

High concentrations of inhibin A and inhibin B in ovarian serous cystadenoma: relationship with oestradiol and nitric oxide metabolites

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Inhibin production has been demonstrated in malignant epithelial ovarian tumours, but secretion of inhibins by benign cystadenoma has not yet been reported. The present study evaluated the concentrations of inhibin A and inhibin B and the relationship with oestradiol and nitric oxide metabolites in fluid collected from benign ovarian serous cystadenomas ($n = 15$). In addition, follicular fluid samples ($n = 14$) from women with regular ovulatory cycles undergoing ovarian stimulation for IVF were studied as a reference group. High concentrations of inhibin A (median = 89.3 ng/ml) and inhibin B (median = 116.1 ng/ml) were found in the cystic fluid of ovarian serous cystadenomas. These inhibin concentrations were even higher than in follicular fluid of stimulated follicles (inhibins A and B = 41.2 and 46.8 ng/ml respectively; $P < 0.001$), whereas oestradiol was ~18-fold lower in cystic fluid than in follicular fluid (median = 34 versus 622 pg/ml, $P < 0.001$). In ovarian cysts, the concentrations of inhibin A and oestradiol were inversely correlated ($r = -0.678$, $P = 0.008$). Cystic fluid samples containing the highest concentrations of $\text{NO}_2^-/\text{NO}_3^-$ (45–60 $\mu\text{mol/l}$) had lower inhibin A and higher oestradiol concentrations than those samples containing lower concentrations (10–25 $\mu\text{mol/l}$) of $\text{NO}_2^-/\text{NO}_3^-$. It is concluded that high amounts of dimeric inhibins are present in ovarian serous cystadenoma. The source of inhibins and the determinants of the inverse association of inhibin A with oestradiol and nitric oxide remain to be determined.

Key words: inhibin/nitric oxide/oestrogen/ovary/ovarian cyst

Introduction

Inhibins are glycoproteins produced mainly by ovarian granulosa cells and corpus luteum across the menstrual cycle. They play a decisive role in the control of FSH secretion by pituitary gonadotrophs (Woodruff and Mather, 1995; Petraglia *et al.*, 1999). There are at least two active molecular forms, which are heterodimers made by an α subunit and either a βA (inhibin A) or a βB (inhibin B) subunit. Inhibins also have paracrine and autocrine effects within the ovary. A major paracrine effect of ovarian inhibins upon steroidogenesis is the augmentation of androgen production (Hillier *et al.*, 1991). Both inhibin A and inhibin B are secreted into follicular fluid, where the concentrations of both inhibins seem to increase during the process of follicle development (Magoffin and Jakimiuk, 1997). Follicles undergoing atresia show a marked decline in inhibin production in the granulosa cell layer (Jaatinen *et al.*, 1994, Uilenbroek *et al.*, 1998).

Inhibins and activins belong to the transforming growth factor β superfamily. In the ovary, these molecules act as local growth factors and may play a role in the development of stromal tumours (Matzuk *et al.*, 1992). Although malignant and borderline tumours derived from the human ovarian surface epithelium express inhibin α , βA and βB subunit mRNA, very few tumour specimens are able to synthesize and release

dimeric inhibins in culture (Welt *et al.*, 1997). It has been suggested that epithelial ovarian neoplasms have an imbalanced expression of inhibin and activin subunits and this may contribute to abnormal cell proliferation (Zheng *et al.*, 1998). Benign serous cystadenoma is the most common type of ovarian neoplasm in premenopausal women (Gerber *et al.*, 1997). Notwithstanding the importance of inhibins in ovarian physiology and pathology, inhibin regulation in benign ovarian cysts is largely unknown. Recently, six specimens of serous cystadenoma were examined (Zheng *et al.*, 1998) and βA but not α subunit expression was detected in the epithelial component of the cysts. The presence of dimeric inhibins in the fluid of cystadenoma has not previously been demonstrated.

In addition to the inhibin family of proteins, a large number of local factors control cell proliferation and hormone secretion within the ovary. One of these factors is nitric oxide (NO), a potent vasodilator formed through the oxidation of L-arginine by nitric oxide synthase (Bredt and Snyder, 1990). Several studies suggested an important role for NO in ovarian physiology. It has been suggested that NO is locally synthesized by the ovary and may play a role during follicular development, ovulation and luteal formation (Ellman *et al.*, 1993; Ben-Shlomo *et al.*, 1994; Zackrisson *et al.*, 1996). Regarding the ovarian NO pathway, no information is currently available

on patients with ovarian cysts. Furthermore, the possible relationship between NO and inhibins remains unexplored, despite the fact that inhibins are sensitive indicators of ovarian function.

The aim of the present study was to investigate the concentrations of inhibin A and inhibin B and their relationship with oestradiol and stable NO metabolites in the fluid of ovarian cystadenomas. The concentrations of these molecules were also determined in the follicular fluid of women undergoing ovarian stimulation in order to characterize possible differences between cystic and follicular fluids.

Materials and methods

Subjects

The study involved 15 patients undergoing surgical removal of benign ovarian serous cystadenomas. Diagnosis was confirmed by cytological and histological examination of the cysts and by negative serum tumour markers (CA-125, CA-19.9, CA-15.3, α fetoprotein). The mean age of patients was 26.7 years (range 16–36 years) and the mean (\pm SEM) volume of cystic fluid recovered was 7.3 ± 0.7 ml (range 3.5–12 ml). Cystic fluid samples were collected by needle aspiration during surgery in the mid- to late follicular phase of menstrual cycle. Samples were then centrifuged at $2000 \times g$ at 4°C for 10 min, and the supernatants were stored at -80°C until further measurements.

In addition to the patients with cystadenoma, samples of ovarian follicular fluid from 14 women with regular ovulatory cycles were studied in parallel with the cystic fluids, as a reference group. These women were undergoing ovarian stimulation (25–35 days) for IVF due to either tubal or unexplained infertility. Their ages ranged between 20 and 36 years (mean age 29.5 years). Patients received a human menopausal gonadotrophin (HMG) treatment protocol (225 IU FSH and 225 IU LH daily; Pergonal; Serono, Geneva, Switzerland) from day 3 of the cycle. After 5 days of HMG administration, the ovarian response was evaluated repeatedly in all patients by serum oestradiol concentrations and vaginal ultrasonographic scans to define ovarian follicular development. The dosage of HMG was adjusted on an individual basis according to clinical judgement as published previously (Dor *et al.*, 1992). When follicles reached a diameter >18 mm and serum oestradiol concentrations were >400 pg/ml, a dose of human chorionic gonadotrophin (HCG; 10 000 IU) was administered. Ultrasound-guided follicular aspiration for oocyte retrieval was performed 34–55 h after HCG administration. Follicular fluid (uncontaminated with blood) from at least three leading follicles containing oocytes was obtained from each woman. One sample at random was centrifuged and the supernatant was frozen at -80°C until biochemical and hormonal measurements.

Informed consent was obtained from all subjects prior to inclusion and the study protocol was approved by the local committee on human research.

Inhibin A and inhibin B assays

Inhibin A and B concentrations in cystic and follicular fluids were measured by using specific two-site enzyme immunoassays purchased from Serotec (Oxford, UK) as previously described (Groome *et al.*, 1994, 1996). Briefly, in each assay, standards and samples were diluted as appropriate and mixed with a half volume of the sample of distilled water containing 10% sodium dodecyl sulphate (SDS). After 3 min at 100°C , tubes were cooled before adding freshly prepared hydrogen peroxide solution. After additional incubation at

room temperature, duplicate aliquots of denatured and oxidized samples and standards were transferred to antibody-coated microtitre plates. Plates were incubated at room temperature, for 2 h (inhibin A) or overnight (inhibin B). After washing with enzyme immunoassay (EIA) wash buffer [0.1 mol/l Tris-HCl, 0.15 mol/l NaCl, 10% (w/v) bovine serum albumin, 5% (v/v) Triton X-100, and 0.1% (w/v) sodium azide, pH 7.5], 50 μ l alkaline phosphatase-conjugated mouse anti-human inhibin α subunit antibody was used. The plates were then incubated for 1 h (inhibin A) or 3 h (inhibin B). Plates were washed and bound alkaline phosphatase was quantified using a commercially available enzyme immunoassay amplification system (Immuno Select ELISA Amplification System, Dako, Milan, Italy), which was used according to the supplier's instructions. The inhibin A and B plates were read at 490 nm on an automated EIA plate reader (BRIO: Basic Radium Immunoassay Operator, Radim spa, Pomezia, Italy). The inhibin A detection limit was 20 pg/ml, and the intra- and inter-assay coefficients of variation for quality control samples were <4.0 and 8.0% respectively. The assay detection limit for inhibin B was <10 pg/ml. Within- and between-plate coefficients of variation were <5.0 and 9.0% respectively. Cross-reactions for each assay with the various inhibin-related proteins were $<0.5\%$.

Oestradiol assay

Cystic and follicular fluid oestradiol concentrations were measured by use of solid phase radioimmunoassay kits provided by Diagnostic Products Corporation (Santa Monica, CA, USA). The intra- and interassay coefficients of variation were 7 and 10% respectively.

Nitrite/nitrate assay

All reagents were purchased from Sigma Chemical Co (St Louis, MO, USA). Cystic fluid and follicular fluid $\text{NO}_2^-/\text{NO}_3^-$ concentrations were measured using the Griess reagent as described elsewhere (Guevara *et al.*, 1998). Briefly, nitrate standards (1–125 $\mu\text{mol/l}$) and undiluted samples were incubated with nitrate reductase from *Aspergillus niger* (0.5 IU/ml) and with NADPH (0.1 mmol/l) for 30 min at room temperature to convert NO_3^- to NO_2^- . To oxidize NADPH, 10 mmol/l of pyruvate and 100 IU/ml of lactate dehydrogenase were added and the mixture was incubated for an additional 10 min at room temperature. Total NO_2^- was then analysed by reacting the samples with Griess reagent and measuring absorbance at 540 nm spectrophotometrically. This assay measures both nitrite derived from the reduction of nitrate and endogenous nitrite, therefore we report our results as $\text{NO}_2^-/\text{NO}_3^-$. The method does not rule out the possibility of underestimation of nitrate concentrations due to the presence of reductase inhibitors in some samples (Anteby *et al.*, 1996). Partial reductase inhibition may be estimated by regression analysis of the nitrite recovery after serial concentrations of exogenous nitrate, but this procedure is insufficient for adjustment of the measures. The recovery of significant amounts of nitrite after reductase reaction in all of our samples further indicates that reductase activity was present, since very little or no nitrite was originally found in the samples. Overall $\text{NO}_2^-/\text{NO}_3^-$ recovery by this method was 88% (Guevara *et al.*, 1998) and the intra-assay coefficient of variation was $<3\%$.

Statistical analysis

Data are reported as medians and ranges. The distribution of $\text{NO}_2^-/\text{NO}_3^-$ concentrations was further explored by K-means cluster analysis. Comparisons between two groups were performed using the Mann-Whitney *U*-test. Spearman's rank correlation coefficients were used to determine the correlation between inhibin concentrations, oestradiol, cyst volume and $\text{NO}_2^-/\text{NO}_3^-$ concentrations. $P < 0.05$ was considered to be statistically significant.

Table I. Concentrations of inhibin A, inhibin B, oestradiol and NO₂⁻/NO₃⁻ in cystic fluid obtained from women undergoing removal of benign ovarian serous cystadenoma and in the follicular fluid obtained by follicle aspiration from ovulatory women undergoing ovarian stimulation for IVF. Values are given as medians with the range shown in parentheses

Group	Cystadenoma (n = 15)	Follicle (n = 14)
Inhibin A (ng/ml)	89.3 (62.5–138.1)*	41.2 (29.8–95.6)
Inhibin B (ng/ml)	116.1 (30.3–182.1)*	46.8 (12.5–126.6)
Oestradiol (pg/ml)	34 (17–79)*	622 (198–1232)
NO ₂ ⁻ /NO ₃ ⁻ (μmol/l)	25.8 (10.7–58.3)	22.0 (6.9–53.2)

*Statistically significant, compared with the follicle group ($P < 0.001$; Mann–Whitney U test).

Results

Inhibin A (median = 89.3 ng/ml) and inhibin B (median = 116.1 ng/ml) were highly concentrated in the cystic fluid of ovarian serous cystadenomas. Inhibin concentrations in the cystic fluid were even higher than in the follicular fluid of stimulated follicles (Table I). Conversely, oestradiol concentrations were low in cystic fluid (median = 34 pg/ml) and were much lower than oestradiol concentrations in follicular fluid of stimulated follicles (median = 622 pg/ml; Table I). The mean concentrations of NO₂⁻/NO₃⁻ in ovarian cysts ranged from 10.7–58.3 μmol/l (median = 25.8 μmol/l) and were similar to those measured in the follicular fluid of women undergoing ovarian stimulation (Table I).

The concentrations of inhibin A in cystic fluid were inversely correlated with oestradiol ($r = -0.678$, $P = 0.008$), while inhibin B was not correlated with either oestradiol or inhibin A concentrations in cystic fluid (Figure 1). Neither inhibin A nor inhibin B concentrations in cystic fluid showed any correlation with the volume of the cysts ($r = 0.05$ and 0.32 respectively).

The distribution of NO₂⁻/NO₃⁻ concentrations in ovarian cysts showed two clusters at 10–25 and 45–60 μmol/l (Figure 2). Cystic fluid samples containing the highest NO₂⁻/NO₃⁻ concentrations had lower inhibin A concentrations (median = 73.6 ng/ml, range = 62.5–100.3 ng/ml) than those samples containing less NO₂⁻/NO₃⁻ (median inhibin A = 97.0 ng/ml, range = 87.2–138.1 ng/ml; $P = 0.06$, Figure 2A), although the difference was not statistically significant. Inhibin B concentrations did not vary as a function of NO₂⁻/NO₃⁻ concentrations in cystic fluid (Figure 2B). The concentrations of NO₂⁻/NO₃⁻ showed no correlation with the volume of the cysts (Figure 3A), whereas oestradiol concentrations were significantly higher in those cysts containing the highest concentrations of NO₂⁻/NO₃⁻ [57 (34–79) pg/ml versus 29 (11–45) pg/ml, $P < 0.01$].

In the gonadotrophin-stimulated women, follicular fluid concentrations of NO₂⁻/NO₃⁻ were not significantly correlated with follicle size, oestradiol, inhibin A or inhibin B concentrations.

Discussion

The main finding of the present study was the observation that both inhibin A and inhibin B concentrations were consistently

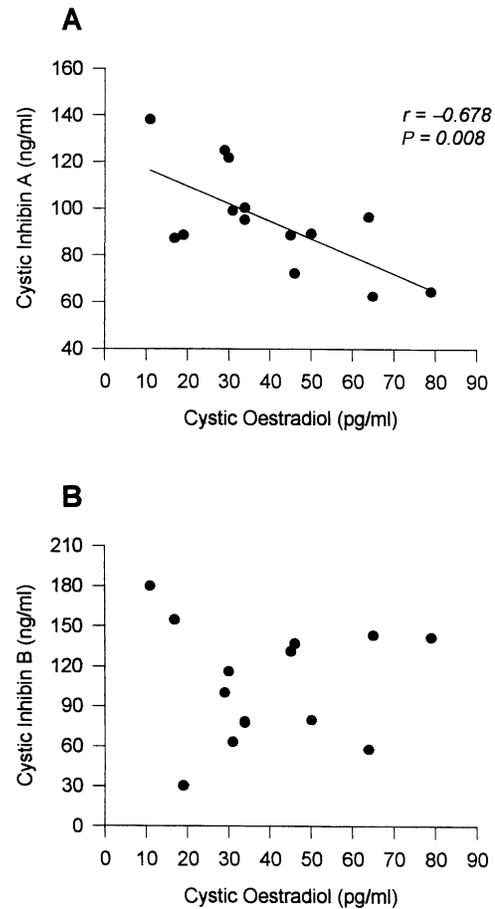


Figure 1. Linear correlation between oestradiol and (A) inhibin A and (B) inhibin B in the cystic fluid of ovarian cystadenomas ($n = 15$). r = Spearman's rank correlation coefficient.

high in cystic fluid of benign ovarian serous cystadenoma. This observation opens the question of which role inhibins would play in the pathogenesis of this type of ovarian cyst: is increased inhibin secretion a cause, consequence, or just an epiphenomenon of cyst formation? At present, no plausible molecular mechanism is known that would support a causal role for inhibins in the genesis of ovarian cysts. Studies using in-vitro stimulation (reviewed by Knight, 1996) have failed to demonstrate a direct effect of inhibins on ovarian granulosa cells, in contrast with the growth-promoting effect of activin. The hypothesis that ovarian cysts have the intrinsic property of secreting large amounts of inhibins cannot be excluded for the moment. Paracrine/autocrine regulators of inhibin secretion in the ovary include oestradiol, androgens, growth factors, and activin (Knight, 1996), and the expression levels of many of these factors in simple ovarian cysts are still unknown. Finally, some mechanism involved in cyst formation might also stimulate inhibin secretion into the cyst.

To test all these possibilities, however, it is important to identify the source or sources of inhibins secreted in the cystic fluid. Serous cystadenomas derive from the ovarian surface epithelium and are typically lined by a ciliated epithelium similar to that of the Fallopian tube. They may also present polypoid excrescences of stromal origin, but they do not contain granulosa cells. As far as we know, the present study

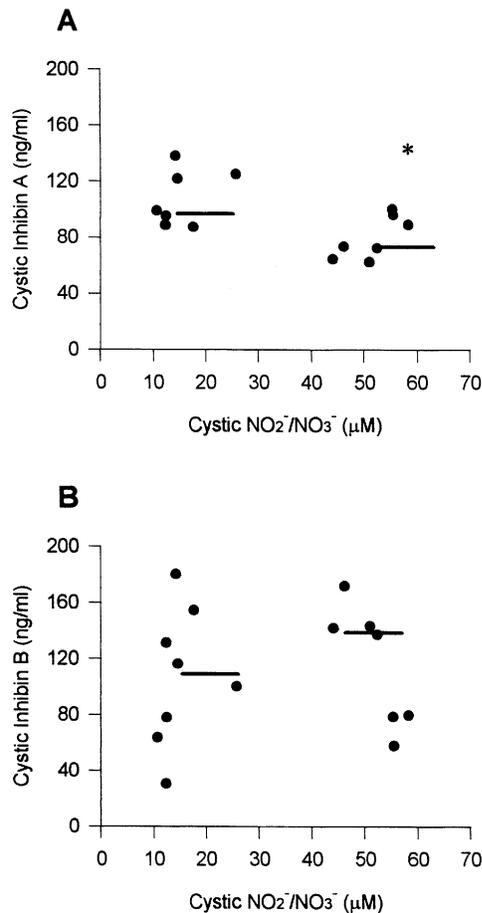


Figure 2. Scatter plot showing the relationship between cystic fluid $\text{NO}_2^-/\text{NO}_3^-$ and cystic fluid (A) inhibin A and (B) inhibin B concentrations in ovarian cystadenomas. The horizontal bars represent the median inhibin concentrations of the high and low $\text{NO}_2^-/\text{NO}_3^-$ clusters. * $P = 0.06$ (Mann–Whitney U -test).

is the first to measure inhibin concentrations in benign tumours of the ovary. A previous study attempting to localize inhibin in ovarian cystadenoma has demonstrated βA but not α subunit expression in the epithelial component of the cysts (Zheng *et al.*, 1998). Therefore it is still uncertain whether inhibins originate in the lining epithelium, the tumour stroma, the peritumoral tissues, or just transudate from blood circulation. The fact that only inhibins are increased compared with the follicular fluid of stimulated follicles while oestradiol concentrations are low suggests that the source of inhibins in ovarian cystadenoma behaves differently from granulosa cells with regard to gonadotrophin dependence and oestrogen synthesis. If this source is the cyst itself, the reason for increased inhibin concentrations in cystic fluid may be simply the cellular hyperplasia. It is also noteworthy that only inhibin A was inversely correlated with oestradiol in cystic fluid, possibly indicating some independent regulation of both inhibins in ovarian cysts, as in normal ovulatory follicles (Welt *et al.*, 1999).

In the present study, we also examined for the first time the relationship between $\text{NO}_2^-/\text{NO}_3^-$ and oestradiol concentrations in ovarian cystadenomas. The association observed may be regarded as an indirect evidence for oestradiol-mediated NO

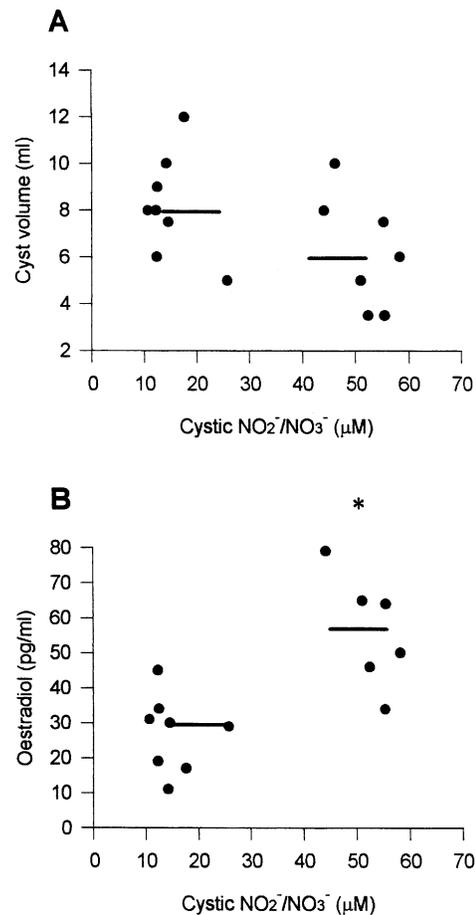


Figure 3. Scatter plot showing the relationship between cystic fluid $\text{NO}_2^-/\text{NO}_3^-$ and (A) cystic volume and (B) oestradiol concentrations in ovarian cystadenomas. The horizontal bars represent the median volume or oestradiol concentrations of the high and low $\text{NO}_2^-/\text{NO}_3^-$ clusters. * $P < 0.05$ (Mann–Whitney U -test).

release, as suggested by the changes in systemic $\text{NO}_2^-/\text{NO}_3^-$ concentrations accompanying follicular growth (Rosselli *et al.*, 1994). Another interpretation, however, is that both NO and oestradiol would come from external sources and concentrations are higher in those cysts which are more vascularized. It is more difficult to speculate about the relationship between $\text{NO}_2^-/\text{NO}_3^-$ and inhibin A concentrations in cystic fluid, since the possible interactions between inhibins and NO remain unexplored.

In the present study, we found no correlation between $\text{NO}_2^-/\text{NO}_3^-$ and oestradiol in follicular fluid. It may be argued that we studied only large pre-ovulatory follicles and therefore it would not be possible to identify changes in oestradiol (and possibly $\text{NO}_2^-/\text{NO}_3^-$) associated with follicular growth; however, when Sugino *et al.* evaluated both leading and secondary follicles no association was again detected between follicular fluid $\text{NO}_2^-/\text{NO}_3^-$ and oestradiol concentrations (Sugino *et al.*, 1996). In contrast, another group (Anteby *et al.*, 1996) found a significant correlation between NO metabolites and oestradiol concentrations in the follicular fluid of women undergoing ovarian stimulation. This apparent discrepancy seems to indicate that the interactions between oestradiol and the NO pathway within human ovary are too complex to be described,

without taking in consideration the microenvironments of individual follicles. However, this interaction is biologically plausible as NO inhibits aromatase activity in cultured human granulosa cells (Kagabu *et al.*, 1999), but it remains unclear due to the lack of an ideal in-vivo model. The main source of NO derivatives in the follicular fluid is uncertain, since part of the NO metabolite pool may be derived from blood plasma and another part may be generated by a local production of NO (Van Voorhis *et al.*, 1994).

In conclusion, our data show that inhibin A and inhibin B are present in high concentrations in the fluid of cystadenomas and that inhibin A concentrations are inversely proportional to those of oestradiol and NO metabolites. The source of inhibins in cystic fluid is still uncertain, but the concurrent observation of low oestrogen concentrations suggests the existence of local, gonadotrophin-independent regulation of inhibin secretion in benign ovarian serous cysts.

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References

Anteby, E.Y., Hurwitz, A., Korach, O. *et al.* (1996) Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. *Hum. Reprod.*, **11**, 1947–1951.

Ben-Shlomo, I., Kokia, E., Jackson, M.J. *et al.* (1994) Interleukin-1 β stimulates nitrite production in the rat ovary, evidence for heterologous cell–cell interaction and for insulin-mediated regulation of the inducible isoform of nitric oxide synthase. *Biol. Reprod.*, **51**, 310–318.

Bredt, D.S. and Snyder, S.H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl Acad. Sci. USA*, **87**, 682–685.

Dor, J., Shulman, A., Pariente, C. *et al.* (1992) The effect of gonadotropin-releasing hormone agonist on the ovarian response and *in vitro* fertilization results in polycystic ovarian syndrome: a prospective study. *Fertil. Steril.*, **57**, 366–371.

Ellman, C., Corbett, J.A., Misko, T.P. *et al.* (1993) Nitric oxide mediates interleukin-1-induced cellular cytotoxicity in the rat ovary. A potential role for nitric oxide in the ovulatory process. *J. Clin. Invest.*, **92**, 3053–3056.

Gerber, B., Muller, H., Kulz, T. *et al.* (1997) Simple ovarian cysts in premenopausal patients. *Int. J. Gynaecol. Obstet.*, **57**, 49–55.

Groome, N.P., Illingworth, P.J., O'Brien, M. *et al.* (1994) Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immunoassay. *Clin. Endocrinol. (Oxf.)*, **40**, 717–723.

Groome, N.P., Illingworth, P.J., O'Brien, M. *et al.* (1996) Measurement of dimeric inhibin B throughout the human menstrual cycle. *J. Clin. Endocrinol. Metab.*, **81**, 1401–1405.

Guevara, I., Iwanejko, J., Dembinska-Kieć, A. *et al.* (1998) Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clin. Chim. Acta*, **274**, 177–188.

Hillier, S.G., Yong, E.L., Illingworth, P.I. *et al.* (1991) Effect of recombinant inhibin on androgen synthesis in cultured human thecal cells. *Mol. Cell. Endocrinol.*, **75**, R1–R6.

Jaatinen, T.A., Penttila, T.L., Kaipia, A. *et al.* (1994) Expression of inhibin alpha, beta A and beta B messenger ribonucleic acids in the normal human ovary and in polycystic ovarian syndrome. *J. Endocrinol.*, **143**, 127–137.

Kagabu, S., Kodama, H., Fukuda, J. *et al.* (1999) Inhibitory effects of nitric oxide on the expression and activity of aromatase in human granulosa cells. *Mol. Hum. Reprod.*, **5**, 396–401.

Knight, P.G. (1996) Roles of inhibins, activins, and follistatin in the female reproductive system. *Front. Neuroendocrinol.*, **17**, 476–509.

Magoffin, D.A. and Jakimiuk, A.J. (1997) Inhibin A, inhibin B and activin A in the follicular fluid of regularly cycling women. *Hum. Reprod.*, **12**, 1714–1719.

Matzuk, M.M., Finegold, M.J., Su, J.J. *et al.* (1992) $\alpha\alpha$ -inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature*, **360**, 313–319.

Petraglia, F., Zanin, E., Faletti, A. and Reis, F.M. (1999) Inhibins: paracrine and endocrine effects in female reproductive function. *Curr. Opin. Obstet. Gynecol.*, **11**, 241–247.

Rosselli, M., Imthurn, B., Macas, E. *et al.* (1994) Circulating nitrite/nitrate levels increase with follicular development: indirect evidence for oestradiol mediated NO release. *Biochem. Biophys. Res. Commun.*, **202**, 1543–1552.

Sugino, N., Takiguchi, S., Ono, M. *et al.* (1996) Nitric oxide concentrations in the follicular fluid and apoptosis of granulosa cells in human follicles. *Hum. Reprod.*, **11**, 2484–2487.

Uilenbroek, J.T., Durlinger, A.L., Tebar, M. *et al.* (1998) Temporal changes in inhibin subunit mRNAs during atresia of preovulatory follicles in the rat. *J. Endocrinol.*, **159**, 331–340.

Van Voorhis, B.J., Dunn, M.S., Snyder, G.D. and Weiner, C.P. (1994) Nitric oxide: an autocrine regulator of human granulosa–luteal cell steroidogenesis. *Endocrinology*, **135**, 1799–806.

Welt, C.K., Adams, J.M., Sluss, P.M. and Hall, J.E. (1999) Inhibin A and inhibin B responses to gonadotropin withdrawal depends on stage of follicle development. *J. Clin. Endocrinol. Metab.*, **84**, 2163–2169.

Welt, C.K., Lambert-Messerlian, G., Zheng, W. *et al.* (1997) Presence of activin, inhibin, and follistatin in epithelial ovarian carcinoma. *J. Clin. Endocrinol. Metab.*, **82**, 3720–3727.

Woodruff, T.K. and Mather, J.P. (1995) Inhibin, activin and the female reproductive axis. *Ann. Rev. Physiol.*, **57**, 219–244.

Zackrisson, U., Mikuni, M., Wallin, A. *et al.* (1996) Cell-specific localization of nitric oxide synthases (NOs) in the rat ovary during follicular development, ovulation and luteal formation. *Hum. Reprod.*, **11**, 2667–2673.

Zheng, W., Luo, M.P., Welt, C. *et al.* (1998) Imbalanced expression of inhibin and activin subunits in primary epithelial ovarian cancer. *Gynecol. Oncol.*, **69**, 23–31.

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