Restoration of human chorionic gonadotropin response in human myometrial smooth muscle cells by treatment with follicle-stimulating hormone (FSH): evidence for the presence of FSH receptors in human myometrium

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Human myometrial smooth muscle cells contain receptors for human chorionic gonadotropin (hCG)/luteinizing hormone (LH). Exogenous hCG and LH can cause a modest hyperplasia in myometrial smooth muscle cells in culture. This response is lost after about the third subculture of the cells. The present study investigated whether the loss of hCG response could be restored by co-culturing with human follicle stimulating hormone (FSH). The results showed that co-culturing with FSH can indeed restore a modest mitogenic response of hCG. However, FSH alone was not mitogenic. The FSH restoration of hCG response can be blocked by antibodies to FSH or hCG but not by non-specific rabbit IgG. The FSH treatment resulted in an increase of steady state levels of hCG/LH receptor mRNA and protein in myometrial smooth muscle cells. Since the FSH actions could be receptor mediated, we investigated the presence of FSH receptor mRNA transcripts and protein in freshly dispersed myometrial smooth muscle cells. Northern blotting demonstrated that myometrial smooth muscle cells, just as rat ovary, a classical target of FSH action, contain multiple FSH receptor mRNA transcripts. Western immunoblotting demonstrated that myometrial smooth muscle cells also contain a 60 kDa FSH receptor protein just as rat ovary and human granulosa cells used as positive control tissues. The immunocytochemistry also demonstrated that myometrial smooth muscle cells, as rat ovary and human granulosa cells, contain FSH receptor immunostaining. In summary, it is novel that FSH could restore the mitogenic response of hCG in human myometrial smooth muscle cells and these cells contain FSH receptors. These findings may have functional implications for direct regulation of human myometrium not only by hCG/LH but also by FSH.

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Human chorionic gonadotropin (HCG) and possibly the other members of this glycoprotein hormone family, belong to a structural superfamily of cystine-knot growth factors (1). Like the growth factors in this family, hCG can exert non-mitogenic as well as mitogenic actions in gonadal and non-gonadal reproductive tissues (2–18). hCG actions are mediated by transmembrane glycoprotein receptors which belong to the family of G-protein coupled receptors (19). The members of this family contain an extracellular ligand binding domain, seven transmembrane spanning regions and a cytoplasmic tail (19).

Our laboratory has recently shown that human myometrium and its blood vessels express human chorionic gonadotropin (hCG)/luteinizing hormone (LH) receptor gene (6, 8, 20, 21). The myometrial receptors increase from proliferative to secretory phase and decrease during postmenopause, suggesting that they are regulated by reproductive hormones (20, 21). The myometrial receptors are also present during pregnancy, with lower levels during labor compared with not in labor in preterm or term pregnancies (22).

The myometrial hCG/LH receptors are functional as treatment with exogenous hCG can modestly increase cell numbers and hypertrophy in a subpopulation of cells (6). In addition, hCG can inhibit oxytocin stimulated myometrial contractions, perhaps by down-regulating myometrial gap junctions (15, 16). The actions of hCG are time, concentration dependent, hormone and tissue specific and mediated by protein kinase A signaling (6, 15, 16).

The mitogenic action of hCG in human myometrial smooth muscle cells is lost after about the third

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subculture of the cells (6). This loss is selective for hCG as EGF continues to be mitogenic in these cells (23). Not knowing the molecular basis of the loss or what could restore the hCG action, we decided to test FSH, as an analogy exists in ovarian Graafian follicles (24–27).

Materials and methods

Highly purified hCG (CR-123, 12780 IU/mg) human FSH (AFP-8792-B = 1683 IU/mg; AFP-4822-B = 3100 IU/mg) and rabbit antisera to human FSH (anti-FSH-6) were obtained from the National Hormone and Pituitary Program supported NICHD, NIH, Rockville, MD. Rat FSH receptor plasmid, pFSH-A-B, was obtained from Drs. M. Griswold and S. Sylvester at Washington State University (Pullman, WA). It is a 729 bp Acc-I/Bam HI fragment within an exon of the genomic clone 7.111, which was subcloned into PGEM 4Z (28). Porcine LH/hCG receptor cDNA in pBluescript (SK+) was obtained from Dr. H. Loosfelt, Hôpital Bicêtre, Kremlin Bicêtre, France (19). Rabbit polyclonal antibody to synthetic N-terminus rat FSH receptor amino acid sequence of 9–30, which has no structural homology to LH or TSH receptors, and the corresponding N-terminus receptor peptide sequence of 9–30 were obtained from Drs. L. Reichert, Jr and B. Dattatreayamurthy at Albany Medical College (Albany, NY) (29). Rabbit polyclonal antibody to synthetic N-terminus rat LH receptor amino acid sequence of 15–38 and the corresponding receptor peptide were obtained from Dr P. Roche (Mayo Clinic, Rochester, MN). The commercial sources of other materials used in this study are the same as previously described (6, 8, 16, 20–23).

Myometrial tissue was obtained from premenopausal women undergoing hysterectomy at University of Louisville affiliated hospitals. The indications were uterine prolapse, fibroids, etc. The fibroid tissue, however, was not used in these studies. None of the patients had a history of hormonal treatment or any other uterine disease. The tissues were placed in ice-cold Hank’s balanced salt solution (pH 7.4) containing 2% HEPEs and 2% antibiotic-antimycotic solution and transported immediately to the laboratory. Cell dispersion, determination of cell purity, cell culture and cell counting are the same as previously described (6, 23). Since the question investigated in the present study was whether FSH treatment could restore hCG response, only the myometrial smooth muscle cells that lost hCG response were used after the sixth subculture. The cells at this stage are still morphologically and functionally (EGF responsive) similar to smooth muscle cells. Freshly dispersed myometrial smooth muscle cells were used for investigation on the expression of FSH receptor gene. Human granulosa cells collected from our in vitro fertilization program and ovaries collected from rats sacrificed for unrelated experiments were used for positive controls in immunocytochemistry and blotting experiments for FSH receptors.

The Northern blotting was performed as previously described using 20 to 25 μg total RNA (30). Briefly, total cellular RNA was isolated, separated on 3% agarose-formaldehyde gels and transferred to nitrocellulose membranes. Prehybridizations were performed for 4 h at either 50°C (FSH receptors) or 60°C (hCG/LH receptors). For the detection of hCG/LH receptor transcripts, the membranes were hybridized for 18 h at 65°C with 5 × 10⁵ cpm/ml of cDNA in 10 ml of diethyl pyrocarbonate (DEPC) treated water containing 50% formamide, 10% dextran, 0.58 g sodium chloride and 1% sodium dodecyl sulphate (SDS). The hCG/LH receptor cDNA was labeled with α-32P-dCTP by the random priming method. For the detection of FSH receptor transcripts, the membranes were hybridized for 18 h at 50°C with 5 × 10⁵ cpm/ml of cDNA in 10 ml of DEPC treated water containing 50% formamide, 5 × SSC (1 × SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), 5 × Denhardt’s solution and 1% SDS. The lower temperature of hybridization for FSH receptors as compared to hCG/LH receptors gave optimal results. The α-32P-CTP labeled antisense riboprobe was synthesized from rat FSH receptor DNA fragment with an exon (729 bp) using the Riboprobe Gemini System and SP6 RNA polymerase after linearizing the plasmid DNA by digestion with Accl. The washing conditions were the same for both the receptors. Briefly, the hybridized membranes were washed twice with 1 × SSC containing 0.1% SDS for 1 h at 60°C; twice with 0.1 × SSC containing 0.1% SDS for 30 min at 60°C; and twice with 0.1 × SSC alone for 20 min at 60°C. The washed blots were exposed to Kodak X-ray film with intensifying screens for 1–3 days at −80°C. The molecular sizes of the transcripts were determined by running an RNA ladder in an adjacent lane.

The immunocytochemistry was performed using an avidin-biotin immunoperoxidase method (20). The dilution of 1 : 300 for hCG/LH receptor antibody and 1 : 30 for FSH receptor antibody was used. The controls consisted of (a) preabsorption of the receptor antibodies overnight at 4°C with 40 μg corresponding receptor peptides, (b) omission of unabsorbed primary antibodies during the immunostaining procedure, and (c) substitution of the unabsorbed primary antibodies with non-specific IgG.

The Western immunoblotting was performed as previously described (31, 32). Briefly, the tissues were homogenized in 10 mM phosphate buffered saline, pH 7.4 containing 5 mM N-ethylmaleimide and 0.2 mM phenylmethylsulfonyl fluoride. The protein content was determined by Bradford’s method using a commercial kit (33). Aliquots of 30 μg protein were dissolved in 2 × loading buffer consisting of 125 mM Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 0.2% bromophenol dye. The samples were heated at 100°C for 5 min before electrophoresis. The proteins were separated by electrophoresis on SDS–polyacrylamide gel, transferred to nitrocellulose membranes, and detected using Enhanced Chemiluminescence (Amersham) and X-ray film. A rat placenta placental membrane, containing 32P-labeled rabbit α-FSH receptor cDNA, was used for hybridization.
blue. Then, the proteins were separated on a discontinuous 10% SDS–polyacrylamide gel electrophoresis under reducing conditions and electroblotted to Immobilon P membranes. The FSH receptors were detected by using 1 : 600 dilution of the FSH receptor antibody, 1 : 1000 dilution of horseradish peroxidase labeled second antibody and enhanced chemiluminescence detection system. The receptor antibody preabsorbed overnight at 4 °C with receptor peptide was used for control. The molecular size of the receptor protein was determined by running standard molecular weight marker proteins in an adjacent lane.

All experiments were repeated at least three times on cells from different tissue specimens. The results, which are expressed as $10^3$ cells/cm$^2$ unit in Figs 1 and 2, are the means and their standard errors. The number of data points used in the calculation of these means ranged from 6 to 10.

Results

Figure 1 shows that culturing with 30 nM hCG alone had no effect on myometrial smooth muscle cell density. Culturing also with 30 nM FSH resulted in a modest and significant increase in cell density ($p < 0.01$). However, culturing with FSH alone had no effect.

The FSH restoration of mitogenic response of hCG was further investigated by using antibodies (Fig. 2). As in Fig. 1, neither 30 nM hCG nor 30 nM FSH alone had any effect on myometrial smooth muscle cell density. However, culture with both hormones together resulted in a significant increase in cell density ($p < 0.01$). This increase was blocked by simultaneous addition of either antibody to hCG or antibody to FSH ($p < 0.01$) but not non-specific anti-rabbit IgG (Fig. 2). The hCG or FSH antibodies alone had no effect.

Since FSH restoration of hCG response could possibly be due to an increase in hCG/LH receptor levels, we investigated hCG/LH receptor protein in myometrial smooth muscle cells cultured with or without 30 nM FSH. Figure 3 shows that FSH treatment resulted in an increase of immunostaining for hCG/LH receptors in myometrial smooth muscle cells (Fig. 3b vs 3a). The receptor immunostaining is specific, as it is absent when the receptor antibody was preabsorbed with excess receptor peptide (Fig. 3c).
Fig. 3. Immunocytochemistry for hCG/LH (a to c) and FSH (d to h) receptors. Panel (a) is myometrial smooth muscle cells cultured for 9 days without FSH; panel (b) is myometrial smooth muscle cells cultured for 9 days with 30 nM FSH; panel (c) is immunostaining control for hCG/LH receptors in myometrial smooth muscle cells; panel (d) is human myometrial smooth muscle cells immunostained for FSH receptors; panel (e) is FSH receptor immunostaining control for human myometrial smooth muscle cells; panel (f) is rat ovary immunostained for FSH receptors; panel (g) is FSH receptor immunostaining control for rat ovary and panel (h) is human granulosa cells immunostained for FSH receptors. Mag. 300×.
transcripts just as rat ovary used as a positive control tissue. Among these transcripts, 2.5 and 5.5 kb receptor transcripts are major in both the tissues.

Next we investigated the presence of FSH receptor protein in myometrial smooth muscle cells by Western immunoblotting. Figure 5 shows that two preparations of myometrial smooth muscle cells (M1 and M2) contained a 60 kDa FSH receptor protein, just as rat ovary (O). The same receptor antibody immunoreacted with a slightly lower molecular size FSH receptor protein in human granulosa cells (G). The receptor protein was not detected in myometrial smooth muscle cells when the receptor antibody was preabsorbed with the receptor peptide (C).

Immunocytochemistry for FSH receptors in myometrial smooth muscle cells showed that myometrial smooth muscle cells immunostain for FSH receptors (Fig. 3d), just as rat ovary (Fig. 3f) and human granulosa cells (Fig. 3h). The FSH receptor immunostaining is specific, as it is absent when the receptor antibody was preabsorbed with the excess receptor peptide (Fig. 3e and 3g).

Discussion

Contrary to one of the conventional beliefs in reproductive endocrinology, numerous recent studies have shown that hCG/LH can directly regulate the functions of several non-gonadal reproductive tissues, including human myometrium (5–16, 18, 34, 35). In human myometrium, for example, exogenous hCG/LH can directly inhibit oxytocin stimulated myometrial contractions (15), down-regulate gap junctions (16), cause hypertrophy in a subpopulation of cells and increase the growth of myometrial smooth muscle cells in culture (6). The last response was not seen in every preparation of non-pregnant myometrial smooth muscle cells; and, when seen, it was lost after about the third subculture (6). Not knowing the physiological significance or the reasons for the loss of hCG response and what could possibly restore it, we decided to try FSH treatment as it regulates the expression and action of LH/hCG receptors in ovarian Graafian follicles (24–27).

To test this hypothesis, we dispersed myometrial smooth muscle cells, cultured and tested for hCG response before the third subculture. Only when the cells showed hCG response did the culturing continue until about the sixth subculture. These cells, which could not respond to hCG, began to respond when they were also cultured with FSH. Culturing with FSH alone or preculturing with FSH had no effect.

The FSH restoration of hCG response is blocked by antibody to hCG which would bind hCG making it unavailable to the cells. The restoration is also blocked by antibody to FSH, which supports the notion that FSH, but not from some contaminant that might be present in FSH preparations, was responsible for the
restoration. We also found that pure FSH used in clinical reproductive medicine, i.e. Metrodin, (Serono Labs, Randolph, MA), also restores hCG response. The hCG and FSH antibodies alone had no effect and their effect with hCG and FSH are specific because non-specific rabbit IgG could not mimic the actions of antibodies to hCG or FSH.

FSH treatment resulted in an increase of hCG/LH receptor protein in myometrial smooth muscle cells. This suggests that restoration of hCG actions by FSH may involve increasing hCG/LH receptor levels. However, the finding that control myometrial smooth muscle cells, which already contain some hCG/LH receptors, cannot respond to hCG unless FSH was present, suggests that FSH may have an additional effect of promoting post-receptor mechanisms.

The FSH action, as the action of other hormones, most likely requires its receptors. The recent cloning of FSH receptor gene (28, 36, 37) and the availability of specific antibodies raised against a synthetic N-terminus of rat FSH receptor sequence (29, 38) have allowed us to probe for the presence of FSH receptors in human myometrial smooth muscle cells. Freshly dispersed rather than subcultured cells were used because of their availability. We figured that if FSH receptors are present in freshly dispersed cells, they should also be present in subcultured cells since they responded to FSH treatment. The results showed that myometrial smooth muscle cells contain FSH receptor mRNA transcripts and receptor protein, just as the rat ovary and human granulosa cells, classical targets of FSH action. The specific FSH receptor immunostaining is also present in myometrial smooth muscle cells, just as in rat ovary and human granulosa cells.

We have not studied the kinetics of loss or restoration of hCG response by FSH. The physiological significance of loss of hCG response or the presence of FSH receptors in myometrial smooth muscle cells is not known. Therefore, the present data should be considered as seminal to further studies investigating these issues in direct gonadotropin regulation of human myometrial smooth muscle cells.

In summary, human myometrial smooth muscle cells contain hCG/LH receptors and respond to exogenous hCG by an increase in cell number. This hCG response is lost after about the third subculture and the loss can be restored by also culturing with FSH. The FSH treatment up-regulates hCG/LH receptor protein levels. Human myometrial smooth muscle cells express FSH receptor mRNA and protein and FSH restoration of hCG response may be mediated by these receptors.

Acknowledgments. We thank numerous Ob/Gyn physicians, pathologists, and other staff at the University of Louisville affiliated hospitals for their cooperation in collecting tissue specimens. Dr Gabor Ambrus for providing human myometrial smooth muscle cells for some of the experiments and Dr Alberto Carrillo for providing human granulosa cells. This work was supported by NIH grant HD-26173.

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Received July 4th, 1995
Accepted October 3rd, 1995

Restoration of hCG action by FSH in human myometrium