Temporal changes in inhibin subunit mRNAs during atresia of preovulatory follicles in the rat

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Abstract

This study aimed to investigate the time course of disappearance of the mRNAs of the various subunits of inhibin in follicles which become atretic. An animal model was used in which atresia of preovulatory follicles could be studied in a chronological order. Injection of gonadotrophin-releasing hormone (GnRH) antagonist (20 μg) at the morning of pro-oestrus (P) blocked ovulation and the 10–12 preovulatory follicles became gradually atretic. A second injection was given the next day to prevent delayed ovulation. The rate of atresia could be delayed by simultaneous administration of a subovulatory dose of human chorionic gonadotrophin (hCG) (0·5 IU) and could be advanced by administration of a fivefold larger amount of GnRH antagonist.

Functional activity of follicles becoming atretic was studied by measuring oestradiol production after incubation of individual follicles for 4 h. Follicles isolated 24 h after the first injection of GnRH antagonist (P+24) already secreted significantly less oestradiol *in vitro* than follicles isolated at pro-oestrus, although they were morphologically not different from pro-oestrous follicles. Follicles isolated at P+24 from hCG-treated rats secreted more oestradiol compared with follicles from rats not treated with hCG. In contrast, follicles isolated at P+24 from rats that were given a fivefold larger amount of GnRH antagonist secreted less oestradiol.

Once this model was validated, temporal changes in inhibin subunit mRNAs in follicles undergoing atresia were measured by *in situ* hybridization and RNase protection assay. *In situ* hybridization showed abundant α- and βA-subunit mRNA in the whole granulosa layer of preovulatory follicles at P and P+24, while βB-subunit mRNA was restricted to the antral layer and cumulus. At P+48 the amount of α- and βA-subunit mRNA had declined and was restricted to the cumulus, whereas βB-subunit mRNA was absent. In the atretic follicles present at P+72 and P+96, mRNAs of all three inhibin subunits were absent. Administration of 0·5 IU hCG delayed the decline in the amount of α, βA and βB mRNA in preovulatory follicles at P+48. RNase protection assay of inhibin subunits in isolated follicles revealed no changes between P and P+24. However, at P+48, the mRNAs of α- and βA-subunits were decreased. Expression of the mRNA of βB-subunit declined gradually from P to P+48.

The present study demonstrates that in follicles which are becoming atretic, mRNAs of α- and βA-subunits decline simultaneously with the appearance of pycnotic cells in the granulosa layer, while βB-subunit mRNA declines earlier, simultaneously with the decrease in the ability to secrete oestradiol *in vitro*.

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Introduction

During the oestrous cycle of the rat, secretion of the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is regulated by steroids and peptides of ovarian origin. While LH secretion is mainly regulated by the steroids oestradiol and progesterone, FSH secretion is mainly regulated by ovarian inhibin. Inhibin is a heterodimer consisting of an α-subunit and a β-subunit. This β-subunit can be βA or βB, resulting in inhibin-A or inhibin-B respectively. The homodimer consisting of two β-subunits is called activin, and may have local actions on ovarian activity (Woodruff & Mayo 1990). The α-subunit is produced by granulosa cells of preantral and antral follicles, whereas the two β-subunits are only produced by granulosa cells of antral follicles (Meunier *et al.* 1988). However, Drummond *et al.* (1996) found βA-subunit mRNA also in preantral follicles.

A decrease in the secretion of inhibin by preovulatory follicles after either ovulation or atresia causes increased FSH secretion and, therefore, growth of the next crop of antral follicles. Indeed, inhibin is low or absent in atretic follicles (Tsonis *et al.* 1983, Meunier *et al.* 1988, Woodruff *et al.* 1993). However, the time course of the

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disappearance of the various inhibin subunits in follicles becoming atretic is not known, since it is impossible to study changes in inhibin subunits in a chronological order. However, temporal changes in preovulatory follicles that are becoming atretic can be studied after blocking ovulation. After inhibition of ovulation by an injection of pentobarbital (Braw & Tsafriri 1980, Uilenbroek *et al.* 1980) on pro-oestrus, the non-ovulated follicles become gradually atretic. At the same time, small antral follicles grow to large antral follicles, which will be preovulatory 4 days after pro-oestrus.

In the present study, a gonadotrophin-releasing hormone (GnRH) antagonist was used to block ovulation. This animal model to study atresia was validated by studying the morphological changes of these follicles and by correlating these changes with a functional characteristic, namely the ability to produce oestradiol *in vitro*. It appeared that the preovulatory follicles became atretic in a predictable order after administration of GnRH antagonist, and that the rate of atresia could be advanced or delayed by changing the endogenous level of LH. Using this model, changes in the mRNAs encoding the α-, βA- and βB-subunits of inhibin were studied during atresia of preovulatory follicles.

Materials and Methods

Animals

Adult female rats of a Wistar substrain (R-Amsterdam) were used. The animals were housed under standard conditions of light (lights on from 0500 h to 1900 h) and temperature (20–23 °C). Vaginal smears were taken daily. Only animals showing at least two successive 5-day cycles were used. The protocol for the handling of animals in this study was approved by the institutional animal ethics committee.

Treatment

At 1000 h on the day of pro-oestrus (P) the animals were given a subcutaneous injection with 20 μg GnRH antagonist (Org 30276, Organon, Oss, The Netherlands). This dose lowered the preovulatory LH surge and blocked ovulation (Meijs-Roelofs *et al.* 1990). To prevent ovulation the next day, a second injection was given at 1000 h the following morning (P+24). Following this treatment, vaginal smears revealed the presence of nucleated cells at P+96 and cornified cells and the presence of ova in the oviduct at P+120.

Animals were killed by an overdose of ether at P, P+24, P+48, P+72 or P+96 between 0900 h and 1000 h. Blood was obtained by puncture of the ophthalmic venous plexus. Serum samples were stored at −20 °C until assayed for LH and FSH. At autopsy, the ovaries were dissected out and fixed overnight in Bouin's fluid for histological examination and for *in situ* hybridization. Separate groups of animals were killed at P, P+24 and P+48. From these animals the ten largest follicles were isolated under a dissection microscope. Only at these days can the preovulatory follicles be distinguished from the new crop of growing follicles. After isolation, the follicles were either snap-frozen in liquid nitrogen and stored at −80 °C for RNA isolation or incubated individually to measure steroid secretion *in vitro*.

To study whether the effects of GnRH antagonist on atresia were due to the suppressed levels of LH, 0·5 IU human chorionic gonadotrophin (hCG) (Pregnyl, Organon) was administered at 1200 h after administration of 20 μg GnRH antagonist at 1000 h. In another experiment, a larger amount of GnRH antagonist (100 μg) was given. Some animals were killed at P+24 for isolation of the largest follicles followed by follicle incubation, other animals were killed at P+48 to collect the ovaries for histological examination of the number of healthy and atretic follicles.

Follicle incubation

Intact follicles were incubated individually in 1 ml Medium-199 (Gibco, Grand Island, NY, USA), pH 7·4, containing 25 mM Hepes (Sigma Chemical Co., St Louis, MO, USA). The incubations were carried out at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 in a shaking waterbath. The responsiveness of the follicles to LH was measured by incubating follicles with 1, 10 or 100 ng LH (NIH-LH S19; LH potency 1·01 NIH-LH-S1, FSH potency <0·05 NIH-FSH-S1). After incubation for 4 h, the follicles were discarded and the medium was stored at −20 °C until assayed for oestradiol.

Histology

For histological examination, the ovaries were embedded in paraffin and serial sections (thickness 10 μm) were stained with haematoxylin and eosin. Healthy and atretic preovulatory follicles were counted. Atresia of antral follicles was classified as described by Osman (1985). Atretic follicles were designated as early atretic (stages 1A and 1B) when degenerative changes were only present in the granulosa cell layer and late atretic (stages 2A and 2B) when the oocyte was also affected. At stage 1A an overall shrinkage of the granulosa wall was apparent and a few pycnotic cells were present in the granulosa layer, while at stage 1B pycnotic cells were widespread in the granulosa layer and could also be found in the antrum. At stage 2 the oocyte showed resumption of meiosis: at stage 2A the degenerating oocyte was still surrounded by cumulus cells, while at stage 2B the oocyte was not surrounded by cumulus cells.

Hormone assays

Serum concentrations of LH and FSH were measured by double-antibody RIA using anti-ovine LH and FSH as antiserum and NIDDK rat LH and FSH as tracer as described earlier (Welschen *et al.* 1975). Concentrations of LH and FSH are expressed in ng NIDDK rat LH RP-2 and FSH RP-3/ml respectively. The sensitivities of the LH and FSH assays were 0·1 ng/ml and 0·4 ng/ml respectively. The intra-assay coefficient of variation for the LH assay was 5% and that for the FSH assay 4%.

Concentrations of oestradiol in the medium were measured by RIA without extraction as described earlier (Uilenbroek *et al.* 1980). The sensitivity of the oestradiol assay was 100 pg/ml and the intra- and interassay coefficients of variation were 7·2% and 8·3% respectively.

cDNA for inhibin subunits

cDNAs encoding parts of the rat inhibin subunits were obtained from Dr F S Esch (Salk Institute, La Jolla, CA, USA). For the inhibin α-subunit probe, a rat cDNA PstI/KpnI fragment corresponding to nucleotides 759–1095 (Esch *et al.* 1987) was subcloned in pBluescript KS(−) (Stratagene, La Jolla, CA, USA). For the inhibin βA-subunit probe, a rat cDNA fragment corresponding to nucleotides 40–1540 (Esch *et al.* 1987) was subcloned in the EcoRI cloning site of pBluescript KS(−). For the inhibin βB-subunit probe, a rat cDNA fragment corresponding to nucleotides 322–632 (Esch *et al.* 1987) was subcloned in the SmaI cloning site of pBluescript KS(+) (Stratagene). All cDNAs were checked by DNA sequencing. For the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe, used as an internal control, a 163 bp AccI/Sau3AI fragment of rat GAPDH cDNA was subcloned in pBluescript KS(−). The GAPDH probe was obtained from Dr P Elfferich (University of Leiden, The Netherlands).

RNA isolation and RNase protection assay

Frozen follicles were pulverized in liquid nitrogen and total RNA was extracted by homogenizing the tissue in 3 M lithium chloride/6 M urea (Auffray & Rougeon 1980). Total RNA was dissolved in RNase-free water and its concentration and purity were determined by optical density measurements at 260 and 280 nm.

RNase protection assays were performed as described previously (van Schaik *et al.* 1997). Hybridization temperature was 45 °C for inhibin α-subunit probe and 50 °C for inhibin βA- and βB-subunit probes. Routinely 5 μg total RNA were analysed. Results were quantified using a Phosphor-Imager (Molecular Dynamics/B&L Systems, Maarsen, The Netherlands) and data are expressed relative to GAPDH.

In situ *hydridization*

In situ hybridization of inhibin α-, βA- and βB-subunit mRNA was essentially the same as described for anti-müllerian hormone mRNA (Baarends *et al.* 1995). Briefly, paraffin sections (8 μm thickness) were mounted on 3-aminopropyl-ethoxysilane-coated slides (Sigma Chemical Co.). After deparaffination and digestion with proteinase K (Boehringer Mannheim, Germany), sections were post-fixed with paraformaldehyde. After blocking non-specific binding with triethanolamine, sections were incubated with ^{35}S-labelled antisense and sense RNA probes specific for inhibin α-, βA- and βB-subunit mRNA as described above. Hybridization was carried out overnight at 55 °C. Slides were washed in 2 × SSC (standard sodium citrate) and 55% formamide at 55 °C, then treated with RNase solution (Boehringer Mannheim) and washed again twice in 2 × SSC and twice in 1 × SSC–55% formamide at 55 °C. After dehydration, slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA) and dried, followed by exposure at 4 °C for 1 week. After developing, the sections were counterstained with haematoxylin and mounted. To minimize variation, duplicate sections of all treatment groups were mounted on the same microscopic slide. Two to four slides per probe (sense and antisense) were used in a single *in situ* hybridization. Results are based on two to three *in situ* hybridizations.

Statistical analysis

Results are expressed as means ± S.E.M. Data were evaluated for statistically significant differences by one-way analysis of variance followed by Duncan's multiple range test. A difference was considered to be significant if $P<0·05$.

Results

Atresia of preovulatory follicles

Numbers of healthy and atretic preovulatory follicles at P, P+24, P+48, P+72 and P+96 in animals in which ovulation was blocked by injecting 20 μg GnRH antagonist are presented in Table 1. At P and P+24 all preovulatory follicles had a healthy appearance. At P+48 and P+72 the preovulatory follicles were in an early stage of atresia (type 1A and 1B), while at P+96 two types of large follicle were present: healthy and atretic follicles. The healthy follicles were recently formed and would be ovulated the next day, while the atretic follicles contained oocytes showing resumption of meiosis, indicative of late atresia (type 2A and 2B).

Administration of a subovulatory dose of hCG (0·5 IU) at P and P+24 to rats treated with 20 μg GnRH antagonist at P and P+24 partially prevented the occurrence of

Table 1 Number of healthy and atretic follicles after blockage of ovulation with GnRH antagonist. GnRH antagonist (20 μg) was given at 1000 h at pro-oestrus (P) and the next day (P+24). Values are means ± S.E.M. of three rats

	Number of follicles				
		Early atretic		Late atretic	
	Healthy	1A	1B	2A	2B
Day of autopsy					
P	11·7 ± 0·3	0	0	0	0
P+24	11·7 ± 0·9	0	0	0	0
P+48	0	4·7 ± 1·8	7·3 ± 1·5	0	0
P+72	0	3·7 ± 2·7	7·0 ± 2·5	0·7 ± 0·3	0
P+96	14·7 ± 0·6	1·0 ± 1·0	0	7·0 ± 1·0	3·3 ± 0·7
P+48(hCG)*	7·0 ± 1·5	1·3 ± 0·3	3·7 ± 0·7	0	0
P+48(100 μg)*	0	0	0	7·7 ± 0·9	5·0 ± 1·0

*0·5 IU hCG or 100 μg GnRH antagonist were given at P and P+24.

atresia at P+48, seen after 20 μg GnRH antagonist alone. When a larger amount of GnRH antagonist (100 μg) was used at P and P+24, resulting in lower concentrations of LH in the serum at P+24 (0·20 ± 0·01 ng/ml versus 0·72 ± 0·09 ng/ml, $P<0·001$), the preovulatory follicles present at P+48 were in a late stage of atresia (Table 1).

Follicle incubation

Follicles isolated at pro-oestrus secreted a 5- to 10-fold larger amount of oestradiol *in vitro* in the absence of LH than follicles isolated at P+24 or P+48 respectively (Fig. 1A). Addition of LH to the culture medium increased oestradiol production in pro-oestrous follicles 6-fold. However, the increase by follicles isolated at P+24 and P+48 was only 3-fold. Follicles isolated at P+24 from rats which were given a subovulatory dose of hCG (0·5 IU) at pro-oestrus produced more oestradiol than follicles isolated at P+24 from animals which were not given hCG. Addition of LH to the culture medium of these follicles resulted in a 15-fold increase in oestradiol production (Fig. 1B). Follicles isolated at P+24 from rats treated with 100 μg GnRH antagonist secreted significantly lower amounts of oestradiol *in vitro* than follicles isolated from rats treated with 20 μg GnRH antagonist (Fig. 1B).

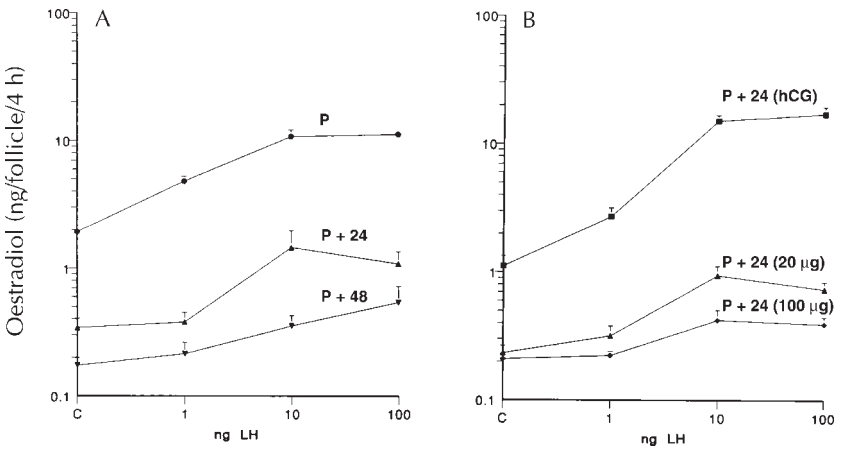

Figure 1 Accumulation of oestradiol in the medium after 4 h of incubation of individual follicles in the absence (C) and presence of 1, 10 or 100 ng LH. (A) Follicles were isolated at pro-oestrus (P, ●), 1 day after blockage of ovulation with 20 μg GnRH antagonist (P+24, ▲) or 2 days after blockage of ovulation at P (P+48, ▼). (B) Follicles were isolated at P+24 from rats treated with 20 μg GNRH antagonist alone (P+24(20 μg), ▲) or in combination with a subovulatory dose of hCG (0·5 IU) (P+24(hCG), ■) and from rats treated with a high dose of GnRH antagonist (100 μg) at pro-oestrus (P+24(100 μg), ◆). Each point represents the mean ± S.E.M. of eight to ten follicles (two follicles per rat).

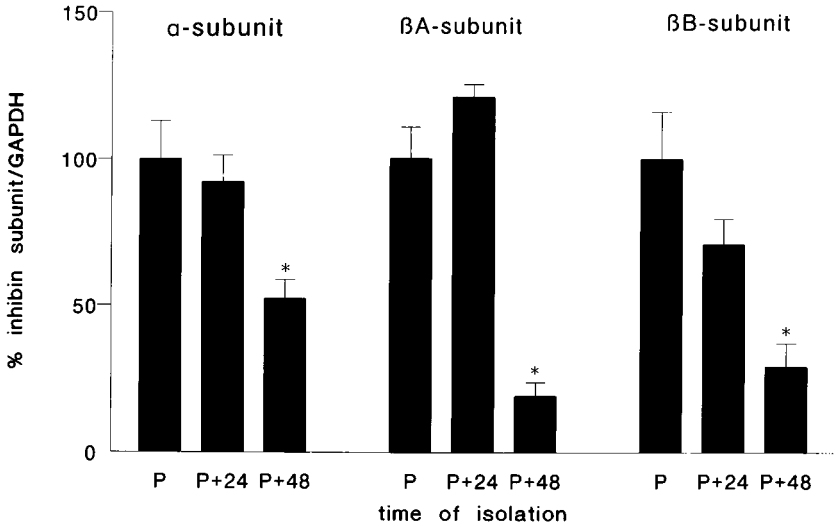

Figure 2 Levels of α-, βA- and βB-subunit mRNA measured by RNase protection assay in the largest follicles isolated at pro-oestrus (P) and 1 day (P+24) and 2 days (P+48) after blockage of ovulation by GnRH antagonist at P. Bars represent means ± S.E.M. of four to five independent samples. One sample contained mRNA from eight to ten follicles. *P<0·05 compared with value at P.

RNase protection assay

The results of the RNase protection assay of the α-, βA- and βB-subunit mRNAs in follicles isolated from rats treated with 20 μg GnRH antagonist are shown in Fig. 2. In follicles isolated at P+24, the levels of α-, βA- and βB-subunit mRNA were statistically not different from the levels in follicles isolated at pro-oestrus. At P+48 the amount of all inhibin subunit mRNAs was significantly lower than that in follicles isolated at P+24.

FSH concentrations in the serum of these animals increased from 6·89 ± 0·39 ng/ml at pro-oestrus to 9·05 ± 0·44 ng/ml at P+24 (P=0·002), and declined again to 6·74 ± 0·53 ng/ml at P+48 (P=0·004).

In situ *hybridization*

Distribution of α-, βA- and βB-subunit mRNAs in various ovarian compartments, as determined by *in situ* hybridization, in rats treated with GnRH antagonist, is presented in Figs 3 and 4 and Tables 2 and 3.

At pro-oestrus, the amount of α-subunit mRNA was high in granulosa cells of preovulatory follicles (Fig. 3B). The level of βA-subunit mRNA was high in the whole granulosa layer of preovulatory follicles (Fig. 3C), in contrast to βB-subunit mRNA which was only present in granulosa cells adjacent to the antrum and in cumulus cells (Fig. 3D). In small antral follicles, α-subunit mRNA was present, but not βA- and βB-subunit mRNAs (Table 2). *In situ* hybridization with sense probes of the three subunits did not show any expression.

At P+24, 24 h after GnRH antagonist at pro-oestrus, the level of α- and βA-subunit mRNA in the preovulatory follicles was not different from the level at pro-oestrus (Fig. 3F and G), while that of βB-subunit mRNA was reduced (Fig. 3H). At P+48, when the preovulatory follicles were in an early stage of atresia, localization of α- and βA-subunit mRNA was limited to the cumulus cells (Fig. 3J and K) and βB-subunit mRNA was absent (Fig. 3L). At P+72 (Fig. 3N, O and P) and P+96 (Fig. 4B, C and D) mRNAs of all three subunits in the granulosa cells were absent.

α-Subunit mRNA was present in small preantral follicles at P+24 (Fig. 3F), whereas βA- and βB-subunit mRNAs were nearly absent (Fig. 3G and H). The small antral follicles present at P+24 (275–350 μm, Table 2) showed moderate expression of all three subunits (not shown in Fig. 3F, G and H). At P+48, granulosa cells of the now medium-sized follicles (300–400 μm) showed the presence of α- and βA-subunit mRNA (Fig. 3J and K), which was further increased at P+72 (Fig. 3N and O) and P+96 (Fig. 4B and C), the day of the next pro-oestrus. The level of βB-subunit mRNA was high at P+48 (Fig. 3L) in the whole granulosa layer, while at P+96 it was restricted to the layer adjacent to the antrum and to the cells surrounding the oocyte (Fig. 4D).

Injection of 0·5 IU hCG at pro-oestrus did not affect the levels of mRNAs of the three subunits at P+24 (Fig. 4F, G and H). However, at P+48 the level of α-, βA- and βB-subunit mRNA (Fig. 4J, K and L) was much higher than the level at P+48 in control rats not injected with hCG. At this time the levels of the three subunits were not different from those at P+24 in control animals (see Table 3).

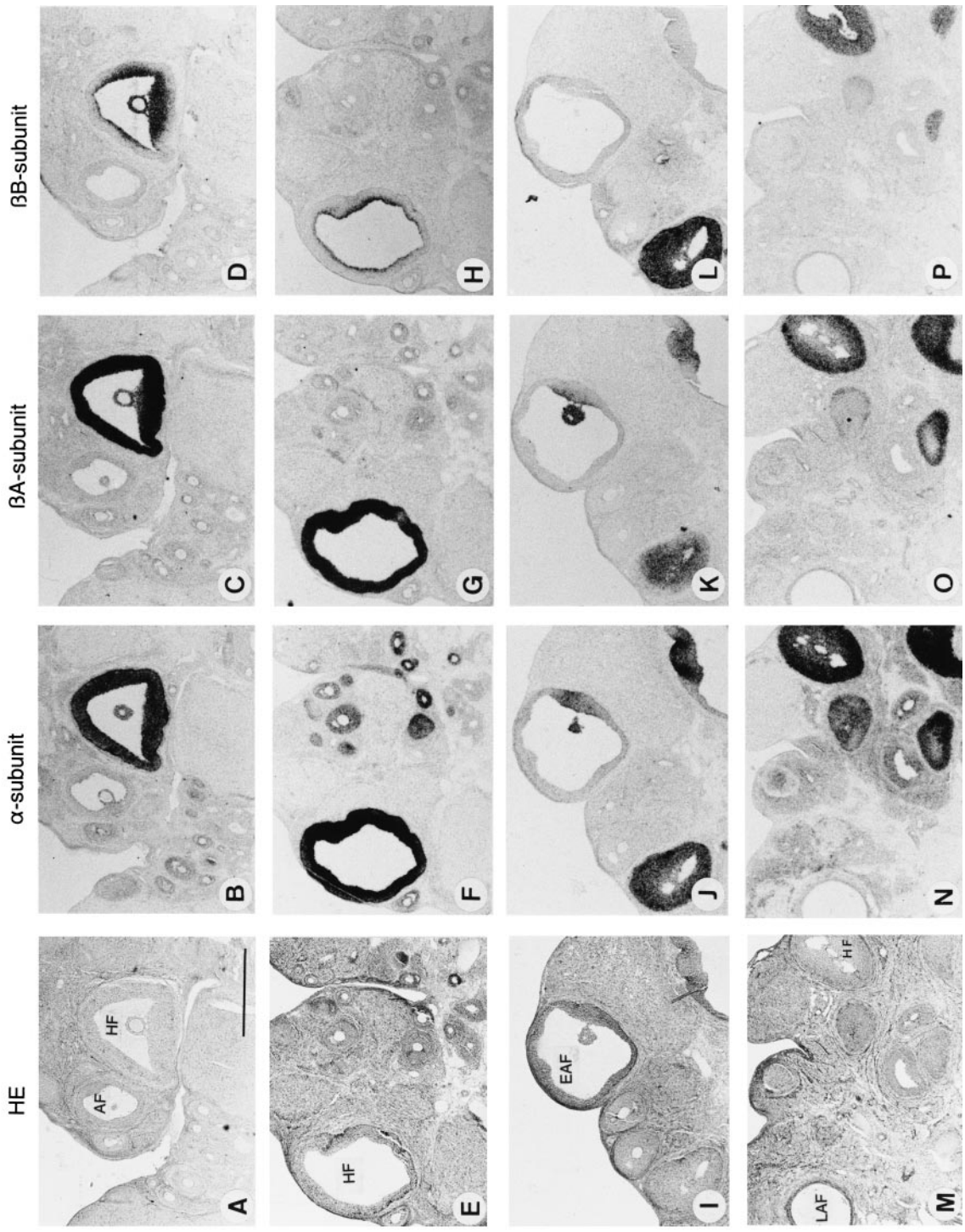

Figure 3 Localization of inhibin α-, βA- and βB-subunit mRNA at pro-oestrus (P) and 1 (P+24), 2 (P+48) and 3 (P+72) days after blockage of ovulation by 20 μg GnRH antagonist at P and the next day (P+24). First column (A, E, I and M) shows ovarian sections stained with haematoxylin and eosin (HE), second column (B, F, J and N) shows α-subunit mRNA, third column (C, G, K and O) shows βA-subunit mRNA and fourth column (D, H, L and P) shows βB-subunit mRNA. Ovarian structures shown: HF, healthy follicle; AF, atretic follicle; EAF, early atretic follicle; LAF, late atretic follicle. Bar=500 μm.

Table 2 Amounts of α-, βA- and βB-subunit mRNA in preovulatory follicles and growing follicles after blockade of ovulation by GnRH antagonist. GnRH antagonist was given at 1000 h at pro-oestrus (P) and the next day (P+24). Results are based on 24–48 follicles (2–3 rats per treatment group)

	P	P+24	P+48	P+72	P+96
Preovulatory follicles					
Morphology	Healthy	Healthy	Atretic (early)	Atretic (early)	Atretic (late)
α-Subunit mRNA	+++	+++	±	−	−
βA-Subunit mRNA	+++	+++	±	−	−
βB-Subunit mRNA	++*	+*	−/±	−	−
New antral follicles					
Morphology	Small 275–350 μm	Small 275–350 μm	Medium 300–400 μm	Large 400–500 μm	Preovulatory >450 μm
α-Subunit mRNA	+	+	++	+++	+++
βA-Subunit mRNA	−	+	++	++	+++
βB-Subunit mRNA	−	+	++	++	++*

(−) no silver grains above background, (±) some, (+) moderate, (++) clear, (+++) high.
*Restricted to the antral layer of granulosa cells.

Discussion

In this study we have used a model that allows the study of atresia of preovulatory follicles in a predictable chronological order. At 24 h after injection of GnRH antagonist on the morning of pro-oestrus (P+24), all preovulatory follicles were morphologically healthy, while 24 h later, at P+48, all preovulatory follicles were in an early stage of atresia. The rate by which the follicles become atretic appears to depend on the circulating level of LH after GnRH antagonist administration. Administration of a fivefold higher dose of GnRH antagonist (100 μg), causing a significantly lower level of LH at P+24, resulted in the presence of late atretic follicles at P+48, whereas administration of 0·5 IU hCG in rats treated with 20 μg GnRH antagonist partly prevented the occurrence of atresia at P+48. This was reflected by the increased secretion of oestradiol *in vitro* by the follicles isolated at day P+24. In an earlier study, in which ovulation was blocked by pentobarbital, the first morphological signs of atresia occurred at P+72 (Uilenbroek *et al.* 1980), while in GnRH antagonist-treated rats this was found at P+48. In pentobarbital-treated rats, serum LH levels at P+24 were significantly higher than those in GnRH antagonist-treated rats (1·57 ± 0·13 ng/ml versus 0·72 ± 0·09 ng/ml, $P<0·001$; J Th J Uilenbroek, unpublished observation). The higher level of LH in pentobarbital-treated rats may also explain why follicles isolated from these rats secreted more oestradiol *in vitro* than follicles isolated from GnRH antagonist-treated rats (Uilenbroek *et al.* 1984). A rapid decline in the ability of preovulatory follicles to secrete oestradiol *in vitro* appears to be an early sign of atresia. Whereas in the present study the first pycnotic granulosa cells were found 48 h after injection of GnRH antagonist, the decline in LH-stimulated oestradiol production by preovulatory follicles *in vitro* was already present at 24 h. This early decline in oestradiol production, before the appearance of morphological signs of atresia, has also been observed in hypophysectomized hamsters, in which follicle development was stimulated by equine chorionic gonadotrophin (eCG). After induction of atresia with an antiserum to eCG, the serum levels of oestradiol decreased earlier than the appearance of pycnotic cells in the granulosa layer (Otsuka *et al.* 1997).

Atresia has been recognised as an apoptotic process (Tilly 1993, Palumbo & Yeh 1994). However, internucleosomal DNA fragmentation, a biochemical characteristic of apoptosis, was found at nearly the same time as the appearance of pycnotic cells in the granulosa layer (A L L Durlinger & J Th J Uilenbroek, unpublished observation). Therefore, we considered the conventional criteria for atresia (pycnosis of granulosa cells) as a reliable marker to follow the process of atresia.

Changes in inhibin subunit mRNAs have been studied during the oestrous cycle (Meunier *et al.* 1988, Woodruff *et al.* 1988) and during reproductive life (Jih *et al.* 1993). mRNA levels of the subunits are regulated by cyclic changes in gonadotrophin secretion and follicular development. Levels of α- and β-subunit mRNA increase when follicles become larger under the influence of FSH and LH. Subunit mRNA levels are maximal in granulosa cells of preovulatory follicles and decrease rapidly after the ovulatory LH surge (Rivier *et al.* 1989, Woodruff *et al.* 1989, Tebar *et al.* 1997). In the absence of the preovulatory LH surge, mRNA levels will not decrease during the late afternoon of pro-oestrus and are still high at 0200 h of oestrus (Rivier *et al.* 1989, Woodruff *et al.* 1989, Tebar *et al.* 1997). The present results with *in situ* hybridization and RNase protection assays in isolated preovulatory follicles demonstrate that mRNA levels of α- and βA-subunits are also not changed on the following day. Only at P+48 are mRNA levels of these subunits decreased. In

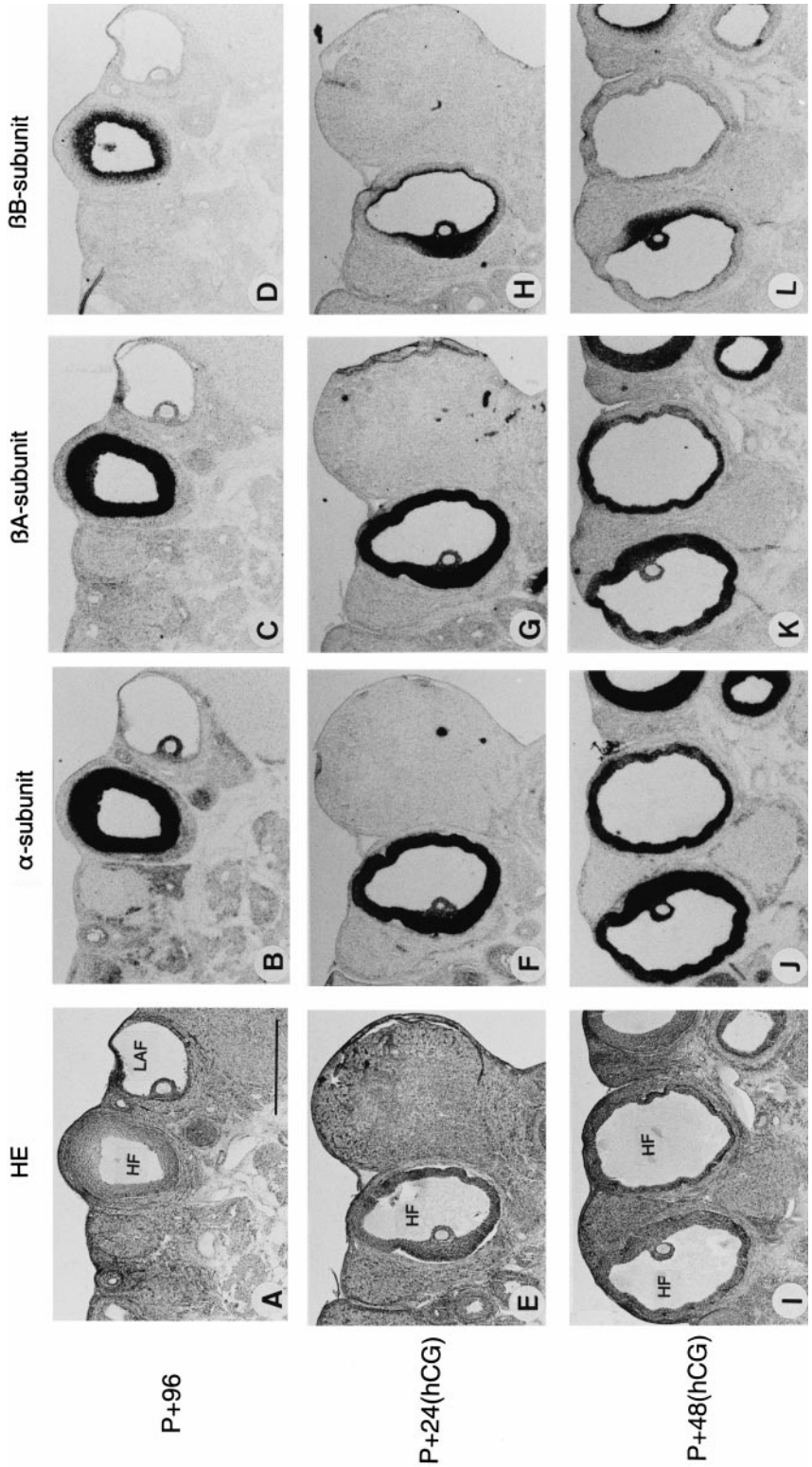

Figure 4 Localization of inhibin α-, βA- and βB-subunit mRNA at 4 days (P+96) after administration of a GnRH antagonist at pro-oestrus (P) and the next day (P+24), and 1 and 2 days after administration of 20 μg GnRH antagonist plus 0·5 IU hCG at pro-oestrus (P+24(hCG) and P+48(hCG) respectively). First column (A, E and I) shows ovarian sections stained with haematoxylin and eosin (HE), second column (B, F and J) shows α-subunit mRNA, third column (C, G and K) shows βA-subunit mRNA and fourth column (D, H and L) show βB-subunit mRNA. Ovarian structures shown: HF, healthy follicle; LAF, late atretic follicle. Bar=500 μm.

Table 3 Effect of hCG on the levels of α-, βA- and βB-subunit mRNAs in preovulatory follicles after blockade of ovulation by GnRH antagonist. GnRH antagonist was given at 1000 h and 0·5 IU hCG at 1200 h at pro-oestrus (P) in the animals killed at P+24, and at P and P+24 in the animals killed at P+48. Results are based on 12–24 follicles (2 rats per treatment group)

	P+24	P+24(hCG)	P+48	P+48(hCG)
Preovulatory follicles				
Morphology	Healthy	Healthy	Atretic (early)	Healthy
α-Subunit mRNA	+++	+++	±	+++
βA-Subunit mRNA	+++	+++	±	++
βB-Subunit mRNA	+*	+*	−/±	+*

(−) no silver grains above background, (±) some, (+) moderate, (++) clear, (+++) high.
*Restricted to the antral layer of granulosa cells.

contrast, the level of βB-subunit mRNA decreased gradually from pro-oestrus onwards. This decrease is inversely related to the serum levels of FSH. Serum FSH levels at P+24 were significantly higher than those at pro-oestrus, and are likely to be responsible for the growth of a new crop of follicles, which were preovulatory at P+96.

Our results with *in situ* hybridization revealed that the α-subunit mRNA was present in granulosa cells of small antral follicles, while β-subunit mRNAs were only present in granulosa cells of antral follicles present at P+24. The high expression of the β-subunit mRNA in large follicles correlates with the presence of biologically active inhibin in such follicles, for biologically active inhibin has been found in isolated large preovulatory follicles, but not, or only at a low concentration, in isolated follicles smaller than 450 μm (van Cappellen *et al.* 1995). This demonstrates that in growing follicles the presence of β-subunits is rate-limiting for the production of inhibin.

The distribution pattern of βB-subunit mRNA is different from that of βA-subunit mRNA. In growing follicles at P+48, mRNAs of both subunits, in the follicles between 350 μm and 450 μm, are equally distributed over the whole granulosa layer, while in follicles larger than 450 μm present at P and P+96, βB-subunit mRNA is restricted to the layer adjacent to the antrum. Furthermore, the βB-subunit mRNA disappears earlier than that of βA in the follicles becoming atretic.

In summary, the present study demonstrates that in preovulatory follicles undergoing atresia after blockage of ovulation by a GnRH antagonist, the levels of α- and βA-subunit mRNA decrease between 24 and 48 h after injection, while the expression of βB-subunit mRNA, which is restricted to the granulosa cells around the antrum, decreases earlier. Furthermore, the ability to secrete oestradiol *in vitro* decreases between P and P+24. The changes in inhibin subunits and the ability to secrete oestradiol appear to depend on the circulating levels of LH.

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