

# Intraovarian Tumor Necrosis Factor-Related Weak Inducer of Apoptosis/Fibroblast Growth Factor-Inducible-14 Ligand-Receptor System Limits Ovarian Preovulatory Follicles from Excessive Luteinization

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In addition to gonadotropins, many ovarian paracrine factors are crucial for optimal follicle rupture, oocyte maturation, and luteinization. Based on DNA microarray analyses, we found that transcripts for the fibroblast growth factor-inducible-14 (Fn14) receptor are increased after LH/human chorionic gonadotropin (hCG) treatment of gonadotropin-primed immature mice or rats. Fn14 is the cognate receptor for TNF-related weak inducer of apoptosis (TWEAK), a TNF superfamily member. TWEAK transcripts also were detected in the ovary; however, their levels were not regulated by gonadotropins. *In situ* hybridization analyses indicated that the Fn14 receptor is expressed in the granulosa and cumulus cells of preovulatory follicles and, to a lesser extent, in theca cells. In contrast, *in situ* hybridization analyses revealed that TWEAK is primarily expressed in theca cells. In cultured granulosa cells pretreated with hCG to induce Fn14 receptor expression, treatment with

TWEAK suppressed progesterone synthesis without accompanying changes in cAMP production. Furthermore, intrabursal injection of TWEAK suppressed ovarian progesterone content in gonadotropin-primed rats. In contrast, preovulatory follicles cultured in the presence of the Fn14 decoy, a recombinant protein containing the ligand-binding domain of Fn14, led to increases in progesterone production, presumably by antagonizing the actions of endogenous TWEAK. Likewise, ip injection of the Fn14 decoy enhanced serum progesterone levels with accompanying increases in transcript levels for several key steroidogenic enzymes. The present findings demonstrate a suppressive role of the TWEAK/Fn14 signaling system in the ovary. Following gonadotropin induction of ovulation, Fn14 is induced and could protect preovulatory follicles from excessive luteinization. (*Molecular Endocrinology* 20: 2528–2538, 2006)

**G**ONADOTROPINS RELEASED FROM the anterior pituitary stimulate ovarian follicle development, ovulation, and luteinization. It has become clear that gonadotropins regulate the expression of a myriad of paracrine and autocrine factors in the ovary (1–3). These intraovarian factors augment and modulate the functions of gonadotropins to allow optimal follicle

rupture, oocyte maturation, and luteinization. Local ovarian mediators of gonadotropin functions during the periovulatory stage include IGF-I (4), epidermal growth factor-like growth factors (5, 6), insulin-like 3 (7), brain-derived neurotrophic factor (BDNF) (8), and vascular endothelial growth factor (9) among others. They exert paracrine effects on diverse ovarian functions including oocyte maturation and cumulus expansion as well as follicular maturation, vascularization, and luteinization. In contrast, potential inhibitors of gonadotropin actions in the ovary are TNF $\alpha$  (10), TGF $\beta$ 1 (11), bone morphogenetic proteins -4, -6, and -7 (BMP-4, BMP-6, and BMP-7) (12, 13). Identification of key ovarian paracrine factors and characterization of their physiological roles are important for understanding cellular mechanisms underlying gonadotropin actions.

TNF-related weak inducer of apoptosis (TWEAK), is a cytokine also known as TNF superfamily member 12. It functions both as a type II transmembrane protein and as a cleaved soluble protein (14, 15). TWEAK transcripts are

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Abbreviations: BDNF, Brain-derived neurotrophic factor; BMP, bone morphogenetic protein; COC, cumulus-oocyte complex; CYP11A, cholesterol side-chain cleavage enzyme; Fn14, fibroblast growth factor inducible-14; hCG, human chorionic gonadotropin; 3- $\beta$ HSD, 3 $\beta$ -hydroxysteroid dehydrogenase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PMSG, pregnant mare serum gonadotropin; StAR, steroidogenic acute regulatory protein; TRAF, TNF receptor-associated factor; TWEAK, TNF-related weak inducer of apoptosis.

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expressed in heart, brain, skeletal muscle, pancreas, immune system, ovary, and placenta (16, 17). TWEAK activates the fibroblast growth factor-inducible-14 (Fn14) receptor, also known as TNF receptor superfamily member 12A or TWEAKR. This receptor is a plasma membrane-anchored protein with a putative TNF receptor-associated factor (TRAF)-binding domain in the cytoplasmic side. Downstream signaling of Fn14 is mediated by the adaptor proteins TRAF1, TRAF2, TRAF3, or TRAF5 (18) in the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) (19–21). Fn14 mRNA is expressed in a variety of tissues including heart, kidney, lung, ovary, and placenta (22, 23). TWEAK binding induces aggregation of Fn14, leading to the stimulation of angiogenesis (24–26), tumor cell apoptosis (16), proinflammatory effects (27–30), cellular proliferation (31, 24), and cell motility (32).

Based on DNA microarray analysis, herein we report substantial increases in the expression of the TWEAK receptor, Fn14, in the ovary after gonadotropin priming. We used *in vitro* cultures of granulosa cells and preovulatory follicles, as well as *in vivo* treatment with a recombinant receptor antagonist, to study the physiological roles of the TWEAK/Fn14 signaling system. The present findings demonstrate a protective function of TWEAK/Fn14 signaling that prevents excessive luteinization after the gonadotropin induction of ovulation.

## RESULTS

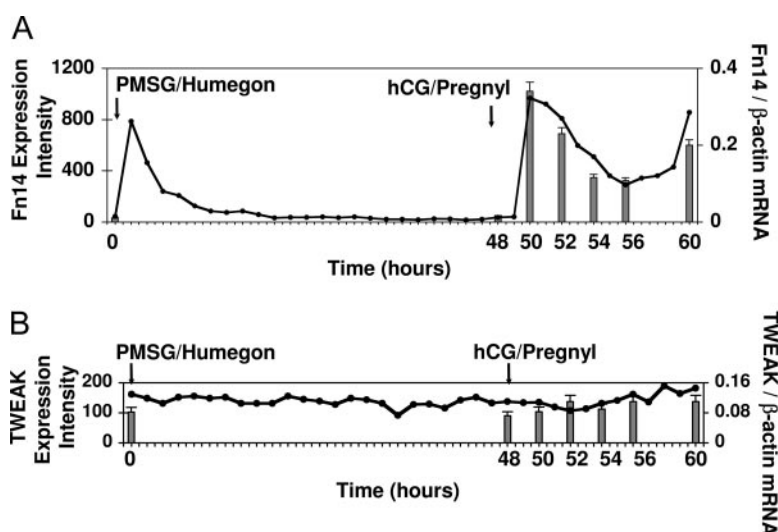
### Expression of Fn14 and Its Ligand TWEAK after Gonadotropin Treatment in Rodent Ovaries

DNA microarray analysis revealed dramatic increases in the expression of the Fn14 receptor in mouse ovaries.

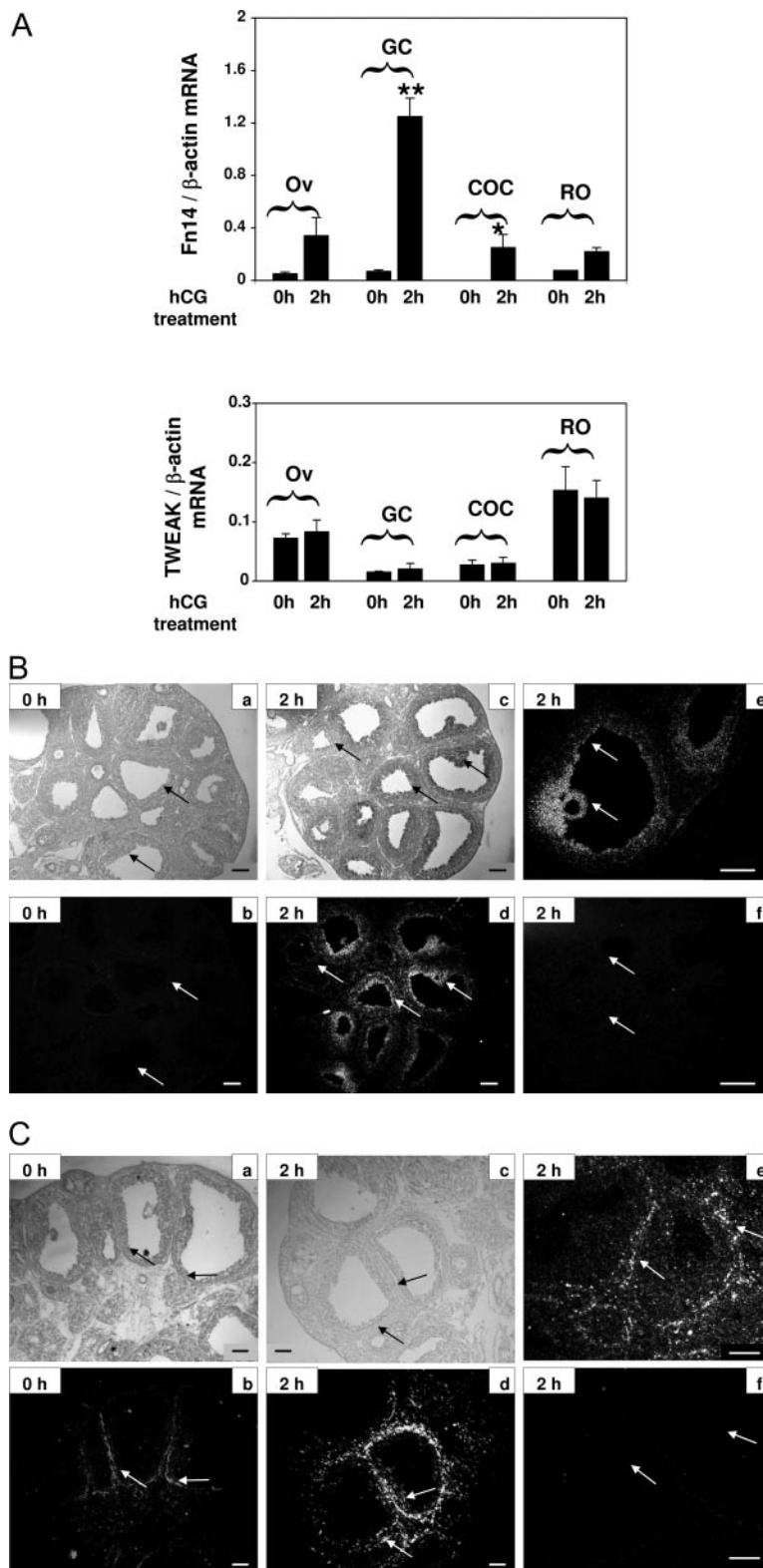
Immediately after injection of Humegon containing both FSH and LH activities to induce follicle maturation in immature mice, there was a rapid and transient increase in Fn14 mRNA levels (Fig. 1A, *line graph*). Furthermore, a sustained increase of the Fn14 transcript was observed 2 h after injection of Pregnyl with LH activities to induce ovulation. In contrast, the transcript levels for the TWEAK ligand did not show obvious regulation after the same treatment (Fig. 1B, *line graph*). DNA array data were confirmed further by performing real-time RT-PCR analysis using immature rats treated with pregnant mare serum gonadotropin (PMSG) (15 IU) to induce follicle growth, and at 48 h later with human chorionic gonadotropin (hCG) (15 IU) to induce ovulation. The Fn14 receptor transcripts increased shortly after hCG treatment and peaked at 2 h, followed by a gradual decrease and then a subsequent increase at 12 h after treatment (Fig. 1A, *bar graph*). In contrast, the TWEAK transcripts showed minimal changes after hCG treatment (Fig. 1B, *bar graph*).

### Localization of Fn14 and TWEAK Transcripts in Different Ovarian Compartments

Granulosa cells and cumulus-oocyte complexes (COCs) were isolated from PMSG-primed ovaries before or at 2 h after hCG treatment. RNA was extracted and individual transcript levels were determined using quantitative RT-PCR. The Fn14 receptor transcripts were found to be stimulated by hCG in different ovarian compartments with highest expression in granulosa cells (Fig. 2A, *upper panel*). In contrast, TWEAK transcripts showed highest expression in the residual



**Fig. 1.** Expression of Fn14 and Its Ligand TWEAK after Gonadotropin Induction of Follicle Maturation and Ovulation in Rodents  
Gonadotropin regulation of Fn14 and TWEAK transcripts in rodent ovaries. *Line graphs* represent DNA microarray data depicting the expression intensity of each transcript in gonadotropin-treated mouse ovaries. Values for expression intensity were derived from integration of hybridization signals from multiple probe sets for individual genes. *Bar graphs* represent quantitative real-time RT-PCR analysis of Fn14 and TWEAK transcript levels in ovaries of gonadotropin-treated rats. A, Fn14; B, TWEAK. Data are represented as the mean  $\pm$  SE of three independent experiments.



**Fig. 2.** Localization of Fn14 and TWEAK Expression in the Rat Ovary

A, Expression of Fn14 and TWEAK mRNAs in whole ovaries (Ov), isolated granulosa cells (GC), COCs, and residual ovaries (RO) obtained from PMSG-primed rats or at 2 h after hCG treatment. Transcript levels were detected using quantitative RT-PCR. All data were normalized based on  $\beta$ -actin levels. Data are represented as mean  $\pm$  SE of four independent experiments. \*,  $P < 0.05$  as compared with respective COCs at 0 h time point; \*\*,  $P < 0.001$  as compared with respective granulosa cells at 0 h time point. B, *In situ* hybridization analysis of Fn14 transcripts from ovaries before (a and b) or at 2 h after (c–f) hCG treatment of

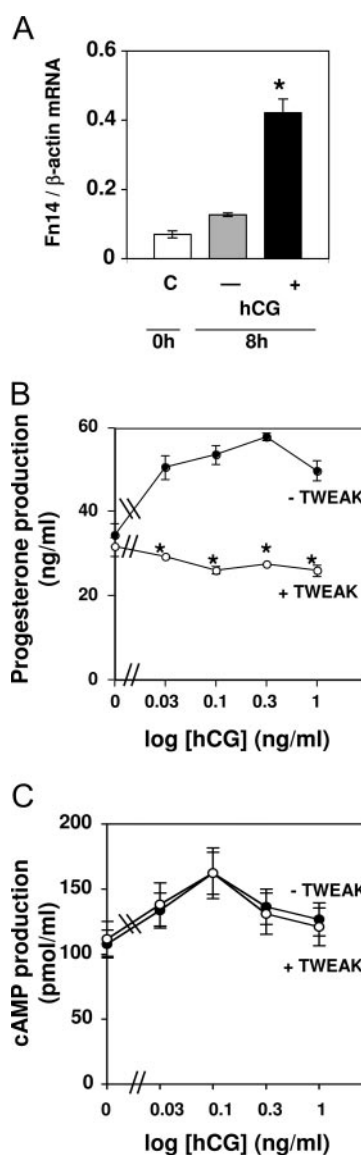
ovary, with lower levels in granulosa cells and COCs (Fig. 2A, lower panel).

*In situ* hybridization analysis further demonstrated Fn14 expression in granulosa cells. The Fn14 transcript was minimally expressed in PMSG-primed ovaries without hCG treatment (Fig. 2B, panel a, bright-field; panel b, dark-field). At 2 h after hCG treatment, the Fn14 signal was detected strongly in granulosa and cumulus cells of preovulatory follicles, but was weaker in theca cells (Fig. 2B, panel c, bright-field; panels d and e, dark-field). *In situ* hybridization using the TWEAK probe further indicated that the ligand was expressed primarily in the theca cells of follicles in PMSG-primed ovaries (Fig. 2C, panel a, bright-field; panel b, dark-field) as well as after 2 h of treatment with hCG (Fig. 2C, panel c, bright-field; panels d and e, dark-field). In contrast, no signal was found in ovarian sections hybridized with the Fn14 or TWEAK sense probes (Fig. 2, B and C, panel f, dark-field). These results indicate that TWEAK is expressed in theca cells and could interact with Fn14 receptors in granulosa and cumulus cells.

### Suppressive Effects of TWEAK on Progesterone Biosynthesis by Cultured Granulosa Cells

Ovaries from PMSG-primed rats were punctured to obtain granulosa cells. Cells were then treated with 3 ng/ml hCG for 8 h to induce expression of the Fn14 receptor. To confirm hCG stimulation of Fn14 transcripts, cells were collected before and after hCG treatment. Levels of the Fn14 transcripts were determined by quantitative RT-PCR. There was a 3.5-fold increase in Fn14 transcript levels after hCG treatment (Fig. 3A). Some of the hCG-pretreated cells were further treated with varying doses of hCG with or without TWEAK (100 ng/ml) for 48 h. Analysis of media progesterone and cAMP content by RIA revealed that there was a dose-dependent increase of progesterone biosynthesis after hCG treatment, whereas cotreatment with TWEAK suppressed hCG actions (Fig. 3B) ( $P < 0.05$ ). In these cultures, treatment with hCG led to minimal increases in cAMP production, and cotreatment with TWEAK did not significantly alter cAMP production (Fig. 3C,  $P > 0.05$ ).

To study a potentially suppressive effect of TWEAK *in vivo*, we performed intrabursal injections of TWEAK in gonadotropin-primed immature rats at 4 h after hCG injection. TWEAK was injected into one ovary whereas the contralateral ovary received saline. After 48 h, ovaries were collected and ovarian progesterone content was determined using RIA. There was no change in



**Fig. 3.** Suppressive Effects of TWEAK on Progesterone Biosynthesis in Cultured Granulosa Cells

Granulosa cells obtained from PMSG-primed rats were treated with hCG (3 ng/ml) for 8 h in culture. Cells then were washed twice with fresh media before treatment with increasing doses of hCG with or without TWEAK (100 ng/ml) for 48 h. A, Fn14 receptor transcripts before or after hCG treatment were determined using quantitative RT-PCR. C, Control group. Media progesterone (B) and cAMP levels (C) were determined using RIA. The experiment was repeated four times, and a representative experiment is shown. Data represent mean  $\pm$  SE of three cultures. \*,  $P < 0.05$  as compared with corresponding cells treated with hCG alone.

PMSG-primed rats. a and c, Bright-field; b and d–f: dark-field. Fn14 was expressed in granulosa and cumulus cells of preovulatory follicles at 2 h after hCG treatment (panels d and e, arrows). No signal was found for an adjacent section hybridized with the sense probe (f). Scale bars = 100  $\mu$ m. C, *In situ* hybridization analysis of TWEAK transcripts from ovaries before (a and b) or at 2 h after (c–f) hCG treatment of PMSG-primed rats. a and c, bright-field; b and d–f, dark-field. TWEAK was expressed in theca cells of follicles at different stages of treatment (panels d and e, arrows). No signal was found for an adjacent section hybridized with the sense probe (f). Scale bars, 100  $\mu$ m.

ovarian weight with or without TWEAK treatment (ovary weight, mean  $\pm$  SE: saline-treated,  $71.2 \pm 2$  mg; TWEAK-treated,  $71 \pm 3$  mg,  $n = 9$ ). However, intrabursal treatment with TWEAK decreased ovarian progesterone content by 30% when compared with the contralateral saline-treated ovaries (Fig. 4). These ovaries also were used to estimate transcript levels for different steroidogenic enzymes. Quantitative RT-PCR analysis indicated that the transcript levels of the enzymes, cholesterol side-chain cleavage enzyme (CYP11A), steroidogenic acute regulatory protein (StAR), and 3 $\beta$ -hydroxysteroid dehydrogenase (3- $\beta$ HSD), were decreased by 28, 26, and 14%, respectively, as compared with the saline-treated contralateral ovaries (Fig. 4C).

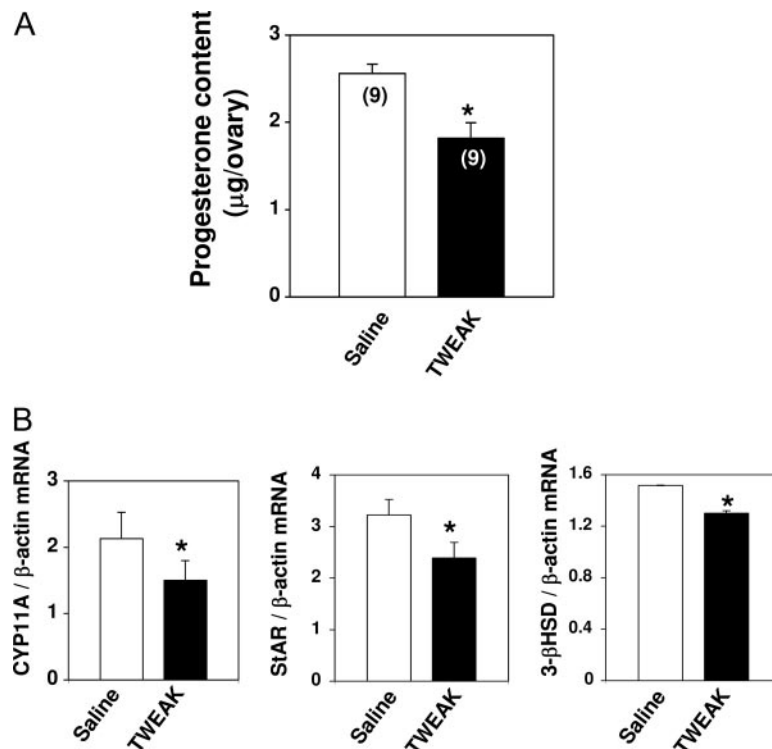
#### Enhancing Effects of the Fn14 Decoy on hCG Stimulation of Luteinization

To further demonstrate a physiological role of the TWEAK/Fn14 signaling system in the ovary, we attempted to block the actions of endogenous TWEAK. We obtained the recombinant Fn14 decoy consisting of the extracellular ligand-binding domain of Fn14 fused to the human immunoglobulin Fc region. Prior studies have demonstrated the potent effects of this

molecule in blocking TWEAK/Fn14 signaling both *in vitro* and *in vivo* (33, 34).

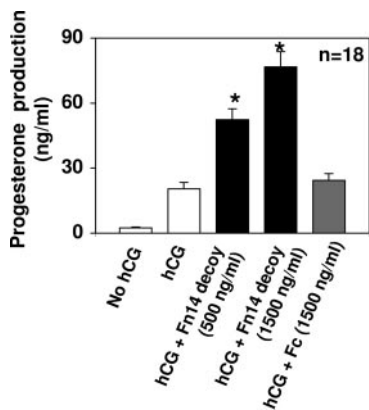
Preovulatory follicles were collected from immature rats primed with PMSG for 48 h and cultured with hCG (1  $\mu$ g/ml) with or without the Fn14 decoy for 18 h. Analysis of media progesterone levels indicated a 8.2-fold increase in progesterone after hCG treatment compared with controls (Fig. 5). Furthermore, cotreatment with the Fn14 decoy led to a dose-dependent increase in progesterone production as compared with hCG treatment alone, showing a 3.7-fold increase at the 1500 ng/ml dose of Fn14 decoy. In contrast, treatment with the human Fc fragment did not significantly alter progesterone production ( $P > 0.05$ ).

To further demonstrate the role of endogenous TWEAK *in vivo*, PMSG-primed immature rats were treated with hCG with or without the Fn14 decoy (2  $\mu$ g, once daily). Animals injected with the Fc fragment (2  $\mu$ g, once daily) served as negative controls. At 48 h after hCG injection, ovaries were collected and weighed. The results showed an increase in ovarian weight 48 h after PMSG treatment compared with control ovaries from immature rats, whereas hCG injection further increased ovarian weight (Table 1). At the 20-h time point, cotreatment with Fn14 decoy



**Fig. 4.** Suppressive Effects of TWEAK on Progesterone Biosynthesis *in Vivo*

PMSG-primed immature rats were given an ip injection of an ovulatory dose of hCG. Three hours later, they were given intrabursal injections of TWEAK (500 ng) in one ovary and saline in the contralateral ovary. Animals were killed 48 h later for ovary collection. Ovarian progesterone content (panel A), and ovarian CYP11A, StAR, and 3- $\beta$ HSD transcript levels (panel B) were determined by quantitative RT-PCR. Numbers inside parentheses represent the number of animals examined. A total of four ovaries were independently analyzed for transcript levels, and the data are represented as mean  $\pm$  SE. \*,  $P < 0.05$  as compared with the saline-treated group.



**Fig. 5.** Enhancing Effects of Fn14 Decoy on the hCG Stimulation of Progesterone Production in Cultured Preovulatory Follicles

Preovulatory follicles were obtained from PMSG-primed immature rats and cultured with hCG (1 μg/ml) with or without the Fn14 decoy. In addition, the Fc fragment was used as a negative control. After 18 h of culture, media were collected and progesterone content was measured by RIA. Data are represented as mean ± SE, and n depicts the number of follicles analyzed per group. \*, P < 0.05; \*\*, P < 0.001 as compared with the hCG-treated group.

further augmented ovarian weights by 29.6% as compared with the saline-treated group. However, no significant changes in ovarian weight were found at 48 h after hCG treatment between the saline- and Fn14 decoy-treated groups (P > 0.05). The ovarian weights of the Fc-treated group were comparable to the saline-treated group. To assess possible changes in the number of corpus lutea at 48 h after hCG injection, ovaries from saline and Fn14 decoy-treated groups were sectioned, and similar numbers of corpus lutea were found (saline group, 19.8 ± 1.5; Fn14 decoy-treated group, 21.2 ± 1.7, n = 5). We further analyzed

**Table 1.** Ovarian Weights in Animals Treated with Gonadotropin with or without the Fn14 Decoy

Treatment	Ovary Weight (mg ± SE)
Control	9.8 ± 0.3 (12)
PMSG-primed	26.69 ± 1.2 (12)
PMSG-primed + 20 h hCG	
Saline	38.02 ± 1.9 (12)
Fn14 decoy	49.27 ± 3 (15) <sup>a</sup>
Fc	43.78 ± 2.5 (12)
PMSG-primed + 48 h hCG	
Saline	70.89 ± 4.5 (12)
Fn14 decoy	81.10 ± 5.4 (15)
Fc	63.48 ± 5.5 (12)

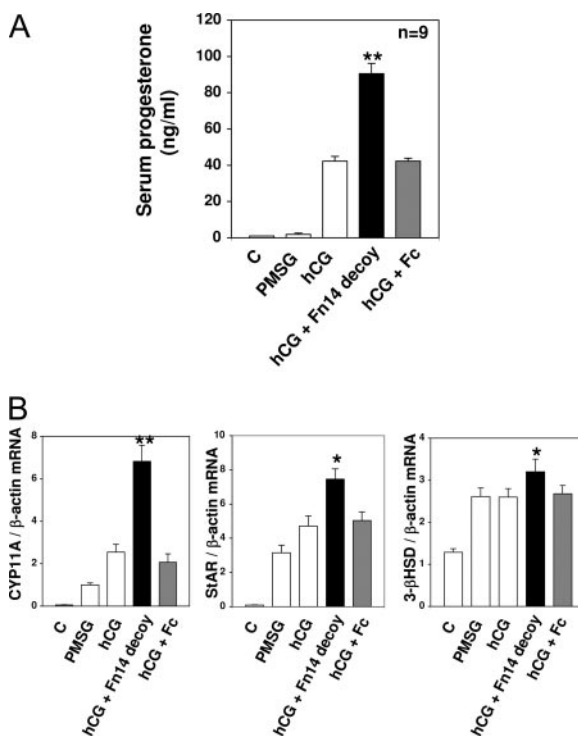
Immature rats were treated with PMSG and, 48 h later, were given a single ovulatory dose of hCG. At the time of hCG treatment, some animals were injected with the Fn14 decoy or the Fc fragment. Ovarian weights were determined at 20 and 48 h after hCG treatment. Number in parentheses indicates the number of animals used.

<sup>a</sup> P < 0.05 as compared to the respective saline-treated group.

serum progesterone levels in these animals. As shown in Fig. 6A, a major increase of serum progesterone was evident at 48 h after hCG treatment compared with those in either immature untreated or PMSG-primed rats. Of interest, cotreatment with the Fn14 decoy led to a 2-fold increase in serum progesterone levels. In contrast, the Fc-treated control group did not show changes in serum progesterone levels. To estimate ovarian transcript levels for key steroidogenic enzymes, RNA was extracted from the ovaries and analyzed by quantitative RT-PCR. CYP11A, StAR, and 3-βHSD transcript levels were significantly higher (168%, 58%, and 23%, respectively) in ovaries from animals treated with the Fn14 decoy (Fig. 6B) as compared with those treated with hCG alone.

**TWEAK Enhances NF-κB Signaling in Granulosa Cells in Vitro**

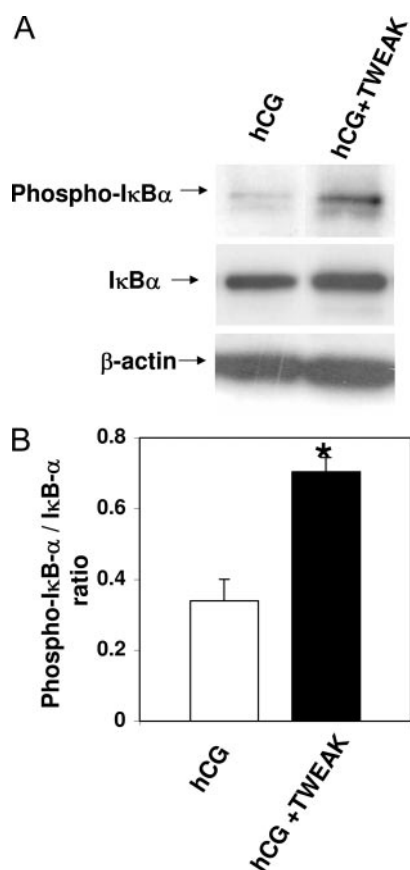
We further evaluated possible signaling pathways for the ovarian TWEAK-Fn14 system. TWEAK has been



**Fig. 6.** Enhancing Effects of the Fn14 Decoy on hCG Stimulation of Luteinization *in Vivo*

Immature rats were primed with 15 IU PMSG and, 48 h later, treated with 15 IU hCG with or without the Fn14 decoy. Animals were killed at 48 h after hCG treatment before determination of serum progesterone levels by RIA (panel A) and transcript levels for steroidogenic enzymes (CYP11A, StAR, and 3-βHSD; panel B) by quantitative RT-PCR. Transcript levels were normalized based on β-actin levels. Four ovaries were analyzed independently for transcript levels. Data are represented as mean ± SE, and n depicts the number of animals analyzed. \*\*, P < 0.001; \*, P < 0.05 as compared with the hCG-treated group. C, Control group.

found to phosphorylate inhibitory I $\kappa$ B- $\alpha$  in diverse tissues, thus leading to activation of the NF- $\kappa$ B pathway (18–21). Granulosa cells were collected from PMSG-primed immature rats and treated with hCG for 8 h to induce Fn14 receptor transcripts. Afterward, some cells were treated with recombinant TWEAK (100 ng/ml) for 30 min. Cell extracts were collected as described in *Materials and Methods* and used for Western blot analysis. As shown in Fig. 7A, there was enhanced phosphorylation of I $\kappa$ B- $\alpha$  in TWEAK-treated granulosa cells compared with control cells. As shown in Fig. 7B, there was a 2-fold increase in the phosphorylation of I $\kappa$ B- $\alpha$  at 30 min after TWEAK treatment as compared with controls (n = 4). This finding suggests activation of the NF- $\kappa$ B pathway in granulosa cells shortly after TWEAK treatment.



**Fig. 7.** TWEAK Treatment Enhances NF- $\kappa$ B Signaling in Granulosa Cells *in Vitro*

A, Immunoblotting of phospho-I $\kappa$ B- $\alpha$  (Ser32/36) protein (*upper panel*), total I $\kappa$ B- $\alpha$  protein (*middle panel*), and  $\beta$ -actin protein (*lower panel*) was performed. Cells were pretreated with hCG for 8 h and then with or without TWEAK for 30 min. Cells that did not receive TWEAK treatment are represented as “hCG.” A representative immunoblot from four independent experiments is shown. B, Densitometric analysis of four independent experiments was performed to estimate the enhancement of phosphorylated I $\kappa$ B- $\alpha$  protein. Data are represented as mean  $\pm$  SE. \*,  $P < 0.05$  as compared with the hCG group.

## DISCUSSION

DNA microarray, quantitative RT-PCR, and *in situ* hybridization analyses indicated that shortly after injection of an ovulatory dose of hCG, there is a major increase in the expression of the Fn14 receptor in granulosa and cumulus cells of preovulatory follicles. When treated both *in vitro* and *in vivo*, the ligand TWEAK exerted inhibitory effects on hCG-stimulated progesterone production, likely mediated through activation of the NF- $\kappa$ B pathway in granulosa cells. Furthermore, studies using the soluble Fn14 decoy indicated that neutralization of the actions of endogenous TWEAK led to increases in progesterone production from cultured preovulatory follicles and elevations of serum progesterone levels in gonadotropin-primed immature rats. The observed effects on progesterone production are likely mediated through regulation of key steroidogenic enzymes such as CYP11A, StAR, and 3- $\beta$ HSD. Because the TWEAK ligand is expressed mainly in theca cells, the present findings are consistent with the hypothesis that TWEAK from thecal cells is an ovarian paracrine factor acting on its receptor, Fn14, in granulosa and cumulus cells to prevent excessive progesterone production during luteinization.

LH/hCG acts exclusively on somatic granulosa and theca cells in the ovary. Several studies have demonstrated the stimulation of paracrine factors after the preovulatory LH surge. For instance, insulin-like 3 expression increases in theca cells after LH/hCG treatment and acts on its receptor LGR8 in the oocyte, thus facilitating oocyte meiotic progression (7). Preovulatory LH increases also stimulate expression of epidermal growth factor-like growth factors in the granulosa cells of preovulatory follicles to facilitate both cumulus expansion and oocyte maturation (5). Additionally, gonadotropin induction of ovulation led to increases in BDNF expression in granulosa and cumulus cells. BDNF, in turn, acts on its receptor, TrkB, which is expressed exclusively on the oocyte to facilitate first polar body extrusion and early embryo development (8).

Whereas in the above examples different ligands were increased after LH/hCG stimulation to mediate facilitatory paracrine effects on ovarian functions, the present study identified another paracrine TWEAK/Fn14 system in which receptor expression was increased after the preovulatory gonadotropin surge. Based on the observation that TWEAK/Fn14 signaling is inhibitory to gonadotropin functions, receptor regulation may prove important in restricting the inhibitory paracrine effects to specific cell types, and thus aid in fine tuning paracrine signaling.

Transcript expression levels for both Fn14 and TWEAK were comparable in rat and mouse species at different time points after gonadotropin treatment (Fig. 1, A and B). Studies using isolated ovarian cell types further revealed an enrichment of Fn14 transcripts in granulosa cells at 2 h after hCG treatment in PMSG-

primed rats. In contrast, the ligand TWEAK was expressed predominantly in the residual ovarian compartment comprised of theca and interstitial cells with minimal levels in granulosa cells and cumulus-oocyte complexes. *In situ* localization studies further supported the increase in Fn14 transcripts after hCG treatment in granulosa and cumulus cells of preovulatory follicles, whereas TWEAK transcripts were found only in theca cells of ovarian follicles at all stages of gonadotropin treatment. Neither the ligand nor the receptor could be detected in the oocyte or interstitial cells at different stages of gonadotropin treatment. Although earlier studies document stimulation of Fn14 receptor expression on endothelial cells by fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor-A (35), as well as the stimulation of TWEAK expression on monocytes after interferon- $\gamma$  treatment (36), we did not detect localization of Fn14 or TWEAK in ovarian blood cells. Taken together, our localization studies demonstrate paracrine signaling of TWEAK from theca cells to its receptor on granulosa and cumulus cells in preovulatory follicles.

In the present study we have demonstrated the inhibitory effect of exogenous TWEAK on granulosa cell progesterone synthesis. The effective dose (100 ng/ml) for TWEAK found for ovarian cells is comparable to that reported for the apoptotic, angiogenic, and proinflammatory effects of TWEAK on colon adenocarcinoma cells, human umbilical vein endothelial cells, and dermal fibroblast cells, respectively (16, 24, 28). Furthermore, intrabursal administration of TWEAK to gonadotropin-primed rats led to decreases in ovarian progesterone content when compared with contralateral saline-treated ovaries. The inhibitory effects of endogenous TWEAK also were demonstrated on the stimulatory effects of the Fn14 decoy on progesterone biosynthesis in cultured preovulatory follicles and *in vivo* studies. The antagonistic effects of the Fn14 decoy are consistent with earlier studies showing the ability of this ligand-binding domain of the Fn14 receptor to block mammary epithelial branching (33) and to reduce infarct volume in a model of murine cerebral ischemia (34).

After an ovulatory dose of LH/hCG, granulosa cells undergo luteinization accompanied by increases in the levels of enzymes for progesterone biosynthesis. TWEAK/Fn14 signaling acts as a potent luteinization inhibitor at this stage of follicle development. Its progesterone suppressing action is similar to those of oocyte-derived factors including GDF-9 (37), BMP-6 (13), and BMP-15 (38) as well as other follicular fluid components such as endothelin-1 (39). Earlier studies have proposed possible roles for these factors to account for the observed luteinization inhibitors in the follicular fluids of preovulatory follicles (40).

TWEAK belongs to the same superfamily as TNF- $\alpha$ , and the observed inhibitory effects of TWEAK on progesterone production are similar to earlier findings on TNF- $\alpha$  (41). In cultures of luteinizing granulosa cells, treatment with TNF- $\alpha$  inhibited gonadotropin-mediated

progesterone synthesis (42). Compared with wild-type mice, TNF receptor-I null mice were found to have higher ovulation rates as well as elevated serum progesterone and estrogen levels after gonadotropin stimulation (43). In contrast to observed increases in the Fn14 receptor in the granulosa cells of preovulatory follicles, earlier studies have shown that TNF- $\alpha$  receptors are increased by LH treatment in bovine theca cells (44). Recently, Fn14 knockout mice have been generated and were found to be fertile (31). Future characterization of ovulatory rates and luteal functions in Fn14 null mice could reveal the precise ovarian roles of the TWEAK/Fn14 paracrine signaling system.

Ovarian hyperstimulation syndrome is an iatrogenic condition that can occur during ovulation induction, leading to massive enlargement of the ovary, multiple cyst formation, and increased capillary permeability. The exogenous application of hCG, or endogenous increases of placenta-derived hCG, has been implicated as a contributing factor to the development of ovarian hyperstimulation syndrome (45). Numerous studies document the possible involvement of cytokines, including interleukin-1, -2, -6, and -8, as well as endothelin-1, in ovarian hyperstimulation (46). The present findings suggest that TWEAK/Fn14 signaling could represent an ovarian paracrine system to moderate LH actions by decreasing the risk of excessive luteinization after the endogenous LH surge.

## MATERIALS AND METHODS

### Animals

Immature Sprague Dawley rats (D = 26) were obtained from Charles River Laboratories (Wilmington, MA). They were injected sc with 15 IU PMSG (Calbiochem, La Jolla, CA) at 26 d of age to induce follicular maturation. Animals were given, 48 h later, ip injections of 15 IU hCG (American Pharmaceutical Partners, Inc., Schaumburg, IL) to induce ovulation. All animals were housed in an environment with controlled light, humidity, and temperature, and they were fed with standard rat chow in accordance with institutional and National Institutes of Health (NIH) animal care guidelines.

### DNA Microarray Analysis

The procedure for DNA microarray has been described previously (8). Briefly, 108 mice at 21 d of age were injected with 7.5 U Humegon (1:1 composition of human FSH and LH; Organon, Oss, The Netherlands) to stimulate follicular growth. Some animals were treated ip, 48 h later, with 5 U Pregnyl (consisting of hCG; Organon) to induce ovulation. Ovaries of three animals were dissected every 2 h during the follicular phase (after Humegon treatment) and every hour during the ovulatory phase (after Pregnyl treatment). Because of the small size of the ovaries in the follicular phase, ovaries of three animals were pooled for RNA extraction. Only one ovary was used from mice treated with Pregnyl or for 48 h with Humegon. To simulate physiological conditions, the gonadotropin treatment protocol induced oocyte release from 20 follicles per animal. RNA samples were extracted using TRIzol reagent (Invitrogen, San Diego, CA) and hybridized to Affymetrix mouse MGU74v2 arrays (Affymetrix, Santa Clara, CA).

### Real-Time PCR Analysis

For assessing TWEAK and Fn14 transcripts, ovaries were collected for total RNA extraction after gonadotropin treatment of immature rats. Some ovaries were dissected and follicles punctured to obtain granulosa cells and COCs. Residual ovaries comprising mostly theca and interstitial cells also were obtained after follicle puncture to minimize granulosa cells. RNA was extracted using an RNeasy Mini Kit (QIAGEN Sciences, Valencia, CA). Total RNA (2  $\mu$ g) from each sample was reverse-transcribed for subsequent PCR analysis. Real-time PCR was performed in 25  $\mu$ l final volume containing 2  $\mu$ l of the reverse transcriptase reaction product, 0.5  $\mu$ M primers, 0.2  $\mu$ M fluorescently labeled probe (3',5'-carboxy tetramethylrhodamine; 5', 6'-carboxy fluorescein), and the PCR reagent mixtures (QuantiTect Probe PCR Kit, QIAGEN Sciences). Standard curves were generated by serial dilution of each plasmid DNA. The primer pairs and fluorescent probes used were as follows: Fn14 forward, 5'-CAGACTCTCCAACCACAAGG-3'; Fn14 reverse, 5'-ACCT-AGCTTGAGGCTCTGTCT-3'; Fn14 probe, 5'-AGGTGGT-GATTCACCTCCAAGGACT-3'. TWEAK forward, 5'-AAAGC-CAGGATGTGGTACCTTT-3'; TWEAK reverse, 5'-GGCCTTT-AGAAACACTTCTTCGAG-3'; TWEAK probe, 5'-CCTGGAA-CACTAGTCCGGC-3'. CYP11A forward, 5'-CCAAGTTCA-ACCTCATCCTGA-3'; CYP11A reverse, 5'-CGTGTGACTGC-AGCCTGCAA-3'; CYP11A probe, 5'-TCTTCAACTCCAGC-CTCTCAAGCA-3'.

StAR forward, 5'-AGATGAAGTGCTAAGTAAGGTGGTG-3'; StAR reverse, 5'-CCAGTCTTCATAGAGTCTGTCCAT-3'; StAR probe, 5'-TCTAGCAGCACCTCCAGTCGGAACA-3'. 3 $\beta$ -HSD forward, 5'-AGACCATCCTAGATGTCAATCTGAA-3'; 3 $\beta$ -HSD reverse, 5'-CAGGATGATCTTCTGTAGGAGT-3'; 3 $\beta$ -HSD probe, 5'-TCTACTGCAGCACAGTTGACGTTGC-3'.  $\beta$ -Actin forward, 5'-TCTGTGTGGATTGGTGGCTCTA-3';  $\beta$ -actin reverse, 5'-CTGCTTGCTGATCCACATCTG-3';  $\beta$ -actin probe, 5'-CCTGGCCTCACTGTCCACCTTCC-3'.

### In Situ Hybridization

Rat ovaries were fixed at 4 C for 6 h in 4% paraformaldehyde in PBS, followed by immersion in 0.5 M sucrose in PBS overnight. The ovaries were embedded in OCT (Tissue-Tek, Redding, CA), sectioned to 10  $\mu$ m thickness, and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA). A 200-bp PCR fragment of rat Fn14 was amplified by primers (forward) 5'-GTTCTGGGGCAGACAGAGAG-3' and (reverse) 5'-AGTGGCATTTCAGTCCATCC-3' before subcloning into the pGEM T Easy vector (Promega Corp., Madison, WI) for probe synthesis. The antisense and sense probes were labeled with [<sup>35</sup>S]UTP (1000 Ci/mmol; Amersham Biosciences, Piscataway, NJ). Tissue sections were hybridized overnight at 65 C in 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 $\times$  Denhardt's solution, 10% dextran sulfate, 1  $\mu$ g/ml carrier transfer RNA, and 10 mM dithiothreitol. Subsequently, sections were treated with RNase A before posthybridization washes with decreasing concentrations of the sodium chloride-sodium citrate solution. Hybridized slides were dehydrated and dried before dipping into the NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) and exposure at 4 C for 1–3 wk. The slides were subsequently counterstained with hematoxylin and eosin Y. After counterstaining, tissues were cleared with xylene, mounted with Permount (Fisher Scientific), and photographed under bright- and dark-field illumination.

### Granulosa Cell Cultures

Granulosa cells were punctured from ovaries of PMSG-primed rats in L-15 Leibovitz media (Invitrogen). Ovarian debris and small follicles were removed and granulosa cells were collected at 500  $\times$  g for 10 min. After repeated washing,

granulosa cells were resuspended in McCoy's 5a media (Invitrogen) supplemented with 10<sup>-7</sup> M androstenedione, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells (200,000/well) were primed with hCG (3 ng/ml, NIH/National Hormone and Peptide Program, Torrance, CA) for 8 h. At the end of incubation, cells were washed twice and incubated for 48 h with varying doses of hCG in the presence or absence of recombinant human TWEAK (Alexis Biochemicals, San Diego, CA). Media were collected for analysis of progesterone content.

### Measurement of Progesterone and cAMP Production

Medium progesterone content was determined by RIA using antisera raised against progesterone-11-hemisuccinate-BSA and the extracellular content of cAMP was determined with antibodies against cAMP (47). The within-assay coefficient of variation, between-assay coefficient of variation, and sensitivity of the progesterone RIA are less than 2%, 5–7%, and 4–8 pg, respectively. The within-assay coefficient of variation, between-assay coefficient of variation, and sensitivity for the cAMP assay are 3.2%, 5.6%, and 0.016 pmol, respectively. To estimate ovarian progesterone content, frozen ovaries were homogenized in water (1 ml), and steroids were extracted with diethyl ether (4 ml). The mixture was snap frozen in dry ice/acetone to remove the aqueous phase. The organic phase was collected and the extraction was repeated once. After extracts were pooled and evaporated to dryness under vacuum, steroids were dissolved in an assay buffer and used for RIA. The recovery of progesterone was assessed by the addition of [1,2,6,7-<sup>3</sup>H] progesterone (1000 cpm, Amersham Biosciences) to some of the homogenates, and average recovery was found to be 73%. Final values were corrected for losses during extraction.

### Intrabursal Injection of TWEAK and ip Injection of Fn14 Decoy

PMSG-primed rats were injected with hCG and anesthetized 3 h later. Two small incisions were made on the peritoneum to externalize the ovaries. Using a 30-gauge needle, recombinant human TWEAK (500 ng/50  $\mu$ l saline) was injected through the fat pad into the bursa surrounding the ovaries. For each animal, one ovary was given TWEAK and the other received saline only. Successful injection was confirmed by bursa swelling. At 48 h after injection, animals were killed and ovaries frozen for steroid or RNA extraction. To evaluate the role of endogenous TWEAK during the periovulatory period, PMSG-primed immature rats were treated ip with hCG with or without Fn14 decoy (2  $\mu$ g daily, Alexis Biochemicals). As a control some animals were administered human Fc fragment (2  $\mu$ g daily, Bethyl Laboratories, Montgomery, TX) via ip injection. Animals were killed at 20 and 48 h after hCG injection before determination of ovarian weight, serum progesterone levels, and ovarian transcript levels. The numbers of corpus lutea also were counted at 48 h after hCG injection. Ovaries were sectioned at 10  $\mu$ m thickness at 50  $\mu$ m intervals before counting. Double counting was avoided by following each corpus luteum through the serial sections. Five ovaries were analyzed per group.

### Culture of Preovulatory Follicles

Preovulatory follicles (>400  $\mu$ m) were collected from immature rats at 48 h after PMSG treatment (48). Three follicles per group were cultured in 400  $\mu$ l media containing  $\alpha$ -MEM (Invitrogen), and 0.1% BSA (Sigma Chemical Co., St. Louis, MO). Cultures were placed in a chamber gassed every 12 h with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Media were collected at different intervals, and progesterone content was determined by RIA. Follicle cultures were incubated for a total of 18 h for optimal progesterone production.

## Western Blot Analysis

Granulosa cells from PMSG-primed immature rats were cultured at a density of 1 million cells per 10-cm plates in McCoy's 5a media. They were treated with hCG (3 ng/ml) for 8 h. At the end of the 8-h incubation, some cells were treated with recombinant human TWEAK (100 ng/ml) for 30 min. After treatment, cells were washed once with chilled PBS and scraped off in Cellytic M Reagent (Sigma) containing a protease inhibitor cocktail (Roche, Palo Alto, CA). After incubation in lysis reagent for 15 min, cell extracts were centrifuged and supernatant was used for Western blot analysis. Protein was loaded on 4–15% SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and electroblotted into Hybond-P membranes (Amersham Biosciences). Membranes were blocked in 5% fat-free milk in Tris-buffered saline containing 0.05% Tween for 1 h at room temperature. After blocking, the membranes were incubated with either anti-phospho-I $\kappa$ B- $\alpha$  (Ser32/36) antibody (1:1000 dilution, Abcam, Cambridge, MA), anti-I $\kappa$ B- $\alpha$  antibody (1:1000 dilution, Cell Signaling Technology, Beverly, MA), or anti- $\beta$ -actin antibody (1:1000 dilution, Abcam) at 4 C overnight. Secondary anti-rabbit or antimouse antibodies were used according to the manufacturer's recommendation (Amersham Biosciences). Signals were developed using enhanced chemiluminescence (ECL kit, Amersham Biosciences), and signal intensity was quantitated using densitometric scanning (IPLab Gel software, BD Biosciences, Rockville, MD).

## Statistical Analysis

Results are presented as mean  $\pm$  SE of three or more independent experiments. Statistical significance was measured by Student's *t* test with *P* < 0.05 being statistically significant.

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