IMMUNOHISTOCHEMICAL DETECTION OF ATRIAL NATRIURETIC FACTOR (ANF) IN DIFFERENT OVARIAN CELL TYPES

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Objective. Atrial natriuretic factor (ANF) and its receptors were identified in various tissues and organs including the female reproductive system. The present studies were undertaken to investigate ANF localization in immature rat ovaries, and the changes of ANF expression in response to different ovarian hormonal conditions.

Methods. ANF was characterized immunocytochemically during different ovarian status using an animal model with synchronized ovarian follicular growth and atresia induced by pregnant mare serum gonadotropin (PMSG). This treatment in prepubescent rats produces a series of ovarian changes mimicking follicular growth and differentiation (48 h after PMSG treatment) and atresia (96 h after PMSG treatment) and permits the synchronization of events for investigation purposes.

Results. Our findings showed that in immature rat ovaries ANF was localized primarily in granulosa cells of all developing follicles (healthy and atretic). Immunocytochemical analysis revealed that during follicular growth and differentiation (48 h after PMSG) ANF was present in all steroid producing cells – interstitial, thecal and granulosa cells. ANF immunoreactivity was also detected in the cytoplasm and nuclear compartment of the growing oocytes. In all atretic follicles ANF staining was detected at 96 h after PMSG injection, but the intensity of the reaction varied with the degree of atresia, the mostly pronounced reaction being observed in the late stage of atresia.

Conclusions. These results indicate that ANF represents a unique peptide, which is expressed in different ovarian cell types and responds to different developmental programming.

Key words: Atrial natriuretic factor – Immunocytochemistry – Ovary – Follicular development – Rat

The growth and development of an ovarian follicle proceed continuously until the follicle either ovulates or undergoes a degenerative process known as atresia. The key factors responsible for promoting the growth and development are pituitary gonadotropins and gonadal steroids (GOLDENBERG et al. 1972). Gonadal steroids are also important intraovarian regulators of follicular atresia and the profile of sex steroid production in healthy follicles differs from that in atretic ones (BILLIG et al. 1993). Several growth factors and intraovarian peptides have been shown to regulate follicular development and steroidogenesis including fibroblast, epidermal and insulin-like growth factors (GOSPODAROWICZ et al. 1977), activin (XIAO et al. 1992), endothelin (KARAM et al. 1999) and others. Recent data suggest a possible participation of ANF in the complex processes of follicular function..

ANF is a member of a family of peptides all shearing a common 17-amino acid ring closed by a disulfide bound between two cysteine residues and varying only in length of their N- and C-terminal extensions (FLYNN et al. 1983). It is synthesized by atrial cardiocytes and secreted into circulation. However, its presence and local synthesis have been described in several extracardiac tissues (GUTKOWSKA

et al. 1989). ANF stimulates the membrane-bound form of guanylate cyclase (Inagami 1989) and inhibits adenylate cyclase resulting in an increase of the intracellular cGMP and a decrease of cAMP in different cell types. The presence of ANF receptors designated GC-A (Chinkers et al. 1989) were established in various tissues and organs including male (Mueller and Middendorf 1997) and female gonads (Gutkowska et al. 1993).

Several studies point to a modulatory role of ANF in the steroidogenesis. ANF has been shown to inhibit aldosterone synthesis and secretion in adrenal glands (Atarashi et al. 1984; De Lean et al. 1984). In mouse Leydig tumor cells ANF inhibits progesterone synthesis (Pandey et al. 1985) and stimulates testosterone production in normal mouse Leydig cells (Pandey et al. 1986) and progesterone secretion in granulosa-luteal cells (Pandey et al. 1987). Increasing evidence suggest that in the ovaries the locally synthesized ANF may act in autocrine or paracrine manner to produce physiological response. ANF binding sites have been detected in bovine ovaries (Saheki et al. 1989) and bovine corpus luteum (Kim et al. 1989). Immunoreactive ANF has been demonstrated in the bovine corpus luteum (Vollmar et al. 1988), in human ovarian follicle and follicular fluid (Steegers et al. 1990) as well as in pig granulosa cells (Kim et al. 1992).

Further studies showed that ANF via cGMP inhibits spontaneous oocyte maturation (Tornell et al. 1990) suggesting a possible involvement of ANF in the control of meiotic process. The presence of both local ANF synthesis and high affinity transducing receptors have been demonstrated in adult rat ovaries (Gutkowska et al. 1993). More recently it has been shown that ovarian ANF system is under gonadotropin regulation particularly by FSH, a crucial hormone of folliculogenesis (Gutkowska et al, 1999), thus suggesting its importance in ovarian function. Moreover, the latter authors demonstrated that ANF has a modulatory effect on gonadotropin-induced ovarian steroidogenesis by inhibiting E_2 production. However, the exact cell type specific localization of ANF during follicular growth, development and atresia and its role in reproductive functions remain unknown.

In the present studies we used an animal model with synchronized follicular growth and atresia induced by gonadothropin treatment. After priming immature rats with PMSG, ovarian follicles develop within 48 h but ovulation is not necessarily induced since an ovulatory surge of LH may be lacking (Peluso et al. 1980). These follicles degenerate in a very predictable manner 96 h after PMSG treatment (Huges and Gorospe 1991). The changes in the follicles correlate with the loss of LH receptors (Peluso et al. 1977) and thereby result in shift in steroidogenesis. Such shift in steroidogenesis from estrogen to progesterone has previously been shown to accompany the onset of follicular atresia (Terranova 1981). To gain further insight into the biology of ANF in female gonads we focused our studies on ANF cell type specific localization during different ovarian status.

Materials and Methods

Monoclonal antibody production. The production and characterization of specificity of Mab 6C3 used in this study have been described previously (Kehayov et al. 1998). Briefly, BALB/c mice were immunized sc with conjugate of ANF to bovine serum albumin (BSA) emulsified in Freund's adjuvant. Immune spleen lymphocytes from the mouse with the highest titer of anti-ANF antibodies were fused with P3U1 mouse myeloma line. Supernatants from wells with growing hybridomas were screened for presence of anti-ANF antibodies by ELISA. As a result Mab 6C3, which was of IgG type, was selected.

Induction of follicular growth and atresia. A total of 10 immature 25 day-old female Wistar rats were injected s.c. with 15 IU PMSG in 0.1 ml PBS. Experimental animals were sacrificed either 48h and 96h after treatment. Ovaries were excised, fixed in Bouin's solution and embedded in paraffin. Immature ovaries were also excised from five 29 day-old untreated control rats. Ovaries from each treatment group were sectioned and stained with hematoxylin and eosin for subsequent morphological analysis.

Immunocytochemistry. Paraffin sections of ovaries from untreated and treated rats were processed with avidin-biotin-peroxidase technique (ABC) of Hsu et al. (1981). In this procedure methanol hydrogen peroxide solution and normal rabbit serum was used to block nonspecific binding of the secondary

antibody (biotinylated rabbit anti-mouse IgG). Sections were incubated with Mab 6C3 for 18 h at 4 °C, then rinsed with phosphate buffered saline (PBS) and incubated for 60 min with biotinylated rabbit anti-mouse IgG (Vector, Burlingam) diluted 1:250 in PBS. After rinsing in PBS avidin-biotin-peroxidase conjugate (Vector, Burlingam) diluted 1:250 in PBS was applied for 60 min. The binding sites were visualized with 3',3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl buffered saline (pH 7.6) 0.01 % H_2O_2, dehydrated and coverslipped. In controls, the primary antibody was replaced with: 1. normal mouse serum; 2. Mab 6C3 absorbed with synthetic ANF for 24 h at 4 °C; 3. PBS instead of the Mab 6C3. Further control was performed with Mab 5G5 (IgG) obtained against rat ovarian antigen, which reacted with granulosa cells and Leydig cell surface (Russinova et al. 1995) as a subclass-matched negative control.

Results

ANF immunoreactivity in immature rat ovaries. In 29-day-old rat ovaries immunocytochemistry revealed that all developing follicles (healthy and atretic) were immunoreactive. The immunoreaction was observed in the cytoplasm of follicular epithelial cells of small primary follicles and in granulosa cells of preantral and early antral stages (Fig. 1). Thecal and interstitial cells were moderately labeled. Little or no ANF labeling was seen in the oocytes.

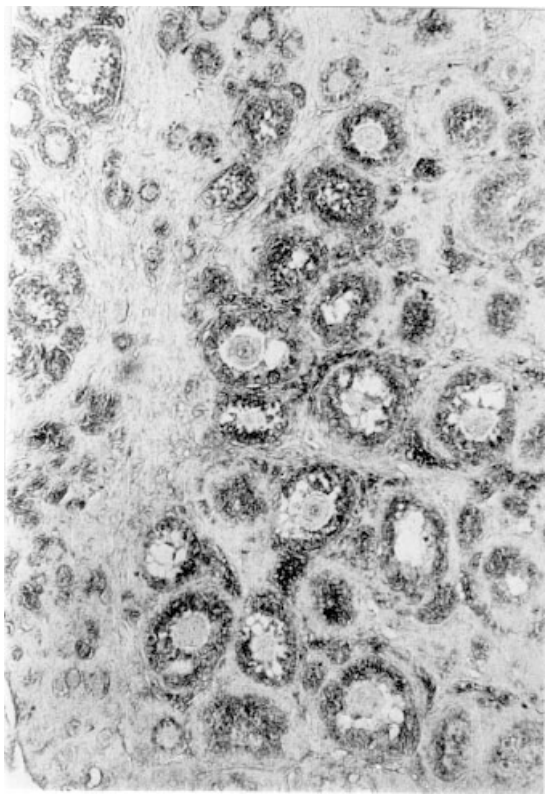

Figure 1
ANF immunoreactivity in immature rat ovary. The immunoreaction was observed in the cytoplasm of follicular epithelial cells of small primary follicles and in granulosa cells of preantral and early antral stages, x 140.

ANF immunoreactivity in rat ovaries during gonadotropin stimulated follicular growth and development. As expected, the majority of the follicules were in preantral to early antral stages 48 h after injection of PMSG (Fig. 2A) The strongest ANF immunoreactivity was observed in the cytoplasm of interstitial cells surrounding the developing follicles (Fig. 3A). ANF immunoreactivity was also detected in thecal and granulosa cells cytoplasm. However, the intensity of immunostaining depended on location of the granulosa cells, since peripheral granulosa cells exhibited little activity while granulosa cells lining the antral cavity and the oocyte were strongly positive (Fig. 3B-E). ANF staining was also observed in cumulus cell cytoplasm (Fig. 3D). The cytoplasm of growing oocytes was moderately stained. The intensity of reaction was stronger near the periphery of the oocyte and in the nuclear compartment (Fig. 3A and 3C). The nuclear labeling in the oocytes seemed to extend around the nucleus. No reaction was observed in control sections (Fig. 3F).

ANF immunoreactivity in rat ovaries during follicular atresia. The majority of ovaries showed definite features of atresia at 96 h after the injection of PMSG. The granulosa layer was thinned and often partly detached from underlying basal lamina. The signs of degeneration were observed both in granulosa cells and oocytes including a degenerative granulosa cell layer and abundance of apoptotic bodies budding off into the antrum (Fig. 2B). Strong ANF staining was detected in all atretic follicles, but the strength of the reaction varied

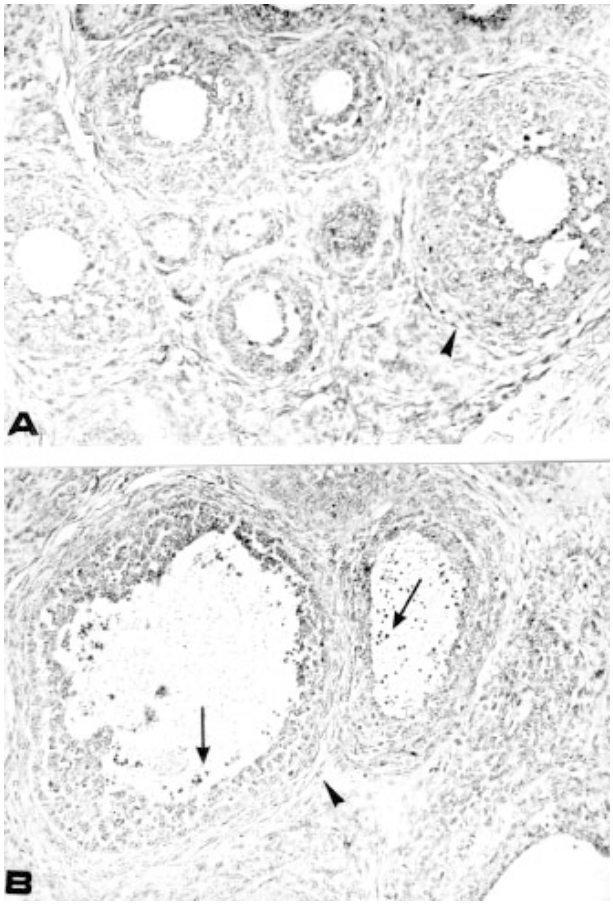

Figure 2
Effects of PMSG treatment on ovarian morphology. Sections were stained with hematoxylin and eosin. (A) 48 h after PMSG treatment. *Arrowhead* indicates a healthy multilaminar granulosa cell layer; (B) 96 h after PMSG treatment. *Arrowhead* indicates a follicle undergoing degeneration. Note the abundance of apoptotic bodies budding off into the antrum *(arrows*). A and B, x 140.

with the degree of atresia. The strongest reaction was observed in late stage of atresia. The interstitial cells were strong labeled. ANF staining was also detected in the oocytes, which often appeared distorted (Fig. 4 A and B).

Discussion

There is increasing evidence that the ovary could be a site of ANF secretion. ANF mRNA and ANF prohormone were detected in pig granulosa cells (Kim et al. 1992). Gutkowska et al. (1993) demonstrated that, in harmony with the presence of biologically active receptors, ANF markedly enhanced cGMP accumulation (by 15-fold) in rat ovaries. Moreover, Johnson (1994) using *in vitro* approaches demonstrated that ANF can modulate progestin steroidogenesis in both the differentiated and undifferentiated granulosa cells and thus may play an important role in granulosa cell differentiation and follicular maturation.

In the present study using immature rats treated with pregnant mare's serum gonadotropin (PMSG) and immunocytochemistry we demonstrated that ANF was present in all steroid producing cells, as well as in developing oocytes. Our immunocytochemical results showed that 48 h after PMSG the strongest ANF immunoreactivity was detected in interstitial cells. The exact role of interstitial cells in ovarian steroidogenesis and/or homeostasis is unclear. ANF staining in rat ovarian interstitial cells has been described previously by Gutkowska et al. (1993). However, the authors revealed ANF staining only in interstitial cells and ovum. We observed ANF immunoreaction in thecal cells, which are known to synthesize renin (Do et al. 1988) and androgens. The latter in turn diffuse to granulosa cells where they are converted to estrogens.

Our immunocytochemical results suggest that the position of the granulosa cells within the follicle modulate their capacity for ANF expression. The strongest reaction was observed in the innermost granulosa cell layer lining the oocyte and antral cavity and in cumulus cells. These findings are consistent with the concept of a heterogeneous granulosa cell population with different origin and function. This was presumed on the basis of different sedimentation properties and hormonal responsiveness (Kasson et al. 1985), the cytochrome P-450 activity (Goldring et al. 1986), and the expression of cell surface antigens (Erikson et al. 1985). Studies carried out on rat have shown that during follicular growth, only granulosa cells in cumulus proliferate (Hirshfield and Midgley 1984) and can be considered as stem cells. Under endogenous FSH the peripheral granulosa cells located near the basal lamina undergo terminal cytodifferentiation, characterized by the acquisition of LH\hCG receptors, occurrence of steroidogenic enzyme and lipid droplets. All of these findings taken together suggest that in developing follicles ANF immunoreactivity was con-

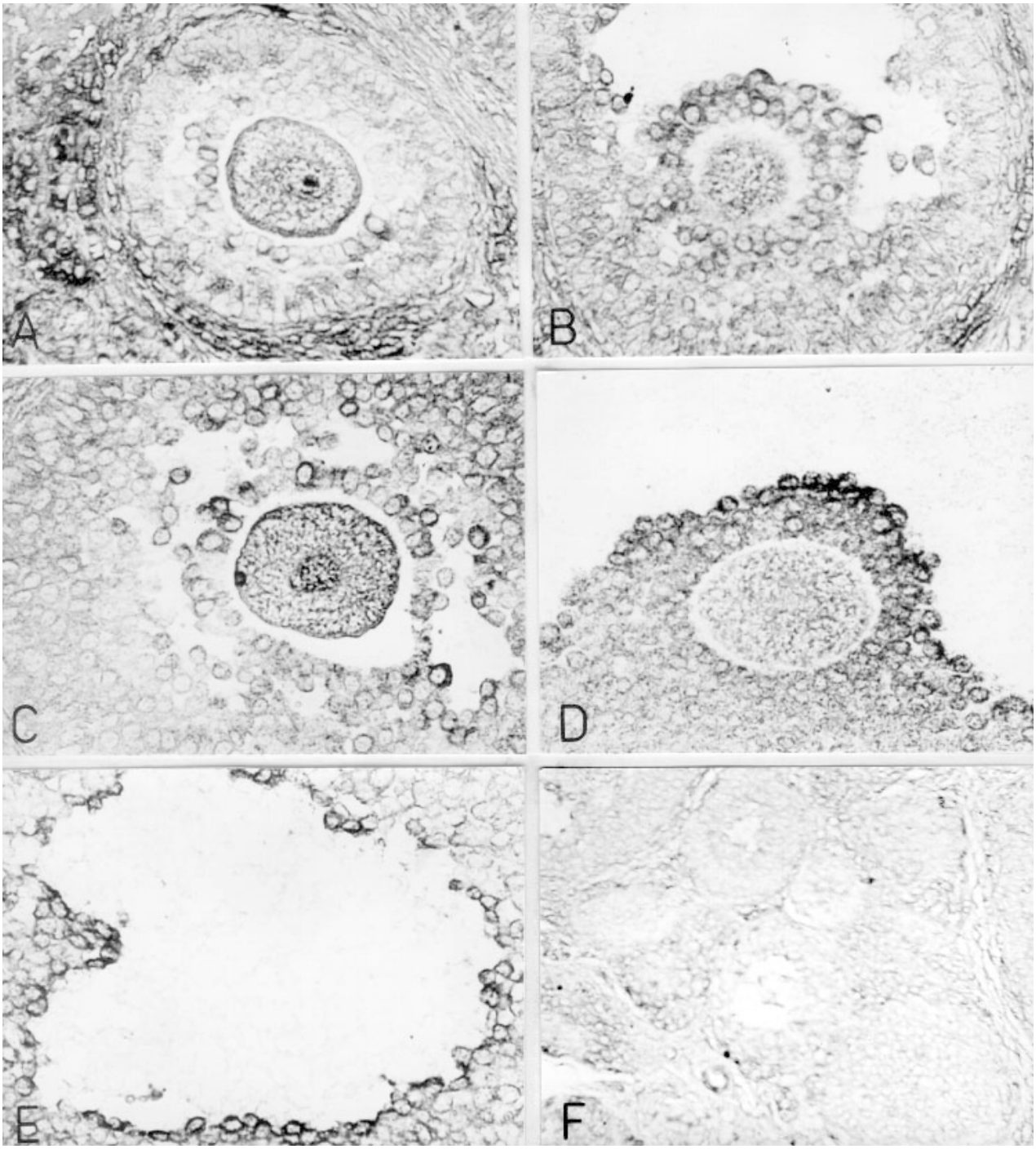

Figure 3
Immunocytochemical localization of ANF in paraffin embedded tissue sections from rat ovaries after PMSG stimulation. Note the dense immunostaining in the cytoplasm of interstitial and thecal cells (A) and immunoreactive gradient in granulosa cells: the immunoreaction was stronger in the granulosa cells lining the oocyte (also cumulus cells) and the antral cavity (B-E). In the growing oocyte the ANF staining was stronger near the periphery of the oocyte and in region of germinal vesicle (A and C). No reaction was observed in control sections after replacement of the anti-ANF antibody with nonimmune serum (F). A, B, C, E: x 400; D: x 240; F: x 140.

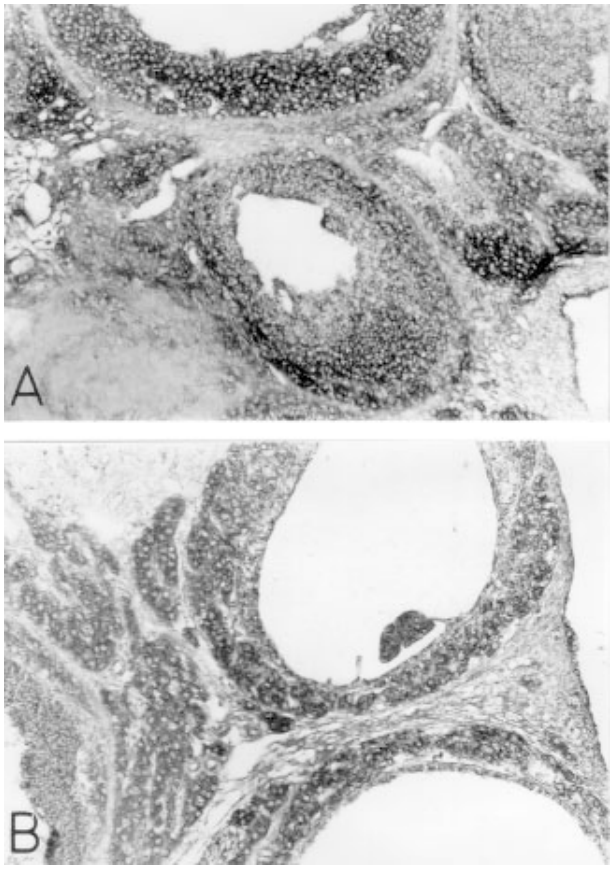

Figure 4
ANF immunoreactivity in rat ovaries during follicular atresia. Strong ANF staining was detected in all atretic follicles (A). The interstitial cells were strong labeled. ANF staining was also detected in the oocyte, which appeared distorted (B). A and B: x 250.

fined primary to stem cell population of granulosa cells uncapable to undergo terminal cytodifferentiation under FSH stimulation. On the other hand, since the oocytes removed from their follicular environment, spontaneously mature in culture, it is believed that they are maintained in its immature stage by a follicular "arrester" of granulosa cell origin (Tsafriri and Pomerantz 1986). Therefore, the cumulus-oocyte communication represents a route by which signals generated in granulosa cells regulate a number of oocyte functions such as growth (Buccione et al. 1987) and meiotic progression (Eppig and Downs 1988). In this regard a functional role of cGMP in inhibition of spontaneous oocyte maturation has been postulated (Tornell et al. 1990).

ANF specific labeling in rat ovum has been described previously in rat (Gutkowska et al. 1993) and in fish vitellogenic oocyte (Mandich et al. 1991). However, we established for the first time ANF labeling in the oocyte nuclear compartment. This finding implies a possible role for ANF in the modulation of oocyte growth and differentiation. In this regard, it is known, that in growing oocyte modification in the nuclear organization occurs coincident with the acquisition of meiotic competence and correlates with the ability to resume meiosis in vitro (Wickramasighe et al. 1991). A precise interpretation of the observed ANF immunoreactivity in developing oocytes is difficult at the present time. It could be due either to a sequestration through the binding to ANF receptor or to local synthesis. The elucidation of ANF intracellular trafficking will be important to understand its source and the mechanism of action.

Our immunocytochemical results showed for the first time ANF labeling during follicular atresia. Earlier studies have shown that the follicle atresia in rodent ovaries is associated with internucleosomal fragmentation of cellular DNA (Huges and Gorospe 1991), a hallmark of apoptotic cell death. The molecular mechanism responsible for follicular atresia and the regulation of granulosa cell endonuclease activity responsible for apoptotic DNA fragmentation are unknown. In this regard (Billig et al. 1993) postulated that the endonuclease is present during all stages of follicular development, but activation of the enzyme is regulated by gonadal steroids. They also found that the estrogens inhibit, whereas the androgens enhance DNA fragmentation in granulosa cells thus suggesting the regulation of endonuclease activity. Recently, Gutkowska et al (1999), demonstrated that ANF has a modulatory effect on CG-induced ovarian steroidogenesis by inhibiting E_2 production. It is known that ANF stimulates the membrane-bound form of guanylate cyclase (Inagami 1989) and inhibits adenylate cyclase resulting in an increase of intracellular cGMP and a decrease of cAMP. In general, little is known concerning the role of cGMP dependent signaling pathways in granulosa cell function. The findings presented by LaPolt and Hong (1995) indicate an inhibitory role of cGMP-dependent signaling pathways on FSH-induced aromatose activity. The functional significance of the

observed elevation in ANF expression in late stage of atresia remains to be elucidated. However, VOLLMAR et al. (1993) demonstrated elevation of ANF expression after X-ray irradiation, which induces apoptosis in thymocytes.

An alternative hypothesis is that ANF in the ovaries may be involved in the control of folliculogenesis by antagonizing the action(s) of one or more growth factors as it was established for aortic endothelial cells, where ANF attenuates the proliferative effects of serum and fibroblast growth factors (ITOH et al. 1992). However, in the absence of detailed information about the biological effect of ANF during follicular development, conformation of the above hypotheses awaits further studies.

The pattern of cytological localization of ANF presented here supports a local origin of ANF. Our results provide additional evidence that ANF represent a unique peptide, which is expressed in different ovarian cell types and probably, responds to different developmental and hormonal programming.

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