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# Induction of Dual Specificity Phosphatase 1 (DUSP1) by Gonadotropin-Releasing Hormone (GnRH) and the Role for Gonadotropin Subunit Gene Expression in Mouse Pituitary Gonadotroph LbetaT2 Cells[1]

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## ABSTRACT

**We examined the expression of dual specificity phosphatase 1 (DUSP1) by gonadotropin-releasing hormone (GnRH) stimulation and investigated the role of DUSP1 on gonadotropin gene expression using LbetaT2 gonadotroph cell line. DUSP1 expression was markedly increased 60 min after GnRH stimulation, and mitogen-activated protein kinase 3/1 (MAPK3/1) activation was gradually decreased after 60 min. GnRH-induced MAPK3/1 activation was completely inhibited by U0126, a MEK inhibitor, whereas GnRH-induced DUSP1 expression was partially inhibited by U0126. GnRH-induced DUSP1 induction was inhibited by triptolide, a diterpenoid triepoxide. In contrast, this compound potentiated MAPK3/1 activation. U0126 prevented GnRH-stimulated gonadotropin subunit promoter activation dose dependently, and 10 μM of U0126 reduced the effects of GnRH on the *Lhb* and *Fshb* promoters to 79.15% and 55.66%, respectively. GnRH-stimulated activation of *Lhb* and *Fshb* promoters as well as serum response factor (*Srf*) promoters were almost completely inhibited by triptolide, suggesting that this component had a nonspecific effect to the cells. *Dusp1* siRNA reduced the expression of DUSP1 and augmented MAPK3/1 phosphorylation, but it did not increase of gonadotropin promoters. By overexpresssion of DUSP1, both GnRH-stimulated *Lhb* and *Fshb* promoters were significantly reduced. We have previously shown that insulin-like growth factor 1 (IGF1) increases MAPK3/1 but does not activate gonadotropin subunit promoters. IGF1 failed to induce DUSP1 expression. In addition, under pulsatile GnRH stimulation, DUSP1 expression was observed following high-frequency GnRH pulses but not following low-frequency pulses. Our study demonstrated that DUSP1, induced by GnRH, functions not only as an MAPK3/1-inactivating phosphatase but also as an important mediator in gonadotropin subunit gene expression regulation.**

*gonadotropin-releasing hormone, neuroendocrinology, phosphatases, signal transduction*

## INTRODUCTION

The gonadotropins LH (luteinizing hormone) and FSH (follicle-stimulating hormone) play key roles in regulating reproductive functions. LH and FSH belong to the glycoprotein family of anterior pituitary hormones and are composed of a common alpha-glycoprotein subunit (CGA) and unique beta subunits. The synthesis of gonadotropins in the gonadotrophs and their secretion are regulated by GnRH. GnRH occupies its receptor at the cell membranes and activates intracellular signaling pathways, which in turn contribute to the regulation of gonadotropin gene expression [1].

GnRH signaling includes the activation of mitogen-activated protein kinase (MAPK) cascades, which link the transmission of signals from the cell surface receptor to the nucleus. GnRH acts on the gonadotroph cells by binding itself to its receptor containing a seven-transmembrane motif, resulting in the induction of membrane phospholipid turnover and the subsequent formation of inositol phosphates and diacylglycerol. This leads to a rapid increase in the intracellular $Ca^{2+}$ and protein kinase C (PKC) [2–4]. To date, four distinct MAPK cascades have been identified, each named after the subgroup of their MAPK components, mitogen-activated kinase 3/1 (MAPK 3/1), also known as extracellular signal-regulated kinase 1 and 2, MAPK8 (c-Jun N-terminal kinase), MAPK14 (p38MAPK), and MAPK7 (BMK, ERK5). The activation of the MAPK 3/1 cascade by GnRH appears to involve mainly activation of Raf1 by PKC. Activation is supported by a pathway that involves dynamin, c-Src, and Ras and a pathway that involves calcium signaling and possibly other signaling components [5]. The MAPK3/1 pathway mediates, at least in part, GnRH-induced gonadotropin hormone subunit gene expression [6–8]. In addition, the PKC pathway is also involved in common alpha glycoprotein (*Cga*) subunit gene expression [9]. The PKC, calcium/calmodulin kinase, and MAPK8 pathways are involved in *Lhb* subunit gene expression [10–12], and the intracellular calcium, c-AMP, MAPK14, and MAPK8 pathways regulate *Fshb* subunit gene expression [13]. The impaired GnRH activation of MAPK3/1, caused by selective down-regulation of the phospholipase C-PKC-$Ca^{2+}$ pathway from desensitization of GnRH-induced signaling, results in reduced *Lhb* synthesis and LH secretion [14]. These observations suggest that MAPK3/1 activation by GnRH plays an important role in gonadotropin regulation.

The dual-specificity phosphatases (DUSP) are a family of protein phosphatases that inactivate MAPKs through dephosphorylation of threonine and/or tyrosine residues. Inactivation of MAPK3/1 is accomplished by its physiologic regulators, the DUSPs. MAPK signaling is integrated at the level of regulation by DUSPs through a negative feedback mechanism [15–17]. DUSP1, the first MAPK phosphatase discovered, belongs to a group comprised of type I DUSPs, which are localized in the nuclear compartment and induced by stimuli that activate MAPK. DUSP1 is required for cell growth and proliferation

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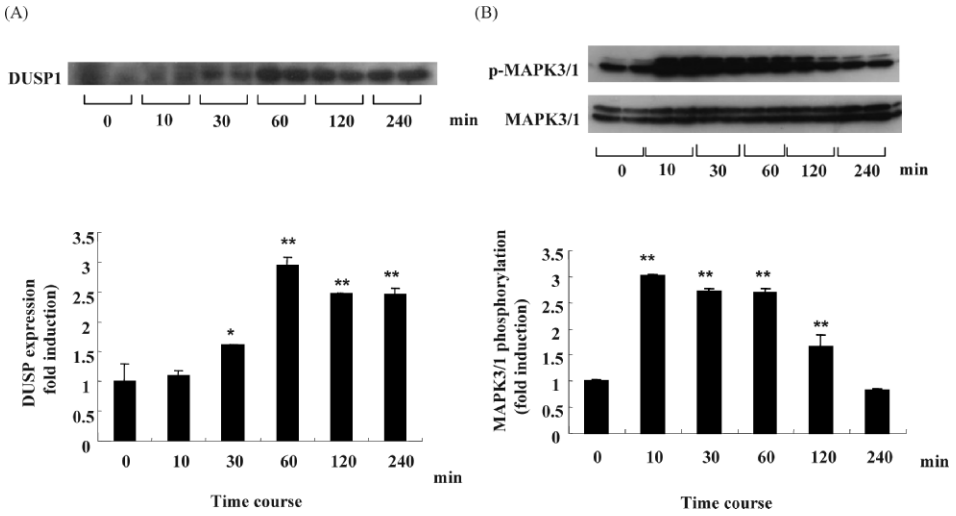


FIG. 1. Time course of GnRH-induced DUSP1 expression (**A**) and MAPK3/1 phosphorylation (**B**). LβT2 cells were treated with 100 nM GnRH for the indicated periods of time. In this experiment, cell lysates (5.0 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with an antibody against DUSP1. To examine MAPK3/1 phosphorylation, the membrane was reincubated with antibody against phosphorylated MAPK3/1. After the antibody was stripped, an immunoblot analysis with MAPK3/1 antibody was carried out. The visualized bands were quantified by scanning densitometry using NIH Image and normalized to total MAPK3/1. Results were expressed as the fold increase over the nonstimulated cells (control) and represent the means ± SEM from three independent experiments. Representative autoradiographs are shown. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control (time 0).

and is expressed in various malignancies [18, 19]. Down-regulation of DUSP1 reduces tumorigenicity in cancer cells [20, 21]. The transcriptional induction of DUSP1 is mediated primarily by MAPK3/1 and modulated by MAPK14; therefore, DUSP1 is thought to play an important role in the feedback control of MAPK in the nucleus, where it attenuates the stimuli [22]. DUSP1 effectively inactivates MAPK8 and MAPK14 [23] and hence has a physiological role as a negative regulator of the synthesis of proinflammatory cytokines in vivo [24]. In fibroblasts, the appearance of DUSP1 coincides with the inactivation of MAPK3/1, while in macrophages, blockade of DUSP1 induction prevents the inactivation of MAPK3/1 [25, 26].

With regard to the pituitary gonadotrophs, DUSPs are increased by GnRH in association with MAPK3/1 and MAPK8 activation [27, 28] in gonadotroph αT3–1 cell lines. EGR1 protein has also proven to be important for the induction of DUSPs by GnRH [29]. Potential feedback regulation between MAPK signaling and DUSPs within the GnRH signaling pathway is also evident in gonadotrophs. DUSPs have overlapping yet unique patterns of tissue distribution, subcellular localization, substrate specificity, and response to mitogenic stimulation [30]. Additionally, in most cell lines studied to date, DUSP1 is activated as an immediate early gene in response to various stimuli including growth factors, insulin, and ultraviolet light [31–33]. These observations suggest that DUSPs may have distinct physiological functions in addition to their role as protein phosphatases.

The observations that MAPK3/1 is involved in gonadotropin subunit gene expression and that DUSP1 is regulated and induced by the MAPK3/1 pathway prompt the question of whether DUSP1 affects downstream signaling molecules in gonadotrophs. In the present study, we examined GnRH-induced DUSP1 expression and investigated how DUSP1 contributes to the expression of gonadotropin subunit genes using pituitary gonadotroph LβT2 cells. The correlation between MAPK3/1 activation and DUSP1 expression was also examined.

## MATERIALS AND METHODS

### Materials

The following chemicals and reagents were obtained from the indicated sources: GnRH, Dubelco modified Eagle medium (DMEM), penicillin-streptomycin, and insulin-like growth factor1 (IGF1) human, recombinant (Sigma Chemical Co., St. Louis, MO); U0126, Triptolide (Calbiochem, La Jolla, CA); Fetal Bovine Serum (FBS) and Trypsin (GIBCO, Invitrogen, Carlsbad, CA), anti-phospho-MAPK3/1 (phosphor-Thr-202/phosphor-Tyr-204) mouse monoclonal $IgG_{2a}$, anti-DUSP1 rabbit polyclonal IgG, and MAPK3/1 rabbit monoclonal antibodies, horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and Matrigel (Becton Dickinson and Co. Labware, Bedford, MA).

### LβT2 Cell Culture

LβT2 cells (kindly provided by Dr. P. Mellon of the University of California (San Diego, CA) were maintained in monolayer culture in high-glucose DMEM supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$ in 95% air [6]. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FCS and 1% penicillin-streptomycin and incubated without (control) or with 100 nM GnRH for the indicated times. When the MEK1/2 inhibitor U0126 (in the indicated concentrations) and the DUSP1 inhibitor triptolide (in the indicated concentrations) were used, these compounds were added 30 min during the preincubation period and during incubation with GnRH.

### Perifusion System

The perifusion system used for this study has been described previously [34]. Briefly, LβT2 cells were plated in perifusion chambers mounted on glass slides, previously coated with Matrigel, and then incubated for 24 h in static culture in high-glucose DMEM containing 10% heat-inactivated FSC and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$. The chambers were then connected to the perifusion system and continuously perifused with high-glucose DMEM containing 1% heat-inactivated FCS and 1% penicillin-streptomycin at a constant flow rate of 0.25 ml/min. During perifusion, cells were treated with either medium alone or pulsatile 10 nM GnRH at either a high frequency (one pulse every 30 min) or a low frequency (one pulse every 2 h) for 12 h. GnRH pulses were delivered by a set of peristaltic pumps controlled by a time controller (Chrontrol XT; Chrontrol Corp., San Diego, CA).

### Transfection and Luciferase Assay

The reporter constructs used in these experiments were generated by fusing −797/+5 of the rat *Lhb* gene (*Lhb*-Luc) or −2000/+698 of the rat *Fshb* gene (*Fshb*-Luc) to the firefly luciferase (Luc) cDNA in pXP2, as previously described [6]. The *Dusp1* gene was inserted into the pWay21 EGFP vector backbone. This construct was provided by Dr. Anton Bennett, Yale University, and obtained from Addgene (Addgene plasmid 13469). The *Dusp1* si-RNA was purchased from Sigma. Cells were transiently transfected by electroporation with 2 μg/well each of *Lhb*-Luc or *Fshb*-Luc. In some experiments, cells were cotransfected with either p*Srf*-Luc (2.0 μg/well) or 0.2 μg/well (or 2.0 μg/well or 4.0 μg/well) of the DUSP1 expression vector, or 4.0 μl *Dusp1* si-RNA and 0.1 μg/well of a PRL-TK vector containing the Renilla luciferase. Cells were then plated in 35-mm culture dishes. After incubation with test reagents,

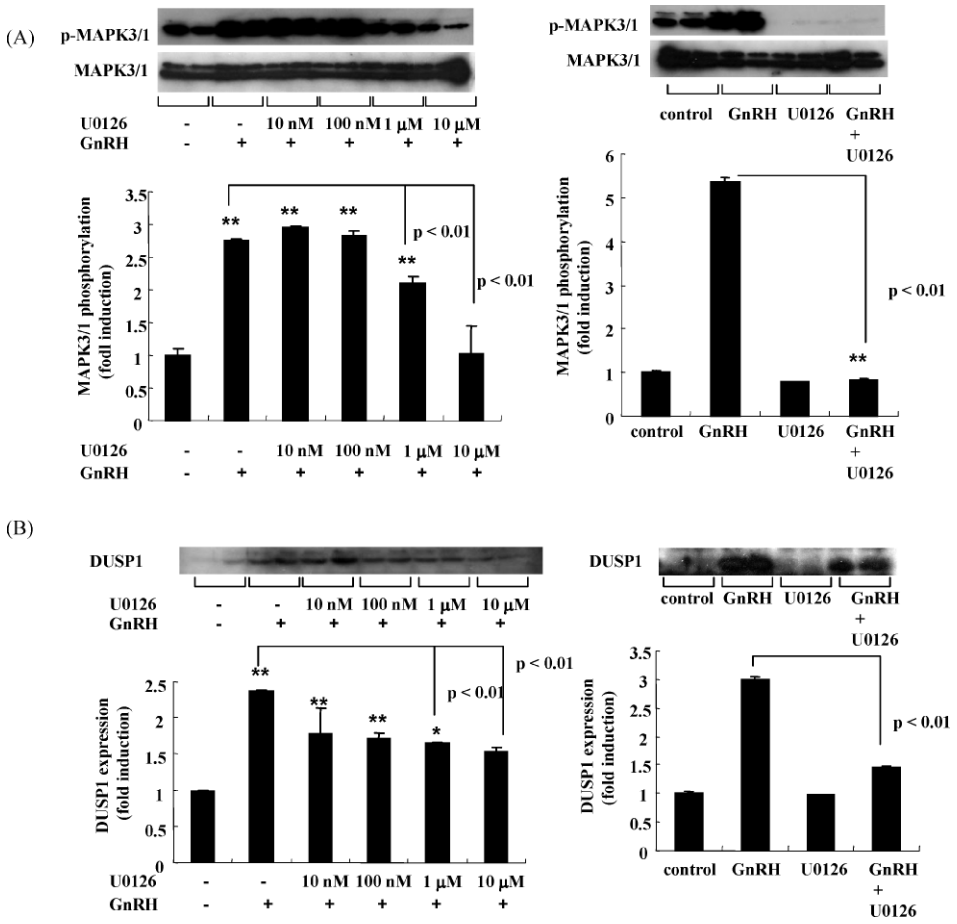


FIG. 2. Effect of U0126 on GnRH-induced MAPK3/1 phosphorylation and DUSP1 expression. **A**) LβT2 cells were preincubated without (control) or with increasing doses of U0126, an MEK inhibitor, for 30 min in DMEM containing 1% FBS; 100 nM GnRH was then added directly, and the cells were incubated for 10 min for MAPK3/1 (**A**) and 2 h for DUSP1 (**B**) before harvesting. In these experiments, cell lysates (5.0 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibodies against phosphorylated MAPK3/1, total MAPK3/1, and DUSP1. The visualized bands were quantified by scanning densitometry using NIH Image and normalized to total MAPK3/1. Results were expressed as the fold increase over the nonstimulated cells (control) and represent the means ± SEM from three independent experiments. Representative autoradiographs are shown. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. The difference between GnRH and GnRH + 1μM and 10 μM of U0126 were statistically significant ($P < 0.01$).

cells were washed with ice-cold PBS and lysed with PLB (Passive Lysis Buffer; Promega). After centrifugation at 150 rpm at 4°C, firefly luciferase and Renilla luciferase activities were measured in the supernatant 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 the results were expressed as the fold increase compared to the unstimulated control.

### *Western Blotting*

LβT2 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, then 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. Ten micrograms of denaturated protein per well were separated on a 10% SDS-PAGE gel according to standard protocols. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF; Amersham Biosciences, Little Chalfont, UK), which were blocked for 2 h at room temperature in Blotto (5% milk in TBS). Membranes were incubated with either anti-DUSP1 antibody (1:100 dilution) or phosphorylated-MAPK3/1 antibody (p-MAPK3/1) (1:250 dilution) in Blotto overnight at 4°C and washed three times for 10 min per wash with TBS/1% Tween. A subsequent incubation with monoclonal HRP-conjugated antibody was carried out for 1 h at room temperature in blotto, and the appropriate additional washes were performed. Following chemiluminescence (ECL) detection (Amersham Biosciences), membranes were exposed onto X-ray film (Fujifilm; Tokyo, Japan). After strip washing (Restore buffer; Pierce Chemical Co., Rockford, IL), membranes were reprobed with MAPK3/1 antibody (1:10 000 dilution) for 1 h at room temperature, followed by incubation with HRP-conjugated secondary antibody and continuation of the procedure as described previously. Films were analyzed by densitometry, and the intensities of either DUSP1 or p-MAPK3/1 were normalized to that of total MAPK3/1 to correct for protein loading. The corrected results were expressed as the fold induction over the controls.

### *Statistical Analysis*

All experiments were independently repeated at least three times. Each experiment was performed with triplicate samples (luciferase assays) or duplicate samples (Western blot) in each experimental group. Values were expressed as means ± SEM. Statistical analysis was performed using the one-way analysis of variance followed by the Dunnett multiple-range test. $P < 0.05$ was considered statistically significant.

## RESULTS

### *Induction of DUSP1 Protein and Activation of MAPK3/1 by GnRH Stimulation*

First, we examined the time course of DUSP1 expression and the activation of MAPK3/1 by GnRH stimulation. The increase in DUSP1 protein was first detected at 30 min after 100 nM GnRH addition and reached its peak level at 60 min (2.95 ± 0.02-fold). DUSP1 protein was still present at 4 h after stimulation (Fig. 1A). In contrast, phosphorylation of MAPK3/1 was rapidly increased at 10 min after GnRH stimulation and then declined gradually (Fig. 1B).

### *Effect of U0126 on DUSP1 Expression and MAPK3/1 Phosphorylation*

The effects of U0126, a MEK inhibitor, on DUSP1 expression and MAPK3/1 phosphorylation in response to GnRH were examined. Cells were preincubated without or with increasing concentrations of the MEK inhibitor U0126 for 30 min, followed by stimulation with GnRH. MAPK3/1 phosphorylation 10 min after GnRH addition significantly increased by 2.23 ± 0.23-fold; however, MAPK3/1 phosphor-

ylation was progressively blocked by increasing doses of U0126. In another experiment using 10μM U0126, GnRH-increased MAPK3/1 phosphorylation was completely inhibited (Fig. 2A). In contrast, following a 2-h incubation of GnRH with or without U0126, DUSP1 expression was reduced in the presence of U0126; 10 μM of U0126 partially inhibited the expression of DUSP1 protein by GnRH (Fig. 2B).

### *Effect of Triptolide on DUSP1 Expression and MAPK3/1 Phosphorylation*

Triptolide, a diterpenoid triepoxide, inhibits DUSP1 induction by LPS in macrophages [26]. We then examined the effect of triptolide on DUSP1 expression and MAPK3/1 phosphorylation by GnRH. Cells were treated with GnRH without or with increasing concentrations of triptolide for 2 h. DUSP1 expression was substantially decreased in a dose-dependent manner in the presence of triptolide (Fig. 3A and B). In contrast, MAPK3/1 phosphorylation was increased in a dose-dependent manner by triptolide and reached up to 5.99 ± 0.08-fold at 10 μM of triptolide (Fig. 3A and C). MAPK3/1 phosphorylation 2 h after GnRH stimulation showed an opposite pattern from that of DUSP1 expression. This finding is in accordance with the general knowledge that DUSP1 is a negative inhibitor of the MAPK3/1 pathway.

### *Effect of U0126 on* Lhb *and* Fshb *Promoter Activity*

The MAPK3/1 pathway is important for the induction of gonadotropin subunit expression. Effects of the MEK inhibitor, U0126 were examined. As expected, GnRH-induced promoter activities of both *Lhb* and *Fshb* subunits were decreased by the presence of U0126, and this decrease was proportional to the concentration of U0126 used. When 10μM U0126 was employed, gonadotropin promoter activity by GnRH was reduced from its maximal value of 9.64 ± 0.64-fold to 2.01 ± 0.01-fold for *Lhb* (Fig. 4A) and from 5.21 ± 0.16-fold to 2.31 ± 0.79-fold for *Fshb* (Fig. 4B). The pharmacological inhibition of MEK by U0126 had a more pronounced effect on *Lhb* subunit promoter activity (79.15%) than on *Fshb* promoter activity (55.66%).

### *Effect of Triptolide on Gonadotropin Promoter Activities and Serum Response Factor (*Srf*) Promoter Activity*

We have shown that inhibition of DUSP1 expression by triptolide increased MAPK3/1 phosphorylation (Fig. 3). In addition, demonstration of the effect of U0126 on gonadotropin promoter activities prompted a further question: whether DUSP1 inhibition, followed by an increase of MAPK3/1 activation, would consequently increase gonadotropin promoter activities. LβT2 cells were preincubated with triptolide and then stimulated with GnRH. Unexpectedly, GnRH-induced promoter activities of both *Lhb* and *Fshb* subunit were significantly decreased almost to the basal level by the presence of increasing doses of triptolide (Fig. 5A and B). To confirm the specificity of triptolide on ERK-dependent target, the serum response factor (*Srf*) luciferase constract was employed. *Srf* is a DNA domain in the promoter region that binds MAPK3/1-mediated transcription factors. We examined how MAPK3/1 inhibition by U0126 and inhibition of DUSP1 by triptolide affected *Srf* promoter activities. Cells were transfected with *Srf*-Luc and then treated with either U0126 or triptolide in combination with GnRH with the exception of the control cells. GnRH stimulation significantly increased *Srf* promoter activity up to 382.42 ± 44.22-fold. Inhibition of the

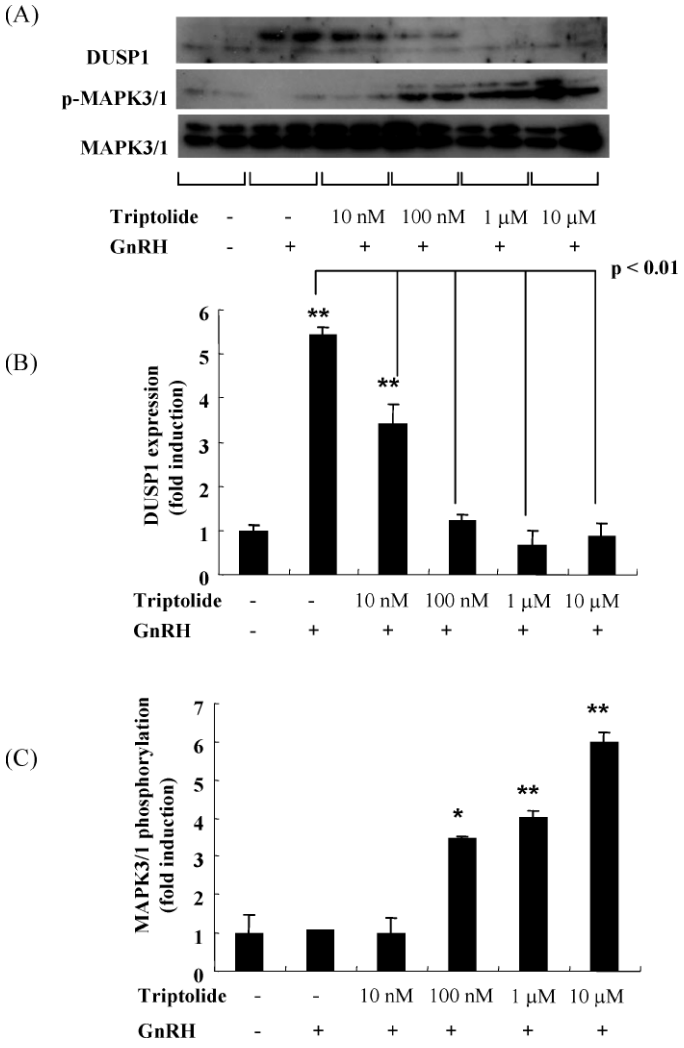


FIG. 3. Effect of triptolide on GnRH-induced DUSP1 expression and MAPK3/1 phosphorylation. LβT2 cells were preincubated with or without increasing concentrations of triptolide for 30 min followed by the addition of 100 nM GnRH and incubation for 2 h. Cell lysates (5.0 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibodies against DUSP1 (**A**, **B**), phosphorylated MAPK3/1 (**A**, **C**), and total MAPK3/1. The visualized bands were quantified by scanning densitometry using NIH Image and normalized to total MAPK3/1. Results were expressed as the fold increase over the nonstimulated cells (control) and represent the means ± SEM from three independent experiments. Representative autoradiographs are shown. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. The difference between GnRH and GnRH + 100 nM to 10 μM of triptolide were statistically significant ($P < 0.01$).

MAPK3/1pathway using U0126 significantly decreased GnRH-induced *Srf* promoter activity by 154.53 ± 23.49-fold, while DUSP1 inhibition using triptolide completely diminished the GnRH effect on *Srf* promoter activity (Fig. 5C). From these results, we had a doubt about the specificity of triptolide.

### *Effect of DUSP1 Knockdown Using siRNA on MAPK3/1 Phosphorylation and Gonadotropin Subunit Promoter Activities*

Pharmacological inhibition of DUSP1 by triptolide did not exert the expected effect by determination of *Srf*-Luc promoter activities. We then employed siRNA method to inhibit DUSP1 expression and examined how it affects gonadotropin subunit promoter activities. Transfection of LβT2 cells with *Dusp1*

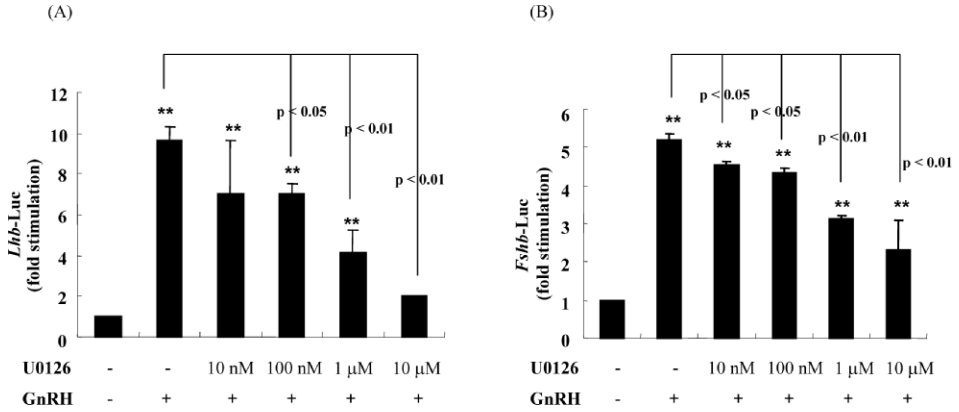


FIG. 4. Effect of U0126 on GnRH induced gonadotropin subunit promoter activity. LβT2 cells were cotransfected with 2.0 μg of either *Lhb*-subunit (*Lhb*-Luc) (**A**) or *Fshb*-subunit (*Fshb*-Luc) (**B**) and PRL-TK (0.1 μg) and incubated for 36 h. Then cells were preincubated in serum-free DMEM for 30 min without or with increasing concentrations of U0126 for 30 min, followed by treatment with 100 nM GnRH for 6 h with the exception of the control cells. A luciferase assay was then performed to examine *Lhb* and *Fshb* promoter activities, which were then normalized to PRL-TK activity and expressed as the fold of activation over the unstimulated controls. Luciferase activity was then measured and expressed as the fold stimulation of the control. Values are means ± SEM (three independent experiments done with triplicate samples). $^{**}P < 0.01$ vs. control. The difference between GnRH and GnRH + 100 nM to 10 μM of U0126 for *Lhb* and the difference between GnRH and GnRH + 10 nM to 10 μM of U0126 for *Fshb* were statistically significant ($P < 0.01$ and $P < 0.05$).

siRNA reduced the expression of DUSP1 (Fig. 6A) and augmented MAPK3/1 phosphorylation (Fig. 6B) by GnRH stimulation. Nevertheless, the increased MAPK3/1 phosphorylation did not result in the increase of GnRH-induced *Lhb* and *Fshb* promoter activities (Fig. 6C and D).

### *Effect of Overexpressing DUSP1 Protein on MAPK3/1 Phosphorylation and Gonadotropin Subunit Promoter Activities*

Next, we examined the role of DUSP1 protein by overexpressing it with the pWay21-DUSP1 FL vector, which was linked with EGFP. Transfection of the cells with the DUSP1 expression vector resulted in the successful expression of EGFP-DUSP1 fusion proteins compared to nontransfected mock (PCI-neo) (Fig. 7A). Interestingly, both exogenous EGFP-DUSP1 protein and endogenous DUSP1 were similarly induced by GnRH stimulation. DUSP1 overexpression almost completely attenuated MAPK3/1 phosphorylation by GnRH (Fig. 7B). The responses to GnRH of *Lhb* and *Fshb* promoter activities were significantly inhibited in 2.0 μg/well DUSP1 overexpressing LβT2 cells compared to the mock transfected cells (Fig. 7C and D).

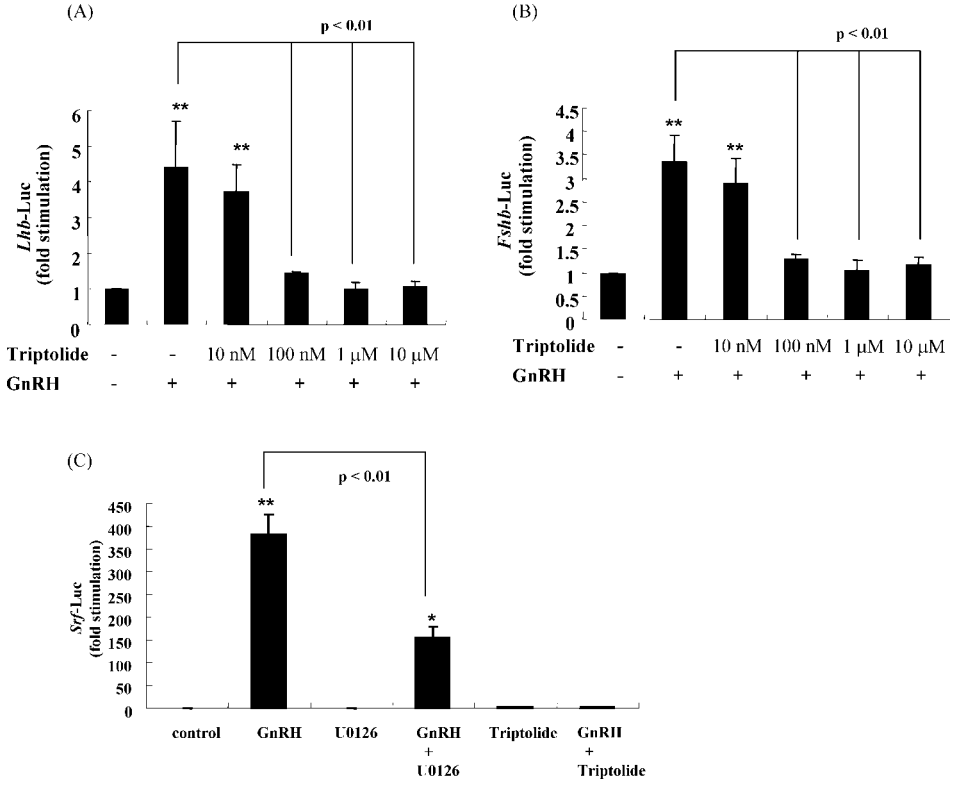


FIG. 5. Effect of triptolide on GnRH induced gonadotropin subunit promoter activity and *Srf* promoter activity. LβT2 cells were cotransfected with 2.0 μg of either *Lhb*-subunit (*Lhb*-Luc) (**A**), *Fshb*-subunit (*Fshb*-Luc) (**B**), or luciferase reporter constructs containing a cis-element, the serum response element promoter (*Srf*-Luc) (**C**), and PRL-TK (0.1 μg) and incubated for 36 h. Then cells were preincubated in serum-free DMEM for 30 min without or with the indicated concentrations of triptolide and U0126 for 30 min, followed by treatment with 100 nM GnRH for 6 h with the exception of the control cells. A luciferase assay was then performed to examine *Lhb* and *Fshb* promoter activities, which were then normalized to PRL-TK activity and expressed as the fold of activation over the unstimulated controls. Luciferase activity was then measured and expressed as the fold stimulation of the control. Values are means ± SEM (three independent experiments done with triplicate samples). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. The difference between GnRH and GnRH + 100 nM to 10 μM of triptolide for both *Lhb* and *Fshb* were statistically significant ($P < 0.01$). The difference between GnRH and GnRH + U0126 for *Srf*-Luc were statistically significant ($P < 0.01$).

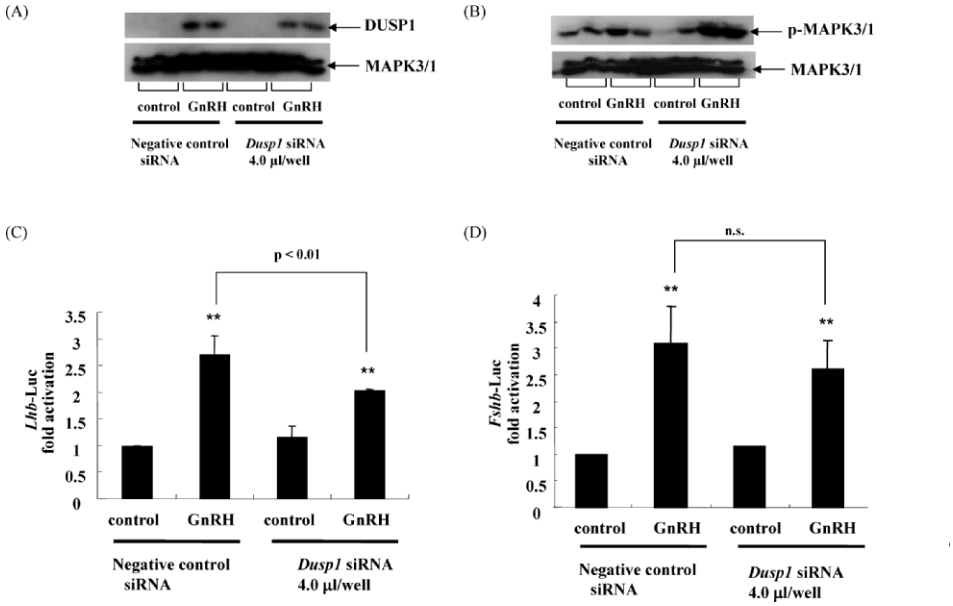


FIG. 6. Effect of *Dusp1* siRNA on gonadotropin subunit promoter activities. LβT2 cells were transfected with 4.0 µl of either negative siRNA or *Dusp1* siRNA. Forty-eight hours after transfection, cells were treated with 100 nM GnRH for 1 h. After cells were harvested, cell lysates (5 µg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibody against DUSP1 (**A**), phosphorylated MAPK3/1 (**B**), and total MAPK3/1. Cells were also transfected with 2.0 µg of either *Lhb*-subunit (*Lhb*-Luc) or *Fshb*-subunit (*Fshb*-Luc) and PRL-TK (0.1 µg). After incubation for 48 h, a luciferase assay was then performed to examine *Lhb* (**C**) and *Fshb* (**D**) promoter activity, which was then normalized to PRL-TK activity and expressed as the fold of activation over the unstimulated controls. Luciferase activity was then measured and expressed as the fold stimulation of the control. Values are means ± SEM (three independent experiments done with triplicate samples). $^{**}P < 0.01$ vs. control. The difference between GnRH stimulation in the negative siRNA and *Dusp1* siRNA transfected cells were statistically significant ($P < 0.01$) for *Lhb*. n.s., fold induction was not statistically significant.

### *Effect of Insulin-Like Growth Factor 1 (IGF1) on DUSP1 Expression and Its Effect on* Lhb *and* Fshb *Promoter Activities*

In our previous study, we reported that IGF1 stimulation activates MAPK3/1 in LβT2 cells [35]. In this study, we examined whether IGF1 increased DUSP1 protein in a manner similar to GnRH. Cells were stimulated with 100 ng/ml IGF1 for 60 min followed by measurement of MAPK3/1 activity and DUSP1 protein expression. As expected, MAPK3/1 was phosphorylated up to 1.70 ± 0.20-fold by IGF1; however, the magnitude of the increase was less than what resulted following GnRH stimulation. IGF1 failed to increase DUSP1 expression (Fig. 8A). Both *Lhb* and *Fshb* promoter activities were not increased by IGF1 stimulation (Fig. 8B), which suggested that MAPK3/1 activation alone was insufficient to promote gonadotropin gene expression.

### *Expression of DUSP1 Protein by Pulsatile GnRH in Perifused LβT2 Cells*

GnRH is released from the hypothalamus in a pulsatile manner in vivo, and frequencies of GnRH pulse decide the specificity of *Lhb* or *Fshb* gene expression. High-frequency GnRH pulses preferentially increase *Lhb*, whereas low-frequency pulses increase *Fshb* [6]. Next, we examined the effect of pulsatile GnRH stimulation on DUSP1 expression. Cells were exposed to pulsatile GnRH (10 nM, 5 min per pulse) at a frequency of once pulse every 30 min (high frequency) or one pulse every 2 h (low frequency) (Fig. 9A). Cells were collected after 12-h exposure to GnRH pulses, and DUSP1 activation was examined by Western blot analysis. DUSP1 was expressed following high-frequency pulsatile GnRH stimulation but not following exposure to low-frequency GnRH pulses (Fig. 9B).

## DISCUSSION

Previous studies have demonstrated that MAPK3/1 mediates GnRH regulation of gonadotropin subunit gene expression [6, 13, 36, 37]. GnRH-activated MAPK3/1 signaling mediates the transcriptional response. MAPK3/1 activity is determined by the balance of upstream specific MAPK3/1 activating kinases, MEK, and inactivating phosphatases. DUSP1 is one dual-specificity phosphatase that dephosphorylates both phosphotyrosine and phosphothreonine residues and inactivates MAPK3/1 in a negative feedback loop [38, 39]. DUSP1 is also a product of a growth factor-induced early response gene [31–33].

The profile and function of GnRH-induced DUSP1 expression in the pituitary gonadotroph cell line αT3–1 has been demonstrated previously [28]. The current study was conducted to identify the correlation of DUSP1 with MAPK3/1 and the contribution of DUSP1 in the regulation of gonadotropin *Lhb* and *Fshb* subunit gene expression. Our results show that GnRH-stimulated MAPK3/1 phosphorylation increases rapidly by 10 min and gradually declines from 2 h, with return to the basal level at approximately 4 h after GnRH stimulation (Fig. 1B). In contrast, DUSP1 protein expression increases 30 min after GnRH addition, with a maximal peak at 1 h, and remains sustained even 4 h after GnRH stimulation (Fig. 1A). From the kinetics of MAPK3/1 phosphorylation and DUSP1 induction by GnRH, we concluded that increased DUSP1 protein following GnRH stimulation dephosphorylated MAPK3/1, which was phosphorylated by GnRH stimulation, Thus, increased and sustained expression of DUSP1 correlates with the decreased activation of MAPK3/1. A similar finding in the temporal pattern of DUSP1 expression and MAPK3/1 phosphorylation has also been observed in αT3–1 cells following GnRH stimulation [28] and in pituitary prolactin-producing cells following TRH stimulation [40, 41]. Our

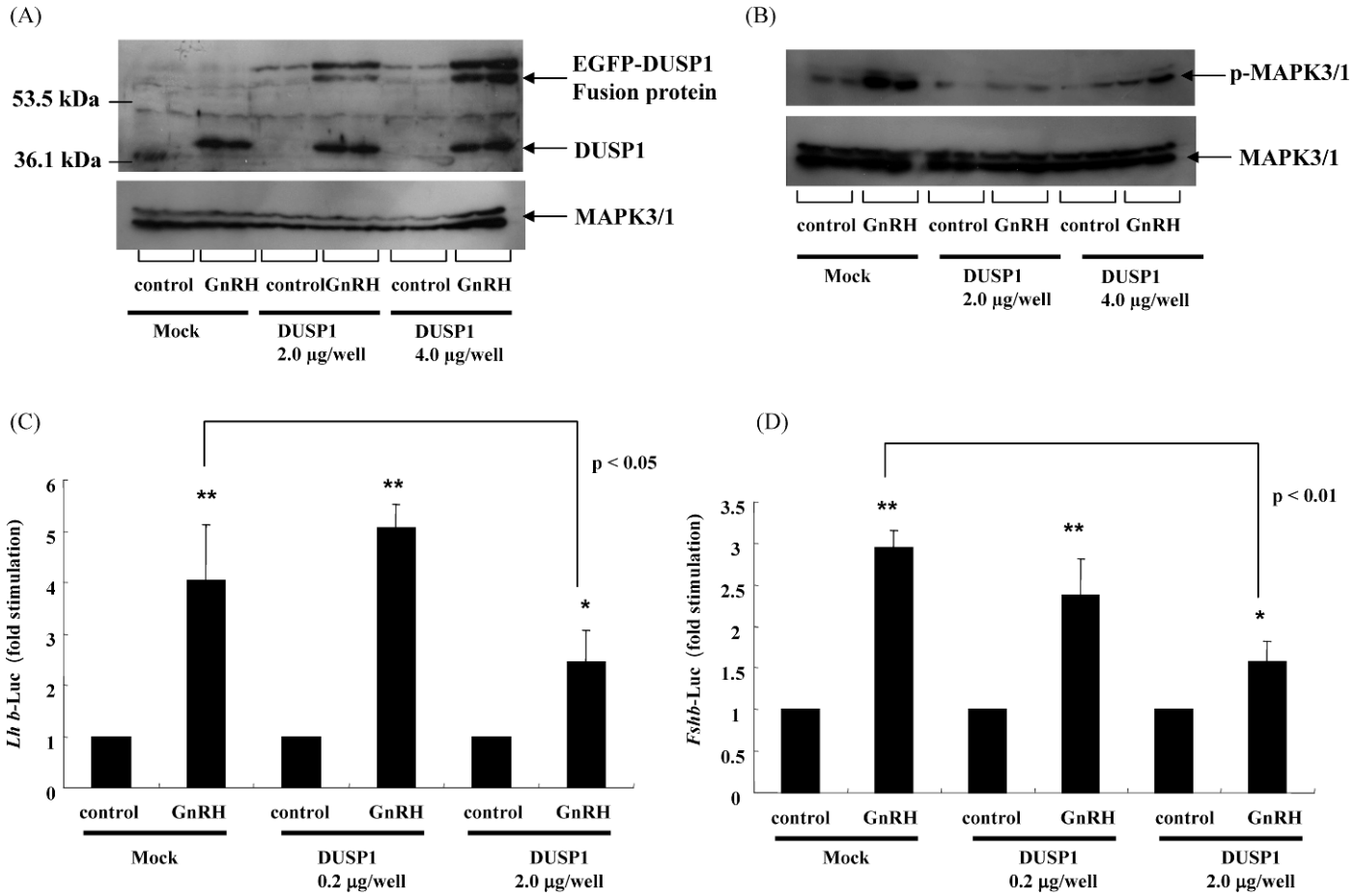


FIG. 7. Effect of overexpression of DUSP1 on MAPK3/1 phosphorylation and gonadotropin subunit promoter activities. LbT2 cells were transfected with 2.0 μg or 4.0 μg of pWay21-DUSP1FL vector, which linked with EGFP. As a mock control, 2.0 μg PCI-neo empty vector was transfected. Forty-eight hours after transfection, cells were treated with 100 nM GnRH for 2 h. After cells were harvested, cell lysates (5 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibody against DUSP1 (**A**), phosphorylated MAPK3/1 (**B**), and total MAPK3/1. Cells transfected with the pWay21-DUSP1 FL vector were also transfected with 2.0 μg of either *Lhb*-subunit (*Lhb*-Luc) or *Fshb*-subunit (*Fshb*-Luc) and PRL-TK (0.1 μg). After incubation for 36 h, a luciferase assay was then performed to examine *Lhb* (**C**) and *Fshb* (**D**) promoter activity, which was then normalized to PRL-TK activity and expressed as the fold of activation over the unstimulated controls. Luciferase activity was then measured and expressed as the fold stimulation of the control. Values are means ± SEM (three independent experiments done with triplicate samples). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. The difference between GnRH stimulation in mock and in 2.0 μg/well DUSP1 transfected cells were statistically significant ($P < 0.05$ for LHβ and $P < 0.01$ for *Fshb*).

current results are consistent with the knowledge that DUSP1 serves as a negative feedback regulator in the MAPK pathway.

We also found that the induction of DUSP1 following GnRH stimulation partially depends on MAPK3/1; 10 μM of U0126, which was enough to inhibit GnRH-increased MAPK3/1 phosphorylation completely, decreased GnRH-induced DUSP1 expression partially (Fig. 2). These results suggest that DUSP1 induction by GnRH occurs via both MAPK3/1-dependent and -independent mechanisms. These results are also observed in the αT3–1 cell, in which induction of MKP-2 by GnRH requires MAPK3/1 and MAPK8 activation as well as calcium-related signals [27]. The regulation of DUSP1 by MAP kinase families has been well described, and the signals that lead to the induction of DUSP1 may be specific to each cell type or type of stimuli [40, 42–44].

Next, we employed triptolide to determine the function of DUSP1. Triptolide induces apoptosis in tumor cells [45], and this apoptotic effect is mediated by the prevention of DUSP1 expression, which results in an increase in MAPK3/1 phosphorylation [46]. In the macrophage, proinflammatory cytokine biosynthesis is prevented by triptolide with the concomitant prevention of DUSP1 expression. This leads to activation of MAPK8 and MAPK14 [26, 47, 48]. In our study, as expected, the increase in DUSP1 expression by GnRH was reduced in the presence of triptolide in a dose-dependent manner. In contrast, concomitant with the inhibition of GnRH-induced DUSP1 expression by triptolide, MAPK3/1 phosphorylation was increased by triptolide, likely through elimination of the negative feedback response (Fig. 3). Regarding gonadotropin subunit gene expression, involvement of MAPK3/1 signaling pathways has been described in previous reports [6, 11, 49]. In this present study, we have again confirmed that both gonadotropin subunit promoter activities are reduced in the presence of the MEK inhibitor U0126 (Fig. 4), suggesting that the activation of the gonadotropin subunit promoter requires the MAPK3/1 pathway. Additionally, we have observed that the decrease in subunit promoter activity by U0126 is more significant in *Lhb* than in *Fshb*. *Lhb* promoters might be more sensitive to the MAPK3/1 signaling pathway compared to *Fshb*. The effect of triptolide, which inhibits DUSP1 expression, on gonadotropin promoters is somewhat unclear. We first expected that inhibition of DUSP1 by triptolide would increase MAPK3/1 activity by preventing MAPK3/1 phosphorylation and ultimately increase gonadotropin promoter activity. The results of DUSP1 expression and MAPK3/1 phosphorylation in the presence of triptolide were as

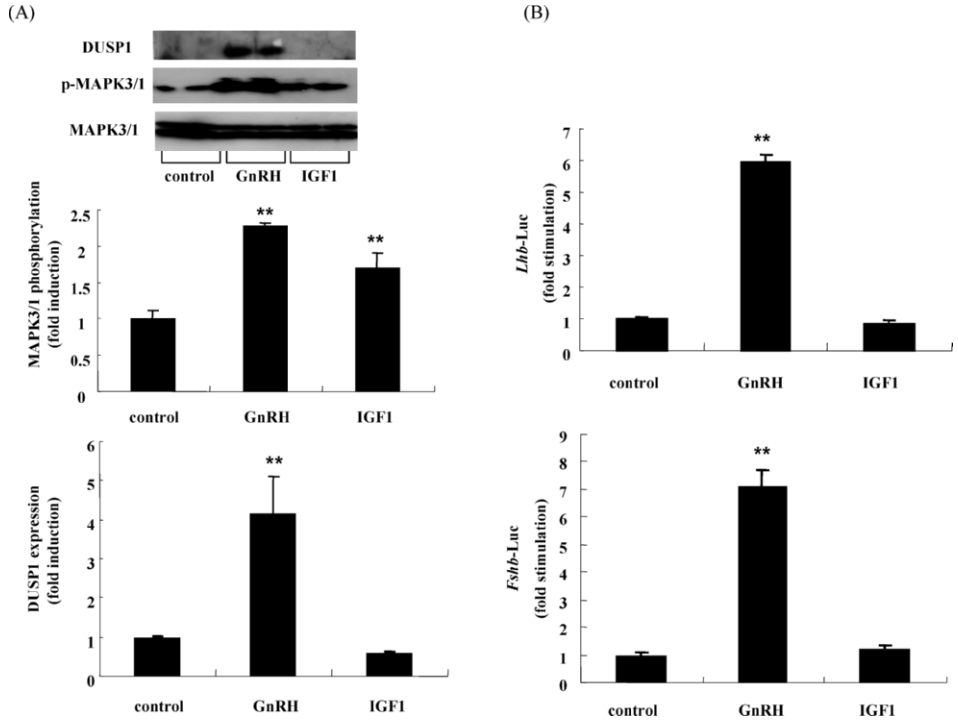


FIG. 8. DUSP1 expression and MAPK3/1 phosphorylation by IGF1 and the effect on gonadotropin subunit promoter activity. **A**) LβT2 cells were stimulated without or with 100 nM GnRH and 100 ng/ml IGF1 for 30 min. Cell lysates (5.0 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibody against DUSP1, phosphorylated MAPK3/1, and total MAPK3/1. The visualized bands were quantified by scanning densitometry using NIH Image and normalized to total MAPK3/1. Results were expressed as the fold increase over the nonstimulated cells (control) and represent the means ± SEM from three independent experiments. Representative autoradiographs are shown. $^{**}P < 0.01$ vs. control. **B**) LβT2 cells were cotransfected with 2.0 μg of either *Lhb*-subunit (*Lhb*-Luc) or *Fshb*-subunit (*Fshb*-Luc) and PRL-TK (0.1 μg) and incubated for 36 h. Cells were then preincubated in serum-free DMEM for 30 min and further treated with 100 nM GnRH for 6 h with the exception of the control cells. A luciferase assay was then performed to examine *Lhb* and *Fshb* promoter activity, which was then normalized to PRL-TK activity and expressed as the fold of activation over the unstimulated controls. Luciferase activity was then measured and expressed as the fold stimulation of the control. Values are means ± SEM (three independent experiments done with triplicate samples). $^{**}P < 0.01$ vs. control.

expected; however, triptolide strongly inhibited the effects of GnRH on gonadotropin promoters (Fig. 5A and B). To verify the effect of triptolide, *Srf*-luciferase reporter constructs were applied. *Srf* is a DNA domain in the promoter region that binds MAPK3/1-mediated transcriptional factors. In the presence of U0126, GnRH-increased *Srf*-Luc promoter activity was significantly inhibited as expected. However, triptolide completely prevented the GnRH action on *Srf* promoters despite an

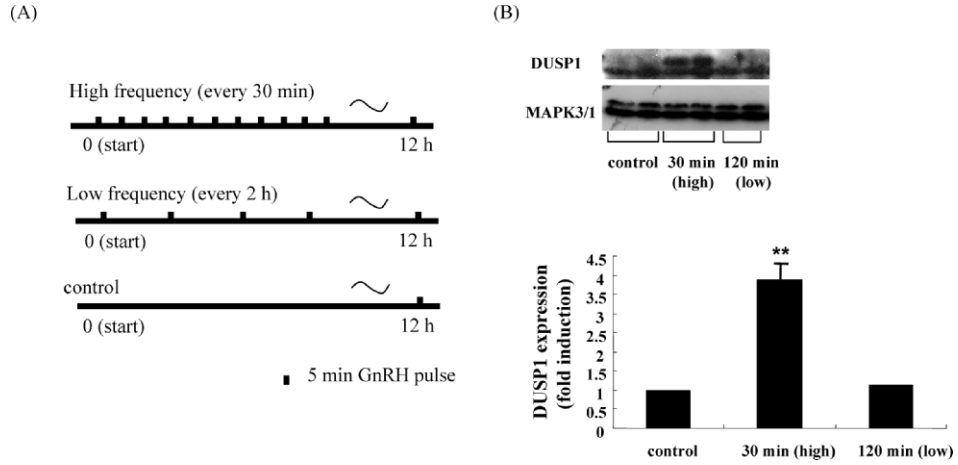


FIG. 9. Pulsatile GnRH stimulation and MKP-1 expression. **A**) LβT2 cells were plated in perifusion chambers and stimulated with pulsatile GnRH (100 nM, 5-min pulses) at a frequency of one pulse every 30 min or one pulse every 2 h. Cells were treated with pulsatile GnRH for 12 h, followed by the harvesting and collection of lysates. **B**) Cell lysates (5.0 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibody against DUSP1, phosphorylated MAPK3/1, and total MAPK3/1. The visualized bands were quantified by scanning densitometry using NIH Image and normalized to total MAPK3/1. Results were expressed as the fold increase over the nonstimulated cells (control) and represent the means ± SEM from three independent experiments. Representative autoradiographs are shown. $^{**}P < 0.01$ vs. control.

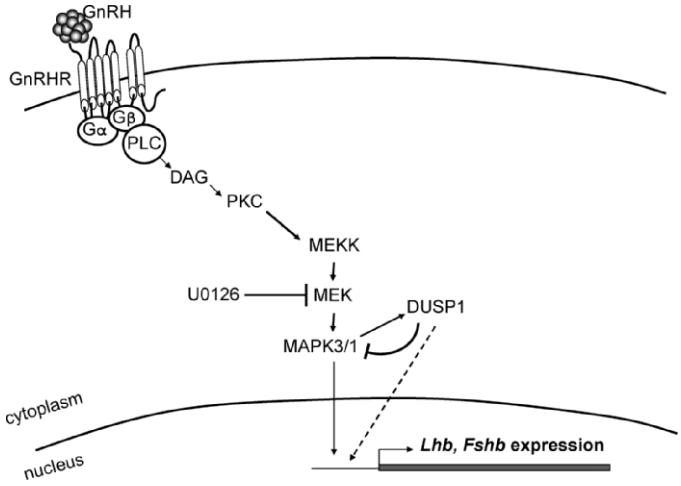


FIG. 10. Schematic summary of the possible role of DUSP1.

increase in MAPK3/1 phosphorylation in the presence of this component (Fig. 5C). If MAPK3/1 phosphorylation is potentiated by triptolide through prevention of DUSP1 expression, one would expect *Srf*-promoter activity to increase. From this determination of *Srf*-promoter activity, we concluded that although triptolide is an inhibitor of DUSP1, while this is the case, this reagent has many other nonspecific effects, including blockade of several types of cellular events.

Several physiologic functions of DUSP1 protein have been described [24, 50–52], but most of these actions are correlated with the role of DUSP1 as a dephosphorylation enzyme against the MAPK pathway. However, DUSP1 has been suspected to have additional functions beyond its role as a phosphatase. Concerning the specific effect of triptolide on DUSP1, we then employed the siRNA method to knockdown DUSP1 expression. The gonadotropin promoter activities were not increased even though MAPK3/1 phosphorylation was augmented as a result of the diminished effect of DUSP1 as MAPK3/1 dephosphorylation enzyme (Fig. 6). This result supports our hypothesis that DUSP1, as an immediate early gene, not only acts as an MAPK3/1 dephosphorylation enzyme but may also play a role in gonadotropin gene expression. In another experiment, overexpression of DUSP1 protein significantly prevented GnRH-induced MAPK3/1 phosphorylation. Both *Lhb* and *Fshb* promoter activities were reduced by the expression of a sufficient amount of DUSP1 following transfection of a DUSP1 expression vector (Fig. 7). These results imply that GnRH-increased MAPK3/1 phosphorylation is negated by overexpression of DUSP1, which ultimately results in a decrease of gonadotropin subunit promoter activities through inhibition of MAPK3/1 activity. Taken together, we suggest that gonadotropin expression somehow requires a balance between MAPK3/1 phosphorylation and DUSP1 expression. In addition, we also observed that *Lhb* subunit gene expression was even significantly reduced when DUSP1 was inhibited by siRNA. This phenomenon was not observed in *Fshb* subunit (Fig. 6). These data support the idea that *Lhb* subunit might be more sensitive to DUSP1 than *Fshb*.

Furthermore, we employed insulin-like growth factor (IGF1), an important neurotrophic hormone in the somatotropic axis, which affects gonadotropin secretion [53]. IGF1 signals are transmitted through phosphatidylinositol 3-kinase (PI3-kinase) which triggers the MAPK signaling cascade [54]. Previously, we have demonstrated that MAPK3/1 is phosphorylated by IGF1 similar to that of GnRH [35]. As shown in Figure 8, although this neuropeptide has the ability to stimulate MAPK3/1 phosphorylation, IGF1 fails to stimulate gonadotropin subunit promoter activity. This result implies that MAPK3/1 is necessary for GnRH-induced gonadotropin gene expression but is not sufficient to control gonadotropin promoters individually. In addition, IGF1 did not increase DUSP1 expression, suggesting that DUSP1 expression was not an automatic sequel of MAPK3/1 phosphorylation. Considering the observation that GnRH increases gonadotropin subunit promoter activity concomitant with MAPK3/1 phosphorylation and DUSP1 expression, IGF1 fails to activate promoters, possibly because of an absence of DUSP1 expression. The experiments using the perifusion system suggest that DUSP1 has a role in gonadotropin expression beyond its function as a phosphatase. GnRH pulse frequency determines specific gonadotropin subunit gene expression in LβT2 cells; that is, the *Lhb* gene promoter is preferentially activated by higher GnRH pulse frequencies, whereas the *Fshb* gene promoter is activated by lower pulse frequencies [34]. DUSP1 expression was only observed following stimulation with high-frequency GnRH pulses (Fig. 9B). Considering the pulse frequency-dependent gonadotropin subunit expression, DUSP1 may be more closely associated with *Lhb* subunit gene expression than with *Fshb* expression. The MEK inhibitor U0126 had a greater effect on the *Lhb* promoter than on the *Fshb* promoter. This suggests that the *Lhb* promoters might be more sensitive to MAPK3/1 and therefore may be more easily affected by DUSP1. In addition, the results of our perifusion experiments are a step toward elucidating the mechanism of the differential mechanism of gonadotropin subunit gene expression following exposure to pulsatile GnRH. One explanation is that the high frequency of GnRH pulses increases MAPK3/1 and DUSP1 activation, which leads to *Lhb* promoter activation. Indeed, *Lhb* expression is more sensitive to the effects of MAPK3/1 and DUSP1. A more detailed study is necessary to completely describe this preliminary observation.

As summarized in Figure 10, this study demonstrated the pattern of DUSP1 expression following GnRH stimulation and demonstrated a mutual interaction of MAPK3/1 phosphorylation and DUSP1 expression. Gonadotropin synthesis required MAPK3/1 phosphorylation; however, DUSP1, as an immediate early gene, may have functions that directly control gene expression besides the dephosphorylation of MAPK3/1.

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