Effect of the human follicle-stimulating hormone-binding inhibitor and its N-terminal fragment on follicle-stimulating hormone-induced progesterone secretion by granulosa cells *in vitro*

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Intrafollicular factors play an important role in folliculogenesis. The follicle-stimulating hormone (FSH)-binding inhibitor (FSHBI), purified by our laboratory from human ovarian follicular fluid, has been shown to suppress ovulation and induce follicular atresia/apoptosis in mice as well as impair fertility in marmosets, the new world monkeys. The octapeptide, a peptide corresponding to the N-terminal region of human FSHBI (hFSHBI), has been synthesized and also shows FSHBI activity *in vitro*. In the present study, we have attempted to identify the mechanism of action of the peptide in granulosa cell cultures. Rat granulosa cell cultures were treated with varying concentrations of the octapeptide or partially purified hFSHBI (gel chromatography fraction hGF_2) in the presence or absence of human FSH (hFSH) and the amount of progesterone (P_4) secreted in the culture supernatants after 3 h/48 h was estimated. Both hGF_2 and the octapeptide failed to alter basal levels as well as 8-bromo cAMP-induced P_4 production, while FSH-induced P_4 secretion was inhibited in a dose-dependent manner. These studies reveal that the octapeptide, a fragment of FSHBI, and the native protein have similar activity *in vitro* and both compounds alter FSH action at the receptor level upstream of cAMP formation.

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1. Introduction

The gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the anterior pituitary are the prime factors responsible for the development of follicles to the preovulatory stage (Vitt *et al* 2000). FSH provides the primary (endocrine) stimulus for folliculogenesis via the activation of cAMP-mediated post-receptor signalling in granulosa cells of the preantral follicles (Hillier 1994). Some of the physiologically important genes that are induced by FSH signalling include the P450 aromatase, LH receptor, steroidogenic acute regulatory protein, P450 side chain cleavage (scc) enzyme, 3β-hydroxysteroid dehydrogenase (3βHSD), inhibin and activin (Richards 1994; Otsuka *et al* 2001). The physiological importance of FSH action is demonstrated by the fact that when FSH is restricted, the developing follicles degenerate by apoptosis and ovulation does not occur (Hsueh *et al* 1994; Nandedkar *et al* 1996; Nandedkar *et al* 1998; Otsuka *et al* 2001).

In the mid-1970s, it became evident that the two gonadotrophins (LH and FSH) and the two steroids (oestrogen and progesterone) are insufficient to provide all the trophic and feedback signals required to orchestrate the myriad events that take place during folliculogenesis.

Keywords. Steroid; progesterone; FSH-binding inhibitor; follicular fluid; granulosa cells; luteinization

Abbreviations used: FSH, follicle-stimulating hormone; FSHBI, FSH-binding inhibitor; hFSH, human FSH; hFSHBI, human FSHBI; LH, luteinizing hormone; IVF, *in vitro* fertilization; RP-HPLC' reverse-phase high-performance liquid chromatography; PMSG, pregnant mare serum gonadotrophin; scc, side chain cleavage; OP, octapeptide; StAR, steroidogenic acute regulatory protein; arom, aromatase; GDF-9, growth differentiation factor-9; TGF, transforming growth factor

Several features of ovarian function are best explained by the participation of local regulatory factors: recruitment for follicular growth, selection of the dominant follicle, atresia of the rest of the cohort and meiotic arrest of oocytes until ovulation. Several non-steroidal factors have been identified in ovarian tissue, postulated to be synthesized and secreted by ovarian cells and proposed to be local regulators of ovarian function (Suter *et al* 1988; Lee *et al* 1990).

Our laboratory has identified one such low molecular-weight peptide (<5 kDa) from sheep and human ovarian follicular fluid termed ovarian follicular fluid peptide. This peptide blocks binding of ^{125}I-hFSH to receptors on granulosa cells in a radioreceptor assay (Nandedkar *et al* 1989) and preliminary studies performed using the partially purified form of the peptide of human origin have indicated that it is secreted by granulosa cells of medium and atretic follicles (Nandedkar *et al* 1994). Administration of the human ovarian follicular fluid peptide, now referred to as FSH-binding inhibitor (FSHBI) and first identified by Darga and Reichert (1978), suppresses ovulation and induces follicular atresia in adult mice (Nandedkar *et al* 1988; 2001). When administered during the preovulatory period in the common marmoset, the peptide induced luteal insufficiency and impaired fertility (Nandedkar *et al* 1989). Our laboratory has purified FSHBI from human ovarian follicular fluid (Nandedkar *et al* 2001; Wadia *et al* 2003). Its N-terminal region was sequenced. Based on this 8 amino acid sequence, a peptide was synthesized which was termed as the octapeptide (OP). The OP also demonstrated FSHBI activity in the radioreceptor assay and induced luteal insufficiency in the common marmoset, as shown by the native peptide (Wadia *et al* 2003). The *modus operandi* of the native peptide as well as the OP is still unknown and is of interest as they are potential agents of contraception due to their ability to block FSH action.

In the present study, rat granulosa cells were cultured for 3 h or 48 h in serum-free medium along with hFSH to stimulate production of progesterone (P_4). Cultures were then treated with hGF_2, the partially purified form of hFSHBI obtained after passing the latter through a Sephadex G-25 column, or the OP to study their effects on FSH-induced steroidogenesis *in vitro*.

2. Materials and methods

2.1 *Animals*

Holtzman rats were maintained in our animal colony at a temperature of 24°C with a controlled 12 h:12 h dark and light schedule, and food and water *ad libitum*. All procedures were first approved by the animal ethics committee of the Institute. Immature rats (24–27 days old) were administered 10 IU pregnant mare serum gonadotrophin (PMSG) in saline subcutaneously and sacrificed 24 h later. Ovaries were harvested at the time of sacrifice.

2.2 *Purification of hGF_2*

Follicular fluid aspirated from patients undergoing *in vitro* fertilization (IVF) was pooled and centrifuged at 633 *g* for 10 min. The supernatant was subjected to ultrafiltration on an Amicon PM-10 filter (cut-off <10 kDa). The filtrate was further purified on a Sephadex G-25 column (2.7 × 35 cm) with 0.2 M acetic acid as eluting buffer and the absorbance read at 280 nm. The active fraction (peak 2) was termed hGF_2. FSHBI activity was assessed by the radioreceptor assay that has been reported earlier (Nandedkar *et al* 2001). hGF_2 was further purified on preparative reverse-phase high-performance liquid chromatography (RP-HPLC) followed by analytical RP-HPLC. The single peak obtained on analytical RP-HPLC was sequenced and the first 8 amino acids at the N-terminal region were deduced (Wadia *et al* 2003) and referred to as the octapeptide (OP-NH_2-AESNEDGY-COOH).

2.3 *Granulosa cell cultures*

The ovaries were placed in medium 199 supplemented with Hank salts (HiMedia, Mumbai, India) containing 0.04 M sodium bicarbonate (Qualigens, Mumbai, India), 2 mM l-glutamine (Sigma Chemicals, CA, USA), 10 mM HEPES buffer (HiMedia) and 0.1% bovine serum albumin (BSA) (Sigma Chemicals, USA). Follicles were punctured and the granulosa cells harvested in the medium were centrifuged at 158 *g* for 5 min. Viability of the cells was determined using trypan blue dye exclusion. The cells (2×10^6 cells/500 μl) containing 10^{-6} M testosterone (Sigma Chemicals) were pre-incubated at 37 °C for 1 h to remove endogenous P_4 in the cells and then incubated for 3 h. 24-well Nunc plates (Nunc, Denmark) were coated with 250 μl/well foetal calf serum (ICN Chemicals, Germany) and incubated for 24 h at 37 °C. The plates were then washed twice with 250 μl 0.01 M phosphate-buffered saline (PBS) and each well was seeded with 4×10^4 viable cells in a total volume of 500 μl/well in medium containing 10^{-6} M testosterone.

In the first set of experiments, hGF_2 (160–1280 μg/ml) or OP (50–3200 μg/ml) was added either alone or along with hFSH (10 ng/ml) to study their effect on basal and FSH-induced P_4 secretion.

In the second set of experiments, P_4 secretion was induced by the addition of 20 ng/ml pregnenolone (P_5; Sigma Chemicals) or 1600 μM/ml 8-bromo-cAMP (Sigma Chemicals); hGF_2/OP was added to the cells in the presence/absence of these P_4-stimulating agents.

The plates were incubated for 3 h/48 h at 37 °C in the presence of 5% CO_2 and the culture supernatants were collected and stored at –20 °C until assayed for P_4.

2.4 *Progesterone radioimmunoassay*

Progesterone was estimated in the spent culture medium by radioimmunoassay using antiserum to P_4 obtained from ICN Chemicals and ^{3}H-P_4 from Amersham, UK. Antiserum to progesterone showed negligible (<0.1%) cross-reactivity with testosterone. Inter- and intra-assay coefficients of variance for progesterone were 12.5% and 7%, respectively.

2.5 *Statistics*

Statistical analyses were done using analysis of variance with Student *t* test to analyse the differences between experimental and control values. Differences with $P<0.05$ were regarded as statistically significant. All results were replicated three times in separate experiments.

3. Results

3.1 *Secretion of progesterone by granulosa cells* in vitro

The presence of hFSH enhanced the production of P_4 by the granulosa cells in a dose-dependent manner. A peak in P_4, 3.02-fold and 2.48-fold over basal levels in the 3 h and 48 h cultures, respectively, was attained at a dose of 20 ng/ml ($P < 0.005$) hFSH and reached a plateau as the concentration of hFSH was increased (data not shown). Hence, further studies were carried out with a dose of 20 ng/ml hFSH. Both 3 h and 48 h cultures revealed a similar trend in P_4 secretion by the granulosa cells.

3.2 *Effect of hGF_2 and OP on basal and FSH-induced progesterone secretion in culture*

The addition of hGF_2 or the OP to granulosa cells did not bring about any change in basal levels of P_4 (Figs 1 and 2). However, co-treatment of the cells with hGF_2 along with hFSH (10 ng/ml) resulted in the suppression of FSH-induced P_4 synthesis in a dose-dependent manner. The data of the 3 h and 48 h cultures have been depicted in figure 1A and 1B, respectively. In the 3 h cultures, hGF_2 (80 μg/ml) significantly decreased ($P<0.05$) hFSH-induced P_4 secretion with a maximum suppression of 68.4% observed at a dose of 320 μg/ml ($P<0.05$) (figure 1A). In 48 h cultures, this action of hGF_2 was observed at a dose of 320 μg/ml and higher, exhibiting a maximum suppression of 35.7% at a dose of 640 μg/ml ($P < 0.05$) (figure 1B). A similar effect was observed in cultures treated with the OP. The hFSH-induced P_4 secretion was significantly suppressed at a dose of 50 μg/ml ($P < 0.05$) and 400 μg/ml ($P < 0.05$) in the 3 h (figure 2A) and 48 h (figure 2B) cultures, respectively. Maximal suppression of P_4 secretion of 55.1% and 76.9% was observed at a dose of 400 μg/ml ($P < 0.005$) and 1600 μg/ml ($P < 0.05$) of the OP in the 3 h and 48 h cultures, respectively (figure 2). Hence, both hGF_2 as well as the OP brought about a suppression of FSH-induced P_4 production by granulosa cells *in vitro*.

3.3 *Effect of hGF_2 and the OP on pregnenolone and 8-bromo-cAMP-induced progesterone secretion*

The addition of P_5 (10 ng/ml), a precursor of P_4, to granulosa cells resulted in increased P_4 secretion by 1.77-fold, and a 1.77–2.25-fold ($P<0.05$) increase over that of basal P_4 levels in the 3 h and 48 h cultures, respectively (figures 3 and 4). The data of the 3 h culture are depicted in figure 3A. Co-treatment of P_5 with hGF_2/OP did not alter the levels of P_4 secretion at both time points (figures 3A and 3B). In other cultures, 8-bromo-cAMP (1600 μM/ml) was added to granulosa cells which stimulated P_4 secretion by 1.67-fold and 1.59-fold ($P<0.05$) over the basal levels in 3 h and 48 h cultures, respectively (figures 3 and 4). Co-treatment with 8-bromo-cAMP and hGF_2/OP resulted in P_4 levels similar to those obtained with 8-bromo-cAMP alone at both time points (figures 3 and 4). Thus, the induction of P_4 synthesis by P_5 or 8-bromo-cAMP remained unaltered following treatment with the hGF_2 or OP.

4. Discussion

We have previously reported (Nandedkar *et al* 1989, 2001) that human ovarian follicular fluid contains a low molecular-weight protein termed FSHBI, which inhibits binding of FSH to its receptors on granulosa cells. A synthetic peptide corresponding to the N-terminal region (OP) of human FSHBI also demonstrated FSHBI activity similar to the native protein. In the present study, we investigated the ability of the hGF_2 fraction, a partially purified form of human FSHBI, as well as the OP, a fragment of FSHBI, to modulate FSH-induced P_4 production by granulosa cells in culture.

P_4 secretion in the culture supernatant rose over basal levels following addition of hFSH to the cultures. Both hGF_2 as well as the OP failed to show an effect on the basal levels of P_4 but suppressed FSH-induced P_4 secretion in a dose-dependent manner. P_4 secretion could also be stimulated by the addition of P_5, a precursor of P_4, or 8-bromo-cAMP, an analogue of cAMP that bypasses the initial step of the signal transduction cascade during FSH–FSH receptor interaction.

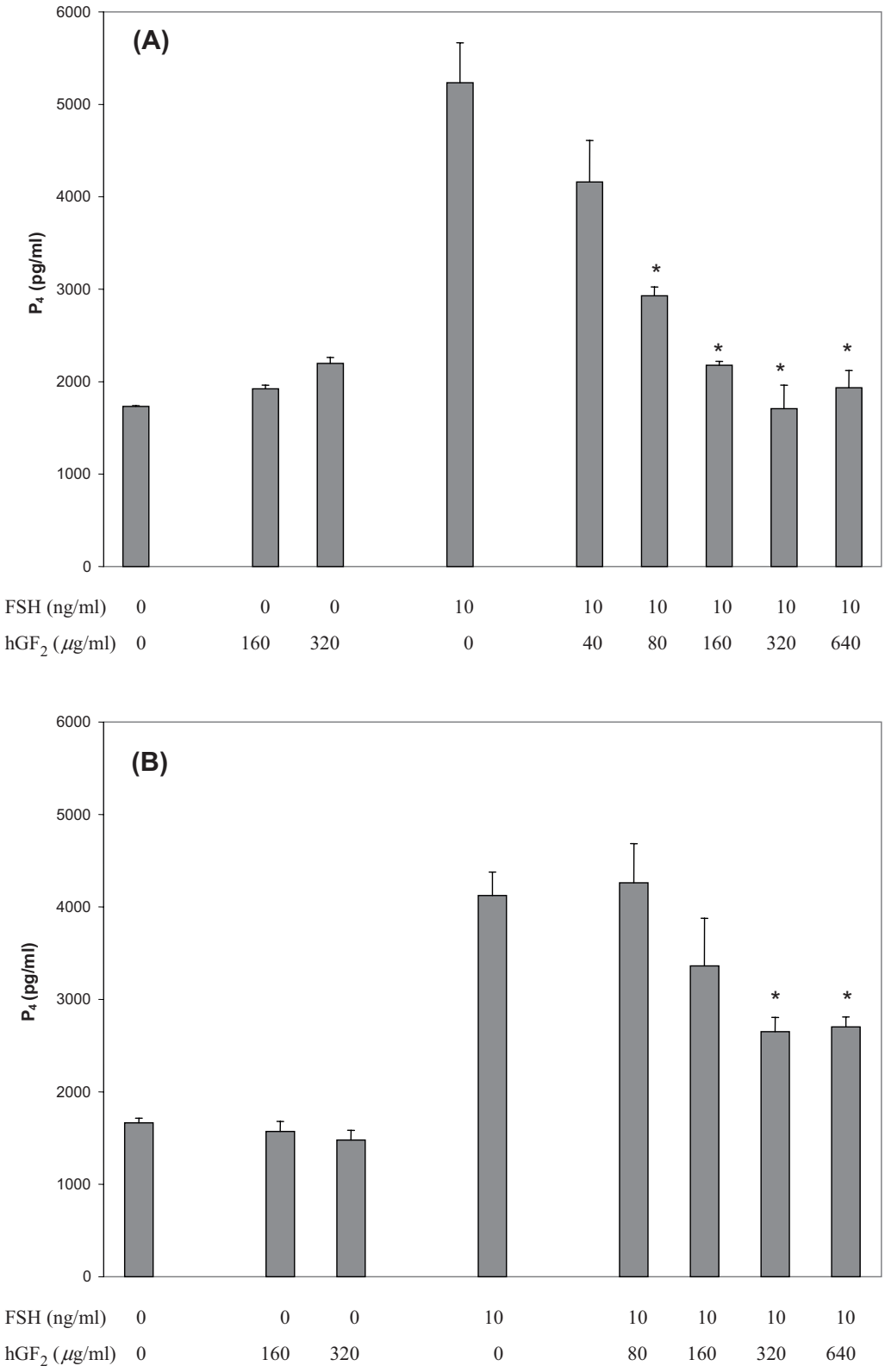

Figure 1. P_4 synthesis in 3 h **(A)** and 48 h **(B)** granulosa cell cultures in the presence of the hGF_2 fraction with/without hFSH (20 ng/ml) (**P*<0.05 with respect to FSH-positive control). Bars indicate P_4 levels ± SEM.

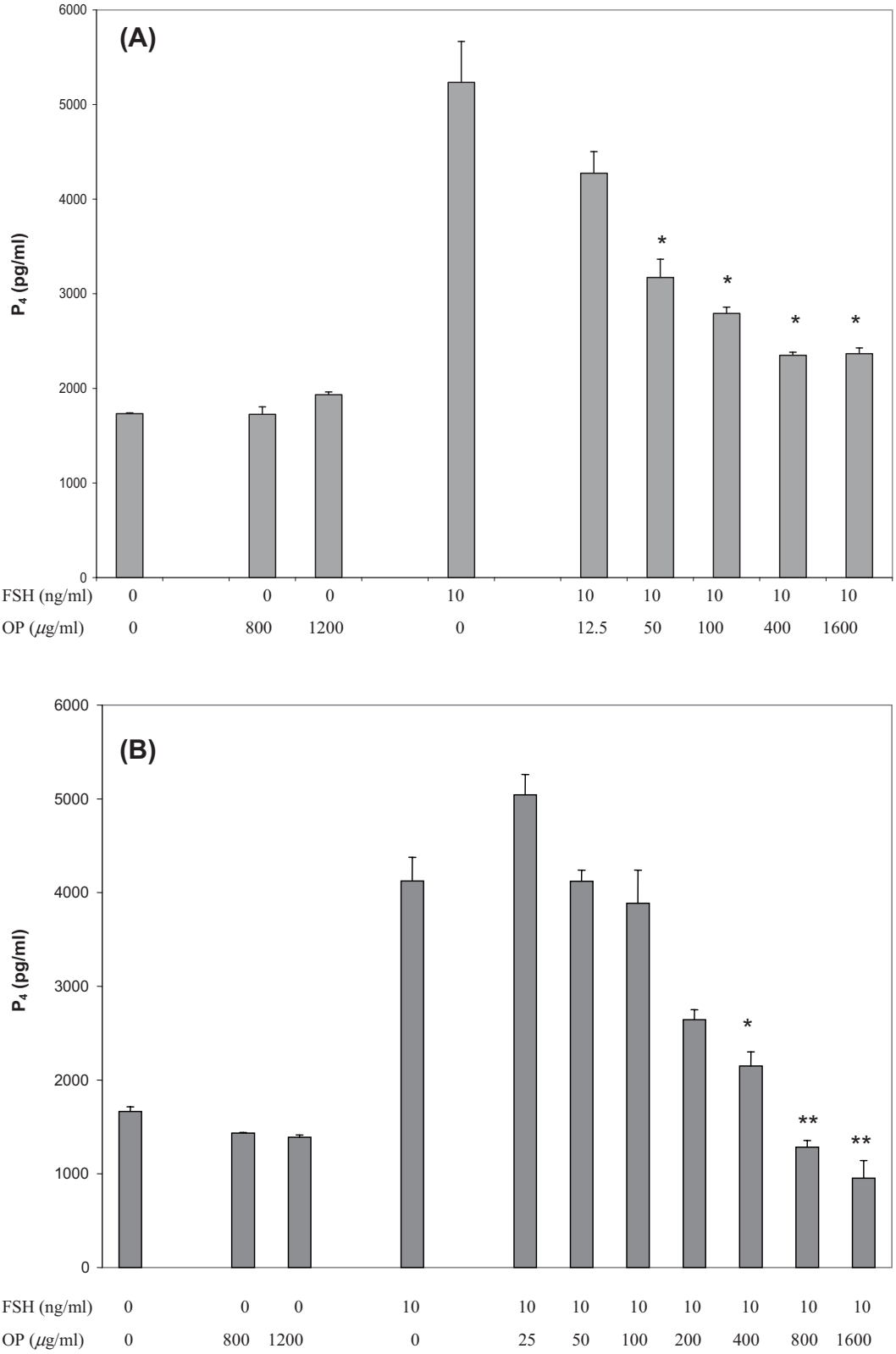

Figure 2. P_4 levels in 3 h **(A)** and 48 h **(B)** cultures modified by treatment with the octapeptide (OP) in the presence/absence of hFSH (20 ng/ml) (**P*<0.05, ***P*<0.005 with respect to FSH-positive control). Bars indicate P_4 levels ± SEM.

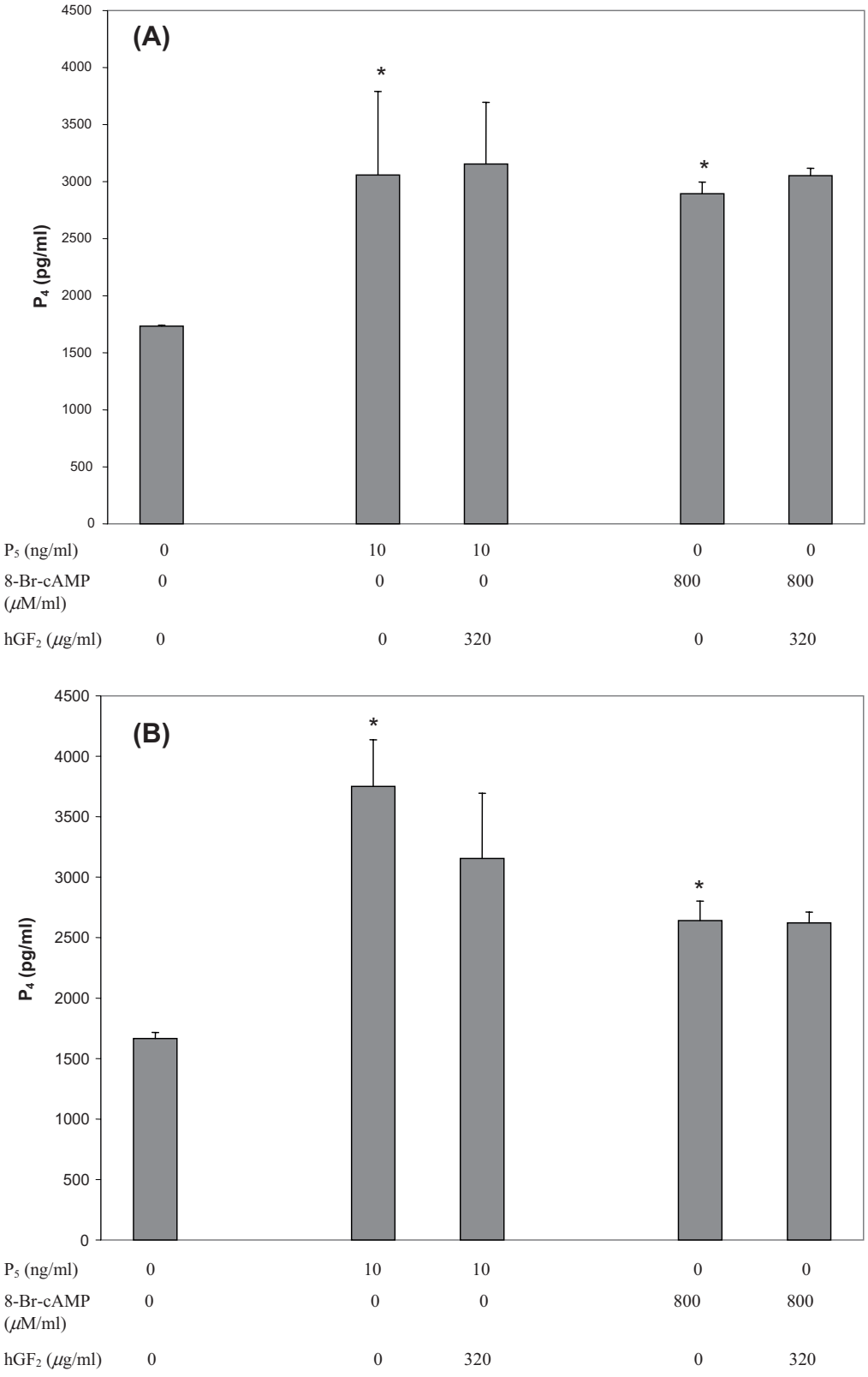

Figure 3. Effect of hGF_2 on P_4 levels in 3 h **(A)** and 48 h **(B)** granulosa cell cultures induced by P_5 or 8-bromo-cAMP. Bars indicate P_4 levels ± SEM (*P<0.05 with respect to basal levels).

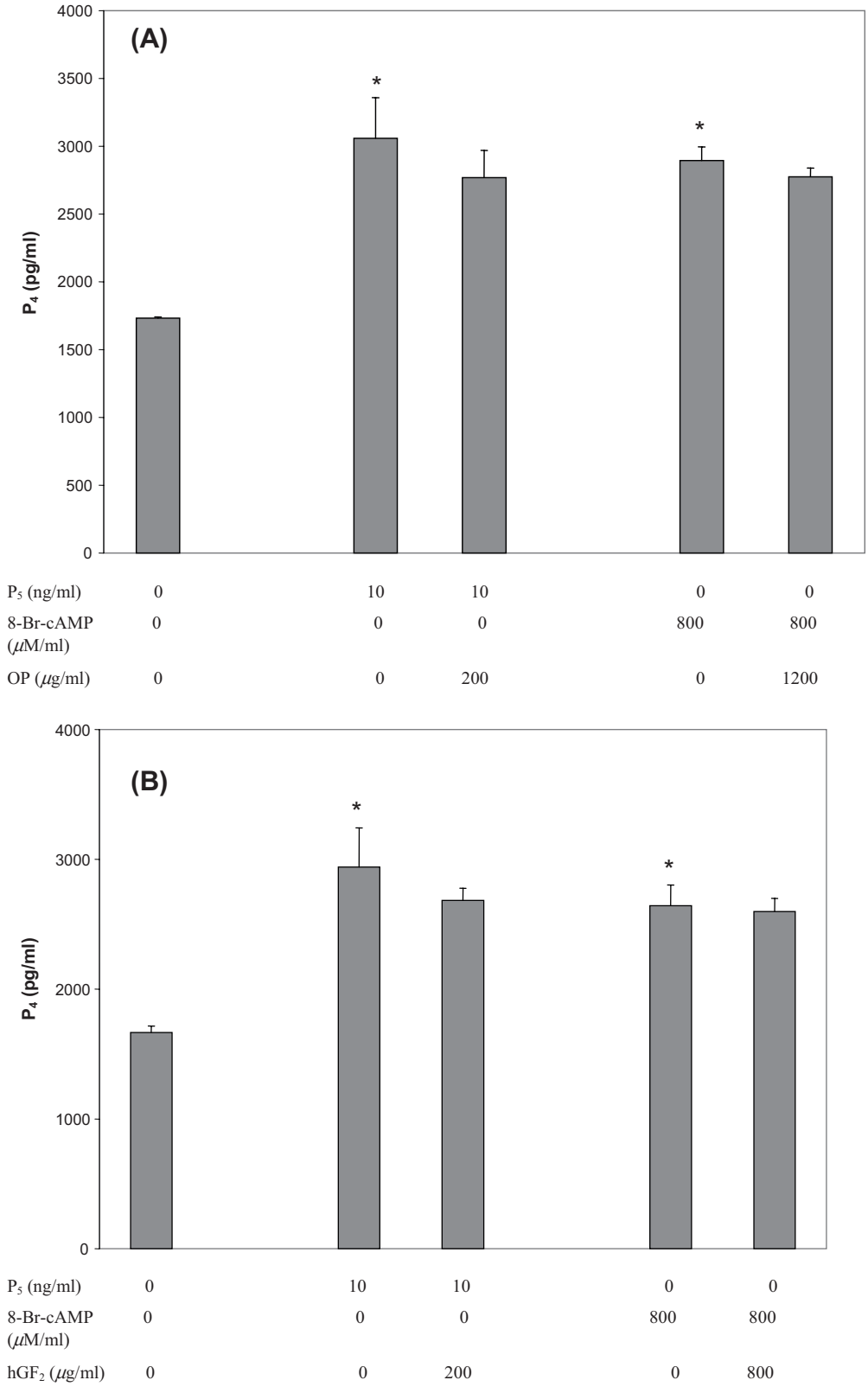

Figure 4. Effect of the octapeptide (OP) on P_4 levels induced by P_5 or 8-bromo-cAMP at 3 h **(A)** and 48 h **(B)**. Bars indicate P_4 levels ± SEM (**P*<0.05 with respect to basal levels).

However, co-treatment with either hGF_2 or the OP failed to enhance or suppress P_4 secretion in the presence of P_5/8-bromo-cAMP in these cultures. These studies indicated that the partially purified hGF_2 as well as the OP did not affect the action of 3βHSD, the enzyme responsible for the conversion of P_5 to P_4. These results confirmed our earlier finding wherein histochemical localization of 3βHSD remained unaltered in the ovarian cells of mice treated with FSHBI compared with those of controls (Nandedkar *et al* 1988, 2001). On the other hand, it was evident that the site of action of the peptide was proximal to the formation of cAMP in the signal transduction cascade activated during FSH receptor signalling. It was thus evident that the effect of FSHBI on steroidogenesis may be primarily due to its ability to block the binding of FSH to its receptors on the granulosa cells (Wadia *et al* 2003). Similar results were obtained in both the 3 h and 48 h cultures, indicating that the early and late effects of the peptide *in vitro* were identical.

Granulosa cells harvested from the follicles before the LH surge spontaneously acquire many of the characteristics of differentiated cells with an enhanced ability to produce progesterone (Hillenjso *et al* 1981; Tambe and Nandedkar 1993). Hence, cells from PMSG-treated mice resulted in the secretion of high levels of P_4 when stimulated with hFSH *in vitro*. The addition of androgens has also been shown to enhance FSH-induced P_4 production (Hillier *et al* 1988). Cells were hence cultured in the presence of 10^{-6} M testosterone in order to enhance P_4 production.

Earlier studies have shown that treatment of female marmosets with the OP led to luteal insufficiency and induced abortion in these animals by lowering P_4 levels during the luteal phase (Nandedkar *et al* 1989; Wadia *et al* 2003). The effect of the OP on P_4 secretion was due to its ability to prevent the action of FSH on the follicles during the follicular phase of the ovarian cycle resulting in luteal insufficiency and premature termination of pregnancy. The results of the present study show that *in vitro* hGF_2/OP modulates the action of FSH on the granulosa cells of the growing follicle, thereby affecting their capacity to luteinize and secrete P_4. These results support those of the study conducted in marmosets.

Until the preantral stages of follicle development, granulosa cells form a relatively homogeneous population of proliferating cells that acquire receptors for FSH and steroid hormones (Richards 1994). Transition to the antral phase occurs under the influence of FSH and is accompanied by differentiation of granulosa cells and the attainment of steroidogenic capacity (Vanderhyden and Macdonald 1998). Further differentiation or luteinization normally occurs only during the preovulatory stage of follicle development. However, granulosa cells removed from the follicular environment before the LH surge spontaneously acquire many of the characteristics of differentiated cells with an enhanced ability to produce progesterone (Hillenjso *et al* 1981). Hence, treatment of granulosa cells with either hGF_2 or the OP, both exhibiting FSHBI activity, led to a decrease in the availability of FSH, hampering both growth and differentiation of the granulosa cells and eventually resulting in a decrease in their steroidogenic potential. Our preliminary studies showed that basal levels of oestradiol were not altered by hGF_2 or the OP suggesting that the major effect of the compound is on luteinization of granulosa cells.

With hGF_2, P_4 was inhibited by only 35.7% while with the OP it was 76.9% in the 48 h cultures. This was probably due to the presence of many other proteins in hGF_2 along with FSHBI. On the other hand, the concentration of the OP required to inhibit FSH binding to the granulosa cells is very high (400–3200 μg/ml). The high concentration of synthetic peptides required to inhibit FSH action has been reported earlier by a number of researchers (Dattatreyamurty and Reichert 1992; Mahale *et al* 2001; Jetley *et al* 2003; Kene *et al* 2004).

Factors secreted by the oocyte have also been shown to exert a similar effect on the steroidogenic potential of granulosa cells. BMP-15 markedly inhibited the FSH-induced increase of mRNAs encoding steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (scc), P450 aromatase (arom) and 3βHSD in granulosa cells. In contrast, BMP-15 did not affect the forskolin-induced levels of these transcripts, indicating that the site of action of BMP-15 was upstream of cAMP signalling. Further studies indicated that BMP-15 severely reduced the levels of FSH receptor mRNA in basal and FSH-stimulated cells (Otsuka *et al* 2000). The overall effect of BMP-15 on steroidogenesis in granulosa cells is similar to that demonstrated by FSHBI. However, unlike BMP-15, FSHBI might not bring about a reduction in the number of FSH receptors on the cells. This is because the FSH-binding inhibitory activity was observed within a span of 2 h during the radioreceptor assay, while transcription and translation of the mRNA along with transport of the FSH-receptor (FSH-R) to the cell surface would take from 6 to 24 h. Another oocyte-derived factor, growth differentiation factor-9 (GDF-9), has the ability to stimulate basal steroidogenesis in granulosa cells. Co-treatment with GDF-9 suppressed FSH-induced P_4, E_2 and cAMP production (Vitt *et al* 2000). Although FSHBI, like GDF-9, has the ability to suppress FSH-induced P_4 production, unlike GDF-9 it does not stimulate basal steroidogenesis in granulosa cell cultures. Hence, FSHBI and GDF-9 are separate entities in the follicles. The latter is a member of the transforming growth factor (TGF)-β superfamily, has a high molecular weight (Hsueh *et al* 2000) and is secreted by the oocyte, while our earlier studies indicate that granulosa cells are the source of FSHBI which has a molecular

weight <5 kDa (Nandedkar *et al* 1994; 1996). The steroid-suppressing activity of GDF-9 has been attributed to the role of the oocyte in inhibiting premature luteinization of the growing follicle (Vitt *et al* 2000). On the other hand, FSHBI may restrict follicular development and subsequently induce follicular atresia in non-dominant follicles, thereby playing an important role in the selection of the dominant follicle.

FSH is essential for ovarian follicular development beyond the antral stage. Female homozygous FSH receptor knock-out mice demonstrate primordial, primary and secondary follicles, but no mature follicles, thus follicular maturity is impaired and leads to infertility (Layman and McDonough 2000). FSHBI appears to function in a similar manner by blocking the action of FSH at the receptor level, thereby suppressing normal steroidogenesis and preventing further growth of the follicle. Hence, this peptide present in the follicular fluid may play a major role as one of the paracrine regulators of FSH action in the human ovary.

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