Regulation of immunoreactive inhibin A and B secretion in cultured human granulosa-luteal cells by gonadotropins, activin A and insulin-like growth factor type-1 receptor

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Abstract

Inhibins are gonadal glycoproteins with endocrine effects on pituitary FSH secretion and para/autocrine effects on ovarian and testicular function. The purpose of this study was to investigate the endocrine and para/autocrine regulation of inhibin A and inhibin B secretion in human ovarian granulosa-luteal cells. The cells were obtained from women undergoing *in vitro* fertilization, and the primary cultures were treated with FSH, LH, human chorionic gonadotropin (hCG), activin A, 8-bromo cyclic AMP (8-BrcAMP), staurosporine (a protein kinase C inhibitor) and an antagonist of IGF action (type-1 IGF receptor antibody αIR3). The secretion of inhibins was measured by ELISA assays capable of reliably distinguishing between inhibin A and B.

FSH, LH, hCG and 8-BrcAMP increased inhibin A secretion on average up to 180% ($P<0{\cdot}01$), 192% ($P<0{\cdot}05$), 210% ($P<0{\cdot}01$) and 243% ($P<0{\cdot}01$) respectively of the control level, while their stimulatory effect on inhibin B secretion was less pronounced (up to 167%, $P<0{\cdot}01$; 139%, $P<0{\cdot}05$; 127%, $P>0{\cdot}05$; 133%, $P>0{\cdot}05$ of the controls respectively). αIR3 decreased inhibin A and B secretion down to 70% ($P<0{\cdot}01$) and 50% ($P<0{\cdot}01$) respectively of the control. Staurosporine decreased inhibin B secretion down to 49% ($P<0{\cdot}01$) of the control; its effect on inhibin A secretion was not significant. Activin A increased inhibin B secretion up to fourfold of the control ($P<0{\cdot}05$) while its effect on inhibin A secretion was insignificant.

We conclude that gonadotropins via the protein kinase A signal transduction pathway are the main positive regulators of inhibin A and B secretion in human granulosa-luteal cells. The protein kinase C signal transduction pathway seems to be important especially for inhibin B secretion. Locally produced IGFs are probably important inducers of the production of both forms of inhibin in human ovaries while activins seem to upregulate inhibin B secretion.

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Introduction

Inhibins are dimeric glycoproteins consisting of an α-subunit and either a βA- (inhibin A) or βB- (inhibin B) subunit. Activins are composed of β-subunits. The homodimer of βA-subunits forms activin A, the homodimer of βB-subunits activin B, and the heterodimer of βA- and βB-subunits activin AB. Circulating inhibins are secreted mainly by ovaries and testes but they are also synthesized in placenta, adrenals and some gonadal tumors (reviewed in Vale *et al.* 1988, Ying 1988, Woodruff 1998).

The main biological function of the inhibins was originally thought to be the suppression of follicle-stimulating hormone (FSH) secretion by the pituitary gland, whereas activins increase pituitary FSH secretion (Vale *et al.* 1988, Ying 1988). In addition to their endocrine effects on pituitary FSH secretion, inhibin and activin peptides have important autocrine and paracrine functions in the organs in which they are produced (Mather *et al.* 1992, Findlay 1993, Woodruff 1998). Inhibins and activins regulate ovarian steroidogenesis in both rodents and primates (Hsueh *et al.* 1987, Miró & Hillier 1992, Rabinovici *et al.* 1992). Inhibins may also have a role in gonadal tumorigenesis (Matzuk *et al.* 1992, 1996).

All inhibin subunit genes (Mason *et al.* 1986) are expressed in human ovaries through the menstrual cycle. The corpus luteum expresses the βA-subunit gene, while βB-subunit gene expression may be minimal or absent (Schwall *et al.* 1990, Roberts *et al.* 1993). Inhibin subunit

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peptides have been localized by immunohistochemistry in human ovaries (Yamoto *et al.* 1992, Arora *et al.* 1997). On the basis of *in situ* hybridization histochemistry it was assumed that inhibin B is the dominant form at the beginning of follicular development and that inhibin A becomes more important during the later stages of folliculogenesis (Schwall *et al.* 1990, Roberts *et al.* 1993). Inhibin A was recently found to be the dominant form of inhibin secreted by cultured human granulosa-luteal cells (Muttukrishna *et al.* 1997).

During the human menstrual cycle, serum immunoreactive inhibin concentrations follow the growth of the developing follicles, which are the main source of circulating inhibins in the follicular phase. At this stage inhibin B is dominant but its concentration decreases rapidly during the luteal phase. Inhibin A concentration is highest in the luteal phase, and it also decreases rapidly before the end of the cycle (Groome *et al.* 1996). Serum inhibin concentrations during the menstrual cycle correlate well with inhibin subunit gene expression in the corpus luteum (Schwall *et al.* 1990, Roberts *et al.* 1993).

Gonadotropin- and protein kinase C-dependent regulation of the expression of inhibin α and βA subunit genes has previously been described in ovarian granulosa cells at mRNA level (Erämaa *et al.* 1994, Tuuri *et al.* 1996) but information about the inhibin peptide secretion by these cells is scant (Muttukrishna *et al.* 1997). The aim of the present work was to shed more light on the endocrine (FSH, luteinizing hormone (LH), human chorionic gonadotropin (hCG)) and local (activins, insulin-like growth factor (IGF) system) regulation of inhibin A and B secretion in human ovaries by using cultured granulosa-luteal cells with highly sensitive and specific inhibin assays. The roles of protein kinase A- and C-dependent signal transduction pathways in inhibin secretion were tested by adding 8-bromo cyclic AMP (8-BrcAMP) (which activates protein kinase A) and staurosporine (which inhibits protein kinase C) to the cell cultures.

Materials and Methods

Human ovarian granulosa cells were obtained by follicular aspiration from women taking part in *in vitro* fertilization programs. The study was approved by the Research Ethics Committees of Kuopio and Helsinki University Hospitals, and the women gave informed written consent. The women were treated with a gonadotropin-releasing hormone (GnRH) analog (Synarela (Searle, Bretigny-Sur-Orge, France), Suprecur (Hoechst Marion Roussel, Frankfurt am Main, Germany) or Zoladex (Zeneca, Alderley Park Macclesfield, Cheshire, UK)) and an FSH preparation (Gonal-F (Serono, Bari, Italy) or Puregon (Organon, Oss, The Netherlands)) to induce the development of multiple follicles. Follicular aspiration was performed 36–38 h after hCG (Pregnyl (Organon) or Profasi (Serono)) administration. After removal of the cumulus–oocyte complex, the granulosa cells from all follicles of each woman were pooled and pelleted. The cells were then dispersed in 0·1% hyaluronidase (Sigma Chemicals Co., St Louis, MO, USA) in Dulbecco's minimal essential medium (DMEM)–Ham's F-12 medium (GIBCO Laboratories, Grand Island, NY, USA) (1:1) for 30 min at 37 °C with intermittent stirring. The granulosa cells were separated from red blood cells by centrifugation in Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) for 15 min at 1000 × **g**. The cells were then washed and plated in DMEM–Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum (GIBCO or Bioclear UK Ltd, Calne, Wilts, UK), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate (GIBCO) at a density of $2–5 \times 10^5$ cells/well on 35-mm six-well Cellstar dishes (Greiner Labortechnik Gmbh, Frickenhausen, Germany). The cells were grown at 37 °C in a 95% air–5% CO_2 humidified environment and the cell culture media were changed every 2–3 days.

In vitro hormonal and other treatments were performed during the 7th to the 11th days of culture when the cells are the most responsive in this culture system (Voutilainen *et al.* 1986). The functional viability of the cells was assessed by measuring progesterone concentrations in selected culture media after different treatments. Recombinant human (rh) FSH (Gonal-F) and rhLH were gifts from Serono, and purified hCG (CR-127) was a gift from the National Hormone and Pituitary Programme, NIDDK, NIH, Bethesda, MA, USA. Recombinant human activin A peptide was generously provided by Dr A F Parlow (NIDDK's National Hormone and Pituitary Programme). 8-BrcAMP was purchased from Sigma, staurosporine from Boehringer Mannheim (Mannheim, Germany), and IGF type-1 receptor antibody αIR3 from Oncogene Sciences (Uniondale, NY, USA).

Inhibin A and B were measured by specific enzyme-linked immunosorbent assay (ELISA) kits (product codes MCA950 KZZ and MCA1312 KZZ respectively; Serotec Ltd, Oxford, Oxon, UK) as described previously (Groome *et al.* 1990, 1996, Groome & Lawrence 1991). The detection limit for the assay was reported to be 2 pg/ml for inhibin A and 15 pg/ml for inhibin B. Both intra- and interassay coefficients of variation were below 10% and 7% for inhibin A and B respectively. The samples were assayed in duplicate. According to the manufacturer, there is minimal cross reaction with inhibin B or activins in the inhibin A assay, and about 1% cross reaction with inhibin A in the inhibin B assay. When activin added into the cell culture medium (up to 40 ng/ml) was measured with the inhibin A and B assays, no cross reaction could be detected. Differences in the inhibin concentrations between treatment groups were assessed by the Mann-Whitney test. The level of significance was chosen as $P<0·05$.

Results

Each experiment was performed with cells derived from the follicles of a single woman, or in some cases with cells pooled from 2–6 women. Both inhibin A and inhibin B secretion was detectable in all conditioned media of cultured human ovarian granulosa-luteal cells. There was considerable variation in the absolute concentrations of both forms of inhibin in the conditioned media of different granulosa cell pools. The mean (± S.E.M.) secretion of inhibin A was 537·9 (± 131·7) pg/ml/24 h (n=16; n means the number of experiments pooled together, each experiment consisted of a single culture with 2–3 parallel wells), and that of inhibin B 433·0 (± 80·4) pg/ml/24 h (n=18) at the stage when the experiments were performed (7th–11th days of culture). The inhibin A/B ratio in the same cultures was 1·24 ± 0·21 (mean ± S.E.M.). As the absolute inhibin values do not have any particular significance in cell culture experiments, and due to the above mentioned considerable variation in the absolute concentrations of the secreted inhibins in different cell pools, the regulation data below are presented as changes compared with the control situation in each experiment. Progesterone was measured in selected conditioned media and its secretion into the medium was about 1500 nmol/l/24 h at the time the experiments were performed.

Both rhFSH and rhLH increased dose dependently inhibin A and B secretion into the culture medium; the maximal effect was reached between 30 and 100 IU/l (dose–response data not shown). In repeated experiments with cells from separate cell pools rhFSH (30 or 100 IU/l) increased inhibin A and B secretion up to 179·9 ± 21·5% and 166·7 ± 27·4% respectively of control (mean ± S.E.M.; P<0·01, n=8 for both). rhLH (30 or 100 IU/l) increased inhibin A and B secretion up to 192·4 ± 14·7% (P<0·05, n=4) and 139·0 ± 20·3% (P<0·05, n=4) respectively of control, and hCG (100 ng/ml) up to 210·1 ± 53·5% (P<0·01, n=6) and 127·4 ± 32·1% (not significant (NS), n=6) respectively of control. 8-BrcAMP (100 μM) increased inhibin A secretion up to 243·3 ± 24·5% (P<0·01, n=16) and inhibin B secretion up to 132·8 ± 15·8% (NS, n=16) of control (presented graphically in Fig. 1). In time-course experiments no increase in inhibin secretion was detectable after four hours of hormonal manipulations but after 24 h rhFSH, rhLH, hCG and 8-BrcAMP had increased inhibin secretion (time-course data not shown).

The protein kinase C inhibitor staurosporine decreased basal inhibin B secretion to 49·2 ± 5·6% (mean ± S.E.M.) (P<0·01, n=7) of control, but it did not have any significant effect on inhibin A secretion. Staurosporine also decreased inhibin B (P<0·05, n=4) but not inhibin A secretion in 8-BrcAMP-treated cultures (Fig. 2). FSH-induced inhibin B secretion was also inhibited by staurosporine in two cultures performed (data not shown).

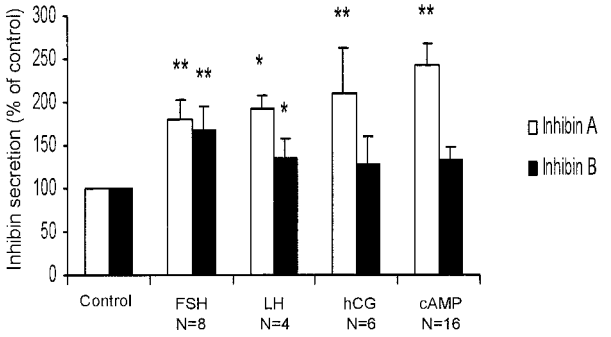

Figure 1 The effects of rhFSH (100 IU/l), rhLH (100 IU/l), hCG (100 ng/ml) and 8-BrcAMP (cAMP, 100 μM) on inhibin secretion in cultured human ovarian granulosa-luteal cells during 24 h of treatment. Means ± S.E.M. from 4–16 different experiments in each group are shown with the control mean adjusted to 100. N=the number of experiments pooled in each treatment group. *P<0·05, **P<0·01 compared with the respective control.

Activin A addition (40 ng/ml) into the culture medium increased basal inhibin B secretion up to 409·2 ± 61·6% of control (mean ± S.E.M.; P<0·05; n=3) and it further stimulated FSH-induced inhibin B secretion (P<0·05; n=3) (Fig. 3). The stimulatory effect of activin A was dose dependent, detectable from 3–10 ng/ml upwards, and reached its maximum between 30 and 100 ng/ml (dose-response data not shown). Activin A addition had no effect on inhibin A secretion (Fig. 3).

Addition of the type-1 IGF receptor antibody αIR3 (which blocks the effect of IGFs through the type-1

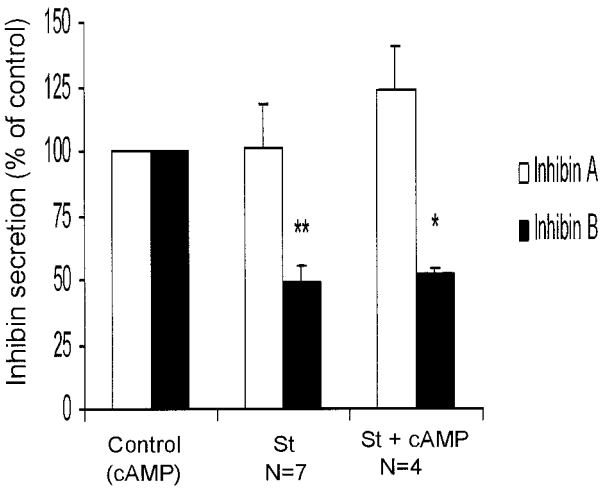

Figure 2 The effect of staurosporine (St, 30 nM) on basal and 8-BrcAMP-induced (cAMP, 100 μM) inhibin secretion in cultured human granulosa-luteal cells during 48 h of treatment. Means ± S.E.M. from 4–7 different experiments in each group are shown with the control mean (both untreated and 8-BrcAMP-treated) adjusted to 100. N=the number of experiments pooled in each treatment group. *P<0·05; **P<0·01 compared with the respective control.

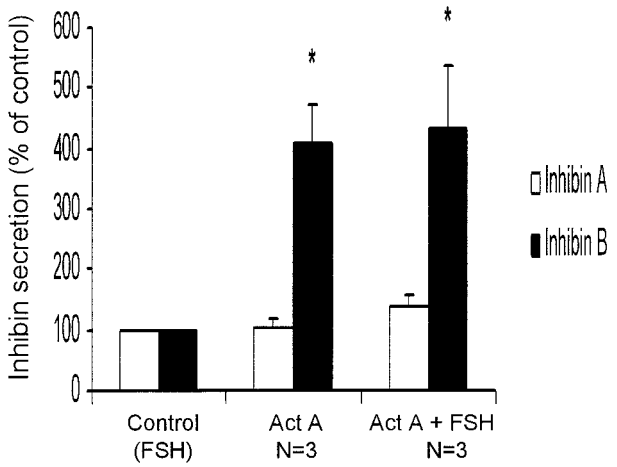

Figure 3 The effect of activin A (Act A, 40 ng/ml) on basal, and rhFSH-induced (100 IU/l) inhibin secretion in cultured human granulosa-luteal cells during 48 h of treatment. Means ± S.E.M. from 3 different experiments in each group are shown with the control mean (both untreated and FSH-treated) adjusted to 100. N=the number of experiments pooled in each treatment group. *P<0·05 compared with the respective control.

receptor) into the culture medium decreased inhibin A secretion to 69·5 ± 3·3% and inhibin B secretion to 49·7 ± 7·9% of control (P<0·01 for both; n=5). In hCG-treated cultures αIR3 decreased inhibin A secretion to 71·9 ± 12·5% and inhibin B to 54·3 ± 4·0% (P<0·05 for both, n=3) of the secretion detected without αIR3 addition. αIR3 decreased slightly inhibin B secretion in 8-BrcAMP-treated cultures (P<0·05; n=5), but it had no significant effect on inhibin A secretion (Fig. 4).

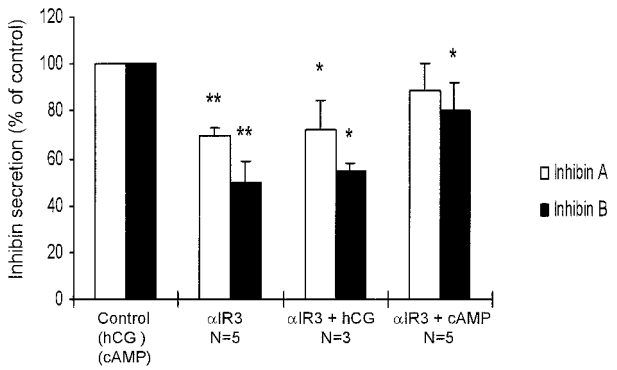

Figure 4 The effect of the type-1 IGF receptor antibody αIR3 (1 μg/ml) on basal, and hCG- (100 ng/ml) and 8-BrcAMP- (cAMP, 100 μM) regulated inhibin secretion in cultured human granulosa-luteal cells during 48 h of treatment. Means ± S.E.M. from 3–5 different experiments in each group are shown with the control mean (untreated, hCG- and 8-BrcAMP-treated) adjusted to 100. N=the number of experiments pooled in each treatment group. *P<0·05; **P<0·01 compared with the respective control.

Discussion

Previous studies using cultured human granulosa-luteal cells have shown that inhibin α- and βA-subunit gene expression is induced by FSH, hCG and cAMP analogs. However, βB-subunit mRNA accumulation is not increased by these agents (Erämaa *et al.* 1994, Tuuri *et al.* 1996). Gonadotropins mediate their effects on human granulosa-luteal cells via increased cAMP production and protein kinase A activation. In this study rhFSH, rhLH, hCG and 8-BrcAMP increased significantly inhibin A secretion while the stimulatory effect of these agents on inhibin B was less pronounced or insignificant. These data fit well with previous mRNA regulation data; induction of α- and βA-subunits leads to increased inhibin A production, and the slightly increased inhibin B production can be explained by increased α-subunit expression assuming that enough βB-subunits are available. Our inhibin A and B secretion data fit fairly well with those of Muttukrishna *et al.* (1997) who recently reported gonadotropin-induced inhibin A and B secretion in cultured human ovarian granulosa-luteal cells.

Staurosporine is a relatively specific protein kinase C inhibitor. It reduced basal and gonadotropin-induced inhibin B secretion, suggesting that the protein kinase C-dependent regulatory pathway is important in the regulation of inhibin B synthesis. To our knowledge there are no convincing data on the effects of protein kinase C regulators on βB-subunit gene expression to explain the mechanism for the clear inhibition of inhibin B secretion caused by staurosporine in these cells. However, protein kinase C has been reported to be involved in the induction of βA-subunit gene expression in human granulosa-luteal cells (Tuuri *et al.* 1996).

Activin A turned out to be a very potent inducer of inhibin B secretion. A similar effect of activin has been found in cultured rat granulosa cells (Lanuza *et al.* 1999). Increased inhibin B secretion can be explained by the activin A-induced βB-subunit gene expression reported previously (Erämaa *et al.* 1995). Activin A is produced by human granulosa-luteal cells (Muttukrishna *et al.* 1997) enabling it to have a physiological autocrine/paracrine role in the regulation of ovarian inhibin production. In our experiments activin A did not increase inhibin A secretion, which is in contrast to rat granulosa cell data (Lanuza *et al.* 1999), but fits well with the human mRNA data showing no induction of α- or βA-subunit gene expression during activin treatment (Erämaa *et al.* 1995).

IGFs are likely to upregulate inhibin secretion in human granulosa-luteal cells. This hypothesis is supported by the clear inhibition of inhibin secretion by αIR3 which blocks the IGF-I- and -II-mediated signaling through the type 1 IGF receptor. Previous studies have shown that endogenous IGF-I production is essential for inhibin α-subunit expression in rat granulosa cells (Kubo *et al.* 1998, Li *et al.* 1998). In addition, IGF-I increased inhibin production by

cultured rat granulosa cells (Lanuza *et al.* 1999). We can assume that IGF-II is the main autocrine/paracrine IGF upregulating inhibin synthesis in human ovaries. This assumption is based on the high and gonadotropin-dependent expression of the IGF-II gene in human ovaries (Voutilainen & Miller 1987, Voutilainen *et al.* 1996).

In summary, our data show that gonadotropins via the protein kinase A signal transduction pathway are the main positive regulators of inhibin A and B secretion in human granulosa-luteal cells. The protein kinase C signal transduction pathway seems to be important especially for inhibin B secretion. Locally produced activins are probably important inducers of inhibin B production, and IGFs seem to upregulate both forms of inhibin in human ovaries.

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