RESEARCH ARTICLE

The effect of 17β estradiol on the expression of eNOS and iNOS in ovariectomized rat uterus

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Özet

Yilmaz O, Yagci A, Ulutas E, Sevimli A, Altunbas K, Bulbul A, Aslan R. 17β östradiolün ovariektomize rat uterusunda eNOS ve iNOS ekspresyonları üzerine etkisi. **Eurasian J Vet Sci, 2013, 29, 2, 65-69**

Amaç: Bu çalışmada ovariektomize rat uterusunda 17β östradiolün doza bağımlı olarak endoteliyal nitrik oksit sentaz (eNOS) ve indüklenebilir NOS (iNOS) enzimlerinin ekspresyon modelleri üzerine etkisinin Western blot yöntemiyle ortaya konulması amaçlandı.

Gereç ve Yöntem: Sprague Dawley ırkı, ovariektomize edilmiş, 3 aylık 40 adet dişi rat kullanıldı. Kontrol grubundaki ratlar (n=10) kasiçi, 3 gün boyunca susam yağı alırken, deneme grubundaki ratlara 3 gün boyunca 25 (n=10), 50 (n=10) ve 100 (n=10) µg/rat/gün dozlarında kas içi 17β östradiol uygulandı. Son uygulamadan 18 saat sonra ratlara servikal dislokasyon uygulandı. Uterus örnekleri vakit kaybetmeden uzaklaştırıldı. Uterus örneklerindeki eNOS ve iNOS enzimlerinin varlığı Western blot ile analiz edilerek elde edilen filmlerde dansitometri gerçekleştirildi.

Bulgular: 25, 50 ve 100 µg/rat/gün 17β östradiol uygulamalarının eNOS ekspiresyonunu kontrol grubuna göre arttırdığı gözlendi. 25 ve 50 µg/rat/gün 17β östradiol gruplarındaki eNOS exspiresyonu relatif dansitesinin 100 µg/rat/gün 17β östradiol grubuna göre yüksek olduğu belirlendi. eNOS'a benzer şekilde, 25 ve 50 µg/rat/gün 17β östradiol gruplarındaki iNOS ekspiresyonunun kontrol ve 100 µg/rat/gün 17β östradiol gruplarına göre yüksek olduğu izlendi.

Öneri: Östrojenin NOS/NO aktivitesine aracılık edebileceği ileri sürülebilir.

Anahtar kelimeler: 17β östradiol, eNOS, iNOS, rat uterus, ovariektomi

Abstract

Yilmaz O, Yagci A, Ulutas E, Sevimli A, Altunbas K, Bulbul A, Aslan R. The effect of 17β estradiol on the expression of eNOS and iNOS in ovariectomized rat uterus. **Eurasian J Vet Sci, 2013, 29, 2, 65-69**

Aim: This study was design to describe the effect of 17β estradiol on the dose-dependent expression patterns of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) enzymes by Western blotting in ovariectomized rat uterus.

Materials and Methods: Female, three months old, ovariectomized, 40 Sprague–Dawley rats were used. Rats in the control group (n=10) received intramuscular injection of sesame oil once daily for 3 days, whereas rats in the experimental groups were treated with intramuscular injection of 17β estradiol 25 (n=10), 50 (n=10) and 100 (n=10) µg/rat/day. The rats were killed by cervical dislocation at 18th hour after the last injection. Immediately after death, the uterine horns were removed. The presence of eNOS and iNOS enzymes in uterine samples were analysed by Western blot and the densitometry of each film were performed.

Results: It was observed that the application of 25, 50 and 100 µg/rat/day 17β estradiol increased the eNOS expression as compared to control group The relative density of eNOS expressions in 25 and 50 µg/rat/day 17β estradiol groups was higher than those in 100 µg/rat/day 17β estradiol group. Similarly to eNOS, 25 and 50 µg/rat/day 17β estradiol groups showed higher iNOS expression as compared to control and 100 µg/rat/day 17β estradiol groups.

Conclusion: Estrogen may mediate the NOS/NO activity in ovariectomized rat uterus.

Keywords: 17β estradiol, eNOS, iNOS, rat uterus, ovariectomy

Introduction

Nitric oxide is synthesized by nitric oxide synthase (NOS) enzyme group (Palmer et al 1988) which has inducible (iNOS) and constitutive (cNOS) forms. Constitutive NOS includes neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms (Förstermann et al 1991, Förstermann et al 1998). The NO has a variety of physiological actions in the reproductive system, such as ovarian function (Jaroszewski et al 2001), secretory and ciliary functions of oviduct (Yilmaz et al 2012), implantation, maintenance of pregnancy and delivery (Maul et al 2003).

A number of studies indicated the presence of NOS enzymes in uterine tissue (Roselli et al 1998, Yallampalli et al 1998). It has been reported that NOS enzymes exist in blood vessels, nerve fibres, glandular epithelium, endometrial stromal cells and smooth muscles cells of uterus (Figueroa and Massmann 1995, Roselli et al 1998). Moreover, it is well documented that umbilical arteria and vein, chorionic villus and trophoblast cells during pregnancy include NOS enzymes and they produce NO via NOS (Rossmanith et al 1999, Taguchi et al 2000).

Changes in reproductive process affect the level of NO in uterus. It has been stated that endothelial NOS (eNOS) in blood vessels of human and rat uterus increases throughout the pregnancy (Weiner et al 1994, Nelson et al 2000). Similarly, eNOS activity in uterine blood vessels during follicular stage is higher than during luteal stage of the oestrous cycle. The activity of eNOS localized in blood vessels of human endometrium increases in the proliferative process of the oestrous cycle and this increment continues during early secretion stage (Taguchi et al 2000). Estrogens increase the capability of NO synthesis of genital tract organs. Therefore, it has been postulated that estrogens are responsible from the enhancement of NO during pregnancy and the follicular stage of the oestrous cycle (Weiner et al 1994, Nelson et al 2000). Moreover, it has been reported that estrogens increase the half-life of NO by augmentation of constitutive NOS (cNOS) expression (Weiner et al 1994, Figueroa and Massmann 1995, Batra and Al Hajji 1998) or discarding the superoxide radicals (Miura et al 1996, Azevedo et al 2001).

This study was design to describe the dose-dependent effect of 17β estradiol on the expression patterns of eNOS and iNOS enzymes by Western blotting in ovariectomized rat uterus.

Materials and Methods

Female, three months old, ovariectomized 40 Sprague–Dawley rats were used in the present study. Rats in all groups were fed ad libitum with the same commercial rat diet. The ethics committee of Afyon Kocatepe University approved all procedures. The ovariectomy procedure was performed at 12 weeks of age (n=40). Rats were anesthetized by an intraperitoneal ketamine (21.2 mg/kg) and xylazine (4.2 mg/kg) combination. Small bilateral incisions were made on the dorsum to expose the ovaries retroperitoneally. The ovarian vessels were then clamped and the ovaries removed. Afterwards, the uterine tubes were ligated and the muscles and skin were sutured. Ten days after operation, the ovariectomized rats were randomly assigned to four groups of 10 rats each. Rats in the control group received intramuscular injection of sesame oil once daily for 3 days, whereas rats in the experimental groups were treated with intramuscular injection of 17β estradiol 25 µg/rat/day, 50 µg/rat/day and 100 µg/rat/day. The rats were killed by cervical dislocation at 18th hour after the last injection. Immediately after death, the uterine horns were removed. Subsequently, the surrounding mesentery and fat tissues were carefully removed from the uterus and the uterine samples were stored at -80 ^{0}C for further analysis.

Western Blotting for eNOS and iNOS in the rat uterus

Uterine samples were homogenizated and centrifugated (10 min, 15000 g) to discard the supernatant. The concentration of proteins in precipitate was determined with Bradford assay (Biorad DC protein assay) and protein preparations were stored at -20 ^{0}C. Immunoblotting was performed according to the procedure of Laemmli (1970). The volume of each protein sample corresponding to 45 µg of protein was supplemented with 50% trichloroacetic acid (POCh, Poland) to a final concentration of 10%, incubated at 0 ^{0}C for 5 min and centrifuged (2 min, 12000 g). The precipitate was washed with ice-cold acetone, air-dried and dissolved in 10 µL of reducing SDS sample buffer (Laemmli 1970). The samples were heated to 95 ^{0}C for 5 min, cooled to room temperature and centrifuged (2 min, 16000 g). The supernatants were loaded on 10% separating SDS-polyacrylamide gel with 5% stacking gel. 1 µL of protein molecular weight marker (Broad Range, Bio Rad, USA) in the reducing sample buffer was run in one lane of the gel. The electrophoresis was done in a MiniProtean II gel apparatus (Bio Rad, USA) at 200 V for 40 min. After electrophoresis the proteins were electroblotted to PVDF membrane (Immobilon, Bio Rad, USA) in a Mini TransBlot apparatus (Bio Rad, USA). Protein bands were stained on the PVDF membrane with 1% Amido Black (Sigma, USA) in 1% acetic acid (POCh, Poland) and positions of molecular weight standard bands were marked with soft pencil. The membrane was blocked with 10% bovine serum albumin (BSA, Sigma, USA) in TRIS-buffered saline (TBS, pH 7.4) at 42 ^{0}C for 1 h and incubated with appropriate primary antibodies (eNOS, Santa Cruz 654 and iNOS, Santa Cruz 651). The membranes were then washed with TBS and incubated with proper biotin-conjugated secondary antibody (Santa Cruz 2030) for 30 min. After incubation membranes were washed with TBS and incubated with alkaline phosphatase-conjugated streptavidin (0.5 µg/mL, Sigma, USA) for 30 min. The membranes were washed with TBS, incubated with the alkaline phosphatase-activating buffer (100 mM NaCl, 100 mM Tris HCl, pH 9.5) and then soaked in CDP Star Ready-to-use (Roche, USA) chemiluminescence detection reagent. The membranes were wrapped in plastic folders and exposed to X-ray film (Retina, Photochemische Werke GmbH, Germany) for 2 min. The X-Ray films were developed with Rodinal (AGFA, Germany) developer. Positions of molecular weight marker bands were transferred to the film.

Table 1. Relative density of eNOS and iNOS expressions in groups.

	Control	25 µg	50 µg	100 µg	P
eNOS	0.02±0.04[c]	0.54±0.02[a]	0.54±0.01[a]	0.21±0.05[b]	0.000
iNOS	0.76±0.01[b]	1.48±0.33[a]	1.22±0.10[a]	0.30±0.08[b]	0.003

[a, b, c]: Different letters in the same line indicate significant differences among the groups.

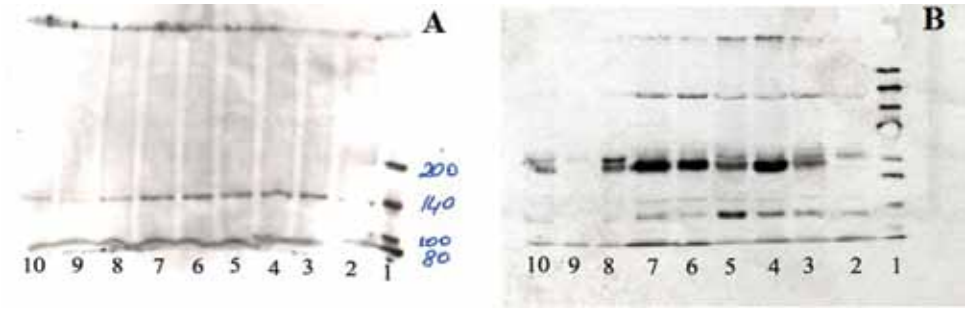

Figure 1 (A, B). Representative images of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) expression patterns of Western blot in ovariectomized rat uterus. 1: Standart Marker, 2: Control, 3, 4, 5, 6: 25 µg/rat/day 17β estradiol, 7, 8: 50 µg/rat/day 17β estradiol, 9, 10: 100 µg/rat/day 17β estradiol.

Densitometry

Each film was photographed and transferred to a computer assisted program "ImageJ" software (http://rsb.info.nih.gov/nih-image/). The images were converted to 8 bit (256 gray shades) for analysis. To define the profiles a rectangular box was defined arbitrarily with a width smaller than the narrowest band on the film and as long as the complete lane. Once defined, the same box was used to measure all lanes in both images. For the measurement of the bands of the expected molecular size individual sample boxes were defined in which the band was fitted as tight as possible. Area under the curve was calculated in pixel values and these values were converted to percent by the software. The percent value of standard marker obtained from expected molecular size was defined as the reference relative density. All the percent value of each experimental band was calculated to diagnose the signalling of uterine samples for eNOS and iNOS.

Statistical analysis

All values are expressed as mean±SE. The results were analyzed by ANOVA and DUNCAN's multible range test (SPSS for Windows 13.0). In all cases, a probability of error less than 0.05 was selected as criterion for significance.

Results

Western blot showed the protein bands which corresponded to expected molecular weight of eNOS (140 kDa) and iNOS (130kDa) (Figure 1A, B). Applications of 25, 50 and 100 µg/rat/day 17β estradiol increased ($p<0.001$) the eNOS expression as compared to control group (Figure 1A). Relative densities of eNOS and iNOS expressions in groups are shown in Table 1. The relative density of eNOS expressions in 25 and 50 µg/rat/day 17β estradiol groups was higher ($p<0.001$) than those in 100 µg/rat/day 17β estradiol group. Injection of 100 µg/rat/day 17β estradiol increased the eNOS expression but the eNOS signalling was lower than other experimental groups. Similarly to eNOS, 25 and 50 µg/rat/day 17β estradiol groups showed higher ($p<0.01$) iNOS expression as compared to control and 100 µg/rat/day 17β estradiol groups (Figure 1B).

Discussion

The data obtained in this study provide evidence that 17β estradiol may play an important role in the activity of NOS enzymes in rat uterus. We have demonstrated that both eNOS and iNOS expressed in ovariectomized rat uterus (Figure 1A, B). Accordingly, Bulbul et al (2007) reported that 17β estradiol, progesterone and the combination of 17β estradiol and progesterone increased the eNOS expression of surface and glandular epithelium of ovariectomized rat uterus, whereas no significant difference was observed in smooth muscle cells, nerve fibres and endothelium of blood vessels. In another studies, it has been reported that estrogens increase the immunoreactivity of eNOS in epithelial and smooth muscle cells of uterine endometrium (Zhang et al 1999, Vagnoni et al 1998, Yallampali et al 1998, Chattarjee et al 1996), while a number of studies report that only progesterone is responsible from the increasing eNOS expression (Ota et al 1998, Farina et al 2001, Ogando et al 2003). Furthermore, it has been indicated that estrogens augment the eNOS expression in smooth muscle cells (Figueroa and Massman 1995, Chattarje et al 1996, Zhang et al 1999, Yallampalli and Dong 2000), unless Ganglua et al (1997) have reported that progesterone increase eNOS expression in smooth muscle cells of myometrium, *in vitro*. However, Bulbul et al (2007) determined that steroid hormones such as 17β estradiol and progesterone did not alter the eNOS expression in smooth muscle cells. In current study, 25 and 50 µg/rat/day 17β estradiol clearly increased the eNOS expression (Table 1). Besides 100 µg/rat/day 17β estradiol augmented the

eNOS expression as compared to control, it is suggested that 17β estradiol effects the eNOS expression in a dose dependent manner in rat uterus, whereas increasing dose of 17β estradiol reduces the expression pattern. Moreover, it is indicated that the discrepancies with other studies (Figueroa and Massman 1995, Chattarje et al 1996, Zhang et al 1999, Yallampalli and Dong 2000, Bulbul 2007) might be a consequence of differences in doses.

Bulbul et al (2007) reported that ovariectomized rat uterus had a weak iNOS expression in surface and glandular epithelium of endometrium and smooth muscle cells of myometrium, whereas 17β estradiol increased iNOS expression in surface epithelium and the combination of 17β estradiol and progesterone augmented the expression in both surface and glandular epithelium. However, Huang et al (1995) indicated that weak iNOS expression occurred in mast cells by 17β estradiol, while progesterone caused weak expression in surface epithelium in ovariectomized rat uterus. In the present study, similarly to eNOS, 25 and 50 µg/rat/day 17β estradiol increased the iNOS expression, while the expression was inhibited by 100 µg/rat/day 17β estradiol (Table 1). This result was consistent with Saxena et al (2000) and Ogando et al (2003). However, Yallampalli and Dong (2000) stated that 17β estradiol inhibited the iNOS mRNA in pregnant rats and ovariectomized rats treated with lipopolysaccaride. In this study, 100 µg/rat/day 17β estradiol inhibited the iNOS expression. It is suggested that iNOS expression may be inhibited by high level of estrogen and consequently, NO production may be decreased.

Conclusions

It was determined the different patterns of eNOS and iNOS mRNA in ovariectomized rat uterus. It seems that 25 and 50 µg/rat/day 17β estradiol increase the both eNOS and iNOS expressions. Moreover, it may be concluded that estrogen mediates the NOS/NO activity in ovariectomized rat uterus.

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