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TIME-DEPENDENT EFFECTS OF FOLLICLE-STIMULATING HORMONE ON PROGESTERONE METABOLISM BY CULTURED RAT GRANULOSA CELLS

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SUMMARY: The effects of FSH on the accumulation of endogenous progesterone and 20α-hydroxy-4-pregnen-3-one as well as on the metabolism of [4-^{14}C]progesterone were studied in 24, 48 and 72 h cultures of rat granulosa cells. FSH stimulated the accumulation of both progesterone and 20α-hydroxy-4-pregnen-3-one by 6-18 fold and 2.5-44 fold, respectively. Short term exposure (24 h) to FSH resulted in the ratio of progesterone to 20α-hydroxy-4-pregnen-3-one accumulations significantly increased (by 2.6 fold), while the reverse was observed for the longer (48 and 72 h) cultures whereby control levels of the ratio of progesterone to 20α-hydroxy-4-pregnen-3-one were significantly greater (by 50% and 270%, respectively) than those of FSH-treated cultures. Effects of FSH on [4-^{14}C]progesterone were also time-dependent. Twenty-four hour cultures were associated with FSH induced inhibition of 20α-reduced metabolites of progesterone (by 55%) while the 48 and 72 h exposures to FSH resulted in a significant increase of the 20α-reduced metabolites above control levels (by 73% and 230%, respectively). Consequently, it is postulated that FSH may exert biphasic time-dependent actions on 20α-hydroxysteroid dehydrogenase activity with short term inhibitory and longer term stimulatory effects.

Stimulatory actions of FSH on P_4 production by granulosa cells in various species are well documented (1-4). Our recent studies have demonstrated that metabolism of radiolabelled P_4 by rat granulosa cells was inhibited by FSH in a dose-dependent fashion by specific inhibition of the 20α-HSD activity (5,6). Consequently, we have postulated that decreased catabolism of P_4 may, in part, contribute to an overall FSH-elicited rise in P_4 accumulation. However, our observation of inhibitory effects of FSH on 20α-HSD activity was difficult to reconcile with findings of others that FSH elicits stimulatory actions on 20α-HSD activity (7,8). The present study was

The abbreviations used are: FSH, follicle-stimulating hormone; P_4, progesterone; 20α-OHP, 20α-hydroxy-4-pregnen-3-one; 20α-HSD, 20α-hydroxysteroid dehydrogenase.

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designed to re-assess the actions of FSH on metabolism of progesterone by rat granulosa cells and especially to determine a time-course of FSH-induced changes of accumulation of endogenously produced P_4 and 20α-OHP as well as conversion of exogenous, radiolabelled, P_4 to its 20α- and 5α-reduced metabolites.

MATERIALS AND METHODS

Granulosa cells were isolated from immature intact Sprague-Dawley rats as described previously (5). The rats were treated with 17β-estradiol (1 mg/mL) for three days prior to sacrifice. Granulosa cells were cultured in McCoy's 5a medium (modified, without serum), supplemented with penicillin (100 U/mL), streptomycin (100 ug/mL), fungizone (250 ng/mL) and L-glutamine (2 mM), all obtained from GIBCO (Grand Island, NY). The cultures were carried out in 12 x 75 mm polystyrene culture tubes (Falcon Plastics, Los Angeles, CA) at 37°C under an atmosphere of 5% CO_2 in humidified air. Cell concentration was adjusted to 3 $x10^5$ cells/mL. Cultures were carried out in the presence or absence of FSH (0.2 ug/mL). The preparation of FSH used (NIADDK-oFSH-16; FSH activity 20 x NIH-FSH-S1 U/mg; LH activity 0.04 x NIH-LH-S1 U/mg; PRL activity less than 0.1 by weight) was kindly donated by the Hormone Distribution Office, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD. The cells were cultured while exposed to the same treatment for up to 72 h with media changed every 24 hours. Spent media were stored at -20°C for subsequent analysis of steroid content. At the end of each 24 h period a portion of cultures (four control and four FSH-treated) was incubated for 4 h with fresh media containing [4-^{14}C]P_4 (0.5 nmol/mL). Following the incubation with radioactive P_4 the cultures were terminated, analysed for protein content by the method of Lowry et al. (9), and the media collected and stored at -20° until further analysis.

All harvested media were extracted twice with diethyl ether; the extracts were evaporated at 35°C under nitrogen. The extracts from all cultures carried out in the absence of radiolabelled P_4 were analysed for P_4 and 20α-OHP content using the technique of radioimmunoassay. Specific antisera were kindly donated by Dr. David T. Armstrong, University of Western Ontario, London, Ontario. [1,2,6,7,16,17-^{3}H]P_4 (sp. act. 110 mCi/mmol) was obtained from Amersham Co. (Arlington Heights, IL); [1,2-^{3}H]20α-OHP (sp. act. 53.4 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA). The extracts from short-term 4 h incubations of cells with radiolabelled P_4 were reconstituted in small volumes of absolute ethanol and supplemented with the following steroids (5 ug each) serving as internal standards: P_4, 20α-OHP, 5α-pregnane-3,20-dione, 20α-hydroxy-5α-pregnan-3-one, 3α-hydroxy-5α-pregnan-20-one and 5α-pregnane-3α,20α-diol. The separation of the above steroids was carried out utilizing thin-layer chromatography (TLC) technique. The extracts were spotted on silica gel coated plastic sheets (Merck, Darmstadt, Federal Republic of Germany) and the separation was performed in a system of chloroform:ethyl acetate:acetone (87.5:10:2.5, v:v:v) at 4°C. Medical no-screen x-ray films (Kodak, Rochester, NY) were exposed to developed chromatograms. Subsequently, the internal steroid standards were visualized by spraying the chromatograms with sulfuric acid:ethanol (1:1, v:v) and charring for 15 min at 120°C. The aligning and transilluminating of the chromatograms with their corresponding radioautograms facilitated the preliminary identification of radioactive metabolites of P_4. Final identification was achieved by recrystallization to constant specific activity (10) of noncharred metabolites eluted from the appropriate zones of the portion of chromatograms. Radioactivities were measured using an LKB 1217 Rackbeta Liquid Scintillation counter (LKB Instruments, Rockville, MD).

Statistical analysis of results was carried out using the Student's t test; the means were considered significantly different for $p<0.01$.

RESULTS

The accumulation of endogenous P_4 and 20α-OHP during each 24 h period of the three-day culture is presented in Fig. 1. For all culture periods, the presence of FSH resulted in stimulation of the production of both P_4 and 20α-OHP. In the presence of FSH, the P_4 production by granulosa cells was 6-18 fold greater than that in the control cultures. Similarly, the presence of FSH resulted in a 2.5-44 fold increase of the production of 20α-OHP. In the absence of FSH, the production of both P_4 and 20α-OHP was declining daily. In contrast, the presence of FSH resulted not only in higher than control but also in increasing daily production of 20α-OHP. The P_4 production in the presence of FSH was the greatest during 24-48 h period and signifi-

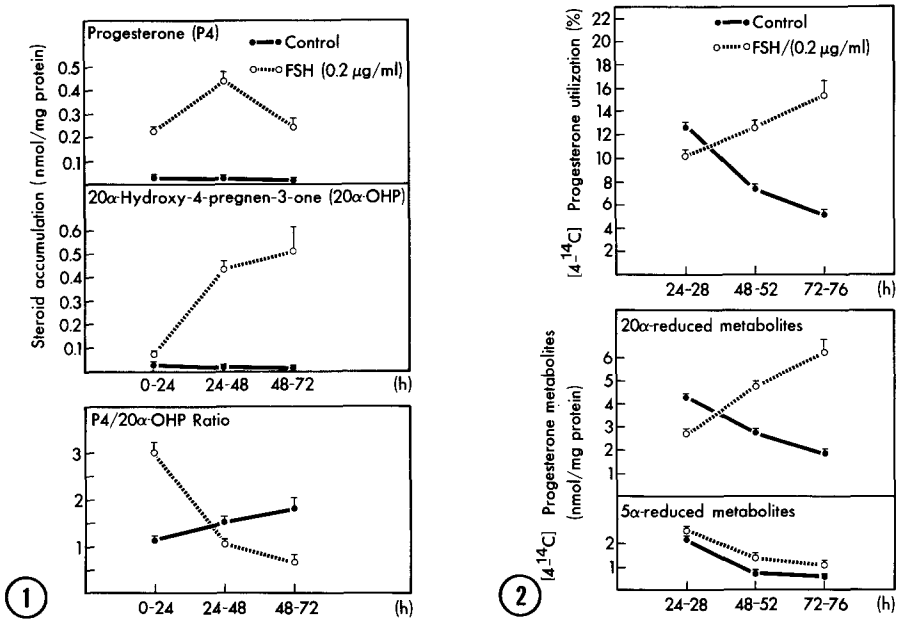

Fig. 1. Effect of FSH on the daily output of P_4 and 20α-OHP (upper panels) and on P_4/20α-OHP ratio (lower panel) by rat granulosa cells in culture. The cells were cultured with or without FSH (0.2 ug/mL) for up to 72 h with media changed every 24 h. Each point represents the mean (±SEM) from four cultures.

Fig. 2. Effect of FSH on the [4-^{14}C]P_4 utilization (upper panel) and on the accumulation of 20α- and 5α-reduced P_4 metabolites by rat granulosa cells in culture. The cells were cultured for 24, 48 and 72 h as described in Fig. 1 and subsequently incubated with [4-^{14}C]P_4 for 4 h. Each point represents the mean (±SEM) from four incubations.

cantly lower during both 0-24 and 48-72 h period. Consequently, the P_4/20α-OHP ratio, as demonstrated in the lower panel of Fig. 1, was significantly declining during each consecutive 24 h culture period in the presence of FSH, but was increasing in the absence of FSH. In the presence of FSH the P_4/20α-OHP ratio was 2.6-fold greater than that of control cultures during the 0-24 h culture period; subsequently, however, during 24-48 h and 48-72 h periods the P_4/20α-OHP ratio was greater for control cultures and exceeded the respective values of FSH-treated cultures by 50% for 24-48 h, and by 2.7-fold for 48-72 h periods.

Fig. 2 presents results of the four-hour incubation of [4-^{14}C]P_4 by granulosa cells following one- two- or three-day culture with or without FSH. The rate of the utilization of radiolabelled P_4 was declining daily for control cultures and, following the 72 h culture, was only 40 of the value observed after 24 h of culture. In contrast, the exposure to FSH resulted in a daily increasing ability of granulosa cells to metabolize [4-^{14}C]P_4 with the rate of the utilization after the 72 h culture being 50% greater than that observed after the 24 h culture. It should be noted that after the 24 h culture the rate of the utilization of [4-^{14}C]P_4 was 25 greater for the control than for the FSH-treated cultures while following prolonged incubations, a reversal of this effect was observed and, after 48 and 72 h, the FSH-treated cultures were utilizing radiolabelled P_4 at rates respectively 70% and 200% greater than those of control cultures.

The utilization of P_4 was associated with its metabolism via two principal enzymatic activities: 20α-hydroxysteroid dehydrogenase (20α-HSD) and 5α-reductase. The lower panel of Fig. 2 presents the accumulation of 20α- and 5α-reduced metabolites reflecting activities of 20α-HSD and 5α-reductase, respectively. The term "20α-reduced metabolites" refers to the following steroids: 20α-OHP, 20α-hydroxy-5α-pregnan-3-one and 5α-pregnane-3α,20α-diol; the term "5α-reduced metabolites" refers to: 5α-pregnane-3,20-dione, 20α-hydroxy-5α-pregnan-3-one, 3α-hydroxy-5α-pregnan-20-one, and 5α-pregnane--3α,20α-diol. The effect of FSH on the 20α-HSD activity (ie. accumulation of

20α-reduced metabolites) was similar to that described for the overall utilization of [4-^{14}C]P_4. Following the 24 h culture, the activity of 20α-HSD was significantly less (by 55%) for FSH-treated cells compared to controls. However, further culture resulted in a continuous decrease of the 20α-HSD activity for control cultures and a simultaneous increase of the 20α-HSD activity for FSH-treated cultures. Consequently, after 48 and 72 h cultures, the activity of 20α-HSD for FSH-treated cultures was exceeding that of controls by 73% and 230%, respectively.

The changes of the 5α-reductase activity (ie. accumulation of 5α-reduced metabolites) were less pronounced. Both control and FSH-treated cultures had significantly greater 5α-reductase activity after 24 h than after 48 and 72 h. FSH treatment resulted in a significant increase of the 5α-reductase activity over the control levels after 48 and 72 h (by 106% and 95%, respectively); however, after 24 h FSH had only slight and statistically insignificant effects.

DISCUSSION

The present study demonstrated that the ability of rat graulosa cells to produce and catabolize P_4 changes in the course of culture and that FSH effects on P_4 synthesis and catabolism are time-dependent. Specifically, it appears that: (i) FSH stimulates P_4 and 20α-OHP synthesis, (ii) FSH initially decreases and subsequently increases 20α-HSD activity, and consequently, (iii) FSH initially decreases and subsequently increases the rate of P_4 catabolism.

The intriguing observation of the biphasic time-course of the FSH effect on the 20α-HSD activity was demonstrated by both the changes of the ratio of endogenous progestins (P_4 to 20α-OHP) and the changes of the accumulation of the 20α-reduced metabolites of [4-^{14}C]P_4.

The present results should be viewed in context of the so far seemingly conflicting reports of either only inhibitory or only stimulatory actions of FSH on 20α-HSD activity in granulosa cells. The present observation of effects of 24 h exposure of the cells to FSH correlate well with our previous findings (5,6) demonstrating FSH induced inhibition of P_4 catabolism

by specific inhibition of 20α-HSD activity. In the previous studies, the maximum inhibition was also observed after 24 h of exposure to FSH (6). Those previous studies, however, were carried out using minimum essential medium (MEM) and cultures were carried out only for up to 48 h at which time the FSH-induced inhibition of 20α-HSD activity was no longer demonstrable. The present studies extended our previous work in two particular aspects. Firstly, the results were obtained on cultures carried out for up to 72 h utilizing McCoy's Medium which is thought to be more suitable than the MEM for longer incubations. Secondly, not only the metabolism of radiolabelled P_4 was monitored, but also the measurements of the endogenous production of P_4 and 20α-OHP were performed allowing thus for two independent techniques measuring 20α-HSD activity. The present data have shown a clearly biphasic pattern of effects of FSH on 20α-HSD whereby subsequently to the above discussed FSH-dependent inhibition of 20α-HSD, there was a significant FSH-dependent induction of 20α-HSD activity. This effect was observable for 48 h and was more prounounced for 72 h cultures. These latter actions of FSH are in agreement with several reports by others demonstrating that FSH stimulates 20α-HSD activity in granulosa cells following both *in vivo* (7) and *in vitro* (8) exposure. Increases of 20α-HSD were also reported for PMSG (11) and hCG (12). Consequently, the present study provides evidence for the presence of both inhibitory and stimulatory time-dependent actions of FSH and thus appears to reconcile previous seemingly irreconcilable reports.

The physiological significance of the above discussed opposing actions of FSH is presently uncertain. Such actions may augment the P_4 accumulation in short term by decreasing P_4 catabolism while providing also a "built in" limiting (?luteolytic) mechanism assuring increased catabolism of P_4 following prolonged exposures of cells to FSH. In addition, the alteration of 20α-HSD activity has obvious impact on the levels of 20α-OHP which is believed to possess certain, potentially important, biological activities; 20α-OHP has been shown to prolong luteinizing hormone production in mated

rabbits (13), to facilitate ovulation in the rat (14) and to modulate serum gonadotropin levels in the rat (15).

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