Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium

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Immunocytochemistry was used to localize endothelial (eNOS) and inducible (iNOS) nitric oxide synthase in human uterine tissues collected at various stages of the menstrual cycle, after exposure to exogenous progestagens, and in early pregnancy. Endothelial NOS-like immunoreactivity was detected in all specimens in endothelial cells lining blood vessels in the myometrium and endometrium, and in endometrial glandular epithelial cells. Inducible NOS-like immunoreactivity was also demonstrated in glandular epithelial cells. For both eNOS and iNOS there was considerable variation in the intensity of epithelial cell staining between samples, which was not related to the stage of the menstrual cycle at which the tissue was collected. Messenger RNA for eNOS and iNOS was detected by reverse transcription–polymerase chain reaction (RT–PCR) using total RNA purified from isolated endometrial gland fragments. Immunoreactivity for eNOS and iNOS was not present in endometrial stroma throughout the menstrual cycle, but iNOS-like immunoreactivity was seen in decidualized stromal cells both following treatment with exogenous progestagen (intrauterine L-norgestrel) and in tissues obtained in the first trimester of pregnancy. The detection of protein and mRNA for eNOS and iNOS in normal human endometrium suggests that NO may play a role in the local control of endometrial function.

Key words: decidua/endometrium/nitric oxide/progestagen

Introduction

Nitric oxide (NO) is a crucial mediator of paracrine interactions, especially within the vascular system. It is a powerful inhibitor of platelet aggregation and a potent vasodilator (Palmer et al., 1987). Nitric oxide also functions as a neurotransmitter and plays a role in cell-mediated cytotoxicity (Lowenstein and Snyder, 1992). This signalling molecule is therefore likely to have an important role within the endometrium where changes in vascular function occur throughout the course of the menstrual cycle and at the time of implantation. Although it is difficult to determine which cells produce NO in vivo, circumstantial evidence of its production by particular cell types can be obtained by localizing the enzymes which synthesize it.

NO is produced by nitric oxide synthases (NOSs), a family of isoenzymes that catalyse the oxidation of L-arginine to nitric oxide (NO) and citrulline. There are two functional classes of NOS, based on the requirement of these enzymes for calcium (Moncada et al., 1991). Inducible NOS (iNOS or type II), which is calcium-independent, binds calmodulin tightly at resting intracellular calcium concentrations. Endothelial NOS (eNOS or type III), originally described in endothelial cells, and neuronal NOS (nNOS or type I) are both dependent on calcium for activity.

The production of NO in the endometrium has not been widely addressed, but iNOS protein has been reported in endometrial epithelial cells in the mouse (Huang et al., 1995) and neuronal NOS protein has been detected in rat endometrial epithelium (Schmidt et al., 1992). We have previously described the presence of eNOS protein and mRNA, and NADPH diaphorase activity, in human endometrium (Telfer et al., 1995). In addition, recent work has shown that human endometrial glandular cells expressed eNOS mRNA throughout the menstrual cycle, whilst the expression of iNOS mRNA was confined to epithelial glands isolated from menstrual endometrium (Tseng et al., 1996).

The onset of menstruation is characterized by intense vasoconstriction (Markee, 1940). Most of the functional endometrium is shed within 20 h of the onset of bleeding, and subsequent haemostasis is thought to be achieved not by the deposition of platelet-fibrin plugs, but by vasoconstriction (Christiaensen et al., 1980). Nitric oxide produced in the human endometrium may play a role in the control of menstruation (and implantation) by virtue of its roles as a vasodilator and an inhibitor of platelet aggregation. Animal studies have suggested that NO, acting via second messenger cGMP, may contribute to the maintenance of uterine quiescence during pregnancy (Natuzzi et al., 1993; Sladek et al., 1993). Thus NO produced in the endometrium may also have an effect on the underlying myometrium.

The aim of the present study was to determine the localization of eNOS and iNOS protein in human endometrium by immunocytochemistry, and to detect the presence of mRNA for eNOS and iNOS using the reverse transcription–polymerase chain reaction (RT–PCR) on enriched preparations of glandular epithelium. The effect of ovarian steroids on the expression...
of NOS in the endometrium was investigated by immunocytochemical analysis of tissue collected both at different time points in the normal menstrual cycle and from women receiving exogenous progestagens (oral norethisterone or intrauterine L-norgestrel) for the treatment of dysfunctional uterine bleeding. Localization of eNOS and iNOS in decidualized stroma was also studied in tissues from women with normal pregnancies undergoing induced abortion in the first trimester.

Materials and methods

Collection of tissue from women with normal menstrual cycles

Tissue was obtained from 34 pre-menopausal women with regular menstrual cycles undergoing hysterectomy for benign disease at the West Glasgow Hospitals University NHS Trust and Glasgow Royal Infirmary University NHS Trust, UK. In nine cases menstrual blood loss was measured by the alkaline haematin method (Hallberg and Nilsson, 1964). Informed consent was obtained in each case and the study was approved by the local ethics committees.

Histological assessment of endometrial morphology was carried out by local pathologists according to standard criteria (Noyes et al., 1950).

Collection of tissue from menorrhagic women treated with exogenous progestagens

Endometrial biopsies were collected from 12 women with regular menstrual cycles and objectively measured menorrhagia (menstrual blood loss >80 ml per month, median 99 ml, range 83–245 ml). Six women subsequently received oral norethisterone (5 mg three times daily from days 5 to 26 of the cycle) and six received L-norgestrel, as the L-norgestrel intrauterine system (LNG-IUS, Mirena®; Leiras Oy, Turku, Finland). The LNG-IUS, which releases 20 µg LNG daily, was inserted within 5 days of the onset of menstruation. Endometrial biopsies were taken using a Z-Sampler endometrial suction curette (Zinnanti, Chatsworth, CA, USA) at a median of 21 days (range 19–25) after the onset of menstruation during a pre-treatment control cycle. A second post-treatment biopsy was taken after a median of 18 days (range 13–24) exposure to L-norgestrel and 17 days (range 13–20) exposure to norethisterone.

Collection of first trimester decidua

Decidual tissue was collected from 10 women with normal pregnancies undergoing surgical termination in the first trimester.

Isolation of endometrial gland fragments

Biopsies of endometrium were transported to the laboratory in cold 1/200 in PBS. Antibody binding was detected using an anti-rabbit IgG peroxidase kit (Vectastain Elite ABC Kit; Vector) and DAB substrate (Sigma), according to the manufacturer’s instructions. Control slides were incubated without primary antibody. Antibody binding was detected using an anti-rabbit immunoglobulin (Ig)G peroxidase kit (Vectastain Elite ABC Kit; Vector) and DAB substrate (Sigma), according to the manufacturer’s instructions. Control slides were incubated without primary antibody. Human DLD-1 colorectal adenocarcinoma cells (European Collection of Animal Cell Cultures, Porton Down, UK), which have previously been shown to express iNOS (Sherman et al., 1993), were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat inactivated fetal calf serum, 100 µg/ml interferon (Sigma), 10 ng/ml tumour necrosis factor (Sigma) and 0.5 ng/ml interleukin-1 (Sigma) for 24 h, and used as a positive control.

Immunocytochemistry for eNOS

Tissue biopsies obtained at hysterectomy, comprising endometrium and myometrium, were fixed in 10% neutral buffered formalin (BDH, Poole, Dorset, UK) and embedded in paraffin. Sections were cut (5 µm thick) and mounted on silane-coated slides, heated to 60°C for 35 min, deparaffinized in xylene and rehydrated in a graded alcohol series. The sections were pre-incubated with 3% (w/v) immunoglobulin-free bovine serum albumin (BSA; Sigma) in PBS (10 mM sodium phosphate, pH 7.5, 120 mM sodium chloride) for 20 min at room temperature. They were then incubated for 1 h at room temperature with a monoclonal antibody against human eNOS (Affinitii, Nottingham, UK), diluted 1/500 in 3% BSA. Sections were then washed in 0.1% Triton-X100, followed by two washes in PBS. Antibody binding was detected with an anti-mouse immunoglobulin peroxidase kit (Vectastain Elite ABC Kit; Vector, Peterborough, UK) in which the biotinylated bridging antibody was diluted in 3% (w/v) BSA and 1.5% (v/v) normal human serum (Sigma). After application of the biotinylated antibody the sections were washed in PBS and placed in 0.3% H₂O₂ (Sigma) in methanol for 30 min at room temperature. The sections were then washed thoroughly in PBS and incubated for 30 min with avidin D/biotinylated horseradish peroxidase H reagent (Vector) in PBS. Immunoreactive eNOS was visualized with 3,3′-diaminobenzidine (DAB; Sigma) and 0.02% H₂O₂ in 50 mM Tris–HCl, pH 7.6. Sections were washed in distilled water, counterstained with Harris haematoxylin and mounted in DPX (BDH).

Negative control sections were incubated without primary antibody, or with an irrelevant IgG1 mouse monoclonal antibody against glucose oxidase from Aspergillus niger (Dako Ltd., High Wycombe, UK), an enzyme which is not present in mammalian tissue.

Immunocytochemistry for iNOS

Immunocytochemistry was performed on parafin-embedded sections using a polyclonal antibody raised against amino acids 1131–1144 of murine iNOS (Cambridge Biosciences, Cambridge, UK) diluted 1/400 in PBS. Antibody binding was detected using an anti-rabbit immunoglobulin (Ig)G peroxidase kit ( Vectastain Elite ABC Kit; Vector) and DAB substrate (Sigma), according to the manufacturer’s instructions. Control slides were incubated without primary antibody. Human DLD-1 colorectal adenocarcinoma cells (European Collection of Animal Cell Cultures, Porton Down, UK), which have previously been shown to express iNOS (Sherman et al., 1993), were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat inactivated fetal calf serum, 100 µg/ml interferon (Sigma), 10 ng/ml tumour necrosis factor (Sigma) and 0.5 ng/ml interleukin-1 (Sigma) for 24 h, and used as a positive control.

Immunocytochemistry was also performed on frozen sections with a polyclonal anti-human iNOS antibody raised against amino acids 1135–1153 (Santa Cruz, Heidelberg, Germany). The antibody was diluted 1/200 in PBS and immunocytochemistry was carried out using an anti-rabbit IgG peroxidase kit ( Vectastain Elite ABC Kit; Vector) according to the manufacturer’s instructions.

Detection of endothelial cells using Ulex europaeus agglutinin 1 (UEA 1) lectin

Lectin binding was carried out on 10 µm frozen sections of endometrium and myometrium mounted on silane-coated slides. The sections were fixed in acetone at room temperature for 20 min, air-dried and rehydrated in 10 mM HEPES, pH 7.5, 0.15 M NaCl, then placed in 0.3% H₂O₂ in methanol for 30 min to remove endogenous peroxidase activity. Biotinylated Ulex europaeus agglutinin 1 (UEA 1) lectin (5 µg/ml, Vector) was applied to the section in HEPES, NaCl buffer for 1 h at room temperature. Avidin–biotin–peroxidase complex

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Identification of decidual stromal cells in first trimester decidua

Cryostat sections were fixed in acetone at 4°C for 10 min, air-dried and rinsed in PBS for 10 min. Decidual cells were identified with a polyclonal antibody against vimentin (Euro-Path Ltd, Bude, UK). Glands and trophoblast were localized using a monoclonal antibody against cytokeratins (clone MNF 116, Dako, High Wycombe, UK). The sections were pre-incubated for 20 min in D-PBS supplemented with 1.5% BSA. The primary antibodies were applied for 60 min (anti-cytokeratin 1:400; anti-vimentin 1:130, diluted in D-PBS supplemented with 1.5% BSA). After washing in D-PBS 3 times for 5 min, biotinylated secondary rabbit anti-mouse antibody (1:300, Dako UK) or biotinylated goat-anti rabbit antibody (Vector) were applied for 30 min. Sections were then incubated in 1% H₂O₂ in absolute methanol for 10 min to inactivate endogenous peroxidase followed by incubation with streptavidin–peroxidase–conjugate (StreptAB-complex/HRP; Dako) for 20 min. The streptavidin–biotin complex was visualized with DAB (Sigma). Negative control sections were treated in the same way as for eNOS.

Reverse transcription-polymerase chain reaction

Endometrial gland fragments and DLD-1 cells were centrifuged at 100 g for 5 min and lyzed in Trizol reagent (Life Technologies). Total RNA was prepared by solvent extraction following the manufacturer’s instructions.

Reverse transcription (RT) was carried out on 2 μg of total RNA extracted from gland fragments of three proliferative and three secretory endometria and DLD-1 cells. The RNA was annealed to 250 ng of pd(T) 19–24 (Pharmacia, Milton Keynes, UK) by heating to 70°C for 10 min. After chilling on ice, 1 IU RNAguard (Pharmacia, Milton Keynes), 1 mM dNTPs, first strand buffer (Life Technologies) and 200 IU of SuperScript II RNase H– reverse transcriptase (Life Technologies) were added, made up to a total volume of 20 μl with water and incubated at 37°C for 1 h. The reaction was terminated by heating to 80°C for 10 min. cDNA products were stored at −20°C until amplification.

Polymerase chain reaction (PCR) was undertaken with primers (Oswell DNA Service, Southampton, UK) for human eNOS which spanned exons 8–11 (Marsden et al., 1993); 5′–CAG TGT CCA ACA TGC TGG AAA TTG–3′ (1004–1030) and 5′–TAA AGG TCT TCT TCG TGG TGA TGC C–3′ (1490–1464, Weiner et al., 1994). Amplification of iNOS was carried out with primers (P.Romanowski, L. Wallman, and D.J. Williamson, St Vincent’s Hospital, Darlinghurst, Australia; personal communication) which spanned exon–intron boundaries (Chartrain et al., 1994); 5′–GGG ATT CAC TCA GCT GTG CAT CG–3′ (1077–1099) and 5′–GTT TCC AGG CCC ATT CTC CTG C–3′ (1433–1412). Amplifications were carried out using a programmable thermal cycler (TLA-2 Hybaid, Teddington, UK) in a total volume of 50 μl containing 60 mM Tris–HCl, pH 8.5, 15 mM (NH₄)₂SO₄, 250 μM dNTPs, 0.5 M of each primer and 5 IU of Taq polymerase. A HotWax Mg²⁺ bead (Invitrogen, Abingdon, UK) was added to each tube. Amplifications were carried out with an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The final cycle was followed by strand extension at 72°C for 10 min. Amplified products were analysed by electrophoresis using 1.5% agarose gels and visualized by ethidium bromide staining.

The PCR products were sequenced with an ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer, Warrington) using AmpliTaq DNA polymerase, FS and the sequencing products were analysed on an automated 373A DNA sequencer (Applied Biosystems, Warrington, UK).

Results

eNOS and iNOS immunocytochemistry across the normal menstrual cycle

Immunocytochemistry for eNOS and iNOS was carried out on paraffin sections from 21 women. Six were in the proliferative phase of the cycle, 11 were in the secretory phase [early secretory (days 14–18) n = 6, mid-secretory (days 19–24) n = 3, late secretory (days 25–28) n = 2] and 4 were in the menstrual phase.

Endothelial NOS protein was detected in endothelial cells lining blood vessels (Figures 1A,B) in the myometrium and endometrium, and in endometrial glandular epithelial cells in all specimens examined. UEA 1 lectin staining confirmed that eNOS antibody stained endothelial cells (Figure 1C), but suggested that eNOS protein was not present in all endothelial cells. There was a difference both in the intensity of staining of endothelial cells and the number of blood vessels which stained positively for eNOS in endometrium and myometrium between different sections. There was also inter-individual variation in the intensity of eNOS antibody staining in glandular epithelial cells which was not related to the stage in the menstrual cycle.

In five of the 21 specimens there was marked variation in the intensity of staining of endometrial epithelial cells in the same specimen, ranging from very faint in the functional layers of the endometrium in four biopsies, two of which were obtained in the proliferative phase and two in the early to mid-secretory phase of the menstrual cycle.

Amongst the nine women in whom menstrual blood loss was measured before hysterectomy, five had a normal monthly blood loss (median 35 ml, range 28–76 ml) and four had menorrhagia (median 202 ml, range 98–265 ml). The intensity of immunostaining for eNOS in these samples did not appear to be related to the degree of menstrual blood loss.

Inducible NOS-like immunoreactivity was investigated in paraffin sections using the murine polyclonal antibody. Immunostaining was not present in endothelial cells, but was detected in glandular epithelial cells (Figure 1D). Once more there was a considerable difference in the intensity of staining between samples which was not related to the stage of the menstrual cycle. As with the eNOS immunocytochemistry, a gradation in staining was also observed between basal and functional layers of the endometrium in four biopsies, two of which were obtained in the proliferative phase and two in the early to mid-secretory phase of the menstrual cycle.

Immunostaining of vascular smooth muscle in the myometrium,
Figure 1. Localization of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) protein in human uterus. (A) eNOS localized to the endothelium of a spiral arteriole (arrows) in a secretory phase specimen and also to the glandular epithelium with variable intensity. (B) eNOS was found in glandular epithelium and endothelial cells of microvessels (arrows) in menstrual tissue. (C) Localization of UEA 1 binding glycans in endometrium and myometrium (m) confirmed the vascular localization of eNOS. (D) iNOS was localized in glandular epithelium and the smooth muscle of spiral arterioles (arrows), but was absent from the microvascular endothelium. (E) iNOS was also expressed in myometrial smooth muscle and vascular smooth muscle within the endometrium. (F) Human DLD-1 adenocarcinoma cells stimulated with cytokines expressed iNOS and acted as a positive control for the anti-murine iNOS antibody. Nuclei were visualized with haematoxylin. The negative controls, without primary antibody or with an irrelevant primary antibody (see Materials and methods) exhibited no reactivity. Bar = 30 µm.

and of the myometrium itself, was seen in tissues from nine women (Figure 1E). Control cultures of human DLD-1 cells treated with cytokines stained intensely with the anti-murine iNOS antibody (Figure 1F).

The pattern of immunolocalization obtained with the anti-human iNOS antibody on cryosections was identical to that obtained with the anti-murine iNOS antibody on paraffin sections.

**Immunocytochemistry for eNOS and iNOS after treatment with exogenous progestagens**

The pattern of localization of eNOS and iNOS protein in the pre-treatment biopsies of 12 menorrhagic women was not distinguishable from that observed in the biopsies from the 21 who underwent hysterectomy for benign disease. Endothelial NOS-like immunoreactivity was confined to vascular endothelium and glandular epithelium, whilst iNOS-like immunoreactivity was seen in glandular epithelium and in some vascular smooth muscle cells. Though immunoreactivity was present in all sections, there was marked variability in the intensity of staining for both vascular and epithelial cells. NOS-like immunoreactivity was not detected in endometrial stroma.

Administration of norethisterone resulted in the appearance of small areas of decidualization in tissues from two of the six patients, whereas a pronounced decidual reaction was seen in all endometrial specimens taken from the six women.
NOS expression in human endometrium

Figure 2. Expression of nitric oxide synthase (NOS) in endometrium after exposure to exogenous progestagens. (A) Endothelial NOS localized to blood vessels after exposure to oral norethisterone for 21 days. The glandular epithelium was reduced in height and in this biopsy did not express eNOS. (B) A highly decidualized patch of endometrial stroma after exposure to LNG-IUS showing weak expression of inducible NOS (iNOS) within the stromal cells. (C) Stromal cells within first trimester decidua also expressed iNOS. The negative controls, without primary antibody or with an irrelevant primary antibody (see Materials and methods) exhibited no reactivity. Bar = 30 µm.

Receiving LNG-IUS. Treatment with progestagen abolished eNOS immunoreactivity in glandular epithelial cells in all of the patients treated with the LNG-IUS and in three of those treated with norethisterone (Figure 2A). Similarly, treatment with the LNG-IUS caused an attenuation or loss of iNOS immunoreactivity within glandular epithelium. By contrast, weak iNOS immunoreactivity appeared in the decidualized stromal cells after exposure to LNG-IUS (Figure 2B).

NOS immunoreactivity in stromal cell of first trimester decidua

Endothelial NOS-like immunoreactivity was localized to endothelial cells in five out of five biopsies of decidual tissue in which eNOS localization was carried out. Localization of iNOS was examined on 10 decidual biopsies using both the murine and human iNOS antibodies. With both antibodies, weak immunoreactivity was observed in decidual stromal cells (Figure 2C) which expressed vimentin but not cytokeratin, and in vascular smooth muscle.

Reverse transcription–polymerase chain reaction

Messenger RNA for both eNOS and iNOS was detected by RT–PCR in total RNA extracted from enriched gland preparations from proliferative and secretory phase endometria (Figure 3). Sequencing of the amplified products revealed 95 and 99% homology with the published sequences of eNOS and iNOS respectively. RT–PCR for iNOS in control cultures of DLD-1 cells produced one intense band of the expected size and equivalent to that observed in isolated gland fragments.

Discussion

This study has demonstrated the presence of mRNA and protein for both eNOS and iNOS in normal human endometrium. Besides localization to the vascular compartment (eNOS to vascular endothelium and iNOS to vascular smooth muscle in some sections), eNOS- and iNOS-like immunoreactivity and mRNA were detected in endometrial glandular epithelium. Whilst the intensity of immunostaining varied markedly between individuals, there was no clear relationship with either the stage of the menstrual cycle at which tissues were obtained, or objectively-measured menstrual blood loss. Endothelial NOS or iNOS mRNA or protein were not found in endometrial stroma, but iNOS immunoreactivity was present in decidualized stroma in specimens obtained after the administration of synthetic progestagen and in the first trimester of pregnancy.

In our previous work which assessed immunoreactivity in six women using a different monoclonal antibody raised in mice against bovine eNOS, staining was detected in endometrial stroma and myometrial blood vessels, with weak staining in glandular epithelium. Whilst the intensity of immunostaining varied markedly between individuals, there was no clear relationship with either the stage of the menstrual cycle at which tissues were obtained, or objectively-measured menstrual blood loss. Endothelial NOS or iNOS mRNA or protein were not found in endometrial stroma, but iNOS immunoreactivity was present in decidualized stroma in specimens obtained after the administration of synthetic progestagen and in the first trimester of pregnancy.

Demonstration of iNOS-like immunoreactivity in endometrial glandular epithelium was also supported by the detection of iNOS mRNA in glandular epithelial cells by RT–PCR. In addition, Tseng et al. (1996) detected mRNA for iNOS in enriched endometrial gland preparations by Northern blot analysis. However, in contrast to eNOS, iNOS message was only found in glandular epithelia derived from menstrual endometrium; the different results obtained with RT–PCR and

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Figure 3. Reverse transcription–polymerase chain reaction (RT–PCR) for (A) endothelial nitric oxide synthase (eNOS) and (B) inducible nitric oxide synthase (iNOS) using total RNA extracted from freshly isolated gland fragments. Lanes 2–4 represent the proliferative phase and lanes 5–7 the secretory phase. Lane 1 is a 123 bp ladder and lane 8 is a negative control from which cDNA was omitted. Five out of six specimens produced an intense band of the appropriate size (486 bp) for the eNOS amplimer. One proliferative phase specimen (lane 4) produced a much fainter band of the same size. RT–PCR with iNOS primers produced a major band of the predicted size (375 bp) in five out of six specimens. This product had 99% sequence homology with the published human iNOS sequence. Products of higher molecular weight were also observed in three secretory phase specimens although their identity is unknown.

Northern blotting might be due to the different sensitivities of these two techniques.

Although not found in stromal cells throughout the normal cycle, iNOS-like immunoreactivity was present in stromal cells which had undergone decidualization following either the administration of synthetic progestagen in vivo, or the establishment of pregnancy. This in-vivo study does not discriminate between a direct effect of progesterone on iNOS expression or a secondary effect of local paracrine mediators that are induced by progesterone. In this study we have obtained no evidence for effects of oestrogen on eNOS and iNOS expression in human endometrium. Similarly in the sheep uterus exogenous oestrogen had no effect on NOS in the endometrium although it increased Ca\(^{2+}\)-dependent NOS in the myometrium (Figueroa and Massmann, 1995).

Identification of eNOS and iNOS in human endometrium suggests that NO may be involved in the local control of uterine function. Moreover, localization of NOS to vascular and non-vascular tissues would not preclude a predominant action on the uterine vascular bed. For example, the renal tubular epithelial cells of the macula densa release NO which dilates the neighbouring afferent artery to increase the glomerular filtration rate (Wilcox et al., 1992). Nitric oxide might participate in both the initiation and control of menstrual bleeding. According to Markee (1940), the onset of menstruation is preceded by endometrial regression, vasoconstriction and then vasodilatation. Nitric oxide could contribute to these events as the most potent known vasodilator. Next, along with the vasodilatory prostanoids, prostaglandin E\(_2\) and prostacyclin (prostaglandin I\(_2\)), NO might play a role in determining the degree of menstrual bleeding (Smith et al., 1981), though a relationship between the intensity of immunostaining and objectively-measured menstrual blood loss was not found in the present study. In addition, NO may play a part in the inhibition of platelet aggregation within the endometrium. During the first few days of bleeding, haemostasis is achieved mainly by vasoconstriction and not by the deposition of platelet-fibrin plugs (Christiaens et al., 1980).

Thus, the endometrium is thought to heal not by clot organization and subsequent scarring, but by vasoconstriction followed by the growth of new blood vessels.

Endometrially-derived NO could also contribute to the local control of myometrial contractility (Izumi et al., 1993; Buhimschi et al., 1995). Much attention has focused on the part that NO might play in maintaining myometrial quiescence during pregnancy, suggesting NO administration as a therapeutic approach for the treatment of pre-term labour (see Norman and Cameron, 1996 for review). Nitric oxide also appears to relax the non-pregnant myometrium, an action which could be exploited for the medical treatment of primary dysmenorrhoea (Pittrof et al., 1996).

Nitric oxide can be released from vascular endothelium by a range of stimuli, including endothelin (ET) binding to the ET\(_B\) receptor (Takayanagi et al., 1991). Endothelin-like immunoreactivity is found on vascular endothelium and glandular epithelium in human endometrium (Cameron et al., 1992; Salamonsen et al., 1992). Furthermore, ET\(_B\) receptors are predominantly localized to the same cell type as NOS-like immunoreactivity in the endometrium, namely glandular epithelium (Collett et al., 1996). The precise relationships between NO, ETs and other locally-produced mediators of endometrial function remain to be elucidated.

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