**Endothelial nitric oxide synthase is differently expressed in human endometrial vessels during the menstrual cycle**

M.Taguchi1,2,4, J.Alfer2, K.Chwalisz3, H.M.Beier2 and I.Classen-Linke2

1Department of Obstetrics and Gynecology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-0034, Japan, 2Department of Anatomy and Reproductive Biology, RWTH Aachen, 52057 Aachen and 3Research Laboratories, Schering AG, 13342 Berlin, Germany

4To whom correspondence should be addressed

Endogenous nitric oxide (NO) can be synthesized by endothelial cells and can act as a potent vasodilator. We investigated endothelial nitric oxide synthase (eNOS), one of the three different enzymes responsible for the synthesis of NO by immunohistochemical methods throughout the menstrual cycle on 34 endometrial samples and compared its detection with the von Willebrand Factor (vWF) as a reliable marker molecule of the endothelium on serial sections. Immunoreactivity for eNOS was clearly localized in various types of arterial and venous endothelial cells as well as in capillaries. In addition, in some samples there was a positive staining in endometrial glandular epithelium. There was no staining in endometrial fibroblasts or in myometrial smooth muscle cells. Whereas the endothelium was constantly stained by the monoclonal antibody against vWF, eNOS was not always expressed in the endothelial lining of the vessels during the menstrual cycle. The number of vessels positively stained for eNOS increased gradually during the proliferation phase and most of the vessels were positive in the early secretory phase. These results suggest that its markedly increased expression during the early secretory phase of the menstrual cycle indicates a physiological significance. Key words: endothelial nitric oxide synthase/human endometrium/immunohistochemistry/menstrual cycle

**Introduction**

Endogenous nitric oxide (NO) is synthesized by a number of different cells, mediates endothelium-dependent relaxation, neurotransmission and cell-mediated immune response and is involved in paracrine and autocrine regulation of neurotransmitter, polypeptide and ion secretion (Schmidt and Walter, 1994). The endothelium-derived relaxing factor, which was first demonstrated by Furchgott and Zawadzki (1980), a locally produced substance that modulates the blood flow, was shown to be NO in subsequent studies (Palmer et al., 1987; Furchgott and Vanhoutte, 1989). Nitric oxide is produced by the nitric oxide synthase (NOS) family of enzymes which oxidizes the amino acid l-arginine to form l-citrulline and NO (Palmer et al., 1988).

NOS is known to exist in at least three isoforms: besides the endothelial NOS (eNOS, type III) there exist a neuronal constitutive form (nNOS, type I) and an inducible NOS (iNOS, type II), which was first purified from macrophages (Xie et al., 1992; Nathan and Xie, 1994; Förstermann and Kleintert, 1995). Types I and III are strongly calcium dependent whereas type II is independent of calcium. Neuronal NOS and endothelial NOS are regarded as constitutive and were initially identified in neurons or in endothelial cells, respectively, while iNOS is generally inducible in almost every cell.

eNOS is capable of synthesizing NO in vascular endothelial cells and plays an important role in the control of vasotension (Janssens et al., 1992). Modifications of uterine blood flow are implicated in many aspects of reproductive physiology and in several of their pathological disorders. These modifications are hormonally regulated but remain poorly understood, and various complex regulations have been proposed (White et al., 1995).

There have been several studies in rat (Schmidt et al., 1992; Biswas et al., 1998), mouse (Huang et al., 1995) and human endometrium (Tseng et al., 1996; Telfer et al., 1997) investigating NOS which have addressed whether the vasodilatory and anti-platelet action of NO may play a role in implantation (Cameron and Campbell, 1998).

We studied eNOS localization in human endometrium by immunohistochemical methods throughout the menstrual cycle to determine whether the expression is related to the stage of the menstrual cycle, which is known to be controlled by steroid hormones and paracrine factors.

**Materials and methods**

**Human endometrial samples**

Endometrial tissue was obtained from 34 patients undergoing hysterec-
tomy due to benign uterine diseases. Specimens were excluded when any bleeding disorders appeared in the patient’s records or the actual diagnosis. The mean age of the women was 43 (range 30–54) years. Seventeen specimens were obtained from the proliferative phase and 17 from the secretory phase. The menstrual cycle was divided into the early to mid proliferative phase (days 3–9), the late proliferative phase (days 10–14), the early secretory phase (days 15–19) and the mid–late secretory phase (days 20–28). The cycle date of each specimen was confirmed by menstrual history, histological examination (Noyes et al., 1950) and assessment for 17β-oestradiol,
progestrone and luteinizing hormone (LH) on the day of hysterectomy by routine laboratory diagnosis. All patients were normally cycling women and none had received medication or hormones for at least 6 months before surgery. The tissue samples were quickly frozen in liquid nitrogen and stored at −20°C until processed.

**Immunohistochemistry on cryostat sections**

Tissue blocks were embedded in Tissue-Tek O.C.T. (Miles, USA). Cryostat sections (4–7 µm) were cut on a cryostat Reichert Jung 2800 Frigocut E, mounted on aminopropyltriethoxysilane-coated slides and fixed for 10 min in acetone at 4°C. Immunohistochemical staining was performed by a streptavidin–biotin–peroxidase method (Histostain-SP Kit; Zymed Laboratories Inc., CA, USA) at room temperature. The sections were rehydrated in PBS and blocked against endogenous peroxidase by incubating with 3% H2O2/methanol for 10 min. After blocking with 5% swine serum (Dako, Denmark), sections were incubated for 1 h with a specific polyclonal rabbit antibody against human eNOS (Berlex, Biosciences, Richmond, CA, USA) which was diluted 1:120 with phosphate-buffered saline (PBS)/1.5% bovine serum albumin (BSA). Lack of cross-reactivity of this antibody with human iNOS and nNOS was confirmed by solid-phase enzyme-linked immunosorbent assay and Western blots (P.Medberry and F.Parkinson, unpublished data). The second antibody, a multi-link biotinylated swine anti-rabbit, mouse, and goat immunoglobulin was incubated for 1 h. From serial sections, immunostaining for von Willebrand Factor (vWF; also called factor VIII-associated antigen) as a reliable marker of human endothelium was carried out simultaneously. After blocking with 10% non-immune goat serum, the cryosections were incubated for 1 h with a monoclonal primary antibody against vWF (Dako) at a concentration of 1:50. The second antibody, a biotinylated goat anti mouse immunoglobulin, was incubated for 30 min. After reaction of streptavidin–peroxidase for 10 min, the antigenic sites were visualized by the chromogen 3,3′-diaminobenzidine (DAB; Sigma, Deisenhofen, Germany) in PBS, pH 7.4. Sections were incubated for 30 min at room temperature. The specificity was controlled omitting the β-NADPH in the incubation mixture.

**Histochemistry**

Enzyme histochemistry of NADPH diaphorase was performed to detect all sites of putative NOS activity using a published protocol (Vincent et al., 1983). The cryostat sections were fixed in acetone at 4°C for 10 min and rinsed in PBS three times. The medium for NADPH diaphorase contained 1.2 mmol/l β-NADPH and 0.25 mmol/l tetranirotrozilium blue chloride (Sigma, Deisenhofen, Germany) in PBS, pH 7.4. Sections were incubated for 30 min at room temperature. The specificity was controlled omitting the β-NADPH in the incubation mixture.

**Evaluation of results**

More than five fields per section and more than five sections per specimen of each patient were assessed and scored separately by three individual investigators (M.T., I.C.L., J.A.). The scores per field, per specimen and per patient were added and the accumulated final scores divided by three. The quantification of coincidental positive immunostaining of vWF and eNOS was calculated on the basis of these scores per patient (see Figure 13). All three investigators were blinded to the final menstrual cycle dating and the hormonal assessment of the patient.

**Results**

Immunoreactivity for eNOS was clearly localized in various types of arterial and venous endothelial cells, i.e. spiral arteries but also small capillaries, which were also stained by the antibody against vWF. In addition, in some samples with strong eNOS-staining in all vessels, glandular epithelial cells were stained as well, but reactions were in general rather weak compared to endothelium.

Whereas the vWF could be detected in all endothelial cells in various types of vessels and did not differ in intensity and degree of staining throughout the endometrial cycle, the staining for eNOS was different (see Figure 1–6). The eNOS expression was very low or negative in the early to mid proliferative phase (day 9, Figure 1) but strongly expressed in the late proliferative phase (day 14, Figure 5) and early (day 18, Figure 11) to mid secretory phase (day 24, Figure 7).

There was no staining in endometrial stromal cells or smooth muscle cells (Figures 7, 8). In the spiral arteries of the secretory phase, the endothelium was clearly stained for eNOS, but the smooth muscle cells surrounding the endothelium were negative (Figure 9). The enzyme histochemical staining for NADPH diaphorase which detects all the three different NOS isoforms was similar to the immunoreactivity of eNOS, but in addition there was always a positive staining for glandular epithelium (Figure 10).

The immunostaining for eNOS was compared with the staining for vWF and the percentage of vessels which were stained by eNOS as well as by vWF was evaluated (Figure 13). In contrast to the immunoreactivity for vWF, which was uniformly positive for endothelial cells at all stages of endometrial development, eNOS immunoreactivity was found to change during the cycle. As shown in Figure 13, the immunostaining of eNOS was found to increase from the late proliferative phase to the early and mid secretory phase, based on the morphometric evaluation of the percentage of vessels stained positively by both eNOS and vWF.

**Discussion**

In addition to its role as a potent vasodilator, NO serves several other functions including activity as an anti-coagulating effector (Schmidt and Walter, 1994), in neurotransmission (Garthwaite et al., 1988) and in cell-mediated immune responses.

In this investigation, we have focused on eNOS immunoreactivity which was clearly localized in the endothelium of spiral arteries, capillaries and veins, and to a weaker extent in glandular epithelium in accordance with earlier results (Telfer et al., 1997).

Studies on placenta, with the same polyclonal antibody we used (Nanaev et al., 1995), revealed positive staining for eNOS in the uteroplacental vessels and trophoblast cells. Endothelial NOS has been detected immunohistochemically in human endometrium by several groups (Telfer et al., 1995, 1997; Ota et al., 1998; Tschugguel et al., 1998) with different antibodies against eNOS but with contrasting results.
Figures 1–6. Comparison of endothelial nitric oxide synthase (eNOS) (Figures 1, 3 and 5) and von Willebrand Factor (vWF) (Figures 2, 4 and 6) immunoreactivity in serial sections throughout the menstrual cycle. (1) Immunoreactivity for eNOS, cycle day 9: endothelial cells showed no positive reaction for eNOS, large negative vessel (arrowhead). No coincidence between eNOS and vWF staining (see Figure 2) of vessels; bar: 100 µm. (2) Immunoreactivity for vWF in a section adjacent to that of Figure 1 (cycle day 9): vWF was detected in various types of vessels which were not stained by eNOS; large positive vessel (arrowhead); bar: 100 µm. (3) Immunoreactivity for eNOS, cycle day 13: some vessels are positively stained but not all compared to an adjacent section (see Figure 4); bar: 50 µm. (4) Immunoreactivity for vWF in a section adjacent to that of Figure 3 (cycle day 13); more vessels are stained with the antibody against the vWF; bar: 50 µm. (5) Immunoreactivity for eNOS in the late proliferative phase (cycle day 14). Strong staining of the endothelium of all vessels and, in addition, an apical staining of the glandular epithelium (*); bar: 50 µm. (6) Immunoreactivity for vWF in a section adjacent to that of Figure 5 (cycle day 14). All vessels positive for eNOS are also positive for vWF; negative glandular epithelium (*); bar: 50 µm.

Whereas one study (Telfer et al. 1997), using a monoclonal antibody against human eNOS (from Affiniti, Nottingham, UK), detected eNOS in endothelial as well as epithelial endometrial cells, another study (Tschugguel et al. 1998), using a different monoclonal antibody (from Transduction Lab., Lexington, KY, USA), localized eNOS on cryostat sections exclusively in endothelial cells. Ota et al. (1998) on the other hand, with the same antibody as Tschugguel and co-workers but using paraffin sections, detected eNOS immunoreactivity on surface and glandular epithelium in addition to a weak staining in the endothelial cells.

Telfer et al. (1997) and Tschugguel et al. (1998) could not find any obvious correlation with the stage of the menstrual cycle, whereas Ota et al. (1998) assessed a similar phase-dependent change in the expression of eNOS as we have shown in our study. Although Telfer et al. (1997) noticed that eNOS staining was not present in all endothelial cells, they could not relate the observed variations to the stage of the menstrual cycle or to the degree of menstrual blood loss. On the other hand, further support for a cyclic expression of eNOS mRNA in the epithelial glands of human endometrium has been provided by Tseng et al. (1996).

In order to elucidate the percentage of vessels positively stained for eNOS in human endometrium, we employed antibodies to vWF which delineates endothelium in human tissue (Mukai et al., 1980). Coincidence between positively stained vessels increased gradually during the proliferative phase. Most vessels were positive in the late proliferative and early to mid secretory phase. During this period of time a complex subepithelial capillary plexus develops (Sheppard and
Figures 7 and 8. Comparison of endothelial nitric oxide synthase (eNOS) immunoreactivity and von Willebrand Factor (vWF) at day 24. (7) Immunoreactivity for eNOS in the secretory phase, cycle day 24. Positive staining of the endothelium in spiral arteries, veins and capillaries; weak staining for glandular epithelium (*); bar: 50 µm. (8) Immunoreactivity for vWF in a section adjacent to that of Figure 7 (cycle day 24). 100% coincidence between localization of eNOS and vWF-staining of vessels; bar: 50 µm.

Figures 9–12. Immunoreactivity of endothelial nitric oxide synthase (eNOS) in capillaries and spiral arteries of the endometrium. (9) Immunoreactivity of eNOS in the endothelium of a spiral artery (arrows), cycle day 24; bar: 25 µm. (10) Histochemical detection of NADPH in the same tissue as in Figure 9 (cycle day 24): positive reaction in endothelium of spiral arteries (arrows), but also in glandular epithelium (*) and in stroma; bar: 25 µm. (11) Staining of eNOS in capillaries in the early secretory phase; weak to negative staining of glandular epithelium (*), cycle day 18; bar: 25 µm. (12) Control reaction with non-immune rabbit serum on a section adjacent to that of Figure 9; no staining can be seen; spiral arteries (arrows), glandular epithelium (*), cycle day 24; bar: 50 µm.

Bonnar, 1980) and a significant dilatation of these vessels during the post-ovulatory phase has been described (Sheppard and Bonnar, 1980; Peek et al., 1992).

The up-regulation of eNOS might be correlated with the 17ß-oestradiol level throughout the menstrual period, particularly the pre-ovulatory oestradiol peak. As we have measured the serum 17ß-oestradiol concentration on the day of hysterec-
tomy, we have tried to correlate this value with the coincidence between eNOS and vWF staining. The mean value of the 17ß-oestradiol concentration of all serum samples with 100%
or >50% coincidence between eNOS and vWF (19 samples) was 101 pg/ml, compared to only 65 pg/ml in the samples with <50% or no coincidence. This assessment reveals supporting evidence for a 17β-oestradiol involvement in eNOS expression; nevertheless there was only one determination of oestradiol for each patient and there was considerable inter-individual variation in steroid concentrations.

However, there are several papers in which the up-regulation of eNOS by oestrogens is described. An increase in eNOS mRNA in the aorta of pregnant rats and after oestrogen treatment has been reported (Goetz et al., 1994). An increase of eNOS in the uterine artery of guinea-pigs in pregnancy as well as after treatment with oestradiol has also been shown (Weiner et al., 1997). Hayashi et al. reported that in-vitro preincubation with a physiological concentration of 17β-oestradiol (10^{-12} to 10^{-8} M) over 8 h significantly enhanced the activity of eNOS in endothelial cells of cultured human umbilical vein and of bovine aorta (Hayashi et al., 1995), and Hishikawa et al. reported that treatment with 17β-oestradiol enhanced calcium-dependent NO production and eNOS protein production in human aortic endothelium analysed by Western blotting (Hishikawa et al., 1995).

Nevertheless the up-regulation of eNOS by oestradiol is still a matter of debate. For example it has been shown (Figueroa and Massmann, 1995) that oestrogen increases NOS activity only in the myometrium, but not in the endometrium of non-pregnant castrated sheep, and Arnal et al. have revealed that oestradiol does not increase eNOS protein in a bovine aortic endothelial cell line (Arnal et al., 1996). Which factors actually may regulate the eNOS concentration in the vasculature during the cycle is not yet clear. Besides the ovarian steroid hormones, there are several paracrine factors, but also shear stress, pathological disorders or blood flow itself which could influence eNOS.

Nitric oxide synthesized by eNOS has a direct vasodilatory impact on vascular smooth muscles, probably ensuring an optimal blood supply which might be beneficial to the endometrium during the phase of attachment and implantation.

For a successful implantation, synchronization between embryo and endometrium is necessary. Various growth factors, cytokines and their receptor molecules are present in the endometrium during the peri-implantation period, and it has been suggested that their role is to provide a suitable microenvironment for the blastocyst expansion and embryonic development and to facilitate the implantation process (Beier et al., 1994; Chwalisz et al., 1996; Beier, 1997; Beier and Beier-Hellwig, 1998; Classen-Linke et al., 1998). NOS is present at the time of implantation to induce NO, and this may have a role not only in increasing blood supply by vasodilatation but also in mediating immunological effects.

In conclusion, we have demonstrated the expression of eNOS in human endometrium with a specific polyclonal rabbit antibody against human eNOS. We found a heterogeneity in the expression patterns of this molecule in the human endometrial vasculature. Increased reactivity was observed in the late proliferative and early to mid secretory phase. The importance of these findings must be further elucidated and assessment of the possible role of eNOS in the regulation of blood flow via NO synthesis in the endometrium, in turn facilitating endometrial receptivity and implantation, needs additional research efforts.

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References


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