

Insulin-like Growth Factor 1 mRNA Expression in the Uterus of Streptozotocin-treated Diabetic Mice

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Abstract. Reproductive functions decline with the onset of diabetes in female mice. Diabetic mice have smaller uteri with an underdeveloped endometrium, suggesting diminished estrogen-induced growth. We aimed to clarify the changes in the estrous cycle and in insulin-like growth factor 1 (IGF1) expression in the uteri of streptozotocin (STZ)-treated diabetic mice, because IGF1 is one of the main growth factors involved in estrogen-induced uterine growth. ICR female mice were intraperitoneally administered STZ (10 mg/100 g BW), and blood glucose levels were determined. Mice with blood glucose levels > 200 mg/dl were classified as diabetic mice. The onset of diabetes was associated with acyclic estrous cycles. Diabetes was also induced with STZ in ovariectomized mice. Uterine *Igf1* mRNA levels were reduced in ovariectomized STZ-treated diabetic mice. Estrogen is known to stimulate *Igf1* mRNA expression in the uterus, but estrogen action was abolished in the uteri of STZ-treated diabetic mice. mRNA expressions of estrogen receptor α ($ER\alpha$) and steroid hormone receptor coactivators (SRC-1/*Ncoa1*, SRC-2/*Ncoa2*, SRC-3/*Ncoa3* and CBP/p300/*Crebbp*) were reduced in the uteri of ovariectomized STZ-treated diabetic mice. The present study demonstrates that diabetes induces a decline in female reproductive functions in mice. *Igf1* expression in ovariectomized diabetic female mice was decreased, and decreased responsiveness to estrogen in the uteri of diabetic mice is probably associated with a reduction in $ER\alpha$ and steroid receptor coactivator mRNA expression.

Key words: Diabetes, Insulin-like growth factor 1 (IGF1), Mouse, Uterus

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Diabetes mellitus is a complex, heterogeneous disorder with common symptoms being hyperglycemia and a relative or absolute lack of insulin. Diabetes mellitus is always fatal if not treated, and even when treated, serious complications such as blindness, kidney disease, and circulatory or circulatory-based problems may develop. We previously found severe disorders in pituitary and reproductive functions in streptozotocin (STZ)-induced diabetic mice, and indicated that diabetes mellitus induces disorders in the pituitary-peripheral target systems [1, 2]. We focused on changes in uterine functions in diabetic mice. STZ was used for pharmacological induction of diabetes.

Diabetic female mice show irregular or acyclic estrous cycles, suggesting a decline in reproductive function [3]. Diminished pituitary function in diabetic rats and mice has also been reported [1–4]. Furthermore, diabetes induces changes in uterine structures and functions in rats [3], and uteri of diabetic mice are smaller in size and atrophic, suggesting a diminished response to ovarian steroid hormones [5]. Interestingly, Garris demonstrated an increase in lipopoptotic endometrial epithelial cells in diabetic (*db/db*) mice [6].

Proliferation of endometrial luminal and glandular epithelial cells is regulated by estrogen, and proliferation of endometrial stromal cells is regulated by estrogen and progesterin [7, 8]. Estrogen and progesterin

stimulate the production of growth factors in endometrial cells, which in turn stimulate DNA synthesis in endometrial cells [9–13]. Insulin-like growth factor 1 (IGF1) is one of the growth factors that govern the proliferation of endometrial cells. IGF1 stimulates DNA synthesis in endometrial luminal epithelial cells and stromal cells through IGF1 receptors (IGF1R) [14–19]. IGF1 expression in the uterus is stimulated by estrogen [16, 20–22]. IGF-binding protein-3 (IGFBP3) modulates the actions of IGF1 by binding to the IGF1 molecule, resulting in obstruction of IGF1 action or prolongation of the half-life of IGF1 [23, 24]. In endometrial stromal cell cultures, IGFBP3 was shown to inhibit IGF1-induced DNA synthesis [25, 26]. In this study, we analyzed three genes of the IGF system, *Igf1*, *Igfbp3* and *Igflr*, to clarify diabetes-related changes in the uterus.

The action of estrogen on *Igf1* mRNA expression is mediated through estrogen receptors (ERs) [27]. Estrogen receptors need several coactivators or corepressors to exert their transcriptional actions [28, 29]. The steroid receptor coactivator (SRC) family includes SRC-1/*NCoA1*, SRC-2/*NCoA2*/TIF2/GRIP1 and SRC-3/*NCoA3*/AIB1/ACTR/RAC3/TRAM-1/pCIP, and is required for transcription of steroid receptors [30–33]. cAMP response element-binding protein (CREB)-binding protein (CBP)/p300 also regulates transcriptional activities of steroid receptors [31, 34, 35]. We also studied the effects of diabetes on SRC and CBP/p300 mRNA expression.

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Materials and Methods

Animals

Adult female mice of the ICR strain were used. Mice were housed in a temperature-controlled animal room and were fed a commercial diet (CA-1; CLEA Japan, Osaka, Japan) and tap water. Vaginal smears were monitored daily to observe estrous cycles. Animal care and experiments were conducted in accordance with the Guidelines for Animal Experimentation of Okayama University, Japan.

STZ treatment

Mice were intraperitoneally administered STZ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 10 mg/100 g body weight three times every two weeks from 3 months of age. STZ was dissolved in 0.05 M citric acid (65 mg/ml). Control mice were given vehicle only. In the other experiment, ovariectomized mice were administered STZ (10 mg/100g body weight) once. All mice were deprived of food for 16 h before STZ treatment.

Blood glucose measurement

Blood samples were obtained from the tail vein under light ether anesthesia. Blood glucose level was determined by the glucose oxidase method. Blood glucose levels were measured every week during experiments. Mice with blood glucose levels above 200 mg/dl were classified as diabetic.

Histological analysis

Eleven weeks after STZ treatment, uteri were collected from diabetic mice at diestrus, and uteri were collected from control mice at estrus or diestrus at the age of 5 months. Uteri were fixed with Bouin's solution. The uteri were embedded in Paraplast, and coronal uterine serial sections (5 μ m thickness) were cut. The sections were stained with hematoxylin and eosin.

Ovariectomy and estradiol-17 β (E2) treatment

Female mice were ovariectomized at 2 months of age under light ether anesthesia. One week after ovariectomy, mice were treated with STZ as described above. Two weeks after the STZ treatment, E2 (0.25 or 2.5 μ g) or sesame oil was given subcutaneously. Uteri were collected 48 h after E2 treatment.

cDNA synthesis and real-time PCR

Total RNA was prepared from uteri using a single-step method [36]. Total RNA was reverse-transcribed using ThermoScript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with random 6-mer primers according to the manufacturer's instructions. Each PCR was performed using primer sets specific for *Igf1*, *Igfbp3*, *Igf1r*, *ERA*, *Ncoa1*, *Ncoa2*, *Ncoa3*, *Crebbp* or *Rpl19* cDNAs (Table 1). Real-time PCR analysis was conducted using an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA, USA) and SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Amplifications were done in 15- μ l aliquots according to the manufacturer's instructions. DNA polymerase was activated by heating at 95 C for 10 sec, followed by 40 cycles of amplification including denaturation at 95 C for 5 sec, and annealing at 60 C for 31 sec. mRNA levels were normalized to ribosomal protein L19 (*Rpl19*) mRNA levels [37].

Table 1. Sequences of primers used in real-time PCR

Name		Sequence	Length (bp)
<i>ERA</i>	Forward	GGCTGGAGATTCTGATGATTGG	55
	Reverse	TTCCCCGGGTGTTCCAT	
<i>Igf1</i>	Forward	AAAGCAGCCCCGCTCTATCC	57
	Reverse	CTTCTGAGTCTTGGGCATGTCA	
<i>Igfbp3</i>	Forward	AAGCACCTACCTCCCCTCCCAA	98
	Reverse	TGCTGGGGACAACCTGGCTTTC	
<i>Igf1r</i>	Forward	GCTTCTGTGAACCCCGAGTATTT	82
	Reverse	TGGTGATCTTCTCTCGAGCTACCT	
<i>Ncoa1</i>	Forward	GCTCCAGCAAACCTCCACCTACTT	44
	Reverse	AATGTTTGCCTTCCACCTG	
<i>Ncoa2</i>	Forward	CCCCGTGGCCTGAGAGCAT	44
	Reverse	GAAGTTCGCAAGGCCAGATACAG	
<i>Ncoa3</i>	Forward	GCTTGGGCCACCTTCTAACG	43
	Reverse	CTTGAGCTGGGTATGTCTGTCC	
<i>Crebbp</i>	Forward	GTCTTTGCCTTTTCGTCAGC	49
	Reverse	CCACATACTGCCAGGGTCT	
<i>Rpl19</i>	Forward	CCCCTCAGCAGATCAGGAA	58
	Reverse	GTCACAGGCTTGCGGATGA	

Statistical analysis

The differences in means among groups were analyzed by analysis of variance followed by Tukey's test.

Results

Blood glucose levels, estrous cycle and uterine histology

Blood glucose levels in STZ-treated mice started to increase 4 to 5 weeks after the first STZ injection, while in vehicle-treated control mice, they did not change (Fig. 1). Vehicle-treated control mice continued to show estrous cycle, but most of the diabetic mice entered a continuous diestrus state, suggesting cessation of ovulation. Eleven weeks after the STZ treatment, the blood glucose levels remained significantly higher in the diabetic mice (386 ± 23 mg/dl, n=5) than in the control mice (107 ± 5 mg/dl, n=5, $P < 0.0001$), and then the uteri of the diabetic mice and control mice were collected for histological analysis and mRNA measurements.

Histological analysis showed that endometrial epithelia and stroma were less developed in the diabetic mice than in the control mice (Fig. 2A and B). The myometrium in the diabetic mice was also less developed. The endometrial luminal epithelial cell layer was significantly thinner in the diabetic mice than in the control mice (Fig. 2C and D).

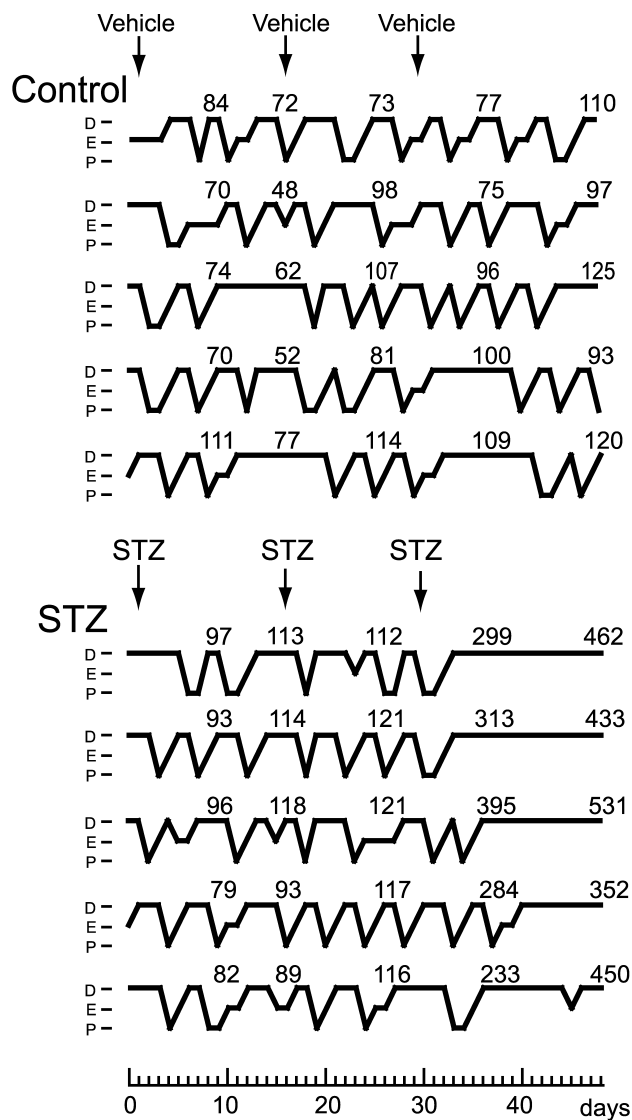


Fig. 1. Effect of STZ-treated diabetes on the estrous cycle and blood glucose levels: a summary of the estrous cycle and blood glucose levels (the number above each line, mg/dl) of diabetic mice and control mice. Intact female mice were intraperitoneally administered STZ at a dose of 10 mg/100 g body weight or the vehicle three times every two weeks at 3 months of age (arrows). Vaginal smears were checked daily (P, proestrus; E, estrus; D, diestrus), and blood glucose levels were measured every week.

Uterine Igf1, Igfbp3 and Igf1r mRNA expression in STZ-treated diabetic mice

Igf1 mRNA levels in control mice were higher during diestrus than during estrus (Fig. 3). *Igf1* mRNA levels in diabetic mice during diestrus appeared to be higher than in control mice at estrus, but the difference was not statistically significant because of the large variation in mRNA levels in the diabetic mice. There were no differences in *Igfbp3* or *Igf1r* mRNA levels between control and diabetic mice.

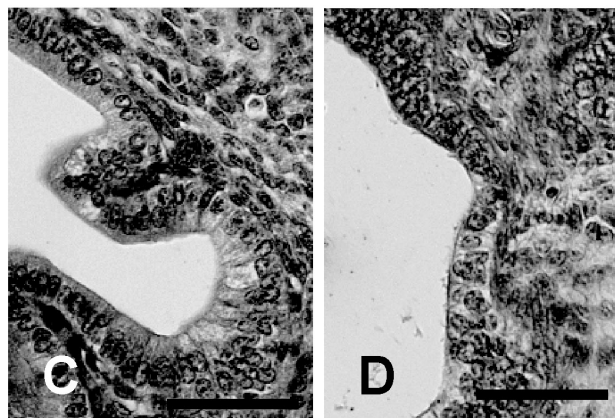
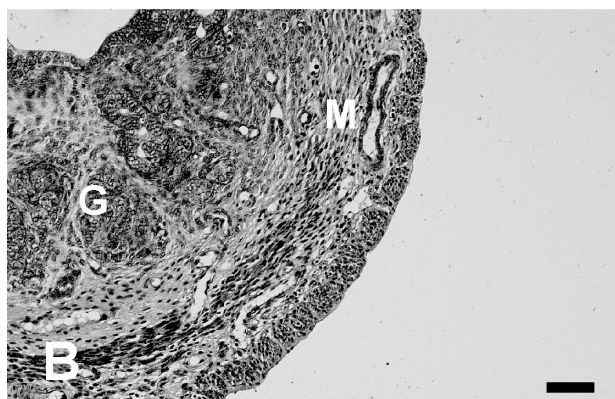
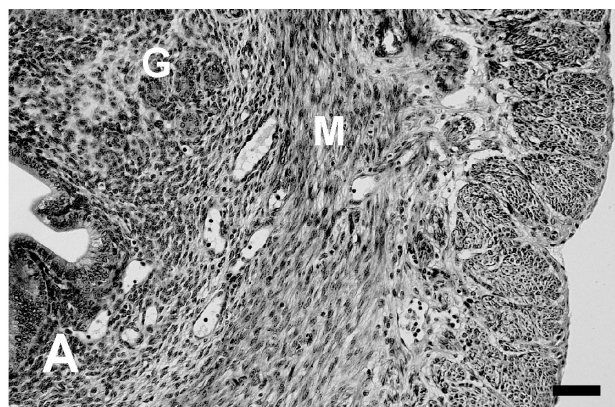


Fig. 2. Effect of STZ-treated diabetes on uterine morphology. Uteri were obtained from adult female mice at 5 months of age (eleven weeks after the STZ treatment). (A, C) Control mice at diestrus; (B, D) diabetic mice at diestrus. G, uterine gland; M, myometrium. Bar = 250 μ m (A, B); bar = 50 μ m (C, D).

Uterine ER α , Ncoa1, Ncoa2, Ncoa3 and Crebbp mRNA expression in the STZ-treated diabetic mice

Era mRNA levels in control mice were higher during diestrus than during estrus (Fig. 4). *Era* mRNA levels in diabetic mice during diestrus were higher than in control mice at diestrus. *Ncoa1* mRNA levels in control mice were higher during diestrus than during estrus. *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebb* mRNA levels during diestrus in diabetic mice were not different from those in control mice at diestrus.

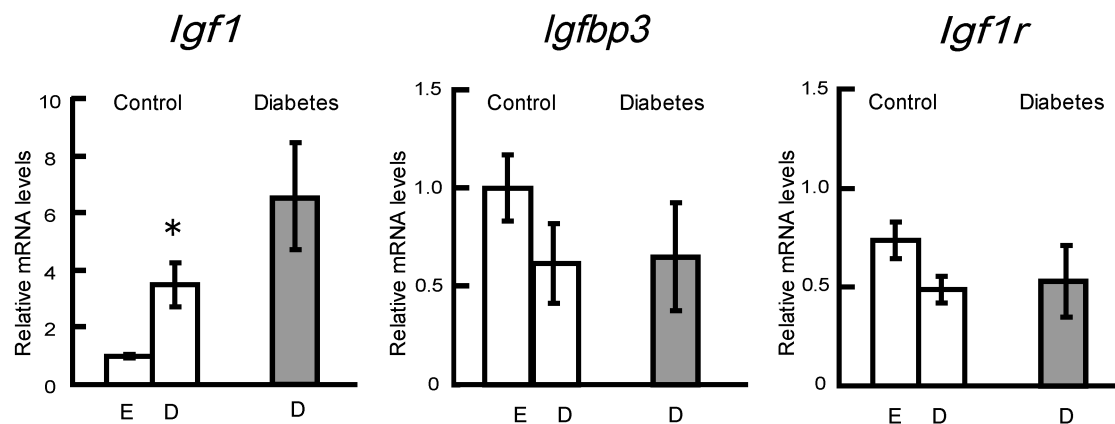


Fig. 3. Effect of STZ-treated diabetes on uterine *Igf1*, *Igfbp3* and *Igf1r* mRNA expressions. Uteri were obtained from adult female mice at 5 months of age. *Igf1*, *Igfbp3* and *Igf1r* mRNA expressions in the uterus of vehicle-treated control mice (E, estrus; D, diestrus) and STZ-treated diabetic mice (diestrus) were analyzed by real-time PCR. Each column represents the mean \pm SEM for 5 animals per group. * $P < 0.05$ compared with control mice at estrus.

E2 effects on uterine *Igf1*, *Igfbp3* and *Igf1r* mRNA expression in ovariectomized STZ-treated diabetic mice

The effect of diabetes on estrogen responsiveness was studied in terms of *Igf1*, *Igfbp3* and *Igf1r* mRNA expression. In control mice, E2 increased *Igf1* mRNA levels, and decreased *Igfbp3* and *Igf1r* mRNA levels, which is in agreement with our previous studies [18, 25, 38]. In the diabetic mice, E2 treatment did not affect *Igf1*, *Igf1r* and *Igfbp3* mRNA levels, and their levels were lower than in the control mice given the corresponding treatment (Fig. 5).

E2 effects on uterine *Era*, *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebbp* mRNA expression in the ovariectomized STZ-treated diabetic mice

Era mRNA levels were lower in the diabetic mice than in the control mice. E2 treatment did not affect uterine *Era* mRNA expression in either the diabetic mice or the control mice (Fig. 6). *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebbp* mRNA levels were lower in the diabetic mice than in the control mice. E2 treatment decreased *Ncoa1*, *Ncoa2* and *Crebbp* mRNA levels in the control mice, but did not in the diabetic mice.

Discussion

Diabetes mellitus affects female reproductive functions in humans. Post-menarcheal women with type 1 diabetes commonly have irregular menstrual cycles, and polycystic ovaries are frequently observed [39, 40]. Severely altered reproductive functions have been reported in diabetic mutant (*db*) and STZ-treated diabetic female mice [3, 41]. The present study clearly demonstrated that mice with high blood glucose levels induced by STZ treatment showed no estrous cycle, indicating cessation of ovulation. Diabetic conditions may affect the hypothalamo-pituitary system that regulates gonadotropin secretion [4, 40]. The uteri of the STZ-treated diabetic mice were less developed, suggesting reduced estrogen secretion or diminished responsiveness to estrogen. Thus, there is strong evidence, both from the present study and previous studies, that female reproductive function is severely affected by diabetes.

The uterus of the diabetic mouse was smaller than that of the control mouse. The endometrial epithelial and stromal cell layers were thinner in the diabetic mouse. The myometrial cell layer was also thinner in the diabetic mouse. Therefore, the regulatory mechanism of uterine growth may be affected under STZ-induced diabetic conditions. The growth of endometrial epithelial and stromal cells is regulated by growth factors. *Igf1* mRNA was localized in endometrial and myometrial tissues in rat and mouse uteri [15, 18, 21]. *Igf1* mRNA levels in the uterus changed during the estrous cycle, and its levels at diestrus were higher than those at estrus, which is consistent with a previous report [42]. IGF1 is one of the growth factors involved in endometrial growth, and possibly works in a paracrine and autocrine manner [13, 15, 17, 18]. *Igf1* mRNA did not differ between control and diabetic mice, indicating that constitutive production of IGF1 may not be affected by diabetes, which was consistent with a previous study done in STZ-treated rats [43]. A similar finding was reported in STZ-treated diabetic mice, although the *Igf1* mRNA levels were determined under human chorionic gonadotropin stimulation [44]. *Igfbp3* mRNA levels did not differ between diabetic mice and control mice, either. The present finding and previous reports may suggest that IGF1 was not a primary factor responsible for the atrophy of uteri in diabetic mice.

Some of the growth factors and related proteins expressed in endometrial epithelial and stromal cells are regulated by estrogen and progesterin. In the uterus, transcription of *Igf1*, *Igfbp3* and *Igf1r* is regulated by estrogen [16, 20, 25, 38]. In the present study, E2 stimulated uterine *Igf1* mRNA expression, and inhibited *Igfbp3* and *Igf1r* mRNA expression in control mice but did not affect uterine *Igf1*, *Igfbp3* and *Igf1r* mRNA expression in the diabetic mice. Thus, the responsiveness to estrogen in terms of IGF1 systems was diminished in diabetic conditions, which was consistent with a previous report in rats [43]. It is highly probable that the reduction or lack of response to estrogen in the uterus of diabetic mice is closely associated with atrophy of the uterus.

An increase in *Era* mRNA levels in diabetic mice was evident, and this was possibly due to low estrogen levels in diabetic mice, if

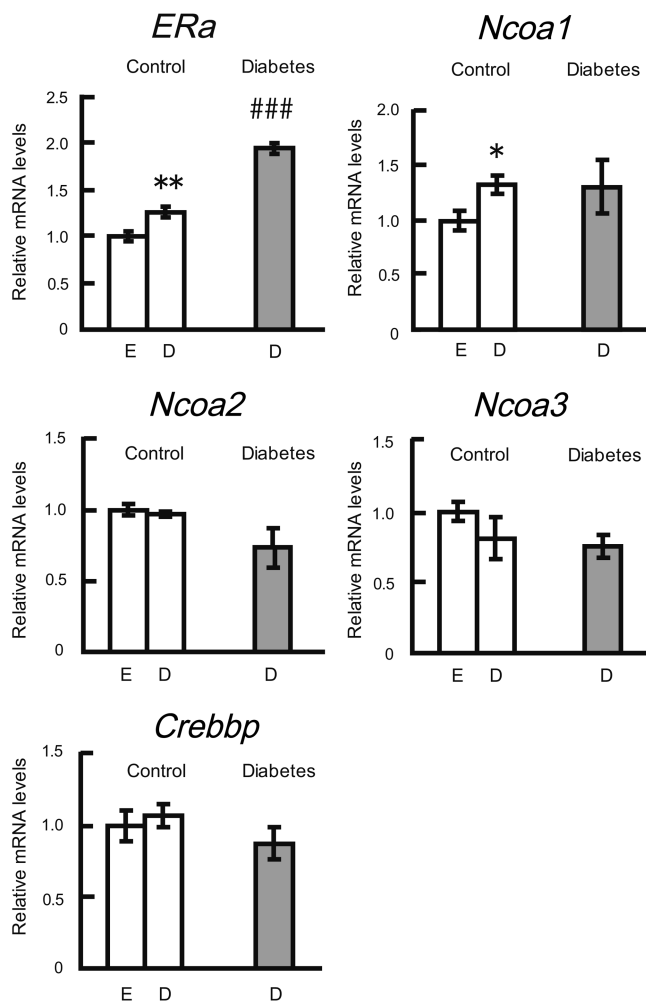


Fig. 4. Effect of STZ-treated diabetes on uterine *ERA*, *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebbp* mRNA expressions. Uteri were obtained from adult female mice at 5 months of age. *ERA*, *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebbp* mRNA expressions in the uterus of vehicle-treated control mice (E, estrus; D, diestrus) and STZ-treated diabetic mice (diestrus) were analyzed by real-time PCR. Each column represents the mean \pm SEM for 5 animals per group. * $P < 0.05$; ** $P < 0.01$ compared with control mice at estrus. ### $P < 0.001$ compared with control mice at diestrus.

the regulatory mechanism of ER gene expression was not affected in the diabetic mice. Estrogen production was decreased in diabetic rats [45], and blood estrogen levels may be decreased in diabetic mice. Uterine ER mRNA expression was upregulated when physiological concentrations of E2 were administered [46] and was downregulated by higher concentrations [47, 48]. In the present study, *ERA* mRNA levels appeared to be decreased by E2 treatment in ovariectomized control mice.

A possible reason for the changes in estrogen responsiveness in the uterus of ovariectomized diabetic mice is the change in estrogen receptor expression. In the uteri of ovariectomized STZ-treated diabetic mice, *ERA* mRNA levels were significantly decreased, which suggests that ER levels were decreased in the uterus, although *ERA*

levels were not determined in the present study. It is probable that the loss of estrogen-induced changes in *Igfl1*, *Igfbp3* and *Igflr* mRNA expression in the uterus is partly due to a decrease in ER expression.

Nuclear receptors are transcription factors, and their actions are aided by coactivators including SRCs and CBP/p300. Several nuclear receptor coactivators are expressed in the uterus [49]. In ovariectomized STZ-treated diabetic mice, mRNA expression of SRCs and CBP/p300 were decreased in the uterus, which may be associated with the loss of responsiveness to estrogen in diabetic mice.

SRC-1 exerts a major role in regulating ER and progesterone receptor (PR) in the uterus [31]. SRC-1 knockout mice (*SRC-1^{-/-}*) are fertile, but their uteri showed less response in the decidualization [50]. SRC-2 is coexpressed with SRC-1 in the uterus during pregnancy, and SRC-2 knockout affects the induction of the decidual reaction under the stimulation of estrogen and progesterin [51], and is required for pregnancy [52]. SRC-2 is also involved in the modulation of estrogen, Wnt, and bone morphogenetic (BMP) growth factor signaling pathways in the uterus [51]. SRC-3 expression is low in the mouse uterus [53, 54], but in the present study, SRC-3 mRNA was detected. The reason for this discrepancy is not clear. SRC-3 knockout female mice showed disrupted reproductive functions, delayed puberty and prolonged estrous cycle [51]. In SRC-3 knockout mice, liver *Igfl1* mRNA levels were reduced [55], suggesting the involvement of SRC-3 in *Igfl1* mRNA expression. In the present study, SRC-1, SRC-2 and SRC-3 mRNA levels were decreased in the uteri of STZ-treated diabetic mice, probably leading to the reduced growth of the uterus, although molecular mechanisms of the interactions of SRCs with ER or PR are not clear.

CBP/p300 acts as essential coactivators for many transcription factors [35, 56]. p300 potentiates ERs through the A/B regions of both ER α and ER β [57]. The decrease in *Crebbp* mRNA expression may lead to the diminished transcriptional activation of ER-dependent genes in endometrial cells.

SRCs mRNA and CBP/p300 mRNA levels were downregulated by E2 treatment over the course of 48 h, which suggests that these expressions may be associated with E2 and ER levels. On the contrary, previous reports showed that E2 did not induce significant changes in SRC mRNA expression in the uterus of ovariectomized rats over the course of 24 h [49]. The discrepancy may be partly due to the difference in the duration of E2 treatment. The decrease in SRC mRNA expression may be not induced by diabetic conditions directly. In diabetic mice, the E2-induced changes in SRC mRNA and CBP/p300 mRNA were not detected. The loss of E2 responsiveness of uterine cells in terms of SRCs and CBP/p300 expression in diabetic mice may be also responsible for the diminished growth of uteri.

The present study clearly demonstrates diabetes-induced declines in female reproductive functions: no ovulation and atrophy of uteri. Several changes in gene expression, such as in the IGF system-related genes, estrogen receptors, and coactivators, were demonstrated in ovariectomized diabetic mice.

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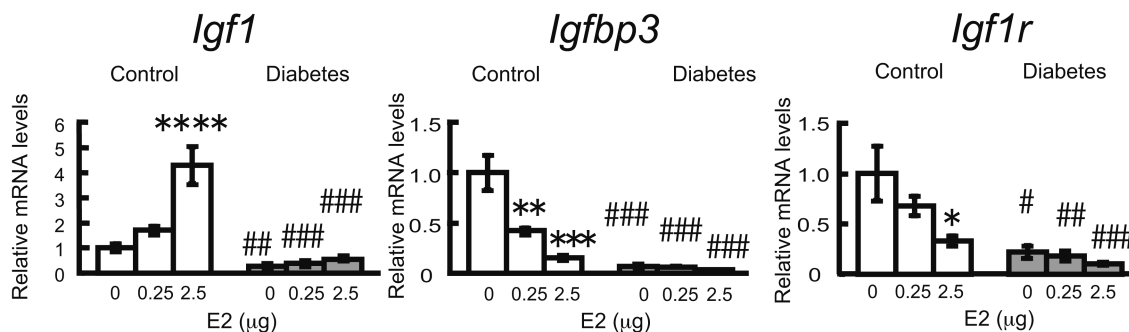


Fig. 5. Effect of E2 on uterine *Igf1*, *Igfbp3* and *Igf1r* mRNA expression in ovarioctomized STZ-induced diabetic mice. Ovarioctomized STZ-induced diabetic mice were given a single injection of E2 (0.25 or 2.5 µg) or sesame oil as the vehicle. Uteri were collected 48 h after treatment, *Igf1*, *Igfbp3* and *Igf1r* mRNA levels were analyzed by real-time PCR. Each column represents the mean ± SEM for 5 animals per group. The mRNA levels are shown as relative values compared with those of control mice given sesame oil. *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001 compared with control mice given sesame oil. #P<0.05; ##P<0.01; ###P<0.001 compared with the control mice given the corresponding treatment.

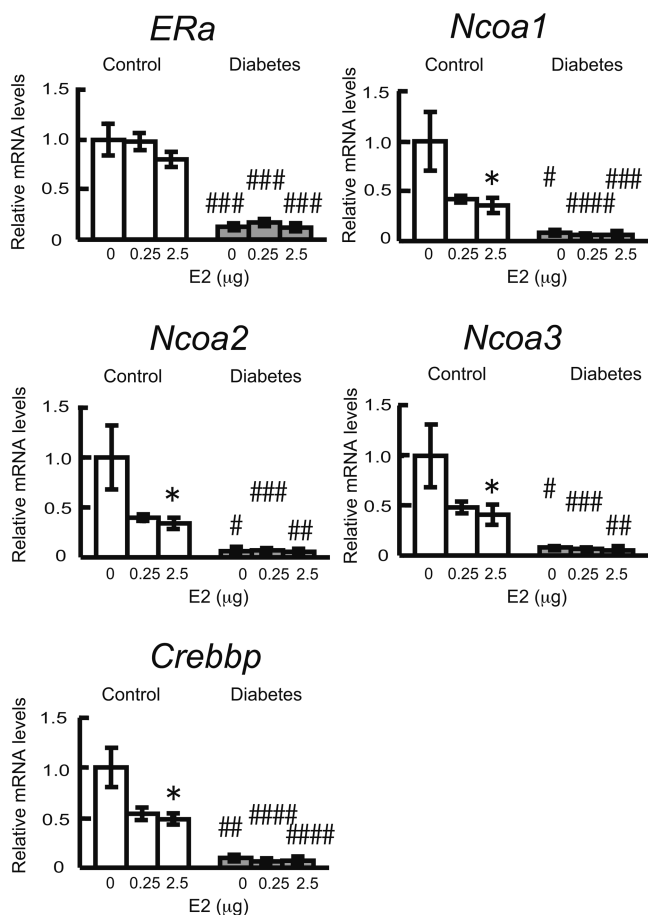


Fig. 6. Effect of E2 on uterine *ERα*, *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebbp* mRNA expression in ovarioctomized STZ-induced diabetic mice. Ovarioctomized and STZ-induced diabetic mice were given a single injection of E2 (0.25 or 2.5 µg) or sesame oil as the vehicle. *ERα*, *Ncoa1*, *Ncoa2*, *Ncoa3* and *Crebbp* mRNA levels were analyzed by real-time PCR. Each column represents the mean ± SEM for 5 animals per group. The mRNA levels are shown as relative values compared with those of control mice given sesame oil. *P<0.05 compared with control mice. #P<0.05; ##P<0.01; ###P<0.001; ####P<0.0001 compared with the control mice given the corresponding treatment.

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