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# Estradiol restores diabetes-induced reductions in sex steroid receptor expression and distribution in the vagina of db/db mouse model☆

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ABSTRACT

Sex steroid hormones and receptors play an important role in maintaining vaginal physiology. Disruptions in steroid receptor signaling adversely impact vaginal function. Limited studies are available investigating the effects of diabetic complications on steroid receptor expression and distribution in the vagina. The goals of this study were to investigate type 2 diabetes-induced changes in expression, localization and distribution of estrogen (ER), progesterone (PR) and androgen receptors (AR) in the vagina and to determine if estradiol treatment ameliorates these changes. Eight-week-old female diabetic (db/db) mice (strain BKS.Cg-m+/+ *Lepr*$^{db}$/J) were divided into two subgroups: untreated diabetic and diabetic animals treated with pellets containing estradiol. Control normoglycemic littermates were subcutaneously implanted with pellets devoid of estradiol. At 16 weeks of age, animals were sacrificed, vaginal tissues excised and analyzed by Western blot and immunohistochemical methods. Diabetes produced marked reductions in protein expression of ER, PR, and AR. Diabetes also resulted in marked differences in the distribution, staining intensity and proportion of immunoreactive cells containing these steroid receptors in the epithelium, lamina propria and muscularis. Treatment of diabetic animals with estradiol restored receptor protein expression and distribution similar to those levels observed in control animals. This study demonstrates that type 2 diabetes markedly reduces steroid receptor protein expression and distribution in the vagina. Estradiol treatment of diabetic animals ameliorates these diabetes-induced changes.


## 1. Introduction

Female genital sexual arousal is a complex neurovascular process which depends on genital tissue structure and the integration of signals from neurotransmitters, locally produced vasoactive substances and sex steroid hormones (estrogens, progestins and androgens). Female genital sexual arousal is characterized by increased genital blood flow, resulting in engorgement of vaginal, clitoral and labial tissues and the appearance of lubricating transudate on the vaginal epithelium (for review cf. [1,2]).

Sex steroid hormones are implicated in modulating female genital sexual arousal [3–7,32]. Androgens and estrogens regulate genital tissue growth and function and modulate genital blood flow [3–6,32]. Thus, androgen and estrogen insufficiency contribute to female genital sexual arousal dysfunction [3–6,32]. Several studies have reported decreased circulating estradiol levels in type 1 and type 2 diabetic animal models [8–14]. We have also reported that type 1 diabetes reduces circulating estradiol levels, alters vaginal tissue morphology, reduces vaginal blood flow and reduces expression of estrogen and androgen receptors, in streptozotocin-treated rats [15]. Women with type 1 diabetes experience decreased sexual arousal function and have significantly reduced estradiol levels compared to control subjects [16]. Limited data are available in type 2 diabetic women. Thus, we propose that diabetes disrupts estrogen signaling. This hypothesis is partially supported by studies showing that estradiol supplementation in diabetic animals ameliorates some of the diabetic complications in several organs and tissues [8,10,12–14].

We have reported that type 2 diabetes alters vaginal histoarchitecture and estradiol treatment ameliorates this pathology [19]. To date, however, no studies have investigated the effects of type 2 diabetes on vaginal physiology and the potential disruption of sex steroid receptor signaling in diabetic females. Type 2 diabetes-induced changes in vaginal functional integrity may be related to

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the regulation of the expression and distribution of sex steroid receptors in the various laminae of the vagina. More importantly, no studies are available on the therapeutic effects of estradiol supplementation in type 2 diabetic animals in ameliorating the changes in sex steroid receptor expression and tissue localization and distribution. For these reasons, we have undertaken this study to investigate the effects of type 2 diabetes on the expression, localization and distribution of estrogen, androgen and progesterone receptors and to determine if estradiol treatment of diabetic animals normalizes these changes.

## 2. Materials and methods

### 2.1. Animals

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Boston University School of Medicine. Eight-week-old female diabetic (db/db) mice (strain BKS.Cg-m+/+ *Lepr*$^{db}$/J) and age-matched db/+ normoglycemic female littermate controls ($n = 8$) were obtained from the Jackson Laboratory (Bar Harbor, ME). Diabetic animals were divided into two subgroups: diabetic treated with vehicle only (untreated group, $n = 8$) and estradiol treated group (treated group, $n = 12$). Mice were housed 2–3 animals per cage with food and water available *ad libitum*. Blood glucose levels were determined at 12 and 16 weeks of age, using an *Accu-check* blood glucose meter (Roche Diagnostics, Basel, Switzerland) as previously described [15,19]. Animals were considered diabetic when blood glucose level exceeded 250 mg/dl (13.86 mmol/L). All animals were sacrificed at 16 weeks of age.

### 2.2. Estradiol administration

At 8 weeks of age, animals in the estradiol treated diabetic group were implanted subcutaneously with pellets containing 17β-estradiol (0.83 μg/day; Innovative Research of America, Sarasota, FL) through a small incision on the nape of the neck. The control and untreated diabetic groups of animals were implanted with pellets devoid of estradiol. At the conclusion of the experiments, animals were sacrificed, blood was drawn from the abdominal aorta and allowed to coagulate at 4 °C and centrifuged. Plasma samples were analyzed for estradiol by immunoassay at the Endocrinology Laboratory, Animal Health Diagnostic Center at the Cornell University College of Veterinary Medicine (Ithaca, NY) as previously described [15,19].

### 2.3. Tissue procurement for biochemical and histological processing

The vagina and uterine horns were removed *en bloc* dissected, weighed and either stored frozen at −80 °C for biochemical analyses or fixed in 10% neutral buffered formalin for histological analyses.

### 2.4. Preparation of vaginal tissue extracts

Vaginal tissue from each control animal and diabetic estradiol-treated animal was processed individually. Vaginal tissue from untreated diabetic animals was insufficient to process individually, due to tissue atrophy, and therefore, tissues from 2 animals were pooled.

Unless otherwise noted, all manipulations were carried out at 0–2 °C. Frozen vaginal tissues were pulverized with a Bessman tissue pulverizer (Spectrum Laboratories, Rancho Dominguez, CA) pre-cooled in liquid nitrogen and placed on dry ice. The tissue powder was transferred into a small test tube and resuspended in ice cold buffer [20 mM HEPES, pH 7.4, 1 mM EDTA containing phenylmethylsulfonylflouride (PMSF; 0.5 mM) and mammalian protease inhibitor cocktail (0.8 μM aprotinin; 20 μM leupeptin; 40 μM bestatin; and 15 μM pepstatin A; Sigma Chemical Co., St. Louis, MO)] and homogenized using a Brinkman PT3000 polytron mini-probe with 10 s bursts and 30 s cooling intervals. The tissue homogenates were mixed with equal volumes of HEPES buffer containing 0.8 M KCl and re-homogenized to extract nuclear receptors [17]. The homogenates were kept at 0–2 °C for 60 min with intermittent vortexing and then centrifuged at 30,000 × *g* for 25 min at 2 °C. The supernatant (total tissue extract) was then transferred to clean tubes and aliquots of the extract were frozen at −75 °C. Additional aliquots of the supernatant were used for protein determination by the method of Lowry.

### 2.5. Western blot analyses

For each vaginal extract sample, we determined protein concentrations by the method of Lowry using various dilutions. Once the protein concentration in each sample was established, an aliquot of each sample was diluted with buffer to give 100 μg protein per 0.1 ml and layered onto sodium dodecyl sulfate polyacrylamide gels [7.5% polyacrylamide; 10% sodium dodecyl sulfate (SDS) under denaturing conditions (5% β-mercaptoethanol)] and electrophoresed for 16 h under cooling conditions. The separated proteins were transferred onto nitrocellulose membranes as described previously [15]. The nitrocellulose membranes were then incubated in MemCode™ Reversible Protein Stain (Pierce Chemical Co., Rockford, IL) to visualize the protein transfer. This staining method results in turquoise-blue protein bands. Following staining with the Reversible Protein Stain, membranes were scanned using an Epson scanner to determine protein loading. The stain was then removed using the MemCode™ Stain Eraser and membranes were washed once again.

The nitrocellulose membranes were then incubated at 4 °C for 16 h in blocking buffer [150 mM NaCl; 10 mM Tris–HCl buffer; pH 7.6 with 0.05% Tween-20 (TBST), 5% milk (TBST-m)] to block non-specific binding sites. The membranes were then washed with TBST and reincubated at 4 °C for 16–20 h with the primary specific antibodies prepared in TBST-m on a rocking platform (anti-ERα (Ab-15), 1:100, LabVision, CA; anti-PR (Ab-5), 1:200, LabVision, CA; anti-AR (N-20), 1:1600, Santa Cruz Biotechnology, CA). The membranes were then washed again and incubated with the secondary antibody prepared in TBST-m [horseradish peroxidase-linked goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody (Pierce Chemical Co., Rockford, IL)], depending on the nature of the primary antibody used. After thorough washing, membranes were incubated with peroxide and Luminol enhancer solution (enhanced chemiluminescence kit; Pierce Chemical Co., Rockford, IL). Immunoreactive bands were visualized by exposure to autoradiographic film (GE Healthcare, Piscataway, NJ).

After gel electrophoresis and immunoblotting, the stained bands were scanned and the band densities were normalized to a prominent non-specific protein band which was consistently observed in all lanes in equal density.

### 2.6. Assessment of ERα, PR, and AR protein band density by densitometry

Exposed autoradiographic films were scanned using an Epson scanner. Scanned images of sex steroid receptor protein band densities were quantified with Image J, a public domain Java image processing program (http://rsb.info.nih.gov/ij); and normalized to densities of the separate band of proteins that was consistently present in each animal in order to normalize for protein loading.

### 2.7. Determination of ERα, PR and AR localization and distribution by immunohistochemical assays

The central portion of the vagina, measuring approximately 3 mm in length was embedded for immunohistological examination. The proximal-most end was labeled with histological dye in order to maintain directionality of the vagina. Tissues were fixed for 24–48 h, dehydrated and embedded in paraffin. Sections were cut at 5 μm on a rotary microtome and mounted on numbered glass slides. Three slides were selected at regular intervals along the sampled portion of the vagina and used for each steroid hormone immunohistochemical assay.

Immunohistochemical assays were performed using the peroxidase-linked Vectastain ABC method (Vector Laboratories, Burlingame, CA) with a Gill's hematoxylin counterstain. Following deparaffinization and rehydration with distilled water, slides were autoclaved for 15 min at 250 °C to facilitate antigen retrieval. Slides then were washed with Tris-buffered saline (TBS; 150 mM NaCl, 11.5 mM Tris–HCl buffer; pH 7.4) containing 0.025% Triton X-100 (TBS/Triton). The slides were immersed in 1.6% hydrogen peroxide in methanol for 30 min at room temperature to quench endogenous peroxidase activity and washed again with TBS/Triton. Non-specific antibody binding was blocked by incubation in 5% normal serum in a humidified chamber for 1 h at 37 °C. Normal serum was always from the species in which the secondary antibody was raised. The sections then were incubated overnight (16–18 h) with primary antibody at 4 °C [ERα (Ab17), 1:500, LabVision, CA; PR (Ab5), 1:200, LabVision, CA; AR (N-20), 1:250, Santa Cruz Biotechnology, CA]. Slides then were washed with TBS/Triton, incubated with biotinylated secondary antibody (ERα, horse anti-mouse; PR and AR, goat anti-rabbit) at a dilution of 1:200 for 30 min at 37 °C. At the end of the incubation, the slides were washed three times with TBS/Triton and re-incubated at 37 °C for 30 min with the avidin–biotin complex (ABC). After another series of washes, the chromogen substrate, diaminobenzidene (DAB), was applied to sections and allowed to develop for 2 min. Slides then were washed with distilled water, counterstained with Gill's hematoxylin, dehydrated with graded alcohols and cover-slipped for light microscopic examination.

To insure that the staining is specific, negative controls were carried out for each immunohistochemical assay to assess the distribution and localization of sex steroid receptors by omitting the primary antibody in the incubation. All other steps were carried out identical to the test samples and positive controls. Sections of mouse uterine tissue were used as positive controls for the presence of ERα and PR antibodies. Sections of mouse prostate tissue were used as positive controls for the presence of AR.

### 2.8. Assessment of sex steroid hormone receptor staining intensity and distribution

Three slides from each animal, containing three to four vaginal sections each, were used to evaluate the distribution of ERα, PR, and AR, by immunohistochemical methods. Three separate areas on one vaginal tissue section were assessed for every slide. Therefore, a total of nine areas per animal were evaluated for each steroid receptor (3 slides/animal × 1 vaginal section/slide × 3 separate areas/vaginal section = 9). Two individual parameters were evaluated for all three steroid receptors: (1) the staining intensity and (2) the proportion of immunoreactive (ir) cells. The intensity of the immunostain was graded on a scale from 0 to 3 where: 0 = no immunostain present, 1 = light brown immunostaining, 2 = moderate brown immunostaining, and 3 = dark/intense brown immunostaining. The proportion of immunoreactive (ir) cells were assessed on a scale of 0–3 where: 0 = no positive ir-cells present, 1 = <50% positively stained ir-cells, 2 = >50% positively stained ir-cells, and 3 = 100% positively stained ir-cells. Cells were considered to be "positively stained" if there was a brown color associated with them beyond that of background staining. Cells that were "negative" for the protein of interest were counterstained blue with Gill's hematoxylin. The epithelium, lamina propria and muscularis layers were each individually assessed for both parameters in order to determine potential differences in each layer of the vaginal wall.

## 3. Statistical analyses

Statistical analyses were performed using GraphPad Prism version 3.0 (San Diego, CA). The staining intensity and proportion of ir-cell grading scales were first validated by a blinded third party. Seventy-five percent of the total number of slides was independently graded. Spearman's rank correlation coefficients were determined and reasonable correlations were assumed when Spearman $r$ values were equal to or greater than 0.75. Immunostained slides were then graded and group differences were tested for statistical analysis using analysis of variance (ANOVA) by the Kruskal–Wallis test for nonparametric data followed by a Dunn's *post hoc* multiple comparison test to determine between group statistical differences.

Relative optical densities from Western blot analyses were averaged within groups and the percent change between groups was calculated and reported.

## 4. Results

### 4.1. Effects of diabetes and estradiol treatment on the estrous cycle

In this study we made no attempt to synchronize the estrous cycle prior to experimentation. However, we assessed the stages of the estrous cycles for each animal at the time of sacrifice in each experimental group by vaginal smears. In the control group, 6 out of 8 animals were in proestrus and 2 out of 8 were in metestrus. In the untreated diabetic group, 3 out of 8 animals were in diestrus and 2 out of 8 were in metestrus, while 1 animal each exhibited proestrus and estrus and the remaining animal demonstrated fungal candidiasis. In the estrogen treated diabetic group, 5 out of 12 animals were in proestrus and 4 of 12 animals were in estrus, while 2 were in diestrus and the remaining animal demonstrated fungal candidiasis.

### 4.2. Effects of diabetes and estradiol treatment on ERα expression and vaginal tissue distribution

Fig. 1(lanes 1–5) shows ERα protein expression in vaginal tissues from 5 separate control animals. In untreated diabetic animals, estrogen receptor alpha expression decreased by 73% (Fig. 1, lanes 6–8) when compared to age-matched control animals. Treatment of diabetic animals with estradiol for 8 weeks did not result in increased ERα band intensity (Fig. 1, lanes 9–14). In fact ERα band density remained low compared to controls, and was similar to untreated diabetics.

In tissue from intact, mature control animals, immunostaining with a specific antibody to ERα demonstrated low to moderate levels of ERα expression in the various laminae of the vaginal wall (Fig. 2). ERα localization was mostly nuclear with minimal cytoplasmic staining. On the basis of immunostaining of ERα, three zones were identified in the epithelium of control animals: (1) a basal zone in which nuclei demonstrated low to moderate staining, (2) an intermediate, or parabasal, zone in which nuclei were minimally immunoreactive, and (3) a superficial, or juxtalumenal, zone in which nuclei were totally unstained. The cytoplasm of the epithelial cells was minimally stained throughout all three zones. Overall,

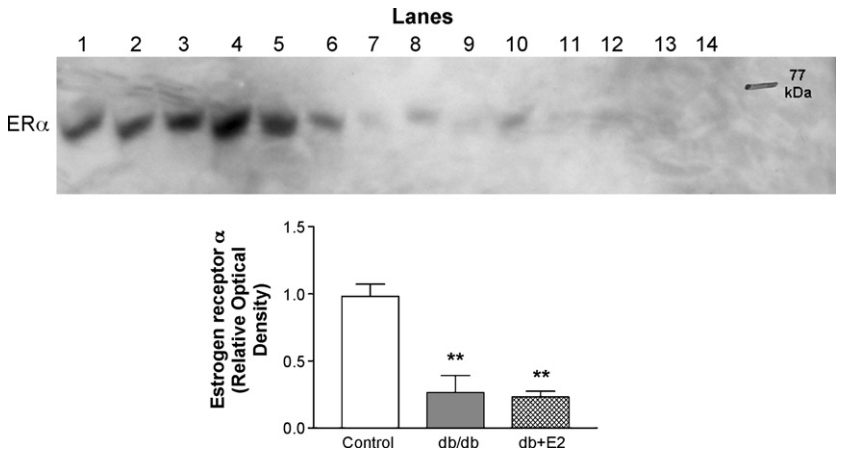


**Fig. 1.** Effects of type 2 diabetes and estradiol supplementation on ERα protein expression in the mouse vagina. Upper panel represents vaginal tissue extracts from controls (lanes 1–5), untreated diabetic (lanes 6–8) and diabetic animals treated with estradiol (lanes 9–14). Lower panel represents the quantitative assessment of the relative optical densities from control, untreated and estradiol treated diabetic animals. Lanes 6 and 7 each represent extracts pooled from two diabetic animals, whereas all other lanes represent extracts from a single animal. $^{**}p \leq 0.001$ vs. control.

more than 50% of epithelial cell nuclei were ERα-immunopositive. Similarly, in the muscularis, more than half of smooth muscle cell nuclei were immunoreactive for ERα. In contrast, fewer than half of fibrocyte nuclei in the lamina propria were immunoreactive.

Type 2 diabetes caused significant atrophy in the vagina and resulted in marked differences in the immunostaining and localization of ERα, especially in the epithelium. As shown in Fig. 2, upper panel B, virtually all epithelial cell nuclei were intensely immunopositive as compared to those in tissue from control animals. Furthermore, the cytoplasm of epithelial cells was also moderately stained. Neither the lamina propria nor the muscularis layers exhibited any significant changes in the intensity or distribution of ERα immunostaining in untreated diabetic animals.

Estrogen supplementation in diabetic animals produced marked hypertrophy and re-stratification of the vaginal epithelium. As can be seen in Fig. 2, estrogen treatment markedly reduced nuclear and cytoplasmic ERα immunostaining, except in very few cells confined to the basal zone of the epithelium. The intensity of ERα

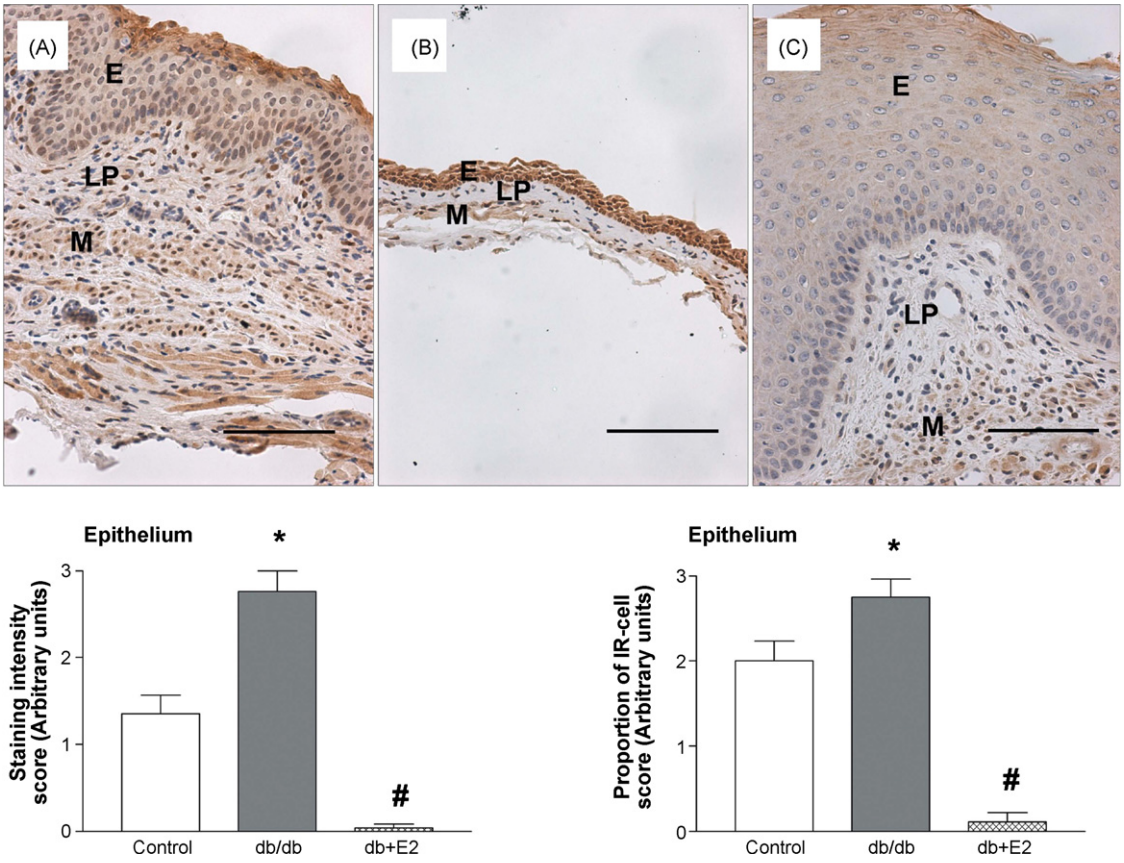


**Fig. 2.** Effects of type 2 diabetes and estradiol supplementation on the localization and distribution of ERα in vaginal tissues. Upper panels represent vaginal tissues from non-diabetic control (A), untreated diabetic (B), and estradiol-treated (C) diabetic animals immunohistochemically probed with an antibody to ERα; lower panels represent semi-quantitative analysis of ERα staining intensity and proportion of immunoreactive cells of the vaginal epithelium. E = epithelium; LP = lamina propria; M = muscularis. Scale bars represent 100 μm. $^{\#}p \leq 0.01$ vs. db; $^{*}p \leq 0.05$ vs. control.

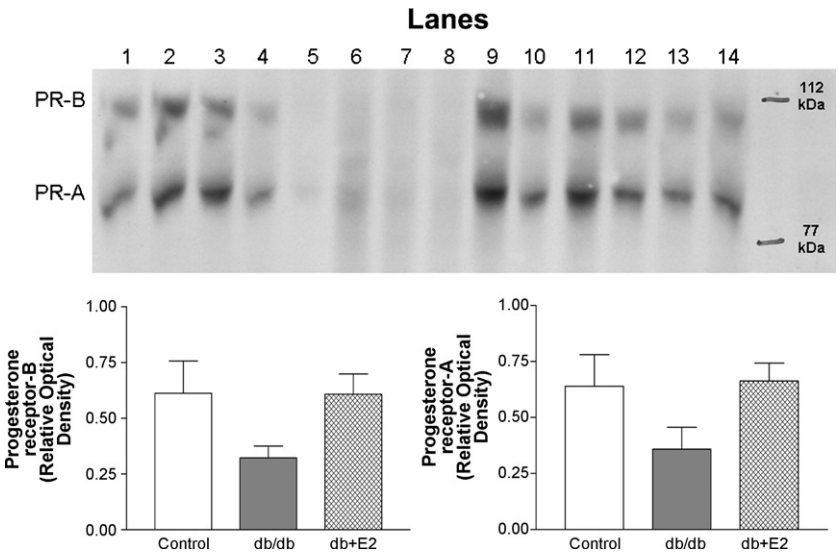


**Fig. 3.** Effects of type 2 diabetes and estradiol supplementation on PR-B and PR-A protein expression in the mouse vagina. Upper panel represents vaginal tissue extracts from controls (lanes 1–5), untreated diabetic (lanes 6–8) and diabetic animals treated with estradiol (lanes 9–14). Lower panels represent the quantitative assessment of the relative optical densities from control, untreated and estradiol treated diabetic animals. Lanes 6 and 7 each represent extracts pooled from two diabetic animals, whereas all other lanes represent extracts from a single animal.

immunostaining in fibrocyte nuclei of the lamina propria was also reduced when compared to control and untreated diabetic animals, although the effect was not statistically significant. Approximately half of all nuclei were immunoreactive and were lightly stained. The staining intensity and proportion of cells immunoreactive for ERα in the muscularis layer of estrogen-treated diabetic animals were comparable to those of control and untreated diabetic animals.

### *4.3. Effects of diabetes and estradiol treatment on PR expression and vaginal tissue distribution*

In vaginal tissue extracts from control animals, two protein bands were identified for progesterone receptor (PR) on Western blot analyses (Fig. 3); PR-A, with an approximate molecular weight of 81–85 kDa and PR-B, with an approximate molecular weight of 110–116 kDa (lanes 1–5). In tissues from untreated diabetic animals (Fig. 3, lanes 6–8), PR protein bands were of lower intensity compared to controls, suggesting down-regulation of PR expression in diabetes. On average, PR-B decreased by 47% and PR-A decreased by 45% in diabetes when compared to bands obtained from controls. Estradiol treatment of diabetic animals resulted in up-regulation of PR protein expression to levels similar to that observed in controls. On average, estrogen treatment of diabetic animals resulted in 90% and 94% increases in PR expression for PR-B and PR-A compared to untreated diabetic animals, respectively.

In vaginal tissue of control animals, immunoreactivity for the progesterone receptor was evident in the epithelium, lamina propria and muscularis. Staining for PR was exclusively nuclear. PR immunostaining in the vaginal epithelium of control animals varied among tissues from different animals ranging from light immunostaining to more moderate staining throughout the epithelial layers. Interestingly, epithelial PR immunostaining intensity paralleled ER immunostaining. Moreover, the proportion of PR immunoreactive cells was similar to the proportion of ER immunoreactive cells throughout the vaginal wall. The highest proportion of PR immunoreactive cells was present in the muscularis, followed by the epithelium and lamina propria layers. Approximately 50% of the smooth muscle and epithelial nuclei were immunoreactive for PR. In contrast, approximately half of the fibrocyte nuclei in the lamina propria were immunostained.

In vaginal tissue from untreated diabetic animals, PR immunostaining was significantly different from that of control tissues. Diabetes-induced immunohistochemical changes in PR staining were most notable in the atrophic diabetic epithelium. The entire basal zone of the diabetic epithelium was intensely stained (Fig. 4). In contrast to the epithelium, the staining intensity and proportion of PR immunoreactive cells in the lamina propria and muscularis layers was reduced when compared to tissue from control animals. Less than half of the nuclei in the lamina propria were PR immunopositive and were lightly stained. Approximately half of all nuclei of the muscularis layer were immunopositive and were lightly stained.

Estradiol treatment of diabetic animals restored PR immunostaining and distribution similar to that observed in tissue from control animals. The staining intensity of epithelial cell nuclei was reduced to levels below that of control animals, except in a small number of scattered, basally located nuclei which were moderately stained. Treatment of diabetic animals with estradiol restored the staining intensity and proportion of immunoreactive cells both in the lamina propria and muscularis layers to levels observed in tissue of control animals.

### *4.4. Effects of diabetes and estradiol treatment on AR expression and vaginal tissue distribution*

Fig. 5(lanes 1–5) shows AR protein expression in vaginal tissue from control animals, as noted by a specific protein band in the 112–116 kDa range. AR protein expression was markedly decreased (76%) in tissues from untreated diabetic animals (lanes 6–8) as compared to controls. Estrogen treatment of diabetic animals restored AR expression by approximately 63% when compared to untreated diabetic animals.

Immunohistochemical assays showed that AR is detected in epithelial, lamina propria and muscularis layers of vaginal tissue from intact control animals (Fig. 6). Staining was primarily nuclear, but there was minimal cytoplasmic staining. The basal zone of the epithelium contained nuclei that were moderately stained while the intermediate and juxtalumenal zones were very lightly immunostained. Approximately half of the fibrocyte nuclei of the lamina propria layer were positively stained for AR and staining was

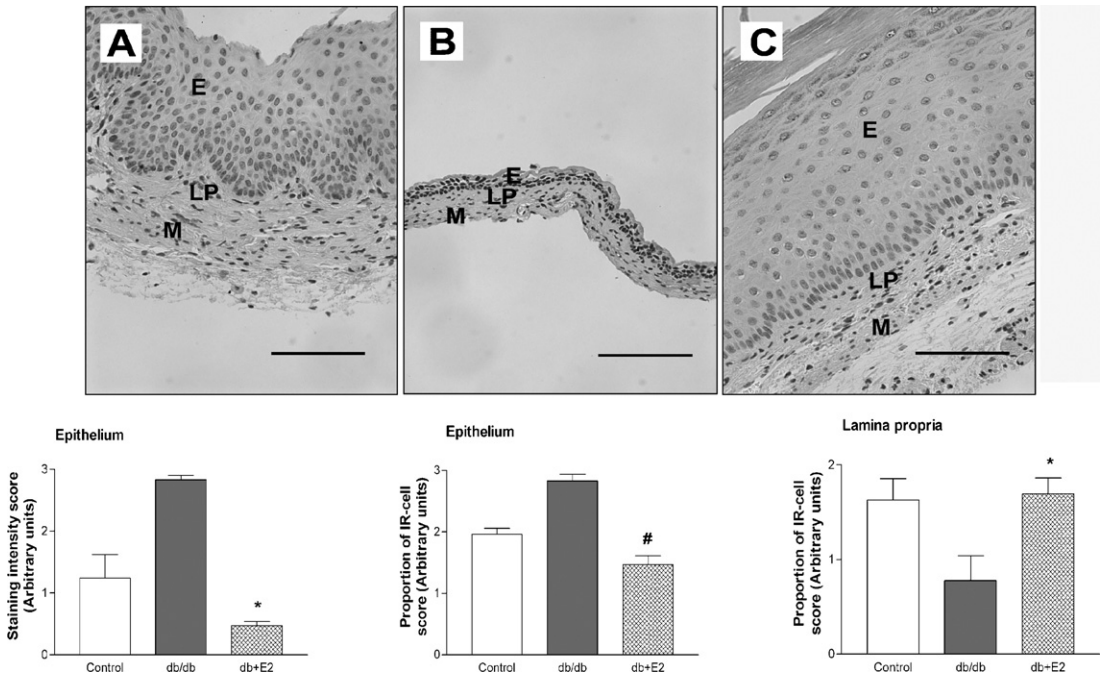


**Fig. 4.** Effects of type 2 diabetes and estradiol supplementation on the localization and distribution of PR in vaginal tissues. Upper panels represent vaginal tissues from non-diabetic control (A), untreated diabetic (B) and estradiol-treated (C) diabetic animals immunohistochemically probed with an antibody to PR; lower panels represent semi-quantitative analysis of PR staining intensity in the vaginal epithelium and proportion of immunoreactive cells in the epithelium and lamina propria. E = epithelium; LP = lamina propria; M = muscularis. Scale bars represent 100 μm. $^{\#}p \leq 0.01$ vs. db; $^{*}p \leq 0.05$ vs. db.

moderate in intensity. The proportion of immunoreactive cells was highest in the muscularis layer of the vaginal wall. Furthermore, AR staining intensity was strongest in the smooth muscle nuclei of the muscularis layer.

Diabetes produced marked changes in AR immunostaining in the vagina. Similar to the observations with ER and PR, the intensity of AR immunoreactivity was distinctly increased in the basal zone of the epithelium of untreated diabetic animals, although this finding was not statistically significant. The proportion of AR immunoreactive cells was significantly reduced in both the muscularis and the lamina propria. The staining within the fibrocyte nuclei of the lamina propria layer was moderate in intensity and was similar in intensity to control animals. AR immunoreactivity in the muscularis layer of untreated diabetic animals was not remarkably different from control animals with regard to the staining intensity. Estrogen treatment of diabetic animals restored the thickness of the epithelium and the immunolocalization and distribution of the AR to control levels (Fig. 6). AR immunostaining in the epithelium of estradiol treated diabetic animals was strongest in the basal zone nuclei, very light in the intermediate zone nuclei and negative in the juxtalumenal nuclei. Cytoplasmic staining was also light in intensity. Almost half of the nuclei residing in the lamina propria layer were immunopositive and were moderately to darkly stained. Smooth muscle nuclei of the muscularis layer, on the other hand, exhibited the highest proportion of immunoreactive cells and were darkly stained similar to control animals.

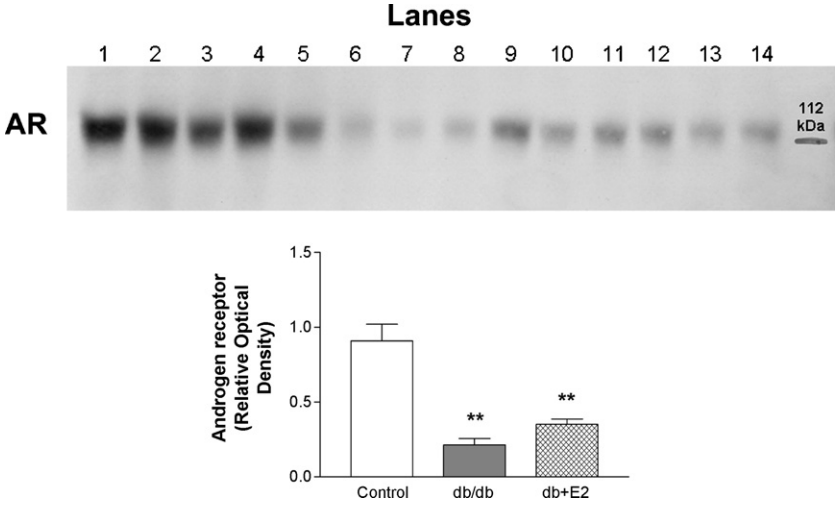


**Fig. 5.** Effects of type 2 diabetes and estradiol supplementation on AR protein expression in the mouse vagina. Upper panel represents vaginal tissue extracts from controls (lanes 1–5), untreated diabetic (lanes 6–8) and diabetic animals treated with estradiol (lanes 9–14). Lower panels represent the quantitative assessment of the relative optical densities from control, untreated and estradiol treated diabetic animals. Lanes 6 and 7 each represent extracts pooled from two diabetic animals, whereas all other lanes represent extracts from a single animal. $^{**}p \leq 0.001$ vs. control.

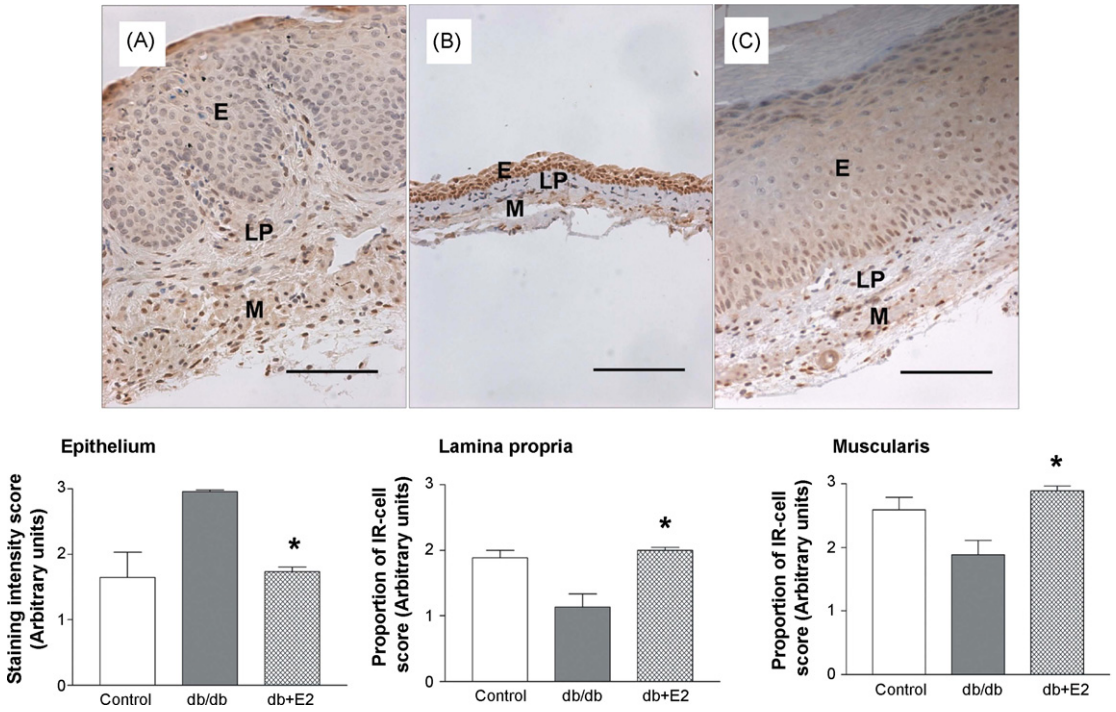


**Fig. 6.** Effects of type 2 diabetes and estradiol supplementation on the localization and distribution of AR in vaginal tissues. Upper panels represent vaginal tissues from non-diabetic control (A), untreated diabetic (B) and estradiol-treated (C) diabetic animals immunohistochemically probed with an antibody to AR, lower panels represent semi-quantitative analysis of AR staining intensity and proportion of immunoreactive cells. E = epithelium; LP = lamina propria; M = muscularis. Scale bars represent 100 μm. * $p \leq 0.05$ vs. db.

## 5. Discussion

In this study, we demonstrated that type 2 diabetes reduced the overall expression of ERα, PR and AR and produced marked changes in their localization and distribution of proteins in the vagina. We further demonstrate that estradiol supplementation in diabetic animals restores most of these diabetes-induced changes in the vagina. Our previous study using the same animal model suggests that the restorative effect of estradiol supplementation occurs despite the persistence of hyperglycemia [19]. Because the vagina is a target tissue for sex steroids, it is likely that diabetes-induced changes in receptor expression impede vaginal tissue physiological function. The significant atrophy of vaginal tissue in untreated diabetic animals suggests that diabetes alters estrogen action either by reducing circulating estradiol levels and/or disrupting the expression and signaling of ERα.

While the overall tissue levels of ERα, AR, and PR were reduced in diabetic animals (as evidenced by Western blots), receptor expression in the epithelium was notably different. Immunostaining intensity and/or proportion of immunopositive cells were increased in this layer of the vagina in diabetic animals. The increased immunolocalization of steroid hormone receptors in the atrophic diabetic epithelium may represent a biological process of compensation to maintain the epithelium at a functioning basal state in the diabetic milieu and prevent any further tissue involution. Normal stratification of the epithelium protects the vaginal wall from abrasions and trauma during coitus and plays a role in permeability and the production of mucinous glycoproteins to lubricate the luminal surface of the vagina [36].

The observation in our study that estradiol supplementation restored steroid hormone immunolocalization in the vagina in a differential manner further suggests disruption of estrogen signaling in diabetes. This comes from several lines of evidence that include the dramatic changes in vaginal tissue structure, as well as the coincident changes in the levels of PR and AR. In a morphometric study, vaginal tissue from diabetic mice exhibited marked reductions of epithelial thickness and muscularis area whereas these diabetes-induced changes were ameliorated by estradiol supplementation [19]. These atrophic changes observed in intact diabetic mice paralleled the structural alterations observed in ovariectomized rats [3,18]. In addition, PR and AR expression has been shown to be regulated by the activity of ERα. Indeed, regulation of the progesterone receptor has been shown to be tightly coupled to estrogen signaling. In ovariectomized but not estrogen receptor knockout (ERKO) animals, estradiol treatment has been shown to increase PR in vaginal epithelial cells via ERα signaling [21]. Similar studies have also reported PR expression levels to decrease after ovariectomy and be restored with estradiol treatment [18,22]. Thus, increased PR expression in type 2 diabetic animals by estradiol supplementation strongly suggests restoration of estrogen receptor signaling.

With respect to the androgen receptor, it has been shown that ovariectomy decreases AR mRNA levels in the mouse uterus and vagina, while estradiol treatment restores AR expression to levels observed in control animals, suggesting a positive estrogenic regulation of AR in these tissues [34]. Furthermore, it has been demonstrated in ovariectomized animals that AR levels were reduced in the proximal and distal vagina, whereas expression was restored in estrogen-treated animals [35]. Therefore, estrogenic restoration of AR expression and distribution also suggests re-establishment of estrogen receptor signaling in diabetic animals.

Our findings are in agreement with previous studies that have correlated changes in steroid hormone receptors with the diabetic state. Ekka et al. showed similar estrogen-induced progesterone receptor expression in uterine tissues in a type 1 diabetic rat animal model [20], and reductions in AR expression have been previously demonstrated in a type 1 diabetic animal model [15]. Interestingly, in our study, estrogen supplementation down-regulated ERα expression in diabetic animals beyond levels detected in untreated diabetic animals. However, this observation is also consistent with studies in female rats in which estradiol treatment of ovariec-

tomized animals down-regulated ERα in vaginal tissues [3,18]. Thus, the data from the present study suggest that type 2 diabetes decreased the expression of ERα as well as PR and AR in the vagina. The changes in receptor expression, therefore, may underlie the observed diabetes-induced changes in the structural integrity of the vagina [19].

Evidence for a role of estradiol in energy metabolism and glucose homeostasis has been suggested by several human and animal studies [23–26]. The exact cellular mechanisms involved in estradiol-mediated glucose metabolism remain to be elucidated. One possible mechanism is ERα regulation of the expression of glucose transporters. Several studies have suggested a possible link between estradiol and the expression of GLUT4 [27]. GLUT4 expression has been shown to be upregulated with estrogen treatment in primate cerebral cortex [28]. ERα null mice showed reduced GLUT4 expression in skeletal muscle [29]. Changes in GLUT4 expression and/or translocation to the plasma membrane may result in insulin resistance [27]. Selective estrogen receptor modulators (SERMs) increase GLUT3 and GLUT4 mRNA expression levels in cerebral cortical neurons and may possess neuroprotective effects due to increased glucose transport [28]. It is therefore possible that decreased estradiol levels and down-regulation of ERα in the untreated diabetic (db/db) mice may contribute to a dysregulation of GLUT4 expression and consequently lead to altered glucose uptake at the level of insulin-sensitive tissues. This, however, remains to be established.

Studies in animal models of diabetic nephropathy suggest estrogen regulation of diabetes-induced fibrosis. Supplementation with 17β-estradiol attenuated diabetic renal disease by regulating extracellular matrix protein expression associated with glomerulosclerosis and tubulointerstitial fibrosis, TGF-β and its downstream regulatory proteins, and the activity of matrix metalloproteinases in renal tissues [12,13]. 17β-Estradiol also suppresses renal mesangial expansion and fibronectin accumulation in diabetic db/db mice [30]. Furthermore, ERα knockout mice (ERKO) exhibited proteinuria and glomerulonephritis suggesting a protective role for estrogen and its actions on renal tissues [31]. Thus, these studies support a role for estradiol in ameliorating diabetes-induced fibrotic pathology.

The importance of estradiol as a regulator of vaginal structure and function has been long recognized. Previous studies have demonstrated that estrogen deprivation by ovariectomy results in marked reductions in vaginal blood flow [4,15], vaginal epithelium thickness [3], vaginal lubrication [5], and ERα expression [4]. Furthermore, these studies demonstrated that estradiol treatment of ovariectomized animals restored the aforementioned structural, physiological, and biochemical parameters to control levels. In a type 1 diabetic animal model, vaginal blood flow was shown to be significantly reduced and the blood flow response was similar to that observed in ovariectomized animals [15]. In conjunction with our previous study [19], decreased levels of plasma estradiol and reduced ERα expression in type 1 and type 2 diabetic animals suggest that estrogen signaling is impaired in the diabetic state. The gross uterine and vaginal atrophy in untreated diabetic animals, coupled with the reduced ERα expression, further suggests that the diabetic state interferes with the estrogen signaling pathway and attenuates vaginal blood flow.

Sex steroid hormone receptor signaling is important in female genital sexual arousal function. Estrogen biosynthesis and receptor signaling are altered in diabetes and therefore, alters vaginal physiological function. The present study suggests that sex steroid hormone receptors are differentially regulated in diabetes with potential pathological consequences on vaginal physiology. Similarly, reduced expression of PR and AR may signify further alterations in vaginal physiology since AR is important in maintaining the adrenergic neural fiber network and nNOS expression [18,33]. These findings further demonstrate that estradiol supplementation provides a protective effect by up-regulating the expression of sex steroid receptor proteins.

## References


[1] A.M. Traish, N.N. Kim, R. Munarriz, R. Moreland, I. Goldstein, Biochemical and physiological mechanisms of female genital sexual arousal, Arch. Sex. Behav. 31 (2002) 393–400.
[2] A.M. Traish, N.N. Kim, R. Munarriz, R. Moreland, I. Goldstein, Female genital sexual arousal: biochemical mediators and potential mechanisms of dysfunction, Drug Discov. Today 1 (2004) 91–97.
[3] M.A. Pessina, R.F. Hoyt Jr., I. Goldstein, A.M. Traish, Differential effects of estradiol, progesterone, and testosterone on vaginal structural integrity, Endocrinology 147 (2006) 61–69.
[4] S.W. Kim, N.N. Kim, S.J. Jeong, R. Munarriz, I. Goldstein, A.M. Traish, Modulation of rat vaginal blood flow and estrogen receptor by estradiol, J. Urol. 172 (2004) 1538–1543.
[5] K. Min, R. Munarriz, N.N. Kim, S. Choi, L. O'Connell, I. Goldstein, A.M. Traish, Effects of ovariectomy and estrogen replacement on basal and pelvic nerve stimulated vaginal lubrication in an animal model, J. Sex Marital Ther. 29 (2003) 77–84.
[6] N.N. Kim, K. Min, M.A. Pessina, R. Munarriz, I. Goldstein, A.M. Traish, Effects of ovariectomy and steroid hormones on vaginal smooth muscle contractility, Int. J. Impot. Res. 16 (2004) 43–50.
[7] F.F. Onol, F. Ercan, T. Tarcan, The effect of ovariectomy on rat vaginal tissue contractility and histomorphology, J. Sex Med. 3 (2006) 233–241.
[8] C.C. Wells, S. Riazi, R.W. Mankhey, F. Bhatti, C. Ecelbarger, C. Maric, Diabetic nephropathy is associated with decreased circulating estradiol levels and imbalance in the expression of renal estrogen receptors, Gend. Med. 2 (2005) 227–237.
[9] D.R. Garris, Diabetes-associated alterations in uterine structure in the C57BL/KsJ mouse: relationship to changes in estradiol accumulation, circulating ovarian steroid levels, and age, Anat. Rec. 211 (1985) 414–419.
[10] D.R. Garris, B.L. Garris, Diabetes (db/db) mutation-induced female reproductive tract hypercytolipidemia: estrogenic restoration of utero-ovarian indices, Reprod. Toxicol. 18 (2004) 641–651.
[11] D.R. Garris, B.L. Garris, Estrogenic restoration of functional pancreatic islet cytoarchitecture in diabetes (db/db) mutant C57BL/KsJ mice: relationship to estradiol localization, systemic glycemia, and persistent hyperinsulinemia, Cell Tissue Res. 319 (2005) 231–242.
[12] R.W. Mankhey, C.C. Wells, F. Bhatti, C. Maric, 17Beta-estradiol supplementation reduces tubulointerstitial fibrosis by increasing MMP activity in the diabetic kidney, Am. J. Physiol. Regul. Integr. Comp. Physiol. 292 (2007) R769–R777.
[13] A. Dixon, C. Maric, 17Beta-estradiol attenuates diabetic kidney disease by regulating extracellular matrix and transforming growth factor-beta protein expression and signaling, Am. J. Physiol. Renal Physiol. 293 (2007) F1678–1690.
[14] R.W. Mankhey, F. Bhatti, C. Maric, 17Beta-estradiol replacement improves renal function and pathology associated with diabetic nephropathy, Am. J. Physiol. Renal Physiol. 288 (2005) F399–F405.
[15] N.N. Kim, M. Stankovic, T.T. Cushman, I. Goldstein, R. Munarriz, A.M. Traish, Streptozotocin-induced diabetes in the rat is associated with changes in vaginal hemodynamics, morphology and biochemical markers, BMC Physiol. 6 (2006) 4.
[16] A. Salonia, R. Lanzi, M. Scavini, M. Pontillo, E. Gatti, G. Petrella, G. Licata, R.E. Nappi, E. Bosi, A. Briganti, P. Rigatti, F. Montorsi, Sexual function and endocrine profile in fertile women with type 1 diabetes, Diabetes Care 29 (2006) 312–316.
[17] A.M. Traish, R.E. Müller, H.H. Wotiz, Comparison of formation, activation, and nuclear translocation of receptor.estradiol (R.E2) complex at 0 degrees C and 37 degrees C in intact uterine cells, J. Biol. Chem. 254 (1979) 6560–6567.
[18] M.A. Pessina, R.F. Hoyt Jr., I. Goldstein, A.M. Traish, Differential regulation of the expression of estrogen, progesterone, and androgen receptors by sex steroid hormones in the vagina: immunohistochemical studies, J. Sex Med. 3 (2006) 804–814.
[19] T.T. Cushman, N.N. Kim, R.F. Hoyt Jr, A.M. Traish, Estradiol ameliorates diabetes-induced changes in vaginal structure of a db/db mouse model, J. Sex. Med., in press.
[20] E. Ekka, I. Vanderheyden, B. Glorieux, R. de Hertogh, Estradiol-induced progesterone receptor synthesis in normal and diabetic ovariectomized rat uterus, J. Steroid Biochem. 28 (1987) 61–64.
[21] T. Kurita, K.J. Lee, P.S. Cooke, J.A. Taylor, D.B. Lubahn, G.R. Cunha, Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract, Biol. Reprod. 62 (2000) 821–830.
[22] W.W. Leavitt, T.J. Chen, T.C. Allen, Regulation of progesterone receptor formation by estrogen action, Ann. N. Y. Acad. Sci. 286 (1977) 210–225.
[23] A. Alonso, R. Fernández, M. Moreno, P. Ordóñez, H. González-Pardo, N.M. Conejo, F. Díaz, C. González, Positive effects of 17beta-estradiol on insulin sensitivity in aged ovariectomized female rats, J. Gerontol. A: Biol. Sci. Med. Sci. 61 (2006) 419–426.
[24] B. Andersson, L.A. Mattsson, L. Hahn, P. Mårin, L. Lapidus, G. Holm, B.A. Bengtsson, P. Björntorp, Estrogen replacement therapy decreases hyperandrogenicity and improves glucose homeostasis and plasma lipids in postmenopausal women with noninsulin-dependent diabetes mellitus, J. Clin. Endocrinol. Metab. 82 (1997) 638–643.

[25] D.A. Araújo, M.L. Farias, A.T. Andrade, Effects of transdermal and oral estrogen replacement on lipids and glucose metabolism in postmenopausal women with type 2 diabetes mellitus, Climacteric 5 (2002) 286–292.
[26] A. Cagnacci, F. Tuveri, R. Cirillo, A.M. Setteneri, G.B. Melis, A. Volpe, The effect of transdermal 17-beta-estradiol on glucose metabolism of postmenopausal women is evident during the oral but not the intravenous glucose administration, Maturitas 28 (1997) 163–167.
[27] R.P. Barros, U.F. Machado, J.A. Gustafsson, Estrogen receptors: new players in diabetes mellitus, Trends Mol. Med. 12 (2006) 425–431.
[28] C.M. Cheng, M. Cohen, J. Wang, C.A. Bondy, Estrogen augments glucose transporter and IGF1 expression in primate cerebral cortex, FASEB J. 15 (2001) 907–915.
[29] R.P. Barros, U.F. Machado, M. Warner, J.A. Gustafsson, Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 1605–1608.
[30] M. Chin, M. Isono, K. Isshiki, S. Araki, T. Sugimoto, B. Guo, H. Sato, M. Haneda, A. Kashiwagi, D. Koya, Estrogen and raloxifene, a selective estrogen receptor modulator, ameliorate renal damage in db/db mice, Am. J. Pathol. 166 (2005) 1629–1636.
[31] G.J. Shim, L.L. Kis, M. Warner, J.A. Gustafsson, Autoimmune glomerulonephritis with spontaneous formation of splenic germinal centers in mice lacking the estrogen receptor alpha gene, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 1720–1724.
[32] A.M. Traish, S.W. Kim, M. Stankovic, I. Goldstein, N.N. Kim, Testosterone increases blood flow and expression of androgen and estrogen receptors in the rat vagina, J. Sex Med. 4 (2007) 609–619.
[33] A.M. Traish, N.N. Kim, Y.H. Yuang, K. Min, R. Munarriz, I. Goldstein, Sex steroid hormones differentially regulate nitric oxide synthase and arginase activities in the proximal and distal rabbit vagina, Int. J. Impot. Res. 15 (2003) 397–404.
[34] G. Pelletier, V. Luu-The, S. Li, F. Labrie, Localization and estrogenic regulation of androgen receptor mRNA expression in the mouse uterus and vagina, J. Endocrinol. 180 (2004) 77–85.
[35] A.M. Traish, N.N. Kim, K. Min, R. Munarriz, I. Goldstein, Role of androgens in female genital sexual arousal: receptor expression, structure, and function, Fertil. Steril. 77 (2002) S11–S18.
[36] G.I. Gorodeski, Estrogen modulation of epithelial permeability in cervical-vaginal cells of premenopausal and postmenopausal women, Menopause 14 (2007) 1012–1019.