connexin 43 protein. Likewise, in the rat, Okuma et al. [36] reported that treatment with another FSH-like gonadotropin, human menopausal gonadotropin, before hCG, had no effect on ovarian connexin 43 protein. However, in this study, the authors did not address the phosphorylation state of connexin 43. It is possible that FSH may enhance the activity of existing gap junction associations via increased protein phosphorylation while having little or no effect on total protein expression. In support of this hypothesis, we observed that in eCG/hCG-primed animals, the intensity of the phosphorylated connexin 43 protein band increased whereas total connexin 43 protein expression was unaffected by gonadotropin treatment. Additionally, the intensity of connexin 43 staining in porcine granulosa cells after in vivo administration of eCG did not appear to increase between 48 and 84 h. These immunohistochemical data support the immunoblot results, which show that total connexin 43 protein expression was not altered by gonadotropin treatment. Taken together, these results are in harmony with those reported in the cyclic rat that link FSH-like activity to gap junction phosphorylation and expression [6, 7, 13].

On the other hand, connexin 43 phosphorylation was significantly attenuated 24 h after administration of hCG, which, like LH, induces follicular maturation and ovulation. This hCG action is in contrast to the stimulatory effect of FSH-like hormones on connexin 43 phosphorylation. In isolated rat follicles, LH has been shown to induce immediate short-term phosphorylation of connexin 43 [16] and to significantly reduce cell-to-cell communication [50]. However, a significant decrease in protein expression was not observed until 8 h after exposure to LH, and total elimination of the protein was not observed until 24 h after treatment [16]. Additionally, in morphological and immunocytochemical studies in the rat and rabbit ovary, no decrease in the number of gap junctions was detected until 8–12 h after in vivo hCG administration [14, 51] or 7 h after the preovulatory LH surge [6]. These studies indicate that LH-induced gap junction phosphorylation in the rat ovary is responsible for the rapid attenuation of cell-to-cell communication, which is then followed by a slower down-regulation of connexin 43 mRNA expression and protein production [6, 16]. We observed similar results with respect to connexin 43 phosphorylation in the porcine ovary but over a longer time frame. Connexin 43 phosphorylation 12 h after hCG administration (84 h post-eCG) was not significantly altered from the 72-h post-eCG (no hCG) levels. However, by 24 h after hCG treatment (96 h post-eCG), there was a significant decline in the relative amount of phosphorylated connexin 43 in the porcine ovary when compared to the peak phosphorylation at 72 h and 84 h.

These differences in connexin 43 phosphorylation in response to eCG and hCG in vivo are in agreement with in vitro studies that show that cell-to-cell communication in the ovary can be reversibly regulated by the dynamic interplay between protein kinase A- and protein kinase C-mediated phosphorylation [16, 23]. These studies suggest that FSH enhances gap junctional activity, aiding follicular development, whereas LH depresses activity, allowing for resumption of oocyte maturation and dissociation of the follicle wall before ovulation.

The absence of connexin 43 staining in small and medium-sized antral follicles from prepubertal gilts before eCG treatment was unexpected for two reasons. First of all, gap junctions are detectable in rabbit follicles when the antrum develops [1] and have been immunolocalized in rat granulosa cells at all developmental stages, including primordial, preantral, and antral follicles [7]. Secondly, we detected connexin 43 by immunoblotting in ovarian homogenates from prepubertal gilts (Fig. 2). While a nonfolicular source of connexin 43 protein was considered, there are no reports of connexin 43 expression in ovarian vascular [36] tissue or the stroma [7, 8, 36] of the ovary during cyclic follicular development. These conflicting results may be due, in part, to differences in the amount of tissue analyzed and the sensitivity of detection between immunohistochemistry and immunoblotting. For example, tissue sections contain only a fraction of the antigen of interest when compared with the antigen content of homogenates prepared from whole tissue. In addition, for the immunoblotting studies we used an ECL detection system that amplifies the immunoreactive signal and enhances visualization of the antigen-antibody complex. The FITC detection system used for immunohistochemistry may not have been as sensitive as the ECL system used for immunoblotting in detecting low-level connexin 43 expression in small and medium-sized antral follicles in the prepubertal pig ovary.

In conclusion, these studies demonstrate that ovarian connexin 43 expression and phosphorylation change during the porcine estrous cycle. We also show that eCG stimulation increases ovarian connexin 43 protein phosphorylation in the pig, while exposure to hCG results in an attenuation of this effect. In addition, we localized connexin 43 immunoreactivity to the granulosa cells of the developing follicle and demonstrated that gonadotropin stimulation enhances the intensity of immunoreactive gap junction plaques. Our findings suggest that, in the porcine ovary, eCG/hCG administration alters the phosphorylation status of existing junctions rather than inducing the production of new protein. In the pig, this increase in connexin 43 phosphorylation during the cycle and in response to exogenous gonadotropins may be important in enhancing cell-to-cell communication for coordination of follicular development.

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