Preantral follicles stimulate luteinizing hormone independent differentiation of ovarian theca-interstitial cells by an intrafollicular paracrine mechanism*

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Ovarian thecal cells are thought to differentiate from fibroblast-like precursor cells in the stroma adjacent to developing follicles. Since the precursor cells do not contain LH receptors, a regulator other than LH must initiate thecal differentiation. These studies were designed to test the hypothesis that preantral follicles secrete substances that can stimulate thecal differentiation. Preantral follicles devoid of theca were obtained by limited enzymatic dispersal of 26-day old rat ovaries. Follicles were cultured (5 follicles/well) in 96-well plates containing serum-free medium to generate follicle-conditioned medium (FCM). Isolated theca-interstitial cells (TIC) were cultured (2 days) in 50% FCM, to bioassay for androgen-stimulating activity. Androsterone production was measured by RIA. FCM from follicles of increasing size with 1, 2, 3, 4 or 5 layers of granulosa cells (GC) stimulated increasing amounts of androsterone suggesting that secretion of androgen-stimulating activity is developmentally regulated in preantral follicles. The androgen-stimulating activity of 7.5-fold concentrated FCM was markedly increased above control levels or the levels stimulated by insulin-like growth factor-I (100 ng/ml), transforming growth factor-α (100 ng/ml), transforming growth-factor-β1 (10 ng/ml), inhibin A (300 ng/ml), activin A (100 ng/ml), or Müllerian inhibiting substance (MIS; 300 ng/ml) suggesting that the bioactive substances were distinct from these intrafollicular growth factors. rFSH stimulated a >10-fold increase in androgen-stimulating activity demonstrating that the bioactivity is hormonally regulated. The bioactivity was sensitive to trypsin digestion but was not inhibited by indomethacin (10 μM) suggesting that it is peptide not prostaglandin in nature. Gel filtration chromatography indicated that the M₄ of the bioactive peptides in FCM ranged from 19 500 to 23 600. Taken together our results demonstrate that preantral follicles secrete thecal differentiating factors (TDFs) that are developmentally and hormonally regulated by FSH. The properties of the TDFs are markedly different from known intrafollicular growth factors and may represent a new paracrine regulator in the ovary that can stimulate LH-independent thecal differentiation.

Keywords theca-interstitial cells; follicle development; androgen production

Introduction

Differentiation of the ovarian theca cells involves the sequential expression of a variety of characteristic genes, some of which are known and others remain to be elucidated. The theca cells are thought to originate from morphologically indeterminate, fibroblast-like precursor cells located in the ovarian stroma adjacent to primordial follicles (Erickson et al., 1985). The undifferentiated pre-theca cells have not yet expressed LH receptors and are therefore not responsive to LH (Magoffin et al., 1994). There is, however, evidence that gene expression is activated in pre-theca cells when primordial follicles are recruited into the pool of growing follicles (Hirshfield, 1991). Genes for LH receptors and steroidogenic enzymes are expressed prior to morphologic differentiation and before the differentiating theca become responsive to LH (Magoffin et al., 1994). The first morphologically identifiable theca cells become associated with developing follicles when the follicle acquires 2–3 layers of granulosa cells (Ingram, 1959) and is coincident with the appearance of LH responsiveness and low levels of steroidogenic enzyme activity in the newly differentiated theca (Magoffin et al., 1994).

The observation that low levels of LH receptor and steroidogenic enzyme gene expression occurs before the differentiating theca can respond to LH suggests the hypothesis that substances other than LH initiate the genetic program of thecal differentiation. Since differentiated theca are only associated with growing follicles, it seems reasonable to propose that the growing follicle secretes one or more substances that regulate thecal differentiation independent of LH.

The purpose of the present studies was to test the hypothesis that preantral follicles secrete one or more substances that can stimulate thecal differentiation. To accomplish this goal we used theca-interstitial cells (TIC) isolated from immature hypophysectomized rats by Percoll gradient centrifugation (Magoffin & Erickson, 1988). The freshly isolated TIC are initially responsive to LH with increased cAMP production but LH does not stimulate steroidogenesis (Magoffin & Erickson, 1988; Magoffin, 1989). Even when substrates such as 25-hydroxycholesterol are provided to the freshly isolated TIC steroidogenesis is not increased demonstrating that the steroidogenic enzymes are not present in sufficient amounts to actively synthesize steroids (Magoffin et al., 1990). Approximately 20 h of continuous stimulation with LH are required before mRNA for cholesterol side chain cleavage cytochrome...
P450 (P450_{ccc}), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase/20 lyase cytochrome P450 (P450_{17α}) mRNAs are expressed and translated into functional proteins (Magoffin, 1989; Magoffin & Weitsman, 1993a,b,c). In this model significant increases in androgen production occur only when steroidogenic enzyme gene expression is induced. In these studies we have used the isolated TIC as a cell culture bioassay for substances secreted by preantral follicles that can stimulate thecal differentiation.

Results

To determine if preantral follicles secrete substances that stimulate androgen production by ovarian theca-interstitial cells (TIC), isolated TIC were cultured in the presence of preantral follicle-conditioned medium (FCM). Follicles of increasing size from one through five layers of granulosa cells (GC) were cultured for 2 days in serum-free medium to generate FCM. TIC were then cultured in 50% FCM for 2 days to bioassay for androgen-stimulating activity. As shown in Figure 1, control TIC cultured in medium incubated without follicles for 2 days produced approximately 3 ng/ml of androsterone. FCM from follicles with two or more layers of GC stimulated androsterone production over control levels. These results demonstrate that preantral follicles secrete substances that stimulate thecal androgen production and that the amount of stimulating activity increases with follicle development.

We next compared the androgen-stimulating capacity of FCM with several substances produced by ovarian follicles that have been shown to regulate TIC androgen production. As shown in Figure 2, LH (0.3 ng/ml) and prostaglandin E₂ (PgE₂; 300 ng/ml) stimulated 30-fold increases in androsterone production (P<0.001). Saturating concentrations of insulin-like growth factor-I (IGF-I; 100 ng/ml), transforming growth factor-α (TGFα; 100 ng/ml), transforming growth factor-β1 (TGF-β1; 10 ng/ml), inhibin A (300 ng/ml), activin A (100 ng/ml), or Müllerian inhibiting substance (MIS; 300 ng/ml) did not alter basal androsterone production. In contrast, FCM from preantral follicles concentrated 7.5-fold caused a marked stimulation (18-fold) of TIC androsterone production over basal levels (P<0.001). These results strongly suggest that the androgen-stimulating substance in FCM is distinct from IGF-I, TGFα, TGF-β1, inhibin A, activin A and MIS but does not rule out the possibility that it is PgE₂ or cAMP.

The only substances previously shown to stimulate large increases in androgen production by ovarian TIC are LH/hCG, PgE₂, and substances that increase intracellular cAMP such as cAMP analogs, forskolin and cholera toxin. Ovarian follicles are not known to secrete gonadotropins, forskolin or cholera toxin, but they can produce prostaglandins and cAMP in response to FSH. To determine if prostaglandins were a component of the androgen-stimulating activity in FCM preantral follicles were cultured in the presence and absence of FSH and indomethacin, an inhibitor of prostaglandin synthesis. As shown in Figure 3, FCM from FSH-treated follicles stimulated a dramatic increase in androsterone production by TIC. Incubation of the preantral follicles with indomethacin (10 μM) did not inhibit the androsterone-stimulating capacity of FCM from control or FSH-treated follicles. These results strongly suggest that products of arachidonic acid metabolism such as prostaglandins are not an important androgen-stimulating component of the FCM.

![Figure 1](image1.png)

**Figure 1** Production of androgen-stimulating activity by preantral follicles. Preantral follicles (5 follicles/well) with 1–5 layers of GC were cultured (2 days) in serum-free medium. Conditioned medium was diluted with an equal volume (100 μl) of fresh medium and used to culture TIC (5×10⁴ viable cells/well) for 2 days. Androsterone in the medium was measured by RIA. The data are the mean ± SEM of three separate experiments with 2–3 replicates/experiment. *P<0.03 vs control; †, P<0.01 vs control

![Figure 2](image2.png)

**Figure 2** Comparison of the androgen-stimulating activity of FCM with intrafollicular regulatory molecules. TIC (5×10⁴ viable cells/well) were cultured (2 days) with medium alone (Control), FCM from follicles with 3–5 layers of GC (100 μl, concentrated 7.5-fold), LH (0.3 ng/ml), PgE₂ (300 ng/ml), IGF-I (100 ng/ml), TGF-α (100 ng/ml), TGF-β1 (10 ng/ml), inhibin A (300 ng/ml), activin A (100 ng/ml) or MIS (300 ng/ml). Androsterone in the medium was measured by RIA. The data are the mean ± SEM of three experiments with 4 replicates/experiment.