Epidermal growth factor receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation

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Communicated by Jean D. Wilson, University of Texas Southwestern Medical Center, Dallas, TX, September 29, 2005 (received for review July 22, 2005)

Female fertility requires ordered follicular development and growth that ultimately leads to ovulation of mature oocytes in response to a midcycle luteinizing hormone (LH) surge. LH triggers multiple processes within the ovary that are all critical for normal ovulation, including steroidogenesis, cumulus-cell expansion, and oocyte maturation. Understanding the signaling pathways induced by LH in the ovary is therefore essential for appreciating how these activities relate to one another. Several pieces of evidence have implicated epidermal growth factor receptor (EGFR) signaling as a potential central pathway coordinating these important LH-mediated events.

First, EGF triggers steroidogenesis in gonadal cells, including Leydig cell lines (1, 2) and possibly granulosa cells (3). Whereas EGF promotes steroidogenesis in these models, an essential role for EGFR and the EGFR in regulating gonadotropin-induced steroidogenesis has not been established. Second, the EGFR regulates oocyte maturation (4, 5). Maturation refers to the meiotic progression of oocytes from prophase I to metaphase II that is required for normal ovulation and fertilization. LH triggers secretion of EGF molecules, which, in turn, signal in a paracrine fashion through the EGFR to stimulate cumulus-cell expansion and oocyte maturation. Notably, EGF cannot directly trigger maturation of denuded oocytes, suggesting that secondary messengers induced by EGFR signaling in the cumulus cells are required for meiotic progression.

Finally, in contrast to EGF, testosterone and estradiol can promote maturation of denuded mouse oocytes held in meiotic arrest in vitro (6). This process is transcription-independent and may be regulated by classical steroid receptors. Although steroids are established physiologic mediators of maturation in frogs and fish (7–10), their role in regulating mammalian-oocyte maturation is controversial. Furthermore, although steroids promote mouse-oocyte maturation in vitro, the physiologic relevance of steroid-mediated maturation in mammals has needed confirmation using models whereby oocytes are held in meiotic arrest by surrounding follicular cells.

These earlier studies raise the possibility that EGFR-mediated signaling may trigger steroidogenesis, which in turn contributes to oocyte maturation. In this study, we used several models to examine EGF-mediated steroidogenesis and maturation, including (i) cultures of oocyte–granulosa cell complexes (OGCs), which are isolated from preantral follicles in 12-day-old mice and grown to meiotic competence; (ii) meiotically competent oocyte–cumulus cell complexes (OCCs), which are isolated from 21- to 24-day-old mice and used immediately; and (iii) intact preovulatory follicles from mice primed with pregnant mare serum gonadotropin (PMSG). In each model, EGF promoted steroid production. In addition, gonadotropin-induced steroidogenesis was regulated in both ovarian follicles and Leydig cells by EGFR-mediated signaling, although the mechanisms activating these receptors appeared different in the two systems. Finally, EGFR signaling mediated sufficient steroid production to promote oocyte maturation in oocyte–granulosa cell models.

Materials and Methods

Steroid Production and Oocyte Maturation in Cultures of OGCs. OGCs were isolated (11) from 12-day-old C57BL/6J × SJL/J F1 mice (The Jackson Laboratory) by using Waymouth’s MB752/1 medium (Invitrogen). EGF-mediated steroid production was measured after 10 days of culture. OGCs were washed twice with Waymouth’s medium containing 3 mg/ml BSA and 5% FBS, followed by incubation with 20 ng/ml EGF (Becton Dickinson). OGCs were placed at 37°C with 5% O₂, 5% CO₂, and 90% N₂. Medium and cells were collected at 16 h, steroids were extracted (12), and RIAs were performed to detect steroids (MP Biomedicals, Irvine, CA). For maturation assays, OGCs were cultured for 10 days and then incubated at 37°C for 16 h with steroid or EGF at the indicated concentrations. Maturation was scored by denuding OGCs and examining oocytes for germinal-vesicle breakdown (GVBD). Results were verified by blinding individuals scoring for GVBD.

Conflict of interest statement: No conflicts declared.

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; GVBD, germinal vesicle breakdown; LH, luteinizing hormone; MMP, matrix metalloproteinase; OCC, oocyte–cumulus cell complex; OGC, oocyte–granulosa cell complex; PMSG, pregnant mare serum gonadotropin; StAR, steroidogenic acute regulatory.

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Steroid Production and Oocyte Maturation in OCCs. Ovaries from unprimed 21- to 24-day-old (C57BL/6J X SJL/J) F1 mice were placed into M2 medium (Chemicon) containing 200 μM 3-isobutyl-1-methylxanthine (IBMX, Calbiochem). Large follicles were punctured with 30-gauge needles, and OCCs were harvested by aspiration with pulled-glass Pasteur pipettes. OCCs were washed with M2 and allocated into four-well culture dishes containing M16/IBMX at 37°C. OCCs were pretreated with EGFR kinase inhibitor AG1478 (Calbiochem), steroid receptor antagonists, or vehicle for 30 min before and throughout the addition of stimulators. Concentrations of DMSO or ethanol were 0.1%. Maturation (GVBD) of denuded oocytes was scored at 8 h. Medium and cells were collected for steroid detection by RIA.

Steroid Production and Oocyte Maturation in Preovulatory Follicles. Twenty-one- to 28-day-old C57BL/6J mice (The Jackson Laboratory) were injected with 5 IU of PMSG (Sigma) into the peritoneum. Follicles were isolated 44–48 h after PMSG injection by vaginal cytology through a minimum of two estrous cycles.

Steroid Production and Oocyte Maturation in MA-10 Cells. The MA-10 mouse Leydig tumor cell line (provided by M. Ascoli, University of Iowa, Iowa City, IA) was grown in Waymouth’s MB752/1 medium supplemented with 15% horse serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin under 5% CO2. Cells were put in 24-well plates 48 h before the experiments. On the day of the study, the cells were washed with PBS and placed in serum-free medium plus 20 mM Hepes and 3 mg/ml BSA. The cells were pretreated with AG1478, Galardin (Calbiochem), or DMSO for 30 min before and throughout the addition of 3 μg/ml LH or 20 μM 22R-hydroxycholesterol. Medium was collected at 4 h, and steroids were extracted and measured by RIA.

Steroidogenic Acute Regulatory (STAR) Protein and CYP11A1 Immunoblotting. Fifteen OCCs were placed in M-16 medium with or without 20 ng/ml EGF and incubated at 37°C for 16 h. OCCs were collected and placed in 2X SDS buffer, pH 6.8, plus 10% β-mercaptoethanol. MA-10 cells were plated in six-well plates 24 h before treatment with 200 ng/ml EGF. After 4 h, 400 μl of 2X SDS buffer plus 10% β-mercaptoethanol was added and the cells sheared with 25-gauge needles. Lysates were separated by electrophoresis and proteins transferred to Immobilon-P membranes (Millipore). The membranes were probed with rabbit anti-STAR (provided by D. M. Stocco, Texas Tech University Health Sciences Center, Lubbock, TX) and rabbit anti-CYP11A1 (provided by B. C. Chung, Institute of Molecular Biology, Taipei, Taiwan) antibodies.

Ovarian Steroid Levels. Steroid levels were measured in 8-week-old C57BL/6 mice maintained on a 12/12 light/dark cycle and tracked by vaginal cytology through a minimum of two estrous cycles. Animals were killed 2 h before the start of the dark cycle, ovaries were removed, and steroids were extracted and measured by RIA.

Immunofluorescence. Immunofluorescence was performed as described in ref. 13. Denuded oocytes from 4-week-old C57BL/6 mice were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Primary antibody concentrations were 0.4 μg/ml for rabbit anti-progesterone receptor (C-19) and rabbit anti-androgen receptor (C-19) (Santa Cruz Biotechnology) and 2 μg/ml rabbit for the anti-estrogen receptor β (Affinity BioReagents, Golden, CO). For controls, the anti-androgen and -progesterone receptor antibodies were preincubated with their target peptides for 16 h. The secondary antibody anti-rabbit Alexa Fluor 488 (Molecular Probes) was used at 8 μg/ml. Images were visualized by using an Optiphot microscope (Nikon) with a UV light source and filter for fluorescein.

Steroid-Extraction Experiment. OCCs (∼150) were placed in M16 with 200 μM IBMX and 20 ng/ml EGF for 16 h at 37°C. Medium was collected, centrifuged at 0.8 relative centrifugal force to remove cells, and added to newly harvested OCCs in four conditions: (i) unmodified, (ii) treated with 20 μM AG1478, (iii) run through a Sep-Pak (Waters) cartridge to remove steroids, and (iv) both Sep-Pak and AG1478 treatment. Sep-Paks removed 95% of the steroid from the medium; however, they may also remove other hydrophobic molecules regulating oocyte maturation. Maturation (GVBD) was scored after 8 h.

Results

EGF Promotes Steroidogenesis in Oocyte–Granulosa Cell Cultures and OCCs by Signaling Through the EGFR. Steroid production in a growing oocyte/follicle cell model system was examined by using cultures of OGCs isolated from preantral follicles of 12-day-old mice (11). To determine whether EGF could stimulate steroid production, 10-day-old OGC cultures were incubated with 20 ng/ml EGF for 16 h. EGF increased progesterone, testosterone, and estradiol levels by 2-fold (absolute concentration ∼10 ng/ml), 3-fold (absolute concentration ∼6 ng/ml), and 2-fold (absolute concentration ∼4 ng/ml), respectively, relative to mock-treated cells (Fig. 14).

The results of tests using OGC cultures were confirmed by using OCCs. Whereas OCCs are cultured from preantral follicles for 10 days before the oocytes are meiotically competent, OCCs are taken from 21- to 24-day-old prepubertal mice and used immediately after isolation. Similar to the results with cultured OGCs, EGF increased progesterone production in freshly isolated OCCs by ∼8-fold (progesterone ∼800 pg/ml) relative to mock treated OCCs (progesterone ∼100 pg/ml) at 8 h. EGF-mediated steroid production required an active EGFR,
were seen in three experiments. (Fig. 2) EGFR signaling is necessary for gonadal LH-induced steroidogenesis. (A) LH is a more potent inducer of steroidogenesis than is EGF. (B) LH-induced steroid production is inhibited by blocking EGFR signaling and MMP activity. Fifteen follicles were incubated with DMSO or 20 ng/ml EGF with or without 20 μM AG1478 or Galardin. Medium progesterone content was measured as an average (± SD) of 10–12 animals. (A) Testosterone and estradiol levels. (B) Progesterone levels.

Normal LH-Mediated Steroidogenesis in MA-10 Leydig Cells Requires Activation of the EGFR but Not Membrane-Bound EGFRs. To determine whether EGFR signaling might be a universal mechanism for regulating gonadal steroidogenesis, we examined LH-induced steroidogenesis in the MA-10 mouse Leydig cell line. LH triggered a 10-fold increase in progesterone production at 4 h (Fig. 2E). Similar to ovarian follicles, LH-induced progesterone production in MA-10 Leydig cells in response to EGF (Fig. 1C). In contrast, neither StAR (Fig. 1C) nor CYP11A1 (Fig. 1D) expression were altered in EGF-treated OOCs relative to mock-treatment. This result suggests that EGF-induced steroidogenesis in OOCs may rely on posttranslational activating modifications of StAR and/or CYP11A1 rather than increased protein expression.

EGF-Induced Steroidogenesis in OOCs Does Not Require Changes in Steroidogenic Acute Regulatory Protein or CYP11A1 Expression. In some cells, EGF-induced steroidogenesis is regulated by increased StAR protein expression (1). StAR is the primary regulator of cholesterol transport into the mitochondria, where the steroid precursor is then converted by CYP11A1 (side-chain cleavage enzyme) to pregnenolone. To determine whether EGF increased StAR or CYP11A1 protein expression, freshly isolated OOCs were incubated with EGF for 16 h, and cell lysates were analyzed for StAR and CYP11A1 expression by Western blot. As predicted in ref. 1, StAR expression increased dramatically in MA-10 Leydig cells in response to EGF (Fig. 1C). In contrast, neither StAR (Fig. 1C) nor CYP11A1 (Fig. 1D) expression were altered in EGF-treated OOCs relative to mock-treatment. This result suggests that EGF-induced steroidogenesis in OOCs may rely on posttranslational activating modifications of StAR and/or CYP11A1 rather than increased protein expression.
production was abrogated by the EGFR kinase inhibitor AG1478; however, unlike follicles, gonadotropin-induced steroidogenesis in MA-10 cells was unaffected by the MMP inhibitor Galardin. Addition of 22R-hydroxycholesterol rescued the suppressive effects of AG1478 on LH-mediated progesterone production (Fig. 4E), confirming that, as in the ovary, EGFR-mediated steroid production in Leydig cells is regulated by increased StAR activity (1).

Steroids Promote Oocyte Maturation in Oocyte–Granulosa Cell Models. EGFR activation is required for oocyte maturation (4). Because the EGFR is also necessary for LH-induced steroidogenesis, and because steroids trigger maturation of denuded mouse oocytes (6), the regulation of EGFR-induced oocyte maturation might, therefore, involve steroids. To determine the steroids that might be important in vivo, ovarian steroid content in cycling mice was measured. The estrous cycles of 8-week-old female mice were tracked by vaginal cytology, ovaries were removed on the indicated days, and steroid content was measured by RIA. Levels of progesterone, testosterone, and estradiol all increased at ovulation and remained high throughout diestrus II (Fig. 3). Ovarian progesterone content was ~100-fold higher than that of testosterone and estradiol; thus, focus was placed on progesterone as a potential physiologic mediator of oocyte maturation.

In cultured OGCs, where oocytes are held in meiotic arrest by the surrounding granulosa cells, both progesterone and testosterone promoted oocyte maturation as well as did EGF (Fig. 4A). Progesterone similarly triggered oocyte maturation in freshly isolated OCCs. In fact, progesterone promoted maturation more rapidly than EGF, suggesting that steroids may act downstream of EGFR signaling (Fig. 4B). Furthermore, unlike EGF, progesterone did not promote cumulus cell expansion in OCCs (Fig. 4D), implying that it acts directly on oocytes rather than on cumulus cells.

Steroids are sufficient to promote oocyte maturation. (A) Progesterone and testosterone promote maturation in cultured OGCs. OGCs were grown for 10 days and incubated with 250 nM steroid or 20 ng/ml EGF for 16 h. The y axis indicates the percent of oocytes that had undergone GVBD. Similar results were seen in three experiments. (B) Time course for oocyte maturation. OCCs were incubated with 250 nM progesterone or 20 ng/ml EGF, and oocytes were scored for GVBD at the indicated times. (C) Steroids rescue AG1478-mediated inhibition of maturation. OCCs were treated with AG1478 or DMSO for 30 min before and after the addition of 20 ng/ml EGF. Progesterone (250 nM) was added to rescue inhibition. GVBD was scored at 6 h. Steroid content is shown below the graph. Twenty OCCs were used in each condition and the experiment repeated twice. (D) Steroids do not promote cumulus-cell expansion in OCCs. Cells were placed in medium containing EtOH, 250 nM progesterone, or 20 ng/ml EGF. Images were taken at 16 h. (E) EGF-mediated steroid production is sufficient to promote oocyte maturation. OCCs (n = 150) were incubated with 20 ng/ml EGF for 16 h. Medium was collected and added to newly harvested OCCs in four conditions: (i) unmodified, (ii) with 20 μM AG1478, (iii) with steroid extraction, and (iv) with AG1478 and steroid extraction. GVBD was scored at 8 h. Results are the average (± range) of two experiments.
factors. (iv) That progesterone acts downstream of the EGFR.

Oocytes were incubated with antibodies against androgen receptor (AR), estrogen receptor (ER), and progesterone receptor (PR). Immunofluorescence demonstrates the expression of all three receptors. (B) Oocytes were incubated with primary antibodies pretreated with peptide to which they were directed (AR and PR) or no primary antibody (ERβ). (C) White-light images of the oocytes in B.

In 100% maturation (Fig. 4E), likely triggered by both steroids and EGF in the medium. (ii) Medium was added to OCCs in the presence of the EGFR kinase inhibitor AG1478, causing 94% maturation, likely regulated by steroids and/or other EGF-induced factors. (iii) Steroids, but not EGF, were extracted from the medium, resulting in 100% maturation. (iv) Steroids were extracted and AG1478 was added, thus inhibiting both steroid- and EGFR-mediated signaling. Under this condition, oocyte maturation was significantly impaired. These results provide proof-in-principle that EGF-induced steroid production is sufficient to mediate oocyte maturation.

**Progestosterone Promotes Oocyte Maturation in Intact Follicles.** Steroid-mediated maturation was examined in preovulatory follicles from PMSG-primed mice. Both progesterone and testosterone promoted oocyte maturation in the preovulatory follicles (Fig. 5A). In addition, progesterone-mediated maturation was unaffected by AG1478 (data not shown) or Galardin (Fig. 5B), again indicating that progesterone acts downstream of the EGFR.

**Classical Steroid Receptors Mediate Steroid-Induced Maturation.** Steroid-induced maturation of denuded mouse oocytes may be mediated by classical steroid receptors (6). To provide additional insight into this issue, steroid-receptor expression was examined in denuded oocytes by using immunofluorescence. Intracellular progestosterone, androgen, and estrogen β-receptors were detected throughout oocytes, as indicated by green fluorescence (Fig. 6A). The α-form of the estrogen receptor (ER) was undetectable by using two different antibodies (data not shown), indicating that the β-form predominates in oocytes.

Pharmacologic experiments were next performed to determine whether classical receptors mediated steroid-induced maturation in OCCs. Care was taken to use agonists that could not be metabolized. First, the synthetic progestin promegestone (RU5020) potently promoted oocyte maturation, and its effects were reduced by the progesterone-receptor antagonist mifepristone (Table 1). Estradiol rescued this inhibition, indicating that the antagonist blocked the progesterone receptor specifically. Second, faslodex (IC1 182, 780), a nonselective ERα and ERβ antagonist, blocked estradiol-mediated maturation, and this inhibition was rescued by promegestone. Finally, the androgen receptor antagonist flutamide blocked maturation induced by dihydrotestosterone, and this inhibition was rescued by estradiol. These experiments support the concept that classical steroid receptors mediate steroid-triggered oocyte maturation.

**Discussion**

Our studies demonstrate that EGF signaling through the EGFR is sufficient to promote steroid production in two models of mouse OGCs. EGFR-mediated signaling is necessary for maximal gonadotropin-induced steroidogenesis in the ovary and testes, because the EGFR kinase inhibitor AG1478 ablated LH-induced steroidogenesis in ovarian follicles and MA-10 Leydig cells. Notably, because EGF was less potent than LH in promoting steroidogenesis in follicles, EGF signaling appears necessary but not sufficient for maximal gonadotropin-induced steroid production.

Our studies also address the mechanisms by which LH-receptor signaling leads to EGFR activation. Based on past and present data, we propose the following model (Fig. 7): LH activates its receptor located on theca cells (20), resulting in the activation of MMPs and cleavage of membrane-bound EGFs. Soluble EGF molecules then bind to EGFRs on cumulus granulosa cells, and perhaps all follicular cells, to enhance steroid production. However, progesterone, the most abundantly produced steroid, likely comes primarily from granulosa cells that lack CYP17 and cannot metabolize the steroid. In contrast, MMPs do not regulate LH-induced ste-

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**Table 1. Classical steroid receptor antagonists inhibit steroid-triggered maturation in OCCs**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>ETOH control, %</th>
<th>Agonist (%)</th>
<th>Inhibitor (%)</th>
<th>Steroid rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>20</td>
<td>Promegestone (90)</td>
<td>Mifepristone (20)</td>
<td>Estradiol (75)</td>
</tr>
<tr>
<td>ER</td>
<td>15</td>
<td>Estradiol (90)</td>
<td>Faslodex (25)</td>
<td>Promegestone (100)</td>
</tr>
<tr>
<td>AR</td>
<td>19</td>
<td>DHT (75)</td>
<td>Flutamide (19)</td>
<td>Estradiol (81)</td>
</tr>
</tbody>
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Percentages represent percent of oocytes that have undergone GVBD after 8 h. OCCs were treated with agonist, agonist plus a corresponding antagonist (inhibitor), or agonist, antagonist, and an alternative steroid (Rescue). Antagonists were added 30 min before and throughout the addition of agonists. Concentrations were 100 nM for agonists, 2.5 μM for antagonists, and 250 nM for rescuing steroids. PR, progesterone receptor; AR, androgen receptor; ER, estrogen receptor.
roidogenesis in MA-10 Leydig cells, suggesting that alternative mechanisms (14) activate the EGFR in these cells. These differences may reflect the greater role of paracrine signaling in the ovary, where multiple cell types are required for steroidogenesis.

How does activation of the EGFR stimulate steroidogenesis? In Leydig cells, EGF increases the expression and activity of StAR protein (1). In ovarian follicles, EGFR-mediated steroidogenesis is also regulated by StAR, because Galardin blocked StAR-dependent LH-induced steroidogenesis but not StAR-independent steroid production from 22R-cholesterol. StAR-protein expression was unchanged by EGF in OCCCs, suggesting that, in granulosa cells, StAR activity rather than expression is increased by EGF signaling, perhaps due to posttranslational modifications, such as phosphorylation (21).

The consequences of EGFR-induced steroidogenesis are significant, because steroids mediate many ovarian functions. We demonstrate here that progesterone, testosterone, and estradiol trigger maturation in models where oocytes are held in meiotic arrest by surrounding follicular cells. Although other receptors might regulate steroid-triggered maturation in fish (22), mouse-oocyte maturation appears to be mediated by classical steroid receptors, because classical receptor antagonists blocked steroid-mediated maturation.

The EGFR regulates oocyte maturation (4–5), but EGF cannot trigger maturation of denuded oocytes, implying that secondary messengers in cumulus cells regulate EGF-mediated maturation. The rapidity of progesterone-mediated maturation relative to EGF, the ability of progesterone to rescue AG1478- and Galardin-resistant oocyte maturation (23), confirming progesterone's potential physiologic role in regulating maturation.

Blockade of follicular steroid production does not completely suppress gonadotropin-induced oocyte maturation (24, 25), suggesting that EGF signaling promotes oocyte maturation by more than one mechanism. Pathways other than steroidogenesis that may promote maturation include EGF-mediated cumulus expansion, which disrupts cumulus cell–oocyte gap junctions (26, 27), and attenuation of G protein-mediated signals holding oocytes in meiotic arrest (28, 29). Each activation pathway may regulate different aspects of follicular and oocyte development. For example, steroid may enhance oocyte maturation in dominant follicles that contain the highest steroid concentrations (30).

Finally, steroid-mediated maturation may be important in disorders of androgen excess, such as polycystic ovarian syndrome (31), which is characterized by anovulation, unregulated follicle growth, and the absence of dominant follicles (32). Treatment with anti-androgens improves ovarian function in these patients (33, 34), demonstrating redundancy in this important physiologic process. However, progesterone is the most abundant ovarian steroid in ovulating mice and, therefore, is likely the primary steroid regulating meiosis in vivo. Interestingly, administration of progesterone to ovulating primates enhances oocyte maturation (25), confirming progesterone's potential physiologic role in regulating maturation.

We thank Jean Wilson for his advice and John Eppig and Marilyn O'Brien for technical assistance in culturing OGCs. S.R.H. is a W.W. Caruth, Jr., Endowed Scholar in Biomedical Research. M.J. was funded, in part, by National Institutes of Health (NIH) Training Grant T32 GM07062-29. This work was supported by NIH Grant DK59913, March of Dimes Grant FY05-78, and Welch Foundation Grant I-1506.