Morphologic effects of the epithelial ion channels on the mouse uterus: differences between raloxifene analogue (LY117018) and estradiol treatments.

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Abstruct 149 words, Text 3000 words

Condensation

LY117018, a raloxifene analogue, regulates epithelial ion channels in mice differently to estradiol, which results in morphological differences in the uterus of ovariectomized mice.

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Abstract

Objectives

Estrogen regulates the expression of epithelial Na^+ channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR). Our purpose was to assess the effects of raloxifene analogue LY117018 on the expression of ENaC and CFTR in ovariectomized mice.

Study design

Three groups of 5 female ovariectomized mice were treated with 17β-estradiol benzoate (E2), LY117018 (LY), or vehicle, respectively, for 4 to 12 weeks. Effects on the mRNA expression levels of ENaC and CFTR channels in the uterus were studied using real-time reverse transcriptase-polymerase chain reaction.

Results

E2 treatment induced CFTR expression, repressed ENaC expression and resulted in fluid accumulation in the uterus. In contrast, LY induced CFTR expression, did not repress ENaC expression, and caused no fluid accumulation.

Conclusions

Estradiol and LY117018 differentially regulate the expression of CFTR and ENaC in ovariectomized mouse uterus. This finding suggests that uterine fluid accumulation can be controlled mainly by targeting the ENaC.

Key words CFTR, ENaC, Fluid accumulation, Mouse uterus, SERM

Introduction

Female reproductive hormones have various effects on the uterus. 17β-estradiol (E2) increases the uterine epithelial cell height, stromal thickness, and luminal fluid accumulation, and occasionally produces endometrial cystic hyperplasia.[1-3] Estrogen mediates the principal proliferative response of the uterus through transcriptional regulation by binding with the estrogen receptor. Several lines of evidence suggest that E2 regulates luminal fluid accumulation through the expression of ion channels in uterus. However, it is unclear how a selective estrogen receptor modulator (SERM) regulates uterine fluid accumulation and the expression of ion channels in the uterus.

Among the effects of E2 on the uterus, fluid accumulation is associated with the induction of the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated Cl^- channel. CFTR is located in the apical membrane of epithelia and is responsible for Cl^- and fluid secretion into the uterine lumen.[4] A rat model of the ovarian hyperstimulation syndrome (OHSS) has demonstrated high levels of E2 and fluid accumulation in the peritoneal cavity and uterine horns with enhanced CFTR

expression.[3] Over-expression of CFTR induces fluid accumulation, such as hydrosalpinx.[5] Moreover, women with CFTR gene mutations are less fertile, suggesting that this gene may play a role in implantation via the regulation of fluid movement.[4, 6]

Fluid accumulation is regulated by the balance between secretion and absorption. Continuous uterine luminal perfusion analysis has demonstrated the secretion of Cl^- into lumen via the CFTR and the absorption of Na^+ into the cell by the amiloride-sensitive epithelial sodium channel (ENaC).[4] ENaC is also located at the apical side of the epithelia and play a critically important role in the movement of Na^+ across epithelial cells. ENaC complex is composed of 3 subunits (α, β, and γ) and consists of 4 subunits ($\alpha_2\beta\gamma$). Only the α-subunit is able to generate a small Na^+ current alone.[7, 8]

In kidney, ENaC regulates the absorption of extracellular Na^+ to control blood pressure. Although aldosterone is the main regulator of ENaC in kidney,[9] the expression of ENaC is up-regulated in E2-treated ovariectomized mouse kidney.[10]

Additionally, the expression of ENaC is repressed and that of CFTR is

induced in estrus period during menstrual cycle in the mouse uterus.[11] Therefore, the regulation of ENaC and CFTR in the uterus by female reproductive hormones has been suggested.

SERM has a high affinity to estrogen receptors and acts as estrogen agonist or antagonist depending on tissue. Raloxifene, a non-steroidal benzothiophene SERM, has estrogen agonistic effects on blood serum lipoproteins and bone mineral density.[12] Although raloxifene has been approved for clinical use to prevent osteoporosis in postmenopausal women, its effects on premenopausal and non-ovariectomized women remain unclear. Raloxifene exhibits little uterotrophic activity in rodents.[12-14]

LY117018 (LY) is a synthetic analogue of raloxifene. It differs from raloxifene at only one site on the molecule, containing a pyrrolidine ring on the basic side chain, compared with a piperidine ring in raloxifene. However this difference does not affect the SERM profile of this molecule.[15] Although E2 has been demonstrated to regulate the uterine morphology and expression of CFTR,[3, 16, 17] little is known about the effects of raloxifene on uterine fluid accumulation through the expression of

CFTR and ENaC in uterus.

To investigate the effect of E2 and LY on the expression of ENaC and CFTR in the ovariectomized mouse uterus, we examined morphological changes and the expression of mRNAs of αENaC (α subunit of ENaC) and CFTR after treatment with E2 and LY.

Materials and Methods

Animals

Seven-week-old female CD-1 (ICR) mice were purchased from Clea Japan (Tokyo, Japan), weighed, and maintained on a 12 hour light, 12 hour dark cycle at constant temperature, and given food and tap water ad libitum. The mice were maintained according to the Guide for the Care and Use of Laboratory Animals established by Hiroshima University. After acclimation for 1 week, bilateral ovariectomies via dorsal approach were performed under ether anesthesia in all mice and were left to rest for 1 week after the operation prior to the start of the experiments.

Materials and treatments

E2 was purchased from Sigma (St Louis, MO). LY, a raloxifene analogue, was kindly donated by Eli Lilly Corp (Indianapolis, IN). The mice were divided into 3 groups (n = 5 in each group). Each group was treated with subcutaneous injections of 0.1 mg/kg E2 with vehicle, 3 mg/kg LY with vehicle, or vehicle alone (50 μL corn oil,

control; Sigma) for 4 to 12 weeks (5 days a week). The doses were selected to be equipotent to those for treating osteoporosis, according to published protocol.[18] We confirmed the efficacy of ovariectomy by the marked atrophy of the uterus in the control group.

The mice were weighed and killed at the end of the treatment. Then the uteruses were removed and weighed. In each case, one uterine horn was flash frozen in liquid nitrogen and stored at -80°C for subsequent RNA analysis, the other horn was prepared for histological analysis.

Serum assays

At the end of the experiments, blood samples were collected from the inferior vena cava after the animals were killed. Serum was obtained by centrifugation at 4°C and stored at -20°C for subsequent analysis of the serum E2, Na^+, and Cl^- concentrations (SRL, Tokyo, Japan). Accumulation of uterine luminal fluid was observed only in the E2-treated mice. Samples of uterine luminal fluid were also analyzed by direct aspiration prior to uterus removal.

Blood pressure measurements

Systolic blood pressure was measured using the tail-cuff method (BP98A; Softron Co, Tokyo, Japan) while the mice were in a conscious and unrestricted state. Systolic blood pressure was measured 3 times in each mouse for the time point before treatment and after treatment.

Morphological analysis

The tissues for morphological analysis were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 3 h, transferred to 20% and 30% sucrose in PBS, embedded in OCT compound (Sakura Finetek, Torrance, CA), and then frozen. The frozen tissue blocks were transverse sectioned (7 μm thick), mounted on glass slides, and then stained with hematoxylin and eosin. Images were taken with Nikon Digital Sight DS-5M-L1 (Nikon, Tokyo, Japan). The uterine epithelial cell height, stromal and myometrial thickness from 5 different sampling sites on the same slide section in each mouse were measured using camera-specific software.

cDNA preparation and real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the uterine horn and kidney using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed with an Omniscript RT Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The cDNA was diluted two-fold with Tris/EDTA buffer prior to PCR amplification. Transcript levels of αENaC, CFTR and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR using a LightCycler system (Roche Diagnostics Ltd, Lewes, UK) with a SYBR Premix Ex Taq (Perfect Real Time; Takara Shuzo, Shiga, Japan) according to the manufacturer's instructions.

The specific oligonucleotide primer pair sets used for the real-time PCR are as follows: mouse GAPDH forward, 5′-ACC CAG AAG ACT GTG GAT GG-3′, reverse, 5′-CAC ATT GGG GGT AGG AAC AC-3′ corresponding to nucleotides with an expected cDNA of 171 bp (Genbank accession no. NM1001303); mouse αENaC forward, 5′-GCC AGA CTT GGA GCT TTG AC-3′, reverse, 5′-GTC CTT GAA

AGC AGT GAG GC-3' corresponding to nucleotides with an expected cDNA of 141 bp (Genbank accession no. NM11324); and mouse CFTR forward, 5'-GGA TGC TGA GGA AGC AAC TC-3', reverse, 5'-CCA GCC TGG AAC TCT CTT TG-3' corresponding to nucleotides with an expected cDNA of 172 bp (Genbank accession no. NM21050).

The amplification program included a denaturation step (10 seconds at 95°C) and an amplification step (5 seconds at 95°C, 12 seconds at 63°C, and 10 seconds at 72°C; 48 cycles), followed by a melting curve program.

To confirm the absence of DNA in each RNA sample, control reaction without reverse transcriptase was carried out. Each sample was analyzed in duplicate. A relative standard curve for the gene of interest was constructed using serial dilutions (1:10 to 1:1000) of cDNA generated from lung obtained in the present study. A melting curve program was also run for each primer pair, and only one melting temperature (Tm) was observed, suggesting one product for each reaction. The PCR products were visualized by electrophoresis on a 1.8% agarose gel in tris/acetate/EDTA (TAE) buffer to confirm that a single product was obtained for

each primer set.

Statistical analysis

The αENaC and CFTR expressions were normalized as a ratio to GAPDH expression in each sample. Differences between the means of different treatment groups were assessed using one-way factorial analysis of variance followed by Tukey-Kramer's multiple comparison test using Statcel software (OMS Publishing Inc, Tokyo, Japan). The results are presented as the mean ± SEM. $P < .05$ was considered significant.

Results

Morphological changes in the uterus following estradiol and LY117018 treatment

To examine the effects of treatment with E2 and LY on the uterus, we performed macroscopic analysis. On macroscopic examination, control mice exhibited atrophic uteruses at 4 weeks after ovariectomy. The E2-treated mice exhibited significantly larger and edematous uteruses and had extensive fluid accumulation in the uterine cavity, compared with the control; the volume of the uterine luminal fluid were greater than 1 mL.

In contrast, LY-treated mice exhibited less atrophic uterine than those of the control (Figure 1, A). Consistently, the uterine wet weights of the E2- and LY-treated mice were more than 10- and 3- to 4-fold heavier, respectively, compared with that of the control ($P < .05$) (Table 1). These findings suggest that LY has a less pronounced effect on uterine morphology than E2.

To further investigate the effects of LY and E2 on the uterus, we performed histological examination of the same samples from E2-treated, LY-treated, and control mice used in the macroscopic analysis. As expected, the uterus in the

E2-treated mice exhibited endometrial cystic hyperplasia, hypertrophic, and hyperplastic changes in endothelial cells and cystic glands, and also displayed edematous, thick stroma. The height of uterine epithelial cells, and the stromal and myometrial thickness were significantly increased in the E2-treated mice, compared with the control (Figure 1, B and Table 1). LY-treated mice also exhibited an increase in uterine stromal thickness; however, the epithelial cell height, although higher, was not significantly different, compared with the control (Figure 1, B and Table 1). Cystic hyperplasia was not observed in the LY-treated mice. These histological data suggest that LY would have less pronounced proliferative effects on uterine endometrial tissue than E2.

Uterine fluid accumulation greater than 1 mL was observed only in the E2-treated mice. To investigate the mechanism of uterine fluid accumulation in the E2-treated mice, we examined the Na^+ and Cl^- concentrations in uterine fluid. The Na^+ and Cl^- concentrations in uterine luminal fluid were 156 ± 4.1 mEq/L and 110 ± 2.0 mEq/L respectively. Importantly, the concentration of Cl^- in uterine luminal fluid was significantly higher than that in serum (100 ± 0.5 mEq/L, $P < .05$). This suggests

the dominant transport of Cl^- through uterine epithelial cells into the uterine lumen in E2-treated mice.

Quantification of epithelial ion channel mRNA expression

The presence of uterine fluid accumulation and the dominant transport of Cl^- into the uterine lumen in E2-treated mice suggest the differential effect of E2 and LY on the regulation of uterine epithelial ion channels. To study the mechanism of uterine fluid accumulation induced by the E2 treatment, we examined the mRNA level of the Cl^- channel-encoding CFTR gene in the the uterus at 4, 8, and 12 weeks after treatment with E2 and LY.

The expression of CFTR mRNA in the E2-treated mice at 4 weeks after treatment was significantly higher, compared with that in the control, as reported previously (Figure 2, A). Surprisingly, the expression of CFTR in LY-treated mice exhibiting no uterine fluid accumulation was significantly higher than that in the E2-treated and control mice (Figure 2, A). In addition, there were no significant differences in the expression of CFTR between each period in each treatment.

Because ENaC, the epithelial Na channel, has been suggested to counteract the effects of CFTR in uterine fluid accumulation by absorbing Na^+ across epithelial cells, we next examined the expression of αENaC, α subunit of the Na channel. The expression of αENaC mRNA was significantly repressed by E2 ($P < .05$, Figure 2, B). In contrast to the E2 treatment, LY treatment failed to repress the expression of αENaC (Figure 2, B). These findings suggest that the uterine luminal fluid in E2-treated mice requires both the induction of CFTR and the repression of αENaC. In LY-treated mice, the increased secretion of fluid by the induction of CFTR could be repressed by absorption through αENaC.

Systemic effects of estradiol and LY117018

E2- and LY-treated mice induced different effects on uterus, including the expression of the epithelial ion channels. However, the difference in the systemic effect of E2 and LY treatment has remained unclear. Therefore, we next examined the systemic effect of E2 and LY on body weight, serum Na^+ and Cl^- concentrations, and systolic blood pressure following treatment with E2 or LY after ovariectomy. The

serum estradiol levels of the E2-treated, LY-treated, and control mice are comparable with those reported previously (data not shown). There were no significant differences in body weight change among the E2-treated, LY-treated, and control mice (Table 1). Serum Na^+ and Cl^- concentrations, systolic blood pressure, and the expression of αENaC mRNA in kidney were also similar in all mice (Table 2 and Figure 3).

Comment

In this study, we demonstrated that E2 and LY differentially regulate the mRNA expression of CFTR and αENaC in ovariectomized mouse uterus. E2-treated mice exhibited macroscopically excessive accumulation of uterine fluid, high expression of CFTR, and low expression of αENaC, compared with control. In contrast, in LY-treated mice, overexpression of CFTR caused no visible uterine fluid accumulation and the expression of αENaC was not repressed.

E2-treated mice with high CFTR expression levels exhibited uterine fluid accumulation. E2-induced CFTR mRNA expression has also been demonstrated in rodent uterus,[16, 17] and resulted in uterine fluid accumulation.[3] Moreover, ion concentration analysis of the uterine luminal fluid of E2-treated mice revealed significantly higher levels of Cl^-, compared with serum. This observation suggests the active transport of Cl^- from epithelial cells into the uterine lumen.

These findings are consistent with previous reports suggesting a relationship between high levels of CFTR and fluid accumulation in hydrosalpinx and OHSS.[3, 5] If only the increased expression of CFTR is responsible for uterine fluid accumulation,

fluid accumulation should be observed in both the E2- and LY-treated mice. However, only the E2-treated mice exhibited uterine fluid accumulation. Importantly, both E2 and LY induced CFTR expression; however, the expression of ENaC was repressed only in the E2-treated mice. Because ENaC is involved in the absorption of Na^+ into cells, absorption of uterine fluid could occur in the LY-treated mice, thereby resulting in an absence of fluid accumulation. Thus, differences in the regulation of CFTR and αENaC expression by E2 and LY could result in uterine fluid accumulation only in the E2-treated mice.

Our findings support the idea that a balance between the functions of CFTR and ENaC could play an important role in the regulation of fluid accumulation in uterus.[4] Therefore, we propose the hypothesis that it is ENaC, and not CFTR, that regulates uterine fluid levels.

Aldosterone has been suggested as a major regulatory factor of ENaC.[9] However, the expressions of ENaC and CFTR change with estrus cycle in mouse uterine endothelium, even under aldosterone stimulating circumstances.[19] In this study we demonstrated the induction of CFTR and repression of ENaC in E2-treated

mouse uterus. These findings suggest that E2 is an important regulator of these ion channel genes in uterus. Interestingly, LY regulates the expression of CFTR and ENaC differently to E2. Moreover, we also found that LY induced an increase in stromal thickness, as has been reported previously in rodent uterus,[12] and that this effect on the stroma is less pronounced in LY-treated mice than in E2-treated mice.

These findings indicate that E2 and LY have different effects on uterus. Although E2 has been demonstrated to act through the estrogen receptor pathway and/or the nonclassical pathway and/or nongenomic pathway,[20, 21] the mechanism by which E2 regulates uterine function has remained unclear. Further study to clarify the differential effects of E2 and LY on the uterus would contribute to understanding the mechanism of E2 regulation in uterus.

ENaC is also found in kidneys and regulates the absorption of extracellular Na^+ to control blood pressure. In this study, compared with the control, treatment of ovariectomized mice with LY did not alter the expression of ENaC in kidney or affect systolic blood pressure over the observation period. This is consistent with a clinical report indicating that raloxifene has no impact on blood pressure, plasma renin

activity, or aldosterone in postmenopausal women.[22]

Long-term therapy with raloxifene or oral conjugated equine estrogen also has no affect on endothelium-dependent vasodilation in healthy postmenopausal women.[23] Moreover, the Multiple Outcomes of Raloxifene Evaluation trial demonstrated that raloxifene decreased the risk of hypertension and hypercholesterolemia.[24] Thus, raloxifene has no significant effects on the regulation of blood pressure in postmenopausal women not only with respect to the main regulator aldosterone and its downstream ENaC expression but also in relation to endothelial function.

To conclude, E2 and LY differentially regulate the expression of epithelial ion channels in the uterus. Based on our observations, we propose the hypothesis that it is ENaC, and not CFTR, that regulates uterine fluid levels. These ion channels contribute to not only fluid accumulation diseases but also implantation environment in uterine and oviduct cavities. Raloxifene does not prevent ovulation in women with normal menstrual cycles;[25] however, the effects of raloxifene in premenopausal and nonovariectomized women have remained unclear.

Further studies, investigating the effects of raloxifene on the expression of epithelial ion channels in uterus and oviduct, would contribute to establishing the efficacy of raloxifene as a drug for the treatment of fluid accumulation diseases such as hydrosalpinx, peritoneal effusion, and infertility.

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Figure legends

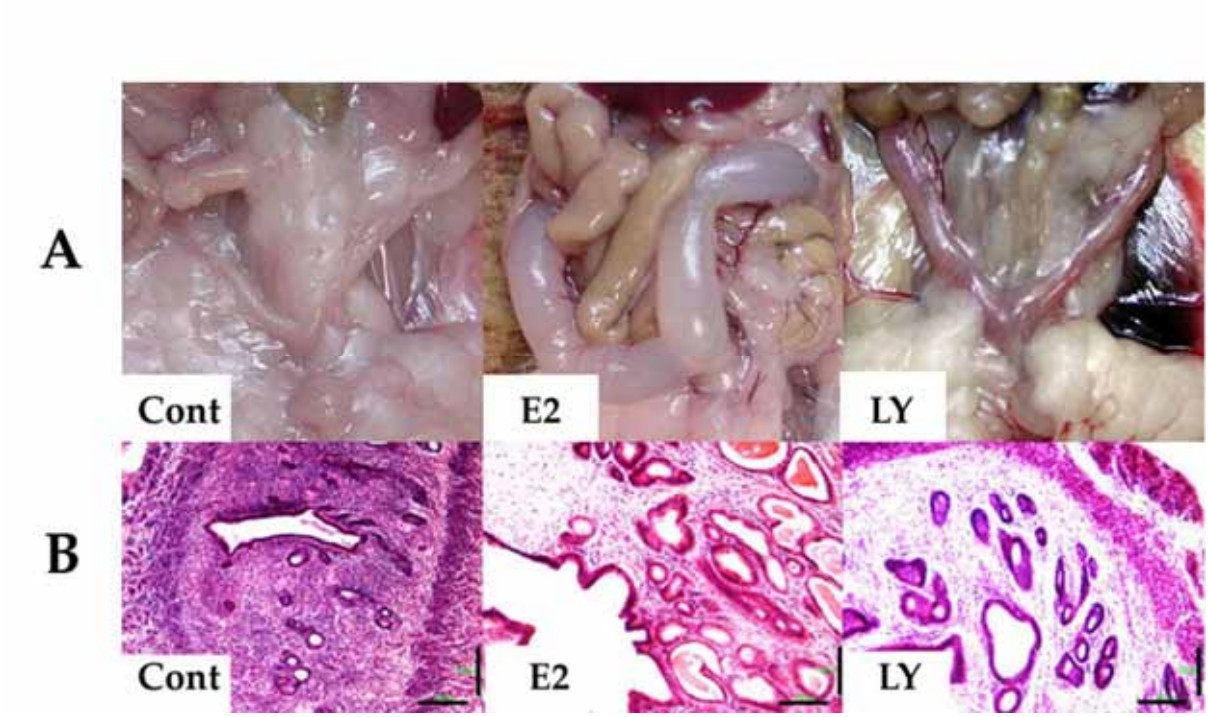

Figure 1. Macroscopic and histological appearance of the uterus after 4 weeks of treatment. The figures show the uterus of control (Cont), estradiol-treated (E2) and LY117018-treated mice (LY). **A** macroscopic appearance, **B** histological appearance. (Hematoxylin and eosin stained). The bars represent 100 μm; magnification, ×20

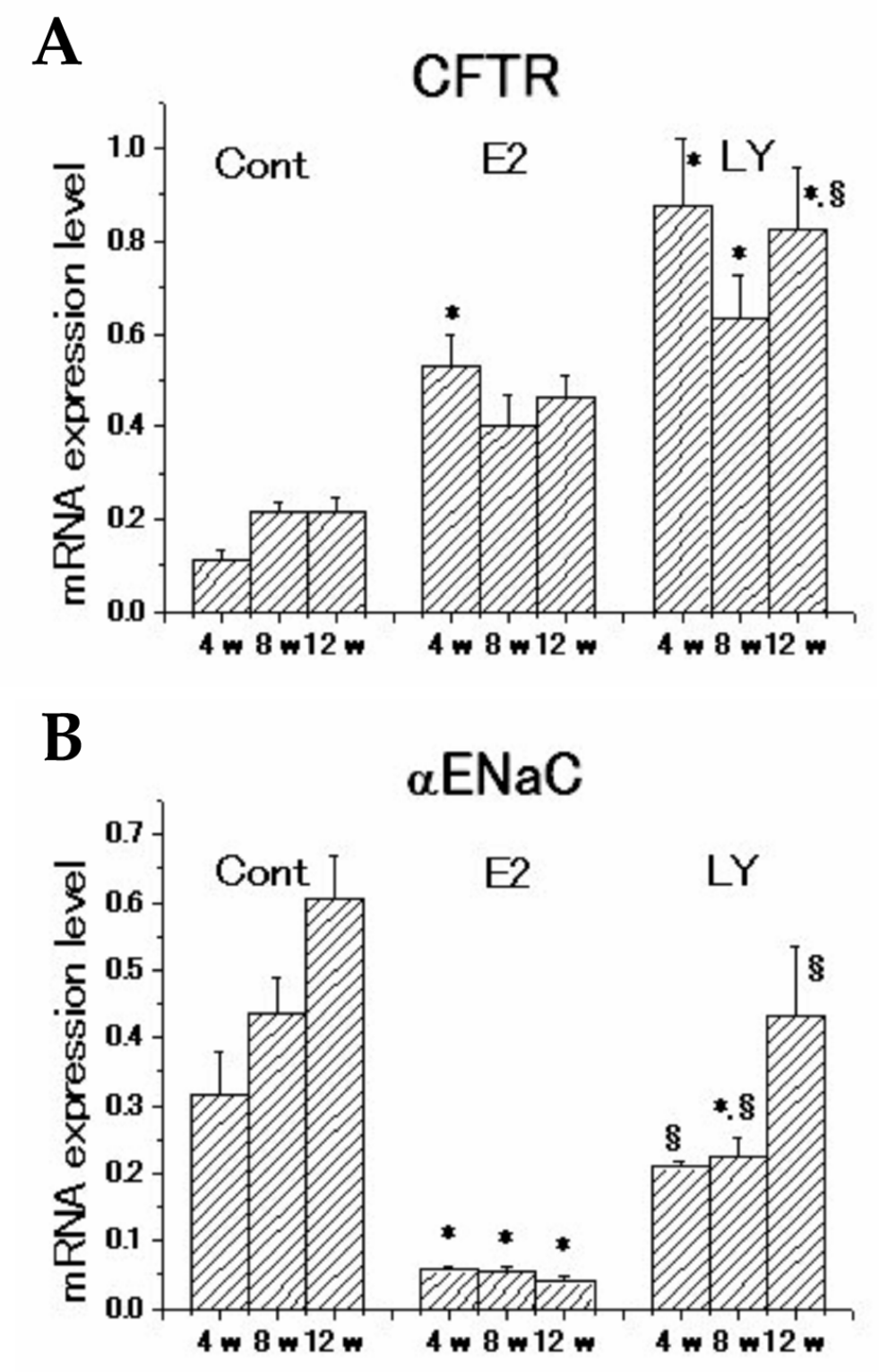

Figure 2. The mRNA expression levels of ion channels in the uterus following treatment. The graphs show mRNA expression levels for **A**, CFTR and **B**, αENaC in

estradiol-treated (E2), LY117018-treated (LY), and control mice (Cont) and analyzed by real-time RT-PCR. The graphs represent data from 5 samples after 4, 8, and 12 weeks of treatment. Data are expressed as the mean ± SEM. * indicates a significant difference from the control. § indicates a significant difference from the E2 value ($P < .05$, analysis of variance followed by Tukey-Kramer's multiple comparison test). No significant differences in CFTR expression were observed between each period in each treatment.

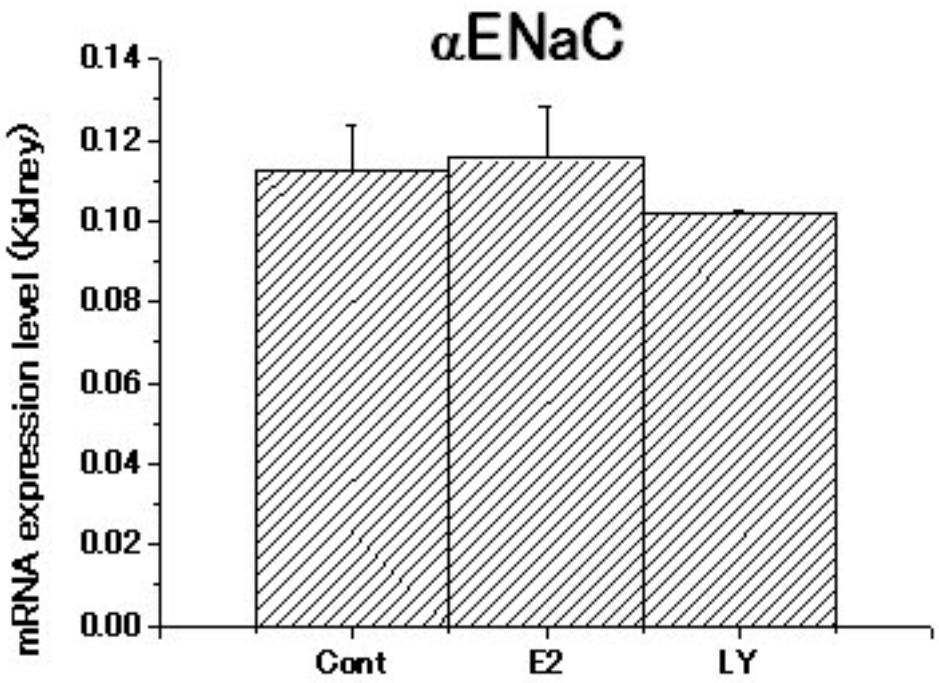

Figure 3. The mRNA expression levels of αENaC in kidney after 4 weeks of

treatment. The graphs show the mRNA expression levels for αENaC in estradiol-treated (E2), LY117018-treated (LY), and control mice (Cont) and analyzed by real-time RT-PCR. Data are expressed as the mean ± SEM.

Table 1 Uterine weight and histological parameter after 4 weeks of treatment.

	Control	E2	LY
Uterine Weight (mg)	22 ± 2.0	250 ± 17 *	64 ± 4.0 *,§
Epithelial cell height (μm)	10.2 ± 0.26	33.3 ± 4.5 *	21.0 ± 2.2 §
Stromal thcikness (μm)	264 ± 24	628 ± 46 *	488 ± 33 *,§
Myometrial thickness (μm)	160 ± 13	257 ± 33 *	215 ± 14

Values are mean ± SEM (n = 5)

*: significant difference from control ($P < 0.05$).

§: significant difference from E2 value ($P < 0.05$)

Table 2 Systemic effects after 4 weeks of treatment.

	Control		E2		LY	
Body weight change (mg)	**3.32 ± 0.52**	**N.S.**	**4.48 ± 0.43**	**N.S.**	**2.76±0.33**	**N.S.**
Serum Na+ (mEq/l)	**151.6 ± 1.4**	**N.S.**	**152.2 ± 1.3**	**N.S.**	**155.6 ± 1.0**	**N.S.**
Systolic blood pressure (mmHg)	**106.8 ± 5.8**	**N.S.**	**105.6 ± 3.8**	**N.S.**	**97.2 ± 2.0**	**N.S.**

Values are mean ± SEM (n = 5)