Up-regulation of gonadotropin α-subunit gene by phosphatidylinositol 3-kinase inhibitors in clonal gonadotroph cells

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Abstract

OBJECTIVE: Phosphatidylinositol-3 kinase (PI3-kinase) has been known to play an important role in cell survival and proliferation by activation of its downstream target, Akt/protein kinase B (PKB). In this present study, we investigated the effects of PI3-kinase inhibitors on gonadotropin α-subunit gene expression in pituitary gonadotrophs.

METHODS: Alpha T3-1 cells, a pituitary gonadotroph cell line, were used in this study. αT3-1 cells were transfected with α-subunit promoter region-linked luciferase vector, and stimulated with GnRH in the presence or abscense of two different PI3-kinase inhibitors, LY 294002 and wortmannin. Dose response effects of these inhibitors were also examined. Extracellular signal-regulated kinase (ERK) phosphorylation were determined by western blotting analysis.

RESULTS: Treatment of αT3-1 cells with PI3-kinase inhibitor, LY 294002, significantly increased α-subunit gene expression up to 6.89 ± 0.26-fold, and showed additive effect with gonadotropin-releasing hormone (GnRH). The increasing effect of LY 294002 on α-subunit gene expression was observed at the concentration more than 1 μM. The experiment using another PI3-kinase inhibitor, wortmannin, showed similar effects, where wortmannin alone increased α-subunit gene expression by dose dependent manner and showed additive effect with GnRH. The inhibitor of PKB failed to modulate basal activity of α-subunit promoter as well as GnRH-induced promoter activities. Western blotting analysis using phosphorylated form specific antibody for ERK demonstrated that both LY 294002 and wortmannin increased ERK phosphorylation.

CONCLUSUON: These results suggested that PI3-kinase inhibitor, LY 294002 and wortmannin increased gonadotropin α-subunit gene expression related with ERK activation.

ORIGINAL ARTICLE

INTRODUCTION

Gonadotropin LH and FSH are produced in pituitary gonadotrophs and these hormones composed of two noncovalently linked subunits, α and β. The α-subunit, called glycoprotein hormone α-subunit (α-GSU) is common to LH and FSH, and the β-subunit is specific to each hormone [1]. LH and FSH are mainly under the control of hypothalamic gonadotropin-releasing hormone (GnRH), and binding of GnRH to its seven-transmembrane G-protein coupled receptor stimulates an increase in inositol phosphate turnover and diacylglycerol levels, both of which ultimately lead to increase intracellular Ca^{2+} concentrations and activation of protein kinase C (PKC) [2–4]. As a result, GnRH activates members of mitogen-activated protein kinase (MAPK) families, including Extracellular signal-regulated kinase (ERK) [5, 6], c-Jun N-terminal kinase (JNK) [7] and P38 MAPK [8]. Cyclic AMP/ protein kinase A [9] and calcium/calmodulin-dependent protein kinase pathways [10] are also involved in GnRH signaling pathways.

Many studies have been performed to evaluate how three gonadotropin subunits, α-, LHβ- and FSHβ- are regulated by downstream signaling pathways evoked by GnRH. Previous studies have demonstrated the involvement of ERK pathways in GnRH-induced α-subunit [5, 11, 12], LHβ [6, 13, 14], and FSHβ [15]. JNK also has been reported to involve in LHβ [7, 13] and FSHβ gene expression [16]. In addition, P38 MAPK was reported to regulate FSHβ as well [16].

Phosphatidylinositol 3-kinase is an enzyme implicated in growth or cell survival by associating with receptor and non receptor tyrosine kinases. Receptors for platelet-derived growth factor receptor [17], insulin [18] and insulin-like growth factor (IGF-1) [19] are known to be associated with PI3-kinase signaling and in addition to that, PI3-kinase activity also has been identified to be related with G-protein-associated receptors [20]. PI3-kinases are family of proteins that phosphorylate phosphoinositides [21]. The resulting lipid products (phosphoinositide 3-phosphate) acts as second messengers and mediate the cellular functions of PI3-kinase. These lipids regulate the location or the activity of target proteins downstream of PI3-kinases. Serine/threonine kinase Akt, also named protein kinase B (PKB) is one of the target and the downstream effector for PI3-kinase. When PKB is completely activated, many cellular events follow by the phosphorylation of downstream targets which involves in apoptotic mechanisms, cell cycle progression, and the control of gene expression [22]. The association of PI3-kinase with a number of growth factors suggests that the enzymes may involve in mitogenic responses, however most of the function of PI3-kinase remain unknown.

Studies on the role of PI3-kinase in association with GnRH receptor and/or pituitary gonadotroph are very limited. Kraus S *et al*. reported that GnRH-induced apoptosis of prostate cancer cells was enhanced by the inhibition of PI3-kinase [23]. In another work, they examined the signaling cascade showing that the PI3-kinase were activated by c-Src through GnRH-induced transactivation of epidermal growth factor receptor and sequentially involved in JNK activation [24]. Similarly, Rose A *et al*. demonstrated that anti-apoptotic activity of IGF-1 was PI3-kinase/PKB dependent in pituitary αT3 cells [25].

Effective inhibitors of PI3-kinase may help to define the role of PI3-kinase and its products. Both 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; LY 294002, and the wortmannin, a microbial secondary metabolite found in a variety of fungal species, are known as specific inhibitors for PI3-kinase and used widely to evaluate the role of PI3-kinase signalings [26, 27]. In the present study using single gonadotroph cell model, αT3-1 cells, we have found that both LY 294002 and wortmannin increased α-subunit of gonadotropins. Furthermore, we observed that both LY 2904002 and wortmannin increased ERK phosphorylation in αT3-1 cells.

MATERIALS AND METHODS

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (Moragate Biotech, Australia & New Zealand); des-Gly^{10}, [D-Ala^{6}]-LH-RH Ethylamide (GnRH), Dulbecco's modified Eagle medium (DMEM) and Penicillin/Streptomycin (Sigma Chemical Co, St. Louis MO); Trypsin (GIBCO, Invitrogen, USA); PI3-kinase inhibitor, LY 294002, wortmannin and Akt/PKB inhibitor V (Triciribine) (Calbiochem, La Jolla, CA); Phospho-ERK1/2 (phospho-Thr-202/phosphor-Tyr-204) mouse monoclonal IgG_{2a} and ERK1/2 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Reporter Genes and Expression Vectors

The reporter constructs used in these experiments were generated by fusing –846/0 of the human α-GSU (alpha glycoprotein subunit) gene (α-Luc) to the firefly luciferase (Luc) cDNA in pXP2 as previously described [28]. Wells contained 2 μg/well of reporter constructs and 0.1 μg/well of the PRL-TK expression vector which contained the Renilla luciferase under the herpes simplex virus thymidine kinase promoter as an internal standard/control.

αT3-1 Cell Culture and Transfection

αT3-1 cells (kindly provided by Dr. P.L. Mellon of the University of California, San Diego, CA) were maintained in monolayer culture in high-glucose DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in 95% air. Cells were transiently transfected by electroporation. In each ex-

periment, αT3-1 cells were suspended in phosphate buffered saline (PBS) plus 5 mM glucose, containing the DNA to be transfected. The cells received a single electrical pulse of 240 V from a total capacitance of 1000 μF, using a Biorad, Gene PulserXCell apparatus [28]. After electroporation, cells were then seeded into 6-well tissue culture plates, incubated for 36 h at 37°C and stimulated for 4 h with GnRH (100 nM) [29] in DMEM without serum. When the PI3-kinase inhibitor LY 294002 (1 nM – 100 μM), wortmannin (1 nM – 1 μM) and the Akt/PKB inhibitor (50 μM) were used, these compounds were added for 60 min during the pre incubation period and during incubation with the test reagents.

Luciferase Assays

Cells were washed with ice-cold PBS and lysed with PLB (Passive Lysis Buffer, Promega). Cells debris were pelleted by centrifugation at 14,000 × g for 10 min at 4°C, and firefly luciferase and Renilla luciferase activities were measured in the supernatants with the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (TD-20/20) (Promega) according to the manufacturer's protocol. Luciferase activity was normalized for Renilla luciferase activity to correct for transfection efficiency and cell number. The results were expressed as the fold stimulation compared to the unstimulated control groups.

Western Blotting

αT3-1 cells were rinsed with PBS, then lysed on ice with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 mg/ml aprotinin, and 1 mM sodium orthovanadate, scraped for 20 sec, and centrifuged at 14,000 × g for 10 min at 4°C. The protein concentration was measured in the cell lysates using the Bradford method of protein quantitation. 50 μg denatured protein/well was separated on a 10% SDS-PAGE gel according to standard protocols. Protein was transferred onto polyvinylidene difluorides membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, UK), which were blocked for 2 h at room temperature in Blotto (TBS with 4.5% milk). Membranes were incubated with anti-phospho-ERK antibody (P-ERK) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:1000 dilution) in Blotto overnight at 4°C and washed 3 x 10 min with TBS/1% NP-40. A subsequent incubation with a monoclonal horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was carried out for 1 h at room temperature in Blotto, and the appropriate additional washes were performed. Following chemiluminescence (ECL) detection (Amersham Biosciences, Little Chalfont, UK), membranes were exposed onto X-ray film (FujiFilm, Tokyo, Japan). After strip washing (Restore buffer, Pierce Chemical Co.), membranes were reprobed with anti-ERK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:1000 dilution), overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody and continuation of the procedure as described above. Films were analyzed by densitometry, and the intensities of P-ERK were normalized to those of total ERK to correct for protein loading in the case of cellular lysates. Corrected results were expressed as the fold activation. Each experiment was repeated at least three times.

Statistical Evaluation

All experiments were independently repeated at least three times. Each experiment was performed with triplicate samples (in luciferase assays) or duplicate samples (in western blot) in each experimental group. Values were expressed means ± SEM. Statistical analysis was performed using the one-way ANOVA plus the Duncan multiple range test. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of LY 294002 on α-subunit promoter activity

The effect of PI3-kinase inhibitor, LY 294002 on α-subunit promoter activity was examined. Inclusion of αT3-1 cells with 50 μM LY 294002 significantly increased the α-subunit promoter luciferase activity up to 6.89 ± 0.26-fold compared to that of control. Co-treatment of the cells with GnRH and LY 294002 significantly increased the α-subunit promoter activity compared to that of GnRH alone, and showed synergistic effect (Fig 1A). In the dose response experiments, LY 294002 showed its stimulatory effect from the concentration of 10 nM, moreover increasing concentration of LY 294002 facilitated the increase of α-subunit promoter activity (Fig 1B).

Effect of wortmannin on α-subunit promoter activity

To confirm the effect of PI3-kinase inhibitor on α-subunit gene expression, another PI3-kinase inhibitor, wortmannin was used. Similar to the effect of LY 294002, wortmanin alone, at concentration of 1 μM increased α-subunit promoter activity up to 2.33 ± 0.06-fold in αT3-1 cells, which showed synergistic effect in combination with GnRH treatment (Fig 2A). The increase of α-subunit promoter activity was significantly noticed at concentration higher than 10 nM (Fig 2B).

Effects of PKB inhibitor on α-subunit promoter activity

PKB has been known as a downstream effector of PI3-kinase. To evaluate the possibility that PI3-kinase act to regulate the α-subunit promoter through the activation of PKB, the effect of PKB inhibitor was examined. Inclusion of PKB inhibitor alone did not affect α-subunit promoter activity as well as GnRH-induced α-subunit promoter (Fig 3). These results suggested

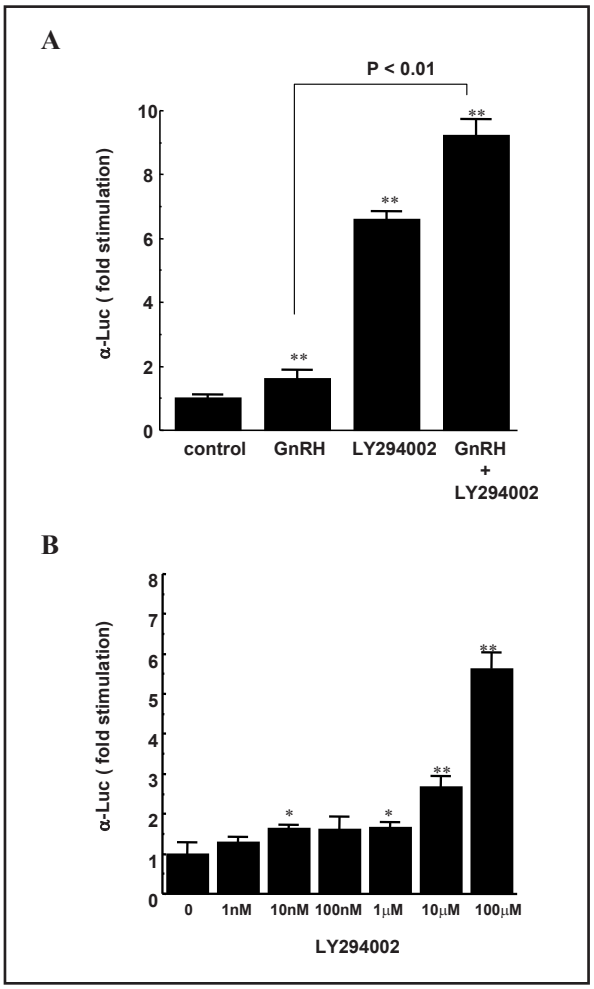

Fig. 1. Effect of PI3-kinase inhibitor, LY 294002 on α-*subunit* promoter activity.
αT3-1 cells were cotransfected with 2.0 μg of *α- subunit* promoter linked luciferase vector (*α*- Luc) and 0.1 μg of pRL-TK. After a 48 h incubation, the cells were treated with GnRH (100 nM), LY 294002 (50 μM) and GnRH + LY 294002 for 4h (A). Cells were treated with increasing concentration of LY 294002 for 4h (B). The luciferase activities were measured and expressed as fold stimulation of control. The data are means ± SEM (three independent experiments done with triplicate samples). *, $p < 0.05$ and **, $p < 0.01$ vs. control luciferase activity. The difference between GnRH and GnRH + LY 294002 were statistically significant ($p < 0.01$).

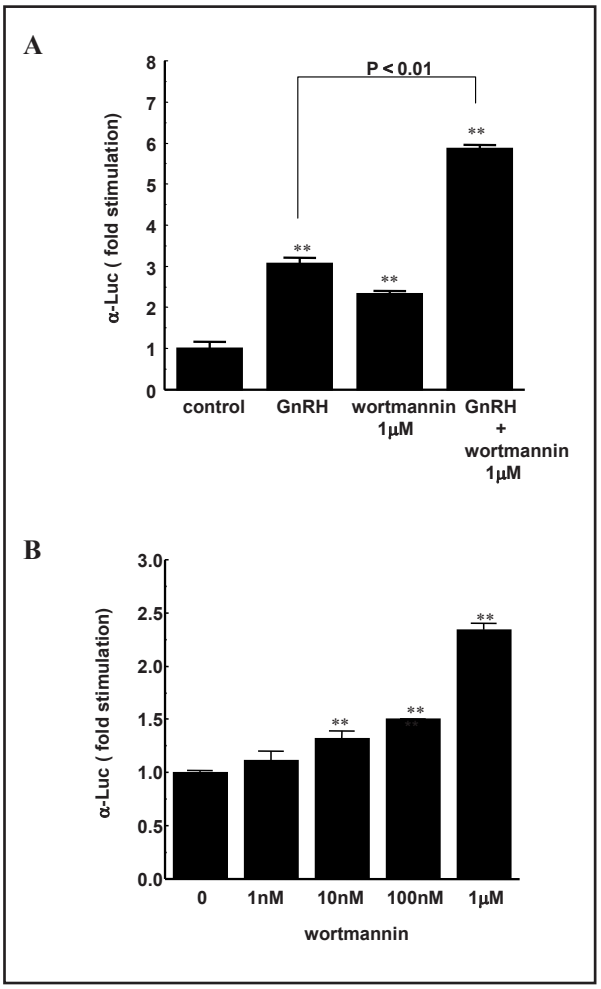

Fig. 2. Effect of PI3-kinase inhibitor, wortmannin on α-*subunit* promoter activity.
αT3-1 cells were cotransfected with 2.0 μg of *α- subunit* promoter linked luciferase vector (*α*- Luc) and 0.1 μg of pRL-TK. After a 48 h incubation, the cells were treated with GnRH (100 nM), wortmannin (1 μM) and GnRH + wortmannin for 4h (A). Cells were treated with increasing concentration of wortmannin for 4h (B). The luciferase activities were measured and expressed as fold stimulation of control. The data are means ± SEM (three independent experiments done with triplicate samples). **, $p < 0.01$ vs. control luciferase activity. The difference between GnRH and GnRH + wortmannin were statistically significant ($p < 0.01$).

that PKB activation did not participate in α-subunit gene expression.

Effects of PI3-kinase inhibitors on ERK activation

Previous reports have suggested the strong involvement of ERK pathways in gonadotropin α-subunit gene expression [5, 11, 12]. We next examined the effect of PI3-kinase inhibitors on ERK pathways. Both LY 294002 (Fig. 4A) and wortmannin (Fig. 4B) strongly increased the ERK phosphorylation at the concentration of 50 μM and 10 μM, respectively.

DISCUSSION

Alpha T3-1 cells, which possess gonadotroph like characteristics, were established by targeted oncogenesis of mouse gonadotrophs [30]. Although αT3-1 cells do not express LH- and FSH-specific β-subunits, this cell line has proven as a useful model for biochemical studies of the regulation of gonadotropin α-subunit, which is regulated by hypothalamic peptides, including GnRH.

The role of PI3-kinase/PKB signaling in pituitary gonadotroph have been studied in association with apoptosis. Fernandez *et al*. clearly demonstrated the anti-apoptotic effect of IGF-1 on serum deprivation-induced cell death in primary rat pituitary culture, in

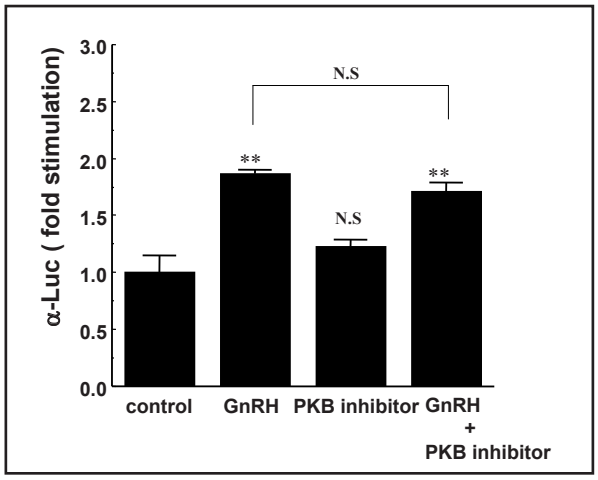

Fig. 3. Effect of PKB inhibitor on α-*subunit* promoter activity.
αT3-1 cells were cotransfected with 2.0 μg of *α- subunit* promoter linked luciferase vector (*α*- Luc) and 0.1 μg of pRL-TK. After a 48 h incubation, the cells were treated with GnRH (100 nM), PKB inhibitor (50 μM) and GnRH + PKB inhibitor for 4h. The luciferase activities were measured and the activity is expressed as fold stimulation of control. The data are means ± SEM (three independent experiments done with triplicate samples). **, $p < 0.01$ vs. control luciferase activity. N.S-fold induction was not statistically significant.

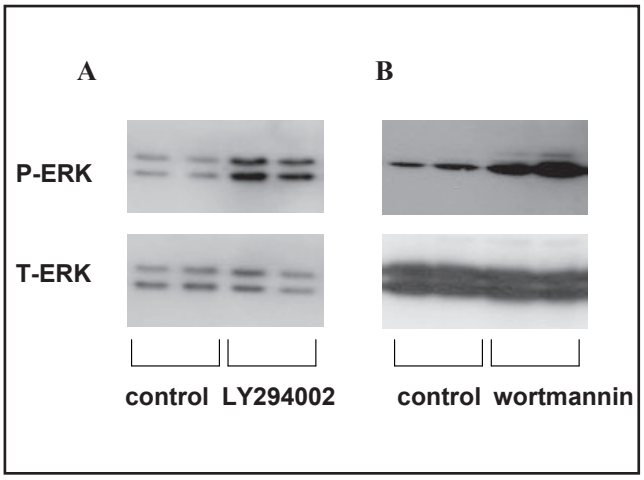

Fig. 4. Effects of PI3-kinase inhibitor, LY 294002 and wortmannin on ERK activation.
αT3-1 cells were stimulated without (control) or with 50 μM LY 294002 (A) and 1 μM wortmannin (B) for 10 min. The cell extracts were then subjected to SDS-PAGE, and immunoblot analysis was performed with anti-phospho ERK antibody (P-ERK). After the antibody was stripped, immunoblot analysis with anti-ERK antibody (T-ERK) was performed. We repeated the same experiments three times with reproducible results, and representative results are shown.

which they showed IGF-1 increased phosphorylation of the pro-apoptotic Bad and the levels of the anti-apoptotic protein Bcl-2 through PI3-kinase pathway [19]. On the other hand, it has been reported that GnRH inhibits anti-apoptotic activity of IGF-1 through inhibition of PKB by PI3-kinase independent mechanism [25]. Based on the fact that PI3-kinase/PKB pathway are activated by tyrosine kinase receptor, such as IGF-1 [19] and activin [31], as well as GnRH [25], which is via G protein coupled receptor, and the fact that both activin [31, 32] and IGF-1 [33] modulate gonadotropin secretion and synthesis, we have speculated that PI3-kinase might be involved in the regulation of gonadotropin α-subunit expression in αT3-1 cells.

The results of this present study showed that pharmacologic inhibition of PI3-kinase by LY 294002 and wortmannin increased gonadotropin α-subunit gene expression in αT3-1 cells. Both LY 294002 and wortmannin significantly increased the α-subunit promoter activities by themselves, and increasing concentration of these inhibitors similarly increased α-subunit gene expressions, suggesting that these chemicals are having the ability to increase gonadotropin α-subunit. This effect emphasized when combination of GnRH and these reagents further induce α-subunit promoter activity (Fig 1A and 2A). Furthermore, we also observed that both LY 294002 and wortmannin increased ERK phosphorylation (Fig. 4). ERK is one of the components of MAPK families, which consist of three major kinases, ERK, JNK and p38MAPK. The signal transduction pathway responsible for GnRH-induced expression of α-subunit has been well characterized and involves Src, PKC and ERK [5, 11]. In general, ERK is activated via cell surface receptor and transduce its signals to conduct appropriate gene expression. However, the mechanism on how does LY 294002 and wortmannin increase ERK is not clear. Several reports have described the effects of PI3-kinase pathway on ERK activation, which seem to depend on the cell type and stimulus [34]. Some studies have shown that ERK activation is PI3-kinase/PKB dependent [35, 36], whereas the activated PI3-kinase/PKB pathway also has been found to be involved in the inhibition of ERK [37, 38]. Using somatolactotroph cell line, Romano D *et al*. have shown that both PI3-kinase and PKB inhibitors enhanced ERK phosphorylation, where they observed that activities of Raf-1 kinase and Rap-1, both of which were components of upstream ERK signaling pathways, were increased. Prolactin release were also increased by the addition of both of these inhibitors [39].

The importance of ERK activation in GnRH-induced α-subunit gene expression has been shown in previous reports [5, 11, 12]. From our results, we could speculated that inhibition of intrinsic PI3-kinase activity by LY 294002 or wortmannin increased ERK phophorylation, and subsequently this activated ERK acts to induce α-subunit gene expression in these cells. Further, our results showed that PKB inhibitor failed to modulate α-subunit gene expression (Fig. 3), which suggested that PI3-kinase, but not PKB had the ability to modulate gonadotropin α-subunit gene expression. The divergent signaling pathways upstream of Akt/PKB might be exist, where PI3-kinase activates and works in association with ERK signaling.

In summary, we have demonstrated that PI3-kinase inhibitors, LY 294002 and wortmannin, increased α-subunit gene expression. PKB inhibitor did not have any effect on α-subunit gene. These inhibitors also increased the activation of ERK, suggest that inhibition of PI3-kinase may involve in α-subunit gene expression in association with ERK activation.

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