Cytodifferentiation of Granulosa Cells Induced by Gonadotropin-Releasing Hormone Promotes Fibronectin Secretion

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ABSTRACT. An analysis of the hormonal regulation of fibronectin production by granulosa cells showed that GnRH stimulated fibronectin secretion. GnRH increased fibronectin production 2.5- to 5-fold over that of control untreated cultures and greater than 20-fold over that of FSH-treated cultures. The GnRH concentration required for a minimal response was $10^{-10}$ M, and that required for a maximal response was $10^{-8}$ M. In contrast to the effects of GnRH, FSH suppressed fibronectin production to low or undetectable levels. In addition, FSH ablated the actions of GnRH on fibronectin production. Treatment of cultured granulosa cells with either (Bu)2cAMP or methylyisobutyryl xanthine to influence cellular cAMP levels mimicked the actions of FSH on fibronectin secretion. Similar to FSH, both (Bu)2cAMP and methylyisobutyryl xanthine ablated the actions of GnRH on fibronectin production. These results indicated that an increase in cAMP levels resulted in suppression of fibronectin secretion by granulosa cells and inhibition of the actions of GnRH. Insulin treatment of granulosa cells also suppressed fibronectin secretion, but to a lesser extent than FSH. In addition, insulin inhibited the actions of GnRH on fibronectin production by approximately 40-50% of stimulated levels. GnRH promoted a state of cytodifferentiation of the granulosa cell which had a high level of fibronectin production and, as shown previously, a low level of steroidogenesis. In contrast, FSH promoted a state of cytodifferentiation which had a low level of fibronectin production and a high level of steroidogenesis. Both fibronectin and steroidogenic enzymes (e.g. aromatase) provide useful markers for an analysis of the cytodifferentiation of granulosa cells between these two distinct differentiated states. Results are discussed in relation to the possible mechanisms controlling granulosa cell cytodifferentiation and the possible functions of fibronectin in the ovary. (Endocrinology 118: 2065–2071, 1986)

The granulosa cell plays an integral role in the maintenance and control of folliculogenesis and oocyte maturation. During the development of the ovarian follicle, the granulosa cell proliferates and undergoes cytodifferentiation to become a steroidogenic cell responsible for the production of estrogen and the support of the maturing oocyte. The follicle can become atretic at several stages of development, which results in an alteration in the cytodifferentiation of the granulosa cells. After ovulation, the granulosa cell continues to differentiate to become a luteal cell. Clearly, the granulosa cell is an actively differentiating cell type which has several distinct differentiated states. Cytodifferentiation is defined here as the transition that occurs when a cell changes from one differentiated state with a set of specific cellular functions, e.g. steroidogenesis for granulosa cells, to a second differentiated state with a different set of cellular functions. This definition applies when the cellular function(s) being examined is not normally being continually regulated (i.e. induced and inhibited). Granulosa cell steroidogenesis (i.e. estrogen biosynthesis) appears to be induced and maintained until the follicle undergoes atresia or ovulation.

FSH promotes steroidogenesis and maturation of the granulosa cell through the apparent actions of cAMP (1). Other factors, including PRL (2), insulin (3), testosterone (4), and estrogen (5), are able to modulate these actions of FSH. Although several substances promote FSH-induced granulosa cell differentiation to a steroidogenic state, one substance that inhibits this action of FSH is GnRH. In vitro, GnRH inhibits FSH-stimulated estrogen and progesterone production (6) through an apparent effect on steroidogenic enzymes (7). GnRH also inhibits FSH-stimulated LH or PRL receptor formation in cultured granulosa cells (8). Although GnRH inhibits the actions of FSH on granulosa cells, GnRH alone has a slight stimulatory effect on estrogen and progesterone production (9), protein synthesis (10), lactate formation (11), prostaglandin production (12), and plasminogen activator activity (13). It is apparent that GnRH pro-

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motes the granulosa cell to differentiate to a state quite different from that promoted by FSH.

Recently, it was demonstrated that granulosa cells synthesize and secrete fibronectin (14). FSH treatment of cultured granulosa cells inhibited the production of fibronectin, which was produced in high amounts in untreated cultures. Quantitation of fibronectin production indicated that FSH and insulin promoted steroidogenesis through an increase in aromatase activity, but suppressed fibronectin production. In addition, while aromatase activity in control untreated cultures decreased, fibronectin production increased (15). It was, therefore, proposed that fibronectin production provides a marker for a differentiated state of the granulosa cell which is distinct from that promoted by FSH (14, 15).

The combined observations that GnRH promotes a differentiated state of the granulosa cell distinct from that promoted by FSH and that fibronectin is a marker for granulosa cell cytodifferentiation raised the question as to the possible influence GnRH may have on fibronectin production. The present study examines the effects of GnRH on fibronectin production by granulosa cells and compares these effects to those of FSH and insulin.

Materials and Methods

Immature Wistar Cr1:(W1)BR rats were obtained from Charles River Canada (Montreal, Canada) and maintained with their mothers under conditions of controlled light and temperature. Female 21-day-old rats were treated daily for 4 days with 1 mg diethylstilbestrol (DES) in 0.1 ml sesame oil by sc injection. DES acts as a granulosa cell mitogen to increase the yield of cells. Ovine FSH (NIADDK oFSH-15; 20 × NIH FSH S1 by the hCG augmentation bioassay of Steelman-Pohley) was provided by the Pituitary Hormone Distribution Program, NIAMDD. Bovine insulin, human fibronectin, and (Bu)3cAMP were purchased from Sigma Chemical Co. (St. Louis, MO), and rat fibronectin and specific antisera were obtained from Calbiochem (La Jolla, CA). The GnRH agonist [d-Ser-(Bu)6, des-Gly-NH2]GnRH ethylamide (GnRH) was kindly provided by Dr. F. Labrie, Le Centre Hospitalier de l'Université Laval (Quebec, Canada).

Preparation of cell cultures

Animals were killed at 25 days of age, and granulosa cells were recovered from the ovaries by puncturing the follicles with a fine needle, as described previously (16). Cells were plated into 24-multiwell Falcon tissue culture plates (Falcon Plastics, Los Angeles, CA) as 1.0-ml aliquots in Eagle's Minimum Essential Medium with Earle's salts and 0.1 mM of the following amino acid supplements: L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine. The medium also contained 4 mM glutamine, 2.5 g/liter NaHCO3, 1.5 mM HEPES, and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 0.6 μg/ml Fungizone). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air to maintain the medium at pH 7.4. The cells were allowed to attach to the surface of the plate for 3 h before the addition of hormones. Unless otherwise specified, the hormone concentration used was 100 ng/ml for FSH, 5 μg/ml for insulin, and 10−7 M for the GnRH agonist. After 24 and 48 h in culture, the medium was removed for the assay of fibronectin content, and the treatments were repeated. The experiments were terminated after 72 h in culture, the medium was removed, and 500 μl 1% sodium dodecyl sulfate containing 1 mM EDTA was added to remove the cell monolayer. After sonication of the cell extract, the protein content was measured by the method of Lowry et al. (17).

Assay for fibronectin

Fibronectin was measured by a competitive enzyme-linked immunoadsorbant assay (ELISA), as previously described (15). Immulon 2 microtiter plates (Dynatech Labs, Alexandria, VA) were coated overnight at 4°C with 200 ng human fibronectin/well in 100 μl 20 mM Na2CO3 buffer, pH 9.6. The standard curve for human fibronectin (0–1000 ng) was set up in triplicate in 100 μl 0.05% (vol/vol) Tween-20 in 100 mM NaH2PO4 containing 0.85% NaCl, pH 7.4 (Tween-PBS), and incubated overnight at 4°C in plastic tubes with 100 μl Tween-PBS containing the goat antiserum raised against rat fibronectin. The final dilution of the antiserum was 1:20,000. Duplicate samples of the medium from the granulosa cell cultures were preincubated as described above. The remainder of the assay was carried out at room temperature. The coating antigen solution was removed from the plate, and the wells were washed once with PBS and then three times with Tween-PBS. The last wash was left on the plates for 5 min. The coated plates were incubated with 200 μl preincubated antigen-antibody mixture/well for 30 min, then washed three times with Tween-PBS. The last wash was left on the plates for 5 min. The coated plates were incubated with 200 μl preincubated antigen-antibody mixture/well for 30 min, then washed three times with Tween-PBS. The last wash was left on the plates for 5 min. The coated plates were incubated with 200 μl preincubated antigen-antibody mixture/well for 30 min, then washed three times with Tween-PBS. Alkaline phosphatase conjugated to goat antimmunglobulin G raised in rabbits was freshly diluted to 1:500 with Tween-PBS, and 100 μl were added to each well. This was incubated for 45 min, and plates were washed three times with Tween-PBS. Plates were then washed once with diethanolamine buffer (5 mM MgCl2 and 10% diethanolamine in water, pH 9.8, stored in the dark). Each well was incubated with 120 μl freshly prepared 4-nitrophenyl disodium orthophosphate (British Drug Houses, Toronto, Canada) and 1 mg/ml diethanolamine buffer. Incubation time averaged 1 h, but varied according to the ambient temperature of the laboratory. Plates were read on a micro-ELISA densitometer (Titertek, Multiskan, Flow Laboratories, Inc., Canada) using a 405-nm filter. The sensitivity of the ELISA assay was 1–5 ng fibronectin. The assay was linear from 5–100 ng, with an interassay coefficient of variation of 5% and an intraassay variation of 10%. Components in culture medium and the hormones used in the study did not cross-react with the fibronectin antibody. As previously reported, the same immunoreactivity was found for rat fibronectin, human fibronectin, and rat granulosa cell fibronectin, as determined with parallel displacement curves in the ELISA (15). Data presented are normalized per mg cell protein and are essentially the same if normalized per μg cell DNA.
Gel electrophoresis

After culturing the cells for 48 h, the monolayer, containing approximately 10⁷ cells, was washed with methionine-free Minimum Essential Medium, and 0.5 ml of the same medium containing 10 μCi [³⁵S]methionine was added to each culture well. After 24 h of incubation, the medium was removed, phenylmethylsulfonyl fluoride was added, and the medium was centrifuged to remove cell debris and frozen. The cell monolayer was washed with 100 μl Dulbecco’s PBS, which was discarded. Sodium dodecyl sulfate (SDS) sample buffer was added to each culture well to remove the cells, which were then frozen. Electrophoresis was performed on 5–15% polyacrylamide gradient slab gels using the Laemmli SDS buffer system (18). All samples were reduced with β-mercaptoethanol before electrophoresis. Gels were fluorographed using diphenylhexazol in acetic acid, as previously described (19).

Statistical analysis

Observations were confirmed in three or more independent experiments, and the same relative effects were observed in each, with minimal variability. A maximum 10% variation was observed in baseline levels. Therefore, all results are presented as the mean ± SE of determinations of cells from three replicate cultures within each treatment group and are derived from a single representative experiment. Mean values were compared using Student’s t test.

Results

Effects of FSH and GnRH on the electrophoretic profiles of secreted radiolabeled proteins

The [³⁵S]methionine-labeled proteins secreted by granulosa cells during the culture period from 48–72 h were electrophoretically analyzed on SDS-polyacrylamide gradient slab gels. The electrophoretic profiles of the radiolabeled proteins secreted by cells under control conditions or when treated with either FSH or GnRH alone or with FSH and GnRH together are shown in Fig. 1. As shown previously, immature granulosa cells cultured under control conditions secreted a major radiolabeled protein with a molecular mass of 220,000 daltons. This protein was identified as fibronectin by immunoprecipitation with a specific antibody and by its ability to displace human fibronectin from antifibronectin antibodies in an ELISA assay (14, 15). In addition, this was the only protein secreted by rat granulosa cells that could be immunoprecipitated with rat serum fibronectin antibody, and the subunit structure was the same as that of serum fibronectin (14). In FSH-treated cultures the intensity of the radiolabeled protein with a mol wt of 220,000 was reduced to a barely detectable level (Fig. 1). In contrast, GnRH alone appeared to increase the intensity of the same protein to an extent even greater than that in control cultures. In the presence of FSH, however, the stimulatory effect of GnRH was abolished, and the

intensity of the radiolabeled 220,000-dalton protein was low. As previously demonstrated, the amount of fibronectin associated with the cellular fraction, including deposited extracellular matrix, was minimal and did not appear to be altered by hormone treatment (14). Similar observations have been found with GnRH-treated granulosa cells (data not shown). Therefore, quantitation of soluble fibronectin after different hormone treatments of granulosa cells does not appear to be affected by or a result of altered deposition.

Levels of fibronectin secreted by FSH and GnRH-treated cultures

To confirm the impression obtained from the electrophoretic profiles that GnRH enhanced the secretion of fibronectin, granulosa cells were cultured with FSH and
GnRH alone and in combination for 72 h, and the fibronectin secreted into the culture medium was quantitated using the ELISA assay. In the presence of GnRH, the amount of fibronectin secreted was greater than that produced by control cultures, as suggested by the electrophoretic patterns of secreted proteins shown in Fig. 1. In contrast, treatment with FSH from the time of plating inhibited the increase in fibronectin secretion observed in the control cultures. FSH was able to suppress GnRH-induced fibronectin secretion to a level approaching that found in FSH-treated cultures (Fig. 2).

The ability of GnRH to stimulate fibronectin secretion above control values was dose dependent; $10^{-10}$ M GnRH caused a significant increase, whereas $10^{-8}$ M GnRH was required to produce a maximal effect (Fig. 3). To demonstrate the specificity of the GnRH effect, a GnRH antagonist and GnRH agonist were simultaneously added to granulosa cell cultures. [AC-$\Delta^3$-Pro$^1$-$\beta$-Phe$^2$-$\beta$-Trp$^3$-$\alpha$]-GnRH has been previously shown to be a potent antagonist of GnRH (20). When this GnRH antagonist ($10^{-6}$ M) and the GnRH agonist ($10^{-7}$ M) were added to the same granulosa cell cultures, fibronectin production between 24 and 48 h of culture was 12 ± 2 µg fibronectin/mg cell protein. This was compared to fibronectin production in control (8.8 ± 0.6 µg/mg cell protein), GnRH agonist ($10^{-7}$ M)-treated (23 ± 3 µg/mg cell protein), and GnRH antagonist ($10^{-6}$ M)-treated (9.5 ± 0.4 µg fibronectin/mg cell protein) granulosa cell cultures. The GnRH antagonist reduced the actions of the GnRH agonist by more than 80% at the concentrations reported. If a lower concentration of agonist was used, the actions of the GnRH agonist were completely abolished by the antagonist (data not shown). These data indicate that the GnRH antagonist can block the actions of the GnRH agonist to stimulate fibronectin production.

The action of FSH was most likely due to its ability to increase the intracellular concentration of cAMP. Treatment of the cells with (Bu)$_3$cAMP or methylisobutylxanthine (MIX), which can influence cAMP levels by inhibiting phosphodiesterase activity, elicited an effect similar to that of FSH (Table 1). As shown in Fig. 1, the addition of FSH inhibited the actions of GnRH on fibronectin secretion by granulosa cells. This effect could also be obtained by treatment of granulosa cells with GnRH and either MIX or (Bu)$_3$cAMP (Table 1). These results indicate that agents that maintain or increase cAMP levels can suppress fibronectin secretion and inhibit the actions of GnRH.

As a comparison to FSH actions, the effects of insulin on GnRH-induced fibronectin production were examined. Insulin also promotes granulosa cell differentiation and concomitantly inhibits fibronectin secretion (Fig. 4), even though this hormone does not directly act by increasing cAMP levels. Nevertheless, insulin was able to suppress the action of GnRH on fibronectin secretion.
TABLE 1. The ability of (Bu)$_3$AMP and MIX to suppress GnRH-stimulated fibronectin secretion by rat granulosa cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibronectin secretion (μg/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14.3 ± 1.2</td>
</tr>
<tr>
<td>GnRH</td>
<td>76.0 ± 2.8</td>
</tr>
<tr>
<td>(Bu)$_3$AMP</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>GnRH + (Bu)$_3$AMP</td>
<td>10.1 ± 1.0</td>
</tr>
<tr>
<td>MIX</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>GnRH + MIX</td>
<td>7.1 ± 1.3</td>
</tr>
</tbody>
</table>

Granulosa cells isolated from 25-day-old DES-primed rats were cultured for 48 h with GnRH (10$^{-7}$ M), (Bu)$_3$AMP (1.0 mM), or MIX (0.2 mM). Fibronectin was assayed in the culture medium collected at 24-h intervals, and the data were expressed as the total fibronectin secreted by 1 mg cell protein in the monolayer over the 48-h culture period. Values are the mean ± SE (n = 4).

Discussion

GnRH was found to stimulate significantly the production of fibronectin by cultured granulosa cells. Electrophoretic analysis demonstrated that after GnRH treatment of granulosa cells, fibronectin was a major secretory product. Quantitation of the influence of GnRH revealed a 2.5- to 5-fold stimulation of fibronectin production compared to that of control untreated cultures and a greater than 20-fold stimulation compared to that of FSH-treated granulosa cell cultures. The effects observed for GnRH were with an analog of GnRH previously shown to be a potent agonist (21). An antagonist of GnRH was found to block the actions of this potent agonist, thus indicating the specificity of the GnRH effects observed. The concentrations of the GnRH agonist required to stimulate fibronectin production were between 10$^{-10}$ and 10$^{-8}$ M, similar to those required for GnRH actions on other cell types (6). In contrast, treatment of granulosa cells with FSH suppressed fibronectin secretion (14, 15). Previous studies have shown that GnRH alone can stimulate the synthesis of progestational steroids and estrogen, but the degree of stimulation is much less than that achieved with FSH (9). In addition, when GnRH and FSH were used simultaneously to treat granulosa cells, GnRH inhibited FSH stimulation of aromatase by 50% and cholesterol side-chain cleavage activity by 25% (9). In the present study, GnRH inhibits FSH suppression of fibronectin secretion by approximately 60%. Because FSH suppresses fibronectin secretion and GnRH stimulates fibronectin secretion, the inhibitory effects of FSH on GnRH stimulation are much greater. It appears that the degree to which GnRH inhibits FSH actions is dependent on the granulosa cell product being examined. In all cases, the effects of simultaneous treatment with GnRH and FSH is between that of GnRH alone and that of FSH alone. In the present study it is thought that the actions of GnRH plus FSH promote a transitional state of differentiation which is between that promoted by either FSH or GnRH alone. Whether such a transitional state may have an important physiological role is at present unknown. Therefore, it is proposed that the differentiated state of the granulosa cell induced by GnRH is characterized by a high level of fibronectin production and a slightly elevated level of steroidogenesis and is distinct from the differentiated state of cells that have a high level of steroidogenic activity and a low level of fibronectin production. High levels of fibronectin secretion thus provide a useful marker for the differentiated state of granulosa cells promoted by GnRH, and low levels provide a marker.

[Graph: Cumulative secretion (72 h) of fibronectin from control and FSH- and insulin-treated granulosa cells cultured in the absence (□) or presence (○) of GnRH. Data are expressed as micrograms of fibronectin secreted per mg granulosa cell protein/72 h of culture and represent the mean ± SE for three replicate cultures.]
for the differentiated state promoted by FSH.

To develop a better understanding of granulosa cell cytodifferentiation, experiments were performed to initiate an analysis of the mechanisms controlling the differentiation of the granulosa cell between these two distinct differentiated states. FSH promotes a steroidogenic state of the granulosa cell and has previously been shown to act by increasing cAMP levels within the cell, which stimulates protein phosphorylation (22). Since FSH abolished the actions of GnRH on fibronectin production, the effect of altering cellular cAMP levels on GnRH actions was examined. Both (Bu)2cAMP and MIX, a phosphodiesterase inhibitor, influence cellular cAMP levels and can mimic or influence the actions of FSH on aromatase activity (22) and fibronectin production. Both MIX and (Bu)2cAMP when added with GnRH abolished the actions of GnRH on fibronectin production. These results imply that an increase in cellular cAMP levels will suppress fibronectin production and stimulate steroidogenesis. It appears that one of GnRH effects on granulosa cells is through an alteration in cellular cAMP levels. This is supported by previous observations of the effects of GnRH on cAMP levels and cAMP-binding protein levels in granulosa cells (9, 23). These studies demonstrate that GnRH can induce a small transient rise in cAMP initially, but that the overall or long term effects of GnRH involve increased cAMP catabolism. MIX inhibition of GnRH actions supports this proposal and indicates the involvement of phosphodiesterase. Insulin was also found to suppress fibronectin production and reduce the stimulatory effects of GnRH. Although the mechanism of action of insulin is not completely understood, clearly a modulation in cAMP levels is not the direct mechanism by which insulin acts. A long term effect of high concentrations of insulin to increase cAMP levels in granulosa cells may be possible. Cross-reactivity with an insulin-like growth factor receptor may also be possible. Whether this or some other more direct action of insulin was responsible for the insulin effects observed on fibronectin secretion remains to be examined.

The mechanism by which GnRH influences granulosa cells has been studied by a number of investigators (1, 6, 9). One study indicated that modulation of phosphodiesterase activity and inhibition of adenylate cyclase activity may play a role (23). The present study supports the observation that modulation of cAMP levels can result in profound effects on granulosa cells. An additional mechanism may be the control of phosphatidylinositol metabolism (24). Further investigation of the mechanism by which GnRH and FSH promote these two distinct differentiated states of the granulosa cell will provide insight into the mechanisms controlling granulosa cell cytodifferentiation. In addition, a better understanding of the mechanism by which increased cAMP levels can suppress fibronectin production would provide insight into the control of fibronectin gene expression.

Specific effects of GnRH on granulosa cell steroidogenesis and ovarian function in vivo have been observed for a number of species (6). However, to demonstrate physiological significance of GnRH effects on ovarian function, a GnRH-like substance is postulated (25). Whether GnRH cross-reacts with a receptor for a GnRH-like ovarian substance remains to be examined. The present study demonstrates that regardless of the physiological ovarian role, GnRH will provide a useful substance to investigate granulosa cell cytodifferentiation.

As previously reported, fibronectin production by granulosa cells does not appear to be an artifact of cell culture (15). This is supported by the observations that fibronectin is a major secretory product and that production is independent of cell density; in addition, fibronectin has previously been shown to be present in the ovary (26). The function of fibronectin secretion by granulosa cells remains to be determined. It is proposed that fibronectin secretion may be high in the primordial follicle, the atretic follicle, and/or, possibly, the corpus luteum. The proposed function is to provide the proper extracellular environment for the granulosa cell, oocyte, and follicle. Previous studies have shown that cells that normally produce fibronectin have decreased levels of production during transdifferentiation and cell proliferation (27). Direct effects of fibronectin on cellular growth (28) and differentiation (29) have been demonstrated. Fibronectin has been shown to influence the growth of a granulosa cell line (30). Whether fibronectin has an indirect or direct effect on granulosa cell growth and/or cytodifferentiation remains to be determined. Any speculations and future studies will need to take into consideration the observations that granulosa cell proliferation and cytodifferentiation may be inversely related (31).

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GnRH-INDUCED FIBRONECTIN SECRETION
