Cross-talk between G Protein-coupled and Epidermal Growth Factor Receptors Regulates Gonadotropin-mediated Steroidogenesis in Leydig Cells*

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Gonadal steroid production is stimulated by gonadotropin binding to G protein-coupled receptors (GPCRs). Although GPCR-mediated increases in intracellular cAMP are known regulators of steroidogenesis, the roles of other signaling pathways in mediating steroid production are not well characterized. Recent studies suggest that luteinizing hormone (LH) receptor activation leads to trans-activation of epidermal growth factor (EGF) receptors in the testes and ovary. This pathway is critical for LH-induced steroid production in ovarian follicles, probably through matrix metalloproteinase (MMP)-mediated release of EGF receptor (EGFR) binding ectodomains. Here we examined LH and EGF receptor cross-talk in testicular steroidogenesis using mouse MLTC-1 Leydig cells. We demonstrated that, similar to the ovary, trans-activation of the EGF receptor was critical for gonadotropin-induced steroid production in Leydig cells. LH-induced increases in cAMP and cAMP-dependent protein kinase (PKA) activity mediated trans-activation of the EGF receptor and subsequent mitogen-activated protein kinase (MAPK) activation, ultimately leading to StAR phosphorylation and mitochondrial translocation. Steroidogenesis in Leydig cells was unaffected by MMP inhibitors, suggesting that cAMP and PKA trans-activated EGF receptors in an intracellular fashion. Interestingly, although cAMP was always needed for steroidogenesis, the EGFR/MAPK pathway was activated and necessary only for early (30–60 min), but not late (120 min or more), LH-induced steroidogenesis in vitro. In contrast, 36-h EGF receptor inhibition in vivo significantly reduced serum testosterone levels in male mice, demonstrating the physiologic importance of this cross-talk. These results suggest that GPCR-EGF receptor cross-talk is a conserved regulator of gonadotropin-induced steroidogenesis in the gonads, although the mechanisms of EGF receptor trans-activation may vary.

Steroid production in the testes begins with gonadotropin-releasing hormone (GnRH)2 secretion from the hypothalamus. GnRH stimulates pulsatile release of luteinizing hormone (LH) from gonadotrophs in the pituitary, followed by LH binding to G protein-coupled LH receptors on testicular Leydig cells to promote steroidogenesis. In males, LH pulsations occur approximately every 2 h, and this steady rhythm is believed to be important for maximum testosterone production (1, 2).

In Leydig cells, LH-induced cAMP production is a critical regulator of steroid production (3–6). One of the major mechanisms by which cAMP promotes steroidogenesis is by increasing expression of the steroidalogenic acute regulatory protein (StAR) (7–9). StAR is needed to bring cholesterol into the mitochondria for conversion to steroid, an event generally believed to be the rate-limiting step in steroid production. Evidence suggests that phosphorylation of StAR is critical for its activation and translocation from the cytoplasm to the mitochondria (10).

In addition to cAMP, several studies have implicated epidermal growth factor receptor (EGFR) signaling as a potential regulator of steroidogenesis in both the ovary and testes. First, EGF increases StAR expression in Leydig cells over the course of several hours (11, 12). Second, human chorionic gonadotropin (hCG) triggers rapid phosphorylation of the EGFR in MA-10 mouse Leydig cells that are overexpressing LH and EGF receptors (13, 14). Finally, inhibition of EGFR signaling blocks LH-induced steroid production in MA-10 Leydig cells, as well as in isolated ovarian follicles (15).

The mechanism by which LH receptor signaling triggers activation of the EGFR is still controversial. Several in vitro studies of other G protein-coupled receptors (GPCRs) have shown that the GPCRs can trans-activate EGFRs through matrix metalloproteinase (MMP)-mediated release of membrane-bound EGFR-activating ectodomains (e.g. HB-EGF, amphiiregulin, and epiregulin) (16–19). In contrast, other studies suggest that such EGFR trans-activation can occur independent of MMPs through intracellular signaling pathways that might include cAMP and/or Src (20, 21). In mouse follicles, MMP inhibitors block EGFR phosphorylation, gonadotropin-induced oocyte

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2 The abbreviations used are: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; LHR, luteinizing hormone receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; hCG, human chorionic gonadotropin; GPCR, G protein-coupled receptor; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; RIA, radioimmunoassay; MEK, MAPK kinase; FGF, fibroblast growth factor.
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maturation, and steroidogenesis, suggesting that extracellular signaling is essential for EGFR trans-activation (15, 22, 23). In MA-10 mouse Leydig cells, MMP inhibitors also reduce phosphorylation of the EGFR (13, 14). However, this reduction in the Leydig cells is only partial, and MMP inhibition does not block gonadotropin-induced steroidogenesis in the same cells (15). Therefore, the importance of MMPs in regulating LH actions in the testes remains uncertain.

To address the role of LH and EGF receptor cross-talk in the physiologic response to gonadotropin signaling in Leydig cells, steroid production and release, we performed detailed signaling and steroidogenesis studies in the mouse MLTC-1 Leydig cell line. These cells express endogenous LH and EGFR receptors and rapidly produce progesterone in response to LH or hCG stimulation. We found that LH receptor activation led to rapid but transient cAMP-dependent activation of the EGFR and downstream mitogen-activated protein kinase (MAPK) cascade. This gonadotropin-induced kinase cascade was essential for short term (~30 min), but not prolonged (~2 h), LH receptor-mediated steroidogenesis. Importantly, both short and long term LH-induced steroidogenesis occurred independent of MMP activation, suggesting that, in Leydig cells, the EGFR pathway was activated through intra- rather than extracellular signals.

**EXPERIMENTAL PROCEDURES**

_Tissue Culture and Materials_—MLTC-1 mouse Leydig cells (ATCC) were maintained in RPMI 1640 medium (Fisher) supplemented with 10 mM Hepes, 5% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen). For each study, cells were plated in either 12- or 6-well plates followed by serum starvation overnight to synchronize the cells and reduce background kinase activity. Notably, most of the steroidogenesis experiments were also performed without serum starvation, and the results were identical. Galardin, TAPI-1, AG1478, H-89, PP2, and U0126 (Calbiochem) were added for 30 min prior to hCG (Sigma) stimulation and kept present throughout each experiment. Erlotinib was a gift from Dr. John Minna (University of Texas Southwestern). Forskolin was from Calbiochem and FGF-2 from PeproTech.

**EGFR/MAPK Activation**—After treatment with hCG ± inhibitors as indicated in the figure legends, cells were washed once with cold PBS followed by addition of 125 µL of Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2 mM NaF, 0.5 mM sodium vanadate, 10 mM sodium phosphate, pH 7.4). The lysis buffer was supplemented with 10 µg/ml pepstatin A (Sigma), 10 µg/ml leupeptin (Sigma), 10 µg/ml aprotinin (Sigma), and 100 µg/ml phenylmethylsulfonyl fluoride. Plates were placed on a shaker at 4°C for 15 min, cells were scraped and microcentrifuged at full speed for 15 min, and supernatants were removed and diluted 1:2 in 2× Laemml sample buffer with 10% β-mercaptoethanol (Sigma-Aldrich). Western blots for phospho- and total-EGFR and extracellular signal-regulated kinase (ERK1/2) were performed as described below.

**StAR Expression**—Mitochondrial isolation was performed as described (12). Briefly, hCG ± inhibitors were added to cells in 6-well plates for the indicated times, and cells were washed one time with cold PBS and then placed in 250 µL of TSE buffer (0.25 mM sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). After 15 min of gentle shaking in the cold room, the cells were scraped from the plates and homogenized using 20 strokes of a pestle pestle (Kimble Kontes). Lysates were then centrifuged at 600 × g for 15 min at 4°C. Finally, supernatants were centrifuged at 10,000 × g for 15 min at 4°C; the mitochondrial pellets were resuspended in 60 µL of TSE, and samples were diluted 1:2 in 2× Laemmli sample buffer with 10% β-mercaptoethanol (Sigma-Aldrich). The BCA protein assay kit (Thermo Scientific) was used to measure protein concentrations, and equal amounts of protein were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis as described below.

**Primary Leydig Culture**—All mice were treated in accord with accepted NIH and University of Texas standards of humane animal care. The primary Leydig cell culture protocol was adapted from Hamra et al. (24). Briefly, 2–4-month-old male C57/B6 mice were used. The testes were removed and added to 20 ml of DMEM:Ham’s F-12 (1:1) (Sigma) medium containing 0.5 mg/ml collagenase 1A (Sigma) and 1.0 mg/ml soybean trypsin inhibitor (Sigma). Samples were incubated at 37°C for 20 min while being rocked by hand every 5 min. After allowing the pellets to settle by gravity for a few minutes, supernatants were filtered through 70-micron mesh (BD Falcon™) twice. The filtrates were centrifuged for 5 min at 200 × g, supernatants were removed, and cellular pellets were resuspended in DMEM:Ham’s F-12 (1:1) medium containing 2% fetal bovine serum. Cells were placed in 6-well plates and stimulated with hCG ± inhibitors for steroidogenesis as described.

**Western Blot**—Samples were separated on 7.5% SDS-polyacrylamide gels for EGFR detection, 10% gels for ERK1/2, or 14% gels for StAR. Proteins were then transferred to Immobilon membranes (Millipore). These membranes were blocked with 5% milk (Carnation) in TBST (100 mM NaCl, 0.1% Tween 20, 50 mM Tris, pH 7.4) for 1 h and incubated overnight at 4°C with primary antibody in TBST plus 5% milk. Blots were then washed three times with TBST, incubated for 1 h at room temperature with 1:4000 horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Bio-Rad) in TBST plus milk, and washed another three times with TBST. Finally, blots were treated with ECL-Plus (GE Healthcare) to visualize the proteins. For EGFR detection, 1:2000 anti-phospho-EGFR Tyr-1173 (Upstate Cell Signaling Solutions) and 1:1000 anti-total-EGFR (Cell Signaling) were used. For ERK signaling, 1:2000 anti-phospho-ERK1/2 or anti-total-ERK1/2 (Cell Signaling) antibodies were used (25). For StAR detection, 1:10,000 anti-StAR antibody (Doug Stocco, Texas Tech Health Sciences Center, Lubbock, TX) and 1:2000 anti-phospho-StAR antibody (Dr. Steven King, Baylor College of Medicine, Houston TX) were used. For StAR detection, 30 µg of total mitochondrial protein was used per well.

**Steroid Radioimmunoassays**—To measure steroid content in the media, a progesterone RIA kit (MP Biomedicals) was used for MLTC-1 cells, whereas a testosterone RIA kit (MP Biomedicals) was used for the primary Leydig cells. Assays were performed as directed in the inserts.
To determine the importance of EGFR signaling in gonadotropin-induced steroidogenesis, MLTC-1 mouse Leydig cells were serum-starved overnight and pretreated for 30 min with the EGFR inhibitors AG1478 or erlotinib. Cells were then incubated with hCG for 30 min or 2 h with the inhibitors still present. EGFR signaling was required for hCG-induced steroidogenesis at 30 min, as both AG1478 and erlotinib almost completely abrogated steroid production (Fig. 2A, open bars). Surprisingly, neither of the EGFR inhibitors had more than a very modest inhibitory effect on hCG-induced steroidogenesis at 2 h (Fig. 2A, closed bars), suggesting that EGFR signaling was no longer required for steroidogenesis at the later time point. Accordingly, gonadotropin-induced EGFR activation was seen only at the early time point, as hCG induced significant phosphorylation of the EGFR at 30 min but virtually none at 2 h (Fig. 2B). This loss of EGFR activation in the setting of persistent steroidogenesis suggested that alternative pathways were being utilized to promote steroid production at 2 h. Notably, both AG1478 and erlotinib abrogated hCG-induced phosphorylation of the EGFR at 30 min and baseline EGFR phosphorylation at 2 h (Fig. 2B), confirming that they were inhibiting EGFR kinase activity as expected and for the duration of the experiment.

Interestingly, EGF alone at a concentration that triggers phosphorylation of the EGFR (data not shown) did not promote significant steroid production at either 30 min or 2 h (Fig. 2C), indicating that EGFR signaling is necessary but not sufficient for early gonadotropin-induced steroidogenesis in MLTC-1 cells.

How is the EGFR being activated in response to hCG? Some studies demonstrate that GPCR-mediated activation of MMPs can promote the shedding of EGFR ligands from the cell surface that then act in an autocrine/paracrine fashion to activate EGFRs (16–19). In fact, recent studies suggest that MMP activation may contribute to LH-induced activation of the EGFR in MA-10 Leydig cells (13, 14), although MMP inhibition did not appear to affect LH-induced steroid production in the same cells (15).

In MLTC-1 mouse Leydig cells, MMP activation was not required for hCG-induced steroidogenesis at any time point, as the broad spectrum MMP inhibitor galardin had no effect on steroidogenesis (Fig. 2A). Notably, this same batch of galardin could inhibit hCG-induced steroid production in ovarian follicles (data not shown), where MMP activation is clearly important for steroidogenesis (15), indicating that the inhibitor was functioning appropriately. Furthermore, the tumor necrosis factor-α converting enzyme (TACE) inhibitor, TAPI-1, had no effect on hCG-induced steroidogenesis in MLTC-1 Leydig cells (data not shown), indicating that, in addition to MMPs, TACE-mediated release of EGFR-activating ectodomains was not regulating gonadotropin-induced steroidogenesis.

To confirm the aforementioned results in a more biologically relevant setting, primary Leydig cells were isolated from adult male mice and similarly treated with hCG and an EGFR inhibitor. Similar to the results in the MLTC-1 Leydig tumor cell line, the EGFR inhibitor AG1478 significantly blocked steroid production at 30 min but not at 2 h in primary Leydig cells (Fig. 2D).
Importantly, although steroidogenesis in primary Leydig cells was not altered by AG1478 at 2 h using an in vitro assay, in vivo treatment of male mice with AG1478 for 36 h significantly reduced serum testosterone levels relative to mock-injected mice (Fig. 2E), confirming the physiologic importance of LH and EGF receptor cross-talk in regulating testicular steroidogenesis.

EGFR Signaling Activates the MAPK Cascade to Regulate Gonadotropin-mediated Steroid Production—To determine the role of other intracellular kinases in the regulation of gonadotropin-induced steroidogenesis, the effects of both Src and MEK signaling is probably downstream of EGFR signaling. Importantly, addition of FGF rescued the inhibitory effects of AG1478 on hCG-mediated steroid production (activation) (Fig. 3D, fourth lane), confirming that AG1478 was not directly blocking MEK signaling. Furthermore, as seen with EGF (Fig. 2C), MEK activation by FGF alone (Fig. 3D, lane E) and MEK signaling was important for testosterone production. In fact, although U0126 almost completely eliminated steroidogenesis at 30 min but had significantly less of an effect at 2 h (Fig. 3B). As with EGFR signaling, MEK activity was dramatically induced by hCG at 30 min but was barely present at 2 h (Fig. 3C). Notably, U0126 abrogated any detectable MEK activity at both time points, confirming that it was functioning appropriately throughout the time course of the experiment. These findings suggested that, like the EGFR, MEK signaling was important for early, but not late, gonadotropin-induced steroid production. Furthermore, the nearly identical patterns of EGFR and MEK signaling suggested that these two kinases may have been functioning together during early gonadotropin-induced steroid production. In contrast, similar to the EGFR kinase inhibitor AG1478, MEK significantly blocked both EGFR activation (Fig. 2A) and MEK signaling (Fig. 3E, third lane). These results indicate that MAPK kinase (MEK) inhibitors on hCG-mediated steroid production were examined. In some systems, Src kinases have been implicated as potential regulators of GPCR-mediated trans-activation of the EGFR (16). However, the Src inhibitor PP2 had no effect on hCG-induced steroid production at either 30 min or 2 h in MLTC-1 cells (Fig. 3A), indicating that Src signaling is not necessary for steroid production in Leydig cells. In contrast, similar to the results using EGFR inhibitors, blockade of MEK signaling with U0126 almost completely eliminated steroidogenesis at 30 min but had significantly less of an effect at 2 h (Fig. 3B). As with EGFR signaling, MEK activity was dramatically induced by hCG at 30 min but was barely present at 2 h (Fig. 3C). Notably, U0126 abrogated any detectable MEK activity at both time points, confirming that it was functioning appropriately throughout the time course of the experiment. These findings suggested that, like the EGFR, MEK signaling was important for early, but not late, gonadotropin-induced steroid production. Furthermore, the nearly identical patterns of EGFR and MEK signaling suggested that these two kinases may have been functioning together during early gonadotropin-induced steroid production. In fact, although U0126 blocked MEK activity at 30 min (Fig. 3C), it had no effect on hCG-induced EGFR activation at the same time point (Fig. 3D). In contrast, the EGFR kinase inhibitor AG1478 significantly blocked both EGFR activation (Fig. 2A) and MEK signaling (Fig. 3E, third lane). These results indicate that
cAMP and Protein Kinase A (PKA) Are Required for Activation of the EGFR during Early Gonadotropin-induced Steroid Production—Because cAMP and PKA are known to be critical regulators of steroid production in all steroidogenic tissues (3–6), the roles of these signaling molecules in regulating steroidogenesis in the context of EGFR trans-activation were examined. As expected, inhibition of PKA signaling with H-89 significantly reduced hCG-induced steroidogenesis in MLTC-1 cells at both 30 min and 2 h (Fig. 4A). Interestingly, H-89 abrogated hCG-induced phosphorylation of both the EGFR and ERK1/2 at 30 min (Fig. 4B and C), suggesting that PKA acted upstream of EGFR signaling. Notably, as seen in Figs. 2 and 3, hCG-induced phosphorylation of the EGFR and ERK1/2 were significantly reduced at 2 h (Fig. 4B and C). Furthermore, H-89 completely abrogated the small amount of remaining hCG-induced MEK activity at 2 h (Fig. 4C), confirming that the drug was still working at the later time point.

To determine whether cAMP and PKA signaling were sufficient to promote activation of the EGFR and downstream MEK, MLTC-1 cells were treated with 10 μM forskolin, which stimulates adenyl cyclase to increase intracellular cAMP levels. This concentration of forskolin was chosen because it promotes similar levels of steroid production as 5 IU/ml hCG. As expected, forskolin promoted significant steroid production at both 30 min and 2 h (Fig. 4D). This forskolin-induced steroidogenesis was PKA-dependent, as H-89 completely abrogated steroid production at both time points (Fig. 4D). Interestingly, similar to hCG stimulation, blockade of EGFR signaling (using erlotinib) or MEK activity (using U0126) almost completely eliminated forskolin-induced steroidogenesis at 30 min but not 2 h. Also similar to hCG, forskolin promoted phosphorylation of the EGFR (Fig. 4E) and activation of MEK (Fig. 4F) at 30 min. Finally, as with hCG stimulation, the EGFR inhibitor erlotinib blocked forskolin-induced MEK activation (Fig. 4F), whereas the MEK inhibitor U0126 had no effect on forskolin-mediated EGFR activation (Fig. 4E). Together, these results confirmed that cAMP/PKA signaling was both upstream of and sufficient to activate EGFR and subsequent MEK signaling and that this pathway was necessary for short term steroidogenesis.

EGFR Signaling Regulates Steroidogenesis by Activating StAR—As mentioned, StAR is an important rate-limiting protein in gonadotropin-induced steroid production, and some evidence suggests that StAR may be regulated by EGFR signaling in gonadal tissues (12, 26). To address the relationship between EGFR signaling and StAR activity in MLTC-1 Leydig cells, the
Effect of the EGFR inhibitor AG1478 on StAR-independent steroidogenesis was examined. Cells were treated for 30 min with 22(R)-cholesterol, which can enter the mitochondria and serve as a substrate for steroidogenesis independent of StAR. 22(R)-Cholesterol stimulated significant steroid production that was unaffected by AG1478 (Fig. 5A), implying that EGFR signaling...
upon both the GPCR and cell type. For example, in COS cells, β2-adrenergic receptor activation triggers activation of the EGFR in an intracellular, ligand-independent, process (20, 27). In contrast, the β2-adrenergic receptor activates the EGFR in an extracellular, ligand-dependent, fashion in cardiac fibroblasts by stimulating matrix metalloproteinases (MMPs) to cleave membrane-bound EGFR ligand ectodomains (19). As another example, lysophosphatidic acid trans-activates the EGFR via MMPs and ectodomain shedding in COS cells (18) but through intracellular mechanisms in adrenal glomerulosa cells (21).

Although in vitro studies of GPCR/EGFR cross-talk have been beautifully characterized, the in vivo importance of this trans-activation is still under investigation. Recent studies in the gonads suggest that MMP-mediated release of EGFR-activating ectodomains from amphiregulin and epiregulin are important for mouse oocyte maturation (22), as well as LH-induced steroidogenesis in ovarian follicles (15). Gonadotropin-mediated activation of the EGFR may also regulate steroidogenesis in Leydig cells; however, the mechanism by which LH receptor activation initiates EGFR signaling in the testes remains controversial. For example, both Src and MMPs may play partial roles in rapidly trans-activating the EGFR in MA-10 mouse Leydig cells that are overexpressing LH and EGF receptors (13, 14). However, MMP inhibition is not critical for LH-induced steroid production in native MA-10 cells (15).

Here we have attempted to reconcile some of the controversies regarding LHR/EGFR cross-talk in Leydig cells. Rather than use partial changes in intracellular signaling as our end point, we instead focused on the physiologically important end point of gonadotropin-induced steroid production. In addition, to avoid complications due to overexpression of receptors, we used native MLTC-1 mouse Leydig cells that express only endogenous LH and EGF receptors.

We found that the EGFR and MEK were activated after 30 min of stimulation with hCG, with MEK signaling occurring downstream of EGFR activation (Fig. 2). Furthermore, EGFR and MEK activation were essential for normal hCG-induced steroidogenesis at 30 min. Similar to previous reports (13, 14), MMP inhibition partially blocked hCG-induced EGFR and MEK activation (data not shown). However, MMP inhibitors, including galardin (Fig. 2A) and the tumor necrosis factor-α converting enzyme inhibitor TAPI-1 (data not shown), had no effect on the final physiologic end point of steroidogenesis, suggesting that both intracellular, as well as MMP-dependent extracellular, activation of the EGFR may be occurring. These observations differ from those in ovarian follicles, where galardin markedly inhibited LH-induced steroid production (15). To explain these results, we propose that LH receptor activation may trigger EGFR trans-activation through both extracellular (MMP-mediated) and intracellular (MMP-independent)
mechanisms in both the ovaries and testes. However, the extracellular pathway may be less important in the testes, where all steps of steroidogenesis occur within individual Leydig cells. In contrast, the gonadotropin-induced extracellular pathway might be essential in the ovarian follicle, where paracrine signaling is required for communication between theca and mural granulosa cells, which express LH receptors, and cumulus granulosa cells, which lack LH receptors.

If MMPs are not involved, then what signals are triggered by LH receptors to trans-activate the EGFR in Leydig cells? As mentioned, Src has been implicated in rapid EGFR trans-activation; however, similar to MMP blockade, inhibition of Src had no effect on the biologic end point of gonadotropin-mediated steroidogenesis (Fig. 3A). Instead, PKA inhibition abrogated hCG-induced EGFR and MEK activation, as well as steroidogenesis, at 30 min (Fig. 4). Furthermore, stimulation of cAMP production with forskolin for 30 min promoted steroidogenesis in an EGFR- and MEK-dependent fashion. These observations indicate that cAMP and PKA are both necessary and sufficient for short term EGFR-mediated steroidogenesis. Interestingly, cAMP/PKA signaling may be a conserved mechanism for regulating LH and EGF receptor cross-talk, as cAMP appears to mediate gonadotropin-induced trans-activation of the EGFR during oocyte maturation (23). How PKA activates the EGFR is still unclear. PKA may be able to directly phosphorylate and activate the EGFR under some conditions (28, 29), and the EGFR contains several potential PKA target sequences (RXX(S/T)). However, attempts to use commercial antibodies against these sequences, as well as direct analysis of EGFR phosphorylation sites in response to hCG or forskolin, have so far been unrevealing.

LHR/EGFR cross-talk occurred at early (30 min) but not late (120 min) time points (Fig. 2). Accordingly, two different EGFR inhibitors (AG1478 and erlotinib), as well as the MEK inhibitor U0126, blocked gonadotropin-induced steroidogenesis at 30, but not 120, min. Together, these data indicate that the EGFR/MEK cascade is essential for short but not long term gonadotropin-induced steroidogenesis.

How might the EGFR/MEK kinase cascade be regulating short term gonadotropin-induced steroidogenesis? The answer may lie in part with StAR, which is the rate-limiting regulator of steroidogenesis in all steroidogenic tissues. As with steroidogenesis, short (30 min) but not long (2 h) term hCG-induced phosphorylation and translocation of StAR to the mitochondria was significantly reduced by the EGFR inhibitor AG1478 (Fig. 5).

What is the biological importance of this bimodal activation? In humans, the pituitary releases LH every 90–120 min, with serum concentrations varying as much as 2-fold (30, 31). Thus, the EGFR/MEK kinase pathway may be important for androgen production in vivo, as Leydig cells might be constantly resensitizing to LH and therefore reactivating the kinase cascade. Consistent with this interpretation, treatment of male mice with AG1478 for 36 h significantly reduced serum testosterone levels relative to mock-treated mice (Fig. 2), confirming a potentially important physiologic role for LH/EGF receptor cross-talk in testicular steroidogenesis. Notably, decreased LH pulsatility in humans is associated with lower Leydig cell sensitivity and reduced testosterone production (30, 32), perhaps due to alterations in the EGFR/MEK kinase pathway.

We present the following model to reconcile the observations regarding gonadotropin-induced steroidogenesis in Leydig cells (Fig. 6). In the short term (less than 60 min), LH induces trans-activation of the EGFR through both extracellular (MMP-mediated) and intracellular (cAMP-mediated) means. However, the extracellular activation seems unnecessary for steroid production. Rapid EGFR signaling leads to activation of the MAPK cascade, resulting in phosphorylation and mitochondrial translocation of small amounts of endogenous StAR protein, followed by increased steroidogenesis. With longer LH stimulation (2 h), LH/EGF receptor cross-talk disappears; however, StAR phosphorylation and translocation to the mitochondria persist. This continued StAR activation might be due to increased StAR production from cAMP/PKA-mediated transcriptional up-regulation, resulting in such high levels of StAR protein expression that phosphorylation and translocation to the mitochondria can occur via other, EGFR/MEK-independent, pathways. Further studies will be necessary to uncover these potential alternative pathways, as well as to confirm the true physiologic importance of LH/EGF receptor cross-talk in testicular androgen production.
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