Estrogen Inhibits Interleukin-18 mRNA Expression in the Mouse Uterus

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Abstract. Interleukin-18 (IL-18) is a proinflammatory cytokine expressed in female reproductive organs in humans, rats and mice. The physiological roles of uterine IL-18 and the regulatory mechanisms of IL-18 gene expression are unclear. The present study aimed to clarify the effects of estradiol-17β (E2) and progesterone (P4) on IL-18 mRNA expression in the mouse uterus. Distribution and expression levels of IL-18 mRNA were studied using an RNase protection assay. Expression of IL-18 mRNA was observed in all organs studied, including testes, ovaries and uteri. The uterine IL-18 mRNA level of estrous mice was higher than that of diestrous mice. E2 treatment (1, 5, 25 or 250 ng/mouse) decreased uterine IL-18 mRNA levels in ovariectomized mice dose-dependently. E2 treatment acutely decreased IL-18 mRNA levels 3 h after injection, but these levels returned to the initial level after 48 h. P4 treatment (1 mg/mouse) decreased uterine IL-18 mRNA levels after 12 h, but levels returned to the initial level after 48 h. Both uterine IL-18 and IL-18Rα mRNAs were detected in cultured endometrial epithelial and stromal cells. These results suggest that uterine IL-18 expression is reduced by sex steroid hormones and that IL-18 acts on endometrial cells in a paracrine or autocrine manner.

Key words: Estrogen, Interleukin-18, Progestin, Uterus (J. Reprod. Dev. 51: 639–647, 2005)

Interleukin-18 (IL-18) is a well-known pleiotropic cytokine that regulates innate and acquired immune responses, and that plays important roles for autoimmune and inflammatory actions and infectious diseases. IL-18 shares functional properties with IL-12 and belongs to the IL-1 family [1]. IL-18 is produced as an inactive 24-kDa precursor molecule, which is cleaved by IL-1-converting enzyme (ICE; caspase-1) into a biologically active 18-kDa molecule [2–4].

IL-18 expression is widespread in various tissues and organs, including the brain [5], astrocytes/ microglia [6], the adrenal cortex and pituitary gland [7], monocytes/macrophages, dendritic cells, Kupffer cells, keratinocytes [1, 8, 9], articular chondrocytes [10], osteoblasts [11], the spleen, intestinal epithelial cells and the liver [12]. In addition, several recent studies have indicated that IL-18 plays roles in the reproductive system of humans and rodents. In mouse immature ovaries, gonadotropin treatment increased IL-18 expression [13]. In human endometrium, IL-18 protein and mRNA were both expressed throughout the menstrual cycle [14]. In human sera and amniotic fluid, IL-18 protein levels increased during pregnancy, and increased further at delivery [15, 16]. On the other hand, IL-18 mRNA levels in human blood decreased during pregnancy [17]. In
pregnant mice, IL-18 was detected in the decidua and placenta [18]. These findings suggest that IL-18 plays important roles in the female reproductive system. However, the regulatory mechanisms of IL-18 gene expression and distribution of IL-18 gene-expressing cells in female reproductive organs are unclear. In the present study, we aimed to clarify the changes of IL-18 mRNA levels during the estrous cycle and the effects of estradiol-17β (E2) and progesterone (P4) on IL-18 mRNA levels in the mouse uterus. IL-18 action is mediated through the IL-18 receptor (IL-18R). IL-18R functions as a heterodimer consisting of a ligand-binding chain, termed the IL-18Rα chain, and a co-receptor, termed the IL-18Rβ chain [19–21]. In addition, both IL-18Rα and IL-18Rβ, which belong to the IL-1R family [22, 23], are essential for the signal transduction of IL-18 [21]. Therefore, we also analyzed the expression of IL-18Rα mRNA.

Materials and Methods

Animals

Male and female mice of the ICR strain (CLEA Japan, Inc., Meguro, Tokyo, Japan) were used. They were kept in a temperature-controlled animal room (20–22 °C; light: 14 h and dark: 10 h) and given a commercial diet (CE-7; CLEA Japan, Inc.) and tap water ad libitum. Vaginal smears were observed daily for one month before the experiment. Seven- to eight-week-old female mice were ovariectomized under light ether anesthesia. About two weeks after the surgery, animals were used for the experiment. All animal care and experiments were performed in accordance with the Guidelines for Animal Experimentation of Okayama University, Japan.

Hormone treatment

E2 (Sigma-Aldrich, Inc., St. Louis, MO, USA) at doses of 1, 5, 25 or 250 ng per mouse, or P4 (Sigma-Aldrich, Inc.) at a dose of 1 mg per mouse was given to ovariectomized mice subcutaneously. Both hormones were dissolved in sesame oil (0.1 ml). Control mice were injected with sesame oil only. After 3, 6, 12, 24, 48, 72 and 96 h, uteri were collected and stored at −80 °C.

Isolation of endometrial epithelial and stromal cells

Isolation of mouse endometrial epithelial and stromal cells was performed according to previous reports [24–26]. Briefly, uteri of 3-week-old mice were excised, and the uterine horn was longitudinally cut to expose the endometrial surface. Tissue fragments were kept in 0.1% trypsin (Sigma-Aldrich, Inc.) in Hanks’ solution containing 20 mM HEPES and 0.3% bovine serum albumin at 4 °C for 1 h, and then at 37 °C for 1 h. Trypsin was inactivated by soybean trypsin inhibitor (STI, Sigma-Aldrich, Inc.) at 37 °C for 10 min. Endometrial epithelial tissues were separated from endometrial stromal tissues with forceps under a stereoscopic microscope, and then tissue fragments were collected by centrifugation. The collected epithelial tissue fragments were further divided into smaller fragments by gentle pipetting. The endometrial stromal and myometrial tissues separated from epithelial tissues were incubated with 0.1% trypsin and 1 mM EDTA at 37 °C for 1 h. They were then treated with 0.1% STI at 37 °C for 10 min. After gentle pipetting and DNase I (Sigma-Aldrich, Inc.) treatment, stromal cells separated from the myometrium were collected by centrifugation. Cell viability was assessed using the trypan blue dye exclusion test (usually more than 95% in each study). Purities of epithelial and stromal cells were 90% or more, which were verified by the method shown in our previous reports [25, 26].

Serum-free culture of isolated endometrial epithelial and stromal cells

A 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium without phenol red and calcium was prepared and supplemented with CaCl2 (0.1 mM for epithelial cells, 1.0 mM for stromal cells), bovine serum albumin (fraction V, 1,000 mg/l, Sigma-Aldrich, Inc.), hydrocortisone (100 µg/l), triiodothyronine (400 ng/l), transferrin (10 mg/l), glucagon (10 ng/l), parathormone (200 ng/l) and sodium selenite (5 µg/l) [25, 26]. Isolated endometrial epithelial cells were seeded at a cell density of 5 × 10⁵ cells/well in collagen-coated 6-well culture plates (Becton Dickinson, Lincoln Park, NJ, USA) and incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C. Isolated endometrial stromal cells were seeded in 6-well tissue culture plates at a density of 6 × 10⁵ cells per well. Culture was maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.
RNA extraction

RNA extraction was performed by the single-step method [27]. RNA samples were dissolved in diethyl-pyrocarbonate (DEPC)-treated water and stored at –80°C until use.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

One μg of total RNAs obtained from the tissues of adult mice and cultured cells were subjected to reverse transcription (RT) reaction using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s instructions. Oligo dT primers were used in the RT reactions. PCR amplifications were performed using the primer pairs specific for IL-18, IL-18Rα, ICE, insulin-like growth factor-I (IGF-I) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs. PCR was carried out using TaKaRa Taq DNA polymerase (TAKARA BIO INC., Otsu, Shiga, Japan) and a thermal cycler (GeneAmp PCR System 9700, PerkinElmer, Inc., Foster, CA, USA). Primer sequences used for analysis are listed in Table 1. Oligonucleotide primers were purchased from Hokkaido System Science (Sapporo, Hokkaido, Japan). PCR were performed as follows (gene, denaturation temperature, extension temperature and number of cycles): IL-18, 95°C, 60°C, 29 cycles; IL-18Rα, 95°C, 65°C, 29 cycles; ICE, 95°C, 65°C, 22 cycles; IGF-I, 95°C, 60°C, 35 cycles; GAPDH, 95°C, 60°C, 22 cycles. Denaturation for 30 s and extension for 60 s were preformed followed by an additional extension at the same temperature for 10 min. For semi-quantitative analysis of IL-18, IGF-I and GAPDH mRNA levels, the amplification cycle numbers used were determined within the range of the exponential amplification phases of each mRNA. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet (UV) illumination. They were then compared with a known standard (100 bp DNA Ladder, Invitrogen Corp.) for size determination.

Riboprobes

Mouse IL-18 and GAPDH riboprobes were generated as follows. DNA fragments encoding a part of the mouse IL-18 and GAPDH were obtained from total RNA prepared from ICR male mice by RT-PCR. The cDNA fragment was subcloned into pGEM3zf (+). One clone was sequenced using fluorescein primers and an automated DNA sequencer (373 DNA Sequencer, PerkinElmer, Inc.), and this was confirmed to represent cDNA encoding a part of the mouse IL-18 (Accession No. D49949, 226–528) and GAPDH (Accession No. M32599, 659–903). Plasmid DNA was linearized using a suitable restriction enzyme. The riboprobe was labeled with [α-32P] rUTP (800 Ci/mmol, Amersham Pharmacia, Buckinghamshire, UK) using the Riboprobe System - T7 (Promega, Madison, WI, USA).

RNase protection assay (RPA)

Total RNA obtained from various tissues was analyzed using an RNase Protection Kit (Roche Diagnostics, Mannheim, Germany). Radiolabeled riboprobes (IL-18: 313 b, GAPDH: 254 b, 3 × 10^5 cpm/reaction) and sample RNAs were hybridized at 45°C for 16 h, and unhybridized RNAs were digested using RNase A and RNase T1 at 30°C for 1.5 h. Samples were electrophoresed on a denaturing 5% acrylamide/8 M urea gel (30 W for 1.5 h). Hybridized signals were detected by autoradiography. GAPDH mRNA levels were

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Table 1. Primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession No.</th>
<th>Sequence</th>
<th>Length (bp)</th>
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<tr>
<td>IL-18</td>
<td>D49949</td>
<td>Forward 5’-ACT GTA CAA CCG CAG TAA TAC GG-3’</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-AGT GAA CAT TAC AGA TTT ATC CC-3’</td>
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</tr>
<tr>
<td>IL-18Rα</td>
<td>U43673</td>
<td>Forward 5’-CCA ACG AAG AAG CCA TAG ACA-3’</td>
<td>261</td>
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<tr>
<td></td>
<td></td>
<td>Reverse 5’-TCA GGA TGA CAC TCT TTC AG-3’</td>
<td></td>
</tr>
<tr>
<td>ICE</td>
<td>U04269</td>
<td>Forward 5’-CAC GTC TTG CCC TCA TTA TC-3’</td>
<td>394</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-CAC TCC TTG TTT CTC TCC AC-3’</td>
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</tr>
<tr>
<td>IGF-I</td>
<td>AK038119</td>
<td>Forward 5’-GCT GGT GGA TGC TCT TCA GTT-3’</td>
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<td></td>
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<td>M32599</td>
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<tr>
<td></td>
<td></td>
<td>Reverse 5’-TGG AAG AGT GGG AGT TGC TGT T-3’</td>
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measured as an internal control. In each sample, IL-18 mRNA levels were normalized relative to the amount of GAPDH mRNA. As a negative control for RPA, yeast tRNA (Roche Diagnostics) was used.

**Statistical analysis**

The results were expressed as means ± SEM, and statistical analysis was performed with ANOVA.

**Results**

**Detection of IL-18 and IL-18Rα mRNAs**

IL-18 mRNA expression was analyzed by RPA. Protected IL-18 mRNA signals, with sizes differing from the riboprobe used, were detected in the cerebella, hypothalami, anterior and neurointermediate pituitary lobes, liver, spleen, adrenals, testes, ovaries and uteri (Fig. 1A). In the ovaries, IL-18 mRNA levels were higher compared with other organs. Expression of IL-18Rα mRNA was analyzed by RT-PCR. IL-18Rα mRNA signals were detected in all organs studied (Fig. 1B).

**Uterine IL-18 mRNA levels during the estrous cycle and the effect of ovariectomy**

Uterine IL-18 mRNA levels were studied by RPA (Fig. 2). Uterine IL-18 mRNA levels at estrus were significantly higher than those at diestrus ($P<0.001$). The effect of ovariectomy on IL-18 mRNA levels in the mouse uterus was also studied. IL-18 mRNA levels at 2 weeks after the surgery did not differ from those at estrus and diestrus.

**The effect of E2 treatment on uterine IL-18 mRNA levels**

The dose-response effect of E2 (1, 5, 25 or 250 ng/mouse) on uterine IL-18 mRNA levels was analyzed by RPA (Fig. 3). Uterine IL-18 mRNA levels were determined 12 h after E2 injection. E2 treatment significantly decreased IL-18 mRNA levels dose-dependently. Uterine IL-18 mRNA levels were significantly lower in E2 (25 or 250 ng/mouse)-treated mice ($P<0.01$).

Time-course analysis of the effect of E2 (25 or 250 ng/mouse) treatment on IL-18 mRNA levels is shown in Fig. 4. Uterine IL-18 mRNA levels were determined 3, 6, 12, 24, 48, 72 and 96 h after E2 injection. IL-18 mRNA levels were significantly decreased at 6 (25 ng/mouse) and 3 h (250 ng/mouse), and the lowest level was detected after 12 h. IL-18 mRNA expression remained low for 24 h with the 250 ng E2 treatment. IL-18 mRNA levels returned to control level 48 h after injection, irrespective of the dose used.

**The effects of E2 and P4 treatment on uterine IL-18 and IGF-I mRNA levels**

The effects of E2 and P4 on uterine IL-18 mRNA levels are shown in Figs. 5A and B. A combination of E2 (250 ng/mouse) and/or P4 (1 mg/mouse) was given, and uteri were collected 12 and 48 h after injection. IL-18 and IGF-I mRNA levels were measured by semi-quantitative RT-PCR. IL-18 mRNA levels were significantly decreased after 12 h in all treated mice (E2 and E2 + P4, $P<0.001$; P4, $P<0.05$). After 48 h, IL-18 mRNA levels were unchanged. IGF-I mRNA levels were also studied (Figs. 5C and D). IGF-I mRNA levels were
significantly increased after 12 h in the E2-treated mice ($P<0.05$), and after 48 h, they returned to control level. The E2 and P4 treatments did not change ICE mRNA levels (data not shown).

Fig. 4. The time-course effect of E2 on uterine IL-18 mRNA levels. Ovariectomized mice were injected with 25 or 250 ng E2. Uterine IL-18 mRNA levels were analyzed by RPA. The values represent the relative levels of IL-18 mRNA compared with the level at 0 h, which was defined as 1.0. Each value is the mean ± SEM ($n=3$). *: $P<0.05$; ***: $P<0.001$, significantly different from the control.

Detection of IL-18, IL-18Rα and ICE mRNAs in cultured endometrial cells

Separated endometrial cells were cultured for 4 d, and expressions of IL-18, IL-18Rα and ICE mRNAs were determined by RT-PCR (Fig. 6). IL-18, IL-18Rα and ICE mRNAs were detected in cultured endometrial epithelial and stromal cells.

Discussion

We showed IL-18 mRNA expression in the mouse testis, ovary and uterus. To our knowledge, the present study is the first to show inhibitory effects of E2 and P4 on uterine IL-18 mRNA expression in the mouse. Expression of ICE mRNA was detected in mouse endometrial cells, suggesting that biologically active molecules of IL-18 were produced and released within the uterus since ICE stimulates the processing of pro-IL-18 to mature IL-18. IL-18Rα mRNA was also detected in the mouse uterus. Therefore, mouse uterus may possess an IL-18 system, and IL-18 plays roles in the regulation of the immune response and uterine...
functions, although it is difficult to specify physiological roles of IL-18 in the mouse uterus.

We investigated IL-18 mRNA expression in the mouse uterus since several recent studies suggested that IL-18 was involved in the regulation of uterine functions [14–18] and there are no reports on sex steroid hormone actions on IL-18 gene expression. In the mouse uterus, expressions of IL-18 and IL-18Ra mRNAs were detected in separately cultured endometrial epithelial and stromal cells by RT-PCR. ICE mRNA was also expressed in endometrial cells. These results suggest that endometrial epithelial and stromal cells both produce functional IL-18, and it is possible that the released IL-18 acted on endometrial cells in a paracrine and/or autocrine manner.

Mouse uterine IL-18 mRNA levels significantly varied at estrus and diestrus. In humans, endometrial IL-18 mRNA levels slightly change through the menstrual cycle and are low during the mid-proliferative phase and high during the late-secretory phase [14]. Changes in mouse uterine IL-18 mRNA levels during the estrous cycle may be due to changes in sex steroid hormone levels since E2 and P4 inhibited uterine IL-18 mRNA expression. In vivo time-course analysis showed that E2 treatment acutely decreased IL-18 mRNA levels after 3 h, and this inhibitory action continued for a maximum of 24 h. IL-18 mRNA levels returned to their initial level 48 h after E2 treatment. P4 alone and in combination with E2 also decreased IL-18 mRNA levels after 12 h, but these effects were not detected after 48 h. The rapid decrease in IL-18 mRNA levels induced by E2 treatment suggests that estrogen acutely inhibited IL-18 mRNA expression. The inhibitory effect of estrogen on IL-18 mRNA expression is in agreement with a recent report showing that in response to bacterial infection, IL-18 production in the spleens of ERα-deficient mice was higher than in wild-type mice [28]. However, the molecular mechanisms of the inhibitory actions of E2 and P4 on IL-18 gene
expression remain unclear. From the above results, it may be reasonable to conclude that IL-18 expression is associated with estrous cycle-dependent reproductive functions in the mouse uterus.

It is well known that estrogen and progestin treatments induce uterine cell proliferation [26, 29]. In the present study, E2 and P4-treatments increased IGF-I mRNA levels in the mouse uterus, as reported in previous reports [30–32]. IGF-I acts as a possible key mediator of sex steroid hormones through proliferative effects on the growth of endometrial cells [25, 33] and inhibits apoptosis of uterine cells [34]. In the present study, concurrent decreases of IL-18 mRNA levels were observed in the uterus of E2- and P4-treated mice. Several reports have indicated that IL-18 induced Fas expression in the human fetal membrane [35], apoptosis of endothelial cells through induction of proapoptotic factors, Fas-L, Bcl-XS, IL-1β and TNF-α [36, 37], and atrophy of lacrimal and salivary glands in the presence of IL-12 [38]. Therefore, it is possible that IL-18 has anti-proliferative and especially proapoptotic functions in the mouse uterus.

In conclusion, we demonstrated that IL-18 mRNA was detected in mouse endometrial epithelial and stromal cells, and that its expression was altered during the estrous cycle and was acutely inhibited by estrogen and progestin. In the mouse uterus, IL-18 may be involved in the regulation of uterine functions.

Acknowledgments

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