Downregulation of Estrogen Receptor Gene Expression by Exogenous 17β-Estradiol in the Mammary Glands of Lactating Mice

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The biological actions of estrogen are mostly conveyed through interaction with the nuclear estrogen receptor (ER). Previous evidence indicated that estrogen participates in self-regulation through the modulation of the expression of its own receptors. However, the self-regulation of estrogen against ER in the mammary gland during established lactation has not yet been investigated. The present study evaluated ER gene expression in the lactating gland activated by large doses of 17β-estradiol (E_2). Repeated E_2 treatments dose-dependently decreased the gene expression of ER, especially its subtype ER-α mRNA, which was decreased to 10% of the vehicle-injected control by 1 μg E_2 injection, whereas it was decreased by 73% for another subtype, ER-β. A single injection of 5 μg of E_2 drastically downregulated both ER genes within 12 hrs of injection, and they did not recover to pretreatment level within 48 hrs. Western blot analysis verified that E_2 treatment inhibited the phosphorylation of Stat5, which is a potent transcriptional regulator for ER mRNA. The present findings demonstrate that E_2 treatment decreases the gene expression of its own receptor in the mammary gland during galactopoesis and induces an apparent transition of the ER profile in the mammary gland during lactation into postlactation. Exp Biol Med 231:311–316, 2006

Key words: ER-α; ER-β; 17β-estradiol; Stat5; mammary gland; lactation

Introduction

The biological actions of estrogen are mostly conveyed through interaction with the nuclear estrogen receptor (ER). Two different subtypes of ER have been identified, termed ER-α and ER-β, which are encoded by different genes. These forms of ER possess different functional properties and show distinct patterns of gene regulation (1–4). Net estrogen sensitivity in the target tissue, including the mammary gland, critically depends on the balanced expression of several ER subtypes and isoforms with distinct functional properties. It has been documented that four distinct stages of the mammary gland in rodents can be defined on the basis of the ER profile: (i) prepuberty—both ER-α and ER-β are present in epithelial cell nuclei, (ii) pregnancy—ER-β is present in the majority of epithelial cells and ER-α expression is low, (iii) lactation—ER-α and ER-β are both expressed in the majority of epithelial cells, and (iv) postlactation—ER-α is extremely low and there is little colocalization of the two receptors (5). During lactation, plasma estradiol (E_2) concentration is maintained at a relatively low level until the weaning period (see Ref. 6), whereas ERs are present in the mammary tissue throughout the lactation period (7–9). In addition, the lack of endogenous E_2 caused by ovariectomy does not affect milk production during lactation (10). These findings complicate the interpretation of the functional significance of estrogen and/or ERs in normal lactation.

The inhibitory effect of exogenous E_2 on the maintenance of lactation has been investigated in a variety of species. The inhibition of lactation by E_2 is attributed mainly to the impairment of milk secretion and the disorganization of secretory mammary morphology (11–15). Mizuno and Sensui (15) and subsequent investigations performed in our laboratory confirmed universally that the litter growth is suppressed gradually with daily systemic injections of 1 μg E_2 to the mother and remarkably with 5–10 μg E_2, but that 0.1 μg E_2 has no effect. In fact, it is not known what occurs in the lactating mammary gland when it is exposed to large doses of E_2, but extraphysiological doses of E_2 can induce the interruption of lactation artificially or pharmacologically. The first goal of the present study was to examine the behavior of both ER genes in the mammary gland of mice in which the interruption of lactation was induced by pharmacological doses of E_2, and to attempt a

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comparison with the aspect during the postlactational period as described above. Under normal conditions, the janus kinase 2 (Jak2)/signal transducer and activator transcription 5 (Stat5) pathway mediating the activation of prolactin receptor (PRL-R) is the major process regulating the expression of the ER gene. The homodimer of Stat5 protein recognizes the DNA binding site or GAS site on the promoter region of ER and regulates the transcription (for a review, see Ref. 16), but has not yet been confirmed in the mammary gland. We also examined whether the aspect of Stat5 in the lactating mammary gland is affected by exogenous E_2.

Materials and Methods

Animals and Treatments. Primiparous lactating mice of the dd strain obtained from our breeding colony were housed in individual cages at 23 ± 2°C under a 14:10-hr light:dark cycle (lights on at 0600 hrs) throughout the experiment. The mice were allowed free access to food and water. Litter size was adjusted to six pups on Day 3 of lactation. The body weight of the mothers and litters, and food and water intake, were measured once daily (0900 hrs) to estimate lactating performance. Mother mice were bilaterally ovariectomized (OVX) or sham-operated (sham) under ether anesthesia on Day 3, and injected ip with 0 (sham, $n=7$; OVX, $n=7$), 1 (sham, $n=5$, OVX; $n=5$) or 5 (sham, $n=5$; OVX, $n=5$) μg/100 μl of E_2 (β-estradiol 3-benzoate; Sigma Chemical Company, St. Louis, MO) dissolved in sesame oil once daily from Day 5 of lactation for 8 days. At 1000 hrs on the final day of the injection (Day 12 of lactation), the mice were killed by cervical dislocation. Abdominal-inguinal mammary glands were removed, weighed, and frozen in liquid nitrogen. Tissues were stored at −80°C before use. The other lactating mice without any surgical operations were single-injected ip with 0 or 5 μg/100 μl of E_2 on Day 10 of lactation, and the mammary glands were removed 0 ($n=5$), 12 ($n=5$), 24 ($n=5$), or 48 hrs ($n=6$; oil-injected control, $n=5$) after injection and stored at −80°C. All experiments conformed to the Guidelines for Animal Experiments, College of Bioresource Sciences, Nihon University.

Semiquantitative RT-PCR. Total RNA from the mammary tissues was isolated using TRIzol Reagent (Invitrogen Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol and quantified spectrophotometrically. The total RNA (1 μg) of each sample was reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) in a final volume of 23 μl, according to the manufacturer's instructions. PCR primers for ER-α were designed to amplify the 583-bp sequence from exon 2 to exon 4 (upstream (US): 5′-TTCTGATGATTGGTCTCGTCTG-3′; downstream (DS): 5′-TGTAAGGAATGTGCTGAAGTGG-3′). The primers for ER-β were designed for the 649-bp sequence from exon 4 to the 3′-untranslated region (US: 5′-GTCAGGCACATCAGTAACAAGG-3′; DS: 5′-TGCGAAACGAGTTGATTGTC-3′). An 865-bp sequence for β-actin was amplified with primers (US: 5′-TAGGCACCAGGGTGTGATGG-3′; DS: 5′-CTTCATGGTGCTAGGAGCC-3′) and used as a control for relative mRNA quantification. For PCRs, 10 μl of Platinum PCR SuperMix (Invitrogen), 1 μl of cDNA, and 0.5 μ*M* gene specific primers, as described above, were mixed in 200 μl PCR tubes. PCR was performed with a thermal cycler, PCR Express (Px2; Thermo Hybaid, Ashford, UK). General PCR conditions consisted of an initial denaturation step at 94°C for 5 mins, followed by additional cycles with a denaturing step at 94°C for 30 secs, an annealing step at 60°C for 30 secs, and an extension step at 72°C for 60 secs. A final extension step was performed at 72°C for 10 mins. The PCR products were electrophoresed on a 1.5% agarose gel, visualized with ethidium bromide, and photographed with a Printgraph (ATTO Corp., Tokyo, Japan) and the images were digitized.

Based on PCR kinetics, a method to calculate the PCR-unamplified initial dose of cDNA templates of a specific gene is effective for the quantification of the mRNA concentration. The amount of PCR products can be expressed in an equation as $Y = I \times E^n$ (Y, PCR products; I, PCR-unamplified cDNA templates; E, efficiency of amplification; n, number of PCR cycles). Each sample was PCR-amplified until 4 distinct cycles, that is, 26, 27, 28, and 29 cycles in ER-α; 28, 29, 30, and 31 cycles in ER-β; and 20, 21, 22, and 23 cycles in β-actin. The optical density of each band was analyzed with NIH Image (version 1.61; NIMH, Research Service Branch, Bethesda, MD). The PCR-unamplified cDNA template of each gene was estimated using a regression equation. All data are represented as relative values to the internal standard, β-actin.

Western Blot for Stat5. The mammary tissues (0.1 g) were homogenized in 1 ml of lysis buffer containing 50 m*M* Tris-HCl (pH 8.0), 0.1% Triton X-100, 5 m*M* EDTA, 150 m*M* NaCl, and 2 m*M* Na_3VO_4. The homogenized tissues were centrifuged at 12,000 *g*, 4°C, for 1 hr, and supernatants were obtained and stored at −80°C. The protein concentration of the supernatants was measured using a DC Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA). Aliquots of protein extract and 2× SDS sample buffer containing 10% glycerol, 4% SDS, 125 m*M* Tris-HCl (pH 6.8), 4% bromophenol blue, 12% 2-mercaptoethanol were mixed and boiled for 5 mins. Aliquots of protein solution (60 μg) were loaded onto each lane of 7.5% polyacrylamide gel at 20 V for 2 hrs. The gel was electrotransferred to nitrocellulose membrane at 100 V for 2 hrs. Nonspecific sites on the membrane were blocked with 5% skim milk in phosphate buffered saline containing 0.2% Tween-20 at room temperature for 1 hr. Following extensive washings in Tween-20/PBS for 5 mins each, the membranes were incubated with the primary antibody to Stat5a (L-20: sc-1081; Santa Cruz, Biotechnology, Santa Cruz, CA), to Stat5a/b (C17: sc-835; Santa Cruz) and antiphospho-Stat5a/b (S5058; Sigma), which was diluted to 1:1000, at room

temperature for 1 hr. Following washes, the membranes were incubated with the secondary antibody (donkey anti-rabbit Ig, HRP-linked whole antibody; Amersham Biosciences, Piscataway, NJ), diluted to 1:1000, at room temperature for 1 hr. Following washes, the membranes were finally developed using enhanced chemiluminescence reagents (ECL Western Blotting Detection System; Amersham). All images were analyzed using NIH Image (version 1.61). The data from the Western blot were used as the representative values from four separate experiments.

Statistics. Statistical significance was based on two-way ANOVA and analysis between groups was tested with subsequent planned comparisons, which contrast the mean comparisons of selected levels of a factor (results for repeated injections). Statistical analyses between the time points were tested by one-way ANOVA with Duncan's new multiple range test (results for single injection), using the statistical software Super ANOVA (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant.

Results

Litter Weight Gain After Repeated Injections of E_2. The profile of the gain in body weight of litter is shown in Figure 1. The body weight of pups increased linearly with age in the vehicle-injected group, whereas repeated E_2 injections diminished litter growth 3–5 days after of injection started. In particular, each litter stopped gaining body weight at Days 5–6 in the 5-μg E_2 injection group. There was no statistical difference between sham and OVX in any injection group.

ER Gene Expression After Repeated Injections of E_2. In lactating mouse mammary glands, the gene expression of both subtypes of ER was observed, and semiquantitative levels of ER-α mRNA were higher by approximately 3.6-fold than those of ER-β. Repeated injections of E_2 dose-dependently decreased both ER-α ($F_{(2,14)} = 54.938$, $P = 0.0001$) and ER-β ($F_{(2,14)} = 12.240$, $P = 0.0008$). One-microgram E_2 treatments reduced gene expression of ER-α to 10% of the control, and 5 μg of E_2 caused the gene expression to almost completely disappear, whereas ER-β gene expression declined gradually with E_2. Ovariectomy did not alter the expression of either ER's mRNA, but suppressed the downregulation of ER-β with a higher dose of E_2 (Fig. 2).

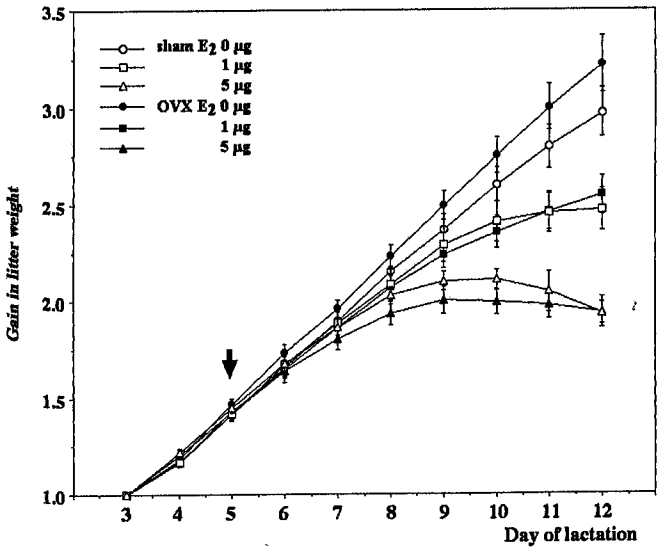

Figure 1. Gain in the litter weight of mother mice with repeated exposure to 0, 1, and 5 μg of E_2 that were bilaterally sham-operated (sham) or ovariectomized (OVX) on Day 3 of lactation. Data are represented as the ratio to the body weight of litter on Day 3 of lactation (means ± SEM). A downward-pointing arrow indicates the beginning day of each injection.

ER Gene Expression After Single Injection of E_2. The mRNA levels of both ER-α and ER-β 48 hrs after vehicle injection were not significantly different when compared to levels at 0 hrs. A single injection of 5 μg of E_2 remarkably decreased both ER-α and ER-β gene expression within 12 hrs. After that, the mRNA levels of ER-α and ER-β partially returned to their initial level as time proceeded (Fig. 3).

Stat5 Protein Expression After Single Injection of E_2. Stat5a and Stat5a/b proteins were detected as a single band (90 kDa). The quantity of proteins recognized by both antibodies was apparently not changed by E_2 injection. The phospho-Stat5 antibody also detected a single band, and the phosphorylation of Stat5 was significantly decreased within 12 hrs after E_2 injection (Fig. 4).

Discussion

The present study demonstrated that exogenous E_2, which has an impact on the lactational ability of mothers, drastically diminishes the gene expression of both ER subtypes in the mammary glands of lactating mice. The downregulation of ER genes arose within 12 hrs after a single injection of 5 μg E_2, and did not recover to pretreatment level within 48 hrs after injection. Furthermore, the repeated treatment of E_2 was followed by the persistence of low ER gene expression in a dose-dependent manner. These results indicate that expression of the ER gene in the mammary gland of lactating mice is under the control of the E_2 level in the plasma or cytoplasm. During lactation, the plasma E_2 level is very low until weaning (6, 17), and the lack of E_2 because of ovariectomy had no noticeable influence on the expression of ER genes. These data also support that the endogenous level of E_2 during lactation is not involved in the regulation of ER gene expression. That is, the downregulation of ER gene expression occurs when the abnormal activation of ERs is induced by exposure to large doses of E_2.

A difference in responsiveness to E_2 among the ER subtypes has been observed. The repeated injection of 1 μg E_2 decreased the expression of ER-α mRNA to 10% of the control, whereas it was 73% in ER-β. It is well known that the both ERs bind to the natural ligand E_2 with high and nearly equal affinity (18). In the ER-α of mice, there are at

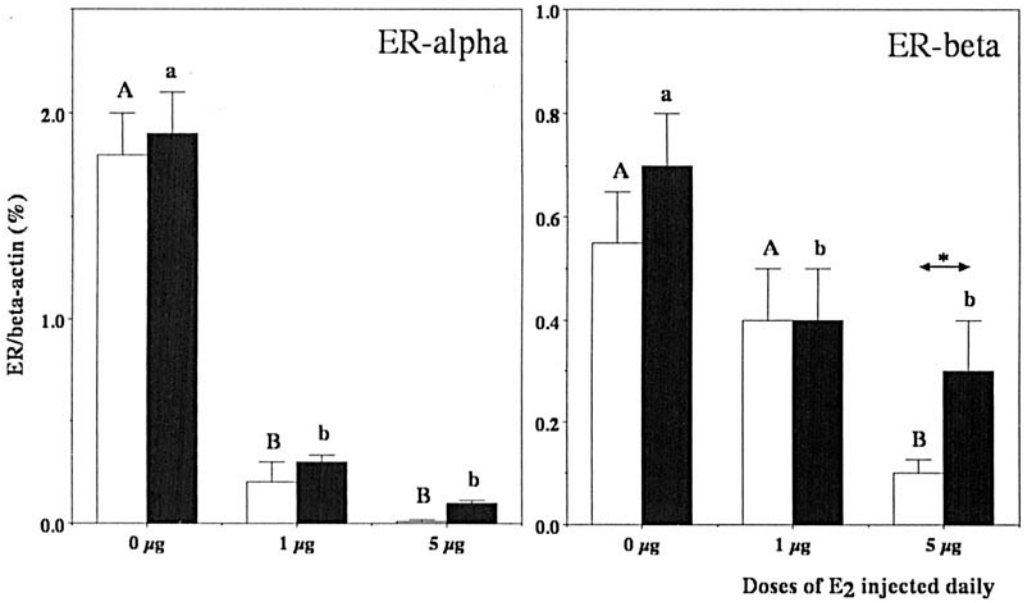

Figure 2. Results from semiquantitative analysis of ER-α and ER-β mRNA in the mammary glands of lactating mice with the repeated injection of 0, 1, and 5 μg of E_2 for 8 days (means ± SEM). Mice were bilaterally sham-operated (□) or ovariectomized (■) on Day 3 of lactation, that is, 2 days before injections started. Represented value was normalized by β-actin mRNA. Different capital letters (sham) or lowercase letters (OVX) indicate a significant difference between treatments ($P < 0.05$). *Values are significantly different between operations ($P < 0.05$).

least seven types of tissue-specific alternative splicing variants, which bind to the front part of the common acceptor splice site (19). To transcribe a variant independently, there should be distinct promoter sites on the 5′ flanking region of each gene. However, the sequence of ER response element (ERE: AGGTCANNNTGACCT) is not present in all promoter regions of those variants. This suggests that the ER-α activated by exogenous E_2 does not directly regulate the expression of the ER-α gene via ERE.

On the other hand, ovariectomy significantly attenuated the decrease in ER-β gene expression by exposure to 5 μg E_2, whereas it did not influence ER-α gene expression. This result indicates that ovarian steroids such as progesterone also participate in the suppression of ER-β gene expression when exposed to large doses of E_2 during lactation. The downregulation of the expression of both ER genes by a large dose of E_2 is likely to be governed by distinct mechanisms or pathways.

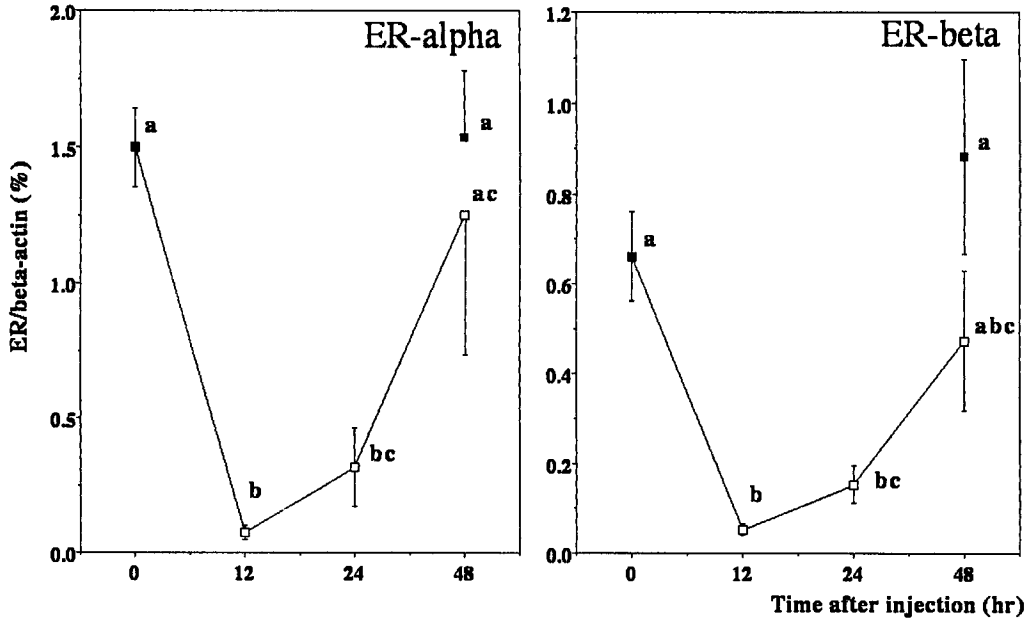

Figure 3. Changes in the ER-α and ER-β mRNA in the lactating mammary gland of mice with a single injection of 5 μg of E_2 (□, E_2-injected; ■, oil-injected controls at 0-hr and 48-hr time points; means ± SEM). The represented value was normalized by β-actin mRNA. Different lowercase letters indicate a significant difference between time points ($P < 0.05$).

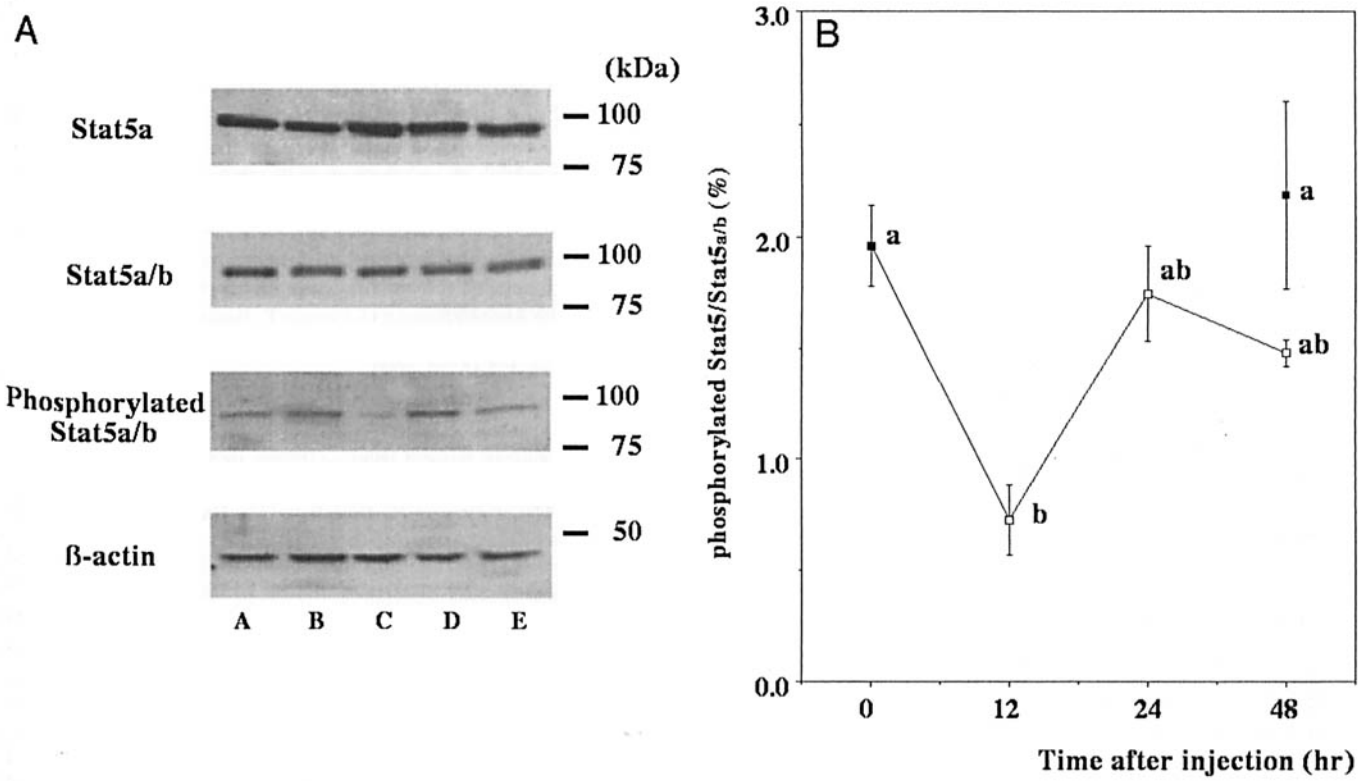

Figure 4. (A) Western blots for Stat5a, Stat5a/b, and phosphorylated Stat5a/b proteins. Each sample was loaded onto 7.5% polyacrylamide gel after reduction treatment by 12% 2-mercaptoethanol. Large letters below each image: A, 0 hrs after oil injection; B, 48-hr oil; C, 12 hrs after 5 μg E_2 injection; D, 24-hrs E_2; E, 48-hr E_2. (B) Changes in the phosphorylated Stat5 protein in the lactating mammary gland of mice with a single injection of 5 μg of E_2 (□, E_2-injected and ■, oil-injected controls at 0-hr and 48-hr time points; means ± SEM). The represented value was normalized by β-actin protein. Different lowercase letters indicate a significant difference between time points ($P < 0.05$).

At present, the major process to stimulate the expression of ER mRNA is the Jak2/Stat5 pathway with the activation of PRL-R (16). The Stat5 proteins, Stat5a and Stat5b, can either homodimerize or heterodimerize with the Src homology domain on the other Stat5, and translocate to the nucleus and regulate the expression of the ER gene to bind to the response element located upstream of the ER-α and ER-β genes. In this process, the phosphorylation of Stat5 is a prerequisite for the dimerization of the proteins. The present results showed that the phosphorylation on Stat5 was suppressed by E_2, whereas there was no effect on the Stat5 protein expression of both subtypes. It seems most probable that the downregulation of ER gene expression by exogenous E_2 depends on the suppression of Stat5 dimerization. When single-injected with E_2, however, ER gene expression gradually returned to the initial level, but the phosphorylation of Stat5 failed to recover within 48 hrs after the injection. Unfortunately, no appropriate finding to interpret this result has yet been reported. This result may indicate the possibility of another pathway regulating the expression of the ER gene during lactation.

A negative cross talk, protein-protein interaction occurring between Stat5s and several nuclear receptors, including ER, has been demonstrated (20). ERs were repressed PRL-induced Stat5 transcriptional activity on a β-casein promoter construct in a ligand-dependent manner (21). The suppression of production of milk components including β-casein via dimerization of ERs and Stat5 may be one of the causes of the inhibition of lactation by exogenous E_2. In the physiological situation, ER-α is extremely low and there is little colocalization of the two receptors during the postlactation period (5). The lactating mammary gland is well known to be estrogen-insensitive, because the progesterone receptor, which is one of the target genes of ER, is not induced by E_2 (22–24). The role of the two ERs during lactation remains unclear. However, a large dose of E_2 induces an apparent transition of the ER profile during lactation into postlactation. Recently, a study supporting our results reported that in the rhesus monkey ER-α, not ER-β, is downregulated when E_2 levels increase and when mammary cells enter the cycle of proliferation (25). The physiological state of these stages conforms to the postlactational stage. The alteration of the ER profile may be a great help in the rapid involution of the mammary gland during established lactation and in remodeling the gland to the next stage.

1. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Hägblad J, Nilsson S, Gustafsson A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. Endocrinology 138:863–870, 1997.
2. Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson A, Kushner

PJ, Scanlan TS. Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. Science 277:1508–1510, 1997.
3. Mitchner NA, Garlick C, Ben-Jonathan N. Cellular distribution and gene regulation of estrogen receptors α and β in the rat pituitary gland. Endocrinology 139:3976–3983, 1998.
4. Tena-Sempere M, Navarro J, Pinilla L, Gonzalez LC, Huhtaniemi I, Aguilar E. Neonatal exposure to estrogen differentially alters estrogen receptor α and β messenger ribonucleic acid expression in rat testis during postnatal development. J Endocrinol 165:345–357, 2000.
5. Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson JA. Estrogen receptors alpha and beta in the rodent mammary gland. Proc Natl Acad Sci U S A 97:337–342, 2000.
6. Tucker HA. Endocrinology of lactation. Semin Perinatol 3:199–233, 1979.
7. Leung BS, Jack WM, Reiney CG. Estrogen receptor in mammary glands and uterus of rats during pregnancy, lactation and involution. J Steroid Biochem 7:89–95, 1976.
8. Moore BP, Forsyth IA. Influence of local vascularity on hormone receptors in mammary gland. Nature 284:77–78, 1980.
9. Hsueh AJW, Peck EJ, Clark JH. Oestrogen receptors in the mammary gland of the lactating rats. J Endocrinol 58:503–511, 1983.
10. Tucker HA, Paape MJ, Sinha YN. Ovariectomy and suckling intensity effects on mammary nucleic acid, prolactin and ACTH. Am J Physiol 213:262–266, 1967.
11. Tucker HA. General endocrinological control of lactation. In: Larson BL, Smith VR, Eds. Lactation: A Comprehensive Treatise. New York: Academic Press, pp277–326, 1974.
12. Llewellyn-Jones D. Inhibition of lactation. Drugs 10:121–129, 1975.
13. Vorherr H. Human lactation and breast feeding. In: Larson BL, Ed. Lactation: A Comprehensive Treatise. New York: Academic Press, Vol 4:pp181–280, 1978.
14. Sankaran L, Qasba P, Topper YJ. Effects of estrogen-depletion on rat casein gene expression. Biochem Biophys Res Commun 125:682–689, 1984.
15. Mizuno H, Sensui N. A counteraction of cortisol on lactation depressing effect of estradiol in the mouse. Endocrinol Japon 20: 167–174, 1973.
16. Frasor J, Gigori G. Prolactin regulation of estrogen receptor expression. Trends Endocrinol Metab 14:118–123, 2003.
17. Sharoni Y, Feldman B, Teuerstein I, Levy J. Protein kinase activity in the rat mammary gland during pregnancy, lactation, and weaning: a correlation with growth but not with progesterone receptor levels. Endocrinology 115:1918–1924, 1984.
18. Bhat RA, Stauffer B, Unwalla RJ, Xu Z, Harris HA, Komm BS. Molecular determinants of ERα and ERβ involved in selectivity of 16α-indo-17β estradiol. J Steroid Biochem Mol Biol 88:17–26, 2004.
19. Kos M, O'Brien S, Flouriot G, Gannon F. Tissue-specific expression of multiple mRNA variants of the mouse estrogen receptor alpha gene. FEBS Lett 477:15–20, 2000.
20. Stoecklin E, Wissler M, Schaetzle D, Pfitzner E, Groner B. Interactions in the transcriptional regulation exerted by Stat5 and by members of the steroid hormone receptor family. J Steroid Biochem Mol Biol 69:195–204, 1999.
21. Faulds MH, Pettersson K, Gustafsson JA, Haldosen LA. Cross-talk between ERs and signal transducer and activator of transcription 5 is E_2 dependent and involves two functionally separate mechanisms. Mol Endocrinol 15:1929–1940, 2001.
22. Haslam SZ, Shyamala G. Effect of oestradiol on progesterone receptors in normal mammary glands and its relationship with lactation. Biochem J 182:127–131, 1979.
23. Mohla S, Clem-Jackson N, Hunter JB. Estrogen receptors and estrogen-induced gene expression in the rat mammary glands and uteri during pregnancy and lactation: changes in progesterone receptor and RNA polymerase activity. J Steroid Biochem 14:501–508, 1981.
24. Shyamala G, Ferenczy A. The nonresponsiveness of lactating mammary gland to estradiol. Endocrinology 110:1249–1256, 1982.
25. Cheng G, Li Y, Omoto Y, Wang Y, Berg T, Nord M, Vihko P, Warner M, Piao YS, Gustafsson JA. Differential regulation of estrogen receptor (ER)alpha and ERbeta in primate mammary gland. J Clin Endocrinol Metab 90:435–444, 2005.