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Change of Steroid Receptor Number of and Bioactivity of Gonadotropins in the Follicular Fluid of Porcine Ovarian Atretic Follicles(Ⅱ) : Testosterone Receptor

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돼지 폐쇄여포내 스테로이드 수용수체의 변화와 여포액내 생식소 자극호르몬의 활성도 변화(Ⅱ) : 테스토스테론 수용체

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국문초록

Estradiol (E)은 난소내 과립세포 (granulosa cell, GC)를 증가시키고 생식소 자극호르몬과 협동으로 배란을 유도한다. Androgen은 E의 작용과 반대의 작용을 나타내며 여포의 폐쇄요인으로 알려지고 있다. Testosterone(T)이 폐쇄여포의 여포액내 다량 존재하는 것이 알려짐에 따라 난소내에도 자기조절분비(autocrine)또는 paracrine regulation에 의해 작용을 나타낼 것으로 가정되어 난소내 여포가 폐쇄됨에 따라 그 수용체의 변화하는 양상을 조사하고저 하였다.

흰쥐의 과립세포의 세포질에는 51.3±6.1 fmol/mg protein의 Estradiol 수용체(ER) ; 153.1±25.3의 Testosterone수용체(TR) ; 또한 35.1±8.1의 Progesterone수용체(PR)가 존재하였다. 과립세포내 ER은 세포질내 E를 제거한 후에 정량이 가능하였고 또한 과립세포내에도 TR이 사람에서는 23.4±7.2 fmol/mg protein, 돼지는 98.5±23.1로 상당량 존재함을 관찰하였다. Dihydrotestosterone Enanthate(DHTE)를 100ug/흰쥐의 농도로 처리한 결과 난소내 TR의 농도는 변화가 없이 ER의 농도만 현저히 저하되고 쥐의 난소무게 역시 감소하는 것을 발견하였다.

위의 결과로 보아 난소내에도 스테로이드 호르몬은 autocrine(자가조절)방법으로 작용하며 Androgen이 난소의 무게를 감소시키는 것은 ER의 수를 감소시켜 E의 작용이 억제되고 여포들이 폐쇄를 일으켜 그 증식이 저하된 때문으로 사료된다.

INTRODUCTION

It has been well known that estrogen(E) plays a major role in ovarian follicular growth and development, proliferation of granulosa cell(GC), and production of progesterone (P). Estrogen also synergizes with gonadotropins to cause maturation of follicles in preparation for ovulation(Richards et al., 1976 ; Richards, 1980). In the mammalian ovary, mainly of human or rat, estrogen receptors(ER) have been found in granulosa cells(Richards, 1975 ; Tonetta and Ireland, 1983 ; Kudolo et al., 1984) as well as in theca cells (TC, Stumpfs, 1969) and luteal cells(Holt et al., 1981). The ER has been reported in hen GC(Kamiyoshi et al., 1986) and progesterone receptor

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(PR) was found in thecal, stroma and GC in hen ovary(Isola et al., 1987). However, to our knowledge, studies of steroid receptor in porcine ovary have not been reported but we can assume without any difficulties that the action mechanism of steroid hormones in the porcine follicular development might be mediated through their receptors. This concept could supported by the fact that there are autocrine and paracrine system in the ovary (Richards, 1980 ; Richards et al., 1987 ; Richards and Hedin, 1988).

It has been known that androgens antagonize the growth promoting actions of estrogens in the ovarian tissue(Payne and Runser, 1958 ; Louvet et al., 1975 ; Azzolin and Saiduddin, 1983) and bring about the reduction in ovarian weight (Saiduddin and Zassenhaus, 1978). Until now it is generally accepted that the higher levels of androgens in ovarian follicular fluid are associated with the atretic phenomena of the mammalian follicles(McNatty et al., 1979 ; Bomsel-Helmreich et al., 1979 ; Tsonis et al., 1984 ; Lee and Yoon 1985 ; Lobo et al., 1985 ; Yoon et al., 1988). Androstenedione is the major steroid hormones synthesized by thecal layer and is converted to testosterone(T) and 5α-dihydrotestosterone(DHT) in bovine follicles(McNatty et al., 1985). However the A concentrations in atretic follicles are not different from that in normal one (Henderson et al., 1984 ; Kruip and Dieleman, 1985 ; Spicer et al., 1987), while A, DHT, and T in human atretic follicles are increased(Lee et al., 1986).

The demonstration of androgen binding protein, T receptor(TR) suggests that the effect of androgen on the ovary also are mediated through its receptors (Saiduddin and Zassenhaus, 1984). However, the changes of the steroid receptors in the mammalian ovary during atresia has not been thoroughly studied.

We report here a comparison of the concentrations of T or DHT receptors in atretic follicles with normal ones and that the androgen decreased the level of ER, not their own level might be one of the mechanism in promoting follicular atresia.

MATERIALS AND METHODS

Chemicals

The following reagents were purchased from Sigma Chem. Co. : Unlabelled steroids and diethystillbesterol(DES), Trizma-HCl, dithiothreitol (DTT), EDTA, phenylmethylsulfonyl-fluoride (PMSF), diisopropylfluorophosphate (DFP), sucrose(grade I), DNA from calf thymus, charcoal(Norit A), flutamide(α, α, α-trifluoro-2-methyl-4'-nitro-m-propionotoluidide). 11β-^{3}H-Moxestrol(R2858, spec, act. 70~87 Ci/mmol), 2, 4, 6, 7, 16, 17-^{3}H-estradiol-17beta(^{3}HE2, 130-170 Ci/mmol), promegestone (R5020 17α 1, 2, -^{3}H, 42.3 Ci/mmol), 1, 2, 6, 7, -^{3}H -progesterone(101 Ci/mmol), testosterone 1, 2, 6, 7, -^{3}H(^{3}HT, 89-101 Ci/mmol) and 1, 2, 4, 5, 6, 7, -^{3}H- 5α-dihydrotestosterone (^{3}HDHT, 110-150 Ci/mmol) and methyltrienolone(17beta-hydroxy-17-methyl-4, 9, 11-estratrien-3-one, R1881, 87 Ci/mmol) and also cold R2858, R5020, R1881 were purchased from New England Nuclear, GmbH. The tritium labelled-tracers were assyed for their purities by ascending thin layer chromatography(Merck, 0.25mm thickness, silica gel)after the addition of unlabelled lignads. The purifying solvent system was benzen/ethyl acetate(3 : 1. v/v). The spots were identified by brief staining over iodine vapors, scraped off and counted. All other chemicals used were analytical grade unless otherwise stated.

Animals

Immature Sprague-Dowley female rats(21 day old) were supplied from a local agent and exposed to 25℃ under 12D : 12L/cycle giving food and water ad libitum. Steroids dissolved in sesame oil were subcutaneously injected. Rat were decapitated between 09 : 00 and 10 : 00 on the morning of 22 to 25 days.

Porcine ovaries were obtained from the slaughter house(Woosung Nonghyub) located at Majangdong, Seoul. The ovaries were grouped to follicular(NCL, corpus luteum absent) or luteal (CL, functional corpus luteum present) phase by the morphological criteria as described in previous papers(Lee and Yoon, 1985 ; Yoon et al., 1989). The ovaraian follicles were dissected out and then classified into small(diameter, less than 3mm, designated as S), medium(3-5mm, M), large (larger than 7mm, L) follicles, after measured by a caliper. The isolated follicles with pale vascularization, large proportion of black or opaque areas, or follicular fluids with floating cell debris under the stereomicroscope (Wild M5A), grouped into atretic ones. The isolated follicles were slit on the wall to release the intrafollicular contents and then cut in a vial with fine scisor. The follicular fluids (pFF) was collected by centrifugation for 20 min at 1,000×g at 4℃ and frozen at -40℃ until assayed. The pelleted porcine granulosa cells(pGC) were resuspended again in TC 199 medium containing 25 mmol/L HEPES. The remaining GC were scrapped out gently from the inner wall of the follicles. The harvested GCs were washed thrice with the medium and then their viability was determined by a method of trypan blue(final dilution, 0.1 %) dye exclusion using haematocytometer. The nuclear phase of the collected ova were examined by the whole mount techniques as described previously(Lee and Yoon, 1985).

The human granulosa cells were harvested from 198 follicles in 63 cycles of 431 women treated with for IVF and ET because of the tubal blockage as described previously (De Geyter, Yoon, Bordt, et al., 1988). Ovarian hyperstimulation was performed by administrating either hMG(Humegon, Organon, Oberschleim, FR Germany of Pergonal, Serono, Freiburg, FRG), or huFSH (Fartinorm, Serono, Freiburg, FRG). The superovulation was induced by an intramuscular injection of 10,000 IU hGC (Pregnyl, Organon). The follicular fluid (hFF) and granulosa cells (hGC)were aspirated by a laparoscopy after 34 to 36 hours later of hCG treatment.

Tissue Preparation

Rat ovaries were quickly denuded with fine scissors of all extra-ovarian tissues, washed and placed in chilled homogenati on buffer as follows. All subsequent procedures were carried out at 0-4℃. The ovaries were placed in embryo dissecting dishes containing the buffer(TEGT), 20 mmol/L Tris-HCl, pH 7.5, 0.3mM-EDTA, 1mM-DTT, 10%-glycerol, 12mM-thioglycerol and freshly prepared 1.0 mM -phenylmethysulfonylfluoride. The follicles randomly punctured with dental 30-gauge needle and the granulosa cells were squeezed out with a pair of fine forceps. Gentle pressure was applied to follicles and the expressed cells were collected after centrifugation at 800×g (20 min, 4℃). The cells and pelleted GCs from 2 ovaries/tube (0.1mM volume, for the estradiol nuclear receptor assay) were washed thrice in TEGT and also the remaining ovarian tissues (ROT) were washed three times in ice cold TEGT.

Cytosol was prepared as follows : For Scatchard analysis, tissue and cells were homogenized in 1 volume of buffer. For the other experiments, homogenization was performed in 4 volume of a 2-fold diluted buffer. In addition, the curde homogenate was treated with 1/4 volume of 1/2%(w/v) dextran-coated charcoal(DCC) for 10 min on ice before high speed. The homogenates were centrifuged at 1,200×g for 10 min at 4℃ to yield a nuclear fractions. The supernatants were further centrifuged at 18,000×g for 20 min and then after removing the floating lipied, the cytosol was prepared by centrifugation again at 35,00×g for 3 hours in a high-speed centrifuge(Beckman J21) or by centrifuging at 108,000×g for 1 hour at 4℃ (Hitachi 70P Ultracentrifuge).

Assay Procedures

Testosterone binding was routinely measured by employing Sephadex G-25 minicolumn (0.6×2.5cm) to separate bound from free steroid. The fine grade, washed Sephadex G-25 was equilibrated in TEGT and poured 1.5ml into siliconized pasteur pipettes plugged with siliconized glass wool. The columns were poured at least a day in advance and stored at 4°C in TEGT containing 0.02% NaN3. Cytosol (adjusting to 1mg/200ul) was incubated for 2 hour on ice with 50 ul-^{3}HT (10 mmol/in TEGT) plus 50 ul of unlabelled T (100 fold excess T in TEGT). All assays were done triplicate. Before loading the samples onto the columns, 10 ul of a blue dextran and phenol red mixture was added to serve as visible dye markers of the excluded volume(blue dextran, bound steroid) and the included volume(phenol red, free steroid). Column chromatography was run in a 4°C cold room. The excluded binding fractions were collected and added to 20ml of scintillation cocktail fluid counted in a Packard Tricarb 4530 counter equipped with a radioreceptor programmes.

To assay the receptors in small quantity, Hydroxyl Apatite method was adopted. HA was equilibrated with TEGT containing 50mM KCl and suspended at 0.1g/ml. After incubation of cytosol with labelled steroids, 1.2ml of the suspension was added to the cytosol and adsorption allowed to continue for 35 min. The HA was then centrifuged at 1,000 ×g for 10 min and washed 4 times with TEGT+50mM KCl. Bound form was extracted 2 times with 1 ml of ethanol and its radioactivity determined after evaporation of the ethanol.

As the reference method, we used the DCC adsorption method. After incubation, 0.25ml of 1/2% (w/v) DCC was added to the cytosol and the samples were left in ice for 10 min. The samples were then centrifuged at 1,000×g for 10 min and the radioactivity of supernatant was counted.

For the measurement of estradiol (ER) or progesterone(PR) in human ovary, the commercial PR-or ER-EIA kits(Abbott Lab GmbH), solid phase enzyme immunoassay kits with sandwich immunoabsorbent types, were used using specimen cytosol (about 1mg soluble cytosol protein/ml). These assays performed according to the methods described by the manufacturer. The color intensity was read in a spectrophotometer at 492nm, which covered the range of standards(0~500 fmol). The intra-assay variation was less than 10% for one sample with 52.5±2.7 fmol/mg protein receptor levels. The inter-assay variation was 18.9 at the same levels.

Other Methods

Protein determination was performed by the method of Lowry et al.,(1958) or by the Bio-Rad kits after precipitation of cytosol aliquots with 10% trichloroacetic acid. Bovine serum albumin was used as a standard. DNA content was determined by a modified method of West et al.,(1985).

Scatchard plots were analyzed by linear regression. Curve linear Scatchard plots were analyzed by our computer programmed(Scatchard et al., 1949).

Statistical analysis of the data obtained from normal distribution was performed by Student's t-test. But the data which was not normally distributed in the samples were performed using the Mann-Whitney U test. All results are expressed as the mean ±1 SD, stand-ard deviation unless otherwise stated.

RESULTS

Assay Methods for Steroid Receptors in Ovary

Three assay methods for steroid receptors were tested to assay multiple samples simultaneously and in order to assay T-high-affinity binding sites(HABS) of cytosolic receptors in rat ovarian homogenates. The results are summarized in Table 1. All procedures yield approximately equivalent values for the concentration of ovarian steroid receptors. How-

Table 1. Measurement of cytosolic steroid receptors in ovarian homogenates of rats

Receptors (fmol/mg cytosol)	DCC adsorption	Methods hydroxyl apatite	Sephadex G-25 column
Estradiol	56.60±13.7	51.3± 6.1	60.5±7.3
Testosterone	*169. 8±49.6	153.1±25.3	–
Progesterone	* 38. 2±13.4	35.1± 8.6	–

*Cytosol negatively skewed. Cytosol (>200mg wet weight) was prepared from rat homogenates of 50 rats. Final protein concentration of cytosol was 2-3mg/ml.

ever, the DCC adsorption assays often gave more variable results(CV>20%), not only for replicates from the same cytosol preparation but also from day-to-day using different cytosol preparation. We found that Sephadex G-25 column procedure was linear to the cytosolic protein concentration as low as 200ug /ml, but unsuitable to analyze a large number of samples. The Hydroxylapatite(HA) method is more reliable and slightly rapid. The DCC assays were employed only when cytosolic protein concentration was greater than 2-4mg/ml.

In the nuclear receptor assay of granulosa cells, we found the present results were in a curvelinear Scatchard by using DTT(0.1nmol) or sodium molybdate(100nmol)in TEGT buffer during the assay and the binding was stable at 37℃ until 90 min and steady state was achieved by 45 min.

Distribution of Steroid Receptors in Ovarian Cells

The receptor contents of rat ovarian tissue are summarized in Table 2. The present results suggested that the majority of ovarian ER was located in GC layer and that of TR are located in the residual ovarian tissues.

Saturation Binding Studies in Granulosa Cells

Table 2. Distribution of steroid receptors in rat ovarian tissue

	Receptor concentration	
	Cytosol (fmol/mg protein)	Nuclear (fmol/mg DNA)
Estradiol		
Whole ovary	180.5± 9.9	115.6± 6.5
Granulosa cell	696.6±36.4	406.0±30.6
Residual ovarian tissues	15.6± 2.6	7.9± 0.9
Testosterone		
Whole ovary	78.9±15.7	115.7±24.8
Granulosa cells	38.6±23.2	28.3±18.9
Residual ovarian tissues	173.5±28.1	193.4±49.9

* Each experiment was repeated 3 times(values are means±SEM).
* The bindings of 10nM-tracers (R2858 for ER, R1881 for TR) in the presence or absence of cold competitors(1 μmol/l DES for ER, DHT for TR) were determined after 18h of incubation at 4℃ (cytosol) and 1h 37℃(nuclear).

Steroid receptors in GC nuclei were apparently saturated at 4nmol of R2858 for ER in rat, 5.0 nmol in porcine GC, and 8.0nmol in human GC, when assayed using DES as competitior. Scatchard analysis revealed a single class of ER and TR with an apparent Kd of 1.64±0.92 nmol/l for ER and 1.82±0.70 nmol/l for TR. The binidng capacity of ER in nucleus of granulosa cells of rat ovary was more than 335.2±161.9 fmol/mg DNA.

Saturation of the granulosa cell cytosol for steroid receptors was observed at 2nmol /l R2858 for ER using DES as competitor, and at 4.0 nmol/l R1881 for TR using DHT as competitor. Apparent Kd was 3.58±1.22 nmol/l, which was similar to that measured in the cytosol of whole ovary, representing a single class of binding sites. The binding capacity of the rat ER receptor was 51.3 fmol /mg cytosol protein and that of TR was

Table 3. Competition of various steroids for porcine granulosa cytosol binding of [^{3}H]-methyltrienolone and moxestrol

Steroids	Displacement ability(%)	
	Testosterone receptor	Estradiol receptor
Methyltrienolone(R1881)	100.0	18.7±3.3
Testosterone	93.7±8.9	13.3±3.9
5α-Dihydrotestosterone	95.6±7.1	26.7±7.9
Androstenedione	67.3±5.4	8.5±7.0
Estradiol	7.6±2.0	100.0
Cortisol	29.4±3.1	11.8±7.1
Triamcinolone acetonide	32.8±6.5	–
Progesterone	26.1±7.1	25.7±4.7
Diethylstillbesterol(DES)	5.5±1.1	95.0±4.1

*The porcine GC pooled cytosol (1 mg/ml) was incubated with 10nM tracers for 18hr at 4℃ in the presence or absence of a 100 fold molar excess of cold steroid. The bound steroids were separated by hydroxylapatitite. The displacement ability is compared for those in the absence of competitors(0%) and in the presence of a 100 fold excess of unlabeled methyltrienolone for TR and DES for ER. Values given are the mean of three separate experiments in triplicate.

153.1 fmol/mg protein.

Ligand Competition Study ; Steroid Specificity

Competition of T binding in ovarian GC cytosol by unlabelled steroids showed a rather broad specificity for the ovarian androgen binding protein (Table 3). Maximum competitition of moxesterol(R2858, E binding to cytosolic receptor) occurred with E_2 and DES, the other hormones examined had little effect on competitition for ER. The most potent natural androgens such as T and appear to compete the best for binding to the receptor whereas the androgen antagonists such as cyproterone acetate and flutamide, etc were only 20% as effective in this competition assay a progesterone, E_2 or 5beta-DHT, which are steroids with no or little androgen activity.

Table 4. Competition of various steroids for triamcinolone acetonide blocked cytosol (testosterone receptor) binding of [^{3}H] methyltrienolone(R1881)

Steroid	Displacing ability of T-receptor(%)
Methyltrienolone	100
Testosterone	90
5α-dihydrotestosterone	85
Androstenedione	85
Estradiol	0
Cortisol	0
Triamcinolone acetonide	0
Progesterone	<1

*The cytosol (1mg protein in 200μl) was preincubated with 1 μM triamcinolone acetonide (TA) for 2h at 0℃ and was further incubated with 10nM-R1881 for 2h at 2℃ in the presence or absence of a 100 fold molar excess of unlabeled steroids (1μM/200μl). The bound steroids were separated by HA and the specificity of R1881 binding in the TA-blocked cytosol. The displacement abiltiy is compared for those in the absence of competitor and in the presence of 100 fold excess of unlabelled R1881. Values are the mean of three separate experiments in duplicate.

These results suggest that R1881 binding to androgen receptor is also displaced by glucocorticoids and triamcinolone acetonides(TA), which has been known to bind glcocorticoid or progesterone receptor. On the other hand, Table 3 shows that an excess of R1881 can completely suppress the TA or cortisol binding to their receptor.

These findings led us to study whether androgen receptor(AR) in porcine GC is not so specific for R1881 and androgens, and whether glucocorticoid receptor and PR interfere with the binding of R1881 to AR, if glucocorticoid and progesterone which have the structure of 3-one and a double bond at C-4, bind to androgen receptor. Table 4 shows that the displacement ability of cortisol, progesterone, and estrogens seemed to be disapperared by treatment of TA. However, andro-

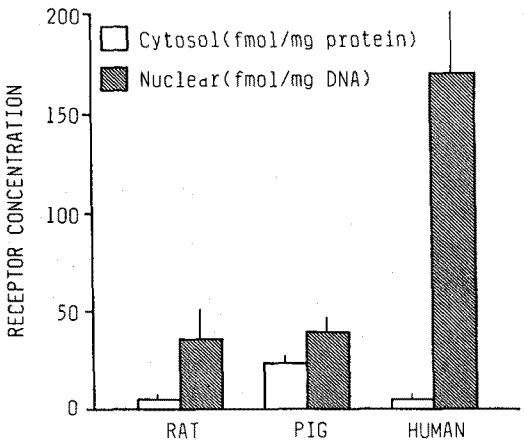

Fig. 1. Estrogen receptor in ovarian granulosa cells. *Cytosol and nuclear suspensions of ovarian granulosa cells were prepared and incubated with labelled R2858(10 nmol) in the presence or absences of 1 μmol DES for 18 hours at 4℃ for cytosol or 1hr at 37℃ for nuclear fraction. Each experiment was repeated three times and expressed as mean±SD.
* : $p<0.05$; ** : $p<0.001$.

stenedione, 5α-DHT had a somewhat less suppressive effect.

Contents of Steroid Receptors in Granulosa Cells

The cytosolic and nuclear ER were determined in the granulosa cells of human, pig, and rat after thorough stripping the endogeneous steroids by dextran-coated charcoals and are summarized in Figure 1. The ratio of ER contents in nucleus(fmon/mg DNA) to cytosolic receptor(fmol/mg protein) of rat and human were significantly higher than that of pig. The cytosolic ER in pGC were significantly higher than those in rGC and hGC, but the nuclear ER in human GC were significantly higher than others.

The concentrations of cytosolic and nuclear testosterone receptor in GC are summarized in Figure 2. On the contrary to ER results, the ratio of nuclear receptor to cytosolic one was less than 2. The amounts of cytosolic and nuclear TR in hGC were significantly lower than those in rGC and pGC.

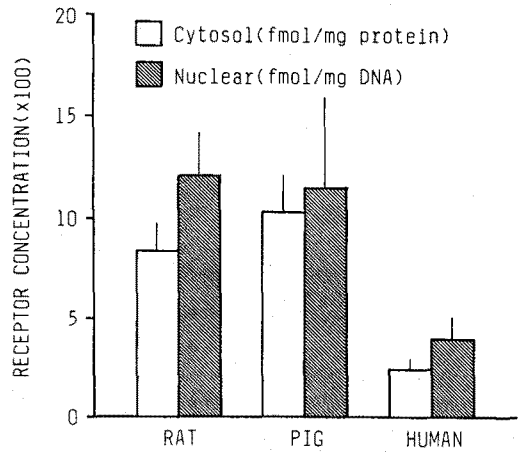

Fig. 2. Testosterone receptor in human and porcine granulosa cells. Values are means± SD. The legends are same as in the Fig. 1 instead of concentrations of tracer, R1881 (10 nmol) and of cold R1881 (1 μmol).

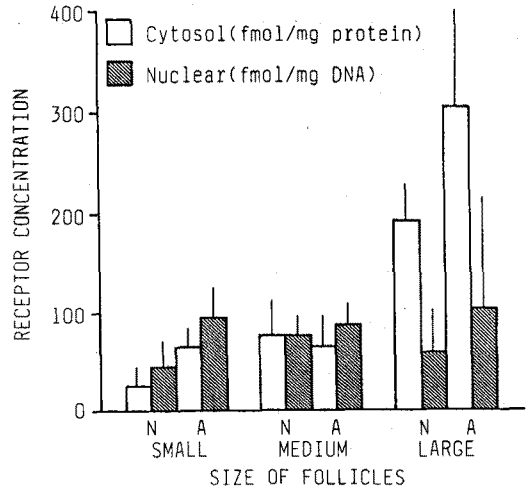

Fig. 3. Testosterone receptor of cytosol of porcine granulosa cells during the follicular maturation and atresia. The values are means ± SD. Each experiment was repeated more than three times. See the criteria of atretic follicle in materials and methods. The bindings of R1881 in the presence or absence of cole R1881 (1 μmol) were determined after 18hrs incubation at 4℃ (cytosol) and 1hr at 37℃(nuclear).

Contents of Testosterone Receptors in Granulosa Cells from Porcine Normal or Atretic Follicles

The cytosolic and nuclear TR in porcine GC were determined to compare the changes

Table 5. Effects of dihydrotestosterone-enanthate on the level of ovarian cytosolic E- and T-receptor in rat.

	Estradiol	Treatment DHTE	t-test
ovarian wet weight (mg)	6.3± 0.9 (n=15)	4.9± 2.3 (n= 5)	p<0.001
ovarian E_2 receptor	169.7±15.5	136.5±20.1*	p<0.01
ovarian T receptor	78.3±20.7	76.3±15.8	

*Rats(3 weeks old) were injected 1mg-estradiol/ml sesame oil by 2 days intervals and on the 4th week, 5 rats were injected with 1mg-dihydrotestosterone enanthate/ml sesame oil. All rats were killed 1 days later after treatment and steroid receptor levels measured and expressed as bound-steroid(fmol)/mg cytosol protein.

of TR during follicle growth and atresia, and summurized in Fig. 7.

The cytosolic TR tends to be increased as the follicle becomes larger, eventhough the significance could not be found because of the small sample sizes and a large variations of the values.

Cytosolic and nuclear TRs in small atretic follicles were 2.1 times and 1.98 times higher than those of normal ones. The same tendency could be observed in the measurement of TR in large follicles. But we found no differences between the concentrations of TR in medium atretic follicles and in normal ones.

Effects of Dihydrotestosterone Enanthate (DHTE) and Estradiol on the Ovarian Receptors

Previously, Saiduddin and Zassenhaus(1984) have shown the presence of E2 receptors in rat ovarian tissue and demonstrated that the action of testosterone may be by blocking of the synthesis of the ER. To confirm this concept, we treated E_2 1mg/ml sesame oil from 3th week age by two day intervals until 30 days. One half of the rats were treated two times the recently synthesized DHTE(100μg/100μl sesame oil) on the 4th week. On 1 day later after the last injection, all animals were killed and the steroid receptor levels in ovarian homogenates were measured and summarized in Table 5.

The cytosolic ER and ovarian wet weight were significantly reduced in rat ovary. At this time, 24 hour later, the follicles became to be atretic by the morphologic criteria. On the contrary, TR in ovarian homogenates of DHTE treated rats were not significantly changed.

DISCUSSION

The present study shows that the DCC adsorption assays often gave more variable results. In part, this variability of DCC method may be due to "stripping" of bound T from the receptor, as has been descried by Peck and Clark(1977) for the uterine estrogen receptor. In particular, the DCC adsorption assay was sensitive to the protein concentration (more than 2mg protein) of cytosol. The DCC assay, therefore, should be employed when cytosolic protein concentration was greater than 2-4mg/ml. The present study also confirmed that hydroxyl apatite method and Sephadex mehtod were more reliable and suitable to use in measurement of steroid receptor in ovarian granulosa cells.

Correlation studies of PR and ER concentratons (data not shown) showed that the commercial Abbott's EIA Kit for PR or ER are useful to determine the low receptor levels of cytosol routinely using monoclonal antibodies(Legros et al., 1988). During the last 10 years, all receptor assays were based on measurement of the capacity of cytosol fractions for binding radiolabelled ligands. Because this approach has the limitation to measure the free receptors and also does not determine the localization of the receptor to specific cells, the method to measure the total receptor number should be developed.

Bulk biochemical assays generally do not

discriminate among various tissue components. Recently, a variety of histochemical techniques including autoradiography, immunocytochemistry and histofluorescene techniques has been tried to identify receptors in different cell types (DeGoeu et al., 1984 ; Perrot-Applanat et al., 1985, 1987). Recent papers measuring steroid receptors using monoclonal antibodies describe exclusively a nuclear distribution of receptor observed in cells of the endometrium, myometrium, oviduct, cervix, vagina pituitary and breast even under the conditions where P_4 is present in only low concentration (king and Greene, 1988 ; Press and Greene, 1988). The present study also demonstrates that a large number of ER(Fig. 1) and TR (Fig. 2) could be determined in the nuclear fractions of granulosa cells.

ER-EIA may be particulary useful when ER is measured in ovarian carcinoma, because the ovaries may contain a huge amount of endogeneous E_2 that may invalidate receptor assays in which radiolabelled ER-ligands are used. (Holt and Bolanos, 1986 Legros et al., 1988). The most potential advantages of these kits will be the fact that endogeneous steroid would not interfere the measurement of concentration of receptors themselves. On the other hand, immunocytochemical study of mammalian steroid receptors might make it possible to visualize small amount of receptors and also their locality. (Perrot-Applanat, et al., 1985, 1988 ; Isola et al., 1987 ; Press and Green 1988). The present study also showed that the endogeneous steroid could be removed by DCC adsorption, and that the tissue isolations and the separation of ovarian granulosa cells could be done without difficulties. The present studies also demonstrated that the steroid receptors in lower amount of cytosol(200μg/ml).

The natural T has a disadvantage as a ligand in the study of androgen receptor, because it is easy to be metabolized to less potent derivatives, so that it can be difficult to measure the accurate dissociation constant and the number of binding sites. The present result confirmed that methyltrienolone could bind to glucocorticoid receptor and to a small portion of PR. Thus, the use of R1881 as ligand in TR measurement, might possibly be overestimated. It has been also known that progesterone also bind glucocorticoid receptor with a lower affinity than corticosterone and TA, which also can bind to PR but not to TR(Kyakumoto et al., 1984). The cross bindings of steroid hormones to their receptors are very important factors in order to study their roles on the ovary because there are a huge amount of steroids. Therefore it is necessary to supress the other steroid receptors and also is necessary to remove the free steroids before assays as shown in this study.

The present results demonstrate that the granulosa cell in porcine and human ovary contain androgen binding activity that has several characteristics of a receptor. The binding affinity (Kd=4-11.8) is similar to other steroid receptors and is saturable. T receptor is comparable to E_2 receptor in the ovarian tissue in quantity. The Scatchard plot analysis of the ovarian androgen receptor showed that at high concentrations of [^{3}H] T, binding activity is detected which is of low affinity and high capacity. However, this binding activity is not detected by the DCC adsorption assay, probably because charcoal "strips" the bound T from these low affinity sites (Saiduddin and zassenhaus, 1984). Indeed, DCC appears to strip bound T from the high affinity sites as well(particularly at low cytosol protein levels). Thus, the assay of ovarian T receptor in dilute cytosol preparations requres a technique such as HA or sephadex G-25 as shown in the present study.

Some data have been published on the steroid receptor content of the human normal ovary since 1973 (Taylor et al., 1973). Wurz et al.(1983) have reported 42% of the normal ovaries to contain both ER and PR. Of

19 healthy ovaries examined by Lantta(1984), 26% contained both ER and PR, and 26% were receptor negative. In 47% the combination ER-/PR+ was found. However, these results suggest that the low ER content of normal ovaries examined is possibly due to the 'endogenous' estrogen of the ovarian tissue. By a recently, developed of commercial kits, receptors content could be found at the level of 3-5 fmol/mg protein. This may be particularly useful when ER is measured in ovary which may contain a huge amount of steroids. Thus, the receptor study might be on the start line for the race. The present result showed that in human and porcine ovary, the detectable amount of TR and ER could be measured after thorough stripping the endogeneous steroids by charcoal treatments, but could not elucidate whether the determined TR or ER is unoccupied or occupied.

The present study showed that after injection of 1mg DHTE ovarian wet weight and the amount of ovarian E_2 receptor have significantly decreased. The table 5 showed that this respones to DHTE is specific for the ovary because the uterine wet weight and amount of E_2 receptor are not significantly changed (data not shown). This result also suggests that the loss of E_2 receptor in the ovary does not appear to be due simply to cell death since the amount of T receptor is unchanged. On the other hand, the follicles from the DHTE treated ovary became atretic at 24hrs later of treatment as shown in the result of Saiduddin and Zassenhaus(1984). Based upon the results that androgen decreases the ovarian weight and ovarian cytosolic ER in rat, and that the contents of TR in granulosa cells of atretic follicles in pig, the present study could demonstrate that androgen might act directly on the ovary and might inhibit the action of estrogen to proliferate the maturation of GC in the ovary by the reduction of estrogen receptor.

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