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Local Regulation of Granulosa Cell Steroidogenic Function and Apoptosis in Human Ovary

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The present study was undertaken to demonstrate the in vitro effects of Inhibin A on apoptotic cell death and steroidogenesis in ovarian granulosa cells of women with different hormonal status. The immunoexpression of apoptotic marker (M30, caspase-3), pro- and antiapoptotic proteins (Bcl-2, Bc-xl, Bak), proliferation marker (Ki67) and steroid production were investigated. Inhibin A inhibits apoptosis rate in GCs and stimulate estradiol production. The results suggest a potential role of Inhibin in the processes of apoptosis, proliferation and steroidogenesis.

Key words: granulosa cells, inhibin, apoptosis, steroid production.

The processes of follicular growth, differentiation and cell death are hormonally regulated processes, depending on peptide and steroid hormones. Even though ovarian steroid hormones are essential for follicular development, steroidogenic functions and degeneration, it has become increasingly apparent that intraovarian locally produced soluble factors may also play an imporant role in the processes that occur in the ovarian granulosa cells (GCs). Recently we have reported that some intraovarian substances (oxytocin, endothelin, relaxin) in physiological doses affected GC differentiation. To clarify the in vitro effects of the locally produced growth factor Inhibin A (I) on steroidogenesis and apoptotic cell death and its mechanisms in ovarian GCs the production of estradiol (E2) and progesterone (P) and the immunoexpression of early and late apoptosis markers (M30; caspase-3), pro- and anti-apoptotic proteins (Bcl-2, Bcl-xl, Bak) and proliferation marker (Ki-67) were evaluated in GCs of ovaries obtained from women of different hormonal status.

Material and Methods

Granulosa cells were isolated from ovarian antral follicles of [1] women participating in an in vitro fertilization (IVF); [2] young (Y) normally cycling, hormonally nontreated women and [3] premenopausal (PrM) women (without any hormonal therapy for at least one year). GCs were cultured in DMEM (Sigma) in the presence of 10% fetal calf serum (FCS, Sigma) either with or without Inhibin A (Sigma, 10 ng/ml) for 72 hours. The steroid levels were estimated by means of of radioimmunoassay (RIA). The cell monolayers were incubated overnight at 4°C with one of the primary monoclonal antibodies: M30 CellDeath; Caspase 3, Bcl-2, Bcl-xL, Bak, Ki-67. To calculate the percentage of immunopositive cells by light microscopy at 400 × magnification, labelled nuclei/labeled cells were counted in 450 randomly selected granulosa cells from three slides per marker studied and per patient.

Results

Apoptotic cells were found in GC cultures of IVF women as is seen by the detected activity of caspase-3 and M30 in the GC cytoplasm (tabl.1). The apoptotic cells were numerous in GC cultures of Y patients, non hormonally treated and were even more abundant in GCs of PrM women. The majority of the nuclei of IVF patient GCs was staining, indicating expression of the Ki67 (Table 1). Percentages of proliferation rate, seen in GCs of Y patients were weakly expressed and diminished in GCs of PrM women. Bak (pro-apoptotic factor) is slightly immunohistochemically detected in GC cytoplasm of IVF patients, enhanced in GCs of Y patients and is markedly increased in GCs of PrM patients. In contrast pro-oncogene Bcl-2 expression is well represented and similar in both groups of Y patients and relatively lower in PrM women. Bcl-xl is well immunoexpressed in GC cytoplasm of IVF patients, slightly weaker in GCs of young patients and almost 2 fold decreased in GC cultures of PrM women. In ovarian GCs from the three experimental groups inhibin A exposition enhanced the nuclear staining with Ki67 and reduced the activation of caspase-3 enzyme and M30 as compared with controls /without I in the culture medium/ (Table 1). Inhibin treatment slightly increased the immunoexpression of pro-oncogenes Bcl-2 and Bcl-xl in all groups. After I addition in the culture medium scattered ovarian

Table. 1. Percentage of caspase-3, M30, Ki67, Bak, Bcl-2, Bcl-xl immunopositive ovarian granulosa cells from young (Y), in vitro fertilized (IVF) and premenopausal (PrM) women (control and after inhibin treatment). Values are mean ± SE of 11 cultures from 2 experiments

Markers	Control (%)			Inhibin (%)		
	IVF	Y	PrM	IVF	Y	PrM
Caspase-3	6.9	15.8	26.6	5.0	11.1	24.5
M30	5.3	12.0	22.4	3.9	9.7	21.6
Ki67	41.3	34.0	28.2	46.2	41.0	31.3
Bak	8.0	11.0	16.0	3.0	4.0	12.0
Bcl-2	24.0	23.0	16.0	27.0	26.0	15.0
Bcl-xl	26.0	25.0	14.0	28.0	27.0	15.0

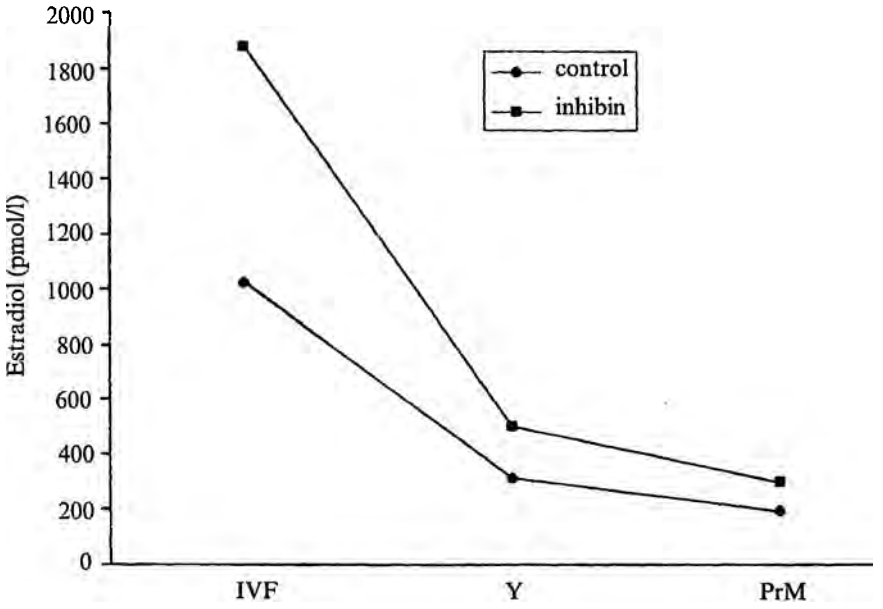

Fig. 1. Inhibin A effect on estradiol production by cultured human ovarian granulosa cells. Values are mean ±SD of 12 cultures from 3 experiments

GCs of Y and IVF patients are with Bak activation, whereas in GCs of PrM patients the expression of apoptosis promoter Bak is better detected. In vitro treatment with inhibin did not change the basal P secretion of GCs from women of the three experimental groups, whereas the E2 production is stimulated especially in GC conditoned media of patients with IVF (Fig. 1).

Discussion

The present data pointed out that the in vitro treatment of GCs from ovaries of women with different hormonal status with inhibin A enhanced estradiol secretion especially in patient with IVF, whereas the progesterone secretion was not affected. Granulosa cell death in "in-vitro" cultures was shown to be apoptotic by the detection of M30 and caspase-3 enzyme activity and by the immunoexpression of pro- and anti-apoptotic proteins respectively. The investigations of several authors [2, 4, 5, 7] have also shown apoptosis in animal and human GCs to be regulated by caspases and Bcl-2 gene family members. The enhancement of GC apoptosis is paralleled by enhanced expression of proapoptotic protein Bak and decreased expression of anti-apoptotic Bcl-2 and Bcl-xl proteins. Estrogen is known to modulate in vitro apoptosis in cultured human GCs [1, 6]. Our results pointed out parallel changes of the stimulated estradiol production after inhibin treatment, supressed apoptosis rate and less pronounced pro-apoptotic proteins. These data are in agreement with the analyses of Campbell and Baird [1] and are contrary to the results of Jimenez et al. [3].

The decrease in the immunoexpression of apoptosis markers and pro-apototic protein (Bak) and the augmentation of Ki76, Bcl-2 and Bcl-xl in GCs from women with different hormonal status after inhibin exposure suggest the involvement of the growth factor I in the processes of proliferation and apoptosis.

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