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The effect of oestradiol and neta on immunohistochemical staining of iNOS and eNOS in coronary arteries of ovariectomized rats

F.M. Koyuncu[1], K. Ozbilgin[2], N.K. Kuscu[1], S. Inan[2], S. Vatansever[2] and E. Ceylan[1]

[1]Celal Bayar University, School of Medicine, Department of Obstetrics & Gynecology and

[2]Celal Bayar University, School of Medicine, Department of Histology & Embryology, Manisa, Turkey

Summary. Aim: The postmenopausal period is associated with increased risk for coronary atherosclerosis, and the effect of hormone replacement therapy in reducing this risk is controversial. Previous studies reported that nitric oxide synthetase (NOS) level might be important for the development of atherosclerosis, but no study has shown the interaction between hormone replacement therapy and endothelial NOS and inducible NOS intensity on coronary arteries yet. Our goal was to find out the immunostaining intensity of endothelial NOS and inducible NOS in ovariectomized rats which received oestradiol and norethisterone treatment. Methods: We performed bilateral ovariectomy in 15, female, 90-day-old Wistar rats with an average weight of 250 grams. After waiting for 4 weeks for the menopausal state, they were divided into 3 groups to receive either placebo, 0.1 mg/day 17-ß-oestradiol (group E2), or 0.1 mg/day 17-ß-oestradiol + 0.1 mg/day norethisterone acetate (group E2-NETA) for 5 weeks. Another group included 5, normal, adult, female intact rats and served as controls. At the end of the treatment, all rats were sacrificed and coronary arteries were stained with inducible NOS and endothelial NOS polyclonal antibodies using streptavidin-biotin technique. Results: The immunostaining of inducible NOS was prominent in perivascular connective tissue of the ovariectomized group but not in the control group. The inducible NOS immunostaining immunoreactivity was not detected in either treated groups. Immunostaining intensity of endothelial NOS did not differ in any 4 groups with similar staining. Conclusion: The present findings indicate that hormone replacement therapy down-regulates iNOS expression in coronary arteries of ovariectomized rats, and reduced iNOS may likely be involved in estrogen's beneficial effects.

Key words: NOS, NETA, Ovariectomy, Oestradiol, Rat

Offprint request to: Dr. Faik Mümtaz Koyuncu, MD, Assoc. Prof. Celal Bayar University, School of Medicine, Department of Obstetrics and Gynecology, Manisa-Turkey 45020. e-mail: faik.koyuncu@bayar.edu.tr

Introduction

Some physiological alterations occur concomitantly in thrombogenesis, blood pressure, fat mass accumulation, insulin levels and lipid metabolism soon after menopause (Glass and Witzum, 2001; Sposito et al., 2001). The lipid changes such as elevated low-density lipoprotein cholesterol, triglycerides and lipoprotein (a), and reduced high-density lipoprotein cholesterol lead to atherosclerosis.

Nitric oxide (NO), a potent vasodilator and a powerful inhibitor of platelet aggregation and leukocyte adhesion to endothelial cells, is produced from L-arginine by 3 nitric oxide synthetase (NOS) isoforms; neural NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Once stimulated, iNOS produces large amounts of nitric oxide with potential consequences in the pathophysiology of atherosclerosis (Behr-Roussel et al., 2000). Increased iNOS may oxidize LDL cholesterol which is an initial, important step in the development of atherosclerosis. In addition, endothelium-derived NO synthesized by a constitutive NOS, is involved in hypertension, atherosclerosis and certain heart diseases (Nava et al., 1995). Loss of endothelial NO activity may contribute to the abnormal vasomotor actions and progression of atherosclerosis observed in coronary artery disease (Cooke, 1998).

The incidence of atherosclerosis appears to be lower in postmenopausal women receiving hormone replacement therapy (HRT), with either estrogen alone or in combination with progesterone (Pines et al., 1997; Mendelssohn et al., 1999; Sitruk-Ware, 2000; Ganz,

2002), but its mechanism of action and the effect on iNOS and eNOS expression is not completely understood. Estrogenic activity may in part be mediated by estrogen receptor (ER) alpha-induced up regulation of eNOS gene expression, and maintenance of endothelial cell function and integrity (Tan et al., 1999; Chen et al., 1999; Hishikawa et al., 1995). 17-ß-oestradiol treatment can counteract the increase in iNOS activity following cytokine treatment (Zancan et al., 2004).

In the present study, we aimed to find out the expression of iNOS and eNOS in the coronary arteries of ovariectomized rats which received oestradiol or oestradiol plus NETA in order to determine the putative protective mechanism of estrogen against atherosclerosis.

Materials and methods

Twenty female, adult, Wistar Albino type rats with an average weight of 250 g were used in this study and general guidelines of the university for animal care were strictly followed. Fifteen of them underwent bilateral ovariectomy under general anesthesia, and then were kept in the Animal Research Laboratory of the medical school for 4 weeks waiting for the menopausal state. At the end of the latent period, they were equally divided into 3 groups to receive either placebo, 0.1 mg/day 17-ß-oestradiol (group E2), or 0.1 mg/day 17-ß-oestradiol + 0.1 mg/day NETA (group E2-NETA) for 5 weeks. Another 5, female, intact rats served as controls. At the end of the treatment period, all rats were sacrificed, coronary arteries were removed and the samples were fixed in 10% formalin solution overnight. They were dehydrated through a series of ethanol, held in a xylene and embedded in paraffin at 60°C to obtain paraffin blocks. Cross sections of 5m were taken from the blocks and prepared for both histological and immunohistochemical staining. For immunohistochemical staining, the samples were first exposed to 60°C overnight and then kept in xylene for 30 minutes and rehydrated through a series of ethanol solutions for 2 minutes each, the sections were washed with distilled water and phosphate buffered saline (PBS) for 10 minutes. Then they were kept in 2% trypsin in Tris buffer at 37°C for 15 min and washed with PBS three times for 5 min. The sections were incubated in 3% hydrogen peroxidase for 15 min to inhibit endogenous peroxidase activity. Then the tissues were washed with PBS three times for 5 min each and stained with primary antibodies; polyclonal anti-iNOS (61-7700, Zymed, California, USA) and monoclonal anti-eNOS (SA-258 Biomol, PA, USA) for 18h. After washing, the secondary antibody (biotinylated goat IgG anti-rabbit/mouse IgG, Histostain-plus bulk kit, Zymed 85-9043, California, USA) was applied for 30 min. followed by three washes in PBS. The streptavidin-peroxidase complex was added for 30 min. and washed in PBS three times. Sections were then stained with diaminobenzidine (DAB, Dako) to detect immunoreactivity and then counter-stained with Mayer's hematoxylin. The presence of a brown precipitate indicated positive findings for the primary antibodies. The negative controls received the same treatment with rabbit IgG or mouse IgG instead of primary antibodies. They were covered with mounting medium and observed under an Olympus BX-40 light microscope.

Three observers blinded to clinical information evaluated the staining scores independently. Staining intensity was graded as mild (1), moderate (2) and strong (3), respectively. ANOVA non parametric test was used to compare the staining intensities, $P<0.05$ was accepted as significant.

Results

Light microscopic evaluation

Histological analysis of the control group with Hematoxylin-Eosin revealed that coronary arteries had a tunica intima which consisted of endothelium and an internal elastic membrane. The media was composed of one to five complete layers of smooth muscle cells and scattered elastic fibrils. The thin adventitia layer was composed of loose connective tissue with longitudinally oriented collagenous and elastic fibers. Histological analysis of these samples did not differ among the 4 groups.

Immunohistochemical staining

Mild iNOS immunoreactivity was seen only in the endothelium of the control group without staining in the perivascular region (Fig. 1a). In the OVX group, strong iNOS immunostaining appeared in the perivascular region of the artery without any differences on endothelial cells (Fig. 1b). In rats which received E2 or E2 plus NETA, iNOS immunoreactivity in the perivascular region was absent or mild respectively; in endothelial cells the immunoreactivity was similar to the control group (Fig. 1c,d). When these immunoreactivities were compared statistically with control and the other groups, iNOS immunoreactivity in perivascular region of OVX group was different ($p<0.01$). Staining intensities and statistical values of iNOS and eNOS were shown in Table 1.

Mild eNOS immunoreactivity was seen in the endothelial cells and the perivascular region of the control group (Fig. 2a). In the OVX group, eNOS immunoreactivity was similar in both endothelium and the perivascular region (Fig. 2b). In rats which received E2 or E2 plus NETA, eNOS intensity was similar to the control group in the endothelium and moderate in the perivascular region (Fig. 2c,d).

Discussion

Atherosclerosis, which leads to coronary artery

disease, is mostly seen in postmenopausal women and can, at least putatively, be delayed with HRT. Previous studies suggested that the protective effects of HRT may be due to NOS level in the coronary arteries, but the exact mechanism of protection has not been completely understood. Increased iNOS levels were seen in the aorta of OVX rats, and treatment with oestradiol alone or oestradiol plus progesterone inhibited this induction (Tamura et al., 2000). Another study has shown increased NOS activity in the coronary artery of rats receiving HRT (Wu et al., 2000), and others have shown NOS activity in human coronary and aortic lesions (Buttery et al., 1996; Wilcox et al., 1997; Behr-Roussel et al., 2000), but so far no study has shown immunohistochemical expression of iNOS and eNOS in coronary arteries of OVX rats.

In this study, mild immunostaining for eNOS was observed in the coronary arteries from both control and OVX groups. This immunoreactivity was similar in the endothelium for all groups, whilst moderate immunoreactivity for eNOS was detected in the perivascular region in both E2 and E2+NETA groups. iNOS immunostaining intensity was similar in the intima/endothelium of the coronary arteries in all groups, this immunoreactivity was detected as mild. Although iNOS immunoreactivity was absent in the perivascular region of coronary arteries from control and E2 groups, strong and mild immunoreactivity of iNOS were detected in OVX and E2+NETA groups, respectively. It is known that excessive production of NO induces development of hypotension, cell damage and atherosclerosis (Kessler et al., 1997; Tamura et al., 2000). In the vessel wall, NO synthesis is regulated by two major types of NOS; eNOS which is normally expressed in endothelial cells, and iNOS, which is mostly expressed in smooth muscle cells after exposure to inflammatory stimuli (cytokines and lipopolysaccharides such as interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) (Zancan et al., 1999). The activity of iNOS is calcium-independent and appears to be controlled predominantly at the transcriptional level through the activation of several transcription factors, including nuclear factor- B and interferon regulatory factor-1 (Kessler et al., 1997).

Table 1. iNOS and eNOS staining intensities are seen in endothelium and perivascular regions of different groups.

GROUPS	iNOS		eNOS	
	Endothelium	Perivascular	Endothelium	Perivascular
Control	0.9±0.1	0.2±0.1	1.1±0.1	1.2±0.1
OVX	1.2±0.1	2.9±0.1* **	1.3±0.2	1.3±0.2
E2	0.9±0.1	0.2±0.1	1.2±0.1	2.0±0.1
E2+NETA	1.1±0.1	1.1±0.1	1.3±0.2	1.7±0.2

ANOVA statistical test were used to compare the staining intensities. Statistical data were shown mean±SEM. *: p<0.01 OVX vs E+NETA. **: p<0.001 OVX vs Control and E2. OVX: Ovariectomized rats; E2: Oestradiol; NETA: Norethisterone acetate.

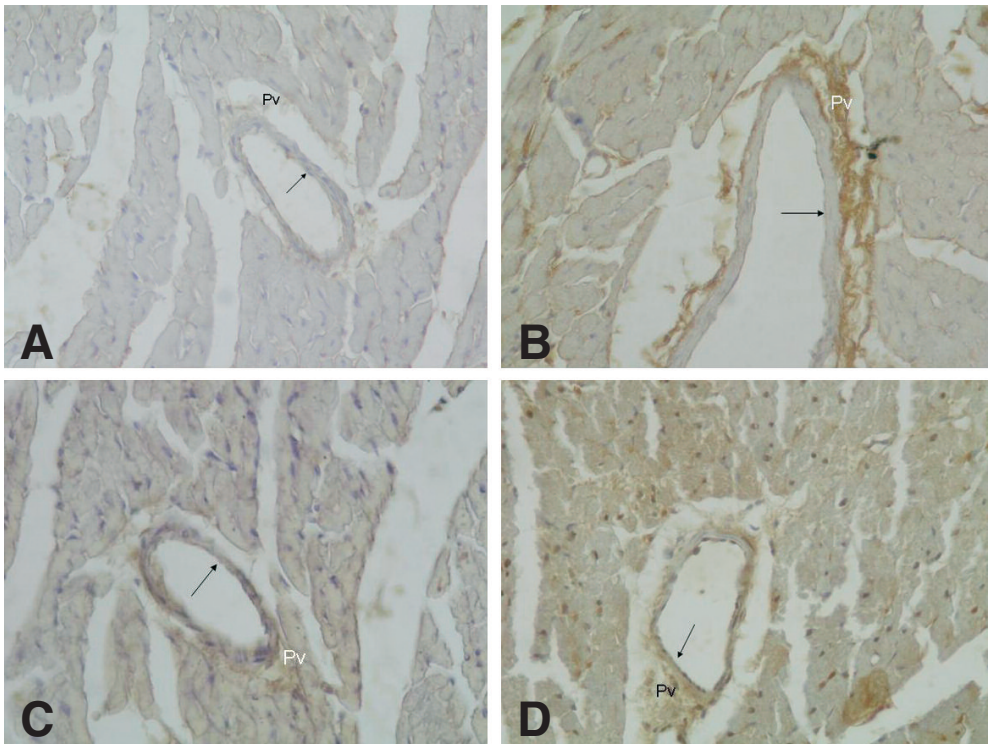

Fig. 1. a. Immunohistochemical staining of iNOS in the coronary arteries of control group shows endothelial staining. Pv: Perivascular region; arrow: Positive immunoreactivity. Original magnification x 200.
b. Immunohistochemical staining of iNOS in the coronary arteries of overiectomy group shows strong perivascular and slight endothelial staining. Pv: Perivascular region; arrow: Positive immunoreactivity. Original magnification x 200.
c. Immunohistochemical staining of iNOS in the coronary arteries of E2 group shows endothelial staining. Pv: Perivascular region; arrow: Positive immunoreactivity. Original magnification x 200.
d. Immunohistochemical staining of iNOS in the coronary arteries of E2+NETA group shows endothelial staining. Pv: Perivascular region; arrow: Positive immunoreactivity. Original magnification x 200

Therefore, once expressed, iNOS can generate NO at a maximal rate over long periods of time and has been associated with atherosclerosis.

In our study, iNOS intensity in coronary arteries was apparently reduced in the estrogen group compared with the OVX group. Similar results were reported in cardiomyocytes and in rat aorta, and anti-atherosclerotic effect of oestradiol was speculated to be related with reduced iNOS level (Medelsohn and Karas, 1999; Nuedling et al., 1999). Estrogen has rapid vasodilative effects partially mediated through NO production. It has been suspected that estrogen may reduce the risk of cardiovascular disease through complicated mechanisms, such as by changing lipid metabolism, endothelial NO generation, vascular cell proliferation and regulation of ion channels (Zancan et al., 1999). Inhibitory effects of estrogen on iNOS protein are due, at least in part, to changes in gene expression regulated by estrogen through ERs, but the mechanism of how estrogen negatively regulates iNOS is not known. Estrogen may likely attenuate iNOS expression by decreasing serum level of IL-1ß and TNF-α which are potent activators of iNOS transcription (Tamura et al., 2000).

Progesterone receptors are present in the arterial wall and it also affects the arterial function. While progesterone can stabilize arteries in the presence of vasomotor instability, the effect of estrogen-progesterone therapy has not completely been understood (Ganz et al., 2002). It was suggested that progesterone might attenuate some of estrogen's beneficial effects on arteries, particularly in the early stages of atherosclerosis (Mueck et al., 2002). In our study, we observed similar eNOS staining intensity in both E2 and E2 + NETA groups, but iNOS staining was slightly higher in the E2 + NETA group. We consider that NETA, probably, does not have any additional benefit than using oestradiol alone, though E2 + NETA combination reduces iNOS intensity when compared with the OVX group. NETA may have positive cardiovascular effects such as increased stroke volume, lowered blood pressure and positive cerebrovascular effect. When the effect of MPA and NETA was compared during continuous combination with E2 on atherosclerotic plaque initiation and plaque formation in human female vascular cell cultures, MPA, but not NETA, antagonized the E2-induced significant reduction of MCP-1 (monocyte chemo attractant protein-1) synthesis. MCP-1 plays an important role in recruitment and activation of monocytes which is the central event in atherosclerosis. In addition, MPA and NETA enhanced the positive E2-effect on pro-MMP-1 which was crucial for

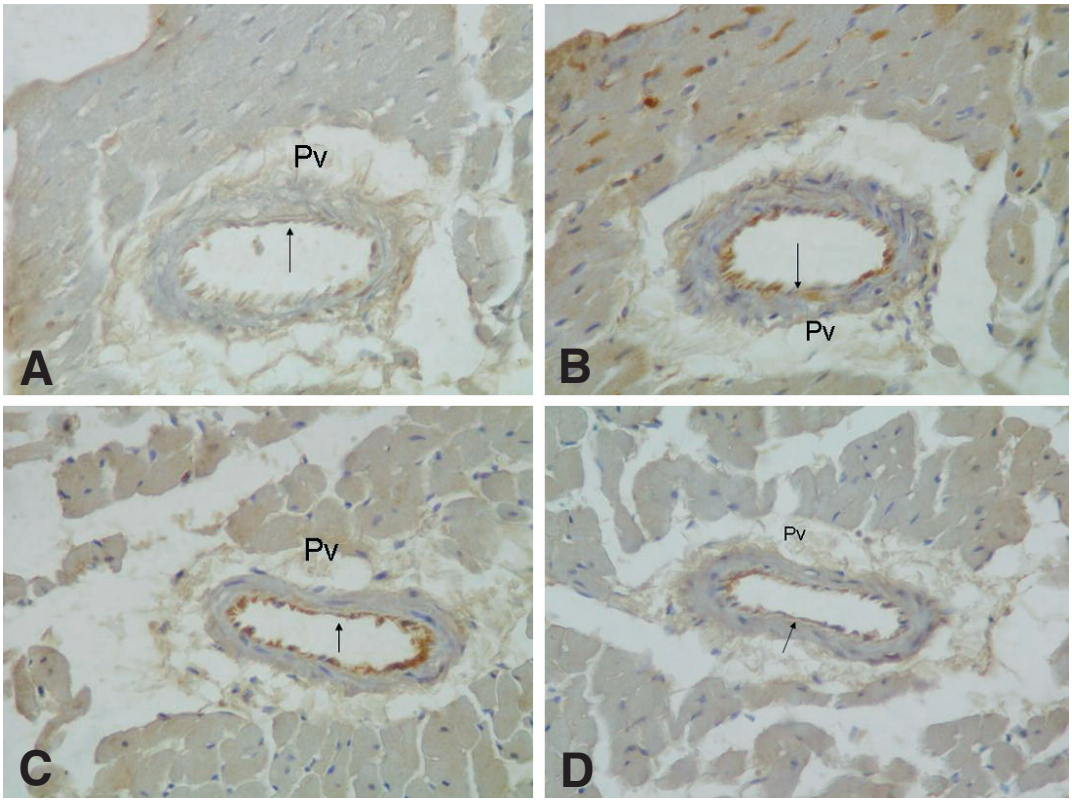

Fig. 2. a. Immunohistochemical staining of eNOS in the coronary arteries of control group shows mild endothelial and perivascular staining. Pv: perivascular region; arrow: positive immunoreactivity. Original magnification x 200. **b.** Immunohistochemical staining of eNOS in the coronary arteries of overiectomy group shows similar endothelial and reduced perivascular staining. Pv: perivascular region; arrow: positive immunoreactivity. Original magnification x 200. **c.** Immunohistochemical staining of eNOS in the coronary arteries of E2 group shows similar endothelial and moderate perivascular staining. Pv: perivascular region; arrow: positive immunoreactivity. Original magnification x 200. **d.** Immunohistochemical staining of eNOS in the coronary arteries of E2+NETA group shows similar endothelial and moderate perivascular staining. Pv: perivascular region; arrow: positive immunoreactivity. Original magnification x 200

atherosclerotic plaque stability and the authors concluded that progestin might differ in their effects, particularly in the early stages of atherosclerosis (Mueck et al., 2002).

In conclusion, E2 treatment reduced the OVX-induced increase in iNOS staining in the coronary arteries, and adding 19-nortestosterone derivative progesterone NETA did not adversely affect this reduction, though iNOS staining was slightly higher in this group than E2 alone. The reduction in iNOS staining may be one of the mechanisms involved in estrogen's protective effect from atherosclerosis.

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