大韓不妊學會雜誌: 第26卷 第2號 1999
Kor. J. Fertil. Steril., Vol. 26, No. 2, 1999, 8

Temporal Changes of c-fos, c-jun, and Heat Shock Protein 25 mRNA in Rat Uterus following Estradiol Treatment

Youngki Lee[1] and Sung Rye Kim[2]

[1]Department of Histology, College of Medicine, Cheju National University, Cheju 690-756, Korea; [2]Department of Medicine and Division of Molecular Biology, Ewha Medical Research Center, College of Medicine, Ewha Womans University, Seoul 158-056, Korea

Estrogen 처리에 따른 흰쥐 자궁조직내 c-fos, c-jun, hsp25 mRNA 발현 변화

제주대학교 의과대학 조직학교실, [1]이화여자대학교 의과대학 의학과 및 의과학연구소 분자생물학부

이 영 기[1] · 김 성 례[2]

= 국문초록 =

포유류의 자궁조직은 발정주기를 통하여 역동적으로 변화하고 있으며 이러한 자궁조직의 분화는 시상하부-뇌하수체-생식소를 잇는 축에 의해 조절되는 스테로이드 호르몬에 의해 이루어진다. 그러나 에스트로겐 (E)이 어떤 유전자를 발현하여 자궁 내의 변화를 일으키는지는 아직 자세히 알려지지 않고 있다. 본 연구는 난소를 절제한 성숙한 흰쥐에 E을 처리한 후 자궁조직 내에서 c-fos, c-jun 및 hsp25 mRNA의 발현 변화를 Northern blot analysis 방법을 사용하여 연구한 것이다. c-fos 및 c-jun 암원유전자의 mRNA 발현은 E 처리 후 1 시간 이전부터 증가하기 시작하며, 3시간 이내에 최고치에 도달한 후 급격히 감소하여 기저수준으로 되돌아 갔다. 반면에 hsp25 mRNA 수준은 E 처리 후 3시간대에서 최고치를 나타내나 증가된 발현량이 서서히 감소하며 12시간이 지난 후 까지도 정상대조군에 비해 높은 수준으로 유지되었다. 이러한 E의 영향이 선별적인지를 검증하기 위하여 E의 길항제인 tamoxifen을 사전처리하고 E을 추가로 처리하여 c-fos, c-jun 및 hsp25 mRNA의 발현이 최고치에 이르렀던 3시간대에 자궁조직을 얻어 각각의 유전자 발현량을 조사한 결과 E에 의해 증가되었던 c-fos, c-jun 및 hsp25 mRNA의 수준이 억제됨을 확인하였다. 이러한 결과는 E이 자궁조직에 영향을 미치는데 초기의 일시적인 변화를 보이는 암원유전자인 c-fos 및 c-jun이 중요한 역할을 하리라는 것을 시사하며 hsp25의 경우는 좀더 늦은 반응에 관여하거나 c-fos 및 c-jun에 의하여 간접적으로 조절을 받을 수도 있음을 보여 주는 것으로 사료된다.

Key Words: Uterus, Northern blot, Estradiol, c-fos, c-jun, hsp25

INTRODUCTION

Estrogen (E) stimulates cell proliferation and differentiation in the female reproductive tract (Martin *et al.*, 1973; Quarmby & Korach, 1984). These hormonal actions are mediated by E receptors. Hormone-occupied E receptor modulates the transcription of target genes by binding to their E responsive elements (Lannigan &

Correspondence: Sung Rye Kim, Ph.D, 서울 양천구 목6동 911-1 이화여자대학교 의과대학 의학과 (우) 158-710, Tel 02) 650-5761, Fax. 02) 650-5761 E-mail: srkim@mm.ewha.ac.kr
This study was supported by Korean Ministry of Education through Research Fund(BM 97-150).

Notides, 1989). Recent studies demonstrated that E treatment induces immediate and transient activation of a number of nuclear protooncogenes in the rodent uterus (Murphy *et al.*, 1987; Kirkland *et al.*, 1992; Webb *et al.*, 1993). Nuclear protooncogenes may play major roles in cell cycle regulation and transformation of normal cells (Hesketh, 1994). The c-Jun proteins are capable of forming dimers, AP-1, with other Jun family proteins, Jun-B and Jun-D and with Fos family proteins, c-Fos, Fos-B, Fra-1, and Fra-2 (Hai & Curran, 1991). The AP-1 regulates the transcription of variety of genes both positively and negatively by binding AP-1 sites and cAMP responsive elements (Ryseck & Bravo, 1991). Alteration of these gene expressions may trigger the proliferation and differentiation of uterine cells by modulating other delayed early genes and even late genes.

The mammalian uterus morphologically and functionally changed during estrous cycle. These changes are regulated by steroid hormones, which are modulated via hypothalamus-pituitary- gonad reproductive endocrine axis. Although there were so many studies about estrogenic regulation of uterine growth and differentiation, a few rather complicated studies have been reported concerning c-fos and c-jun gene expression. Therefore, employing Northern blot analysis, we carried out a study to demonstrate the temporal expressions of c-fos and c-jun mRNA after E-treatment in the uterus of ovariectomized adult rat.

In the recent report, a particular heat shock proteins (hsp), hsp27, which in human tissues was highly expressed in E target organs of the female reproductive tract, and the biological and clinical significance of this protein has been reviewed elsewhere (Ciocca *et al.*, 1993). Thus changes of hsp25, the counter part of human hsp27 in the rat, mRNA levels were also examined after E-treatment in the present study.

MATERIALS AND METHODS

Animals and tissue preparation

Female Sprague-Dawley rats (weighing 230~250 g) were housed in temperature-controlled conditions under a 14 h light and 10 h dark photocycle (light on at 06:00 h) with food and water supplied *ad libitum*. Rats bilaterally ovariectomized (OVX) under light ether anesthesia and two weeks later, these OVX rats were used the following experiments.

Experiment 1. To elucidate the time-course effect of E treatment on c-fos, c-jun, and hsp25 mRNA levels, a single injection of E (10 μg/0.1 ml in sesame oil) was given s.c. to OVX rats at 10:00 h in the morning and sacrificed by decapitation at 0, 1, 3, 6, 12, and 24 h after the injection. Uteri were immediately dissected and kept in 70℃ until use.

Experiment 2. To determine whether antagonist of E is able to counteract the action of E on the changes in c-fos, c-jun, and hsp25 mRNA levels, we used tamoxifen (ICI Pharmaceuticals, UK). Tamoxifen is non-steroidal antiestrogen known to inhibit the binding of E to its receptor in target tissues and thus preventing its estrogenic action (Black & Goode, 1980; Lee *et al.*, 1990). Animals were administered with E (10 μg/0.1 ml) alone or 2 h prior treatment with tamoxifen (20 μg/0.1 ml). Tamoxifen was initially dissolved in absolute ethanol and diluted with distilled water when injected into the animals. Animals were sacrificed 3 h after E injection. When this time, c-fos, c-jun, and hsp25 mRNA levels were peaked in our study (Fig. 1-3).

Total RNA isolation

Total cytoplasmic RNA was extracted from uterine tissues by an acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, two uterine tissues (from 1 rat) were homogenized in 600 μl of denaturing solution containing 4 M guan-

idinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauroyl sarcosine and 0.1 M 2-mercaptoethanol. Sixty μl of 2 M sodium acetate (pH 4), 600 μl of water-saturated phenol, and 120 μl of chloroform-isoamyl alcohol mixture (49:1) were added. After cooling on ice for 15 min, samples were centrifuged at 10,000 xg at 4℃ for 20 min and precipitated with ethanol. After washing with 75% ethanol, the RNA pellet was dried under vacuum and dissolved in 20 μl of sterilized distilled water. RNA content was then quantified at A_{260} absorbance. The optical density (OD) ratio of A_{260} to A_{280} ranged between 1.8 to 2.0.

Northern blot analysis

Samples of uterus total RNA (20 μg) were dissolved in distilled water and denatured in 50% formamide, 6.2% formaldehyde, 20 mM MOPS (3-[N-morpholino]-propanesulfonic acid), 5 mM sodium acetate, and 1 mM EDTA at 60℃ for 5 min. Electrophoresis was performed at 100 V for 1.5 h in a submarine 1.2% agarose gel. After RNA was transferred to a Nytran membrane (pore size: 0.45 μm, Schleicher & Schuell) for 18 h by diffusion blotting, the membrane was dried and baked at 80℃ for 2 h. Prehybridization was carried out at 42℃ for 2 h in a heat-sealable plastic bag (Kapak) with hybridization buffer consisting of 50% deionized formamide, 5x SSPE, 5x Denhardt's solution (1x Denhardt's solution: 0.02% polyvinylpyrrolidone, 0.02% ficoll, and 0.02% BSA), 0.1% SDS, and 2 mg of heat-denatured salmon sperm DNA. After addition of each ^{32}P-labeled c-fos, c-jun, and hsp25 cDNA probe as made by the random hexamer extension method (Feinberg and Vogelstein, 1984), hybridization was performed at 42℃ for 20 hr. The Nytran membrane was washed twice with 2x SSC and 0.1% SDS at room temperature for 20 min, followed by the second washing with 0.07x SSC, 0.5% SDS, and 5 mM EDTA (pH 8) at 42℃ for 10 min for three times. The membrane was then dried and exposed to X-ray film (Fuji) at -70℃ for 2 days. The same membrane was then rehybridized with 18S cDNA probe under the same conditions.

Data analysis

The amount of RNA applied to the gel was normalized by 18S rRNA control valus. The hybridization signals were determined by scanning the appropriately exposed autoradiogram with densitometry (Hofer Scientific Ins.), and c-fos, c-jun, and hsp25 mRNA levels were ex-

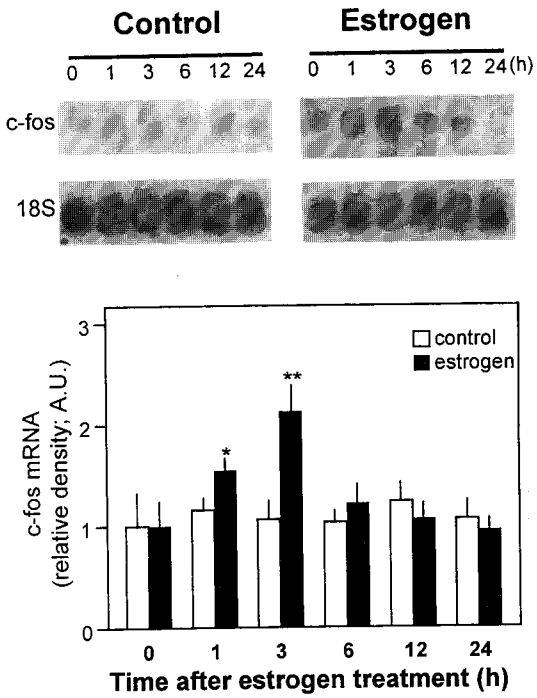

Fig. 1. Time course changes of E-induced c-fos mRNA levels in the uterine tissues of OVX rats. Upper panel shows representative autoradiograms of c-fos mRNA by Northern blot hybridization. A single injection of E (10 μg/rat) or vehicle was administered and animals were sacrificed at 0, 1, 3, 6, 12, and 24 h after E administration. Total RNA (20 μg/lane) was isolated from uterine tissues, electrophoresed on 1.2% agarose denaturing gels, blotted on the nylon membranes, and hybridized with c-fos cDNA probes. Lower panel shows autoradiograms quantitated by densitometric scanning and the data are expressed as arbitrary units (set to 1 at 0 h). Error bar represented the mean ±SEM. Values are significantly different from control values at $p<0.01$ (**) and $p<0.05$ (*).

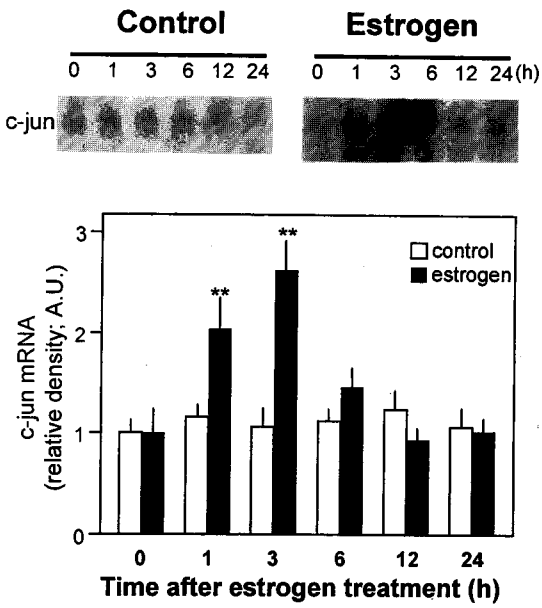

Fig. 2. Time course changes of E-induced c-jun mRNA levels in the uterine tissues of OVX rats. Upper panel shows representative autoradiograms of c-jun mRNA by Northern blot hybridization. Lower panel shows autoradiograms quantitated by densitometric scanning and the data are expressed as arbitrary units (set to 1 at 0 h). Error bar represented the mean ± SEM. Values are significantly different from control values at $p<0.01$ (**) and $p<0.05$ (*).

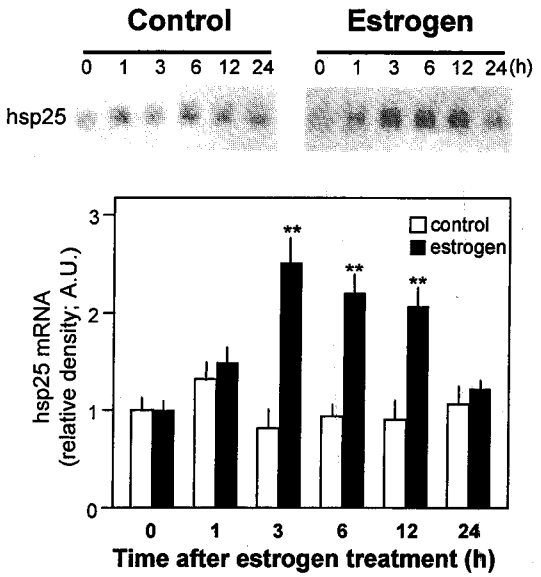

Fig. 3. Time course changes of E-induced hsp25 mRNA levels in the uterine tissues of OVX rats. Upper panel shows representative autoradiograms of hsp25 mRNA by Northern blot hybridization. Lower panel shows autoradiograms quantitated by densitometric scanning and the data are expressed as arbitrary units (set to 1 at 0 h). Error bar represented the mean ±SEM. Values are significantly different from control values at $p<0.01$ (**) and $p<0.05$ (*).

pressed as arbitrary unit. Experiments with multiple RNA samples were replicated independently 3 or 4 times. Data expressed as the mean ± SEM were analysed by one-way analysis of variance (ANOVA), followed by Fisher's least significance difference test for post hoc comparison with $p<0.05$ required for a statistical significance.

RESULTS

Changes in c-fos, c-jun, and hsp25 mRNA levels in the uterine tissues of OVX rats after single injection of E

One of the important actions of E in the mammalian reproductive tissue is to stimulate proliferation of uterine cells (Martin *et al.*, 1976). These actions of E primarily appear through activation of protooncogenes. In the present study, a single injection of E (10 μg/rat) or vehicle was subcutaneously administered and uterine tissues were isolated at 0, 1, 3, 6, 12, and 24 h after E treatment. Time course changes of c-fos (Fig. 1), c-jun (Fig. 2), and hsp25 (Fig. 3) mRNA levels were monitored by Northern blot analysis. In the control group (vehicle treated), there were no significant changes in c-fos, c-jun, and hsp25 mRNA levels at any time points. On the other hand, in the E-treated group, c-fos, c-jun, and hsp25 mRNA levels peaked at 3 h and c-fos and c-jun mRNA levels rapidly declined to the basal levels at 6 h while, hsp25 mRNA level was sustained until 12 h after E injection.

Effects of tamoxifen, a E antagonist, on the changes in c-fos, c-jun, and hsp25 mRNA levels

The biological action of E is mediated by its

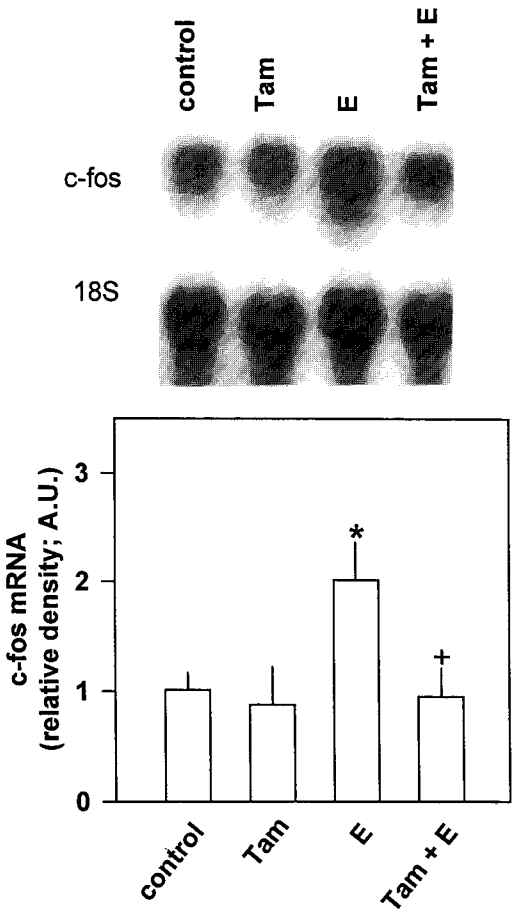

Fig. 4. Effect of antiestrogen (Tamoxifen) on E-induced c-fos gene expression in the uterine tissues of OVX rats. Upper panel shows the representative autoradiograms of c-fos mRNAs. OVX rats were administered with vehicle, E (10 μg/rat), or tamoxifen (20 μg/rat) with or without E. Animals were sacrificed 3 h after E injection. Lower panel shows autoradiograms quantitated by densitometric scanning and the data are expressed as arbitrary units (set to 1 at CTL). Error bar represented the mean ±SEM. Values are significantly different from control values at $p<0.01$ (*), and significantly different from E-treated group at $p<0.0$ (+).

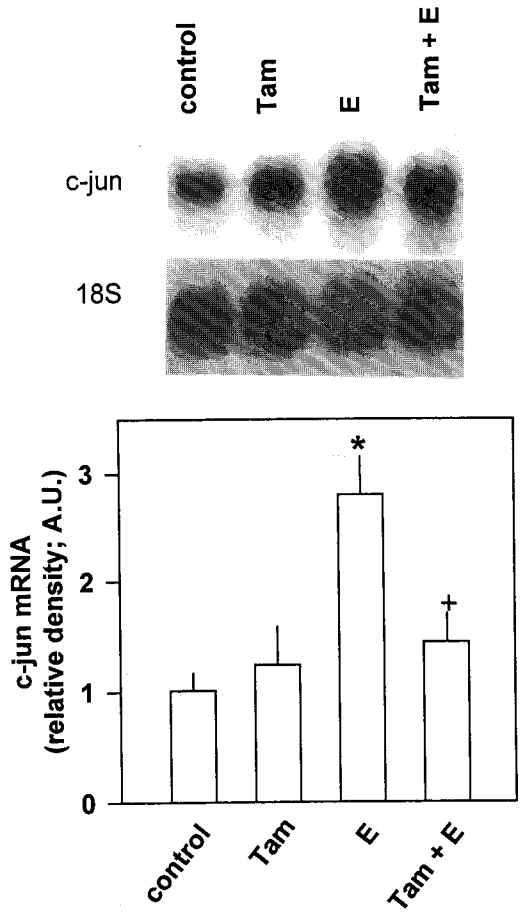

Fig. 5. Effect of antiestrogen (Tamoxifen) on E-induced c-jun gene expression in the uterine tissues of OVX rats. Upper panel shows the representative autoradiograms of c-jun mRNAs. Lower panel shows autoradiograms quantitated by densitometric scanning and the data are expressed as arbitrary units (set to 1 at CTL). Error bar represented the mean ±SEM. Values are significantly different from control values at $p<0.01$ (*), and significantly different from E-treated group at $p<0.05$ (+).

specific high affinity receptor in target cells, and anti-E are known to inhibit its action by interfering with receptor dimerization and/or receptor-DNA binding (Dauvios *et al.*, 1993; Fawell *et al.*, 1990). We examined the effect of anti-E, such as tamoxifen, on E-induced c-fos (Fig. 4), c-jun (Fig. 5), and hsp25 (Fig. 6) gene expression. OVX rats were administered i.p. with tamoxifen (20 μg/rat in 0.9% saline) with or without s.c. E (10 mg/rat). C-fos, c-jun, and hsp25 mRNA levels were examined in the uterine tissues 3 h after E treatment (5 h after tamoxifen treatment). Treatment with tamoxifen alone failed to alter c-fos, c-jun, and hsp25 mRNA levels. However, when tamoxifen was co-administered with E, E-induced increases of c-fos, c-jun, and hsp25 mRNA levels were effectively blocked ($p<0.05$, n=3). These results

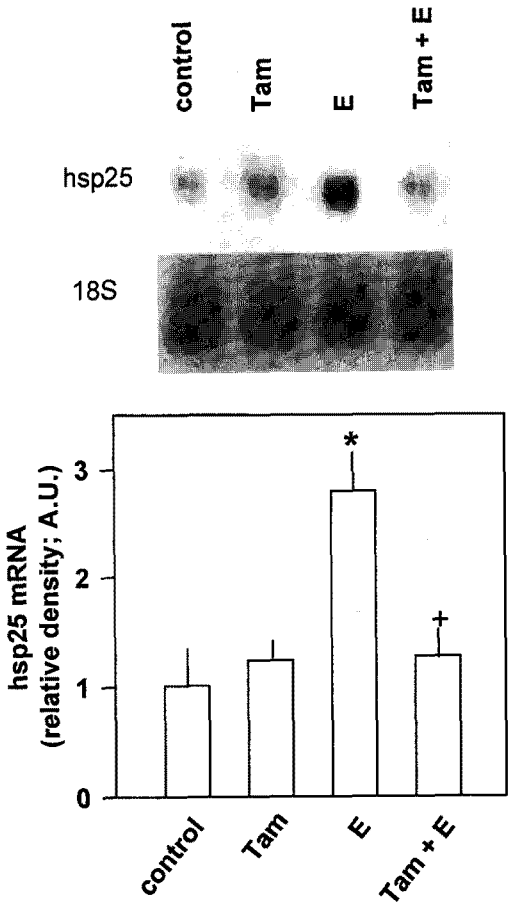

Fig. 6. Effect of antiestrogen (Tamoxifen) on E-induced hsp25 gene expression in the uterine tissues of OVX rats. Upper panel shows the representative autoradiograms of hsp25 mRNAs. Lower panel shows autoradiograms quantitated by densitometric scanning and the data are expressed as arbitrary units (set to 1 at CTL). Error bar represented the mean ±SEM. Values are significantly different from control values at $p<0.01$ (*), and significantly different from E-treated group at $p<0.05$ (+).

indicate that E-induced increase of c-fos, c-jun, and hsp25 mRNA levels are specific and mediated by E receptor.

DISCUSSION

The ovarian steroid hormones E and progesterone (P) are the primary uterotropic hormones, and their effects are reflected by the cyclic patterns of uterine cell proliferation, vascular growth, and blood flow that occur throughout the nonpregnant cycle (Johnson *et al.*, 1997). These uterotropic effects of the ovarian steroids are thought to serve primarily to prepare the uterus for implantation and subsequent placentation (Reynolds & Redmer, 1995). However, the action mechanism of steroid hormones has not been fully understood.

Treatment of OVX animals with E stimulates uterine growth and cell proliferation (Magness & Rosenfeld, 1989). As described in results part, one of the important actions of E in the mammalian reproductive tissue is to stimulate proliferation of uterine cells (Martin *et al.*, 1976). The actions of E primarily appear through the activation of protooncogenes in the uterine cells. However, there were few studies concerning the precise temporal changes of protooncogenes after E treatment. In the present study, a single injection of E into OVX adult rats increased c-fos and c-jun mRNA levels in the uterus within 1 h and peaked at 3 h post E treatment (Fig. 1-2) and rapidly declined to the basal levels at 6 h. Rapid and transient estrogenic induction of c-fos and c-jun mRNAs also reported employing Northern blot analysis of RNA from the whole uterus of immature rats (Bigsby & Li, 1994). The present study confirmed that the expression of c-fos and c-jun transcript reaches a peak at 3 h, and then rapidly decreases over several hours in the uterine tissue following E treatment. The rapid increases of protooncogene thought to regulate the downstream events following E treatment. Thus, E acts on early regulatory genes that code for transcription factors required for the transcription of late structural genes. These early genes respond within minute to hour to steroids and enter the nucleus to regulate, either positively or negatively, transcription of the late genes encoding the bulk of structural proteins and biosynthetic enzymes that are involved more directly with cell proliferation, preparing the cell for DNA synthesis, and general cellular functions. How-

ever, the requirement of protooncogenes for cellular proliferation or their relation to differentiation or death of uterine cells has not been firmly established.

In the present study, we also investigated the expression of hsp25 mRNA which was highly expressed in E target organs of the female reproductive tract of human tissues following E treatment (Ciocca et *al.*, 1993). As shown in Figure 3, hsp25 mRNA levels reached in a peak at 3 h such as c-fos and c-jun, but the elevated level was sustained until 12 h after E treatment. It has been known that hsp activation occurs in response to heat shock as well as other environmental and pathophysiological stress conditions. Moreover, diverse hsp are expressed in normal unstressed cells. Hsp family is known to involve in many cell functions, act in various case as molecular chaperons (Georgopoulos & Welch, 1993), and the transcription of the hsp25 gene is under regulation both by heat shock element and by estrogen-responsive elements (Ciocca *et al.*, 1993). Although a clear role for hsp25 in reproductive physiology has not been established at present, our result demonstrates that hsp25 mRNA is expressed in uterine tissues and regulated by E. The long-lasting expression of hsp25 mRNA compare to the expression of c-fos and c-jun mRNAs suggests that hsp25 may relate to the late effects of E in the uterine tissues. It also presumable that hsp25 is one of the downstream genes of protooncogene in respond to E.

We also examined the effect of progesterone (P) on the expression of c-fos, c-jun, and hsp25 mRNA from the E-primed animals (data not shown). However, there were no significant changes in the expression of these genes. Thus, the concomitant effect of E and P needs further evaluation.

In conclusion, E affects the uterine physiology, at least in part, through the regulation of c-fos and c-jun protooncogenes as well as hsp25.

SUMMARY

Steroid hormone is known to cause the dynamic changes of mammalian uterus during reproductive cycle, which are modulated via hypothalamus-pituitary -gonad reproductive endocrine axis. Although there were so many studies about estrogenic regulation of uterine growth and differentiation. There is little information about the effect of estrogen on the expression of various transcription factors involved in gene expression. Thus the present study was designed to demonstrate E induced expression of c-fos, c-jun, hsp25 mRNA in rat uterus.

Employing Northern blot analysis, we studied the temporal expressions of c-fos, c-jun, and hsp25 messenger RNAs (mRNAs) elicited by a single 17beta-estradiol (E) treatment in the uteri of bilaterally ovariectomized adult rats. c-fos, c-jun, and hsp25 mRNA levels were increased and peaked at 3 h after E administration, and then c-fos and c-jun mRNA levels were rapidly decreased to basal control level while, increased hsp25 mRNA levels were sustained till 12 h post E treatment. To test the estrogenic effect on the increase of c-fos, c-jun, and hsp25 mRNA levels, we also examined the effects of antiestrogen (tamoxifen). Pretreatment with tamoxifen effectively blocked the E-induced increase of c-fos, c-jun, and hsp25 mRNA levels at 3 h post E treatment. Present results suggest that transient increase of c-fos and c-jun protooncogene mRNA at the early time and simultaneous expression of hsp25 mRNA contribute to the response of uterine tissues to E in adult female rats.

REFERENCES

Bigsby RM, Li A: Differentially regulated immediate early genes in the rat uterus. *Endocrinology* 1994, 134, 1820-1826.

Black LJ, Goode RL: Uterine bioassay of tamoxifen,

trioxifene and a newestrogen antagonist (LY-117018) in rats and mice. *Life Sci* 1980, 26, 1453-1458.

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid-guanidiumthiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162, 156-159.

Ciocca DR, Oesterreich S, Chamness GC, McGuire WL, Fuqua SA: Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J Natl Cancer Inst* 1993, 85, 1558-1570.

Dauvois S, White R, Parker MG: The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *J Cell Sci* 1993, 106, 1377-1388.

Fawell SE, White R, Hoare S, Sydenham M, Page M, Parker MG: Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. *Proc Natl Acad Sci USA* 1990, 87, 6883-6687.

Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1984, 137, 266-267.

Georgopoulos C, Welch WJ: Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* 1993, 9, 601-634.

Hai T, Curran T: Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Nat Acad Sci USA* 1991, 88, 3720-3724.

Hesketh R: The oncogene handbook. New York: Academic Press, 1994.

Johnson ML, Redmer DA, Reynolds LP: Uterine growth, cell proliferation, and c-fos proto-oncogene expression throughout the estrous cycle in ewes. *Biol Reprod* 1997, 56, 393-401.

Kirkland JL, Murthy L, Stancel GM: Progesterone inhibits the estrogen-induced expression of c-fos messenger ribonucleic acid in the uterus. *Endocrinology* 1992, 130, 3223-3230.

Lannigan DA, Notides AC: Estrogen receptor selectively binds the "coding strand" of an estrogen responsive element. *Proc Natl Acad Sci USA* 1989, 86, 863-867.

Lee JB, Yoon M, Kim C, Hong SS, Ryu K: Action mechanism of antiestrogens on uterine growth in immature rats. *Kor J Pharmacol* 1990, 26, 167-175.

Magness RR, Rosenfeld CR: Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. *Am J Physiol* 1989, 256, 536-542.

Martin L, Finn CA, Trinder G: Hypertrophy and hyperplasia in the mouse uterus after oestrogen treatment: an autoradiographic study. *J Endocrinol* 1973, 56, 133-44.

Martin L, Pollard JW, Fragg B: Oestradiol, oestradiol-17 and the proliferation and death of uterine cells. *J Endocrinol* 1976, 69, 103-110.

Murphy LJ, Murphy LC, Friesen HG: Estrogen induction of N-myc and c-myc proto-oncogene expression in the rat uterus. *Endocrinology* 1987, 120, 1882-1888.

Quarmby VE, Korach KS: The influence of 17 beta-estradiol on patterns of cell division in the uterus. *Endocrinology* 1984, 114, 694-702.

Reynolds LP, Redmer DA: Utero-placental vascular development and placental function. *J Anim Sci* 1995, 73, 1839-1851.

Ryseck RP, Bravo R: c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene* 1991, 6, 533-542.

Webb DK, Moulton BC, Khan SA: Estrogen induces expression of c-jun and jun-B protooncogenes in specific rat uterine cells. *Endocrinology*, 1993, 133, 20-28.