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LOCAL REGULATORY EFFECTS OF ACTIVIN A AND FOLLISTATIN ON MEIOTIC MATURATION OF RAT OOCYTES

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SUMMARY: Activin A and follistatin identified in follicular fluids have been shown to regulate follicular development. Activin A, in a dose responsive manner, increased the percentage of germinal vesicle breakdown that served as a marker for the resumption of meiosis of oocytes in vitro, especially these obtained from immature follicles. This effect of activin A was inhibited by the activin-binding protein, follistatin. Follistatin alone exerted an inhibitory effect on oocyte maturation. Reverse transcription and polymerase chain reaction revealed the expression of activin βA and activin type IIA receptor gene transcripts in the oocytes. Activin A, along with follistatin, appears to play a local regulatory role in the meiotic maturation of rat oocytes.

Activin A, a dimer of the inhibin β-subunit, was originally identified in porcine ovarian fluid. This agent exerts biological effects on the release of FSH by pituitary cells in vitro which oppose those of inhibin; while inhibin suppresses FSH release, activin A stimulates it (1, 2). Activin A acts on thecal and granulosa cells (3-5) and oocytes (6), suggesting that it has an intraovarian paracrine effect. In addition to inhibin and activin, follicular fluid contains the polypeptide follistatin, a binding protein of activin A that suppresses FSH release in a manner similar to inhibin (7,8).

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The development and maturation of oocytes are initiated by the secretion of FSH from the pituitary glands followed by a surge of luteinizing hormone (LH) that is mediated by follicle cells (9). Nuclear maturation events, such as breakdown of germinal vesicles, progress spontaneously in vitro when oocytes are obtained from mature follicles (10,11). Although activin and follistatin are believed to regulate follicular development, little information is available about the roles played by these and other factors in mammalian oocytes. Understanding the hormonal interactions and possible factor(s) that are involved in the developmental process called maturation may be important in improving techniques used for *in vitro* fertilization in man, since maturation is essential to fertilization and subsequent development (12).

We assessed the roles of activin A and follistatin in oocyte maturation and investigated the expressions of activin and its receptor in the oocytes themselves.

MATERIALS AND METHODS

Rats and oocytes: Immature (25-day-old) female Wistar rats were injected subcutaneously with 10 IU of pregnant mare serum gonadotropin (PMS). Ovarian oocytes were collected by puncturing the growing follicles of the ovaries either 24, 36, or 48 h after PMS treatment; the yield was 20 to 25 oocytes per ovary. Cumulus-oocyte complexes were incubated in modified Biggers-Whitten-Wittingham (mBWW) medium (13) supplemented with either recombinant activin A, a kind gift from Dr. Eto, produced from Chinese hamster ovary cells (14), follistatin, a generous gift from Dr. Sugino, purified from porcine follicular fluids (15), or both, in a humidified atmosphere of 95% air and 5% CO2 at 37℃. At the indicated times the oocytes were recovered and the cumulus cells were removed by repeatedly drawing each oocyte-cumulus cell complex into a micropipette having a bore slightly larger than an oocyte, then expelling it. The medium was in 1 ml of phosphate-buffered saline (PBS), pH 7.4 containing 300 IU hyaluronidase/ml. Denuded oocytes were washed twice in 1 ml of ice-chilled PBS without hyaluronidase. Oocytes were examined by interference contrast microscopy for evidence of maturation. Meiotically arrested oocytes were marked by the presence of the nuclear structure or germinal vesicle (GV). Breakdown of the germinal vesicle (GVBD) served as a marker for the resumption of meiosis or oocyte maturation. Each treatment group consisted of approximately 100 oocytes from several independent experiments.

RNA extraction: A total of 100 cumulus-oocyte complexes or denuded oocytes obtained from the ovaries of PMS-treated rats were washed five times with PBS. PolyA$^+$ RNA was extracted by use of the Micro-Fast Track mRNA isolation kit (Invitrogen, San Diego), which allowed the direct isolation of polyA$^+$ RNA from a

very small number of cells. To offset the efficiency difference in RNA extraction, an internal standard, i.e., 10^6 copies of a synthetic polyA$^+$ RNA pAW109 that was transcribed from the plasmid pAW109, was added to each batch that was lysed in a detergent-based buffer. The lysates of 100 oocytes or cumulus-oocyte complexes and internal standard RNA were then applied directly to oligo (dT) cellulose for adsorption. The resulting polyA$^+$ RNA pellet was resuspended in 6 μl of 10 mM Tris-HCl, pH 7.6, 1 mM EDTA and the two 2 μl aliquots were used for each RT-PCR reaction, one for the activin βA mRNA assay and the other for assay of activin type IIA receptor mRNA and internal standard RNA.

Primer pairs: Primers used for amplification of activin βA sequence were ATCA-TCACCTTTGCCGAGTCAG (residues 529-550; sense strand) and TCTTACAGC-AAATGTTGACCTT (residues 1105-1126; antisense strand), as derived from the rat βA cDNA sequence (16). Primers for amplification of activin type IIA receptor sequence, i.e., GCAAGGGGAAGATTTGGTTGTGTC (residues 595-618; sense strand) and GTCTGACAGTGAGCCCTTTTCATG (residues 808-831; antisense strand), were constructed from the cDNA sequence described by Mathews and Vale (17). The other pair of primers for internal standard RNA (pAW109) was obtained from Perkin-Elmer/Cetus (Norwalk), as pAW109 primers. PCR with these activin βA and activin type IIA receptor primers yielded 598 base pairs (bp) and 237 bp products, respectively.

RT-PCR: Each 2 μl of polyA$^+$ RNA aliquot was incubated with 5 units of rTth DNA polymerase (Perkin-Elmer/Cetus, Norwalk) for 15 min at 70℃ in a total reaction volume of 20 μl containing 1×rTth reverse transcriptase buffer (10 mM Tris-HCl, pH 8.3, 90 mM KCl), 0.2 mM of each dNTP, 1 mM $MnCl_2$, and 10 pmol of the appropriate antisense primer. The reverse transcription reaction was stopped by placing the tube on ice. PCR was performed in the same tube after the addition of 80 μl of chelating buffer (5% glycerol, 10 mM Tris-HCl, pH 8.3, 100 mM KCl, 0.75 mM EGTA, 0.05% Tween 20) containing 1.8 mM $MgCl_2$ and 10 pmol of the appropriate sense primer. PCR was performed in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk) according to the following protocol: an initial denaturation at 95℃ for 2 min was followed by 50 cycles of 1-min denaturation at 95℃, annealing and extending the primers for 1 min at 60℃. A further 7-min extension at 60℃ was performed to extend any remaining single-strand products. Ten microliters of the PCR-amplified products were electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide and were then photographed.

Statistical analysis: The data were analyzed by χ^2 test, with $P<0.05$ considered to be significant.

RESULTS

Oocytes in mature follicles are induced to undergo spontaneous meiotic maturation when incubated as cumulus-oocyte complexes. As shown in Table 1, 35.8% of the oocytes from mature follicles obtained 48 h after PMS treatment lost their GV in 1 h of incubation. Resumption of meiosis was also observed in oocytes

Table 1. Germinal vesicle breakdown as a function of time after PMS treatment and the effects of activin A on cumulus-oocyte complexes

Hours after PMS	Percentage of Germinal Vesicle Breakdown	
	Control	Activin A, 10 ng/ml
24	15.9	31.7*
36	17.8	33.0*
48	35.8$	47.1

*, $p<0.05$ compared with untreated controls.
$, $p<0.05$ compared with that obtained 12 h earlier.

from maturing follicles. Percentages of GVBD in oocytes obtained 24 and 36 h after PMS, however, were significantly lower than that in oocytes obtained 48 h after PMS. Activin A, at a dose of 10 ng/ml, served as a potent inducer of oocyte maturation, increasing the percentage of GVBD in the oocytes; the increase was significantly greater in oocytes obtained from immature follicles, i.e., 24 and 36 h after PMS treatment. Induction of oocyte maturation by activin A was dose dependent (Fig. 1). The effect was significant and maximal at a dose of 1 ng/ml.

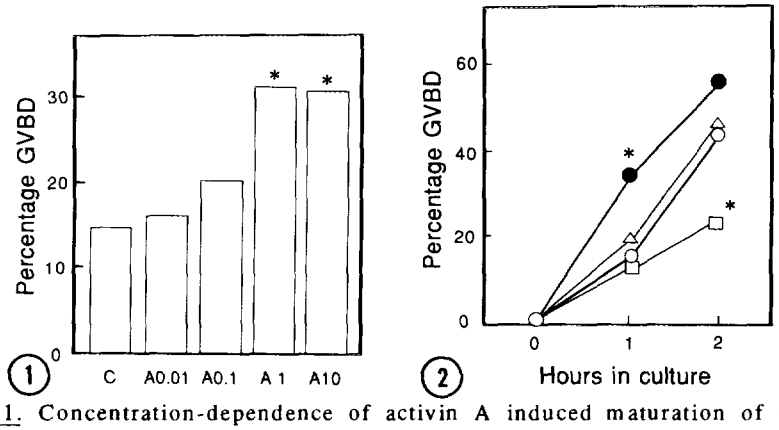

Figure 1. Concentration-dependence of activin A induced maturation of cumulus cell-enclosed oocytes. GVBD was analyzed in oocytes obtained 24 h after PMS treatment and incubated for 1 h in the presence of the indicated concentrations (ng/ml) of activin A (A). *, $p<0.05$ compared with untreated controls.

Figure 2. Time course of meiotic maturation in cumulus cell-enclosed oocytes. *, $p<0.05$ compared with untreated controls. ○, controls; ●, activin A 10 ng/ml; □, follistatin 10 ng/ml; △, activin 10 ng/ml+follistatin 10 ng/ml.

Follistatin blocks the maturation-inducing action of activin A when both are added to oocytes in vitro. As shown in Fig. 2, activin A increased the percentage of GVBD significantly in 1 h; the addition of follistatin at doses of 10 ng/ml to the incubation medium in the presence of activin A, 10 ng/ml, inhibited its stimulatory action. The addition of follistatin alone to the medium significantly decreased the percentage of GVBD in 2 h of culture (Fig. 2).

RT-PCR was performed on mRNA samples from cumulus-oocyte complexes and from denuded oocytes. The identity of the amplified products was confirmed by correct sizing on agarose gel. As shown in Fig. 3, both cumulus-oocyte complexes and denuded oocytes expressed activin β A and activin type IIA receptor transcripts as evidenced by the presence of amplified cDNA fragments of the correct sizes. The specificity of these bands was determined by cloning and sequencing the PCR products, which showed a 100% nucleotide sequence identity with the cloned sequences reported.

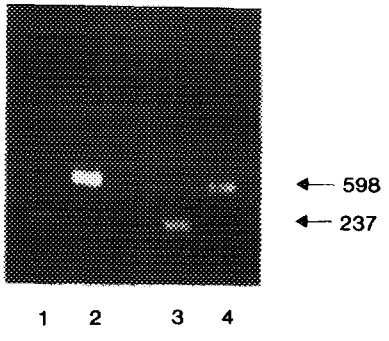

Figure 3. Detection of activin β A and activin type IIA receptor transcripts in cumulus-oocyte complexes and denuded oocytes by RT-PCR. Poly A^+ RNA was extracted from 100 cumulus oocyte complexes and denuded oocytes, one-third of the extraction was reverse transcribed and amplified by 50 cycles of PCR, as described in MATERIALS AND METHODS. The gel stained by ethidium bromide was photographed under UV-irradiation. Lane 1, activin type IIA receptor in denuded oocytes; lane 2, activin β A in denuded oocytes; lane 3, activin type IIA receptor in cumulus-oocyte complexes; lane 4, activin β A in cumulus-oocyte complexes. The positions of the expected 237-bp and 598-bp PCR products for activin type IIA receptor and activin β A, respectively, are indicated.

DISCUSSION

This study demonstrated that activin A stimulates meiotic maturation in rat cumulus cell-enclosed oocytes and that activin-induced oocyte maturation is inhibited by follistatin. Since activin A regulates follicular development (3-5,18), these data provide a close link between follicular development and oocyte maturation. The stimulatory action of activin A on oocytes was remarkable and significant both at 24 and 36 h after PMS treatment, when the follicles were developing; no significant difference was apparent 48 h after PMS, when the follicles had developed fully (Table 1). The dose study of activin A (Fig. 1) revealed that activin A exerts its stimulatory effects at 1 ng/ml, which is less than the physiological concentrations found in follicular fluids of several species (19,20). It is possible that activin A is a paracrine factor in the acquisition of meiotic competence of oocytes, a process that depends primarily on FSH.

Follistatin, a putative binding protein of activin A, at a dose of 10 ng/ml almost fully inhibited the effect of a like dose of activin A (Fig. 2). Follistatin is capable of binding same dose of activin A (15) and it antagonizes the effects of activin on steroidogenesis of rat granulosa cells, presumably by competing with activin for binding to its receptors (18). Follistatin may have inhibitory effects on cumulus-oocyte complexes in the same manner. Interestingly, follistatin alone inhibited the meiotic maturation of oocytes (Fig. 2). This finding raises the possibility of a source(s) of activin A in the cumulus-oocytes complexes other than the granulosa cells shown to produce activin A (21,22).

We demonstrated the expression of activin receptor gene in mammalian oocytes for the first time (Fig. 2). It is likely that activin A present in follicular fluids directly stimulates oocyte maturation via its receptors on the oocytes. In this report, we also present evidence that activin βA is expressed in both cumulus-oocyte complexes and in the oocytes themselves. Since activin A is a homodimer of the βA subunits, while inhibin is a heterodimer consisting of the βA subunit together with an α subunit (1,2), our data indicate that the oocyte contains mRNA encoding both activin A and inhibin. Based on our present data that follistatin

added to the medium exerts inhibitory effects on oocyte maturation, it may be at least in part activin A that is present, reflecting the presence of βA subunit. A recent study on activin expression during early mouse development reported agreement with our idea that α subunit protein cannot be detected from the fertilized egg stage onwards, and that the presence of β subunits reflects the presence of activin rather than of inhibin (23). These results suggest that activin A, produced by cumulus-oocyte complexes along with granulosa cells under modulation by follistatin, may play an important role in oocyte growth and maturation leading finally to acquisition of fertilization competence.

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REFERENCES

1. Vale,W., Rivier,J., Vaughan,J., McClintock,R., Corrigann,A., Woo,W., Karr, D., and Spiess,J. (1986) Nature 321, 776-779.
2. Ling,N., Ying,S.Y., Ueno,N., Shimasaki,S., Esch,F., Hotta,M., and Guillemin, R. (1986) Nature 321, 779-782.
3. Hsueh,A.J.W., Dahl,K.D., Vaughan,J., Tucker,E., Rivier,J., Bardin,C., and Vale,W. (1987) Proc. Natl. Acad. Sci. USA 84, 5082-5086.
4. Shukovski,L., and Findlay,J. (1990) Endocrinology 126, 2222-2224.
5. Xiao,S., Findlay,JK., and Robertson,D. (1990) Mol. Cell. Endocrinol. 69, 1-8.
6. Itoh,M., Igarashi,M., Yamada,K., Hasegawa,Y., Seki,M., Eto,Y., and Shibai,H. (1990) Biochem. Biophys. Res. Commun. 166, 1479-1484.
7. Robertson,D.M., Klein,R., de Vos,F.L., McLachlan,R.I., Salamonsen,L.A., Hearn, M.T.W., Burger H.G., and de Kretser,D.M. (1987) Biochem. Biophys. Res. Commun. 149, 744-749.
8. Ueno,N., Ling,N., Ying,S.Y., Esch,F., Shimasaki,S. and Guillemin,R. (1987) Proc. Natl. Acad. Sci. USA 84, 8282-8286.
9. Tsafriri,A., Dekel,N., and Bar-Ami,S. (1982) J. Reprod. Fert. 64, 541-551.
10. Bachvarova, R., Baran, M.M., Tejblum,A. (1980) J. Exp. Zool. 211, 159-169.
11. Eppig,J.J., and Downs, S.M. (1987) Dev. Biol. 119, 313-321.
12. Tsafriri,A. (1985):In Biology of Fertilization (C.B. Metz and A. Monroy, Eds) Vol. 1, pp.221-252. Academic Press, New York.
13. Biggers,J.D., Whitten,W.K., and Whittingham,D.S. (1971): In Methods in Mammalian Embryology (J.E.Jr.Daniel, Ed), p.85. W.H.Freeman and Co., San Francisco.
14. Murata,M., Eto,Y., Shibai,H., Sakai,M., and Muramatsu,M. (1988) Proc. Natl. Acad. Sci. USA 85, 2434-2438.

15. Nakamura,T., Takio,K., Eto,Y., Shibai,H., Titani,K., and Sugino,H. (1990) Science 247, 836-838.
16. Woodruff,T.K., Meunier,H., Jones,P.B.C., Hsueh,A.J.W., and Mayo,K.E. (1987) Mol. Endocrinol. 1, 561-568.
17. Mathews,L.S., and Vale,W. (1991) Cell 65, 973-982.
18. Xiao,S., and Findlay,JK. (1991) Mol. Cell. Endocrinol. 79, 99-107.
19. Robertson,D.M., Foulds,L.M., Prisk,M., and Hedger,MP. (1992) Endocrinology 130, 1680-1687.
20. Sadatsuki,M., Tsutsumi,O., Sakai,R., Eto,Y., Hayashi,N., and Taketani,Y. (1993) Hum. Reprod. (in press).
21. Schwall,R., Mason,A.J., Wilcox,J., Bassett,S., and Zeleznick,A. (1990) Mol. Endocrinol. 4, 75-79.
22. Rabinovici,J., Spencer,S.J., Doldi,N., Goldsmith,P.C., Schwall,R. and Jaffe,R.B. (1992) J. Clin. Invest. 89, 1528-1536.
23. Albano,R.M., Groome,N., and Smith,J.C. (1993) Development 117, 711-723.