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Regulation of Steroidogenic Acute Regulatory Protein and Luteinizing Hormone Receptor Messenger Ribonucleic Acid in Hen Granulosa Cells*

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ABSTRACT

The regulation of steroidogenic acute regulatory protein (StAR) *in vitro* by gonadotropins was investigated in granulosa cells from prehierarchal and preovulatory hen follicles. Basal levels of StAR messenger RNA (mRNA) in undifferentiated granulosa cells from prehierarchal (6- to 8-mm) follicles were consistently low, but detectable, and were significantly increased by treatment with 8-bromo-cAMP and FSH (but not LH) within 3–6 h of culture. After 20 h of culture, 8-bromo-cAMP, FSH, and LH each increased StAR mRNA levels above those in control cultured cells, and the delayed response to LH treatment was associated with increased levels of LH receptor (LH-R) mRNA. On the other hand, inhibition of mitogen-activated protein (MAP) kinase signaling, using the MAP kinase kinase inhibitors U0126 and PD98059, in the presence of FSH further increased StAR mRNA and protein levels, LH-R mRNA levels, and progesterone synthesis compared with those in cells cultured with FSH alone. The highest basal expression of StAR mRNA during follicle development was found in granulosa from the largest (F1) preovulatory follicle, with comparatively lower levels in granulosa from less mature (F2 plus F3) preovulatory follicles. Treatment with LH rapidly increased StAR mRNA and protein (but not LH-R mRNA) expression in cultures of F1 granulosa and in combined F2 plus F3 granulosa within 3 h, although the magnitude of stimulation was greater in F2 plus F3 granulosa. Compared with results from granulosa cells from prehierarchal follicles cultured for 20 h, inhibition of MAP kinase signaling in the presence of LH for 1 h failed to further enhance levels of StAR or LH-R expression or progesterone production in F2 plus F3 follicle granulosa compared with the effect of LH treatment alone. These results demonstrate that StAR expression in the hen ovary is up-regulated by gonadotropins at least in part via cAMP signaling. The ability of MAP kinase kinase inhibitors to potentiate gonadotropin-induced StAR and LH-R expression plus progesterone synthesis in prehierarchal follicle granulosa cells *in vitro* suggests that inhibition of paracrine or autocrine factor-mediated MAP kinase signaling *in vivo* may be a prerequisite for the full potentiation of granulosa cell steroidogenesis that occurs after recruitment into the preovulatory hierarchy. Finally, these results fail to support a role for MAP kinase signaling in acutely modulating LH-mediated StAR expression or progesterone production in hierarchal follicles, such as occurs during the preovulatory surge of progesterone. (*Endocrinology* **142:** 3116–3124, 2001)

SINCE THE FIRST cloning and characterization of the steroidogenic acute regulatory protein (StAR) in mammals, numerous studies have implicated the expression and function of this mitochondrial-localized protein to be a major rate-limiting step in the process of steroidogenesis within the gonads, adrenal gland, and other steroidogenic tissues (for reviews, see Refs. 1 and 2). A major role for StAR protein has been determined to involve cholesterol transport from the outer to the inner mitochondrial membrane, the site of the cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme. As in mammals, the conversion of cholesterol to pregnenolone is the rate-limiting enzymatic step within granulosa cells of the hen ovary (3). In the mammalian ovary, StAR expression and function are rapidly up-regulated by gonadotropic hormones (*e.g.* LH and FSH) acting primarily via cAMP/protein kinase A signaling. The exact downstream mechanisms that promote StAR transcription have yet to be unequivocally identified, but probably involve transcription factors such as steroidogenic factor-1 (SF-1), the CCAAT/enhancer binding proteins (C/EBPs), and DAX-1 (4–6). More recently, the cloning and characterization of the full-length StAR complementary DNAs (cDNAs) from *Xenopus*, zebrafish, and chicken have provided evidence that the deduced amino acid sequences and patterns of messenger RNA (mRNA) expression are highly conserved across vertebrate species (7).

It has previously been reported that during follicle development, cells from the hen granulosa layer first become competent to produce significant amounts of progesterone only at or shortly after a prehierarchal follicle (defined as a follicle ≤8 mm) has been selected into the preovulatory hierarchy (at the 9–12 mm stage of development) (8). Prehierarchal follicles are highly susceptible to undergoing atresia, and granulosa cells from such follicles are considered undifferentiated. By comparison, preovulatory follicles rarely become atretic under normal physiological conditions. Follicle recruitment into the preovulatory hierarchy is accompanied by the first evidence of FSH-induced cAMP accumulation (8) and increased basal levels of LH receptor (LH-R) mRNA (9) within the rapidly differentiating granulosa cell layer. Of additional significance is that although granulosa from prehierarchal follicles express low, but detectable, levels of P450scc mRNA and immunoreactive protein, cells fail to actively convert cell-permeable 25-hydroxycholesterol to pregnenolone *in vitro* (3). On the other hand, preculture of 6- to 8-mm follicle granulosa cells with forskolin or FSH for 24 h

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renders cells competent to synthesize progesterone in response to a challenge with LH or 8-bromo-cAMP during a subsequent 3-h incubation. This induction of steroidogenesis can be blocked by coculture with the growth factors, transforming growth factor-α (TGFα) and epidermal growth factor (EGF) (10). The latter finding suggests that the differentiation of granulosa cells from prehierarchal follicles is tonically suppressed by paracrine and/or autocrine factors up to the time at which the follicle is selected into the preovulatory follicle hierarchy. In fact, it has been hypothesized that the removal of this inhibitory influence may be a prerequisite for follicle selection and the subsequent progression of granulosa cell differentiation (11).

We recently reported expression of very low levels of StAR mRNA (by Northern blot analysis) in undifferentiated granulosa cells from prehierarchal follicles. By comparison, readily detectable levels of the transcript are present in granulosa from the third (F3) and second (F2) largest follicles, and the highest basal levels expressed within the granulosa layer during follicle development are consistently found in the largest (F1) preovulatory follicle (7).

Taken together, the above observations led to the working hypothesis, tested herein, that gonadotropin-induced StAR expression is linked to the initial differentiation of granulosa from prehierarchal follicles and to the full potentiation of progesterone production in preovulatory follicles. The results demonstrate that StAR expression in granulosa cells from prehierarchal follicles is promoted initially by FSH and subsequently by LH over a 20-h culture period, and preexisting elevated levels of StAR expression in preovulatory follicle granulosa are enhanced by LH treatment. Furthermore, inhibition of mitogen-activated protein (MAP) kinase signaling (using the MAP kinase kinase inhibitors U0126 and PD98059) potentiates gonadotropin-induced StAR and LH-R expression and progesterone synthesis in granulosa cells from prehierarchal follicles. These findings further support a balance between positive and negative regulators of StAR expression in hen granulosa cells and are consistent with the proposal that follicle selection and the subsequent progression of granulosa cell differentiation are initiated only after the removal of differentiation-inhibiting signals targeted at the granulosa layer.

Materials and Methods

Animals and reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–35 weeks of age and laying regular sequences of five or more eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash, Ralston Purina Co., St. Louis, MO) and water, and were exposed to a photoperiod of 15 h of light, 9 h of darkness, with lights on at midnight. Individual laying cycles were monitored for each hen throughout the laying sequence. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. All procedures described herein were reviewed and approved by the University of Notre Dame institutional animal care and use committee, and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Recombinant human TGFα and insulin-like growth factor I (IGF-I) were obtained from PeproTech, Inc. (Rocky Hill, NJ), and 8-bromo-cAMP (8-br-cAMP) was acquired from Sigma (St. Louis, MO). Ovine LH (lot 26) and recombinant human FSH (lot AP8468A) were provided by the National Hormone and Pituitary Program. The MAP kinase kinase inhibitors U0126 and PD98059 were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). These agents, at the doses used, are reported to be selective for the MAP kinase pathway and do not attenuate signaling via protein kinase A or B in hen granulosa cells (12).

Granulosa cell cultures

Granulosa cells from the F1 follicle and from the combined F2 plus F3 follicle layers were studied separately because it had previously been established that levels of StAR mRNA were dramatically higher in F1 compared with F2 or F3 follicles (7). In addition, granulosa layers from 8–12 prehierarchal (6–8 mm) follicles were pooled and prepared for culture as previously described (8, 13). Portions of granulosa cell layers from each follicle group were immediately frozen at −70 C (T0 controls) or were dispersed with 0.3% collagenase (type 2; Worthington Biochemical Corp., Freehold, NJ) and plated in 6-well polystyrene culture plates (Falcon 3046, Fisher Scientific, Pittsburgh, PA) at a density of approximately 10^6/well in 1 ml medium 199-HEPES supplemented with Hanks' salts (Life Technologies, Inc., Gaithersburg, MD) plus 1 ml DMEM containing 5% FBS (Life Technologies, Inc.).

In the first set of experiments, granulosa cells from 6- to 8-mm follicles were cultured for 0 (T0), 1, 3, 6, or 20 h in the absence or presence of 8-br-cAMP (1 mM), LH (100 ng/ml), FSH (100 ng/ml), IGF-I (50 ng/ml), or FSH plus IGF-I. The agents and doses chosen for study were based upon previous experiments that evaluated steroidogenesis in hen granulosa cells (8, 10). After the appropriate culture time, only the plated cells were collected and frozen at −70 C until prepared for total cellular mRNA.

In addition, 6- to 8-mm follicle granulosa cells were prepared and immediately cultured in the absence or presence of FSH (100 ng/ml), LH (100 ng/ml), U0126 (50 μM), FSH plus U0126, LH plus U0126, PD98059 (50 μM), or FSH plus PD98059 for 20 h. After culture, plated cells were collected and frozen for analysis of StAR mRNA and protein, and LH-R mRNA, and media were assayed for progesterone. In a related experiment the effectiveness of these inhibitors to block MAP kinase signaling through Erk1/2 was tested by pretreating freshly cultured granulosa cells from prehierarchal follicles for 1 h in the absence or presence of U0126 (50 μM) or PD98059 (50 μM), then culturing cells for an additional 20 min in the absence or presence of 50 ng TGFα/ml. Samples were processed for the analysis of phosphorylated Erk (Erk-P). Treatment with this dose of TGFα has previously been demonstrated to be an effective inducer of Erk-P in hen granulosa cells (12). Freshly plated cells were also pretreated without or with TGFα (50 ng/ml) for 30 min, then cultured for 20 h in the absence or presence of FSH (100 ng/ml). Media were removed and assayed for progesterone, whereas the plated cells were collected and analyzed for levels of StAR and LH-R mRNA.

In a third set of experiments, granulosa cells from F1 or F2 plus F3 follicles were plated in the absence and presence of 1 mM 8-br-cAMP or LH (100 ng/ml) and cultured for 1, 3, or 6 h. At the end of each culture period, cells were collected and rapidly frozen at −70 C until prepared for total cellular RNA and analysis of StAR mRNA and protein and LH-R mRNA.

In the final experiments, granulosa cells from F2 plus F3 preovulatory follicles were precultured for 1 h in the absence or presence of U0126 (50 μM) or PD98059 (50 μM), then treated for an additional 1 h in the absence (Con) or presence of 100 ng/ml LH. Cells were collected and rapidly frozen at −70 for analysis of StAR and LH-R mRNA, and in some instances the media were analyzed for progesterone. In addition, F2 and F3 follicle granulosa cells were preplated for 6 h, then pretreated for 1 h in the absence or presence of U0126 or PD98059 and cultured for an additional 20 min in the absence or presence of TGFα (50 ng/ml). Cells were collected and frozen at −70 for the analysis of Erk-P.

Northern blot analysis

The chicken StAR cDNA used for probing Northern blots was recently described by Bauer *et al.* (7), and the chicken LH-R cDNA was described by Johnson *et al.* (9). Each blot was probed separately for StAR mRNA and LH-R mRNA and was finally probed with a chicken 18S ribosomal RNA cDNA to standardize for equal loading of RNA samples. Conditions for conducting Northern blot analysis have been previously detailed (13), and all blots were visualized on phosphor screens using

a Storm 840 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Analysis of blots was conducted using the ImageQuant data reduction system (Molecular Dynamics, Inc.).

Western blot analysis

The phospho-specific Erk1/2 monoclonal antiserum was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and the α-tubulin monoclonal antibody used for standardization was purchased from Sigma. The StAR polyclonal antibody was provided by Dr. D. B. Hales (University of Illinois, Chicago, IL) (14). Western blot analyses were conducted essentially as previously described (12, 13). Briefly, tissues were homogenized in a protein lysis buffer containing an enzyme and phosphatase inhibitor cocktail (Sigma). Proteins were separated on a 10% polyacrylamide gel under denaturing conditions, then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Fisher Scientific, Hanover Park, IL). Incubations with primary antibodies (phospho-Erk and phospho-Akt at 1:1000 dilution; StAR at 1:5000 dilution) were conducted overnight at 4 C, whereas those for the horseradish peroxidase-conjugated antimouse IgG secondary antibody (for phospho-Erk; Pierce Chemical Co., Rockford, IL) or horseradish peroxidase -conjugated antirabbit IgG secondary antibody (for StAR; Pierce Chemical Co.) were performed for 1 h at room temperature. Blots were incubated with ECL Western blotting agent (Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 min and exposed to x-ray film for 1–15 min. The extent of antibody binding was quantitated by densitometry (UltraScan XL laser densitometer, Pharmacia LKB, Piscataway, NJ).

Progesterone assays

Progesterone levels in medium samples were quantitated using an enzyme immunoassay (Diagnostics Systems Laboratories, Inc., Webster TX) according to the manufacturer's protocol.

Data analysis

All experiments were repeated a minimum total of three times unless otherwise stated. Levels of StAR and LH-R mRNA, Erk-P, and progesterone were expressed as the fold difference (mean ± SEM) *vs.* a designated reference treatment such as freshly collected (T0) or cultured control cells (value for reference treatment arbitrarily set at 1). Data were analyzed by one-way ANOVA (analysis of fold difference data did not include the reference treatment) and the Fisher protected least significant difference multiple range test. *Post-hoc* analysis of selected data was conducted by paired *t* test using original (untransformed) data (*e.g.* LH-R mRNA, T20 control *vs.* T0; Fig. 1).

Results

Cultures of granulosa from prehierarchal (6- to 8-mm diameter) follicles

StAR transcript levels were increased in granulosa cells from prehierarchal follicles treated with 8-br-cAMP within 3 h of culture (5.7 ± 2.6-fold compared with the T0 control), and remained elevated or further increased after 6 and 20 h of culture (5.9 ± 1.8- and 38.9 ± 3.0-fold T0, respectively; Fig. 1, *top panel*). Within 6 h of culture, FSH, but not LH, treatment increased StAR mRNA levels (10.4 ± 3.5-fold increase *vs.* T0), and by 20 h of culture, treatment with both LH and FSH stimulated increased StAR mRNA levels (by 5.0 ± 1.2- and 4.3 ± 0.9-fold, respectively). By comparison, there was a small, but significant, increase in LH-R mRNA levels within control cultured cells after 20 h of culture ($P < 0.05$ *vs.* T0, by paired *t* test; Fig. 1, *bottom panel*). Treatment with 8-br-cAMP, LH, or FSH further increased LH-R mRNA levels above those detected in T20 control cultured cells ($P < 0.05$). On the other hand, IGF-I treatment for 20 h failed to increase levels of StAR or LH-R mRNA compared with those in T20 control cells, and IGF-I had no additive effect on StAR or LH-R expression when combined with FSH compared with the FSH treatment alone ($P > 0.10$; Fig. 2). As a positive control for biological activity, IGF-I was determined to induce a greater than 10-fold increase in Akt phosphorylation after a 20-min treatment

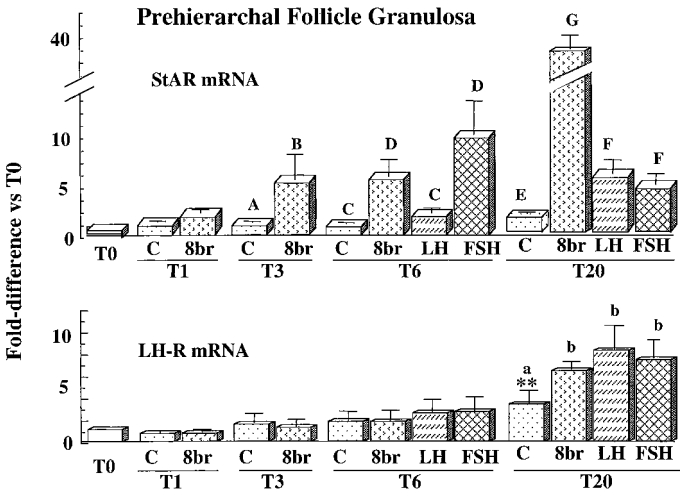

FIG. 1. Time frame to induction of StAR (*top panel*) and LH-R (*bottom panel*) mRNA in granulosa cells from prehierarchal (6- to 8-mm) follicles. Cells were plated in the absence (C) or presence of 8-br-cAMP (8br; 1 mM), LH (100 ng/ml), or FSH (100 ng/ml) for 1 h (T1), 3 h (T3), 6 h (T6), or 20 h (T20). Data are expressed as the fold difference *vs.* freshly collected (T0) cells and represent the mean ± SEM from three to five experiments. ANOVA was conducted on treatments within each culture interval. A, B; C, D; E, F, G; a, b: $P < 0.05$; **, $P < 0.05$ (*vs.* T0, by paired *t* test).

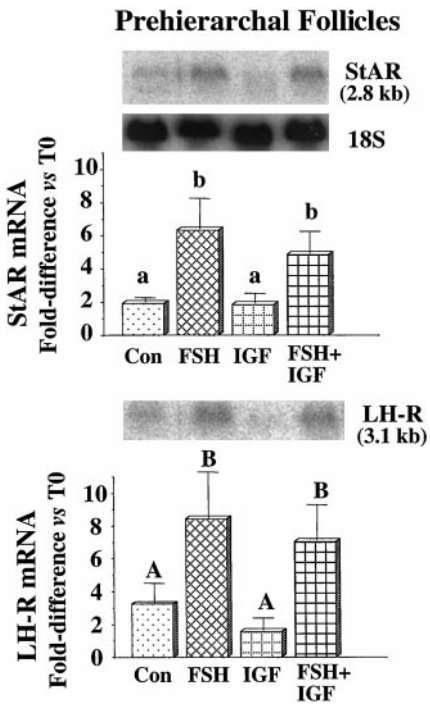

FIG. 2. IGF-I (50 ng/ml) treatment fails to increase StAR (*top panel*) or LH-R (*bottom panel*) mRNA levels after a 20-h culture compared with control (Con) cultured cells or to potentiate expression of either transcript in the presence of FSH (100 ng/ml). n = 3–5 experiments. a, b; A, B: $P < 0.05$.

of granulosa cells from prehierarchal follicles (data not shown).

Consistent with previous reports using chicken cells (12, 15), phosphorylated Erk appeared as a single, approximately 32-kDa band (Fig. 3). Whereas a 20-min treatment with TGFα induced a 2.6 $\pm$ 0.6-fold increase in levels of Erk-P within prehierarchal follicle granulosa cells ($P < 0.05$, by paired t test), pretreatment with the MAP kinase kinase inhibitors U0126 and PD98059 effectively blocked this increase.

Levels of StAR mRNA were not altered by culture for 20 h with either U0126 or PD98059 compared with those in control cultured cells (Fig. 4, *top panel*). However, although FSH and LH induced 3.4- and 6.4-fold increases, respectively, in StAR mRNA levels compared with control cultured cells ($P < 0.05$), 20 h of culture with these gonadotropins in the presence of U0126 or PD98059 induced a further 5.1- to 6.9-fold increase in StAR mRNA compared with FSH or LH treatment alone. By comparison, FSH, LH, U0126, and PD98059 each induced a significant increase in LH-R mRNA compared with levels in control cultured cells ($P < 0.05$; Fig. 4, *bottom panel*). Furthermore, cultures containing FSH or LH combined with U0126 or PD98059 expressed 2.2- to 5.4-fold higher levels of LH-R mRNA than cultures with either gonadotropin alone. The synergistic effects of U0126 and PD98059 on FSH-induced StAR mRNA levels were paralleled by increases in the mature form of the 30-kDa StAR protein (Fig. 5).

Low, but detectable, levels of progesterone (1.1 $\pm$ 0.3 ng/well) were measured in culture medium from control cells after 20 h of culture. Treatment with FSH or LH increased progesterone production by modest 2.3 $\pm$ 0.3- and 2.9 $\pm$ 0.6-fold, respectively, compared with that in the T20 control ($P < 0.05$, by paired t test; Fig. 6). Neither U0126 nor PD98059 alone had any discernable effect on progesterone levels after 20 h of culture; however, each inhibitor in combination with FSH or LH produced a synergistic effect on progesterone synthesis (a 2.2- to 9.9-fold increase) compared with gonadotropin treatment alone.

Finally, a 20-h culture with TGFα did not alter levels of StAR or LH-R mRNA or progesterone production compared with those in control cultures; however, TGFα effectively blocked FSH-induced StAR and LH-R mRNA as well as the initiation of progesterone synthesis (Fig. 7).

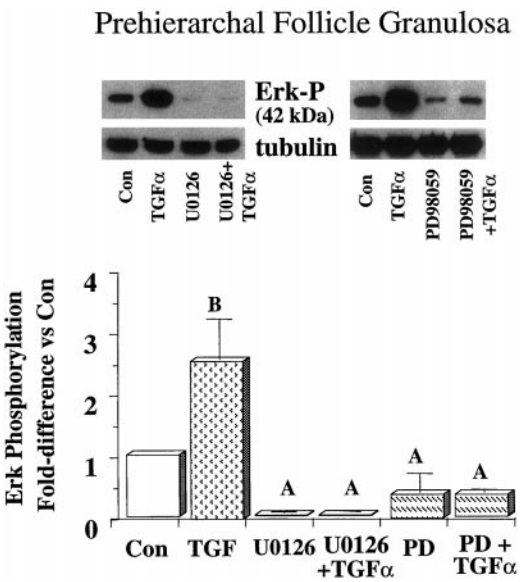

FIG. 3. Inhibition of TGFα-induced Erk phosphorylation (Erk-P) in preovulatory follicle granulosa cells by MAP kinase kinase inhibitors. Freshly plated granulosa cells were pretreated for 1 h in the absence or presence of U0126 (50 μM) or PD98059 (50 μM), then cultured for 20 min in the absence or presence of TGFα (50 ng/ml). Total cellular proteins were analyzed for Erk-P and standardized to α-tubulin. n = 3 or 4 experiments. A, B: $P < 0.05$.

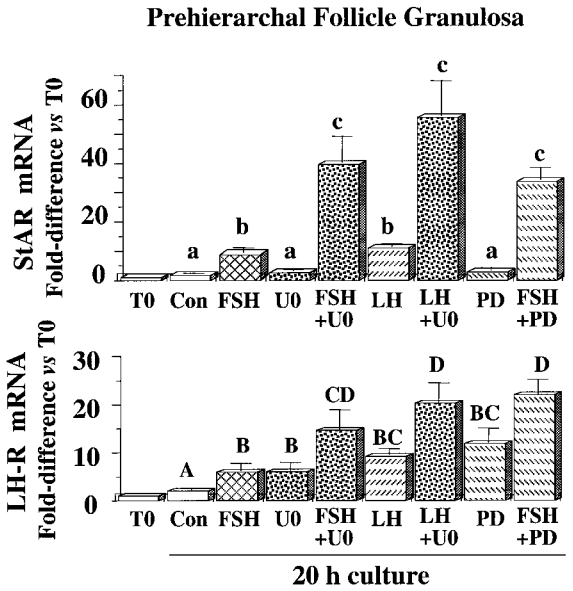

FIG. 4. Evidence for MAP kinase signaling as a negative regulator of StAR (*top panel*) and LH-R (*bottom panel*) mRNA expression in cultured granulosa cells from prehierarchal follicles. Granulosa cells were cultured for 20 h in the absence or presence of FSH (100 ng/ml), LH (100 ng/ml), U0126 (UO; 50 μM), PD98059 (PD; 50 μM), or combinations of these factors. Only adhering (viable) cells were collected for total cellular RNA. Values for StAR and LH-R mRNA are expressed as the fold difference *vs.* freshly collected (T0) cells. Data represent the mean $\pm$ SEM from three to six experiments. a, b, c; A, B, C, D: $P < 0.05$.

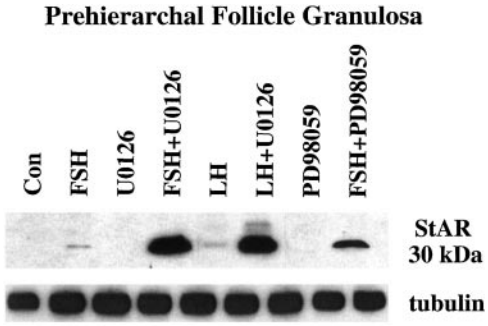

FIG. 5. Representative Western blot for StAR protein in granulosa cells from prehierarchal follicles cultured for 20 h in the absence or presence of U0126 (50 μM), PD98059 (50 μM), FSH (100 ng/ml), or LH (100 ng/ml). Levels of α-tubulin are provided as evidence for equal loading. This experiment was replicated twice with similar results.

Short-term cultures of granulosa from preovulatory follicles

Freshly collected (T0) granulosa cells from F1 follicles express higher levels of StAR mRNA than combined granulosa

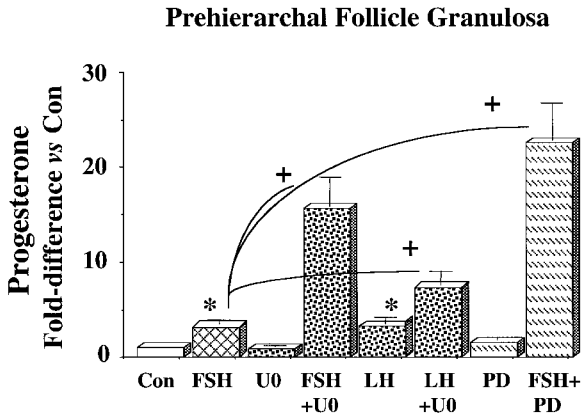

FIG. 6. Progesterone secretion in cultured granulosa cells from prehierarchal follicles after a 20-h culture. Inhibition of MAP kinase signaling by pretreatment with U0126 (UO; 50 μM) or PD98059 (PD; 50 μM) potentiates FSH- and LH-induced progesterone production. Progesterone values are from three to six experiments, and are expressed as the fold difference compared with control cultured (Con) cells. *, $P < 0.05$ *vs.* Con, by paired *t* test; +, $P < 0.05$ *vs.* FSH treated.

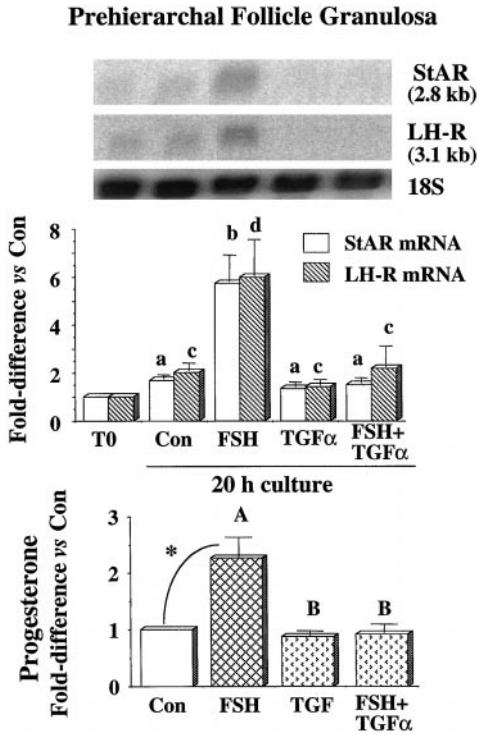

FIG. 7. TGFα-mediated inhibition of FSH-induced StAR and LH-R mRNA levels (*top panels*) plus progesterone synthesis (*bottom panel*) in granulosa cells from prehierarchal follicles after a 20-h culture. Data represent the mean fold difference (±SEM) *vs.* T0 (StAR and LH-R mRNA) or *vs.* control (Con) cultured cells from four to six replicate experiments. a, b; c, d; A, B: $P < 0.05$; *, $P < 0.05$ (by paired *t* test).

from the F2 and F3 follicles (Fig. 8, *inset*). Levels of StAR mRNA were increased in F2/F3 follicle granulosa cells after treatment with 8-br-cAMP or LH for 1 h, whereas in F1 follicle granulosa the first significant increase in response to each treatment was not observed until 3 h (Fig. 8, *top panel*). Both 8-br-cAMP and LH stimulated a greater fold increase in F2 plus F3 granulosa cells compared with F1 granulosa at

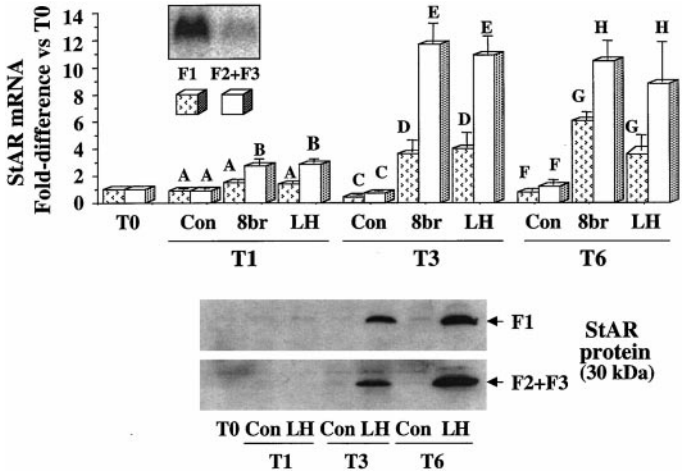

FIG. 8. Levels of StAR mRNA (*top panel*) after culture of preovulatory follicle granulosa cells for 1 (T1), 3 (T3), or 6 (T6) h in the presence of 8-br-cAMP (8-br; 1 mM) or LH (100 ng/ml). F1, Granulosa collected from the largest preovulatory follicle; F2+F3, pooled cells from the second plus third largest preovulatory follicles. *Top panel inset*, Representative blot depicting the relative levels of StAR mRNA in freshly collected (T0) cells from F1 compared with pooled F2 plus F3 granulosa. Data are expressed as the fold difference *vs.* T0 cells and represent the mean ± SEM from three to five experiments. ANOVA was conducted on treatments within each culture interval. A, B; C, D, E; F, G, H: $P < 0.05$. *Bottom panel*, Representative Western blots for StAR protein from F1 or F2+F3 granulosa cultured in the absence or presence of LH for 1, 3, or 6 h. This blot was replicated once with similar results.

each culture interval investigated. The rapid increase in LH-induced StAR mRNA levels was subsequently reflected by increased StAR protein (Fig. 8, *bottom panel*). By contrast, neither 8-br-cAMP nor LH treatment altered levels of LH-R mRNA after any of these relatively short culture periods compared with the respective control value ($P > 0.10$; data not shown).

Similar to findings in granulosa from prehierarchal follicles, a 20-min treatment with TGFα induced a significant (4.4 ± 0.9-fold) increase in Erk-P within granulosa from F2 plus F3 preovulatory follicles compared with the control, whereas pretreatment with U0126 or PD98509 effectively prevented (U0126) or significantly reduced (PD98059) this response (Fig. 9). However, unlike granulosa from prehierarchal follicles, neither U0126 nor PD98059 further increased levels of LH-induced StAR mRNA after 1 h of culture beyond those found in LH-treated cells (Fig. 10). Furthermore, treatment with PD98059 plus LH did not stimulate progesterone secretion above levels found in cells treated with LH alone (a 9.0 ± 1.6- *vs.* 11.1 ± 2.5-fold increase, respectively, compared with control cultured cells; $P > 0.05$). Not unexpectedly, levels of LH-R mRNA were not affected by any treatment after only a 1-h culture period ($P > 0.10$; data not shown).

Discussion

The significant findings of the present studies include the demonstration of gonadotropin-mediated induction of StAR mRNA and protein expression in both undifferentiated (steroidogenically inactive) and differentiated (steroidogenically

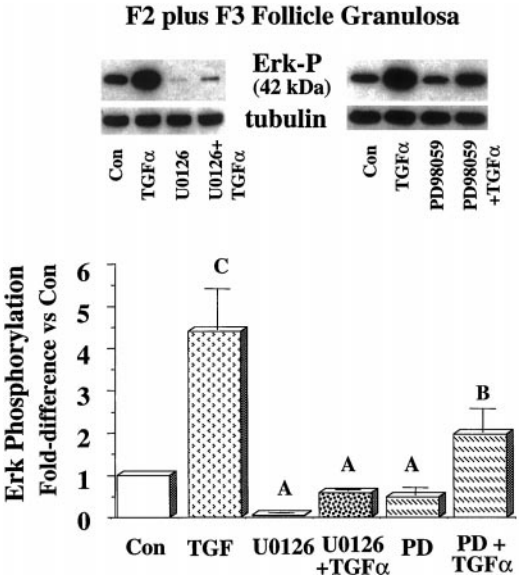

FIG. 9. Inhibition of TGFα-induced Erk-P in pooled F2 plus F3 preovulatory follicle granulosa cells by MAP kinase kinase inhibitors. Freshly plated granulosa cells were pretreated for 1 h in the absence or presence of U0126 (50 μM) or PD98059 (PD; 50 μM), then cultured for 20 min in the absence or presence of TGFα (50 ng/ml). Total cellular proteins were analyzed for Erk-P. *Top panel*, Representative Western blots of Erk-P together with levels of tubulin (used for standardization). *Bottom panel*, Summarized data are expressed as the fold difference *vs.* the control (Con) and represent four to six experiments. A, B, C: $P < 0.05$.

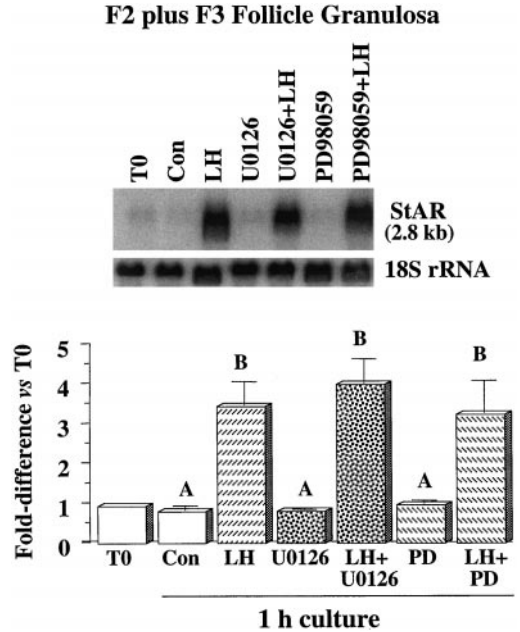

FIG. 10. Inhibition of MAP kinase signaling fails to enhance StAR mRNA expression after a short-term culture of preovulatory follicle granulosa cells. Granulosa cells were precultured for 1 h in the absence or presence of U0126 (U0; 50 μM) or PD98059 (PD; 50 μM), then cultured for an additional 1 h in the absence or presence of LH (100 ng/ml). Data are expressed as the fold difference *vs.* freshly collected (T0) cells and are the mean ± SEM from four replicate experiments. A, B: $P < 0.05$.

active) hen granulosa cells as well as the potential for modulation of such responsiveness in prehierarchal granulosa cells by MAP kinase signaling. These data suggest that the attenuation of StAR and LH-R expression by the MAP kinase pathway is relevant to processes leading to the differentiation of granulosa cells. Moreover, it is proposed that the ability of LH to promote a rapid induction of StAR expression in preovulatory follicle granulosa cells represents a mechanism to ensure the full potentiation of progesterone synthesis during the preovulatory surge *in vivo*.

Expression of both StAR and LH-R mRNA in freshly collected granulosa from prehierarchal follicles is extremely low, and this reflects the undifferentiated state of the follicles used. The finding that after 6 h of culture, FSH, but not LH, induces StAR mRNA expression reflects the fact that granulosa cells from prehierarchal follicles predominantly express the FSH receptor and considerably lesser amounts of the LH receptor (9, 16, 17). Significantly, LH-R mRNA levels are increased by 2- to 3-fold in adhering, viable control cells cultured for 20 h, whereas LH treatment induced a further doubling of LH-R mRNA expression. These results indicate that isolated granulosa cells from undifferentiated follicles begin the process of differentiation during culture, possibly due to the release from inhibitory influences that exist *in vivo*, and in addition that this initial increase in LH-R expression is sufficient to mediate StAR mRNA transcription in the presence of LH. Bypassing gonadotropin receptors with the relatively stable cAMP analog, 8-br-cAMP, results in the greatest induction of StAR mRNA and protein (data not shown) after 20 h of culture, and this provides direct evidence for the involvement of protein kinase A signaling.

It is of interest to note that the increase in StAR mRNA levels after 3–6 h of culture in prehierarchal follicle granulosa cells in response to 8-br-cAMP or FSH occurs more rapidly than the 8- to 16-h culture period required for the increased expression of P450scc mRNA (10). Although this finding emphasizes the acute nature of StAR induction via the protein kinase A signaling pathway in hen granulosa, it is interesting to note that P450scc expression appears to precede that of StAR during follicle development (3). By comparison, in PMSG-primed prepubertal rats the first detectable expression of StAR within granulosa was found almost 2 days after an increase in P450scc (18). Given this observed relationship, it is likely that the low levels of P450scc protein previously determined to be expressed within mitochondria from prehierarchal follicle granulosa cells (3) are sufficient to initiate low, but physiologically relevant, amounts of steroid production immediately after the initial induction of StAR expression. The potential for a rapid initiation of steroid production is probably a critical component of the follicle selection process. This proposal is supported by the fact that FSH induced a small, but significant, increase in progesterone production after 20 h of culture.

In contrast to prehierarchal follicles, granulosa cells from F3 through F1 follicles express similar and comparatively high levels of LH receptor mRNA, and LH represents the primary gonadotropin responsible for promoting granulosa cell steroidogenesis (predominantly progesterone) during the final stages of follicle development (17). Thus, it is not surprising that LH or 8-br-cAMP treatment rapidly induces StAR mRNA expression (within 1–3 h of culture), as a rapid

up-regulation of StAR by gonadotropins via the protein kinase A signaling pathway has been well documented in mammalian species (2, 5, 19). It is also noteworthy that inhibition of MAP kinase signaling failed to enhance LH-induced StAR mRNA levels after short-term culture, suggesting that MAP kinase signaling is not involved in acute regulation of steroidogenesis (*e.g.* as would occur during the rapid and transient preovulatory surge of serum progesterone).

It was recently established that constitutive expression of StAR mRNA is dramatically increased in granulosa during the transition from the F2 stage to the F1 stage of development (7). Thus, it is reasonable to speculate that the increased potential for F1 follicle granulosa to synthesize progesterone compared with those from the remaining hierarchal follicles (previously reviewed in Ref. 20) may be linked to this preexisting elevation in StAR expression. In general, high levels of preexisting StAR mRNA expression within preovulatory follicle granulosa ensure rapid protein translation to facilitate the 8- to 10-fold increase in serum progesterone levels that occurs after initiation of the LH surge (21). Furthermore, the present data suggest that an additional mechanism to provide maximal transport of steroid precursor to the inner mitochondrial membrane during the LH surge occurs via new transcription of StAR. It is reasonable to conclude that the comparatively smaller fold increase in StAR mRNA levels after short-term culture of granulosa from F1 (compared with F2 plus F3 follicles) with 8-br-cAMP or LH probably reflects an already near-maximal rate of StAR transcription by F1 follicle granulosa.

One additional point worthy of discussion is the inability to reliably detect basal (T0) levels of StAR protein in F1 follicle granulosa by Western blot analysis despite the relatively high preexisting levels of the StAR transcript. One possible explanation is a lack of sensitivity to basal levels of StAR protein due to the use of a heterologous antibody. However, alternative explanations include the possibility that StAR protein expression is controlled at the level of translation, and/or that StAR protein may be rapidly turned over on a continuing basis such that elevated levels of LH (such as those that occur during the preovulatory LH surge) are obligatory for the production of sufficient StAR protein to enable the generation of the preovulatory progesterone surge. Further experiments to evaluate these possibilities are currently underway. In any event, the rapid translational response, and subsequent elimination, of StAR would allow for the precise regulation of steroidogenesis required for a species, such as the hen, that contains a hierarchy of ovarian follicles differing in steroidogenic potential. The proposed requirement for newly synthesized StAR protein during the 4- to 6-h preovulatory LH surge is supported by the finding that in the absence of continued LH stimulation *in vitro*, protein levels in whole cell extracts rapidly decline by 95% after 3 h of culture (data not shown).

Furthermore, it is significant to note that despite the high level of LH receptor mRNA expression previously reported in F2 and F3 follicle granulosa *in vivo* (9), and the ability of LH treatment to markedly increase StAR expression in cultured F2 plus F3 granulosa *in vitro*, basal levels of StAR mRNA in F2 and F3 follicle granulosa are markedly lower than those found within granulosa from the F1 follicle (7). This relationship suggests that gonadotropin-mediated StAR mRNA transcription in F2 and F3 follicles may be tonically suppressed *in vivo*, and that a putative inhibitory signal (via a paracrine and/or autocrine factor) is lost during the transition to the F1 stage of development. Of relevance is a previous report of a suppressive effect of the F2, but not F1, thecal layer on granulosa cell progesterone production *in vitro* (22). Accordingly, it is reasonable to speculate that factors produced by the thecal layer from hierarchal follicles other than the F1 follicle may be responsible for tonic suppression of StAR transcription, and thus the attenuation of steroidogenesis. The subsequent loss of or acquired insensitivity to such putative factors within F1 follicle granulosa could explain both the increase in StAR expression (7) and the heightened potential for progesterone production from the F1 follicle (20).

Similarly, it is of interest to speculate regarding mechanisms that contribute to the relative absence of constitutively expressed StAR mRNA in granulosa cells from prehierarchal follicles (7). As noted above, such cells predominantly express FSH-R and are exposed to fluctuating levels of serum FSH on a continual basis. We previously reported that the increase in FSH-promoted P450scc mRNA levels and the initiation of steroidogenesis in undifferentiated granulosa cells *in vitro* are blocked by coculture with TGFα (10). Furthermore, inhibition of MAP kinase kinase signaling (with U0126 or PD98059) enhanced gonadotropin-induced StAR and LH-R mRNA levels, StAR protein levels, and progesterone synthesis in cultured granulosa cells from prehierarchal follicles. The comparatively lower net production of progesterone in response to LH plus U0126 (compared with FSH plus U0126) probably reflects the delay in expression of the LH-R after the initiation of culture.

Results presented in Fig. 7 further demonstrate that the inhibition of FSH-induced initiation of steroidogenesis by TGFα is associated with the complete inhibition of StAR expression. TGFα treatment has previously been reported to stimulate DNA synthesis and promote cell proliferation in cultured granulosa cells (23, 24). Taken together, these results implicate MAP kinase signaling via TGFα (and possibly additional growth factors) as a mechanism to promote granulosa cell proliferation in prehierarchal follicles while at the same time preventing premature differentiation. Significantly, both immunoreactive TGFα and the TGFα/EGF receptor have been localized to cells within the granulosa and thecal layers in follicles throughout development, yet levels of both are reported to decrease with follicular maturation (25). It remains to be unequivocally demonstrated whether the decline in the expression of either the ligand or receptor is directly related to the maintenance of undifferentiated prehierarchal follicles and subsequently follicle selection and/or the progression of differentiation *in vivo*. Finally, it is noted that the structurally related growth factor, EGF, has previously been reported to dramatically decrease steady state expression of StAR in the porcine corpus luteum (26).

It has been proposed that the nuclear transcription factor SF-1 is an essential regulator of StAR expression and that activation of SF-1-dependent transcription requires MAP kinase-dependent phosphorylation (27). Nevertheless, the results presented herein clearly demonstrate that inhibition of

MAP kinase signaling potentiates gonadotropin-induced StAR expression and steroidogenesis in granulosa cells from prehierarchal follicles. This finding argues against a role for phosphorylated SF-1 in mediating gonadotropin-induced StAR expression in hen granulosa cells. Although this is consistent with results derived from site-directed mutagenesis studies in mice, which demonstrate that activation of protein kinase A signaling (using 8-br-cAMP) can fully potentiate StAR transcription after deletion of one or both SF-1 promoter elements (19), the requirement for SF-1 appears to be species specific (5).

Inhibition of MAP kinase signaling with U0126 or PD98059 alone was found to increase LH-R (but not StAR) mRNA levels in granulosa cells from prehierarchal follicles, and the combination of FSH or LH plus MAP kinase kinase inhibitor further enhanced this increase in LH-R transcript. Although the activation of MAP kinase signaling via gonadotropins has previously been described (28), the present results are among the first to implicate MAP kinase signaling in the regulation of a gonadotropin receptor. Moreover, this result further supports a role for MAP kinase signaling in the regulation of granulosa cell differentiation.

The absence of an additive effect of IGF-I on FSH-induced StAR and LH-R mRNA after a 20-h culture is consistent with previous findings regarding a lack of synergism by the combination of these same factors on P450scc enzyme mRNA levels or steroid production in granulosa cells from prehierarchal follicles (10). This inactivity cannot be attributed to an absence of bioactivity, as recombinant human IGF-I has been found here and previously to rapidly activate Akt/protein kinase B signaling in this model system (12). By contrast, IGF-I has been demonstrated to promote a direct effect on StAR expression and a synergistic effect on FSH-induced StAR expression in porcine granulosa cells after 24–48 h of culture (29). Although cultures in the present studies were not maintained beyond a duration of 20 h, it is possible that longer cultures in the presence of IGF-I would facilitate gonadotropin-induced StAR expression. If so, the mechanisms mediating this long-term effect are proposed to be more complex than those mediated directly by FSH or LH (within 1–6 h) or those promoted by MAP kinase inhibition ($<$20 h).

In summary, gonadotropin-induced expression of StAR is linked to the earliest stages of granulosa cell differentiation and the initiation of steroidogenesis in prehierarchal follicles as well as the potentiation of progesterone production in preovulatory follicles. Moreover, results suggest that cell signaling via the MAP kinase pathway may act to tonically suppress gonadotropin-mediated StAR and LH-R expression in undifferentiated granulosa cells; thus, the removal of such inhibition may be critical for follicle recruitment into the preovulatory hierarchy as well as for the progression of granulosa cell differentiation. On the other hand, LH-induced StAR expression in preovulatory follicle granulosa cells, although not potentiated by inhibition of MAP kinase signaling, is probably required for full potentiation of progesterone synthesis such as occurs in response to the preovulatory LH surge *in vivo*. The dramatic increase in StAR protein in response to LH treatment is predicted to result from the initiation of translation from preexisting mRNA and is supported by the induction of new transcription.

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