BIOCHEMISTRY AND BIOPHYSICS

Disorders of Folliculogenesis Are Associated with Abnormal Expression of Peripheral Benzodiazepine Receptors in Granulosa Cells

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We studied apoptosis and expression of peripheral benzodiazepine receptors in granulosa cells of dominant follicles from women with endocrine sterility. The expression of peripheral benzodiazepine receptors in granulosa cells depends on the degree of granulosa cell apoptosis and type of disorders in follicular steroidogenesis, which suggests the involvement of peripheral benzodiazepine receptors into the regulation of folliculogenesis in health and disease.

Key Words: *granulosa cells; apoptosis; peripheral benzodiazepine receptors; steroidogenesis; folliculogenesis*

Peripheral benzodiazepine receptors (PBR) are evolutionally conservative proteins with a molecular weight of 18 kDa initially identified on the outer mitochondrial membrane [5]. These receptors were detected virtually in all organs and tissues, including the lungs, hemopoietic cells, peritoneum, intestine, thyroid, adrenals, pancreas, brain, prostate, and ovaries of mammals, including humans [4,5]. In mitochondria PBR form a complex with apoptosis-regulating proteins (bcl-2, bax) and components of megachannels of the outer mitochondrial membrane, which attests to their involvement in the regulation of programmed cell death [8].

PBR ligands regulate cell proliferation and differentiation, fatty acid and cholesterol metabolism in cells, energy function of mitochondria, porphyrin transport, heme synthesis, and anion transport [7,9,11]. Endozepine (acyl-CoA-binding protein or diazepam-binding inhibitor) and thrombocytosis-activating factor are endogenous ligands of PBR [5]. Subcellular location of PBR can essentially modify their activity; for example, receptors located on the nuclear membrane stimulate cell proliferation, while PBR on mitochondrial membrane mainly modulate steroidogenesis [6]. In addition, PBR were detected in Golgi complex and peroxisome membrane [10].

The role of PBR in the regulation of functional activity of cells of the reproductive system is little studied. At the same time, intensive steroidogenesis in these cells implies important contribution of PBR into regulation of physiological and pathological processes in the reproductive system. The expression of PBR in ovarian follicular cells was demonstrated; it was found that the number of PBR on steroid-producing cells in human ovaries is higher during the reproductive age and gestation, while in animals it depends on the phase of the estrous cycle

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[7,12]. Exogenous steroids exhibit a dose-dependent effect on PBR [8].

We studied the relationship between apoptosis intensity and PBR expression in human follicular granulosa cells under conditions of steroidogenesis in health and disease.

MATERIALS AND METHODS

The study was carried out on granulosa cells of dominant follicles (n=44) of stimulated ovaries in infertile patients (mean age 29.8±2.5 years) during therapeutic cycles of *in vitro* fertilization. Control group consisted of patients with tuboperitoneal sterility and normal steroidogenesis (n=20). Experimental groups consisted of patients with abnormal steroid production associated with hyperprolactinemia (HP; n=15) and hyperandrogenism (HA; n=9).

Ovulation in therapeutic cycles of *in vitro* fertilization was stimulated according to standard protocols using gonadotropin-releasing hormone agonists in combination with human menopausal gonadotropin (humegon; Organon). Pregnyl (Organon) served as the ovulation inductor.

Granulosa cells and fluid from the antral cavity of dominant follicles were collected during puncture of stimulated ovaries in accordance with the therapeutic cycle protocol, after which the cells were treated with hyaluronidase (Caltag; 80 U/ml, 1 min), resuspended, and washed in phosphate buffer (pH 7.4; ICN). The viability of granulosa cells was evaluated by standard vital trypan blue (ICN) staining. Cell concentration of at least 3000/ml was used.

Aliquots of aspirated follicular fluid were placed into disposable plastic tubes and frozen for subsequent studies. Only one dominant follicle was analyzed in each patient.

Granulosa cells of human dominant follicles and specimens of follicular fluid were a gracious gift from Laboratory of Embryology, Krasnoyarsk Center of Reproductive Medicine.

Apoptosis of granulosa cell was assessed by phosphatidylserine externalization recorded by fluorescent microscopy with FITC-labeled annexin V (Caltag). Viable cells and cells in the state of necrosis, apoptosis, and secondary necrosis were counted.

Peripheral benzodiazepine receptors were detected by immunocytochemical method using polyclonal antibodies to PBR (kind gift from Prof. V. Papadopoulos, Georgetown University Medical Center, Washington, USA). The results were registered in the Super Stain System (HRP) — DAB detection system (ID Labs Inc.).

The contents of luteinizing hormone, follicle-stimulating hormone, testosterone, progesterone, and estradiol in follicular fluid were measured using standard kits (Immunotech) according to manufacturer's instructions.

The results were statistically processed using Statistica 6.0 software (StatSoft). Mean values, mean square deviations, and errors of the means were evaluated for each sampling. If the data corresponded to normal distribution, the significance of differences was evaluated using Student's t test.

RESULTS

During folliculogenesis, the oocyte develops under conditions of local microenvironment containing various humoral factors (cytokines, growth factors, hormones) realizing paracrine regulation [1]. Steroid hormones play the key role in this regulation; their main source is granulosa cells expressing the key steroidogenesis enzymes (P450scc, 3β-HSD, P450c17, P450arom) [3]. We showed that the levels of steroid hormones in the follicles were changed significantly in different variants of pathological steroidogenesis (Table 1).

Cholesterol transport through the mitochondrial intermembrane space with participation of PBR is the rate-limiting reaction of steroidogenesis [11]. Expression of PBR in granulosa cells with diffuse

TABLE 1. Hormone Levels in Follicular Fluid of Dominant Follicles in HA, HP, and Normal Steroidogenesis (M±m)

Parameter	Control group (n=20)	HA (n=9)	HP (n=15)
Luteinizing hormone, mU/ml	0.19±0.04	0.23±0.05	0.35±0.03*
Follicle stimulating hormone, mU/ml	4.8±0.7	3.7±0.7	4.9±0.5
Estradiol, nmol/liter	3636.0±45.1	3465.0±58.5*	3532.0±64.7
Testosterone, nmol/liter	12.3±1.3	14.4±2.8	17.3±3.7
Estradiol/testosterone	295.6±7.5	240.6±6.2*	204.1±8.8*
Progesterone, nmol/liter	848.6±30.1	782.3±36.1	882.4±35.6

Note. Here and in Table 2: *p<0.01 compared to the control group.

TABLE 2. PBR Expression in Granulosa Cells

Parameter	Control group (*n*=20)	HA (*n*=9)	HP (*n*=15)
PBR$^-$ cells, %	25.0±4.2	26.6±5.5	45.2±10.9
PBR$^-$ cells with pyknotic nuclei, %	0.7±0.5	0.5±0.5$^+$	7.0±4.6$^+$
PBR$^+$, diffusely stained cells, %	40.0±2.0	21.0±10.3*	13.5±5.7*
PBR$^+$, mosaically stained cells, nucleus unstained, %	4.0±2.0	36.0±17.1$^+$	9.7±9.4
PBR$^+$, perinuclear location, %	22.5±3.5	14.0±7.7	23.0±13.9

Note. $^+p<0.01$: significant differences in the group.

staining predominant in normal steroidogenesis was significantly reduced ($p<0.01$) in patients with HA and HP (Table 2). This can be attributed to the fact that steroidogenesis disorders in both conditions are qualitatively similar (according to pathophysiological classification proposed by S. S. K. Yen, these disorders belong to the group of anovulatory sterility caused by excessive hormone secretion). In addition, experiments on rodent ovarian cells showed that excessive and monotonous release of androgens into peripheral blood induced by prolonged treatment with exogenous androgens reduced PBR expression in steroid-producing tissues [8].

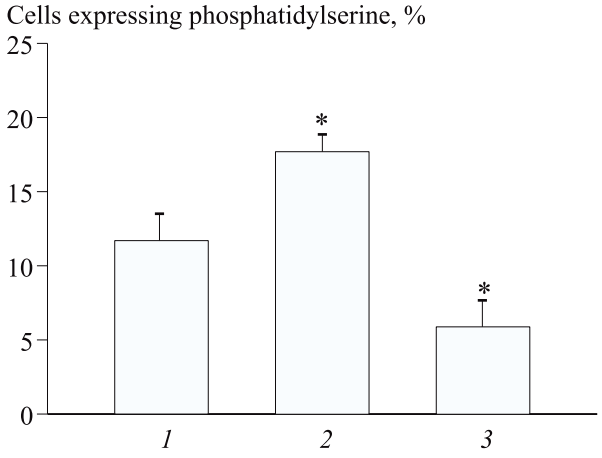

Fig. 1. Intensity of initial apoptosis phenomena in follicular granulosa cells in control group (*1*), HP (*2*), and HA (*3*). Here and in Fig. 2: *$p<0.05$ compared to the control group.

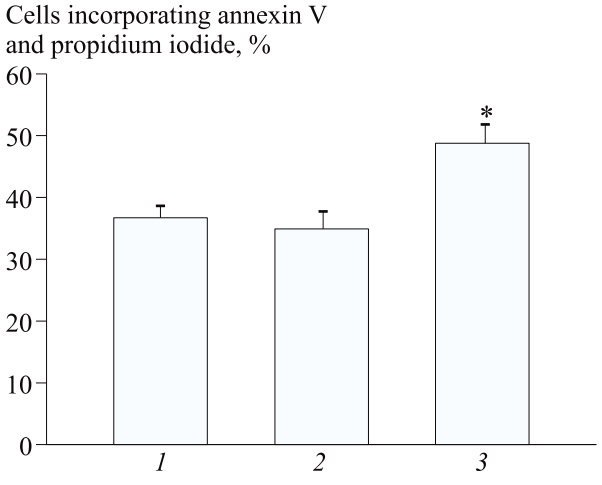

Fig. 2. Intensity of secondary necrosis in follicular granulosa cells in control group (*1*), HP (*2*), and HA (*3*).

Groups with pathological steroidogenesis differed by intracellular location of PBR. In HP, the receptors were located mainly perinuclearly, while in the HA group they were located diffusely or mosaically in the cytoplasm. In control samples diffuse staining predominated. Changed intracellular location of PBR in HP is in line with published data indicating excessive proliferation and hypofunction of granulosa cells in this condition [2].

In addition, perinuclear location of mitochondria in cells with initiated apoptosis program reflects their preserved steroid-producing function as vital function for these cells until the final stages of cell death [8]. In our study, the number of PBR$^-$ cells with pyknotic nuclei in the HP group tended to increase (Table 2), which agrees with published data [8]. In light of this, evaluation of apoptosis at its early stage preceding DNA fragmentation seemed to be most interesting. The number of cells in a state of initial apoptosis increased significantly in HP, while HA was characterized by performance of secondary necrosis (Fig. 1).

Reduced intensity of apoptosis in follicles in HA can be caused by hyperexpression of androgen receptors on granulosa cells positively correlating with proliferation and negatively with apoptosis [1,15]. The number of cells in a state of secondary necrosis significantly increased in HA (Fig. 2). Hyperexpression of PBR in these cells probably attests to antiapoptotic effect of their endogenous ligands; realization of this effect terminates apoptosis program and provokes the development of secondary necrosis.

Thus, we detected a feedback relationship between the level of PBR expression and develop-

ment of granulosa cell apoptosis. On the other hand, intensive apoptosis in HP can be associated with higher proliferation rate caused by regulatory activity of perinuclear or nuclear PBR. Changed expression of PBR in granulosa cells corresponds to the intensity of cell death processes and disorders in the steroid-producing function of cells in HP and HA.

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