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Tumor Necrosis Factor Alpha Inhibition of Follicle-Stimulating Hormone-Induced Granulosa Cell Estradiol Secretion in the Human Does Not Involve Reduction of cAMP Secretion but Inhibition at Post-cAMP Site(s)

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Tumor necrosis factor α (TNF) inhibits follicle-stimulating hormone- (FSH)induced estradiol secretion by granulosa cells in several species, including humans. One major inhibitory effect of TNF in rat granulosa cells is at the level of stimulatable adenylyl cyclase, resulting in reduced cAMP concentrations. The purpose of the present study was to investigate whether a reduction in cAMP secretion could account for the inhibitory effects of TNF on FSH-induced estradiol in human granulosa cells. Granulosa cells were taken from ovaries of premenopausal women undergoing oophorectomy for reasons unrelated to ovarian pathology. Women in this study were in various stages of the menstrual cycle or exhibited irregular cycles. Granulosa cells from follicles ranging from 5 to10 mm diameter were subjected to culture for 48 and 96 h. Granulosa cells were cultured with human FSH (2 ng/mL) and testosterone (1 μ*M*) in the presence and absence of human TNF (20 ng/mL). Media were collected at 48 h, fresh media and hormones added, and cultures continued for an additional 48 h. Accumulation of cAMP, progesterone, and estradiol in media were determined by radioimmunoassay (RIA). FSH induced significant increases in cAMP, progesterone, and estradiol by 96 h of culture. TNF inhibited the secretion of estradiol at 96 h without reducing the accumulation of cAMP and progesterone in media. Similar results were observed in the presence of 0.1 m*M* isobutylmethylxanthine (D3MX), a phosphodiesterase inhibitor that would prevent metabolism of cAMP to AMP. To determine whether TNF would inhibit the ability of cAMP to induce estradiol and progesterone secretion, granulosa cells were incubated with 0.1 m*M* cAMP in the presence and absence of TNF. TNF consistently inhibited the ability of cAMP to increase estradiol secretion. These results indicate that a pathway for TNF inhibition of FSH- or cAMP-induced estradiol secretion in human granulosa cells is at post-cAMP sites rather than inhibition of FSH-stimulatable adenylyl cyclase.

Key Words: cAMP; granulosa cell; estradiol; progesterone; tumor necrosis factor.

Introduction

There is increasing evidence of tumor necrosis factor (TNF) in the ovaries of several species, including humans *(1)*. Human granulosa cells *(2)* and follicular fluid *(2,3)* of antral follicles are sources of immunoreactive TNF. Large granulosa-lutein and small paraluteal cells of the human corpus luteum also exhibit immunoreactive TNF *(2)*, and recently, it has been shown that TNF mRNA is present in large luteal cells of the pig *(4)*, irnmunoreactive protein is present in pig corpora lutea *(5)*, bioactive TNF is present in ovine corpora lutea *(6)*, and bovine corpora lutea contain *(7)*, and secrete TNF *(8)*. TNF has also been observed in conditioned media of granulosa cells *(9)* and oocyte corona complexes *(10)* from women undergoing in vitro fertilization. Recently, it was hypothesized that luteal TNF may be a factor in suppressing estradiol secretion and follicle development in the luteal phase of the menstrual cycle *(11)*.

TNF inhibits gonadotropin-stimulated steroidogenesis in granulosa and theca cells (1). Proposed mechanisms for these effects include activation of protein kinase C (PKC) *(12–15)*, a reduction in ovarian gonadotropin receptors, cAMP, protein kinase A (PKA) activity *(14,16–19)*, and

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17αhydroxylase activity *(20)*. However, a recent study using human granulosa cells from cyclic women indicated that TNF inhibited follice stimulating-hormone- (FSH) stimulated estradiol secretion did not correlate with a decline in cAMP *(21)*. Thus, mechanisms other than a decline in FSH stimulatable cAMP must explain the inhibitory effects of TNF on estradiol secretion. In contrast, a study using human luteinized granulosa cells revealed that TNP-inhibited FSH-stimulated estradiol secretion was associated with a decline in cAMP generation, but that TNF could also inhibit at post-cAMP sites *(22)*. The purpose of the present study was to investigate whether a reduction in cAMP secretion could account for the inhibitory effects of TNF on FSH-induced estradiol secretion in human granulosa cells obtained from women not treated with exogenous gonadotropins. If TNF did not inhibit FSH-induced cAMP accumulation, then a post-cAMP site of TNF action would be investigated.

Results

Effects of FSN on cAMP, Progesterone, and Estradiol Accumulation by Granulosa Cells (Table 1)

FSH stimulated significant increases in cAMP accumulation within 48 h, and this was also evident at 96 h. However, increases in estradiol and progesterone induced by FSH were not statistically evident ($p \leq 0.05$) until 96 h.

Effects of TNF on FSH-Stimulated cAMP, Progesterone, and Estradiol (Tables 2 and 3)

TNF had no significant effect on FSH-stimulated cAMP and progesterone accumulation ($p > 0.05$ in Table 2). However, at 96 h of culture, TNF inhibited FSH-stimulated accumulation of estradiol (Table 2). Similarly, in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), TNF had no effect on accumulation of cAMP and progesterone, but TNF reduced estradiol secretion at 48 and 96 h of culture (Table 3).

Effects of TNF on cAMP-Stimulated Accumulation of Progesterone and Estradiol (Table 4)

TNF had no effect on cAMP stimulated progesterone secretion. However, TNF reduced cAMP-stimulated secretion of estradiol at 48 and 96 h of culture.

Discussion

These data indicate that TNF specificaLly inhibits FSH stimulated estradiol secretion (but not progesterone and cAMP accumulation) in human granulosa cells taken from follicles <10 mm diameter (Tables 2 and 3). Although estradiol secretion is reduced, cAMP levels are not altered by TNF treatment. In addition, when the cAMP analog, 8-bromo-cAMP, is added to human granulosa cells, bypassing the FSH receptor/G-protein portion of the pathway, TNF retains its ability to inhibit estradiol production (Table 4). These data indicate that the mechanism whereby TNF inhibits FSH-stimulated estradiol is at a post-cAMP site. The next key factor in FSH receptor messenger system after cAMP is PKA; however, to date, no studies have measured effects of TNF on PKA activity in human or rat granulosa cells.

The present study revealing a post-cAMP action of TNF supports a previous study using luteinized human granulosa cells in which TNF inhibited cAMP-stimulated estradiol accumulation *(22)*. However, studies in the human differ from results obtained using rat granulosa cells in which TNF inhibited steroidogenesis at the level of stirnulatable adenylyl cyclase *(16)*. In those studies, TNF dose-dependently inhibited the accumulation of cAMP induced by FSH in rat granulosa cells, but TNF apparently did not have effects at sites distal to cAMP generation. Similarly, TNF has been shown to inhibit LH-stimulated cAMP accumulation by rat thecalinterstitial cells in vitro, but, in contrast, TNF was shown to also act at PKA, a post-cAMP site *(14)*. The latter was demonstrated by stimulating thecal-interstitial cells with TNF and cAMP analogs that selectively activate cAMP-dependent protein kinase types I and II. At 48 and 96 h of culture, TNF blocked androstenedione production stimulated by all combinations of cAMP analogs. Peak PKA activity in theca-interstitial cells was observed at 30 min in the presence of LH or cAMP analogs, and that activity was inhibited after concomitant exposure to TNF.

Another site of action of TNF in inhibition of estradiol secretion would be at the level of aromatase gene expression. Studies have shown that aromatase activity in human granulosa-lutein cells is correlated to changes in the levels of aromatase mRNA *(26,27)*. Expression of the aromatase gene is dependent on binding of steroidogenic factor-1 (SF-1) and cAMP response element binding protein (CREB) to the promoter of aromatase. The effects of SF-1 and CREB on aromatase gene expression appear to be additive *(28)*. The absence of either one will reduce aromatase expression. Thus, the rationale for this hypothesis is based on two observations. First. the activity of aromatase is largely regulated Iby expression of the gene *(26,28)*, and SF-1 and CREB regulate expression of the gene, at least in part. Second, using a human blood mononuclear cell model, TNF has been shown to inhibit the binding of CREB to DNA *(29)*. Thus, TNF inhibition of FSH-stimulated estradiol production may be related to a reduced ability of CREB to bind to and/or activate the aromatase promoter.

The reasons for the lack of TNF inhibition of cAMP accumulation in the human granulosa cell are unknown. Possibly it is because of differences in TNF signaling mechanisms, types of adenylyl cyclase and/or the state of differentiation of the granulosa cells (taken from human antral follicles ≤10 mm diameter and rat preantral follicles). The specificity of the effect for aromatase is also enigmatic. TNF did not inhibit FSH-stimulated progesterone accumulation.

Table 1
Effects of FSH (2 ng/mL) on Accumulation of cAMP, Progesterone, and Estradiol in Media at 48 and 96 h[a]

	cAMP, pmol/mL		Progesterone, ng/mL		Estradiol, pa/mL	
	Control	FSH	Control	FSH	Control	FSH
48 h	1.13 ± 0.27	5.45 ± 1.50[b]	12.4 ± 8.9	27.2 ± 10.7	886 ± 262	2366 ± 859
96 h	0.26 ± 0.05	0.77 ± 0.20[b]	19.9 ± 4.8	866 ± 407[b]	363 ± 154	1648 ± 486

[a]Fifteen patients were studied.
[b]$p \leq 0.05$ compared to control by paired *t*-test.

Table 2
Effects of TNF (20 ng/mL) on FSH-Stimulated Accumulation of cAMP, Progesterone, and Estradiol in Media at 48 and 96 h[a]

	cAMP, pmol/mL		Progesterone, ng/mL		Estradiol, pa/mL	
	FSH	FSH + TNF	FSH	FSH + TNF	FSH	FSH + TNF
48 h	5.82 ± 1.27	5.48 ± 1.04	35.6 ± 21.8	34.7 ± 23.2	6261 ± 2460	4778 ± 1797
96 h	0.78 ± 0.20	1.13 ± 0.29	93.9 ± 54.4	91.9 ± 50.6	3634 ± 1348	2064 ± 1244[b]

[a]Exogenous testosterone (1 μ*M*) was present in the media. Data from 17 patients are presented for each.
[b]$p < 0.001$.

Table 3
Effects of TNF (20 ng/mL) on FSH-Stimulated Accumulation of cAMP, Progesterone, and Estradiol in Media at 48 and 96 h in the Presence of the Phosphodiesterase Inhibitor, Isobutylmethylxanthine (0.1 m*M*)[a]

	cAMP, pmol/mL		Progesterone, ng/mL		Estradiol, pg/mL	
	FSH	FSH + TNF	FSH	FSH + TNF	FSH	FSH + TNF
48 h	3.82 ± 1.24	4.31 ± 1.90	8.46 ± 4.32	6.48 ± 3.00	1105 ± 417	729 ± 986[b]
96 h	0.22 ± 0.12	0.28 ± 0.26	37.3± 17.8	40.8 ± 17.4	3270 ± 1436	609 ± 280[b]

[a]Exogenous testosterone (1 μ*M*) was present in the media. Data from three patients are presented.
[b]$p < 0.05$.

Table 4
Effects of TNF (20 ng/mL) on cAMP-Stimulated Accumulation of Progesterone and Estradiol in Media at 48 and 96 h[a]

	Progesterone, ng/mL		Estradiol, pg/mL	
	cAMP	cAMP + TNF	cAMP	cAMP + TNF
48 h	24.5 ± 11.5	25.1 ± 13.3	8904 ± 6358	1519 ± 592[b]
96 h	145 ± 67	202 ± 1O9	26,180 ± 22,735	12,422 ± 11,540[b]

[a]Exogenous testosterone (1 μ*M*) was present in the media. Data from three patients are presented.
[b]$p < 0.05$ by paired *t*-test.

Table 5
Medical Data on Patients Used in This Study[a]

Age	Weight	Surgical indication	Cycle	Reproductive medications
39 ± 1 (23)	171 ± 9 (23)	Endometriosis (6) Endometrial sarcoma (1) Pelvic pain (2) Uterine prolapse (1) Fibroids (10) Cervical cancer (2) Polycystie ovary (1)	Follicular (5) Luteal (8) Irregular (9) Menstrual (1)	None (19) Climera Patch/Provera (2) Depo-lupron (2)

[a]Number of patients is in parentheses.

In the presence of TNF, long-term cultures of human granulosa cells *(30)* increased secretion of estradiol *(30)*. Similarly, in adipose stromal cells *(31)*, TNF increased aromatase expression. Thus, in differentiated cells, it appears that TNF has a stimulatory action on estradiol secretion. The molecular mechanisms accounting for this difference is apparently at the level of the AP-1 site, which binds c-*fos* and c-*jun*. These act in cooperation with ligand activated glucocorticoid receptor in the induction of aromatase gene expression in the adipose stromal cells *(31)*. Thus, TNF can stimulate aromatase, and this action may depend on the cell type and its state of differentiation.

In summary, TNF inhibited FSH-stimulated secretion of estradiol without reducing the accumulation of cAMP and progesterone. Similar results were observed in the presence a phosphodiesterase inhibitor. In addition, TNF consistently inhibited the ability of cAMP to increase estradiol secretion. These results indicate that a pathway for TNF inhibition of FSH- or cAMP-induced estradiol secretion in human granulosa cells is at post-cAMP sites.

Materials and Methods

Patients

Patients undergoing total abdominal hysterectomy with salpingo-oophorectomy or bilateral salpingo-oophorectomy were used in this study. Ovaries were removed owing to reasons unrelated to ovarian pathology. Clinical conditions for which ovaries were removed are presented in Table 5. Stage of cycle was determined from endometrial biopsy as presented in medical records of each patient. The Institutional Human Studies Committee of the University of Kansas Medical Center approved this study. It was given exemption status because of the discarded nature of the tissue.

Granulosa Cell Collection

Granulosa cells were collected as previously described *(11)*. Briefly, granulosa cells were collected in the surgical room within 15 min of removal of the ovaries from the abdominal cavity. Follicular diameter was measured using a millimeter rule. Granulosa cells were aspirated from each follicle using a 21-gage needle attached to a 1-cc syringe. Follicular aspirates containing blood were not used. Straw colored follicular aspirates were pooled from 3–12 follicles/patient. Aspirates were placed in stedle, capped, plastic 12 × 75 mm culture tubes and transported to the laboratory at room temperature.

Granulosa Cell Culture

Follicular aspirates were washed twice with 2 mL of culture medium (Medium 199 containing Hank's salts, 25 m*M N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer, 2 m*M* L-glutamine, 50 mg/mL streptomycin, 0.1% [wt/vol] bovine serum albumin, and 1.0% fetal bovine serum) as previously described *(23)* and then centrifuged at 1000*g* for 5 min. The supernatant fluid was discarded, and the cells were resuspended in a known volume of culture medium. Cell viability and counting were determined by adding 2% trypan blue to an aliquot of the cells. Cells were diluted with culture medium so that 20,000 viable cells/well were added in 0.5 mL medium in individual wells of a 24-well culture plate. Treatments and control vehicle were added to each well in 10-μL aliquots. Treatments include 2.0 ng human recombinant FSH/mL, 1 μ*M* testosterone, 20 ng human recombinant TNF/mL, 0, 0.1, and 1.0 m*M* cAMP, and 0, 0.1, and 0.5 m*M* IBMX. The doses of FSH and testosterone *(23)* and TNF *(11)* were chosen based on effective stimulating doses previously reported. In addition, 20 ng TNF/mL was used as a dose that would adequately stimulate granulosa cells based on a Kd of 0.17 n*M (19)*. The doses of cAMP and IBMX used were based on previous studies *(24)*. Cells were incubated for 96 h at 37°C in a humid)fied incubator, with 95% air and 5% CO_2. Media were collected for RIA after 48 and 96 h. At 48 h fresh media and treatments were added.

Radioimmunoassay (RIA)

Progesterone, estradiol *(25)*, and cAMP *(14)* from unextracted media were analyzed as previously described.

Statistics

The data were transformed to log to reduce variance and then analyzed by paired *t*-test. Differences were considered significant if $p \leq 0.05$.

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