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Activin Signaling through Type IB Activin Receptor Stimulates Aromatase Activity in the Ovarian Granulosa Cell-Like Human Granulosa (KGN) Cells

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In addition to a stimulatory effect on FSH production by the pituitary gland, activin is thought to have a paracrine or autocrine role in follicular development in the ovary, where it is produced. Recently, we established a human ovarian granulosa tumor cell line, KGN, which possesses *in vivo* characteristics of granulosa cells, namely the expression of functional FSH receptors and cytochrome P-450 aromatase. Here, we have demonstrated the activin signaling pathway and its role in KGN cells. A series of transient transfection experiments revealed that activin type IB receptor (ActRIB) is an essential component of the activin signaling pathway in KGN cells. Smad2 was found to act downstream of ActRIB as an intracellular signal transmitter. Smad7, but not Smad6, was an inhibitory Smad in the pathway. Finally, we show that FSH receptor expression and cytochrome P-450 (P-450) aromatase activity was up-regulated by activin stimulation through ActRIB in KGN cells. These results show that we have clarified the signaling mechanisms and the roles of activin in the human granulosa cell line, KGN. Activin signaling mediated by ActRIB-Smad2 system in the ovary may thus be essential for the regulation of follicular differentiation. (*Endocrinology* 144: 1603–1611, 2003)

OVARIAN FOLLICULOGENESIS is controlled by a number of factors including the pituitary gonadotropin and the intraovarian TGF-β family members such as activin, inhibin, bone morphogenetic protein (BMP), growth differentiation factor 9 (GDF9), and Müllerian-inhibiting substance (1). Therefore, it is necessary to elucidate the mechanism of how these local factors function and interact with the gonadotropin in the follicle to fully understand ovarian folliculogenesis.

Activin, a TGF-β family member, was initially identified as a secreted protein from ovarian follicular fluid, which stimulates FSH production by the pituitary gland (2, 3). A growing number of studies have demonstrated its involvement in a wide variety of physiological processes, including embryogenesis and carcinogenesis, as well as reproduction (4). In the ovary, activin is expressed predominantly in the granulosa cell layer of follicles, suggesting important roles in processes such as folliculogenesis, steroid hormone production, and oocyte maturation as a paracrine or autocrine factor (5). The role of gonadotropin in ovarian follicle development is well established (6). It is apparent that both paracrine and autocrine growth factors have important roles in follicle development. Many *in vitro* studies using primary cultures of granulosa cells have shown that activin A has a direct effect on many ovarian granulosa cell functions. For example, it has a mitogenic effect on immature granulosa cells, as demonstrated by an increase in [^{3}H]thymidine uptake *in vitro* (7). With respect to the relationship between activin and pituitary FSH, activin A has been demonstrated to induce FSH receptor mRNA (8, 9) and potentiate FSH-stimulated aromatase activity as well as progesterone and inhibin production in rat immature granulosa cells (10). These results strongly suggest the importance of activin in the regulation of both maturation and proliferation of granulosa cells through paracrine or autocrine mechanisms. Although many of these studies have provided valuable insights, the signaling mechanism of activin in ovarian granulosa cells is still not well understood, mainly because of the limitations in molecular biological analyses that use a primary culture system.

The basal components of the TGF-β family signaling pathways are the type II and type I transmembrane serine and threonine kinase receptors and the cytoplasmic Smad proteins (11–13). Upon ligand binding, the type II receptor recruits and transphosphorylates the type I receptor. The type I receptor acts downstream of the type II receptor and has been shown to determine signaling specificity within the heteromeric receptor complex (14, 15). The type I receptor, activated by phosphorylation, subsequently propagates signals to the Smad pathway. Once phosphorylated, the receptor-regulated Smads (R-Smads) dissociate from the receptor complex, bind to the common Smad (Smad4) and enter the nucleus as a complex where they participate in the transcriptional regulation of the target genes. On the other hand,

Abbreviations: ActRIA, Activin type IA receptor; ActRIB, activin type IB receptor; BMP, bone morphogenetic protein; FCS, fetal calf serum; GDF9, growth differentiation factor 9; H89, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; hFSH, human FSH; IRES, internal ribosome-entry site; I-Smad, inhibitory Smad; P-450, cytochrome P-450; RNase, ribonuclease; R-Smads, receptor-regulated Smads; SARA, Smad anchor for receptor activation.

inhibitory Smads have been shown to block the phosphorylation of R-Smad by the type I receptor, or to block the hetero-oligomerization of R-Smad with Smad4 by binding to the type I receptor or R-Smad, thus interfering with the TGF-β signaling (16–18). In addition to these Smad proteins, SARA (Smad anchor for receptor activation) has been identified as an essential intracellular component for activation of the Smad proteins by the type I receptor (19). Activin, like most of the TGF-β family members, mediates its action by binding to a complex of type I and type II receptors. The type I receptors comprise type IA (ActRIA) and type IB (ActRIB), also known as the activin receptor-like kinases Alk2 and Alk4, respectively. The type II receptors comprise type IIA (ActRIIA) and type IIB (ActRIIB). Activated type I receptors propagate the signal to specific intracellular Smad proteins, namely Smad2 and/or Smad3. The precise roles for the different activin receptors and Smads are still not clear. The different usage of the type I and type II receptors and Smads may be responsible for the different actions of activin on different tissues and target genes, explaining their functional diversity. Thus, the delineation of the cellular components of the system may be necessary for understanding the regulation of numerous biological systems.

Recently, we established the human granulosa cell line, KGN. As reported previously (20), this cell line expresses the functional FSH receptors and has aromatase activity, reminiscent of ovarian granulosa cells *in vivo*. Thus, the KGN cell line could prove to be a very useful model for the study of the role of activin in the regulation of granulosa cell differentiation during folliculogenesis in the ovary. To clarify the signaling pathway and the role of activin in the ovarian granulosa cells, we have performed a detailed analysis of the KGN cells. We found that activin signaling was mainly transmitted though the ActRIB-Smad2 system, leading to the upregulation of FSH receptor expression and aromatase activity in KGN cells. The activin signaling through the ActRIB-Smad2 may thus be essential for ovarian granulosa cell function and differentiation.

Materials and Methods

Cell culture

The human ovarian granulosa-like tumor cell line, KGN, was cultured in DMEM and Ham's F-12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sera Laboratories Ltd., Sussex, UK), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere at 37 C, as described previously (20).

Plasmids

The expression vectors for rat ActRIA, human ActRIB, and human ActRIBCA were kindly provided by Dr. Lawrence S. Mathews (University of Michigan, Ann Arbor, MI). A constitutively active form of ActRIB, designated ActRIBCA, was generated by the amino acid substitution of the threonine at position 206 with glutamine in human ActRIB cDNA (21). The expression vectors for Smad2, Smad3, and Smad4 were constructed by inserting their entire coding sequences into the pCDNA3 vector. The reporter plasmid 3TP-Lux, containing the three consecutive TPA responsive elements and TGF-β responsive elements of the plasminogen activator inhibitor 1 (PAI-1) promoter, was kindly provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY). The reporter plasmid AR3-Lux, containing the activin-responsive elements of the Mix 1 promoter and the expression vector for FAST1 were kindly provided by Dr. M. Whitman (Harvard Medical School, Boston, MA). The ActRIBCA expression vector was constructed by inserting a 1.6-kb cDNA fragment, which contains the entire human ActRIBCA coding sequence, between the *Hin*dIII and *Xba*I sites of the plasmid pEF-BOS (22). The ActRIBCA gene was tagged with a triple HA influenza virus hemagglutinin epitope at the carboxyl terminus and its expression was controlled by the human elongation factor-1α promoter. A 5.0-kb *Sal*I-*Sal*I fragment containing an internal ribosome-entry site (IRES)-βgeo cassette (23) was inserted next to the HA epitope for G418 selection as described (24).

Luciferase assays

1×10^5 cells/well were transfected using 3 μl/well Superfect Transfection Reagents (QIAGEN, Hilden, Germany) with 1 μg/well 3TP-Lux reporter construct and 1 ng/well pRL-CMV (Promega Corp., Madison, WI) as an internal control. After incubation overnight in the optimum medium, the medium was changed to serum-containing culture medium. After another 24-h incubation of the cells in the presence or absence of activin A (100 ng/ml) or TGF-β1 (50 ng/ml, Sigma, St. Louis, MO), luciferase activity was measured using the dual luciferase assay system (Promega Corp.) in a microLumat LB9507 luminometer. The luciferase activity was normalized for transfection efficiency using the *Renilla* luciferase activity from pRL-CMV. In other experiments, cells were cotransfected in the same way with the AR3-Lux reporter construct and the FAST1 expression vector.

mRNA analysis

KGN cells were cultured in a 10-cm dish containing 1.7×10^6 viable cells in 10 ml of medium. The cells were transfected with the appropriate expression vectors and cultured in the presence or absence of activin A (100 ng/ml) for 24 h followed by preparation of the total RNA using ISOGEN solution (Molecular Research Center, Inc., Tokyo, Japan). The final RNA pellet was dissolved in Tris-EDTA buffer. Total RNA was quantified by measuring the absorbance of the samples at 260 nm, and stored at −80 C until the assay. The first strand cDNA was synthesized using 1 μg total RNA using an RT-PCR kit (Stratagene, La Jolla, CA). To analyze the expression of the activin signaling components, including type I and type II activin receptors, Smads, and SARA, a sensitive RT-PCR was performed. PCR was carried out in a 50-μl reaction mixture containing $MgCl_2$ (2.5 mM), deoxynucleotide triphosphate (0.3 mM) and 2.5 U of Taq DNA polymerase (Life Technologies, Inc.) under the following conditions: 30 or 35 cycles of denaturation at 93 C for 30 sec, annealing at 60 C for 30 sec and extension at 72 C for 1 min. Primer sets used in this study were as follows: ActRIA, 5′-AATGTTGCCGTGAAGATCTTC-3′/5′-CTGAGAACCATCTGTTGGGTA-3′ (700 bp); ActRIB, 5′-CTGGCTGTCCGTCATGATGCA-3′/5′-CAATTCGCTCTCAGAGTCTCC-3′ (684 bp); ActRIIA, 5′-ACCAGTGTTGATGTGGATCTT-3′/5′-TACAGGTCCATCTGCAGCAGT-3′ (456 bp); ActRIIB, 5′-TTCTGCTGCTGTGAAGGCAAC-3′/5′-GAGGTCGCTCTTCAGCAAT ACA-3′ (699 bp); Smad2, 5′-AGAAGTCAGCTGGTGGGTCTG-3′/5′-TCATGATGACTGTGAAGATCAGG-3′ (370 bp); Smad3, 5′-GCTGGAAGAAGGGCGAGCAGA-3′/5′-CTTCATATTGAAGGCGAACTCAC-3′ (299 bp); Smad4, 5′-TCTGGAGGTGGCCTGATCTTC-3′/5′-AAGTTGGCAGTGCTGGTAGCAT-3′ (349 bp); Smad6, 5′-ACTGGATCTGTCCGATTCCAC-3′/5′-CGAAGTCGAACACCTTGATGG-3′ (455 bp); Smad7, 5′-TGTGCAAAGTGTTCAGGTGGC-3′/5′-GTCCGAATTGAGCTGTCCGAG-3′ (437 bp); SARA, 5′-AGAACATGCCTAATGGGTCTGG-3′/5′-CTGGGTCTTGCATTCCATAGG-3′ (431 bp); FSH receptor, 5′-CTCAGGCTAGGGGTCAGAGA-3′/5′-CTGGTAGTTAGGATCACTAGC-3′ (256 bp). The number in *parentheses* indicates the size of the products. Aliquots of the PCR products were electrophoresed in 2% agarose gels containing 0.5 mg/ml ethidium bromide and photographed under UV light using a positive/negative instant film (Polaroid 665, Nippon-Polaroid, Tokyo, Japan). The authenticity of the PCR products was confirmed by sequencing.

Quantitative analyses of the expressions of the activin receptors were performed using a LightCycler Roche (Mannheim, Germany) as described (25). The primer set for human β-actin was supplied in the LightCycler-Primer set (Roche). The other primer sets used in this study were described above. RNA extraction, cDNA preparation and quantitative PCR were all performed in triplicate. For PCR, 2 μl each of the

standard cDNA pool diluted 1:2, 1:20, 1:200, and 1:2000 (arbitrarily designated 1.0, 0.1, 0.01, and 0.001, respectively), the quality control cDNA pool diluted 1:25, and the sample cDNAs diluted 1:10 in sterile water or calibrator, were added to individual capillaries. *Taq* enzyme, deoxynucleotide triphosphate, reaction buffer, and SYBR GREEN I dye were supplied in the FastStart DNA Master SYBR Green I kit (Roche), of which 2 μl/capillary was added. Primer concentrations of 0.5 μM were added to each capillary. Magnesium concentrations (2–4 mM), annealing temperatures (58–62 C), and extension time (number of seconds = product size/25 plus 3) were determined for individual primer sets. The capillary volume was made up to 20 μl with sterile water. Forty cycles of PCR were programmed to ensure the threshold crossing point (cycle number) was attained. Fluorescence emission was monitored continuously during cycling. At the completion of cycling, melting curve analysis was carried out to establish the specificity of the amplicons produced. The level of expression of each mRNA and their estimated crossing points in each sample were determined relative to the standard preparation using the LightCycler computer software. The relative abundance of the mRNAs, expressed as fold changes, was extrapolated from crossing point data. A difference of 1 PCR cycle in crossing point number translates into a 2-fold change in mRNA expression.

The RT-PCR fragment of human FSH receptor cDNA was subcloned into the pGEM-T Easy Vector (Promega Corp.). The plasmid was linearized and used as a template for [^{32}P]-labeled riboprobe synthesis using an *in vitro* transcription system (Riboprobe System-SP6, Promega Corp.). The ribonuclease (RNase) protection assay was carried out according to the manufacturer's instructions. Briefly, 20 μg of each total RNA and the riboprobe were hybridized followed by digestion with RNasese. The RNase-resistant hybrids were separated by electrophoresis in 5% polyacrylamide/8 M urea gels. The results were visualized by autoradiography and the signal intensities of the protected RNA bands were quantified by densitometric scanning (FUSIX BAS2000, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Western blot analyses

Proteins were extracted by cell lysis in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 tablet/10 ml of protease inhibitor mix (Roche). Extracts were subjected to 10% SDS-PAGE, blotted, and probed with specific antibodies. The anti-HA antibody, 12CA5, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Chemiluminescent signals were generated by incubation with the ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). The antihuman P-450 aromatase antibody was kindly provided by Dr. N. Harada (Fujita Health School of Medicine, Toyoake, Japan).

Aromatase assay

The aromatase activity of KGN cells was determined as previously described (20). Briefly, KGN cells were transfected with the ActRIB expression plasmid and then plated on a 12-well multidish (Nalge Nunc International, Rochester, NY) in DMEM/Ham's F-12 containing 10% fetal calf serum (FCS). At confluency, the culture medium was replaced with medium containing 10% dextran-coated charcoal-treated FCS (steroid-free FCS), and the cells were then incubated for another 24 h in the presence or absence of human FSH (hFSH; Sigma) or activin A at the concentration indicated in the figures. After treatment, the cells were further incubated with 11.4 nM [1β-^{3}H] androstenedione (NEN Life Science Products, Boston, MA; specific activity, 25.9 Ci/mmol) for 6 h. After incubation, the medium (1.0 ml) was transferred to tubes containing 0.5 ml ice-cold 30% (wt/vol) trichloroacetic acid, and then centrifuged to remove the precipitated protein. The cells were harvested using 0.25% trypsin-1 mM EDTA to determine the protein concentration. The amount of radioactivity in the [^{3}H] H_2O was corrected by subtracting the blank values from each sample. The cell protein content was determined using a micro bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL) after the cells were dissolved in cell lysis buffer. The aromatase activity was expressed as picomoles per mg cell protein per hr incubation. Protein kinase inhibitor H89 (*N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) was purchased from Seikagaku Corp. Co. Ltd. (Tokyo, Japan).

Statistics

All experiments were carried out at least three times. In the luciferase and the aromatase assays, each independent experiment was run in triplicate or duplicate plates and these were used to generate a single mean value, which was then used to generate the mean ± SD shown in the figures. All values represent the mean ± SD. Statistical significance was determined by one-factor ANOVA followed by a *post hoc* test (Fisher's protected least significant difference test).

Results

Expression of activin signaling pathway components in KGN cells

To investigate the activin signaling pathway in KGN cells, we first investigated the expression of its components by a RT-PCR. As shown in Fig. 1, each product of the activin receptors and Smads amplified by RT-PCR was detected as a single band, and the size of each fragment was found to correspond to the predicted size. The authenticity of the PCR products was further confirmed by sequencing. All of the subtypes of activin type I and type II receptors, ActRIA, ActRIB, ActRIIA, and ActRIIB, were detected at 35 cycles of RT-PCR, consistent with previous reports that human granulosa cells express these four subtypes of receptors (26, 27). At 30 cycles, however, neither ActRIB nor ActRIIB were

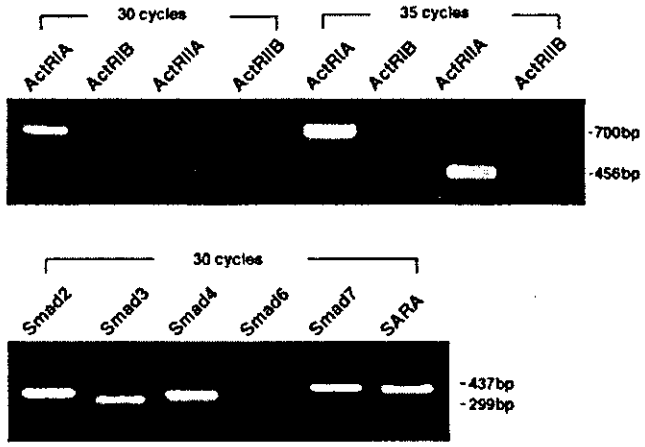

FIG. 1. RT-PCR amplification of the known components of the activin signaling pathway. Total RNA from KGN cells was prepared and RT-PCR was performed as described in *Materials and Methods*. Ethidium bromide-stained PCR products were separated in a 2% agarose gel. Each of the products is listed above the panels. A, Activin receptors. PCR products at 30 or 35 cycles are shown. Note that all the subtypes of activin type I and type II receptors, ActRIA, ActRIB, ActRIIA, and ActRIIB, were detected at 35 cycles of RT-PCR. B, Smads and SARA. All the components except Smad6 are detected at 30 cycles.

detected under the conditions employed in this study. To complete the signal transduction from the cell membrane to the nucleus, intracellular signaling molecules such as Smad proteins and SARA are required. In KGN cells, the mRNAs for these intracellular components, including Smad2, Smad3, Smad4, and SARA, were amplified as shown in Fig. 1. With regard to the inhibitory Smads, Smad7 was detected at 30 cycles, whereas Smad6 could not be amplified even at 40 cycles (data not shown). Taken together, we have detected mRNAs encoding all the known components required for activin signaling in KGN cells, suggesting the potential for activin signal transduction in KGN cells.

Relative expression of the activin receptors in KGN cells

To quantitatively assess the relative expression levels of the activin receptors, crossing point comparisons of real-time PCR were performed and fold differences estimated. As summarized in Table 1, ActRIA and ActRIIA mRNAs were more highly expressed as the type I and type II activin receptors, respectively. The amounts of these receptors were 8-fold higher than those of ActRIB and ActRIIB, consistent with the results shown in Fig. 1. Next, the type I receptor employed for the functional receptor complexes and mediation of the action of activin in the granulosa cells was investigated.

Identification of ActRIB as the type I receptor for activin in KGN cells

To address the presence of the activin signaling pathway, KGN cells were transiently transfected with 3TP-Lux, a TGF-β/activin-responsive reporter construct, and stimulated with either activin A or TGF-β1 for 24 h before being measured for luciferase activity. Unexpectedly, activin had little effect on the luciferase activity, whereas TGF-β increased it more than 3-fold as shown in Fig. 2, demonstrating a defective activin signaling pathway in KGN cells. Two isoforms of the type I receptors, ActRIA and ActRIB, have been identified by their ability to bind activin in the presence of the type II receptor (28). However, as these type I receptors appear to have different functions, it has been suggested that ActRIA transmits a BMP-like signal (29). Thus, we speculated that the low level of expression of ActRIB might be a reason for the defective activin signaling pathway in KGN cells. To address this possibility, KGN cells were transfected with each type I receptor along with the reporter construct as indicated in Fig. 2. As we expected, activin stimulation of KGN cells resulted in a 4-fold induction of the 3TP-Lux luciferase activity when the cells were cotransfected with ActRIB. The KGN cells became competent to activin stimulation by cotransfection of the ActRIB expression plasmid. These results suggest that activin signaling requires ActRIB, but not ActRIA, as a type I receptor in KGN cells.

TABLE 1. Quantitative analyses of the activin receptors by real-time PCR

mRNA	Crossing point	Relative expression (fold difference)
ActRIA	24.18 ± 0.36	8
ActRIB	27.10 ± 0.80	1
ActRIIA	24.64 ± 0.19	8
ActRIIB	27.51 ± 1.09	1[a]
β-actin	19.12 ± 0.70	256

Mean ± SD, n = 3. The crossing point of PCR cycles required for individual mRNA to reach the LightCycler detection threshold is shown. One PCR cycle equates to a 2-fold change in mRNA. The fewer PCR cycles required to achieve the crossing point, the more abundant the mRNA. The level of expression was determined relative to the expression of the least abundant.

[a] mRNA.

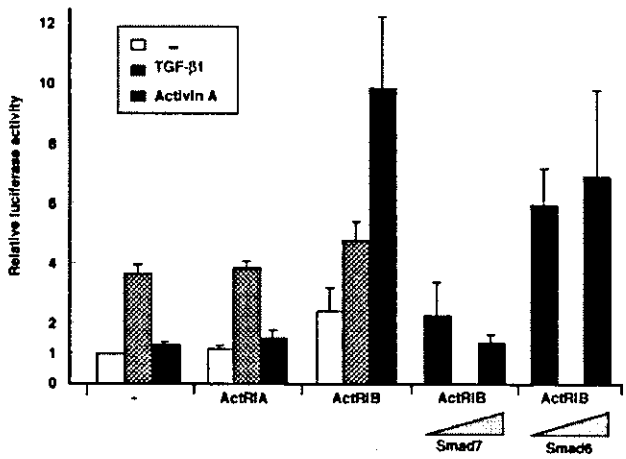

FIG. 2. ActRIB is required for the activin signaling in KGN cells. 1 × 10^5 KGN cells were transiently transfected with 3TP-Lux (0.5 μg) with or without the indicated type I receptor (0.5 μg) and treated with either activin A (100 ng/ml) or TGF-β1 (50 ng/ml) for 24 h before being measured for luciferase activity. In the presence of an increasing dosage of Smad7 (0.1–0.5 μg), the luciferase activity of the cells transfected with ActRIB followed by treatment with activin A (100 ng/ml) is decreased in a dose-dependent manner. The fold induction relative to the luciferase activity in the cells transfected with the reporter luciferase plasmid alone are shown. Each value indicates the mean ± SD of at least three separate experiments, with triplicate plates per point.

Smad2 as a downstream signal transmitter of the activin/ActRIB signaling pathway

Currently, the intracellular signaling pathway of activin is indistinguishable from that of TGF-β. Both Smad2 and Smad3 have been shown to mediate the activin signal as well as TGF-β (30). It is widely accepted that R-Smads are activated through phosphorylation by their ligand-activated type I receptors. Thus, it is conceivable to speculate that the type I receptors specify the downstream R-Smad proteins. To assess the involvement of Smad proteins in activin signaling, KGN cells were transfected with AR3-Lux, an activin-responsive reporter construct, and the FAST1 expression plasmid together with different combinations of Smads and ActRIB as indicated in Fig. 3. When AR3-Lux was used as a reporter construct, activin stimulation without ActRIB transfection resulted in a slight induction of the luciferase activity (significantly different, $P < 0.05$). AR3-Lux seems to be more sensitive than 3TP-Lux to activin stimulation, consistent with a previous observation (31). To confirm the requirement for ActRIB in activin signaling, the luciferase activity from

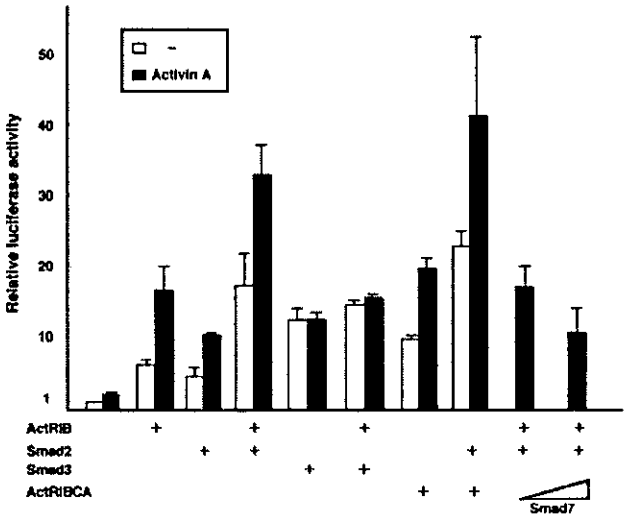

FIG. 3. Smad2 and ActRIB function cooperatively in the activin signaling pathway. Luciferase assays were performed on the lysates from cells transfected with AR3-Lux (0.5 μg) together with FAST1 (0.1 μg) and the indicated receptor and/or Smad in the presence or absence of activin A (100 ng/ml). In the presence of an increasing dosage of Smad7 (0.1–0.5 μg), the luciferase activity of the cells transfected with ActRIB followed by treatment with activin A (100 ng/ml) is decreased in a dose-dependent manner. The fold induction relative to the luciferase activity in the cells transfected with the reporter luciferase plasmid alone in the absence of activin A are shown. Each value indicates the mean ± SD of three experiments, with triplicate plates per point.

AR3Lux was measured with or without cotransfection of ActRIB. As shown in Fig. 3, the luciferase activity was increased more than 5-fold by cotransfection of ActRIB alone and was further increased up to 18-fold by additional activin stimulation. Overexpression of ActRIB may result in a slight activation of downstream signal leading to an increase of luciferase activity. Together, these results further confirmed that ActRIB is essential for activin signaling in KGN cells. To define the Smad proteins involved in the pathway downstream to ActRIB, the cells were transfected with each of the Smad expression vectors with or without ActRIB as indicated in Fig. 3. When the cells were transfected with Smad2, a 5-fold increase in basal luciferase activity and a 2-fold induction by activin stimulation were observed. This increase of the activity by the Smad2 expression vector was further enhanced by the presence of ActRIB as shown in Fig. 3, suggesting that ActRIB activates Smad2 as a downstream R-Smad. When the cells were transfected with Smad3, a further induction of luciferase activity by the treatment with activin A and cotransfection of ActRIB was not observed, although the basal luciferase activity was increased more than 10-fold. Thus, Smad3 is unlikely to be a substrate for ActRIB in KGN cells. Because the luciferase activity from AR3-Lux in these transfected cells was increased by TGF-β stimulation (data not shown), Smad3 may be phosphorylated by the TGF-β type I receptor in KGN cells. Taken together, activin signaling mediated by ActRIB is likely to employ Smad2, but not Smad3, as a downstream signal transmitter.

Smad7 inhibits the activin-induced transcriptional activity in the presence of ActRIB

So far, two inhibitory Smads (I-Smads), Smad6 and Smad7, have been identified by their ability to block either the TGF-β/activin or BMP signaling pathways. While Smad6 seems to inhibit the BMP signal preferentially, Smad 7 acts as a general inhibitor of the TGF-β family signaling pathways (16–18). One of the mechanisms by which I-Smad blocks the TGF-β family signaling pathway is through their efficient interaction with the activated type I receptor, thereby preventing phosphorylation of R-Smads by the activated type I receptor. As shown in Fig. 2, in the presence of ActRIB, Smad7 inhibited the 3TP-Lux activity in a dose-dependent manner. On the other hand, Smad6 had no inhibitory effect on the activin signaling pathway in KGN cells. This inhibitory action of Smad7 was also observed on the transcription of AR3-Lux as shown in Fig. 3.

FSH receptor mRNA is induced by activin signaling

Activin has been shown to be involved in the regulation of FSH receptor expression in rat granulosa cells (8, 9). To determine whether the expression of the FSH receptor is regulated in a manner similar to that in primary cultures of rat granulosa cells, the FSH receptor expression in KGN cells was analyzed by both RT-PCR and a RNase protection assay. As shown in Fig. 4A, RT-PCR revealed that the amount of FSH receptor mRNA was increased by the activation of the activin signaling pathway. To confirm the induction of the FSH receptor mRNA, an RNase protection assay was performed as shown in Fig. 4B. The intensity of each protected band in Fig. 4B was quantified by densitometric scanning. As summarized in Fig. 4C, activation of the activin signaling pathway in KGN cells resulted in more than a 2-fold increase in FSH receptor mRNA.

Activin signaling through ActRIB stimulates aromatase activity in KGN cells

To investigate whether activin signaling is involved in the change of cell fate, such as follicular maturation, the effect of activin stimulation on aromatase activity was analyzed in KGN cells. As shown in Fig. 5, transient transfection of the ActRIB expression vector followed by an addition of activin A in the medium resulted in a more than 2-fold increase of the aromatase activity in KGN cells. Next, we examined the possible interaction between FSH and the activin signaling pathways in the regulation of aromatase activity. FSH is well known to regulate aromatase expression through the A-kinase pathway, and indeed increased the aromatase activity by more than 4-fold in KGN cells as shown in Fig. 5. When the cells were treated with both FSH and activin A, a further increase in aromatase activity was observed. Although the mechanisms underlying the increase of aromatase activity by activin signaling remain to be clarified, the evidence that activin increases the FSH receptor level may provide one possible explanation. However, the fold induction of aromatase activity by the stimulation of the activin signaling pathway is still less extensive than the previous observations obtained using a primary culture of marmoset granulosa

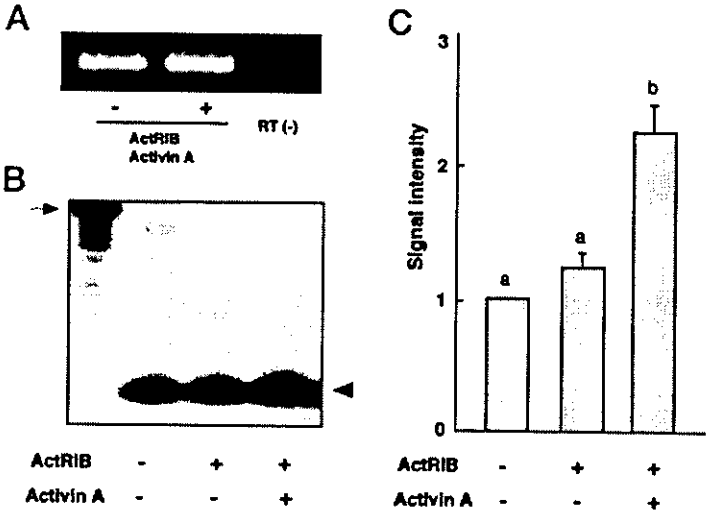

FIG. 4. FSH receptor mRNA is increased by activin signaling in KGN cells. Total RNA was prepared from cells transfected with or without ActRIB, in the presence or absence of activin A as indicated. A, RT-PCR was performed with specific primers for the FSH receptor. The same procedure without reverse transcriptase gives rise to no amplified signal. B, RNase protection assays (20 μg total RNA per lane) of RNA extracted from KGN cells cultured without treatment (control, lane 2), with transfection of ActRIB in the absence (lane 3) or presence (lane 4) of activin A. The riboprobe was hybridized with each total RNA followed by digestion with RNases. The RNase-resistant hybrids were separated by electrophoresis in a 5% polyacrylamide/8 M urea gel as indicated by the *arrowhead*. An undigested riboprobe is shown with an *arrow* (lane 1). The same experiments were carried out three times with the RNA prepared independently from triplicate plates. C, Intensities of protected RNA bands in (B) were quantified by densitometric scanning. The data are represented as mean ± SD. Histograms *without common letters* are statistically different, $P < 0.01$.

cells that showed induction by 10-fold (32). Because the expression of ActRIB is essential for activin responsiveness as described above, the transfection efficiency of the ActRIB expression plasmid, usually less than 20%, is likely to be the reason of the lower induction in this experiment. Therefore, we established stable cell lines expressing a constitutively active form of the ActRIB cDNA for further investigation of the role of activin signaling in KGN cells.

KGN-ActRIBCA cells show high aromatase activity

The expression vector for ActRIBCA was constructed as described in Fig. 6A. The expression of ActRIBCA is under the control of the EF1α promoter and a selectable marker is translated from a single fusion transcript. The IRES sequence provides cap-independent translation of a fusion protein of Neo^r and β-galactosidase. The KGN cells were transfected with the construct and cultured for 2 wk in the presence of G418 at the concentration of 200 μg/ml. Several colonies were picked and cultured individually for another 2 wk under the same conditions to create cell lines. Finally, we established three independent clones, which fundamentally had the same characteristics (data not shown). For a detailed analysis, we used one of the stable transformants, designated KGN-ActRIBCA. Firstly, the expression of ActRIBCA was confirmed by Western blotting using the anti-HA antibody and by lacZ staining. As shown in Fig. 6B, KGN-ActRIBCA cells expressed triple HA-tagged ActRIBCA protein and β-galactosidase, indicating a stable expression of the constitutively active form of ActRIB in KGN cells. As shown in Fig. 6D, the aromatase activity of KGN-ActRIBCA at a basal level was dramatically increased by about 20-fold and reached a level much higher than that of the KGN cells treated with FSH. High expression of aromatase in KGN-ActRIBCA cells was also confirmed by western blotting as shown in Fig. 6C. Interestingly, FSH treatment failed to increase the aromatase activity in KGN-ActRIBCA cells to the extent seen in KGN cells. These results suggested that activation of activin signaling stimulates aromatase activity not only by the increase of FSH receptor expression but also by a mechanism inde-

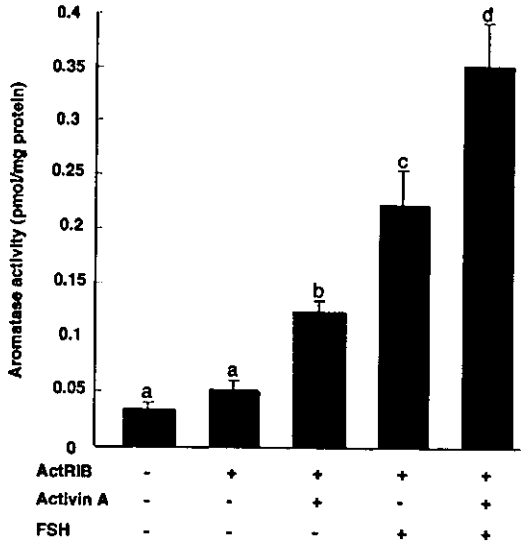

FIG. 5. Effect of activin A and FSH on aromatase activity in KGN cells. KGN cells were transfected with or without ActRIB and preincubated with or without activin A (100 ng/ml) or hFSH (50 ng/ml) for 12 h, and aromatase activity was assayed as described in *Materials and Methods*. Each value indicates the mean ± SD of four separate experiments, with duplicate plates per point. Histograms *without common letters* are statistically different, $P < 0.05$ (a *vs.* b), $P < 0.01$ (b *vs.* c), $P < 0.01$ (c *vs.* d).

pendent of FSH signaling. To ensure this explanation, aromatase activity was examined in the presence of an inhibitor of adenylate cyclase, H89. As shown in Fig. 6D, 5×10^{-6} M of H89 completely abolished the increase of aromatase activity induced by FSH stimulation in KGN cells. On the other hand, the aromatase activity in KGN-ActRIBCA cells was not completely suppressed by the same concentration of H89. The aromatase activity in KGN-ActRIBCA cells in the presence of 10^{-5} M H89 remained about 10-fold higher than that in KGN cells, suggesting that the stimulatory effect of activin on aromatase activity is likely to be independent of the A-kinase pathway.

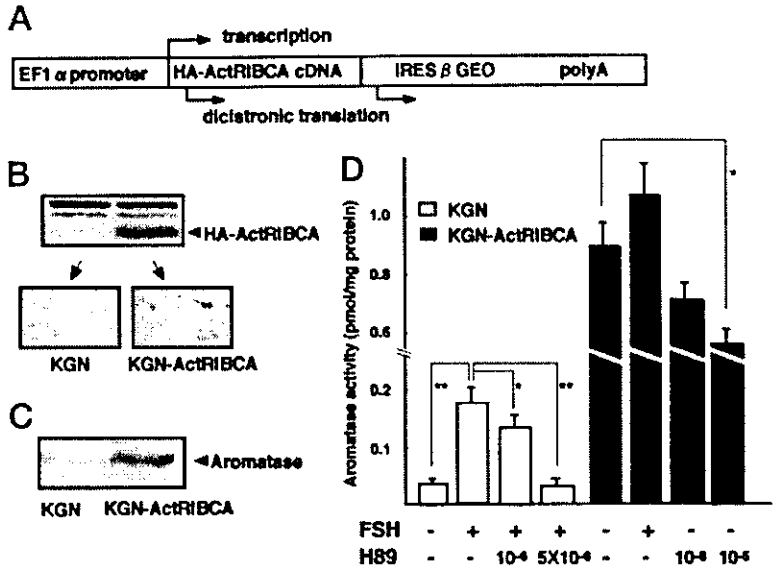

FIG. 6. Expression of the ActRIBCA transgene in KGN cells. A, Diagram of the ActRIBCA transgene expression vector. The human ActRIBCA cDNA tagged with a triple HA epitope at the 3′ end of the coding sequence was inserted between the EF-1α promoter and IRESβgeo cassette. B, Expression of the HA-ActRIBCA protein in the transgenic KGN-ActRIBCA cells was analyzed by immunoblotting the cell extracts with 12CA5, a monoclonal antibody against the HA epitope. The *arrowhead* indicates the HA-ActRIBCA band (*upper panel*). β-Galactosidase staining further confirmed the transgene expression in the KGN-ActRIBCA cells (*lower panels*). C, Expression of the P-450 aromatase protein was analyzed by immunoblotting the cell extracts with an antibody against human P-450 aromatase. The *arrowhead* indicates the P-450 aromatase band. D, Effect of H89 on aromatase activity in KGN cells and KGN-ActRIBCA cells. KGN cells and KGN-ActRIBCA cells were preincubated with or without hFSH (50 ng/ml) and H89 (10^{-6} to 10^{-5} M), as indicated in the figure, for 12 h, and aromatase activity was assayed as described in *Materials and Methods*. Each value indicates the mean ± SD of three experiments, with duplicate plates per point. *, $P < 0.05$; **, $P < 0.01$.

Discussion

To investigate the signaling pathway of activin, we first characterized the expression of the components of the activin signaling pathway, including the type I and type II activin receptors, Smads and SARA, in KGN cells. However, despite the presence of these molecules, the transient transfection assay using the 3TP-Lux plasmid revealed that the cells are almost refractory to activin stimulation. Very importantly, cotransfection of the ActRIB expression plasmid was found to be required for activation of the reporter gene, although ActRIB is expressed at a level that can be detected by an agarose electrophoresis after 35 cycles of amplification. On the other hand, ActRIA, another type I receptor for activin, is more abundantly expressed than ActRIB by 8-fold. These results clearly demonstrate that ActRIB, but not ActRIA, is the type I receptor that functionally mediates activin signaling in granulosa cells. Defective activin signaling in KGN cells may thus be explained by an insufficient expression of ActRIB. The targeted disruption of the genes encoding the activin signal components, such as type II activin receptors and Smad2, in mice have revealed their haploinsufficient phenotypes. This genetic evidence strongly suggests that the dosage of these signaling molecules is important for normal embryonic development (33, 34). Therefore, it is conceivable that the dosage of activin type IB receptor is also critical for the activin signaling pathway. In granulosa cells, there may be a threshold of activin signal required for the exertion of its function. Alternatively, the abundance of the signaling components may be an important determinant for the relative responsiveness of the granulosa cells to the growth factor. Indeed, this concept has been recently addressed by Drummond *et al.* (25). Namely, in the rat ovary, the abundance of activin receptors and Smad proteins in the granulosa cells has been shown to change during follicular development and also change dramatically during postnatal days. It is of interest whether the expression levels of the activin receptors and Smad2 are altered at different stages of follicular development in humans. In KGN-ActRIBCA cells, the amount of endogenous Smad2 is not changed, but it is activated and found to be preferentially located in the nucleus (data not shown).

Both activin and TGF-β are known to activate both Smad2 and Smad3 (35). A recent study in KAR6 cells demonstrated that activin specifically induces the association of both Smad2 and Smad3 with the activin receptor complex consisting of ActRIB and ActRIIA (30). However, our study suggests a functional preference for Smad2 for activation by activin in KGN cells. Furthermore, our functional study suggested that activation of Smad2 by activin occurred in the presence of ActRIB. There may be a preference for usage of either Smad2 or Smad3 or both, depending on the cell type. It is clear from our study that Smad2 functions downstream of ActRIB in the activin signaling pathway in KGN cells.

Two I-Smads, Smad6 and Smad7, have been so far identified as antagonists of either the TGF-β/activin or the BMP signaling pathway (16–18). Inhibitory Smads have been shown to elicit their antagonistic effects by interacting with activated type I receptors and thereby preventing phosphorylation of R-Smads, or by competing with activated R-Smads for complex formation with Smad4. Recently, another mechanism by which I-Smads block TGF-β family signaling has been described. Smad7 was found to recruit Smurfs (Smad ubiquitination regulatory factors), members of the HECT family of E3 ubiquitin ligases, to the TGF-β type I receptor, resulting in the degradation of the TGF-β type I receptor protein (36, 37). We demonstrated, for the first time, the inhibitory effect of Smad7 on the activin signaling through ActRIB in KGN cells, namely that Smad7 was found to block the activin-induced 3TP-Lux response in a dose-dependent manner, in good agreement with a previous report that Smad7 can prevent the association of the pathway-specific Smads with ActRIB (30). Further study will be needed to elucidate which mechanism described above takes place in granulosa cells.

Ovarian granulosa cells undergo a complete differentiation process during the growth and maturation of ovarian follicles that depends on pituitary gonadotropin. In this regard, the maintenance of the FSH receptor expression in the granulosa cells is important for the follicular maturation, and thus fertility. However, very few factors have been shown to regulate the expression of the FSH receptor. Those increasing the expression are FSH and activin. FSH has been shown to increase the number of its own receptors on the cell surface, implying a positive regulatory loop in the FSH signaling pathway in the granulosa cells (38). This may explain, at least in part, the mechanism by which FSH receptor expression is maintained during follicular maturation. On the other hand, activin has also been shown to increase the number of FSH receptors (8, 9) and extend the half-life of the FSH receptor mRNA (39), thereby enhancing the FSH signal in granulosa cells from diethylstilbestrol-treated immature rats. It is noted that the fold induction of FSH receptor mRNA observed in KGN cells is comparable to these observations in rat granulosa cells. The response of granulosa cells to FSH changes dramatically during follicular growth *in vivo*. In small follicles, FSH regulates the proliferation of granulosa cells, whereas, as follicles mature to a preovulatory stage, FSH induces the expression of differentiation-specific genes such as *CYP19* encoding P-450 aromatase. Although the precise molecular mechanism involved in this switch remains unknown, it has been suggested that there may be a signal(s) that interacts with the FSH signal and directs the cells to differentiation rather than proliferation. Activin A has been shown to be present exclusively in the granulosa cells of mature follicles and in the corpus luteum in the human ovary (40). On the basis of the expression profile of activin A in granulosa cells, this may be one of the signals that modulates the FSH responsiveness and promotes the maturation of granulosa cells (10). To address the effect of activin on the differentiation of granulosa cells, we investigated P-450 aromatase activity in KGN cells. Activin A treatment together with transient transfection of ActRIB resulted in a 2-fold increase of aromatase activity, which was further increased by additional FSH treatment. These results suggest that the activin signal through ActRIB has not only a pivotal role but also a synergism with the FSH signal on the expression of P-450 aromatase in granulosa cells. The molecular mechanism underlying the synergism of activin with the FSH signal on the expression of FSH receptor and P-450 aromatase is not well understood; however, studies of KGN cells will help clarify the molecular mechanism of a synergism between activin and FSH signals.

FSH receptor activation by binding its ligand results in an intracellular cAMP increase followed by an activation of the A-kinase (PKA) pathway, which is well known as a major signaling pathway for the stimulation of the P-450 aromatase gene expression. Indeed, the increase of aromatase activity induced by FSH in KGN cells was completely blocked by pretreatment with the PKA inhibitor H89 at 5×10^6 M. On the other hand, the aromatase activity of KGN-ActRIBCA cells was suppressed only 40% by pretreatment with H89 at even 10^5 M, becoming a level higher than that of KGN cells indicating that the activin signal, at least in part, stimulates aromatase activity through a PKA-independent pathway. One possible mechanism may be that the FSH and activin signaling pathways converge at the promoter of the aromatase gene to regulate its expression. To address this possibility, an analysis of the transcriptional regulation of the Ic promoter, used in the human granulosa cells as well as in KGN cells (41), will be required.

Several kinases other than PKA have recently been shown to be activated by FSH receptor stimulation in granulosa cells. Those intracellular kinases activated downstream of the FSH receptor are protein kinase B (PKB/Akt), serum and glucocorticoid-induced kinase, p42-p44 ERK, MAPK and p38 mitogen-activated protein kinase (42–45). On the other hand, recent progress has revealed that Smad signaling is not merely determined by the activation of the class of TGF-β receptors, but is also regulated through cross-talk with other kinase signaling cascades (for reviews, see Refs. 46–48). For example, TGF-β has been demonstrated to phosphorylate p38MAPK in various types of cells (49, 50). Both TGF-β and activin signaling share a subset of receptor-regulated Smads, Smad2 and Smad3, and thus it is conceivable that activin signaling also has cross-talk with the p38MAPK pathway. Recently, activin signaling was found to require the p38MAPK pathway to exert its inhibitory effect on cell growth in breast cancer cells (51). Interestingly, in the KGN-ActRIBCA cells, but not in the KGN cells, p38MAPK is strongly phosphorylated (data not shown), suggesting activin signal cross-talk with the p38MAPK pathway in granulosa cells. It is noted that both activin and FSH receptor signals converge on p38 MAPK. Future studies in the KGN cells will provide a better understanding of the cross-talk network between these kinase pathways as well as P-450 aromatase gene regulation.

In conclusion, we propose that activin produced by granulosa cells plays a pivotal role in the maturation of follicles by activating the ActRIB-Smad2 signaling pathway. The results of our current study clearly show that activin A can stimulate P-450 aromatase activity through pathways both dependent and independent of PKA phosphorylation.

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Interleukin-8 gene and protein expression are up-regulated by interleukin-1β in normal human ovarian cells and a granulosa tumor cell line

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Objective: To evaluate the expression, regulation, and role of interleukin (IL)-8 in human ovary.

Design: Prospective study.

Setting: University hospital.

Patient(s): Sixteen premenopausal women.

Intervention(s): Follicular fluid and granulosa lutein cells (GLCs) were collected during IVF cycles. Ovarian stromal and theca cells were obtained from women underwent surgery. KGN cells, the human granulosa cell tumor cell line, were also used.

Main Outcome Measure(s): The levels of IL-8 and IL-1β in follicular fluid and IL-8 protein production were determined using ELISA. Interleukin-8 and IL-8 receptor gene expression in ovarian cells and the effect of IL-8 on the proliferation of stromal cells were determined. The expression of pIκB was evaluated by Western blot, and the effect of NF-κB inhibitor APDC was examined by Northern blot analysis and ELISA in KGN cells.

Result(s): The levels of IL-8 and IL-1β in follicular fluid; each concentration and the volume showed a positive correlation. Reverse transcription polymerase chain reaction showed the presence of IL-8 mRNA in all ovarian cells. In contrast, IL-8 receptor mRNA was only detected in stromal cells. The expression of IL-8 in GLCs and KGN cells was increased by addition of IL-1β and TNFα. Interleukin-8 increased the proliferation of ovarian stromal cells. The expression of pIκB in KGN cells was induced by IL-1β, and the effects were reduced by APDC.

Conclusion(s): Interleukin 8 induced by IL-1β via activation of NF-κB in granulosa cells may have a role in the periovulatory period of follicular maturation. (Fertil Steril® 2003;79:151–7. ©2003 by American Society for Reproductive Medicine.)

Key Words: IL-8, IL-1β, granulosa lutein cells, KGN cells, NF-κB, IκB

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Ovulation and luteinization are intricate processes that occur within a complex network of regulatory mechanisms involving steroid hormones, gonadotropins, growth factors, cytokines, and their cognate receptors. A growing body of evidence reveals the mammalian ovary to be a site of cytokine production and action. Cytokines are primarily active in inflammatory and inflammatory-like phenomena. Therefore, they are hypothesized to be involved in several aspects of ovulatory processes that resemble an inflammatory reaction (1). In ovarian physiology, interleukin (IL)-1, IL-8, tumor necrosis factor (TNF)-α, and colony-stimulating factor may be of particular significance.

Interleukin-8 is a chemotactic cytokine involved in the recruitment and activation of neutrophils as well as in cell proliferation and angiogenesis (2). Because several reports showed the presence and production of IL-8 in rabbit (3, 4), rat (5), and human ovary (6, 7), IL-8 also has been implicated in ovarian follicular development, ovulation, steroidogenesis, and corpus luteum function. However, the role of IL-8 in the ovarian function and mutual regulation of cytokines during follicle development has not been fully elucidated.

In humans, the involvement of cytokines in ovarian function has been exclusively studied

on cultured granulosa-lutein cells (GLCs) derived from follicles of patients undergoing IVF. Because GLCs collected from preovulatory follicles were already luteinized after an injection of ovulatory dose of hCG, we also used a steroidogenic human granulosa-like tumor cell line, KGN cells, as a model for granulosa cells collected during the follicular phase (8).

The objective of the present study was to investigate the potential role of IL-8 in human ovary. We examined the expression of IL-8 receptor type A mRNA in human ovarian cells and of IL-8 protein in follicular fluid. We also investigated hormonal and cytokine modulation of IL-8 gene and protein expression in human GLCs and KGN cells. Additionally, activation of NF-κB during induction of IL-8 by IL-1β was examined. The data may extend our understanding about the intraovarian regulators that modulate follicular development and ovulation.

MATERIALS AND METHODS

Aspiration of Follicular Fluid

Follicular fluid was collected from 14 women who had undergone oocyte retrieval for IVF-ET and who had given informed consent. The patients were stimulated with a combination of GnRH analog (Sprecur; Hoechst Marion Roussel, Tokyo, Japan) and hMG. A dose of 5,000 IU of hCG was administered when the majority of follicles had reached 16 mm in diameter and the concentration of serum E_2 for each large follicle was >200 pg/mL. Follicular fluid was aspirated 36 hours after hCG was administered (9). The average number of aspirated follicles was 11.6 (range, 4–18), and 63.8% of these follicles included oocytes. The follicles from which no oocyte was retrieved were not included in the study. The volume of each follicular fluid was measured, then the fluid was processed by centrifuge at 200 × *g* for 10 minutes to remove cells and subsequently stored at −30°C until assayed.

Cell Preparation and Culture

Granulosa Lutein Cells and KGN Cells

Granulosa lutein cells were collected from preovulatory follicles during oocyte retrieval for IVF, then processed for study. Briefly, GLCs were recovered from follicular aspirates by centrifugation at 200 × *g* for 10 minutes. The pellet was resuspended in a minimal volume of Dulbecco's modified Eagle's medium (DMEM), layered over a 50% Percoll gradient (Pharmacia Fine Chemicals Ltd, Milton Keynes Bucks, UK), and processed by centrifuge for 15 minutes at 78 × *g*. Granulosa lutein cells were recovered from the interface and resuspended in DMEM culture medium. Granulosa lutein cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2 nmol/L L-glutamine, and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours. The medium was changed to a serum-free medium and contained with 1 mg/mL BSA. Cells were treated with various hormones and cytokines. The supernatants were stored at −80°C until IL-8 and IL-1β concentrations were measured.

The number of cells was determined by hemocytometer, and cell viability (83.3–94.4%) was determined by trypan blue exclusion. To confirm the purification of the GLCs, immunocytochemical analysis of isolated granulosa cells was performed with cytokeratin (DAKO Japan Co. Ltd, Kyoto, Japan) as a marker of epithelial cells and vimentin (DAKO) as a marker of stromal cells. The results showed that the purity of granulosa cells was ≥98% (10).

KGN, a steroidogenic human ovarian granulosa-like tumor cell line, was established by Nishi et al. (8) from a patient with invasive ovarian granulosa cell carcinoma.

Theca and Stromal Cells

Ovarian tissue was obtained from women with regular menstrual cycles who underwent benign gynecological surgery unrelated to ovarian pathology and who had given informed consent. Theca cells were prepared as described elsewhere (11). Briefly, under a microscope, the follicles were cut with a fine scissors into hemispheres and gently scraped to remove the granulosa cells. After removing the granulosa cells, theca internal layers were microdissected from the follicle wall and enzymatically dispersed with 2 mg/mL of collagenase (Sigma, St. Louis, MO). Theca and stromal cells were cultured in DMEM-Ham's F-12 medium (1:1; vol/vol) supplemented with 10% FCS, 2 nM/L L-glutamine, and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Assay for IL-8 and IL-1β

The concentrations of IL-8 in the follicular fluid and in the supernatants of cultured ovarian cells were determined by means of the human IL-8 ELISA kit (Genzyme TECHNE, Minneapolis, MN). The concentrations of IL-1β in the follicular fluid were measured by the IL-1β human ELISA system (Amersham Pharmacia Biotech, Amersham Place, UK). The interassay and intra-assay variations were <5% in IL-8 and IL-1β assay.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from the GLC, KGN, theca, and stromal cells by the guanidium thiocyanate method. The detailed procedures followed the guidelines provided by the manufacturer (Isogen; Nippon Gene Co. Ltd., Tokyo, Japan). Reverse transcription of RNA from these cells into complementary DNA and PCR amplification was performed by using a Gene Amp RNA and PCR Core Kit (Perkin Elmer Corp., Branchburg, NJ), as detailed elsewhere (12).

Samples were amplified for 32 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 60°C), and synthesis (90 seconds at 72°C), followed by primer extension for 5 minutes at 75°C after each cycle.

For PCR analysis, the following specific primers for human IL-8 and IL-8 receptor were used: IL-8 sense was 5′-TCA CTG GCA TCT TCA CTG ATT-3′; IL-8 antisense was 5′-ACT TCC AAG CTG GCC GTG GCT-3′; IL-8 receptor type A sense was 5′-TGG CAT GCC ACC TGC AGA TG-3′; and IL-8 receptor type A antisense was 5′-CAG GTA ACG GTC CAC ACT GA-3′. The distances between primers, including the primers, were 345 and 359 base pairs, respectively.

Interleukin-8 Production by Granulosa and KGN Cells

Granulosa lutein cells and KGN cells were plated in 24-well plates coated with human fibronectin (Becton Dickinson Labware, Bedford, MA) at a density of 3×10^4 viable cells per well and were cultured. After 24 hours of incubation, the medium was replaced with and without E_2 (200 pg/mL), P (5 μg/mL), LH (100 mU/mL), FSH (20 mU/mL), TNFα (0.1–10 ng/mL), and IL-1β (0.1–10 ng/mL) in triplicate wells. The supernatants were collected after 60 hours' incubation and determined by means of the human IL-8 ELISA kit (Genzyme TECHNE).

Proliferation of Stromal Cells

Proliferation of the ovarian stromal cells was determined spectrophotometrically by measuring the incorporation of tetrazolium dye. The tetrazolium dye assay system used in this study was described elsewhere (2).

Briefly, stromal cells in culture medium (DMEM with 10% FCS) to a seeding density of $5–6 \times 10^3$ per well, suspended in 96-well tissue culture plates (120 μL/well), and incubated at 37°C for 12 hours. The medium was changed to a serum-free medium and contained with 1 mg/mL BSA. Cells were treated continuously with 80 μL of various concentrations of IL-8 (0–400 pg/mL, recombinant human IL-8; PeproTech, London, UK), respectively.

Each plate had one control column (six wells) containing IL-8–free medium. After the cells were incubated for 72 hours, 20 μL of 93-(4,5-dimethylthiazol-y-yl)2,5-diphenyl tetrazolium bromide (MTT; Sigma) solution (2.5 mg/mL) was added to each well, and the plates were incubated for another 4 hours. Dimethyl sulfoxide (150 μL) was added, and the plates were vigorously shaken on a plate shaker to solubilize the MTT-formazan product. Absorbance was measured at 590 nm with a microplate reader (Model 550, Bio-Rad Laboratories, Inc., Richmond, CA).

Western Blotting of Phosphorylated IκB in KGN Cells

NF-κB exists in the cytoplasm in an inactive form associated with antagonistic regulatory proteins called inhibitors of κB (IκB). The activation of NF-κB is associated with phosphorylation of IκB, followed by its degradation and release from NF-κB and nuclear translocation of NF-κB.

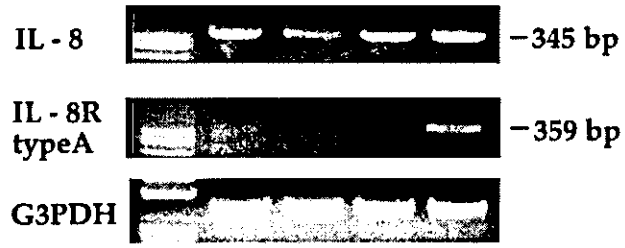
We determined the protein expression of phosphorylated IκB (pIκB) by Western blot analysis. After 0, 10, and 20 minutes' exposure of IL-1β, KGN cells were solubilized on ice in lysis buffer (50 mM Tris-HCL, 125 mM NaCl, 0.1% NP40, 5 mM ethylenediamine tetra-acetic acid, 50 mM NaF, 0.1% phenylmethyl sulfonyl fluoride, and protease inhibitors including 1% leupeptin, 10% soybean trypsin inhibitor, 1% aprotinin, and 10% tosylphenylaranine chloromethyl ketone) and processed by centrifuge at 25,000 × *g* for 30 minutes. The total protein concentration in the supernatant was measured, and samples of 60 μg of protein were separated by electrophoresis on a 4–20% gradient polyacrylamide gel. The separated proteins were transferred onto a polyvinylnylidene difluoride membrane (Millipore Co., Bedford, MA). Proteins were visualized with anti-rabbit IgG coupled to horseradish peroxidase, using enhanced chemiluminescence according to the manufacturer's recommendation. The primary and secondary anti-pIκB antibodies were IκB-α(Ser32) antibody kit (Cell Signaling Technology, Inc. Beverly, MA).

Northern Blot Analysis of IL-8 Gene in KGN Cells

KGN cells were plated in 60-mm culture dishes at a concentration of 5×10^5 cells per dish and incubated until they were confluent, with the medium exchanged every 24 hours. After the cells were preincubated in medium without serum for 24 hours at 37°C, either media alone or media with 10.0 ng/mL of IL-1β was added, and incubation was continued for another 6 hours. One of the inhibitors of NF-κB, 100 ng/mL of ammonium pyrrolidinedithiocarbamate (APDC; Wako, Osaka, Japan), was added to a culture dish 2 hours before the addition of IL-1β. Total RNA was extracted from cells subjected to different treatments by the guanidium thiocyanate method, as described previously. The detailed procedures followed the guidelines provided by the manufacturer (Isogen; Nippon Gene). Total RNA (5 μg per sample) was size fractionated by electrophoresis on 1% formaldehyde-agarose gels and transferred to Hybond-NT membrane (Amersham Life Science, Buckinghamshire, UK). The partial-length human IL-8–specific cDNA (Genbank no. Y00787; 291 base pairs, 102–393) was labeled with [α-32P] dCTP using a random prime labeling system (Amersham). The nylon membranes containing total RNA were incubated with the cDNA probe in hybridization buffer (TOYOBO, Osaka, Japan) for 3 hours at 65°C. Thereafter, the blots were washed once with 2× standard saline citrate and 0.1% sodium dodecyl sulfate for 20 minutes at 65°C, and twice with 0.2× standard saline citrate and 0.1% sodium dodecyl sulfate for 20 minutes at 65°C. Autoradiography of the membranes was performed at −80°C using Kodak BIOMAX Film (Eastman Kodak Company, Rochester, NY). The

FIGURE 1

Reverse transcription-polymerase chain reaction analysis of IL-8 and IL-8 receptor type A (IL-8RA) gene expression in human ovarian cells. G3PDH = glucose 3 phosphate dehydrogenase; KGN = KGN cell; GLC = granulosa lutein cell.



Fujii. IL-8 is regulated by IL-1β in KGN cells. Fertil Steril 2003.

visualization of ethidium bromide–stained 28S ribosomal RNA subunits was used for normalization (13).

Statistical Analysis

Correlation analysis was performed using linear regression analysis. The SD of the absorbance of tetrazolium dye assay (percentage of control values) was analyzed by one-way analysis of variance, followed by Fisher's protected least significant difference test. The data are presented as means ± SE. $P<.05$ was accepted as indicating statistical significance.

RESULTS

Concentrations of IL-8 and IL-1β in Follicular Fluid

The mean values of IL-8 and IL-1β in follicular fluid were 159.5 pg/mL (mean, 143.3 ± 16.1; range, 4.9–736.8 pg/mL) and 245.6 pg/mL (mean, 252.1 ± 60.5; range, 153.0–392.4 pg/mL), respectively. We observed significant positive correlation between the levels of IL-8 and IL-1β in the follicular fluid ($y = 0.97x - 78.48$: $r = 0.34$; $P=.0018$; 95% confidence interval, 0.13–0.53). We also found positive correlations between the levels of IL-8 and IL-1β and the follicular volume (IL-8: $y = 18.64x + 70.47$: $r = 0.37$; $P=.011$, IL-1β: $y = 6.04x + 216.56$: $r = 0.34$; $P=.03$).

Expression of IL-8 and IL-8 Receptor in Human Ovary

The gene expression of IL-8 and IL-8 receptor type A in the granulosa (GLCs and KGN), theca, and stromal cells of the human ovary were examined by means of RT-PCR. Reverse transcription polymerase chain reaction analysis showed the presence of IL-8 mRNA in all cell types. On the other hand, IL-8 receptor type A mRNA was detected in stromal cells (Fig. 1).

Effects of Sex Hormones, Gonadotropins, and Proinflammatory Cytokines on IL-8 Production in Cultured Granulosa Cells

We examined the basal secretion of IL-8 in GLCs and KGN cells. The concentrations of IL-8 were increased in a time-dependent manner after 12 to 60 hours of incubation (data not shown). Therefore, we incubated the cells for 60 hours and examined the effects of various hormones and gonadotropins on IL-8 production in GLCs and KGN cells. The addition of E_2 and FSH significantly increased the IL-8 protein production in KGN cells (231.78% and 156.56% vs. control), whereas there were no effects on the IL-8 protein production in GLCs (Fig. 2A).

Interleukin-1β and TNFα have been shown to be involved in the ovulatory process in animals and human ovary. We examined the effects of IL-1β and TNFα on IL-8 production in GLCs and KGN cells. The addition of TNFα and IL-1β stimulated IL-8 secretion by GLCs and KGN cells in a dose-dependent fashion (Fig. 2B). Tumor necrosis factor-α (10 ng/mL) stimulated IL-8 secretion 80 times more than control value. The stimulatory effects of IL-1β were more pronounced. Interleukin-1β (10 ng/mL) stimulated IL-8 secretion 130 times more than that of control value. The effects of IL-1β on IL-8 secretion were neutralized and decreased by the addition of IL-1 receptor antagonist (IL-1Ra).

Effect of IL-8 on Ovarian Stromal Cell Proliferation

The tetrazolium dye assay showed that the number of stromal cells was increased in the presence of 50–200 pg/mL IL-8 (Fig. 3). Adding anti–IL-8 antibody abolished the stimulatory effects. The mouse IgG did not influence the effect of IL-8.

Detection of Phosphorylated IκB in KGN Cells

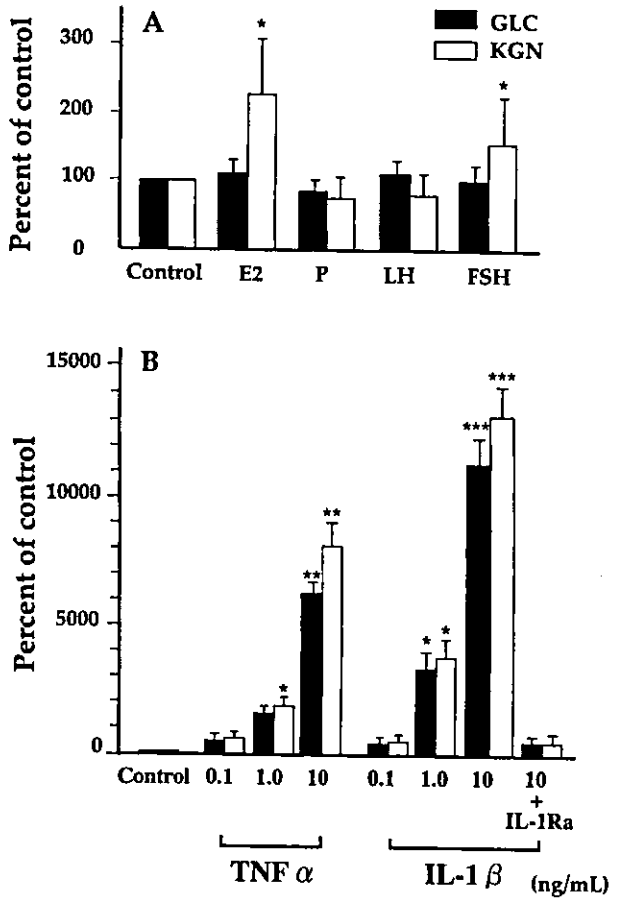
The activation of NF-κB is usually associated with induction of phosphorylation of IκB, followed by its degradation by the proteasome and NF-κB nuclear translocation. Therefore, to determine the activation of NF-κB in KGN cells during inducible expression of IL-8 by IL-1β, Western blotting was performed using an antibody for phosphorylated IκB (pIκB). The pIκB protein was rapidly induced by the addition of IL-1β (Fig. 4). The expression of pIκB was reduced by the addition of IL-1RA.

Reduction of the Effects of IL-1β by NF-κB Inhibitor

We tried to verify the participation of NF-κB during the up-regulation of IL-8 expression by IL-1β. The effects of APDC on IL-8 protein and gene expression were examined using ELISA and Northern blot analysis, respectively. The IL-8 protein production induced by IL-1β in KGN cells was reduced by the addition of APDC (Fig. 5A). Interleukin-8 gene expression consistently was enhanced by addition of IL-1β and suppressed by APDC (Fig. 5B).

FIGURE 2

The concentration of IL-8 protein in culture medium of GLCs (*black bars*) and KGN cells (*white bars*) was evaluated by ELISA. (**A**), Effect of sex hormones and gonadotropins on IL-8 protein production (*$P<.05$, **$P<.01$). (**B**), Effect of TNFα and IL-1β on IL-8 protein production in GLCs and KGN cells. Cells were incubated in medium alone (control) or in medium containing TNFα (0.1–10 ng/mL), or IL-1β (0.1–10 ng/mL), or IL-1β (10 ng/mL) and IL-1Ra (IL-1 receptor antagonist, 10 ng/mL) for 48 hours (*$P=.002$, **$P=.0003$, ***$P<.0001$). All comparisons were made vs. controls.



Fuji. IL-8 is regulated by IL-1β in KGN cells. Fertil Steril 2003.

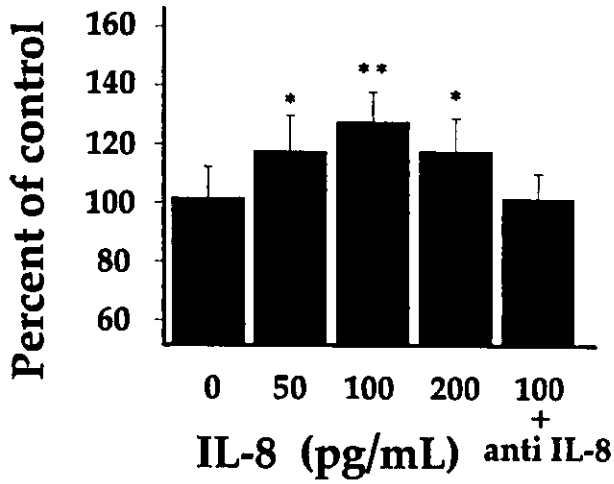
DISCUSSION

It has been reported that IL-8 is present in follicular fluid and is expressed in human ovarian cells (6, 7). However, little information about the role of IL-8 in human follicular development is available. The current results demonstrate the presence of IL-8 protein in human follicular fluid and show that concentration of IL-8 protein correlates with follicular fluid volume and IL-1β concentration, both of which increase during follicle and oocyte maturation (14, 15).

A growing body of direct and indirect evidence supports the concept that intraovarian IL-1β may constitute an inter-

FIGURE 3

Effect of IL-8 on the growth of ovarian stromal cells. Cellular proliferation was determined spectrophotometrically by the incorporation of tetrazolium dye. Results are expressed as the percentage of control values (in the absence of IL-8). Anti–IL-8 antibody (20 ng/mL) was added to the culture medium containing IL-8 (100 pg/mL). *$P<.05$; **$P<.001$; both are as compared with control.

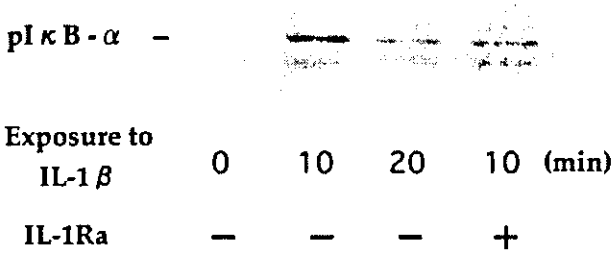


Fuji. IL-8 is regulated by IL-1β in KGN cells. Fertil Steril 2003.

mediary in the ovulatory process (16). First, addition of IL-1β has been shown to induce ovulation in the in vitro perfused ovary (17). Second, the addition of an IL-1RA has been shown to attenuate LH-supported ovulation (18, 19). Third, IL-1β has been shown to induce a series of ovulation-associated phenomena such as the promotion of ovarian prostaglandin production (20), the stimulation of ovarian

FIGURE 4

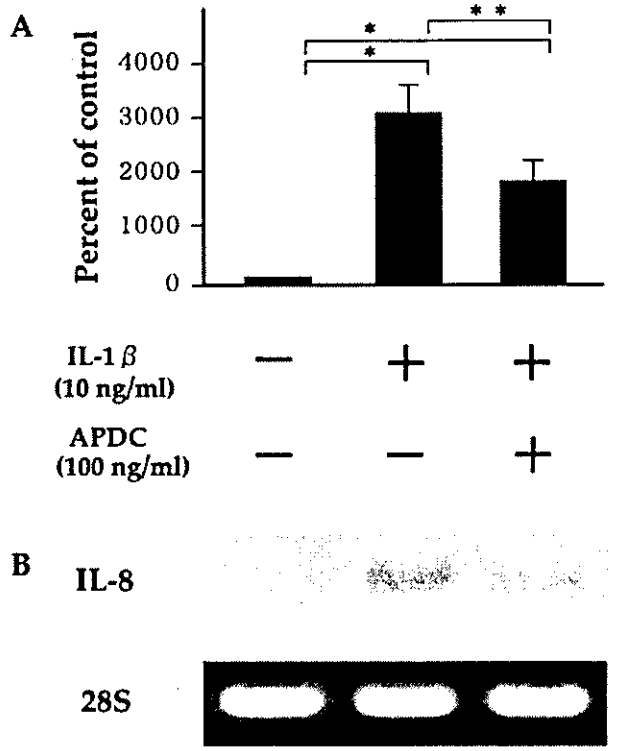
The expression of phosphorylated IκB proteins in KGN cells by Western blotting. Cells were treated with medium alone (control) or medium containing IL-1β (10 ng/mL) for 10 minutes, 20 minutes, or 10 minutes after a 1-hour pretreatment of IL-1Ra (10 ng/mL).



Fuji. IL-8 is regulated by IL-1β in KGN cells. Fertil Steril 2003.

FIGURE 5

Inhibitory effect of APDC, NF-κB inhibitor, on the stimulated IL-8 production induced by IL-1β in KGN cells. Confluent cell cultures were incubated in medium containing IL-1β (0 or 10 ng/mL). The cells were also preincubated with 100 ng/mL of APDC for 2 hours before addition of IL-1β. (**A**), The concentration of IL-8 protein in culture medium was evaluated by ELISA (*P<.0001 vs. control; **P=.003). (**B**), A representative Northern blot with its corresponding densitometric scanning data are shown. Relative densities of IL-8–specific mRNA signals were normalized with the corresponding ethidium bromide–stained 28S ribosomal RNA subunits.



Fuji. IL-8 is regulated by IL-1β in KGN cells. Fertil Steril 2003.

hyaluronic acid biosynthesis (21), the induction of ovarian collagenase activity (22), and the modulation of ovarian plasminogen activation (23).

A significant correlation between IL-8 and IL-1β suggests that IL-1β may regulate IL-8 expression in human ovarian follicles. In an in vitro study, IL-1β induced the expression and production of IL-8 in various types of cells (24), including granulosa cells (12). A recent study demonstrated that IL-1β stimulated IL-8 production in human granulosa and theca cells (7). We also showed that adding IL-1β into a culture of human GLCs and KGN cells stimulated the production of IL-8 protein and gene expression in a dose-dependent fashion. Moreover, the effect of IL-1β on IL-8 protein production was much greater than that of the E_2 and FSH.

Interleukin-8, which is recognized as a chemoattractant for neutrophils and an angiogenic agent, induces the proliferation of human melanoma (25), glioma (26), and endometrial cells (2). It is postulated that IL-8 may be involved in periovulatory events not only by attracting and activating neutrophils that would play a role in timely follicular rupture but also by stimulating new blood vessel formation for a healthy ovulatory follicle. The results of the present study suggest that IL-8 enhanced the proliferation of stromal layers surrounding the leading follicle just before the time of ovulation. The growth-promoting factors including IL-8 are important because the rapid and exponential growth of cells in surrounding tissues of ovulatory follicles may be mandatory.

Interleukin-8 is also important in a pathological condition of ovary. Ito et al. (27) showed that extremely high IL-8 concentrations were found in the serum and ascitic fluid in patients with severe ovarian hyperstimulation syndrome. This finding suggests that IL-8 plays a role in the intraperitoneal acute inflammatory process by inducing the transendothelial migration of neutrophils and rapid expansion of ovary by promoting stromal cell proliferation.

NF-κB was originally identified as a transcription factor that bound to an enhancer element in the gene for the Igκ light chain (28). Subsequent studies revealed that it is ubiquitously expressed and plays a central role in regulating the expression of many genes involved in immune and inflammatory processes (28). NF-κB can be activated by different stimuli such as microbial products, proinflammatory cytokines, T- and B-cell mitogens, and physical and chemical stresses (28). NF-κB in turn regulates the inducible expression of many cytokines, chemokines, adhesion molecules, and acute phase proteins (28).

The up-regulation of IL-8 production by IL-1β in human GLCs are thought to be exerted through activation of NF-κB, but it has not been examined. We showed that phosphorylation of IκB was induced in KGN cells after addition of IL-1β and APDC, which inhibits NF-κB activity, and partially inhibits the inducive effect of IL-1β on IL-8 gene expression and protein production.

These findings suggest that NF-κB was activated during IL-8 induction by IL-1β in KGN cells. Human granulosa cells are obtained mainly from IVF programs. However, they are only obtainable in small numbers, and they do not survive in culture for extended cell generations. Difficulties in maintaining primary culture systems and also in preparing uniform cell populations in sizable amounts often have prevented the performance of detailed analysis of characters of the cells. Although several human granulosa cell lines have been established (29–33), none was reported to express functional FSH receptor. Here we used the KGN cells that were established from a tumor specimen enucleated from a patient who showed a local recurrence of a granulosa cell tumor after menopause (8).

We characterized that KGN cells had steroidogenic activities similar to those of normal granulosa cells and expressed functional FSH receptor and relatively higher aromatase levels. The developmental stage of KGN cells is thought to be close to that of immature granulosa cells. The response pattern of aromatase activity to gonadotropin in human granulosa cells has been reported as different, depending on the developmental stage of the follicle. In granulosa cells from immature follicles, treatment with FSH, but not LH, increased aromatase activity, whereas in mature granulosa cells, both treatment markedly stimulated aromatase activity (34).

In the present study, responsiveness of IL-8 production to TNFα or IL-1β is quite similar between the GLCs and KGN cells (Fig. 3B), although the responsiveness to E_2 and FSH was increased only in KGN cells. Therefore, this KGN cell line is useful for studying cytokine regulation in human granulosa cells as a model of immature granulosa cells.

In conclusion, the current results suggested that IL-8 induced by IL-1β via activation of NF-κB may have a role in the periovulatory period of follicular maturation.

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Functional characterization of a new human Ad4BP/SF-1 variation, G146A☆

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Abstract

Ad4BP/SF-1 plays key roles at all levels of the hypothalamic–pituitary–steroidogenic organ axis and its functional disruption causes endocrine disorders of these organs. However, only three human subjects with Ad4BP/SF-1 mutations have been reported to date, suggesting limited clinical significance as a cause of inborn adrenal or sexual abnormalities. We report the first functional characterization of a new variation found in the hinge region of human Ad4BP/SF-1, G146A. Resulting from a single nucleotide shift (GGG → GCG), G146A bears slightly diminished transactivation activity evidenced by both adrenal specific *cyp11A* promoter and ovary specific *cyp19* promoter II. The variation does not affect protein expression or stability, exhibiting no dominant negative effect. G146A has a normal interaction pattern with standard co-regulators and subnuclear distribution pattern, and can be considered as a nonsynonymous single nucleotide polymorphism, since it occurs in normals and patients with adrenal diseases. In normal Japanese the allele C frequency is 8%, while in a preliminary population of patients with adrenal diseases it is elevated to 30%; suggesting the G146A variation might be of clinical importance.

Keywords: Ad4BP/SF-1; DAX-1; Polymorphism

The adrenal glands and gonads are essential tissues, particularly because of their crucial function of synthesizing steroid hormones. Dysfunction of these tissues leads to severe pathological conditions, including various types of abnormalities in sexual differentiation. Ad4BP, also known as SF-1, and officially designated NR5A1, is a transcription factor belonging to the nuclear receptor superfamily and contains a characteristic zinc finger DNA-binding domain, an intervening hinge region, and a carboxyl-terminal putative ligand-binding domain, although the ligand for Ad4BP/SF-1 is still unknown. A variety of genes involved in steroidogenesis throughout the hypothalamic–pituitary–adrenal/gonadal axis are transcriptionally regulated by Ad4BP/SF-1, such as the steroid hydroxylase genes, luteinizing hormone receptor, adrenocorticotropin receptor, prolactin receptor, and star protein [1–7]. In addition, disruption of the *ad4bp/sf-1* gene in mice leads to complete agenesis of the adrenal glands and gonads, male-to-female sex reversal, and persistence of Müllerian structures in males. There is also a virtual absence of the VMH and

☆ *Abbreviations:* Ad4BP, adrenal 4 binding protein; AF-2 domain, activation function-2 domain; AHC+HHG, adrenal hypoplasia congenital with hypogonadotropic hypogonadism; CREB, cAMP-response element-binding protein; DAX-1, dosage sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome gene 1; GFP, green fluorescence protein; LBD, ligand-binding domain; NR5A1, nuclear receptor subfamily 5, group A, member 1; PCR, polymerase chain reaction; PKA, protein kinase A; 17α-OHD, 17α-hydroxylase deficiency; P450 SCC, cytochrome P450 side chain cleavage; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein; VMH, ventromedial nucleus of hypothalamus.

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the gonadotropin levels of pituitary gonadotropes are decreased [8–11].

Achermann et al. [12] first reported a human Ad4BP/SF-1 mutation in a patient with primary adrenal failure, XY sex reversal, and persistent Müllerian structure. A heterozygous G35E mutation was revealed in the "P"-box of the Ad4BP/SF-1 DNA-binding domain in this genotypically male patient. A homozygous mutation in the A-box of Ad4BP/SF-1 (R92Q) in a baby born to consanguineous parents was reported by the same group [13]. R92Q affects a region of Ad4BP/SF-1 that modulates DNA binding by monomers and produces a phenotype with an autosomal recessive mode of inheritance. This A-box change produces a partial loss of function, compared with the P-box mutation. These results confirmed the critical role of Ad4BP/SF-1 in the adrenal gland and gonads in human, and also highlight the importance of the quantitative effects of transcription factors that control development. In addition, Biason-Lauber et al. [14] reported a heterozygous R255L mutation in the hinge region of Ad4BP/SF-1 protein in a phenotypically and genotypically normal girl. The girl had typical signs and symptoms of adrenal insufficiency but no apparent defects in ovarian maturation.

While Ad4BP/SF-1 is essential to the hypothalamic–pituitary–adrenal/gonadal axis, few occurrences of mutation of the gene restrict its clinical importance as compared with its closely related partner-DAX-1, which is also an orphan nuclear receptor, but one that plays a key role in the development and function of the adrenal glands and the hypothalamic–pituitary–gonadal axis. DAX-1 mutations, which cause X-linked AHC plus HHG, occur more frequently and are thus of greater clinical importance [15]. DAX-1 interacts physiologically with [15–18] and antagonizes Ad4BP/SF-1-mediated transcription by recruiting the corepressor NcoR [19] or Alien [20] to the Ad4BP/SF-1 complex.

In the present study, we found a nonsynonymous single nucleotide polymorphism in the hinge region of human Ad4BP/SF-1, G146A, which was found to occur frequently in Japanese. The protein function of this polymorphism variant was explored with respect to transactivation activity, interaction properties with co-activators like TIF2 and CREB, interaction with the specific repressor DAX-1, and subnuclear localization in living cells. We also analyzed the frequency of this polymorphism in a preliminary population of patients with adrenal diseases and compared this with a sample Japanese population as control.

Materials and methods

Subjects. Thirty patients with several adrenal disorders and 55 normal individuals as control were analyzed in this study of the genotype of the single nucleotide polymorphism of G146A (GGG to GCG) in human Ad4BP/SF-1. Eight patients showed typical phenotypes of Cushing's syndrome (CS) like moon face, central obesity, buffalo hump, hypertension, and hypokalemia. In these patients, autonomous secretion of cortisol was observed as evidenced by unsuppressed secretion of cortisol even by p.o. administration of 8 mg dexamethasone overnight. Three patients were diagnosed as having preclinical Cushing's syndrome (PCS) because of the absence of typical Cushing's appearance, despite the autonomous secretion of cortisol to some extent. Four patients were diagnosed as having nonfunctioning adrenocortical adenoma (NF). Six patients were diagnosed as having primary aldosteronism because of hypokalemia, hypertension, hyperaldosteronemia, and low plasma rennin activity (PA). All of the above 21 patients were finally operated on and each diagnosis was pathologically confirmed to be adrenal adenoma. Three patients were typical cases of adrenal hypoplasia congenital (AHC) complicated with hypogonadotropic hypogonadism. One case is a female patient affected by adrenal insuficiency since birth; and though diagnosed as having congenital Addison's disease, the etiology of her adrenal insufficiency is not completely understood. However, we found no *Ad4BP/SF-1* mutation in this patient's coding region. Five patients had 17α-hydroxylase deficiency (17α-OHD).

PCR, sequencing, and restriction enzymes digestion. After written consent was obtained from all subjects, genomic DNA was extracted from each subject's peripheral leukocytes following the manufacturer's protocol (QIAamp DNA Blood Maxi Kit, Qiagen, Valencia, CA). PCR was performed using an automated thermocycler (Whatman Biometra, Göttingen, Germany). Primers were designed by DNASIS MAC v3.0 to amplify each exon and the intron–exon boundaries of human Ad4BP/SF-1, including the 5′ flanking regulatory region of noncoding exon 1 (from −290 to +180) based on the human *Ad4BP/SF-1* gene sequence we previously clarified [21]. PCR conditions are available on request. Direct sequencing of each PCR segment in both directions was performed using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems). Digestion by *Sph*I or *Pma*CI (*Bbr*PI) endonuclease restriction enzymes was used to screen for sequence variations that caused changes in the target sites. Digests were separated in a 2% agarose gel (Sigma, St. Louis, MO).

Plasmid construction. A full-length human Ad4BP/SF-1 cDNA was cloned from a human spleen cDNA library (Bioscience Clontech, Palo Alto, CA) by PCR using primers based on the human Ad4BP/SF-1 cDNA sequence (GenBank Accession No. NM 004959.2). PCR was performed using an Advantage cDNA PCR Kit (BD Bioscience Clontech) and an automated thermocycler (Whatman Biometra, Göttingen, Germany) with an appropriate program. The PCR product was first subcloned to the pGEM-T-Easy vector (Promega, Madison, WI), and sequenced to validate its sequence using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems). Finally, Ad4BP/SF-1 cDNA was subcloned into the expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA), at the *Not*I and *Xba*I restriction sites to produce pcDNA 3.1-Ad4BP/SF-1. Both the polymorphism (G146A, GGG → GCG) and the mutant (G35E, GGC → GAA) pcDNA 3.1(+)-Ad4BP/SF-1 were made using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and subsequently confirmed by sequencing. To study the subnuclear localization, cDNAs of wild-type and polymorphism Ad4BP/SF-1 were subcloned into the green fluorescent protein vector pEGFP-C1 (Clontech, Palo Alto, CA), downstream of the humanized GFP sequence at the *Sac*II site. The human DAX-1 expression vector (pRc/RSV-DAX-1), the human cytochrome P450scc gene (*CYP11A*) promoter luciferase reporter plasmid pGL3-hSCCprom (0.6 kb) and the human cytochrome P450arom gene (*CYP19*) promoter II luciferase reporter plasmid pGL3-ArPII were as constructed previously [22]. The expression vector of TIF2 was a generous gift from Professor Kato S (Tokyo University). CREB expression vector was purchased from Clontech (Palo Alto, CA).

Cell culture. CV1 monkey kidney cells were purchased from the Japanese Cell Research Bank (Tokyo) and maintained in DMEM (high glucose) supplemented with 10% FBS at 37 °C. The human

ovarian granulose-like tumor cell line, KGN, was originally established by our group and expresses a relatively high level of aromatase activity which is PKA-dependent [23]. The cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 10 U/L penicillin, and 10 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37 °C.

Luciferase assay. For the luciferase assays, 1.5×10^5 cells/well were seeded into 12-well plates in 1 ml growth medium the day before transfection. As much as 0.8 μg pGL3-hSCCprom or pGL3-ArPII, 0.4 μg pcDNA-Ad4BP/SF-1, and 3 ng pRL-CMV were transiently cotransfected to each well using the Superfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. The respective dosages of DAX-1, TIF2 or CREB were also cotransfected as indicated in the figures. The total amount of plasmid was kept constant by compensating with an empty expression vector. Forty hours after transfection, cells were lysed in 100 μl/well lysis buffer and luciferase assay was performed according to the protocol of the Dual-Luciferase Reporter assay system (Promega, Madison WI) using a Lumat LB 9507 luminometer (Berthold Technologies). Firefly luciferase activity produced by PGL3-hSCCprom in identically treated triplicate samples was normalized for *Renilla* luciferase activity produced by pRL-CMV. The data shown are representative of at least three independent experiments.

Western blotting. CV1 cells were grown to subconfluent phase on 60 mm dishes and transfected with Ad4BP/SF-1-WT (wild type) or Ad4BP/SF-1-P (polymorphism); after 24 and 48 h, respectively, cells were washed with PBS and actively lysed in 500 μl lysis buffer. Samples were subjected to electrophoresis on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were incubated with polyclonal anti-SF-1 antibody (a generous gift from Professor Morohashi K, National Institute of Basic Biology, Okazaki, Japan) and subsequently with an HRP-linked goat anti-rabbit IgG secondary antibody (Cell Signaling). Detection was carried out using the ECL+Plus Western Blotting Detection System (Amersham Biosciences, UK). Membrane was then scanned by a Molecular Dynamics STORM 860 scanner and subsequently analyzed by ImageQuaNT software.

Laser confocal fluorescence microscopy. The subnuclear distributions of wild-type and polymorphism Ad4BP/SF-1 were observed in living KGN cells using an LSM 510 META microscope (Carl Zciss). Briefly, KGN cells were seeded in 35 mm glass-based dishes and a total amount of 0.5 μg/dish of pEGFP-Ad4BP/SF-1-WT or pEGFP-Ad4BP/SF-1-P were transfected into cells using Superfect. Four hours post-transfection, the culture medium was replaced with fresh medium in the presence or absence of 10^{-6} mol/L forskolin. After overnight incubation (12 h), cells were observed using an LSM 510 META microscope equipped with a Plan-Apochromat 100× 1.4 oil objective. GFP fluorescence was excited by the 488 nm laser line from an air-cooled fiber-coupled argon laser. All images obtained represent the average of eight sequentially obtained images. LSM images were exported as TIF files and final figures were generated using Adobe Illustrator and Adobe Photoshop (Adobe Systems, Buffalo, NY).

Results

Ad4BP/SF-1 polymorphism

Direct sequencing of the human Ad4BP/SF-1 gene in individuals for screening revealed a G–C heterozygous substitution in exon 4 (Fig. 1A). This one-base shift resulted in a Gly146 to Ala (GGG–GCG) missense mutation. No other variations were found in any other regions, including the 5′-flanking noncoding regulatory

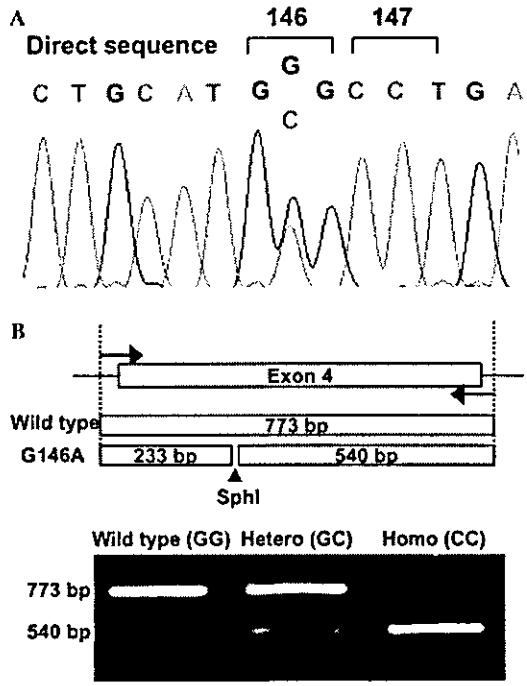

Fig. 1. G146A variant of human Ad4BP/SF-1. (A) Direct sequencing of the PCR products of exon 4 (including the flanking boundary regions) reveals an altered base at the second (G → C) position of codon 146, resulting in a substitution of Gly (GGG) with Ala (GCG, G146A). (B) This one-base shift introduces a novel *Sph*I site (GCATGC). Digestion by this enzyme was applied for further testing 30 cases of patients with adrenal diseases and totally 55 normal control cases. The digestion pattern is shown; the 233 bp fragment is not visible.

exon 1. The G–C shift created a restriction site for *Sph*I (Fig. 1B), and digestion by this enzyme was applied for further testing of 30 adrenal disease cases and 55 normal control cases. Genotypes of patients with adrenal diseases were as follows; CS group, 3cases with G/G, 3 cases with G/C, and 2 cases with C/C; PCS group, 2 cases with G/G and 1 case with G/C; NF group, 1 case with G/G, 2 cases with G/C, and 1 case with C/C; PA group, 5 cases with G/G and 1 case with G/C; AHC group, one case of congenital Addison's disease, 2 cases with G/G, and 2 cases with G/C; and 17α-OHD group, 3 cases with G/G, 1 case with G/C, and 1 case with C/C. As a total, 14 of 30 patients (46.7%) had genotypes G/C (heterozygous, 10 cases) or C/C (homozygous, 4 cases). The frequency of allele C was 30%. In the control group, only 7 of 55 (12.7%) contained allele C (5 heterozygotes and 2 homozygotes), a frequency of 8.2%. Among the total of 21 allele C-possessing cases (15 G/C, 6 C/C), 66.7% (14 of 21) were adrenal disease patients. In contrast, only 26.5% (17 of 64) GG cases were adrenal disease patients.

Our previous reports [22,24] show that the class B HLH (helix–loop–helix) family recognition site, E-box, which is located about −80 bp from the transcriptional