Induction of inducible nitric oxide synthase expression in human secretory endometrium

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The endometrial secretory phase is characterized by stromal oedema, a premenstrual increase in stromal macrophages and an increased cytokine production as menstruation approaches. Nitric oxide (NO) is a mediator of vasodilatation and cytotoxicity which is synthesized from L-arginine by NO synthases (NOS). These enzymes are either constitutively expressed or induced by lipopolysaccharides and/or cytokines. The presence and function of the inducible isoform of NOS (iNOS) in normal human endometrium has not been fully elucidated until recently. Frozen tissue sections taken from 22 women who underwent hysterectomy and adnexectomy for benign disease were immunostained with antibodies raised against the different NOS isoforms to investigate the presence of NOS in human endometrium. iNOS stained positive in the glandular epithelial cells of the secretory endometrium. Staining was either weak or absent in the proliferative and inactive endometrium, as well as in the oviduct and the glandular epithelium of the endocervix. The stroma remained uniformly negative. Immunoreactivity for endothelial constitutive NOS (eNOS) was confined exclusively to endothelial cells. Furthermore, epithelial cells from endometrium, oviduct and endocervix and all endothelial cells showed positive staining for reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity which is a histochemical marker for NOS activity. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in order to assess the presence of NOS mRNA. Abundant expression of iNOS mRNA was detected in the secretory phase endometrium only. The strong expression of inducible NO synthase in human secretory phase endometrium suggests that the increased production of NO, probably induced by cytokines, may be relevant to the process of menstruation.

Key words: endocervix/endometrium/nitric oxide/oviduct

Introduction

Nitric oxide (NO), derived from L-arginine by the action of nitric oxide synthase (NOS), is a powerful inhibitor of platelet aggregation and a potent vasodilator (Palmer et al., 1987). It also functions as a neurotransmitter and is a crucial mediator of macrophage and neutrophil cytotoxicity (Lowenstein and Snyder, 1992). NOS exists in a variety of isoforms. A constitutive, calcium-dependent enzyme is found in endothelial cells (eNOS) and the brain (bNOS). Macrophages, neutrophils, endothelial and epithelial cells express an inducible, calcium-independent form of the enzyme (iNOS) (Knowles and Moncada, 1994; Robbins et al., 1994). The deduced amino acid sequences of the different NOS forms share 50–57% homology.

Until now, the expression pattern of NOS isoforms in human endometrium has not been fully elucidated. Recent work revealed nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity which detects all known NOS isoforms (Tracey et al., 1993), and NOS mRNA expression in endometrial glandular epithelium and stroma (Telfer et al., 1995). NO, by acting as an alternative inhibitor of platelet aggregation, could theoretically play a role in the local control of the uterine vascular bed, since treatment of women with an inhibitor of endometrial prostaglandin production was not followed by platelet plug formation within the endometrium (van Eijkeren et al., 1992). Moreover, following treatment of ovariectomized mice with 17β-oestradiol for 24 h, the resultant iNOS expression was only found in myometrial macrophages but not in uterine epithelial cells. By contrast, treatment with progesterone, which mimics endometrial secretion, resulted in a switch of staining from myometrial macrophages to uterine epithelial cells (Huang et al., 1995). Since iNOS expression in macrophages or epithelial cells requires induction with cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interferon-γ, or bacterial lipopolysaccharide (LPS) (Nathan, 1992), its expression in the secretory endometrium of mice is probably caused by cytokine induction (Huang et al., 1995). This is further supported by increasing evidence demonstrating that cytokines secreted from lymphoid cells within the endometrial stroma are likely to mediate – at least in part – effects of steroid hormones on the endometrium (Pacifici et al., 1989; Ralston et al., 1990; Tabibzadeh, 1991, 1994; Tabibzadeh and Sun, 1992). In particular, TNF-α and IL-1 increased during the transition from proliferative to secretory endometrium and further increased as menstruation approached (Tabibzadeh et al., 1994). This increase in cytokine production, together with a premenstrual increase of stromal macrophages, may contribute to menstrual disintegration and subsequent regeneration of the endometrium (Morris et al., 1985; Kamat and Isaacsor, 1987; Tabibzadeh and Sun, 1992). However, iNOS expression in the human endometrium with
respect to the phases of the menstrual cycle has not yet been clearly established.

The presence of iNOS compared with eNOS protein and mRNA was evaluated by immunohistochemical staining procedures and reverse transcriptase polymerase chain reaction in order to ascertain whether or not iNOS is detectable in the human endometrium and if its expression depends on the menstrual cycle or age-related changes in endometrial morphology. Our results from within the endometrium were compared with those obtained from within the oviduct and the endocervix uteri. In addition, immunolabelling of NOS isoforms was compared with immunolabelling of lymphoid cells. Biochemical enzyme activity of NOS isoforms was further evaluated by NADPH-diaphorase staining. In addition, the bNOS antigen was sought because a positive NADPH reaction was observed which was associated neither with iNOS nor eNOS antigens.

Materials and methods

Patients

Uteri and their corresponding oviducts (n = 22) were from hysterectomy specimens obtained from patients in their pre- and post-menopausal years. The uteri were removed for benign abnormalities which were not endometrial in origin, including uterine leiomyomas and ovarian lesions. None of these patients was under the influence of hormone replacement therapy. Informed consent was given in each case, and the study was approved by the local ethics committee. Cross-sections of the endometrium, the ampullary segment of the oviduct, and the endocervix were excised within 10 min of clamping the blood flow. Each of the specimens was separated into two smaller specimens for both immunohistochemistry and isolation of RNA. Specimens were frozen immediately in liquid nitrogen and were then transported to the laboratory, where they were stored at −70°C.

Immunohistochemistry and Western blotting analysis

Frozen sections of 6 μm were mounted onto silane-coated slides. The sections were then fixed in formalin, 3.7% for 10 min then washed in Tris-buffered saline (TBS) buffer (5 mM Tris-HCl, pH 7.4, 0.9% NaCl). Thereafter, the slides were incubated in 3% hydrogen peroxide in TBS to block endogenous peroxidase activity. Sections were then blocked with human AB serum (Behring, Marburg, Germany) at 1/4 dilution for 20 min at room temperature. Next, sections were incubated for 1 h at room temperature with a commercially available murine monoclonal antibody raised against amino acid fragment 961–1144 of mouse iNOS at 1/25 dilution, a murine monoclonal antibody raised against amino acid fragment 1030–1209 of human eNOS at 1/100 dilution (both antibodies were from Transduction Laboratories, Lexington, KY, USA) and a rabbit polyclonal antibody raised against amino acid fragment 724–739 of rat brain NOS (bNOS) synthetic peptide at 1/4000 dilution (Biomol, Plymouth, PA, USA). The immunostaining against leukocyte common antigen (LCA) (Bio Genex, San Ramon, CA, USA) was employed at a dilution of 1/40.

Negative controls were applied for all tissue sections by replacement of primary antibodies by appropriately diluted isotype immunoglobulins, namely IgG1 instead of eNOS and LCA antibody, and IgG2a instead of iNOS antibody. As an additional control, polyclonal von Willebrand factor antibody (Bio Genex) was used at 1/200 dilution to visualize vascular endothelium.

A high performance biotin–streptavidin detection system was used for immunodetection (Bio Genex). Aminoethylcarbazol, which forms a reddish brown colour, was utilized as a chromogen. The sections were finally counterstained using Harris haematoxylin and mounted in an aqueous medium.

Specificity of the staining for eNOS was evident from its elimination by preabsorption with the eNOS peptide and the absence of staining with isotype IgG, as was described recently (Dinerman et al., 1994). The specificity of the iNOS antibody was confirmed by Western blot analysis of 15 μg total cell lysate from mouse macrophages (Transduction Laboratories) treated with IFN-γ and lipopolysaccharide (LPS) for 12 h. The lysate was kept at 100°C for 5 min. The whole tissue lysate which contained 15 μg of protein, was subjected to SDS–PAGE (7.5% gradient). The separated proteins were electrophoretically transferred to membranes, then incubated with the iNOS antibody at a 1/500 dilution or the respective isotype control antibody at an equivalent dilution for 1 h. The bound antibody was detected using a chemiluminescent detection kit (ECL Western blotting detection system, Amersham Corp., Arlington Heights, IL, USA) according to the manufacturer’s instructions. Staining specificity for bNOS was evident from its elimination by preabsorption with purified bNOS peptide as described previously (Bredt et al., 1990).

Vascular endothelial cells in the samples, Western blot analysis on the mouse macrophage lysate and tissue sections from human cerebellum and tonsils were used as respective positive controls for the antibodies against eNOS, iNOS, bNOS and LCA peptide (not shown).

Western blot analysis was performed by using the eNOS antibody with mouse macrophage lysate to exclude eNOS antibody cross-reactivity with iNOS. No appropriately sized protein was detectable (data not shown).

An additional control to exclude cross reactivity of the eNOS antibody with bNOS was recently described where eNOS and bNOS immunostaining were performed on human cerebral arteries (Dinerman et al., 1994). eNOS immunostaining was prominent in the endothelial layer of the middle cerebral artery but not in the adventitia, whereas bNOS immunoreactivity was confined to nerve fibres of the adventitia but not vascular endothelium (Dinerman et al., 1994).

Reduced NADPH-diaphorase histochemistry

Sections 6 μm thick were cut from the same tissue blocks as for immunohistochemistry, and were mounted on glass slides. NADPH-diaphorase activity was identified by incubating the slides with 50 mM Tris-buffered saline (pH 7.5) containing 1 mM NADPH (Sigma Chemical Co., St Louis, MO, USA), 0.5 mM nitroblue tetrazolium (NBT), and 0.2% Triton X-100 at 37°C for 30 min as described previously by Hope and Vincent (1989). Control sections were exposed to the staining solution without NADPH.

All the slides were randomized and coded and then examined and assessed independently for immunohistochemical and histochemical staining by four observers (W.T., G.U., K.C. and W.W.).

PCR

Isolation of RNA and reverse transcription

Total RNA was isolated from frozen tissue by using the Tri Reagent method of MRC (Molecular Research Center, Inc., OH, USA) and quantified by measuring the optical density at 260 nm. cDNA was synthesized in a total volume of 25 μl containing a commercially available Random Primed Reverse Transcription Reaction Mix (ViennaLab, Vienna, Austria), 20 units RNasin, 100 units Mu-MLV Reverse Transcriptase (ViennaLab) and 1 μg of a total RNA sample. Reactions were incubated at room temperature for 10 min followed by 50 min at 37°C and 5 min at 95°C.

DNA amplification

All RT-PCR products were designed to span one or more introns. eNOS primers map to exon 3 (3′-primer) and exon 5 (3′-primer),
iNOS primers map to exon 13 (5'-primer) and exon 15 (3'-primer), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers map to exon 3 (5'-primer) and exon 4 (3'-primer) of the respective genomic sequences. This enabled us to check for genomic contamination in the isolated RNA samples. We did not observe any PCR product originating from genomic DNA templates in the samples.

Polymerase chain reactions

These were performed on a Perkin-Elmer GeneAmp PCR System 9600. PCR was carried out in a total volume of 50 µl containing 5 µl cDNA template, 25 pmol of each primer, 250 µM nucleotide triphosphates (dNTPs), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM MgCl₂, 0.1% Triton X-100, and 0.1 units Hi-Taq DNA polymerase (ViennaLab). The amplification profiles were as follows: 94°C for 30 s, 50°C (eNOS- and GAPDH-PCR) or 46°C (iNOS-PCR) for 30 s and 72°C for 45 s; 30 (eNOS PCR) or 35 (iNOS-PCR) or 27 (GAPDH-PCR) amplification cycles. To simplify the performance and to increase the reproducibility of PCR, PCR mastermixes for eNOS as well as for iNOS- and GAPDH-containing primers, dNTPs and buffer were prepared and used in all amplification reactions. In addition, tubes containing all PCR components and distilled water instead of cDNA served as negative controls to check for the presence of DNA that may have been carried over from prior reactions. To ensure that we amplified eNOS, iNOS and GAPDH specific fragments, we sequenced our amplification products and compared them with published sequences (BLAST Similarity Search; National Center for Biological Information; http://www.ncbi.nlm.nih.gov). We found 99% homology with the respective published sequences in all three cases.

PCR product analysis

PCR products were separated by agarose gel electrophoresis (7 µl PCR product: 4% SR Fine Gel Agarose (Severn Biotech Ltd, UK)). Reaction products were visualized by performing the electrophoresis on SYBR Green I (Molecular Probes Inc., WI, USA) containing gels. Quantitative analysis was performed by densitometric scanning of the gels using a CCD Video Camera System and the Bioimage Whole Band Analyzer software (Bioimage Inc.). Semi-quantitative eNOS and iNOS mRNA expression levels were determined by calculating the eNOS/GAPDH and iNOS/GAPDH ratios.

Results

Cellular localization of NOS isoforms

Endometria were dated with respect to both histopathological criteria according to Noyes and Hertig (1950) and the patients’ testimony as described in Table I. Endometria, oviducts and endocervices used in this study showed normal morphology.

The 22 tissue blocks studied showed the following typical staining characteristics agreed upon by all observers (W.T., G.U., K.C. and W.W.).

iNOS

Immunolocalization of iNOS was confined to the endometrial luminal and glandular epithelium with no difference in staining intensity between the functional and basal layers throughout all sections. However, the intensity of staining in epithelial cells was different with respect to both menstrual cycle-related and age-related changes in endometrial morphology and was semi-quantitatively scored on a three point scale as described (Table I). The immunostaining for iNOS was weak or not detectable in the proliferative (Figure 1) and inactive endometrium (Figure 3A). In the secretory phase, however, epithelial immunoreactivity for iNOS was strong (Figure 2A), with the exception of one patient, a 42 year old woman with early secretory phase endometrium, who underwent an operation for adenomyosis uteri. This specimen expressed rather weak iNOS immunoreactivity [‘(+)’ in Table I]. The staining pattern within the glands was uniform: all the cells were positively stained. Staining was distributed evenly within the cytoplasm and was occasionally granular and perinuclear. The stroma remained uniformly negative. No reaction product was present in control sections on substitution of the primary antibody with the appropriately diluted isotype antibody (Figure 2B).

iNOS-like immunoreactivity was weak and only diffusely scattered in epithelial cells lining the ampullary segment of the oviducts, and in cases of weak immunoreactivity there was no relationship to the secretory endometrium. No immunoreactivity was observed in the underlying stroma containing the mucosal blood and lymph vessels (Figure 4A). Only little

| Table I. Summary of the immunohistochemical experiments identifying iNOS protein in endometrial epithelial cells |
|-----------------------------------------------|-----------------|---------------|
| Intensity of staining                        | Proliferative endometrium | Secretory endometrium | Inactive endometrium |
| VIT /+/-+/-                                 | PRC (+)(+)(+)+     | KUB --/--/--     |
| KAL -/-/-/-                                 | SCH +/+ + + + + +  | KLE --/--/--     |
| HOL -/-/-/-                                 | SEI + + + + + + + + | HAG --/--/--     |
| MUE -/+ + /+                                  | DJO ++ + + + + + + | SAD +/+ + + + |
| SPI -/+ + +                                  | MOT + /+ + + + + + | MAY +/+ + + + |
| CAS -/+ + +                                  | WAN -/+ + + + + + + | NOV +/+ + + + |
| SUZ + + + +                                  | WIE + + + + + + +   |               |
| TIM + + + +                                  | BRE + + + + + + +   |               |

Designations of -- (no staining), + (moderate staining) and ++ (strong staining) indicate the relative intensities of the immunostaining for iNOS protein. Variability between observers is indicated with a slash mark. Based on the patient’s testimony, 10 were in the proliferative phase of the cycle, one (PRC) was in the early-secretory phase (day 16), one (SCH) was in the mid-secretory phase (day 20) and four (SEI, DJO, MOT, WAB) were in the late secretory phase (days 25, 26, 28, 28). Six patients were postmenopausal, as additionally evaluated by serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH) and oestradiol.

Figure 1. Proliferative endometrium: iNOS immunostaining; no immunoreactivity in any of the cells (original magnification ×200).
Figure 2. Late secretory endometrium: (A) iNOS immunostaining: strong iNOS immunoreactivity is confined to the cytoplasm of endometrial epithelial cells; (B) negative control for iNOS immunostaining using IgG2a isotype immunoglobulin; (C) eNOS immunostaining: positive staining in endothelial cells, but lack of any staining in epithelial and stromal cells. Endothelial cells are photographed in a longitudinal (arrows) or transversal direction (arrowheads); (D) bNOS immunostaining: no staining in any of the cells; (E) LCA immunostaining: abundance of LCA labelled cells in the stroma which, in part, appear to indent the epithelium (arrows); (F, G) NADPH-diaphorase histochemistry: positive staining in the luminal and abluminal compartments of the endometrial glands’ cytoplasm (arrows) and vascular endothelial cells exhibiting their typical face to face morphology (arrowheads) (A–F: original magnification ×200, G: original magnification ×1000).
Inactive endometrium: (A) iNOS immunostaining: only a few, weakly labelled cells are visible in the epithelium (arrows); (B) LCA immunostaining: scarce positive cells in the stroma (arrows) (original magnification ×200).

Figure 3. Inactive endometrium: (A) iNOS immunostaining: only a few, weakly labelled cells are visible in the epithelium (arrows); (B) LCA immunostaining: scarce positive cells in the stroma (arrows) (original magnification ×200).

Cervix: iNOS immunostaining (original magnification ×200).

Figure 5. Cervix: iNOS immunostaining (original magnification ×200).

(not shown) or no (Figure 5) iNOS-like immunoreactivity was detectable in specimens of glandular epithelium within the endocervix uteri and underlying stroma.

Specificity of the monoclonal iNOS antibody was confirmed in Western blots by detecting a protein of approximately 130 kDa corresponding to the size expected for the iNOS antigen (data not shown). No specific reaction was observed when an isotype-matched control antibody was used instead of the anti-iNOS reagent (data not shown).

eNOS eNOS like-immunoreactivity was uniformly restricted to the endometrial (Figure 2C), oviductal, and endocervical (data not shown) endothelial cells, lining the respective blood vessels. Endothelial cells were strongly stained throughout, irrespective of the phase of the menstrual cycle or age (not shown). Epithelial cells and non-endothelial cells of the stroma remained negative, irrespective of their anatomical location. Vascularization is more abundant in secretory than in proliferative or inactive endometrium. Detection of von Willebrand factor antigenicity in adjacent sections permitted identification of endothelial cells (data not shown).

No specific immunostaining was detectable when the respective isotype controls were performed (data not shown).

bNOS bNOS immunoreactivity was weak in epithelial cells (Figure 2D), whereas cells of the underlying stroma remained negative.

LCA LCA labelled cells (leukocytes, lymphoid cells, and macrophages), scarce during the proliferative (not shown) and inactive (Figure 3B) phase and used as an internal positive control, were abundant in the stroma of the secretory phase (Figure 2E). Here, these cells were distributed either scattered
lymphoid cells within the stroma and modulate the functions in all the cases studied, the amplified product for GAPDH was positive control in all RT-PCR reactions. In all reactions, and paracrine acting cytokines. These cytokines are derived from endometrium are indirectly mediated, at least in part, by 3-phosphate dehydrogenase (GAPDH) mRNA served as a amplification of the constitutively expressed glyceraldehyde-
actions on Steroid was already stated by Telfer due to the different sensitivities of these two techniques, as results obtained with RT-PCR and Northern blots might be PCR Non-specific products were not observed in any of the RT­ eDNA, followed by amplification of the respective eDNA specimens by the reverse transcription of their mRNA to eNOS is reduced to the water-insoluble dye NBT formazan. The reaction product was identified in endometrial, luminal, and glandular epithelium (Figure 2F, G). Strong staining was observed in glandular epithelial cells of the endometrium, irrespective of the phase of menstrual cycle or age. Only weak staining was observed in the stroma. This weak histochemical staining pattern could be interpreted to be non-specific, considering that all known NOS isoforms represent only a fraction of the total cellular NADPH-diaphorase activity (Tracey et al., 1993). Moreover, in the stroma no corresponding specific NOS immunoreactivity was detectable. In the epithelium lining the oviduct (Figure 4B), and in the endocervical glands staining was only weak (data not shown). In addition, reaction in vascular endothelial cells was widespread throughout all specimens without any exception, but was not as strong as reaction in epithelial cells (Figure 2F). Staining was not detectable in control sections without any NADPH (not shown).

Demonstration of NOS gene expression

iNOS and eNOS gene expression was demonstrated in all specimens by the reverse transcription of their mRNA to cDNA, followed by amplification of the respective cDNA fragments, using iNOS- and eNOS-specific primers (Table II). 

Amplicons of both NOS products were observed in all cases studied. Amplified products from RT-PCR reactions for the eNOS and iNOS isoforms were of the expected lengths (Table II, Figure 6) (Charles et al., 1993; Miyahara et al., 1994). Non-specific products were not observed in any of the RT-PCR reactions (Figure 6). Relative mRNA expression levels were determined as described and summarized in Table III. The amplification of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as a positive control in all RT-PCR reactions. In all reactions, and in all the cases studied, the amplified product for GAPDH was observed in nearly equal amounts and thereby permits semi-quantitative assessment of iNOS and eNOS mRNA (Figure 6). In addition, amplified products were not observed in PCR negative control reactions, where all the PCR components were used except for the addition of the RT reaction mixture.

Discussion

The question addressed by the present study was concerned with the expression of iNOS in the human endometrium and the determination of a probable dependency of this isof orm of NOS on the menstrual cycle.

iNOS protein and gene expression was particularly strong in secretory endometrium but only weak or absent in proliferative and inactive endometrium. The pattern of iNOS labelling in secretory endometrium was also correlated with an increasing number of lymphoid cells in the respective stroma.

Localization of iNOS protein and mRNA to endometrial epithelia is further supported by two recent reports (Tseng et al., 1996; Telfer et al., 1997). However, whereas RT-PCR data obtained here and reported by Telfer et al. (1997) consistently match, no clear relationship was demonstrable between iNOS protein expression and the stage of the menstrual cycle (Telfer et al., 1997). Since the latter investigators utilized polyclonal iNOS antibodies, this could reflect the discrepant results on iNOS protein expression obtained here and by Telfer et al. (1997). Additionally, Tseng et al. (1996) detected iNOS mRNA only in glandular epithelia derived from menstrual endometria by means of Northern blot analysis. The different results obtained with RT-PCR and Northern blots might be due to the different sensitivities of these two techniques, as was already stated by Telfer et al. (1997). Steroid actions on endometrium are indirectly mediated, at least in part, by paracrine acting cytokines. These cytokines are derived from lymphoid cells within the stroma and modulate the functions.

Table II. The primer sequences and PCR product size

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Product size</th>
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<tbody>
<tr>
<td>eNOS-5'</td>
<td>GGACTTCTACCAACCAGTAC</td>
<td>250 bp</td>
</tr>
<tr>
<td>eNOS-3'</td>
<td>GATGTAGGTTGAAACATTCCC</td>
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</tr>
<tr>
<td>iNOS-5'</td>
<td>CCATTGAAGTCTTTGTC</td>
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</tr>
<tr>
<td>iNOS-3'</td>
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</tr>
<tr>
<td>GAPDH-5'</td>
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<tr>
<td>GAPDH-3'</td>
<td>GATGGTTAGGGATTCCC</td>
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This paraffin-embedded immunohistochemistry or absence endometria, secretory in by reactivity eNOS for sensitivity a higher to contrast. In studies. require and may be lacking results on the release nitriteinitrate or eNOS on NOS on endothelial in the process cytokines (Tabibzadeh and Sun, 1992). In particular, TNF-α and IL-1, which also have been reported to be strong inducers of iNOS (Nathan, 1992), show an increased expression in the secretory endometrium when menstruation approaches (Hunt et al., 1992; Philippeaux and Piguet, 1993; Tabibzadeh et al., 1994), and theoretically may contribute to necrosis within endometrial glands resulting in menstrual shedding (Tabibzadeh et al., 1994). In airway epithelia, NO synthesis may result in detachment of epithelial cells in the asthmatic state (Montfort et al., 1992) because of the known cytotoxic effects of NO (Mulligan et al., 1991). Additionally, in menstrual endometrium iNOS mRNA concentrations were shown to be increased compared with the other stages of the menstrual cycle (Tseng et al., 1996). Based on these data, it is tempting to speculate that iNOS-derived NO may also exert its cytotoxic effects on endometria, thereby leading, at least in part, to the physiological process of menstrual disintegration. However, this is still a matter for speculation, since data on NO activity or nitrate/nitrite release in secretory endometria are still lacking and will therefore require further studies. In contrast to the strong iNOS expression in secretory endometria, absence or only weak iNOS expression together with the absence of lymphoid cells within inactive endometria may reflect lack of endometrium exposure to sex steroid hormones in the post-reproductive period of life.

eNOS protein was visible in vascular endothelial cells, but was absent from any of the other cells in all specimens studied. Herein, our results are consistent with those obtained by Telfer et al. (1995), where eNOS protein was located mainly in the stroma. However, our results further support those obtained from Telfer et al. by demonstrating the localization of eNOS protein in stromal endothelial but not non-vascular stromal cells. A more recent study by Telfer et al. (1997) revealed the presence of eNOS immunoreactivity in endothelial, myometrial and endometrial cells in paraffin-embedded sections obtained from hysterectomy specimens. However, though not shown in that paper, the authors state that eNOS immunoreactivity on additional cryosections was present in vascular endothelium and glandular epithelium, albeit staining intensity was less extensive within the cytoplasm of individual epithelial cells (Telfer et al., 1997). That latter finding is strongly consistent with our results on cryosections that revealed eNOS immunoreactivity in vascular endothelial cells and the absence of eNOS immunoreactivity from epithelial cells, but in turn makes the results on paraffin sections obtained by Telfer et al. (1997) more difficult to interpret. The different results on eNOS staining obtained by using either paraffin- or cryosections may suggest a higher sensitivity for eNOS reactivity by performing immunohistochemistry on paraffin-embedded sections. This

Table III. Summary of eNOS and iNOS RT-PCR experiments showing the relative mRNA concentrations of both isoforms

<table>
<thead>
<tr>
<th>eNOS</th>
<th>Proliferative phase</th>
<th>Secretory phase</th>
<th>Inactive phase</th>
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iNOS

<table>
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<tr>
<th>Proliferative phase</th>
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Relative mRNA expression levels were determined by calculating band intensity ratios of eNOS/GAPDH and iNOS/GAPDH. − indicates low (0–0.5), + indicates moderate (0.5–2) and ++ indicates strong (2–7) expression levels. (A = endocervix; B = endometrium; C = oviduct).
could result from additional antigen moieties unmasked by microwave treatment. However, the synthesis of NO might be important in maintaining myometrial quiescence during pregnancy (Norman and Cameron, 1996; Bansal et al., 1997). Moreover, the increase of calcium-dependent NOS activity in the pregnant myometrium correlated negatively with myometrial contractility (Natuzzi et al., 1993; Yallamalli et al., 1993). In the light of these previous findings, constitutive eNOS expression at very low levels in the myometrial and endometrial cells of the non-pregnant uterus might reflect basal levels that can be activated by agonists when pregnancy occurs. Moderate increase of eNOS gene expression in the endometrium compared with the oviduct and the endocervix may reflect growth of the arterial system in the endometrium, and parallels an increase in DNA synthesis in the vascular endothelial cells (Ferency z et al., 1979).

The pattern of NADPH diaphorase activity that has been shown to co-localize with all known NOS isoforms is similar to that recently reported in human uteri, where the enzyme was localized in small blood vessels and endometrial epithelium (Telfer et al., 1995). In accordance with this previously published report, the intensity of staining in our sections did not vary with respect to the phase of the menstrual cycle. Since iNOS or eNOS expression was almost or completely absent from proliferative or inactive endometrial epithelial cells, staining for NADPH-diaphorase in both proliferative and inactive endometria may either identify sites where NO can also be synthesized by the action of the bNOS isoform of NOS, or otherwise may represent a NOS-independent fraction of NADPH-diaphorase activity (Tracey et al., 1993), but this remains to be further clarified. However, our results on bNOS expression throughout all endometrial epithelia confirm a previous report, demonstrating the presence of NADPH-diaphorase positive nerve fibres in rat endometrium (Shew et al., 1993). Thus NO, possibly synthesized by the action of bNOS, was suggested to act as a effector molecule of myometrial contractility (Shew et al., 1993).

Our further results obtained from within human oviducts indicate only a weak and diffusely scattered distribution of iNOS protein in epithelial cells. eNOS protein was confined exclusively to vascular endothelial cells. Thus our results suggest that neither iNOS nor eNOS expression is regulated in the human oviduct epithelium under non-pathological conditions. By contrast, we found weak but consistent bNOS immunostaining in epithelial cells throughout all oviductal specimens. These findings support the hypothesis of Rosselli et al., suggesting that the basal synthesis of NO within the oviduct may play a vital role as a local relaxing factor that may contribute to the rhythmic contraction of the oviduct, essential for regulating the transport of gametes and embryos (Rosselli et al., 1996).

Our findings demonstrating lack of iNOS or eNOS immuno-reactivity, and only moderate bNOS immunoreactivity in the glands of the human endocervix under non-pathological conditions, are applicable to the oviduct, and consistent with the additional observation demonstrating the paucity of lymphoid cells in the underlying submucosa. These data are pertinent to previous findings that indicate only moderate numbers of lymphoid cells in the submucosa of the rhesus monkey endocervix (Miller et al., 1992). Our findings of bNOS expression in the endocervical epithelial cells confirm a previous report demonstrating NADPH-diaphorase positive nerve fibres in the epithelial layer of the rat endocervix and suggest that NO could influence functions of the uterine cervix in the reproductive process (Papka and McNeill, 1992).

In conclusion, this study substantiates mRNA and protein expression of inducible nitric oxide synthase, being induced definitively in secretory glandular epithelial cells of the human endometrium under non-pathological conditions. This may suggest a possible role of inducibly released nitric oxide, as a result of, or together with, cytokine expression in the process of menstrual disintegration of the endometrium.

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