Microfilaments and FSH stimulation of rat granulosa cell steroidogenesis *in vitro*

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Summary. The secretion of progesterone and 20 α -hydroxypregn-4-en-3-one (20 α -dihydroprogesterone) by granulosa cells from 30-day-old rats pretreated with PMSG (4 i.u.; i.p.) was significantly increased in a time- and concentration-dependent manner by FSH or cytochalasin B. Whereas FSH markedly stimulated progestagen secretion during 3 h of incubation, a significant enhancement of the steroidogenic response was not noted until 12 h of exposure to the inhibitor *in vitro*. Although cytochalasin B also enhanced the submaximal stimulation of progestagen production by FSH (15 ng/ml), it was ineffective in the presence of maximal stimulatory concentration of the gonadotrophin (150 ng/ml). With increasing concentrations of cytochalasin B, the ability of FSH to further stimulate progestagen secretion was progressively reduced. Granulosa cells cultured in medium alone contained a prominent cytoplasmic array of microfilaments which was markedly reduced by FSH or cytochalasin B. FSH and, to a greater extent, cytochalasin B elicited concentration-dependent reductions in the mean area occupied by the cells on the culture surface, the contour index (a size-independent representation of cell profile irregularity) and cell perimeter, indicating that the cells underwent less spreading and were more spherical and regular in outline in the presence of either agent. The FSH-induced reductions in the three shape-related parameters were augmented by cytochalasin B although the influence of the FSH on the mean area and perimeter was progressively reduced in the presence of higher concentrations of cytochalasin B. These findings are consistent with the concept that microfilaments influence cell shape and steroidogenesis in granulosa cells *in vitro* and that FSH alters microfilament distribution and shape of cultured granulosa cells in eliciting its steroidogenic influence.

Keywords: microfilaments; steroidogenesis; FSH; granulosa cells

Introduction

The gonadotrophic control of ovarian steroidogenesis is a complex process involving the regulation of steroidogenic enzyme activities as well as steroid precursor availability (Marsh, 1976). Whereas FSH is known to increase the activities of a number of key steroidogenic enzymes in granulosa cells (Hsueh *et al.*, 1984), how the gonadotrophin influences substrate availability at the enzymic sites is unclear. The production of steroid is believed to involve considerable movement of substrate and intermediates from one intracellular locale to another. While cholesterol, normally stored as esters in lipid droplets, is transported to and taken up by the mitochondria for the synthesis of pregnenolone, further metabolism to progesterone and 20 α -hydroxy-pregn-4-en-3-one (20 α -dihydroprogesterone)

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by 3β -hydroxysteroid dehydrogenase and 20α -hydroxysteroid dehydrogenase, respectively, requires the translocation of the pregnenolone to smooth endoplasmic reticulum where the enzymes are situated (Marsh, 1976; Hsueh *et al.*, 1984). Previous studies from our laboratory have demonstrated that granulosa cells cultured in the presence of FSH, cyclic AMP, a calcium ionophore, cytoskeleton-perturbing agents or a supporting collagen matrix retained a primarily spherical configuration with minimal cytoplasmic spreading and secreted significantly more progesterone than did control cells (Carnegie *et al.*, 1987, 1988; Carnegie & Tsang, 1987, 1988; S. K. Jindal, J. A. Carnegie & B. K. Tsang, unpublished data). It was also noted that depolymerization of microtubules or microfilaments with colchicine or cytochalasin B, respectively, increased basal progesterone and 20α -dihydroprogesterone production (Carnegie *et al.*, 1987; Carnegie & Tsang, 1987, 1988). Since the cytoskeleton is believed to be important for the development and maintenance of cell shape as well as the control of the intracellular distribution and/or movement of various organelles, secretory proteins and hormones (Buckley & Porter, 1967; Olmsted & Borisy, 1973; Snyder & McIntosh, 1976), it has been suggested that, in cultured granulosa cells stimulated by FSH, mitochondrial uptake of cholesterol is enhanced due to a closer association between these organelles and lipid droplets, facilitated in part by cytoskeleton-mediated rounding of the cells (Carnegie & Tsang, 1988).

In the present study we have further examined the relationship between microfilaments and steroidogenesis by investigating the influence of cytochalasin B on basal and FSH-stimulated granulosa cell production of progesterone and 20α -dihydroprogesterone *in vitro* and the effects of these agents on cellular morphology and microfilament distribution.

Materials and Methods

Ovarian follicular development was initiated in immature Sprague-Dawley rats with an intraperitoneal injection of 4 i.u. MSG (Equinex: Ayerst Labs, Montreal, Quebec) on the morning of Day 28 $\pm$ 1. Most follicles had reached the antral stage of development when the ovaries were removed 48 h later. Granulosa cells, released into Eagle's Minimal Essential Medium (MEM; GIBCO Laboratories, Mississauga, Ontario) by follicle puncture, were collected by centrifugation, as described previously (Tsang & Carnegie, 1983). Removal of non-viable cells by a sequential trypsin-DNase treatment (Farookhi, 1982) increased the percentage viability of the granulosa cell preparations from an initial value of 20–30% to a final level of 90–95%. Cells were incubated for 0, 3, 6, 12 and 24 h (on coverslips if to be examined subsequently by microscopy) at 36°C under an atmosphere of 5% CO₂ and 95% air in 1 ml MEM containing various concentrations of FSH (0–400 ng NIAMDD-oFSH-13/ml) and/or cytochalasin B (0–10 µg/ml).

Medium was collected at the end of the incubation period for extraction with ether and determination of progesterone and 20α -dihydroprogesterone by specific RIA (Orczyk *et al.*, 1979). The antiserum for the progesterone assay has previously been characterized (Leung & Armstrong, 1979). With the exception of 8.7% cross-reactivity with 20β -hydroxy-pregn-4-en-3-one, the antiserum to 20α -dihydroprogesterone cross-reacts minimally (<0.1%) with other progestagens, oestrogens and androgens (Morley *et al.*, 1987). For both assays, the intra- and interassay coefficients of variation were <10% and <20%, respectively.

Cells grown on coverslips for subsequent investigations of microfilament organization/distribution and cell shape were prepared according to the following protocols.

Morphometric analysis of cell shape changes. Granulosa cells were fixed overnight at 4°C in 2% glutaraldehyde, washed and stained with haematoxylin and eosin. Changes in various cell shape-related characteristics were quantitated using a previously documented procedure (Carnegie *et al.*, 1987). Briefly, random areas of isolated cells were photographed and the film was projected onto a digitizer pad (Summagraphics Corporation, Fairfield, CT, U.S.A.) to enable individual cell outlines to be traced with a cursor. The measures for which data were collected with the use of an MSA II computer program (Atlantic Scientific Systems Group, Ottawa, Canada) and an Apple II Plus microcomputer were: cell perimeter, the surface area occupied by each cell on the coverslip, and the contour index. Contour index is a size-independent measurement of the shape of a profile whereby increases in profile irregularity are represented by higher numerical values (Schrek, 1972). A cell taking the shape of a perfect circle is associated with a contour index of 3.54.

Microfilament organization/distribution. Microfilament organization/distribution in granulosa cells was examined using 7-nitrobenz-2-oxa-1,3-diazole-phalloidin (NBD-phalloidin) and a modification of the protocol of Barak *et al.* (1980), as provided by Molecular Probes Inc. (Eugene, OR, U.S.A.). Cells were washed twice with phosphate-buffered saline (PBS; pH 7.0), fixed (10 min at room temperature) in 3.7% paraformaldehyde and then washed twice more with PBS. After extraction (4 min at –20°C) of the cells with acetone, they were air dried and stained (20 min at room temperature) with 220 nM-NBD-phalloidin in PBS. The coverslips were then rapidly washed twice with PBS and

mounted on slides in a 1:1 (v/v) solution of PBS and glycerol before observation with a Carl Zeiss microscope equipped with epifluorescence optics.

Statistical analysis. Results were treated by analysis of variance and the significance of differences between treatment groups was determined by Duncan's new multiple range test or Scheffe's post-hoc comparison test. When there was evidence of heterogeneity of variance, statistical analysis was performed on logarithmically transformed data.

Results

FSH and cytochalasin B significantly ($P < 0.01$) increased progesterone and 20α -dihydroprogesterone secretion by granulosa cells *in vitro* (Fig. 1). The concentrations of FSH and cytochalasin B which elicited half-maximal stimulation of total progestagen secretion were approximately 45 ng/ml and $0.4 \mu\text{g/ml}$, respectively (Fig. 1). By 3 h of incubation (earliest time examined), FSH (15 ng/ml) had markedly stimulated progesterone and 20α -dihydroprogesterone secretion by 10- and 6-fold, respectively. However, a significant increase in progestagen secretion by cytochalasin B was not noted until 6–12 h of culture (Fig. 2).

Fig. 1. Concentration-dependent stimulation of basal granulosa cell secretion of progesterone (P), 20α -dihydroprogesterone (20α -OH-P) and total progestagen (P + 20α -OH-P) by (a) FSH and (b) cytochalasin B during 24 h of culture. Each point represents the mean $\pm$ s.e.m. of 8 (a, 2 experiments) or 21 (b, 6 experiments) cultures.

Cytochalasin B significantly enhanced the submaximal stimulation of both progestagens by FSH (15 ng/ml) in a concentration-dependent manner but was ineffective in the presence of a maximally stimulatory concentration of the gonadotrophin (150 ng/ml). However, with increasing concentrations of cytochalasin B the ability of FSH to further stimulate progestagen secretion was markedly reduced (Fig. 3).

Control cells underwent considerable spreading during 24 h of culture, and staining of the cells with NBD-phalloidin indicated the presence of parallel bundles of microfilaments (stress fibres) close to the plasma membrane and in the narrow cytoplasmic extensions (Fig. 4a). On the other hand, cells cultured with $10 \mu\text{g}$ cytochalasin B/ml remained rounded *in vitro* and, while positive staining for filamentous actin was still observed, it was reduced in extent and confined to small,

Fig. 2. Effects of incubation time on progesterone (P), 20 α -dihydroprogesterone (20 α -OH-P) and total progestagen (P + 20 α -OH-P) secretion by granulosa cells in the absence (control) or presence of FSH (15 ng/ml) or cytochalasin B (CB; 10 μ g/ml). Each point represents the mean $\pm$ s.e.m. of 12 cultures (4 experiments).

Fig. 3. The influence of various concentrations of cytochalasin B (CB) on basal and FSH-stimulated progestagen secretion by granulosa cells during a 24-h culture period. Each histogram represents the mean $\pm$ s.e.m. of 9 cultures (3 experiments).

Fig. 4. Granulosa cells stained with NBD-phalloidin to demonstrate the subcellular distribution of actin-containing microfilaments following 24 h of culture: (a) controls, (b) cytochalasin B (10 µg/ml), (c) FSH (150 ng/ml), or (d) FSH + cytochalasin B (150 ng/ml and 10 µg/ml, respectively).

peripheral regions of the granulosa cells (Fig. 4b). Similarly, culture of cells in the presence of 150 ng FSH/ml was associated with a marked decrease in cellular staining with NBD-phalloidin, although the cells were not as uniformly rounded as those cultured with cytochalasin B (Fig. 4c). In the presence of FSH and cytochalasin B, staining for actin filaments was reduced to such an extent that it was not detectable using this histochemical approach (Fig. 4d).

Fig. 5. Changes in the mean area on the coverslips, contour index and perimeter of granulosa cells after 24 h of culture in the presence of various concentrations of FSH and/or cytochalasin B (CB). Each histogram represents the mean $\pm$ s.e.m. of 99–110 cells.

Fig. 6. Concentration-dependent effects of FSH and/or cytochalasin B (CB) on granulosa cell distribution based on area of the culture surface occupied by each cell (a) and on contour index (b) after 24 h of culture. In (a), each histogram represents 45 μm²; in (b), each histogram represents a range of 0.3 units. Data were derived from measurements of 94–107 cells per treatment group.

Figure 5 illustrates that the stimulation of progesteragen secretion by cytochalasin B or FSH was accompanied by a significant and concentration-dependent decrease ($P < 0.001$) in cell shape measures as outlined below. When cytochalasin B and FSH were both present in the granulosa cell

cultures, the changes in these shape-related values were greater than those noted in the presence of either agent alone (Fig. 5). However, when granulosa cells were incubated with concentrations of cytochalasin B which maximally stimulated basal progesterin secretion (10 µg/ml, Fig. 1b), FSH was no longer effective in eliciting a further decrease in the three shape-related values.

The influence of FSH and cytochalasin B, alone or in combination, on the distribution of the granulosa cell population according to cell areas and contour indices is illustrated in Fig. 6. A mean cell area of $303 \pm 15 \mu\text{m}^2$ for the control group (Fig. 5) represented a broad range of areas with 73% of the granulosa cell population distributed within the range of 135 to 405 µm² (Fig. 6a). In the presence of increasing concentrations of FSH or cytochalasin B, the proportion of cells with an area of < 135 µm² increased markedly. At 150 ng FSH/ml or 10 µg cytochalasin B/ml, over 80% or 90% of the cells were < 90 µm², respectively. Whereas FSH (150 ng/ml) significantly potentiated the submaximal effect of cytochalasin B (0.4 µg/ml) on cell area it was ineffective in the presence of 10 µg cytochalasin B/ml (Fig. 6a).

Likewise, contour indices of the control cells were also variable, with the largest division (> 20% of the population) observable between 4.7 and 5.0 and with approximately 10% of the cells associated with other unit intervals between 4.1 and 5.1 (Fig. 6b). FSH or cytochalasin B elicited a concentration-dependent shift in the cell population such that 64% or 98% of the cells exhibited contour indices between 3.5 and 4.0 at 150 ng/ml or 10 µg/ml, respectively. Moreover, FSH caused further shifts in contour index distribution of the cell population in the presence of 0.4 but not 10 µg cytochalasin B/ml: at 10 µg/ml, over 90% of the cells had contour indices of < 4.0.

Discussion

In the current investigation, we have found that concentration-dependent inhibition of microfilament polymerization was associated with a corresponding stimulation of the secretion of both progesterone and 20α-dihydroprogesterone in cultured granulosa cells. While such a stimulatory effect of cytochalasin B has been previously reported (Carnegie & Tsang, 1988), this is the first study in which a time course of the induction of progestagen secretion by this agent and its interaction(s) with FSH in the regulation of granulosa cell steroidogenesis have been examined *in vitro*. In contrast to the rapid stimulation by FSH, a steroidogenic response of the granulosa cells to the cytoskeletal-perturbing agent was associated with a time lag of 6–12 h. Similarly, a response of ovarian or adrenal cells to the stimulation of steroidogenesis by colchicine, an inhibitor of microtubule polymerization, has been observed during cultures of 6–24 h but not in short-term incubations of less than 4 h (Temple & Wolff, 1973; Ray & Strott, 1978; Carnegie *et al.*, 1987). Furthermore, the time-dependency of this effect may, at least in part, explain why previous studies using incubations of up to 3 h have reported variable effects of cytochalasins on basal steroidogenesis in ovarian cells (Azhar & Menon, 1981; Gwynne & Condon, 1982; Silavin *et al.*, 1984). The existence of a time lag also lends support to the concept that microfilament- and microtubule-dependent regulation of granulosa cell steroidogenesis is closely associated with changes in cellular morphology. Although all granulosa cells were rounded in shape at the beginning of the culture period, the cytochalasin B-treated cells remained rounded while the control cells underwent considerable spreading with increasing time in culture. Hence the two treatment groups became increasingly different in terms of both cell shape and the levels of steroid hormone secreted. We have previously suggested that, in granulosa cells in which a rounded cell shape was maintained by the use of microtubule-depolymerizing agents, the stimulation of progestagen production resulted from increased mitochondrial uptake of cholesterol due to a closer association between these organelles and lipid droplets (Carnegie *et al.*, 1987). Further support for this concept comes from our studies in which granulosa cells suspended in collagen matrices remained more rounded and secreted more progesterone than did cells cultured in medium alone (Carnegie *et al.*, 1988). However, the rounded cells cultured in collagen gels were unresponsive to colchicine in terms of progesterone secretion, implying that perturbation of the

cytoskeleton increased granulosa cell progesterone production only under those conditions in which the treatment also altered cell shape.

Several lines of evidence suggest that microfilaments are involved in the gonadotrophic regulation of granulosa cell steroidogenesis. In this study we found that those concentrations of FSH that gave dose-dependent stimulation of progesterone production reduced the apparent synthesis and bundling of microfilaments into stress fibres and, correspondingly, decreased cell spreading *in vitro*. Similarly, it has been reported that granulosa cell monolayers incubated with various gonadotrophins underwent transient rounding which was accompanied by a reduction in cellular microfilament content, primarily within the rounded cell bodies rather than the anchoring cytoplasmic extensions (Lawrence *et al.*, 1979; Soto *et al.*, 1986). Furthermore, we have shown in this investigation that inhibition of microfilament polymerization with cytochalasin B significantly enhanced the steroidogenic response of the granulosa cells to a submaximally- but not a maximally-stimulatory concentration of FSH. Conversely, with progressive inhibition of microfilament elongation and accompanying increases in steroidogenesis by cytochalasin B, the ability of FSH to stimulate further these responses was correspondingly reduced. These findings suggest that the action of FSH on granulosa cell progesterone secretion involves in part the regulation of microfilament organization. It is of interest that the stimulation of adrenal cells by ACTH *in vitro* is accompanied by a reduction in cytoplasmic actin content over a 12-h period (Cheitlin & Ramachandran, 1981).

Inhibitory effects of cytochalasins on LH- or hCG-stimulated progesterone production during short-term (up to 3 h) incubation of granulosa cells or luteal slices have been reported (Silavin *et al.*, 1980, 1984; Azhar & Menon, 1981; Gwynne & Condon, 1982). While these results appear to be at variance with those obtained in this study, a comparison of the culture conditions suggests another interpretation. Since cytochalasin did not inhibit gonadotrophin-induced cyclic AMP production (Silavin *et al.*, 1980; Azhar & Menon, 1981), it did not appear to have influenced hormone-receptor binding or actin-regulated movement of the hormone-receptor complex within the membrane (Geiger, 1985). Cells in suspension or incubated as slices would not undergo the intense spreading and exhibit stress fibre formation, as noted in cells cultured for 24 h. Hence the stimulatory action of cytochalasin on granulosa cell steroidogenesis induced by the lower concentration of FSH during 24 h may reflect the ability of both agents to reduce stress fibre formation and the generation of an elongated, flattened cell form. It would be of interest to evaluate the influence of cytochalasin on FSH-regulated progesterone production by granulosa cells cultured under conditions which allowed them to remain rounded.

The regulation by FSH of microfilament polymerization and organization could profoundly influence organelle distribution within granulosa cells. Microfilaments have been shown to be linked to mitochondria in granulosa cells (Anderson & Batten, 1980) and to influence their subcellular organization (Soto *et al.*, 1986). The gonadotrophic stimulation of mitochondrial cholesterol uptake is well-established (Marsh, 1976) and may be accomplished in part by bringing mitochondria into closer association with lipid droplets. The present studies suggest that microfilaments are important in the regulation of granulosa cell steroidogenesis by FSH *in vitro*. The gonadotrophic stimulation of steroidogenesis is accompanied by a loss of these cytoplasmic polymers which may function to increase mitochondrial uptake of cholesterol by interacting with microtubules to bring these organelles into closer proximity with lipid droplets or simply by providing a more pliant cytoplasmic environment favouring organelle redistribution.

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