—Original Article—

Estrogen induces phosphorylation of prolactin through p21-activated kinase 2 activation in the mouse pituitary gland

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Abstract. A phosphorylated prolactin is a kind of modified prolactin, which is produced through phosphorylation of native prolactin (PRL) by p21-activated kinase 2 (PAK2) in secretory granules in lactotrophs. Phosphorylated prolactin is involved in the regulation of the estrous cycle and in apoptosis in cancer cells, which seem to have important physiological and pathological roles, respectively. In previous research, it has been reported that estrogen induced the phosphorylation of prolactin in the mouse pituitary gland. However, the relationship between estrogen and PAK2 in the production of phosphorylated PRL has not been clarified yet. In order to examine whether PAK2 is involved in PRL phosphorylation by estrogen, we analyzed PAK2 protein levels in mice and phosphorylated prolactin levels in mouse pituitary cells by western blot analysis. The ratio of phosphorylated PAK2/total PAK2 was increased in estrogen implanted mice, but PAK2 protein and gene expression levels were decreased. In addition, the ratio of phosphorylated prolactin/non-phosphorylated prolactin was decreased in primary pituitary cells with introduced siPAK2. These findings suggest that estrogen could induce the phosphorylation of PRL through PAK2 activation. Therefore, this study contributes to better understanding of the mechanism of phosphorylated PRL production in physiological and pathological conditions associated with estrogen.
Key words: Estrogen, PAK2, Phosphorylation, Pituitary, Prolactin

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Prolactin (PRL) is a 23 kDa peptide hormone, which is mainly produced in, and secreted from, lactotrophs in the pituitary gland. Its main functions are milk production, mammary gland development and maintaining pregnancy, although over 300 different functions have been reported [1, 2]. The diversity of its functions is thought to be due to the existence of modified PRL; phosphorylated, glycosylated and cleaved PRL. One of these modified PRL is a phosphorylated prolactin (PPRL) that is modified by some kinases: protein kinase A, casein and p21-activated kinase 2 (PAK2) [3, 4]. The phosphorylation sites of PRL are specified in each species; phosphorylation of rat PRL occurs on serine 177, while the equivalent site for human PRL is on serine 179 [5]. Other reported phosphorylation sites are threonine 58 or 63 for rat PRL and serine 179 or 180 for bovine PRL [6, 7]. In addition, mouse PRL is phosphorylated on serine 133 and threonine 58 or 63, which can produce serine phosphorylated PRL (PPRLS) and serine/ threonine phosphorylated PRL (PPRLT) [8]. The pI of native PRL and PPRLT (the most acidic) are 5.93 and 5.67, respectively. The quantity of PRL phosphorylation is physiologically regulated during the estrous cycle, pseudopregnancy and pregnancy [9]. The functions of PPRL are diverse; inhibiting cell proliferation [10] and angiogenesis [11] and inducing cell differentiation [12]. PPRL also induces apoptosis in prostate cancer cells [13]. Therefore, PPRL seems to have important physiological and pathological roles. Phosphorylation of PRL occurs in secretory granules prior to exocytosis which contain PAK2 that can phosphorylate PRL [14, 15]. PAK2 is a serine/threonine protein kinase, which is an effector protein for the Rho protein. It is known that PAK2 has an active form. Also, PAK2 has an autoinhibitory domain (AID) which plays an important role in the activation of PAK2. In dimerizing PAK2, AID prevents autophosphorylation and the subsequent activation of PAK2 kinase activity. Once PAK2 becomes monomer, it is autophosphorylated and activated, and is known as phosphorylated PAK2 (pPAK2).

It has been reported that estrogen induces two kinds of phosphorylated PRL, PPRLS and PPRLT, in mice pituitary glands [8]. However, the relationship between PAK2 and estrogen in the production of PPRL has not been clarified. So, in this study we examined whether estrogen altered the expression of PAK2 and phosphorylated PAK2, and if PAK2 could be involved in phosphorylation of PRL.

Materials and Methods

Animals

ICR mice were housed under controlled temperature conditions (22 ± 2ºC) in an artificially illuminated room (12h-light/12h-dark). Food and tap water were available *ad libitum*. Females were mated with males at 70–90 days. The day of birth was designated as day 0. At 8–9 weeks, the female mice were ovariectomized (OVX) under isoflurane anesthesia. A silastic tube (Kaneka, Tokyo, Japan) containing 10 mg of 17β-estradiol (E2; TOKYO CHEMICAL INDUSTRY, Tokyo, Japan) was subcutaneously implanted into OVX female mice 7 days after OVX and the tube was left in place for 7 days. Control mice were treated with an implanted silastic tube without

E2. After 7 days anterior pituitary glands were collected and lysed in Tissue-Protein Extract Reagent (Thermo Fisher Scientific, Waltham, MA, USA) to extract the protein. The total RNA was extracted from the anterior pituitary glands using an RNeasy mini Kit (Qiagen, Venlo, The Netherlands) and reverse-transcribed into cDNA using a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol.

Tissue preparation

Perfusion fixation was performed under isoflurane anesthesia. After anesthetizing, the mice were transcardially perfused with 10 ml PBS, and then perfused with 10 ml 4% paraformaldehyde (PFA). The pituitary glands of the mice were collected and immersed in 4% PFA for 2 h. Then the pituitary glands were dehydrated and embedded in paraffin wax, using an automated embedding system. Subsequently, tissue sectioning was performed, with the thickness of the tissue sections fixed at 6 μm. The sections were mounted on glass slides (MATSUNAMI, Osaka, Japan). The mounted sections were used for HE staining and immunohistochemistry.

Immunohistochemistry

Prior to immunohistochemistry, the tissue sections were deparaffinized in xylene and ethanol. Sections were treated with Block Ace for 1 h at room temperature and incubated with PRL monoclonal antibody (Abcam, Cambridge, UK) at a 1:5000 dilution over night at 4ºC. Then, the sections were incubated with a cy3 conjugated-anti rabbit secondary antibody at 1:500 dilution for 1 h and treated with 10% rabbit serum for 1 h. After blocking, the sections were incubated with an alexafluor 647 conjugated-PAK2 antibody at 1:250 dilution for 1 h at room temperature. Coverslips were mounted in FULUORSHIELD with DAPI (Vector laboratories, Burlingame, CA, USA) and images were acquired on a FLUOVIEW FV-1000 microscope (Olympus, Tokyo, Japan). Ten fields were selected randomly from each section under a 200 × field, and the PRL, PAK2 and DAPI positive cells in the field were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Real Time PCR

Total cDNA of the mouse anterior pituitary gland was used as the template for real-time PCR with SYBR select master mix and the 7500 real-time PCR system (Thermo Fisher Scientific). Specific primers for *Prl* and *Gapdh* genes are presented in Table 1. The expression level of *Gapdh* was used for reference to quantify the relative target gene expression levels.

Western Blotting.

Equivalent amounts of mouse pituitary protein were electrophoresed on SDS-polyacrylamide gels. Western C (BIO-RAD, Hercules, CA, USA) were used to determine molecular weight. Then, the separated proteins were electrophoretically transferred onto a PVDF membrane (Merck Millipore, Darmstadt, Germany), which was treated with EzBlock Chemi (ATTO, Tokyo, Japan). Subsequently, the membrane was incubated with an anti PAK2 antibody (Cell Signaling Technology, Denvers, MA, USA) at 1:2500 dilution and anti-pPAK2 antibody (Sigma Aldrich, St. Louis, MO, USA) at 1:2500 dilution overnight at 4ºC. After the incubation, the membrane was incubated with a

Table 1. Sequences of oligonucleotide primers used for Real Time PCR

Targeted gene		Primer sequence (5'-3')	Product size (bp)
Pak2	Forward	ACACCAGCACTGAACACCAA	82
	Reverse	GAGGAGCAGCGTCTTCATCA	
Gapdh	Forward	AGGTCGGTGTGAACGGATTTG	123
	Reverse	TGTAGACCATGTAGTTGAGGTCA	

peroxidase-labeled anti-rabbit IgG antibody (Vector laboratories) at 1:10000 dilution for 30 min at room temperature. After washing the membrane, the immunoreactive bands were detected by immobilon western chemiluminescent HRP substrate (Merck Millipore). The bands were quantified with ImageJ software (NIH)

Primary pituitary cell culture

Female mice (8–9 weeks old) were ovariectomized under isoflurane anesthesia and anterior pituitary glands were collected 7 days after OVX. The anterior pituitary glands were incubated in solution 1 (1% Trypsin, 0.2% collagenase in Hanks' Balanced Salt Solution, HBSS) for 15 min. After centrifugation at 2,000 *g* for 5 min, the supernatant was removed by aspiration, and solution 2 (1% Trypsin, 0.2% collagenase, 0.5 μg/ml DNase in HBSS) was added and incubated for 5 min. After a further centrifugation at 2,000 *g* for 5 min, the supernatant was removed by aspiration, and solution 3 (5 mM EDTA in HBSS) was added and incubated for 5 min. The disassociated cells were filtered by cell strainer and counted with a hemocytometer. The primary anterior pituitary cells were seeded onto an 8-well slide chamber at a density of 1.0×10^5 cells/well and cultured overnight in Medium 199 (Thermo Fisher Scientific) with 10% FBS. siPAK2 (100 nM, SI01368647, Qiagen) or siControl (100 nM, AllStars Negative Control siRNA; Qiagen) were introduced into the cells with INTERFERin (Polyplus transfection, Illkirch, France). The cells were treated with 1.0×10^{-8} M E2 for 48 h. Then, the cells were lysed in 9.8 M Urea Buffer. The total RNA was extracted from these cells using an RNeasy mini Kit (Qiagen) and reverse-transcribed into cDNA using a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol.

Immunocytochemistry of primary pituitary cells

The primary anterior pituitary cells were fixed with 4% PFA for 15 min at room temperature, then treated with Block Ace for 30 min at room temperature followed by incubation with PRL monoclonal antibody (Abcam) at a 1:5000 dilution overnight at 4ºC. Then, the sections were incubated with an anti-rabbit cy3 secondary antibody at 1:500 dilution for 1 h and blocked with 10% rabbit serum for 1 h. After blocking, the sections were incubated with an alexafluor 647 conjugated PAK2 antibody at 1:250 dilution for 1 h at room temperature. Coverslips were mounted in FULUORSHIELD with DAPI (Vector laboratories) and images were acquired on a FLUOVIEW FV-1000 microscope (Olympus).

Two-dimensional electrophoresis

The anterior pituitary proteins with rehydration buffer were applied

by in-gel rehydration (IPG pH 3–10, GE HealthCare, Chicago, IL, USA) for 12 h according to the manufacturer's instructions. The proteins were then focused for up to 56,000 Vh at a maximum voltage of 3,500 V. The second dimension of electrophoresis was resolved on 12.5% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were subjected to western blotting with PRL monoclonal antibody (Abcam).

Statistics analysis

Experimental data were expressed as mean ± SEM. Tukey-Kramer test (when the number of groups was more than two) and T-test (when the number of groups was two) were used for statistical analysis (P<0.05 was considered statistically significant).

Ethical Statement

All animal experiments were carried out in accordance with the guidelines of Institutional Animal Care and Use Committee of Meiji University that approved the protocol for this study (IACUC16-0011).

Results

Co-localization of PRL and PAK2

In order to confirm whether E2 replacement worked adequately, we observed the size of the pituitary gland and measured the weight of the uterus at day 7 after E2 replacement. The pituitary of the mice with E2 was enlarged compared to that of the control mice (Fig. 1A). The uterus of mice with E2 was also enlarged (Fig. 1B), and was approximately 5 times heavier than that of the control mice (Fig. 1C).

Co-localization of PRL and PAK2 was observed in the anterior pituitary gland of both the E2 and control mice (Fig. 1D, closed arrowhead). Some cells expressed only PAK2 (Fig. 1D, open arrowhead) but all PRL positive cells expressed PAK2 (Fig. 1D, open arrowhead). E2 replacement increased the ratio of PRL positive cells per total cells (Fig. 1E), but it did not alter the ratio of PAK2 positive cells in PRL positive cells (Fig. 1F).

The effect of E2 on PAK2 expression

In order to examine whether estrogen altered the expression of PAK2 in the mouse anterior pituitary gland, western blotting and Real Time PCR analysis were performed on E2 treated OVX mice. Real Time PCR analysis showed that E2 replacement decreased the gene expression of *Pak2* in female mice (Fig. 2A). Similarly, western blotting analysis showed that the protein level of PAK2 was decreased in female mice with E2 (Fig. 2B).

We also analyzed the effect of E2 replacement on the protein level of pPAK2 in the mouse anterior pituitary gland. E2 replacement decreased the protein level of pPAK2 in female mice (Fig. 2C). However, the ratio of pPAK2 per total PAK2 in the mouse anterior pituitary gland of mice with E2 was significantly higher than that of control mice (Fig. 2D).

The role of PAK2 in PRL phosphorylation by E2

In order to confirm that PAK2 was induced by E2 phosphorylated PRL, we introduced siPAK2 into primary anterior pituitary cells. *Pak2* mRNA levels were lower in siPAK2 introduced cells than in control cells, in both cells with and without E2 (Fig. 3A). However,

the *Pak2* mRNA level in siControl introduced cells with E2 was not decreased (Fig. 3A). In addition, the fluorescence intensity of PAK2 was attenuated by siPAK2 (Fig. 3B, PAK2), and the fluorescence intensity of PRL was increased by E2 treatment (Fig. 3B, PRL).

The phosphorylated PRL and non-phosphorylated PRL in the primary anterior pituitary cells was detected by 2-DE (Fig. 4A). The pI of native PRL and PPRLT (the most acidic) were 5.93 and 5.67, respectively, and the PRL between native PRL and PPRLT was PPRLS (Fig. 4A). E2 induced PPRLS and PPRLT in the primary anterior pituitary cells, resulting in an increase in the ratio of each phosphorylated PRL/non-phosphorylated PRL (Fig. 4B, C). siPAK2 suppressed PPRLS induced by E2, resulting in a significant decrease in the ratio of PPRLS/PRL (Fig. 4A, B). The dotblots of PPRLT induced by E2 were suppressed by the introduction of siPAK2, causing a decrease in the PPRLT/PRL ratio (Fig. 4A, C).

Discussion

We showed that E2 decreased *Pak2* mRNA and protein levels in primary anterior pituitary cells, but that the ratio of pPAK2 increased *in vivo* and induced the phosphorylation of PRL. We also showed that siPAK2 suppressed the phosphorylation of PRL induced by E2.

Lactotroph hyperplasia is observed in rodents and humans exposed to high estrogen doses. High doses of estrogen induce rat lactotroph hyperplasia and pituitary adenoma formation [16]. Estrogen also induces the proliferation of endometrium resulting uterus enlargement. In the current study, pituitary hyperplasia and uterus enlargement were observed in E2 mice, indicating that E2 replacement worked properly.

E2 treatment decreased the expression of PAK2 in the mouse anterior pituitary gland *in vivo*, but there was no change in cultured anterior pituitary cells. The reason for this difference may be because the pituitary and hypothalamus are closely connected meaning that that various hormones are regulated. The expression of E2 receptor and its signaling in the hypothalamus has been widely reported [17, 18]. Therefore, the down regulation of PAK2 in mice with E2 seems to be regulated via hypothalamic signaling. In our study, we also showed that PRL phosphorylation occurred in the primary anterior pituitary cells with E2. It has been reported that pituitary cells express E2 receptor [19], so E2 could act on the pituitary cells directly to induce the phosphorylation of PRL through the E2 receptor. In our study, E2 tubes were left in OVX mice for 7 days whereas mouse anterior pituitary cells were treated with E2 for only 48 h. This difference in treatment time might have produced the conflicting results. Therefore, further research is needed to clarify whether longer E2 treatment decreases the expression of PAK2.

In this study, we showed that E2 induced the phosphorylation of PRL in primary anterior pituitary cells. This result corresponded with a previous study that reported that estrogen induced the phosphorylation of PRL *in vivo* [8]. These results suggest that mouse primary anterior pituitary cells could be used for phosphorylated PRL research. As mentioned above, since the pituitary and hypothalamus are closely connected, hypothalamic signaling cannot be ignored in the *in vivo* study. However, in the study of the cultured anterior pituitary cells, that were isolated from the hypothalamus, we could observe the direct effect of phosphorylated prolactin in the pituitary gland.
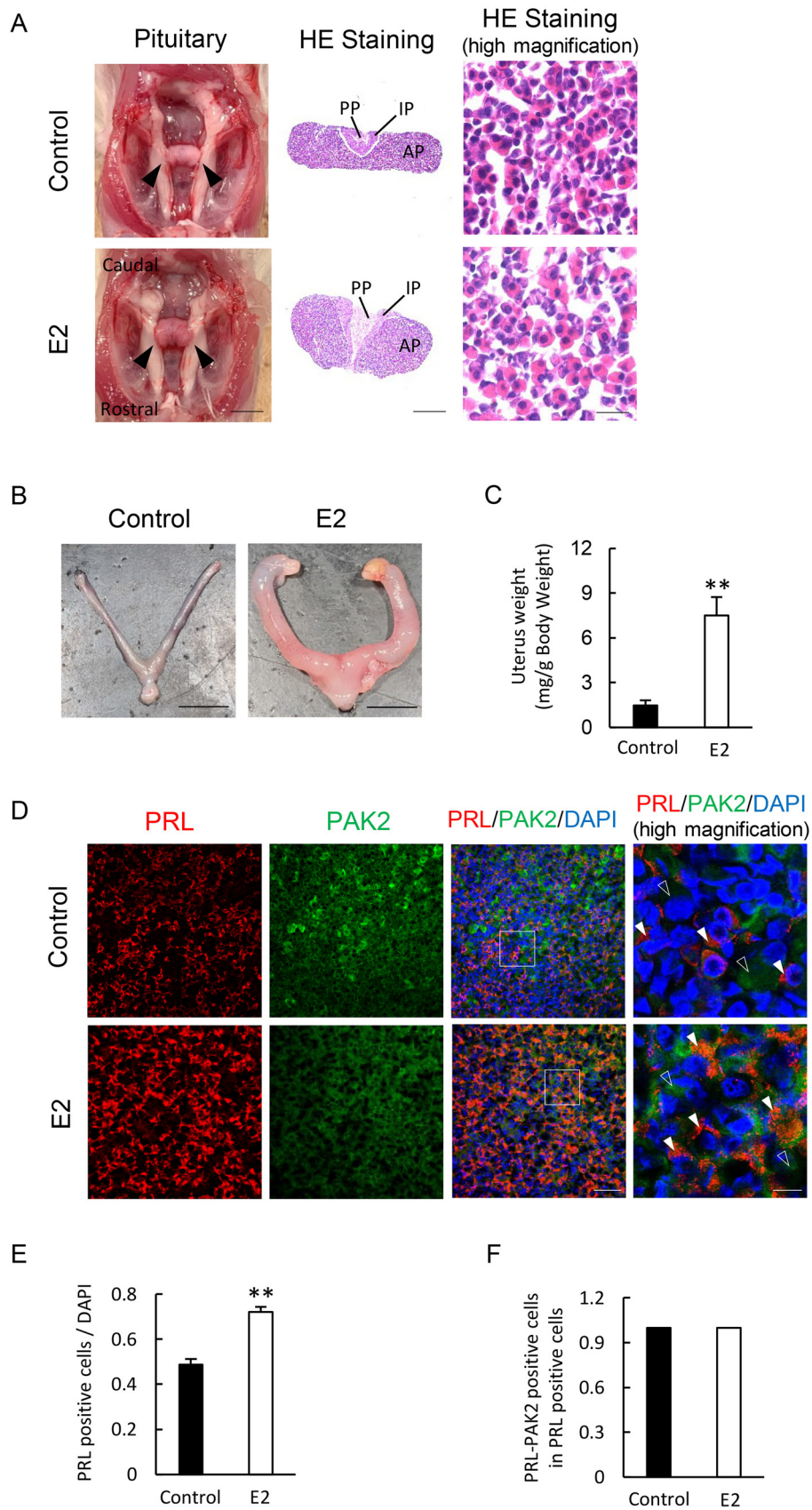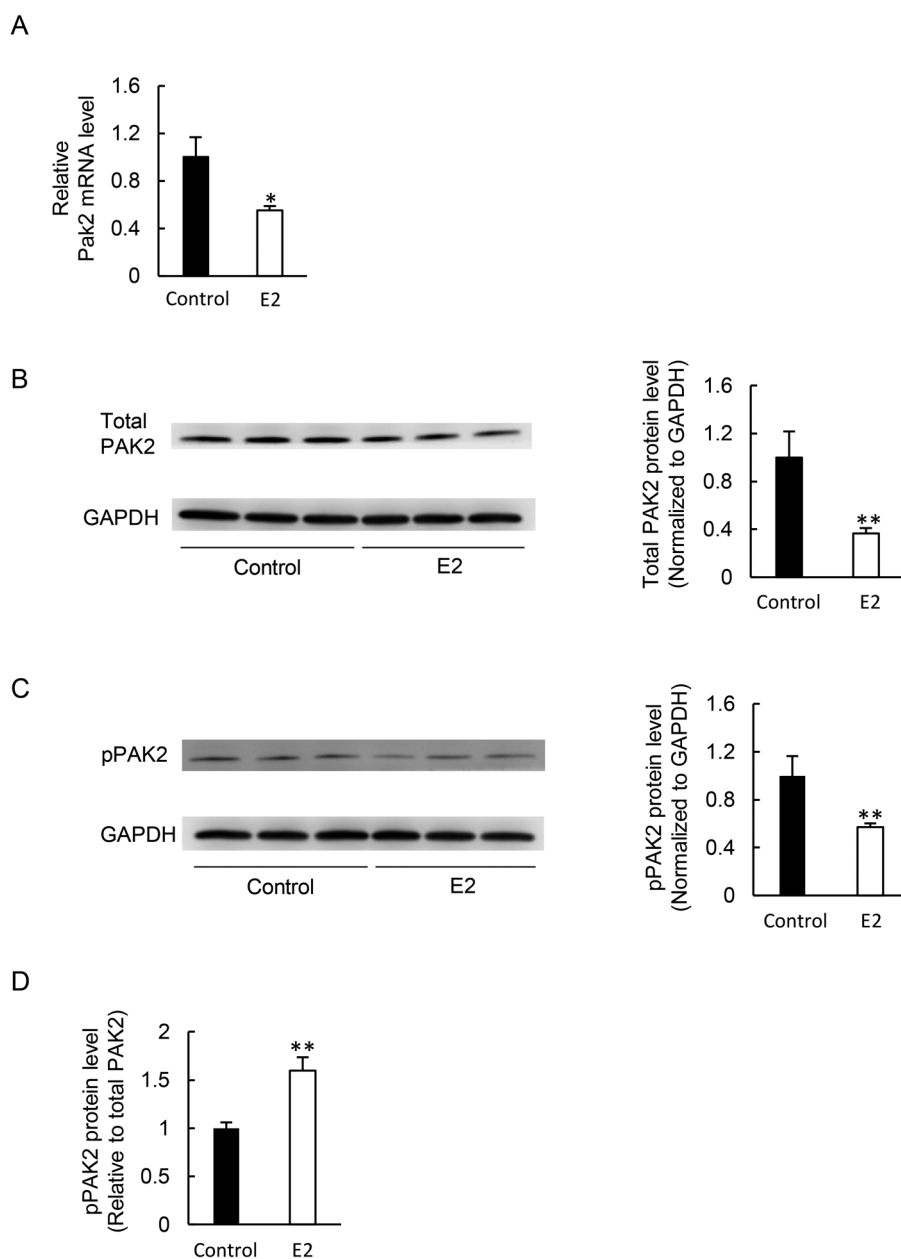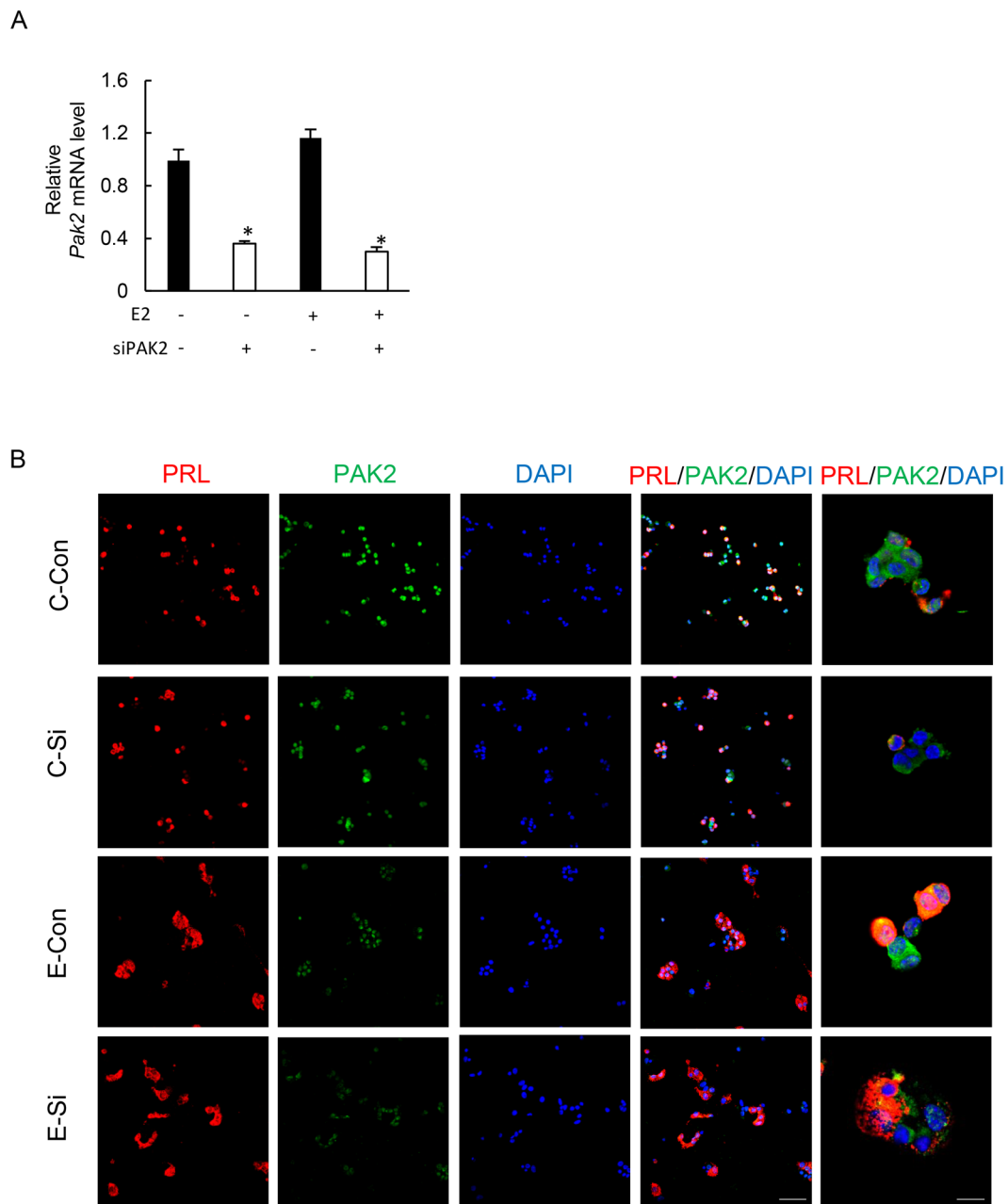
Fig. 1.

Fig. 2. Effect of estrogen on p21-activated kinase 2 (PAK2) mRNA and protein levels in mouse anterior pituitary gland. (A) Relative mRNA level of *Pak2* (normalized to *Gapdh*) in mouse anterior pituitary gland with 17β-estradiol (E2). (B) Western blotting analysis of total PAK2 and relative protein levels of total PAK2 (normalized to GAPDH) in mouse anterior pituitary gland with E2. (C) Western blotting analysis of phosphorylated PAK2 (pPAK2) and relative protein levels of pPAK2 (normalized to GAPDH) in the mouse anterior pituitary gland with E2. (D) Ratio of pPAK2 / total PAK2 in mouse anterior pituitary with E2. Data represent mean ± SEM of 3 independent experiments, asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$).

Fig. 1. Co-localization of prolactin (PRL) and p21-activated kinase 2 (PAK2). (A) Size of mouse pituitary gland with and without 17β-estradiol (E2), and HE Staining. AP, anterior pituitary gland; PP, posterior pituitary gland; IP, intermediate pituitary gland. Right panel shows high magnification of AP. Scale bars indicate 2 mm (left panel), 500 μm (middle panel) and 20 μm (right panel). (B) Mouse uterus with and without E2. Scale bar indicates 1 cm. (C) Weight of mouse uterus with and without E2. Data represent mean ± SEM of 3 independent experiments, double asterisk indicates significant difference (** $P < 0.01$). (D) Immunohistochemistry of anterior pituitary gland with or without E2. Right panels show high magnification of boxed area in PRL/PAK2/DAPI. Closed arrowheads indicate PRL and PAK2 positive cells. Open arrowheads indicate only PAK2 positive cells. Scale bar indicates 50 μm (PRL/PAK2/DAPI) and 10 μm (high magnification). (E) Ratio of PRL positive cells to DAPI positive cells in mouse anterior pituitary gland with E2. Data represent mean ± SEM of 3 independent experiments, double asterisk indicates significant difference (** $P < 0.01$). (F) Ratio of PRL + PAK2 positive cells to PRL positive cells in mouse anterior pituitary gland with E2. Data represent mean ± SEM of 3 independent experiments.

A

B

Fig. 3. siPAK2 suppressed p21-activated kinase 2 (PAK2) mRNA and protein levels. (A) Relative mRNA level of *Pak2* (normalized to *Gapdh*) in primary anterior pituitary cells with introduced siPAK2. This data shows the expression fold change compared to control + siControl. (B) Immunocytochemistry of primary anterior pituitary cells. Scale bar indicates 50 μm (DAPI/PRL/PAK2) and 10 μm (higher magnification). C-Con, control + siControl; C-Si, control + siPAK2; E-Con, E2+siControl; E-Si, E2+siPAK2. Data represent mean ± SEM of 3 independent experiments, asterisks indicate significant differences (* $P < 0.05$).

The mRNA expression of *Pak2* was decreased in the primary anterior pituitary cells with introduced siPAK2, but not in the siControl cells. This suggests that siPAK2 suppresses PAK2 expression. The immunocytochemistry of the primary anterior pituitary cells, showed that introducing siPAK2 did not change the PRL immunostainability, indicating that the introduction of siPAK2 did not affect PRL expression.

In this study, we showed that E2 increased the ratio of PPRL/PRL in mouse primary anterior pituitary cells. However, we also found that E2 decreased the expression of PAK2 in mice pituitary glands. Hence, the phosphorylation of PRL could be independent of the quantity of PAK2. This result agrees with the results of a previous report
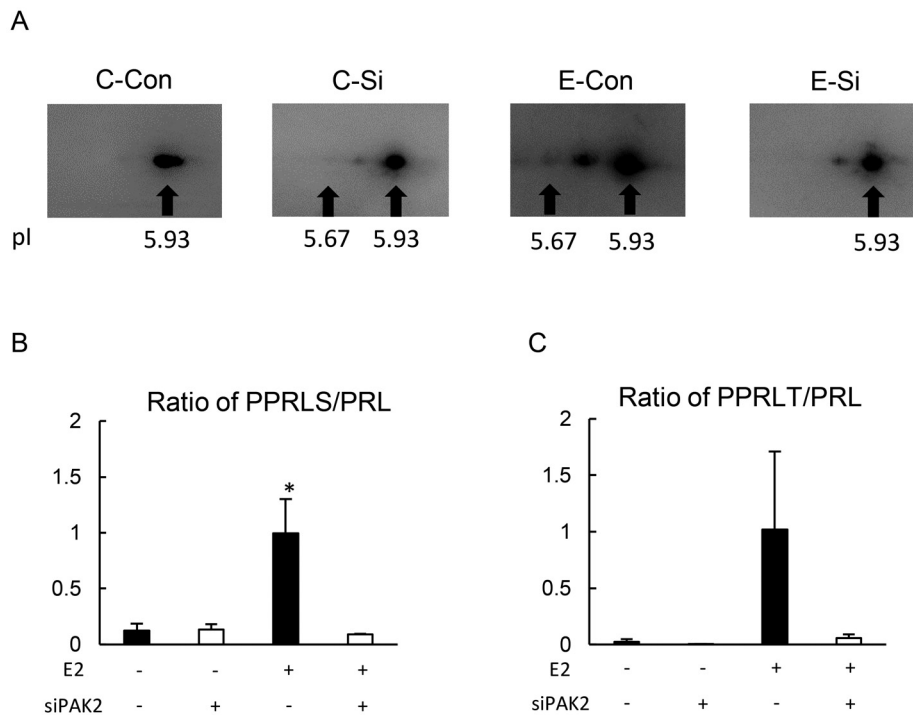
Fig. 4. siPAK2 suppressed expression of phosphorylated prolactin (PPRL) in primary anterior pituitary cells with 17β-estradiol (E2). (A) Dotblots of primary anterior pituitary cells treated with siPAK2 and E2. Dots were probed with monoclonal PRL antibody. C-Con, control + siControl; C-Si, control + siPAK2; E-Con, E2 + siControl; E-Si, E2 + siPAK2. (B) Ratio of serine phosphorylated PRL (PPRLS)/PRL in primary anterior pituitary cells treated with siPAK2 and E2. (C) Ratio of PPRLT/PRL in primary anterior pituitary cells treated with siPAK2 and E2. Data represent mean ± SEM of 3 independent experiments, asterisks indicate significant differences (* P < 0.05).

which showed that E2 suppressed phosphorylation in the pituitary gland [20]. In our study, we also showed the activation of PAK2 by E2. E2 decreased the quantity of pPAK2 but increased the ratio of pPAK2/total PAK2. These results suggest that the phosphorylation ratio could be more important than the quantity of pPAK2 with regard to the phosphorylation of PRL.

We demonstrated that the introduction of siPAK2 suppressed the serine phosphorylation of PRL by E2 in anterior pituitary cells. In addition, PRL and PAK2 co-localization were detected by immunohistochemical observation. These results show that PAK2 is involved in the serine phosphorylation of PRL. However, PPRLS has been detected in anterior pituitary cells without E2, therefore other kinases could also play a role of the serine phosphorylation of PRL, in addition to PAK2. We also demonstrated that the suppression of PAK2 induced the suppression of the serine/threonine phosphorylation of PRL by E2. This suggests that PAK2 mainly regulates the serine/threonine phosphorylation of PRL.

PRL is aggregated by Zn^{2+} and stored in secretory granules in lactotrophs [21, 22]. Treating pituitary with Ca^{2+} has been shown not to increase the quantity of PPRL whereas treating it with Zn^{2+} does increase PPRL [15]. Therefore, kinases which are activated by Zn^{2+} in secretory granules might be other candidates for phosphorylation of PRL, in addition to PAK2.

In conclusion, we showed that E2 induced the phosphorylation of PRL in anterior pituitary cells and that the phosphorylation was inhibited by siPAK2. Our findings suggest that E2 induces the phosphorylation of PRL through PAK2 activation in the mouse pituitary gland.

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