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Role of nitric oxide in implantation and menstruation

Kristof Chwalisz[1,3] and Robert E.Garfield[2]

[1]Jenapharm GmbH & Co. KG, Jena, Germany and [2]University of Texas Medical Branch, Galveston, Texas, USA

[3]To whom correspondence should be addressed at: Women's Health Care, Jenapharm GmbH & Co. KG, Otto-Schott-Strasse 15, 07745 Jena, Germany.
E-mail: Kristof.Chwalisz@Jenapharm.DE

Nitric oxide (NO) is a major paracrine mediator of various biological processes, including vascular functions and inflammation. In blood vessels, NO is produced by the low-input constitutive endothelial NO synthase (eNOS) and is a potent vasodilator and platelet aggregation inhibitor. The inducible NOS isoform (iNOS) is capable of producing NO at high concentrations which have pro-inflammatory properties. Immunohistochemical and molecular studies of endometrial NOS expression, as well as animal experiments with NOS inhibitors, indicate that NO plays an important role in endometrial functions such as endometrial receptivity, implantation and menstruation. In rodents, both iNOS and eNOS are highly up-regulated in the implantation sites, and NOS inhibitors show synergistic effects with antiprogestins in inhibiting the establishment of pregnancy. In the human endometrium, eNOS have been localized in the glandular epithelium and in endometrial microvascular endothelium, primarily during the luteal phase. iNOS has been found in the endometrial epithelium during menstruation, in immunocompetent endometrial cells, and in decidualized stromal cells. In primates, NO may be involved in the initiation and maintenance of menstrual bleeding by inducing tissue breakdown and vascular relaxation as well as by inhibiting platelet aggregation. Endometrium-derived NO may also play a role in myometrial relaxation during menstruation. These studies open up new applications for NO-donating and -inhibiting agents in uterine disorders. NO donors may be useful in the treatment of dysmenorrhoea and for promoting fertility. Antiprogestins, progesterone receptor modulators and iNOS inhibitors may find applications in the treatment and prevention of abnormal uterine bleeding.

Key words: antiprogestins/dysfunctional bleeding/implantation/nitric oxide/progesterone

Introduction

The primate endometrium is a highly specialized organ composed of different cell components, including luminal and glandular epithelium, endometrial stroma, lymphoid and non-lymphoid cells and vessels. During the reproductive age, the entire endometrium undergoes cyclic changes in order to assure optimum conditions for implantation. These changes are closely controlled by the ovarian hormones 17β-oestradiol and progesterone, which regulate, directly or indirectly, endometrial proliferation and differentiation as well as endometrial angiogenesis. If implantation does not occur, the upper part of the endometrium, the functionalis, is shed during menstruation in response to progesterone withdrawal at the end of the luteal phase. This process is accompanied by leukocyte subpopulations infiltrating the endometrium, the dissolution of the extracellular matrix, epithelial apoptosis, and dramatic vascular changes similar to inflammation (Finn, 1986). The endometrium represents a unique

tissue with regard to vascular changes during the cycle. Cyclic changes of the endometrial blood vessels such as their damage during menstruation, neovascularization and an increase in the growth and number of blood vessels occur within a single month. During implantation, the endometrial stroma undergoes a dramatic proliferation and differentiation into a well-vascularized decidual tissue in order to provide an optimum environment for the embryo. This process is accompanied by a substantial remodelling of the extracellular matrix, intensive blood vessel growth and dilatation, similar to inflammation. However, the exact regulation of endometrial blood vessels during the menstrual cycle and implantation still remains poorly understood.

Steroid hormones may act directly on both stromal and epithelial cells, as well as on endometrial vessels (spiral arterioles) during the proliferative and luteal phases of the menstrual cycle. However, many steroid hormone effects—particularly during menstruation and implantation—are mediated by local factors, including growth factors, cytokines, chemokines, and vasoactive agents such as prostaglandins (PG), endothelin (ET), vascular endothelial growth factor (VEGF), platelet-activating factor (PAF) and nitric oxide (NO). Since NO relaxes the myometrial and vascular smooth muscles, inhibits platelet aggregation, and plays a role in inflammation, it is a strong candidate for mediating various steroid hormone and cytokine effects in the endometrium and decidua. In this brief review we aim to discuss the role of NO in controlling such endometrial functions as menstruation and implantation. We will also address the potential involvement of NO during pathological conditions such as dysfunctional uterine bleeding and dysmenorrhoea.

The nitric oxide generating system

The discovery of an endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980), and its later identification as NO, have to be considered as one of the most exciting discoveries in medicine in the 1980s. NO is a small uncharged gas molecule that is a highly reactive free radical. Although NO has an extremely short half-life *in vivo* of ~4 s, it can penetrate the surrounding tissues and can activate a variety of targets, including enzymes such as guanylate cyclase, cytochrome P450 enzymes, protein kinases and phosphatases, as well as other cellular signalling pathways. Consequently, NO functions as a major mediator of numerous biological processes, including vascular homeostasis, smooth muscle relaxation and neurotransmission. NO has also been shown to inhibit platelet aggregation, stimulate angiogenesis, reduce blood pressure, and alter the function of the immune system (Ignarro, 1989; Moncada and Higgs, 1993).

NO was recently implicated as an important regulatory agent in various female reproductive processes, such as ovulation, implantation, pregnancy maintenance, labour and delivery (reviewed by Chwalisz *et al.*, 1996; Rosselli, 1997; Cameron and Campbell, 1998). Animal studies clearly indicate that during pregnancy, NO is up-regulated in the myometrium and placenta. It contributes to uterine quiescence and controls utero-feto-placental blood flow (Izumi *et al.*, 1993; Sladek *et al.*, 1993; Buhimschi *et al.*, 1996; Liao *et al.*, 1997). NO is also involved in cervical ripening during labour (Chwalisz *et al.*, 1997; Chwalisz and Garfield, 1998). Moreover, these studies also indicate that the regulation of NO production in the female reproductive tract is mainly controlled by steroid hormones in a tissue-specific manner.

Nitric oxide synthases and their functions

NO is synthesized by a family of nitric oxide synthases (NOS), which are enzymes that convert the amino acid L-arginine to citrulline and NO. Thus far, three related NOS enzymes whose genes are localized on different chromosomes have been isolated and characterized. These include neuronal constitutive NOS (nNOS, b-NOS, type I), inducible NOS (iNOS, type II), and endothelial constitutive NOS (eNOS, type III) (Nathan and Qia-wen, 1994). The constitutive isoforms eNOS and nNOS were originally identified in endothelial and neuronal tissues, respectively. They require calcium/calmodulin for activation, and can rapidly and transiently produce small amounts of NO under basal conditions. The nNOS was the first NOS isoform to be isolated and studied at the molecular level (Huang *et al.*, 1993). It can be found in neuronal tissues

of the central and peripheral nervous systems, and is thought to act as a neurotransmitter. The eNOS-derived NO is the most important vasodilator and platelet aggregation inhibitor. It maintains a constant vasorelaxation, normal blood pressure and adequate tissue perfusion (Huang *et al.*, 1995a).

The iNOS isoform can be induced by either interleukins (IL-1, IL-2, IL-12), tumour necrosis factor alpha (TNF-α), interferon gamma (IF-γ) or endotoxin (LPS), and produces large quantities of NO in a calcium-independent manner. NO production under the influence of iNOS occurs with a delay of 6–8 h after stimulation. However, once induced, iNOS is active for hours or even days and produces NO in 1000-fold larger quantities than the constitutive isoforms (Moncada and Higgs, 1993; Beck *et al.*, 1999). iNOS was first identified in immune cells (macrophages), but has meanwhile also been discovered in other cells, including epithelial cells, hepatocytes, myocytes, fibroblasts, chondrocytes and bone-forming cells (osteoblasts and osteoclasts). This system also plays a pivotal role in connective tissue remodelling during acute and chronic inflammation. The binding of the nuclear factor κB (NF-κB) to DNA seems to be essential for iNOS expression by pro-inflammatory cytokines (Eberhard *et al.*, 1998). Agents inhibiting NF-κB activation or binding have been shown to diminish the cytokine-stimulated iNOS production. Large quantities of iNOS-derived NO can kill parasites and bacteria, and it has been postulated that this pathway is the most significant in controlling the invasion of pathogens (MacMicking *et al.*, 1995).

Molecular targets of NO actions

Guanylate cyclase, which triggers the formation of cyclic guanosine monophosphate (cGMP), seems to be the most relevant target of NO at low concentrations. cGMP is an important cellular messenger regulating intracellular calcium concentrations which mediate physiological functions of NO such as smooth muscle relaxation and platelet aggregation. The cGMP increase activates, in turn, at least three critically important signalling pathways, including: (i) cGMP-regulated ion channels; (ii) cGMP-regulated protein kinases; and (iii) cGMP-dependent protein kinases and phosphatases (reviewed by Beck *et al.*, 1999). It has been shown recently that NO activates the mitogen-activated protein kinase (MAPK) cascades, the key transduction mechanisms of growth factors and cytokines involved in cellular proliferation and differentiation (Lander *et al.*, 1996; Beck *et al.*, 1999).

There is increasing evidence that NO can directly regulate gene expression by modulating the activity of transcription factors such as NF-κB and the activator protein 1 (AP-1) (Sen and Packer, 1996). Since NF-κB inhibits progesterone receptor (PR) action via protein–protein interaction (Kalkhoven *et al.*, 1996), NO may, therefore, modulate progesterone responses in the reproductive tract. The list of genes under the regulatory control of NO is expanding, including those involved in extracellular matrix protein synthesis and their degrading enzymes (Chatziantoniou *et al.*, 1998; Sasaki *et al.*, 1998), cytokines and chemokines, such as interleukin 8 (IL-8) (Villarete and Remick, 1995). Higher concentrations of NO produced by iNOS may also directly interact with various metal-containing proteins, including matrix metalloproteinases (MMP). Indeed, NO has been shown to stimulate gelatinase activity in rat mesangial cells (Trachtman *et al.*, 1996). Similarly, in articular chondrocytes, IL-1β-stimulated MMP-9 (92 kDa metalloproteinase) expression is mediated by NO (Tamora *et al.*, 1996; Sasaki *et al.*, 1998).

NO can interact with other vasoactive and angiogenic agents, including PG, ET, VEGF and PAF. It was discovered in various inflammation models that NO is a powerful inducer of the inducible cyclooxygenase 2 (COX-2), and elevates local PGE_2 and PGI_2 concentrations in inflamed tissues, while the inhibitors of NO synthesis block PG production during chronic and acute inflammation (Salvemini *et al.*, 1995). On the other hand, ET after binding to ET_B receptors was shown to be a potent stimulus of NO release from the vascular endothelium, suggesting the existence of a feedback mechanism between these two molecules of opposing functions (Takayanagi *et al.*, 1991). Since the endometrium is a well-known source of PG, and ET_B receptors are expressed in the glandular epithelium (Collet *et al.*, 1996), interaction between NO, PG and ET may well be of physiological importance. NO is also involved in angiogenesis

by modulating VEGF expression. It has recently been demonstrated that a VEGF increase in response to endothelial injury is inhibited by NO (Tsurumi *et al.*, 1997), a mechanism which may represent a negative feed-back loop between NO and VEGF during angiogenesis. On the other hand, VEGF stimulates NO release from endothelial cells, a mechanism contributing to the vascular effects of VEGF (Papapetropoulos *et al.*, 1997). In the endometrium, NO may also interact with PAF, a potent inflammatory lipid mediator. PAF is known to increase vascular permeability and vasodilatation in various tissues — effects which may be, at least in part, mediated by NO. PAF receptor (PAF-R) immunoreactivity and mRNA were detected in proliferative and secretory endometrium (Ahmed *et al.*, 1998). This study also demonstrates that endometrial explants release NO in response to physiological concentrations of PAF. Interestingly, the maximum NO release was observed in endometrial tissues obtained during the late luteal phase.

Finally, at high concentrations NO plays a role in apoptotic cell death. An increased apoptosis following exogenous application of NO donors or iNOS induction has been described in different cell types, such as macrophages and mesangial cells (Brüne *et al.*, 1998). NO-induced apoptosis was accompanied by the accumulation of the tumour suppressor protein p53 and activation of caspases (Meßmer and Brüne, 1996).

In summary, low eNOS- and nNOS-produced concentrations of NO are involved in maintaining basic physiological functions such as vascular homeostasis and neurotransmission, and generally exhibit anti-inflammatory effects. Conversely, high concentrations of NO, i.e. iNOS-derived, mediate various pro-inflammatory responses, including extracellular matrix remodelling, apoptotic cell death and tissue destruction.

NOS regulation by steroid hormones

It has been postulated that oestrogen is the chief hormonal factor regulating NO production in the vasculature and reproductive tract. Indeed, numerous experimental and clinical studies indicate that oestrogens stimulate vascular NO production by up-regulating eNOS and/or inhibiting superoxide anion production (Van Buren *et al.*, 1992; Weiner *et al.*, 1994; Hishikawa *et al.*, 1995). In fact, the protective effects of oestrogen replacement therapy on cardiovascular disease are attributed to the up-regulation of NO production in the endothelial cells (Collins, 1996). On the other hand, oestrogen was shown to inhibit iNOS synthesis in rat vascular smooth muscle cells via oestrogen receptor activation (Zancan *et al.*, 1999). This finding may indicate a novel anti-inflammatory mechanism of a protective oestrogen effect on cardiovascular disease by preventing excessive NO production during atherosclerosis. Oestrogen treatment is also thought to elevate NO production rapidly in various tissues of the female reproductive tract, such as the uterus (Van Buren *et al.*, 1992; Weiner *et al.*, 1994). In the latter, the oestrogen effects on NOS synthesis seem to be tissue-specific. Oestrogen administered to ovariectomized sheep up-regulated eNOS in the myometrium, but not in the endometrium (Figueroa and Massman, 1995). In addition NO mediates, at least in part, the oestrogen-induced uterine oedema—an acute reaction to oestrogen treatment in rodents (Chaves *et al.*, 1993).

Previous experimental and clinical studies of myometrial, cervical and endometrial NOS expression, as well as our own functional studies with antiprogestins, clearly indicate however that progesterone also regulates NO production and NOS expression. During pregnancy, when both oestrogen and progesterone blood concentrations are elevated, progesterone may in fact be the chief hormonal factor regulating NO production in the female reproductive tract. Our previous studies in rats have shown that iNOS is the dominant NOS isoform in the pregnant uterus, cervix and placenta (Buhimschi *et al.*, 1996; Ali *et al.*, 1997; Purcell *et al.*, 1997). In pregnant rats, uterine, cervical and placental iNOS expression is gestationally regulated at protein and mRNA levels, with progesterone as the key physiological regulating agent. These results are consistent with studies in mice. In this species combined oestrogen and progesterone treatment has elevated both eNOS myometrial expression and iNOS expression in the endometrial glands, the stroma and myometrium compared to treatment with oestrogen alone (Huang *et al.*, 1995b). The effects of progesterone on uterine and

cervical iNOS expression are, however, tissue-dependent. Experimental studies with antiprogestins in rats indicate that in the uterus and placenta progesterone up-regulates iNOS expression and NO production, while exerting opposite effects in the cervix (Buhimschi *et al.*, 1996; Chwalisz *et al.*, 1996; Ali *et al.*, 1997).

The role of progesterone in regulating NO production in the primate uterus has been poorly investigated to date. However, studies of eNOS and iNOS expression in both the human and baboon endometrium and decidua, discussed below, suggest that progesterone may also up-regulate uterine NO production in primates.

NOS in the endometrium

Animal studies

In the initial investigations, nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity was employed to study NOS localization in the rat endometrium (Schmidt *et al.*, 1992; Shew *et al.*, 1993). This method can, however, detect other enzymes besides NOS. More recently, NOS localization in the endometrium was examined via both NADPH-diaphorase and immunohistochemistry. In rats, both diaphorase activity and nNOS-like immunoreactivity was found in uterine nerves, whereas iNOS staining was positive in macrophage-like cells in the vicinity of the uterine lumen (Suburo *et al.*, 1995). In the endometrium of mice, iNOS immunoreactivity could be observed in uterine epithelial cells, macrophage-like stromal cells, mast cells and in myometrial cells (Huang *et al.*, 1995b). The latter study also indicates a differential regulation of iNOS expression in uterine mast and epithelial cells by ovarian steroids. After progesterone treatment, iNOS staining was found in endometrial epithelial cells but not in mast cells, whereas the reverse occurred after oestradiol treatment. In mice and baboons, dramatic changes in the pattern of eNOS and iNOS expression were observed at peri-implantation. These studies are discussed below.

Human endometrium

A number of recent studies have identified both eNOS and iNOS in the human endometrium (Telfer *et al.*, 1995, 1997; Tseng *et al.*, 1996; Ota *et al.*, 1998; Tschugguel *et al.*, 1998, 1999; Khorram *et al.*, 1999; Taguchi *et al.*, 2000). The results of these investigations—particularly those addressing steroid hormone regulation — are somewhat divergent, perhaps due to various techniques of different sensitivities used in these studies.

Initial studies of NOS activity were carried out in premenopausal women via NADPH-diaphorase and Northern blot analysis (Tseng *et al.*, 1996). There was an increased NADPH-diaphorase activity in epithelial cells and blood vessels during the secretory phase of the menstrual cycle. Northern blot analysis revealed the presence of both eNOS and iNOS mRNA in epithelial cells. In Tseng's study an increased expression of eNOS mRNA was found in glandular epithelial cells obtained from the late secretory phase, whereas iNOS mRNA was only observed in the glandular epithelium obtained during menstruation. Subsequently, the presence of iNOS and eNOS was confirmed in endometrial glandular epithelial cells by applying immunohistochemistry and reverse transcriptase–polymerase chain reaction (RT–PCR) (Telfer *et al.*, 1997). However, contrary to the observations by Tseng *et al.*, the study of Telfer and colleagues did not reveal any cycle-dependent NOS variations, nor any correlation between NOS expression and menstrual blood loss. In addition, in this study neither eNOS nor iNOS immunostaining could be detected in stromal cells during the menstrual cycle of non-pregnant women. Further studies employing NADPH-diaphorase and iNOS and eNOS immunohistochemistry (Tschugguel *et al.*, 1998) reported the presence of iNOS immunoreactivity exclusively in the secretory endometrium, whereas eNOS immunoreactivity could be detected only in endothelial cells.

The results of more recent studies provide further evidence suggesting that eNOS may be a dominant isoform in the non-pregnant human endometrium. Both iNOS and eNOS expression were studied (Western blot analysis and immunohistochemistry) in the endometrium and myometrium of premenopausal and postmenopausal women undergoing hysterectomy for benign gynaecological reasons (Khorram *et al.*, 1999). This study clearly revealed eNOS to be predominant in the human uterus. eNOS immunostaining was localized primarily in

the glandular epithelium and endometrial microvascular endothelium, whereas iNOS labelling was detectable only in occasional specimens obtained during the secretory phase. Western immunoblot analysis, however, showed a differential menstrual cycle-dependent eNOS expression in the endometrium and myometrium. The secretory endometrium revealed a greater eNOS expression then the proliferative endometrium. The eNOS expression pattern in the myometrium was reversed, with the up-regulation of eNOS during the proliferative phase. In postmenopausal women, hormone replacement therapy (HRT) produced an increased expression of eNOS in both the endometrium and myometrium. Similar results were obtained later when cycle-dependent eNOS immunostaining on cryostat endometrial sections was studied (Taguchi *et al.*, 2000). Immunoreactivity for eNOS was clearly localized in various types of arterial and endothelial cells, as well as in the capillaries. Furthermore, in some samples there was positive eNOS staining in endometrial glandular epithelium. Interestingly, the number of vessels which were positively stained for eNOS increased gradually during the proliferative phase — indeed, most of the vessels were positive in the early secretory phase.

There is growing evidence, however, indicating that iNOS turns into a dominant NOS isoform at peri-menstruation and during early pregnancy. A very elegant study recently showed a six-fold increase of iNOS activity in endometrial samples obtained during menstruation compared with those from the proliferative or secretory phases, whereas the constitutive NOS enzymes remained unchanged (Tschugguel *et al.*, 1999). In this study, a cRT–PCR reaction revealed an increase in endometrial iNOS mRNA expression during the secretory phase and during menstruation, contrary to the proliferative phase. Immunohistochemistry demonstrated intense iNOS immunostaining in epithelial cells during menstruation. The authors concluded that iNOS is highly elevated in the human endometrium during menstruation and plays a role in the signal transduction mechanisms, leading to endometrial breakdown and apoptosis. An increased iNOS immunoreactivity was previously found in decidual cells obtained from early pregnant women and those exposed to synthetic progestagen treatment (Telfer *et al.*, 1997). This observation, which is consistent with our morphological and functional studies in mice and rats (Chwalisz *et al.*, 1999a; Purcell *et al.*, 1999a), suggests that decidualization may generally be accompanied by the up-regulation of iNOS. Furthermore, increased activity in superoxide dismutase (SOD), an enzyme enhancing NO actions by reducing superoxide-mediated NO inactivation, could be detected in decidual cells (Sugino *et al.*, 1996).

In conclusion, both eNOS and iNOS have been identified in the human endometrium. Although some of the results of the studies reviewed above tend to be rather divergent, the majority of them demonstrate the presence of eNOS in the glandular and epithelial endometrium and in the endometrial microvasculature, accompanied by greater eNOS expression during the secretory phase which suggests progesterone regulation of this particular isoform. The menstrual cycle-dependent regulation of endometrial eNOS indicates that this isoform is not strictly constitutive. It may indeed be regulated by steroid hormones and may regulate endometrial glandular function and blood flow during endometrial receptivity. iNOS seems to be present in various immunocompetent endometrial cells, in decidualized stromal cells, as well as in epithelial cells during menstruation. Unfortunately, systematic studies of NOS activity and expression in women exhibiting abnormal uterine bleeding are presently not available.

NO and the uterine immune system

The regulation of leukocyte migration to specific targets of the female reproductive tract in response to the local production of chemokines, subsequent to the leukocyte secretion of specific paracrine factors, may be one of the ways in which key events of female reproduction such as ovulation, menstruation, implantation, cervical ripening and labour are controlled. Since NO is a major product of activated macrophages and other bone marrow-derived cells, it may mediate the pro-inflammatory effects of these cells. We suggested that infiltrating leukocytes might be the major source of NO during cervical ripening during both term and preterm labour (Chwalisz *et al.*, 1997; Chwalisz and Garfield, 1998).

Compared with other tissues of the female reproductive tract, the endometrium is unique in this respect. It contains a large number of bone marrow-derived cells, leukocytes, which may play a pivotal role during menstruation, endometrial proliferation and differentiation, neovascularization and implantation. Normal human endometrium contains numerous leukocytes in both stromal and intra-epithelial sites, the quantity of which changes during both the menstrual cycle and pregnancy. During the follicular phase they account for approximately 5–10% of the total stromal cell population, whereas in the late luteal phase leukocytes make up 20–25% of stromal cells (Bulmer *et al.*, 1991; Jones *et al.*, 1998). This large increase in the endometrial leukocyte population is due to the mucosal infiltration of phenotypically unusual uterus-specific natural killer (NK) cells also called endometrial large granular lymphocytes (LGL; reviewed by King *et al.*, 1998; see also Jones *et al.*, 1998). Both leukocyte subtypes persist in early pregnant decidua (Loke and King, 1995). In addition, except immediately before and during menstruation, only a small number of polymorphonuclear leukocytes are present in the endometrium during the proliferative and secretory phases (Poropatich *et al.*, 1987). Mast cells do not change in number throughout the menstrual cycle, but a dramatic mast cell activation occurs immediately before and during menstruation (Jeziorska *et al.*, 1995). The exact function of the endometrial population of leukocytes is still unknown, although a role in the regulation of key endometrial functions, including implantation and menstruation has been previously suggested (Bulmer *et al.*, 1991; King *et al.*, 1998).

Moreover, the non-pregnant endometrium is also the synthesis site of multiple cytokines and chemokines, including TNF-α, IL-1 and IL-8 (Tabibzadeh, 1996), which are very potent inducers of iNOS in macrophages and other leukocytes. It is also well known that the pro-inflammatory cytokines are up-regulated in the uterus during implantation (Simón *et al.*, 1993; Chard, 1995; Tazuke and Giudice, 1996). In the endometrium and decidua the leukocytes may in fact represent the most important source of NO during menstruation, implantation and early pregnancy. In mice, a strong iNOS expression was found in the uterine leukocytes, including mast cells, macrophage-like cells, and uterine natural killer cells during early and mid-pregnancy (Hunt *et al.*, 1997). Similar findings were reported in rats (Sladek *et al.*, 1998). Furthermore, progesterone is most likely an important endocrine factor controlling directly or indirectly both leukocyte infiltration and NO production in macrophages and other leukocytes. Experimental studies in mice and in-vitro studies employing RAW264.7 mouse macrophages clearly indicate that, despite the absence of detectable PR mRNA, progesterone down-regulates NO production and iNOS mRNA expression in uterine macrophages (Miller and Hunt, 1996; Hunt *et al.*, 1998). Recently, in an in-vivo model of progesterone withdrawal and maintenance in women, there was evidence indicating that progesterone withdrawal is a signal for an influx of macrophages and LGL into the endometrium (Critchley *et al.*, 1999). This study also showed an up-regulation of various pro-inflammatory mediators, including IL-8 and COX-2 in the endometrium in response to progesterone withdrawal.

Role of NO during implantation

In all mammals, the endometrium is receptive to blastocyst implantation only during a specific period after ovulation. This stage of the luteal phase is called 'implantation window' (Chang, 1950; McLaren, 1973; Psychoyos, 1973). During early pregnancy under the influence of progesterone (humans) and both progesterone and embryonic signals (rodents), the endometrial stroma undergoes a dramatic differentiation into the decidua, a specialized, well-vascularized tissue that encapsulates the developing embryo (Schlafke and Enders, 1975). Decidual cells are believed to play a key role in providing nutrients to the embryo, and in controlling the trophoblast invasion (Parr and Parr, 1989; Loke and King, 1995). A localized increase in endometrial vascular permeability seems to be the first sign of impending implantation in all mammals studied to date, including humans (reviewed by Rogers, 1996). In rodents, the decidual cell reaction occurs in response to either blastocysts or artificial stimuli, and is always preceded by an increase in endometrial vascular

permeability which is normally initiated at the antimesometrial sites where blastocysts implant (Psychoyos, 1973). Similarly, in humans an adequate uterine blood supply is essential for embryo development, and an impaired blood flow to the uterus can jeopardize the establishment of pregnancy (Edwards, 1995). Furthermore, uterine blood flow, as measured by colour Doppler, was proposed as the physiological parameter to assess endometrial receptivity to blastocyst implantation following assisted reproduction treatments (Achiron *et al.*, 1995; Friedler *et al.*, 1996).

The exact mechanism of the vascular reaction at peri-implantation is unclear. There is ample evidence indicating that prostaglandins mediate, at least in part, the changes in endometrial vascular permeability and subsequent decidualization in pregnant as well as in pseudopregnant animals (Evans and Kennedy, 1978; Kennedy, 1977, 1980). However, there is growing evidence that NO may play an important role in implantation and decidualization. In guinea pigs, dilatation of uteroplacental arteries was observed when invading trophoblast cells co-expressing eNOS and iNOS were present in the extravillous trophoblast (Nanaev *et al.*, 1995), suggesting that NO mediates spiral arterial changes occurring during pregnancy. We also found an intense iNOS and eNOS labelling in the connective tissue surrounding spiral arterioles in the baboon endometrium during implantation (Purcell *et al.*, 1999b). This study also revealed a very prominent iNOS immunostaining in the cytotrophoblasts during early pregnancy, while eNOS labelling was mainly associated with the glandular endometrium and decidua. In both studies the pattern of NOS localization suggests the involvement of NO in vasodilatation during the initial stages of trophoblast migration.

Recently, we performed immunohistochemical, molecular and functional studies in mice and rats to address the question of whether or not NO is involved in implantation (Chwalisz *et al.*, 1999a; Purcell *et al.*, 1999a). All three NOS isoforms were present within the mouse implantation site, with iNOS and eNOS being the most prominent within the ectoplacental cone as well as the layers of the myometrium and the primary decidual zone respectively (Purcell *et al.*, 1999a). In this study,

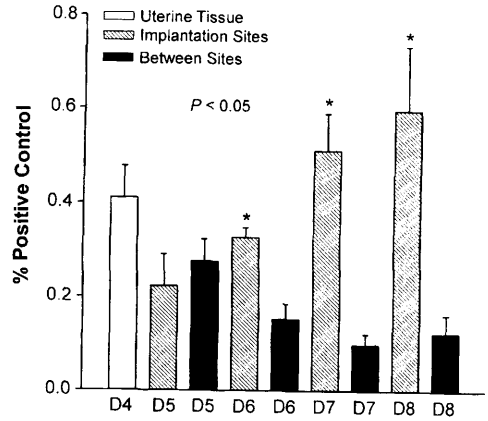

Figure 1. Inducible nitric oxide synthase (iNOS) expression pattern during the peri-implantation phase of mice pregnancy. iNOS values on day 4 were higher than non-pregnant and inter-implantation tissue, and were comparable with day 5 tissue. On days 6–8, iNOS values were significantly higher in implantation sites compared with inter-implantation site regions. This difference increased with gestational age. D = days. (Reproduced from Purcell *et al.*, 1999a, with permission from *Molecular Human Reproduction*).

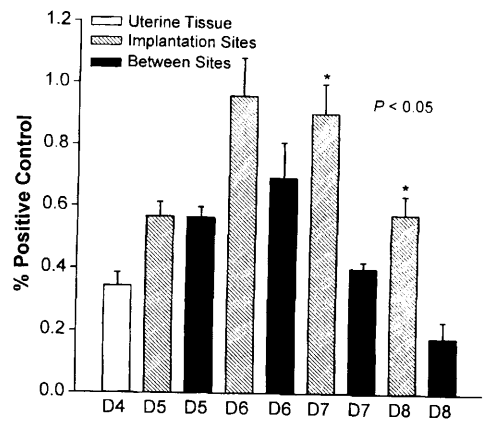

Figure 2. Endothelial nitric oxide synthase (eNOS) expression during the peri-implantation phase of mice pregnancy. Implantation site tissue contained higher values of eNOS than inter-implantation regions, with differences that were statistically significant on day 6 and later. Values of eNOS within implantation sites peaked at day 6, remained elevated on day 7, and declined on day 8 of pregnancy. D = days. (Reproduced from Purcell *et al.*, 1999a, with permission from *Molecular Human Reproduction*).

Western blot analysis of iNOS and eNOS in conjunction with trace optical density readings revealed a significant elevation of iNOS and eNOS expression in implantation sites versus intersite tissues on days 6, 7 and 8 of mice pregnancy (Figures 1 and 2). This study also demonstrated

an inhibitory effect of L-NAME (non-specific NOS inhibitor) on mice implantation after treatment during the peri-implantation phase. The results of our studies in rats clearly demonstrate that NO inhibition with aminoguanidine (iNOS inhibitor) and L-NAME markedly increases the inhibitory effects of the antiprogestin onapristone: (i) on the establishment of pregnancy after treatment during pre-implantation; (ii) on implantation and decidualization; and (iii) on successful pregnancy outcome during the peri-implantation phase (Chwalisz *et al.*, 1999a). These effects were synergistic, as either treatment alone only marginally affected implantation and pregnancy outcome. Implantation is subject to the interaction of the trophoblast cells with the decidua. The results of this study also suggest that NO is involved in decidualization since NOS inhibitors, in particular L-NAME, reduced the extent of decidualization after treatment at peri-implantation even in the absence of onapristone.

Overall, our molecular and functional studies in mice and rats demonstrate that: (i) both iNOS and eNOS proteins are up-regulated in the implantation sites; (ii) NOS inhibitors block both decidualization and the establishment of pregnancy; and (iii) NOS inhibitors act synergistically with antiprogestins. The dramatic increase in iNOS and eNOS in the decidua at peri-implantation suggests, however, that besides progesterone, additional signals of embryonic origin are involved in NOS regulation during implantation. Since IL-1β, a very potent inducer of iNOS, is produced by the embryo (Simón *et al.*, 1993; Kruessel *et al.*, 1997), this cytokine may also act as an embryonic signal promoting implantation. It could in fact, via iNOS and COX-2 induction, initiate the vascular reaction, decidualization, and the remodelling of the extracellular matrix during trophoblast invasion. Since NO may directly regulate the activity of MMP, it may play a role in extracellular matrix changes occurring during trophoblast invasion.

Role of NO during uterine bleeding

According to earlier classic experiments (Markee, 1940), in which the morphological changes of the autologous endometrial tissue transplanted to the anterior eye of the rhesus monkey were examined, initial changes that occurred after progesterone withdrawal were endometrial tissue regression followed by vasoconstriction of the spiral arterioles and vasodilatation. More recent studies have shown that the widespread degeneration accompanied by reduced cell-to-cell contacts occurs in endometrial stroma and in the lamina basalis before the onset of menstruation, but that the endothelial cells remain relatively intact during this time (Roberts *et al.*, 1992). These changes, which are accompanied by leukocytic infiltration and MMP activation, suggest that the menstrual process is initiated by changes in the extracellular matrix and the stromal and epithelial cells, rather than in the vascular network (Osteen *et al.*, 1994; Rogers, 1996; Salamonsen and Woolley, 1996). Since endometrial bleeding may last for several days, specific mechanisms have to operate to maintain blood flow. There are currently two hypotheses to explain the maintenance of uterine bleeding during menstruation: (i) an enhanced vasodilatation of endometrial arterioles and capillaries (Markee, 1940); and (ii) the coagulation theory based on increased fibrinolytic activity (Lockwood *et al.*, 1994). The former hypothesis is supported by the finding of high tissue-type plasminogen activator (tPA) concentrations in the endometrium of menorrhagic women, and can be seen in the efficacy of antifibrinolytic drugs administered in this condition (Bonnar and Sheppard, 1996).

Although the precise role of NO during menstruation is still unclear and studies of NOS inhibitors on uterine bleeding are not yet available, the existing data on endometrial NOS expression and NO production during the menstrual cycle strongly suggest that NO plays a central role in controlling both the initiation and maintenance of uterine bleeding (Figure 3). Since NO is both a powerful inhibitor of platelet aggregation and a potent vasodilator, the concept of endometrial iNOS induction during menstruation bridges somewhat the vascular and coagulation theories of menstruation. In addition, iNOS-derived NO can promote tissue destruction by activating MMP and inducing apoptosis.

It is still unclear which mechanisms are responsible for the initial vasoconstriction of spiral arterioles in response to progesterone withdrawal. It is likely that an up-regulation of the ET system (increase in ET synthesis and decrease in degrada-

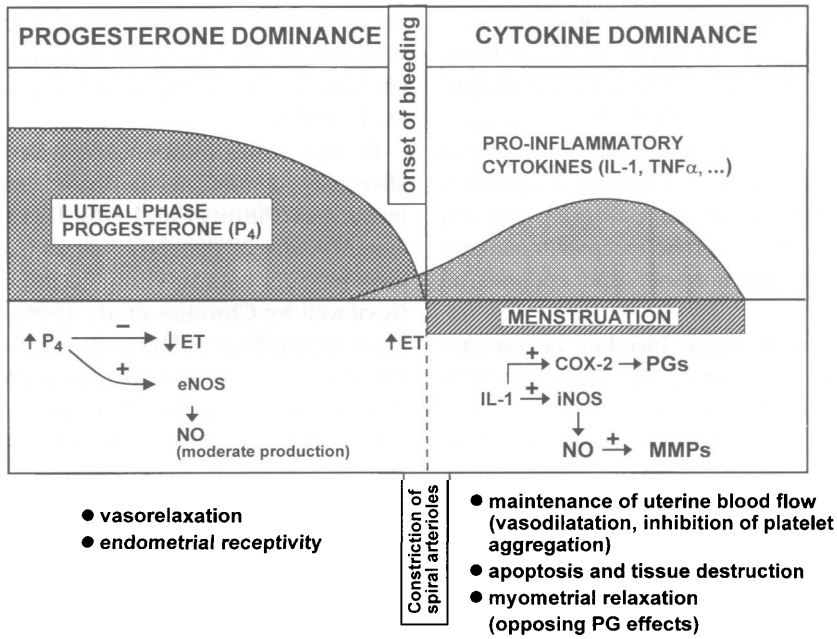

Figure 3. Proposed model of nitric oxide (NO) action during the peri-menstrual phase of the cycle and during menstruation. During the luteal phase, under the influence of both progesterone and oestrogen, there is an up-regulation of eNOS in endometrial glands and blood vessels, thus precipitating a moderate local elevation in NO. An increased NO production may play a role in both the dilatation of endometrial blood vessels and receptivity. Luteal phase progesterone also down-regulates the endometrial endothelin (ET) system. Progesterone withdrawal at the end of the luteal phase leads to an elevation in endometrial ET expression while simultaneously inhibiting its major inactivator, enkephalinase. These effects may induce vasoconstriction of spiral arterioles. At peri-menstruation there is also an increased endometrial expression of pro-inflammatory cytokines, including IL-1 and TNF-α, which in turn induce iNOS and cyclooxygenase-2 (COX-2) in uterine leukocytes and epithelial and stromal cells. High concentrations of iNOS-derived NO may contribute to the maintenance of uterine bleeding during menstruation. Furthermore, it may activate endometrial metalloproteinases (MMPs) which play a key role during tissue destruction at peri-menstruation. In addition, high NO concentrations can induce endometrial apoptosis, an effect which can be observed during menstruation. The effects of NO on the endometrial arterioles and platelets may be enhanced by COX-2-derived vasodilatory prostaglandins (PGs), such as prostacyclin (PGI_2) and PGE_2.

tion by enkephalinase) plays a pivotal role in this respect (Casey *et al.*, 1992; Economos *et al.*, 1992; Marsh *et al.*, 1996). However, a down-regulation of eNOS in spiral arterioles at peri-menstruation cannot be completely ruled out.

In the non-pregnant primate uterus under progesterone dominance, during the luteal phase there is proliferation of spiral arterioles (Koji *et al.*, 1994), a down-regulation of the endothelin system (Casey *et al.*, 1992; Economos *et al.*, 1992), and an up-regulation of eNOS activity in both endometrial vessels and glands (Khorram *et al.*, 1999; Taguchi *et al.*, 2000). These physiological changes may provide optimal conditions for embryo implantation. During this phase, progesterone may be the primary factor up-regulating NO production via eNOS and, in turn, leading to vasodilatation of spiral arterioles. During the secretory phase, eNOS-derived NO from both glandular epithelium and blood vessels may play a role in endometrial receptivity. Conversely, progesterone seems to suppress endometrial ET production, and up-regulates endometrial enkephalinase, a key enzyme responsible for ET degradation (Casey *et al.*, 1992; Economos *et al.*, 1992).

The concept of enhanced angiogenesis and vasorelaxation by progesterone is consistent with the well-known phenomenon of unscheduled bleeding during chronic administration of synthetic progestins. On the other hand, antiprogestins not only block progesterone action but also inhibit oestrogen-dependent endometrial proliferation and induce amenorrhoea in primates through unknown mechanisms (Wolf *et al.*, 1989; Brenner and

Slayden, 1994; Williams *et al.*, 1994; Zelinski-Wooten *et al.*, 1998). More recent studies in rhesus monkeys (Slayden *et al.*, 1998) and cynomolgus monkeys (Chwalisz *et al.*, 1999b) strongly suggest that vasoconstriction of spiral arterioles and/or inhibition of endometrial angiogenesis is a primary event of treatment with antiprogestins (RU 486, ZK 137 316) and the progesterone receptor modulator J1042, leading to amenorrhoea and endometrial atrophy.

After the onset of uterine bleeding in response to progesterone withdrawal, an increased iNOS activity (Tschugguel *et al.*, 1999) may lead to a local release of NO at high concentrations. It is likely that during this phase iNOS is induced by pro-inflammatory cytokines which are elevated at peri-menstruation (Figure 3). An increased endometrial production of IL-1 and TNF-α, during the late luteal phase and during menstruation has been previously reported (Tabibzadeh, 1996). As a result, elevated local NO production may on the one hand induce vasodilatation of endometrial arterioles, and on the other hand contribute to the reduction of platelet fibrin plug formation. Since NO can activate MMP and enhance COX-2 activity leading to an increase in PGE_2 and PGI_2, both of these actions would be expected to promote both extracellular matrix remodelling and vasodilatation. Finally, NO may play a role in endometrial apoptosis which is increased in various components of the functional layer during the late secretory phase and menstruation (Kokawa *et al.*, 1996).

The role of NO in menorrhagia and other uterine bleeding disorders remains poorly understood. Unfortunately, systematic studies on NO production and NOS expression in menorrhagic women are not available to date. We believe that the possibility of increased local NO production in this condition merits consideration. In fact, a recently published study (Hurskainen *et al.*, 1999) clearly showed an association between uterine artery blood flow (measured by transvaginal colour Doppler) and menstrual blood loss. A significant inverse correlation was found between uterine pulsatility index and the amount of blood loss. This study suggests that local concentrations of vasoactive agent(s) simultaneously decrease uterine blood flow resistance as well as promoting uterine bleeding. Hence, NO could be a good candidate to exert both effects, since it is well-known that NO donors can increase uterine blood flow in humans (Ramsay *et al.*, 1994).

In this context, endometrium-derived NO can also play a role in the pathogenesis of dysmenorrhea (Pittrof *et al.*, 1996). There is meanwhile ample evidence that NO relaxes uterine smooth muscle under both in-vitro and in-vivo conditions (reviewed by Chwalisz *et al.*, 1996). On the other hand, local effects of NO on spiral arterioles during menstruation may be beneficial in preventing pain due to anoxia. Hence, NO under physiological conditions may exhibit a dual action — a paracrine tocolytic effect on the myometrium, and a direct action on the endometrial blood vessels.

Therapeutic perspectives

The data reviewed above indicate that NOS, in particular iNOS, may represent a new target for novel therapeutic agents capable of both promoting and inhibiting implantation. NO donors or NO substrates may also have implications for the management of early pregnancy disorders, including recurrent abortions, treatment of dysmenorrhoea and chronic pelvic pain. On the other hand, iNOS inhibitors may find application in the treatment of uterine bleeding disorders.

Up-regulating uterine NO production with either the NO substrate L-arginine or NO donors alone or in combination with progesterone may have beneficial effects on pregnancy outcome during assisted conception. In fact, it has been shown recently (by Doppler measurement) that administration of L-arginine improved uterine and follicular flow in poor responder patients during in-vitro fertilization (Battaglia *et al.*, 1999). However, larger clinical studies are needed to address the question of whether the up-regulation of NO will improve the pregnancy rate during assisted reproduction. On the other hand, the effects of NOS inhibitors in combination with antiprogestins point to a novel method for controlling fertility, particularly by enhancing the efficacy of antiprogestins used for endometrial contraception, menstrual induction and postcoital contraception.

Abnormal uterine bleeding is a common gynaecological problem which has an enormous impact

on the lives of many women. Furthermore, unscheduled bleeding, which is frequently associated with chronic progestin administration or continuous HRT regimens, represents one of the major problems leading to the discontinuance of medication. The role of NO in this respect is likely to be of considerable interest for the understanding of the mechanisms of uterine bleeding under normal and pathological conditions. Both experimental and clinical evidence is emerging pointing to cross-talk between progesterone and NO in the endometrium. Further clinical studies are clearly needed to explore the potential of antiprogestins, other progesterone receptor modulators, and NOS inhibitors for the treatment of abnormal uterine bleeding.

Finally, NO donors may find application in the treatment of dysmenorrhea. In fact, preliminary studies employing transdermal nitroglycerine suggest its efficacy in severe dysmenorrhea (Pittrof *et al.*, 1996). However, larger, controlled studies are needed to establish the potential of NO donors for the treatment of dysmenorrhea.

References

Achiron, R., Levran, D., Sivan, E. *et al.* (1995) Endometrial blood flow response to hormone replacement therapy in women with premature ovarian failure: a transvaginal Doppler study. *Fertil. Steril.*, **63**, 5050–5054.

Ahmed, A., Dearn, S., Shams, M. *et al.* (1998) Localization, quantification, and activation of platelet-activating factor receptor in human endometrium during the menstrual cycle: PAF stimulates NO, VEGF, and FAK^{pp125}. *FASEB J.*, **12**, 831–843.

Ali, M., Buhimschi, I., Chwalisz, K. and Garfield, R.E. (1997) Changes in expression of nitric oxide synthase isoforms in rat uterus and cervix during pregnancy and parturition. *Mol. Hum. Reprod.*, **3**, 995–1003.

Battaglia, C., Salvatori, M., Maxia, N. *et al.* (1999) Adjuvant L-arginine treatment for in-vitro fertilization in poor responder patients. *Hum. Reprod.*, **14**, 1690–1697.

Beck, K.-F., Eberhardt, W., Frank, S. *et al.* (1999) Inducible NO synthase: role in cellular signalling. *J. Exp. Biol.*, **202**, 645–653.

Bonnar, J. and Sheppard, B.L. (1996) Treatment of menorrhagia during menstruation: randomised, controlled trial of ethamsylate, mefenamic acid, and tranexemic acid. *Br. Med. J.*, **313**, 579–582.

Brenner, R.M. and Slayden, O.D. (1994) Oestrogen action in the endometrium and oviduct of rhesus monkeys during RU 486 treatment. *Hum. Reprod.*, **9** (Suppl. 1), 82–97.

Brüne, B., von Knethen, A. and Sandau, K.B. (1998) Nitric oxide and its role in apoptosis. *Eur. J. Pharmacol.*, **351**, 261–272

Buhimschi, I., Ali, M., Jain, V. *et al.* (1996) Differential regulation of nitric oxide in the uterus and cervix during pregnancy and labour. *Hum. Reprod.*, **11**, 1755–1766.

Bulmer, J.N., Morisson, L., Longfellow, M. *et al.* (1991) Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Hum. Reprod.*, **6**, 791–798.

Cameron, I.T. and Campbell, S.C. (1998) Nitric oxide in the endometrium. *Hum. Reprod. Update*, **4**, 565–569.

Casey, M.L., Smith, J.W., Nagai, K. *et al.* (1992) Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase) in human endometrium. *J. Biol. Chem.*, **266**, 23041–23047.

Chang, M.C. (1950) Development and fate of transferred rabbit ova or blastocyst in relation to the ovulation time of recipients. *J. Exp. Zool.*, **114**, 197–225.

Chatziantoniou, C., Boffa, J.L., Ardaillou, R. and Dussaule, J.C. (1998) Nitric oxide inhibition induces early activation of type I collagen gene in renal resistance vessels and glomeruli in transgenic mice. *J. Clin. Invest.*, **101**, 2780–2789.

Chard, T. (1995) Cytokines in implantation. *Hum. Reprod. Update*, **1**, 385–396.

Chaves, M.C., Ribeiro, R.A. and Rao, V.S. (1993) Possible involvement of nitric oxide in estrogen-induced uterine edema in the immature rat. *Braz. J. Med. Biol. Res.*, **26**, 853–857.

Chwalisz, K. and Garfield, R.E. (1998) Nitric oxide as a final metabolic mediator of cervical ripening. *Hum. Reprod.*, **13**, 245–257.

Chwalisz, K., Buhimschi, I. and Garfield, R.E. (1996) Role of nitric oxide in obstetrics. *Prenat. Neonat. Med.*, **1**, 292–329.

Chwalisz, K., Shi Shao-Qing, Garfield R.E. and Beier, H.M. (1997) Cervical ripening in guinea-pigs after local application of nitric oxide. *Hum. Reprod.*, **12**, 101–109.

Chwalisz, K., Winterhager, E. Thienel, T. and Garfield, R.E. (1999a) Synergistic role of nitric oxide and progesterone during the establishment of pregnancy in rats. *Hum. Reprod.*, **14**, 542–552.

Chwalisz, K., Brenner, R.M., Fuhrmann, U. and Elger, W. (1999b) Antiproliferative effects of progesterone receptor modulators on the endometrium. Progesterone, progestins and antiprogestins in the next millennium. Jerusalem, Israel, August 31–September 3, 1999. Program and Abstracts, p. 55.

Collet, G.P., Kohnen, G., Campbell, S. *et al.* (1996) Localization of endothelin receptors in human uterus throughout the menstrual cycle. *Mol. Hum. Reprod.*, **2**, 439–444.

Collins P. (1996) Vascular effects of oestrogen. *Maturitas*, **23**, 217–226.

Critchley, H.O.D., Jones, R.L., Lea, G. *et al.* (1999) Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. *J. Clin. Endocrinol. Metab.*, **84**, 240–248.

Eberhardt, W., Plüss, C., Hummel, R. *et al.* (1998) Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J. Immunol.*, **160**, 4961–4969.

Economos, K., MacDonald, P.C. and Casey, M.L. (1992) Endothelin-1 gene expression and protein biosynthesis in human endometrium. Potential modulator of endometrial blood flow. *J. Clin. Endocrinol. Metab.*, **74**, 14–19.

Edwards, R.G. (1995) Clinical approaches to increasing uterine receptivity during human implantation. *Hum. Reprod.*, **10** (Suppl. 3), 60–67.

Evans, C.A. and Kennedy, T.G. (1978) The importance of prostaglandin synthesis for the initiation of blastocyst implantation in the hamster. *J. Reprod. Fertil.*, **54**, 255–261.

Figueroa, J.P. and Massman, G.A. (1995) Estrogen increases nitric oxide synthase activity in the uterus of nonpregnant sheep. *Am. J. Obstet. Gynecol.*, **173**, 1539–1545.

Finn, C.A. (1986) Implantation, menstruation and inflammation. *Biol. Rev.*, **61**, 313–328.

Friedler, S., Schenker, J.G., Herman, A. and Levin, A. (1996) The role of ultrasonography in the evaluation of endometrial receptivity following assisted reproductive treatments: a critical review. *Hum. Reprod. Update*, **2**, 323–335.

Furchgott, R.F. and Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

Hishikawa, K., Nakai, T., Marumo, T. *et al.* (1995) Up-regulation of nitric oxide synthase by oestradiol in human aortic endothelial cells. *FEBS Lett.*, **360**, 291–293.

Huang, P.L., Dawson, T.D., Bredt, D.S. *et al.* (1993) Targeted disruption of the neuronal nitric oxide synthase gene. *Cell*, **75**, 1273–1286.

Huang, P.L., Huang, Z., Mashimo, H. *et al.* (1995a) Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*, **377**, 239–242.

Huang, J., Roby, K.F., Pace, J.L. *et al.* (1995b) Cellular localization of hormonal regulation of inducible nitric oxide synthase in cycling mouse uterus. *J. Leukocyte Biol.*, **57**, 27–35.

Hunt, J.S., Miller, L., Vassmer, D. and Croy, B.A. (1997) Expression of the inducible nitric oxide synthase gene in mouse uterine leukocytes and potential relationship with uterine function during pregnancy. *Biol. Reprod.*, **57**, 827–836.

Hunt, J., Miller, L. and Platt, J.S. (1998) Hormonal regulation of uterine macrophages. *Dev. Immunol.*, **6**, 105–110.

Hurskainen, R., Teperi, J., Paavonen, J. and Cacciatore, B. (1999) Menorrhagia and uterine artery blood flow. *Hum. Reprod.*, **14**, 186–189.

Ignarro, L.G. (1989) Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ. Res.*, **65**, 1–21.

Izumi, H., Yallampalli, C. and Garfield, R.E. (1993) Gestational changes in L-arginine-induced relaxation of pregnant rat and human myometrial smooth muscle. *Am. J. Obstet. Gynecol.*, **169**, 1327–1337.

Jeziorska, M., Salamonsen, L.A. and Woolley, D.E. (1995) Mast cells and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. *Biol. Reprod.*, **53**, 312–320.

Jones, R.K., Bulmer, J.N. and Searle, R.F. (1998) Phenotypic and functional studies of leukocytes in human endometrium and endometriosis. *Hum. Reprod. Update*, **4**, 702–709.

Kalkhoven, E., Wissink, S., van der Saag, P.T. and van der Burg, B. (1996) Negative interaction between RelA(p65) subunit of NF-κB and the progesterone receptor. *J. Biol. Chem.*, **271**, 6217–6224.

Kennedy, T.G. (1977) Evidence for a role for prostaglandins in the initiation of blastocyst implantation in the rat. *Biol. Reprod.*, **16**, 289–291.

Kennedy, T.G. (1980) Timing of uterine sensitivity for the decidual cell reaction: role of prostaglandins. *Biol. Reprod.*, **22**, 519–525.

Khorram, O., Garthwaite, M. and Magness, R.R. (1999) Endometrial and myometrial expression of nitric oxide synthase isoforms in pre- and postmenopausal women. *J. Clin. Endocrinol. Metab.*, **84**, 2226–2232.

King, A., Burrows, T., Verma, S. *et al.* (1998) Human uterine lymphocytes. *Hum. Reprod. Update*, **4**, 480–485.

Koji, T., Chedid, M., Rubin, J.S. *et al.* (1994) Progesterone-dependent expression of keratinocyte growth factor mRNA in stromal cells of the primate endometrium: keratinocyte growth factor as a progestomedin. *J. Cell Biol.*, **125**, 393–401.

Kokawa, K., Shikone, T. and Nakano, R. (1996) Apoptosis in the human uterine endometrium during the menstrual cycle. *J. Clin. Endocrinol. Metab.*, **81**, 4144–4147.

Kruessel, J.K., Huang, H.Y., Wen, Y. *et al.* (1997) Different pattern of interleukin-1β-(IL-1β), interleukin-1 receptor antagonist-(IL-1ra) and interleukin-1 receptor type I-(IL-1R tI) mRNA-expression in single preimplantation mouse embryos

at various developmental stages. *J. Reprod. Immunol.*, **34**, 103–120.

Lander, H.M., Jacovina, A.T., Davis, R.J. *et al.* (1996) Differential activation of mitogen-activated protein kinases by nitric oxide-related species. *J. Biol. Chem.*, **271**, 19705–19709.

Liao, Q.P., Buhimschi, I.A., Saade, G. *et al.* (1997) Regulation of vascular adaptation during pregnancy and post-partum: effects of nitric oxide inhibition and steroid hormones. *Hum. Reprod.*, **12**, 2777–2784.

Lockwood, C.J., Krikun, G., Papp, C. *et al.* (1994) The role of progestationally regulated stromal cell tissue factor and type-1 plasminogen activator inhibitor (PAI-1) in endometrial hemostasis and menstruation. *Ann. N. Y. Acad. Sci.*, **734**, 57–79.

Loke, Y.W. and King, A. (1995) Uterine mucosal leukocytes. In Loke, Y.W. and King, A. (eds), *Human Implantation. Cell Biology and Immunology.* Cambridge University Press. Cambridge, pp. 102–129.

MacMicking, J.D., Nathan, C., Hom, G. *et al.* (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell*, **81**, 1273–1283.

Markee, J.E. (1940) Menstruation in intraocular endometrial transplants in the rhesus monkey. *Contrib. Embryol.*, **28**, 219–308.

Marsh, M.M., Findlay, J.K. and Salamonsen, L.A. (1996) Endothelin and menstruation. *Hum. Reprod.*, **11**, 83–89.

McLaren, A. (1973) Blastocyst activation. In Segal, S.J. *et al.* (eds), *The Regulation of Mammalian Reproduction.* Thomas, Springfeld, IL, pp. 321–334.

Meßmer, U.K. and Brüne, B. (1996) Nitric-oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem. J.*, **319**, 299–305.

Miller, L. and Hunt, J.S. (1996) Sex steroid hormones and macrophage function. *Life Sci.*, **59**, 1–14.

Moncada, S. and Higgs, E.A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.*, **329**, 2002–2012.

Nanaev, A., Chwalisz, K., Frank, H.-G. *et al.* (1995) Physiological dilation of uteroplacental arteries in the guinea pig depends upon nitric oxide synthase activity of extravillous trophoblast. *Cell Tissue Res.*, **282**, 407–421.

Nathan, C. and Qia-wen, X. (1994) Nitric oxide synthases: roles, tolls, and controls. *Cell*, **78**, 915–918.

Osteen, K.G., Rodgers, W.H., Gaire, M. *et al.* (1994) Stromal-epithelial interaction mediates steroidal regulation of metalloproteinase expression in human endometrium. *Proc. Natl Acad. Sci. USA*, **91**, 10129–10133.

Ota, H., Igarashi, S., Hatazawa, J. and Tanaka, T. (1998) Endothelial nitric oxide synthase in the endometrium during the menstrual cycle in patients with endometriosis and adenomyosis. *Fertil. Steril.*, **69**, 303–308.

Papapetropoulos, A., Garcia-Cardena, G., Madri, J.A. and Sessa, W.C. (1997) Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.*, **100**, 3131–3139.

Parr, M.B. and Parr, E.L. (1989) The implantation reaction. In Wynn, R.M. and Jollie, W.P. (eds), *Biology of the Uterus.* 2nd edn. Plenum Medical Book Company, New York, London, pp. 233–267.

Pittrof, R., Lees, C., Thompson, C. *et al.* (1996) Crossover study of glycerol trinitrate patches for controlling pain in women with severe dysmenorrhea. *Br. Med. J.*, **312**, 884.

Poropatich, C., Rojas, M. and Silverberg, S.G. (1987) Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle. *Int. J. Gynecol. Pathol.*, **6**, 230–234.

Psychoyos, A. (1973) Hormonal control of ovoimplantation. *Vitam. Horm.*, **31**, 201–256.

Psychoyos, A., Nikas, G. and Gravanis, A. (1995) The role of prostaglandins in blastocyst implantation. *Hum. Reprod.*, **10** (Suppl. 2), 30–42.

Purcell, T.L., Buhimschi, I.A., Given, R. *et al.* (1997) Inducible nitric oxide synthase (iNOS) is present in rat placenta at the fetal-maternal interface and decrease prior to labor. *Mol. Hum. Reprod.*, **3**, 485–491.

Purcell, T.L., Given, R., Chwalisz, K. and Garfield, R.E. (1999a) Nitric oxide synthase distribution during implantation in the mouse. *Mol. Hum. Reprod.*, **5**, 467–475.

Purcell, T., Fazleabas, A.T., Chwalisz, K. *et al.* (1999b) Localization of nitric oxide synthase in the endometrium during implantation in the baboon. *J. Soc. Gynecol. Invest.*, **6**, 215A (abstract).

Ramsay, B., De Belder, A., Campbell, S. *et al.* (1994) A nitric oxide donor improves uterine artery diastolic blood flow in normal early pregnancy and in women at high risk of preeclampsia. *Eur. J. Clin. Invest.*, **24**, 76–78.

Roberts, D.K., Parmley, T.H., Walker, N.J. *et al.* (1992) Ultrastructure of the microvasculature in the human endometrium throughout the normal menstrual cycle. *Am. J. Obstet. Gynecol.*, **166**, 1391–1406.

Rogers P.A. (1996) Structure and function of endometrial blood vessels. *Hum. Reprod. Update*, **2**, 57–62.

Rosselli, M. (1997) Nitric oxide and reproduction. *Mol. Hum. Reprod.*, **3**, 639–641.

Salamonsen, L.A. and Woolley, D.E. (1996) Matrix metalloproteinases in normal menstruation. *Hum. Reprod.*, **11** (Suppl. 2), 124–133.

Salvemini, D., Misko, T.P., Masferrer, J. *et al.* (1995) Role of nitric oxide in the regulation of cyclooxygenase. In Moncada, S., Feelish, M. and Busse, R. (eds), *Biology of Nitric Oxide. 4 Enzymology,*

Biochemistry and Immunology. Portland Press, London, pp. 304–309.

Sasaki, K., Hattori, T., Fujisawa, T. *et al.* (1998) Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit chondrocytes. *J. Biochem.*, **123**, 431–439.

Schlafke, S. and Enders, A.C. (1975) Cellular basis of interaction between trophoblast and uterus at implantation. *Biol. Reprod.*, **12**, 41–65.

Schmidt, H.H., Gagne, G.D., Nakane, M. *et al.* (1992) Mapping of neuronal nitric oxide synthase in rats suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase, a novel paraneural functions for nitrogenic signal transduction. *J. Histochem. Cytochem.*, **40**, 1439–1456.

Sen, C.K. and Packer L. (1996) Antioxidant and redox regulation of gene transcription. *FASEB J.*, **10**, 709–720.

Shew, R.L., Papka, R.E., McNeil, D.L. and Yee, J.A. (1993) NADPH-diaphorase-positive nerves and the role of nitric oxide in CGRP relaxation of uterine contractions. *Peptides*, **14**, 637–641.

Simón, C., Piquette, G.N., Frances, A. and Polan, M.L. (1993) Localization of interleukin-1 receptor type I and interleukin-1β in human endometrium throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.*, **77**, 549–555.

Sladek, S.M., Regenstrin, A.C., Lykins, D. *et al.* (1993) NO synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am. J. Obstet. Gynecol.*, **169**, 1285–1291.

Sladek, S.M., Kanbour-Shakir, A., Watkins, S. *et al.* (1998) Granulated metrial gland cells contain nitric oxide synthases during pregnancy in the rat. *Placenta*, **19**, 55–65.

Slayden, O.D., Zelinski-Wooten, M.B., Chwalisz, K. *et al.* (1998) Chronic treatment of cycling rhesus monkeys with low doses of the antiprogestin ZK 137 316: morphometric assessment of the uterus and oviduct. *Hum. Reprod.*, **13**, 269–277.

Suburo, A.M., Chaud, M., Franchi, A. *et al.* (1995) Distribution of neuronal and non-neuronal NADPH diaphorases and nitric oxide synthases in rat uterine horns under different hormonal conditions. *Biol. Reprod.*, **52**, 631–637.

Sugino, N., Shimamura, K., Takiguchi, S. *et al.* (1996) Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and early pregnancy. *Hum. Reprod.*, **11**, 11073–11078.

Tabibzadeh, S.S. (1996) The signals and molecular pathways involved in human menstruation, a unique process of tissue destruction and remodelling. *Mol. Hum. Reprod.*, **2**, 77–92.

Taguchi, M., Alfer, J., Chwalisz, K. *et al.* (2000) Endothelial nitric oxide synthase (eNOS) is differently expressed in human endometrial vessels during the menstrual cycle. *Hum. Reprod.* (in press).

Takayanagi, R., Kitazumi, K., Takasaki, C. *et al.* (1991) Presence of non-selective endothelin receptor on vascular endothelium and its linkage to vasodilatation. *FEBS Lett.*, **282**, 103–106.

Tamora, T., Nakanishi, T., Kimura, Y. *et al.* (1996) Nitric oxide mediates interleukin-1-induced matrix degradation and basic fibroblast growth factor release in cultured rabbit articular chondrocytes: a possible mechanism of pathological neovascularization in arthritis. *Endocrinology*, **137**, 3729–3737.

Tazuke, S.I. and Giudice, L.C. (1996) Growth factors and cytokines in endometrium, embryonic development, and maternal:embryonic interactions. *Semin. Reprod. Endocrinol.*, **14**, 231–245.

Telfer, J.F., Lyall, F., Norman, J.E. and Cameron, I.T. (1995) Identification of nitric oxide synthase in human uterus. *Hum. Reprod.*, **10**, 19–23.

Telfer, J.F., Irvine, G.T.A, Kohnen, G. *et al.* (1997) Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium. *Mol. Hum. Endom.*, **3**, 2546–2552.

Trachtman, H., Futterweit, S., Garg, P. *et al.* (1996) Nitric oxide stimulates the activity of a 72-kDa neutral matrix metalloproteinase in cultured rat mesangial cells. *Biochem. Biophys. Res. Commun.*, **218**, 704–708.

Tschugguel, W., Schneeberger, C., Unfried, G. *et al.* (1998) Induction of inducible nitric oxide synthase expression in human secretory endometrium. *Hum. Reprod.*, **13**, 436–444.

Tschugguel, W., Schneeberger, C., Unfried, G. *et al.* (1999) Elevation of inducible nitric oxide synthase activity in human endometrium during menstruation. *Biol. Reprod.*, **60**, 297–304.

Tseng, I., Zhang, J.Z., Peresleni, T.Y. *et al.* (1996) Cyclic expression of endothelial nitric oxide synthase mRNA in the epithelial glands of human endometrium. *J. Soc. Gynecol. Invest.*, **3**, 33–39.

Tsurumi, Y., Murohara, T., Krasinski, K. *et al.* (1997) Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. *Nature Med.*, **3**, 879–886.

Van Buren, G.B., Yang, D. and Clark, K.E. (1992) Estrogen-induced uterine vasodilatation is antagonized by L-nitroarginine methyl ester, an inhibitor of nitric oxide synthesis. *Am. J. Obstet. Gynecol.*, **167**, 828–833.

Villarete, L.H. and Remick, D.G. (1995) Nitric oxide regulation of IL-8 expression in human endothelial cells. *Biochem. Biophys. Res. Commun.*, **211**, 671–676.

Weiner, C.P., Lizasoain, I., Baylis, S.A. *et al.* (1994) Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc. Natl Acad. Sci. USA*, **91**, 5212–5216.

Williams, R.F., Chwalisz, K., Kim, S. *et al.* (1994) Intermittent antiprogestin reduces unscheduled (breakthrough) bleeding during estrogen-progestin hormonal therapy in ovariectomized monkeys. Society of Gynecological Investigation, 41st Annual Meeting, 1994, Abstract P8.

Wolf, J.P., Hsiu, J.G., Anderson, T.L. *et al.* (1989) Noncompetitive antiestrogenic effect of RU 486 in blocking the estrogen-stimulated luteinizing hormone surge and the proliferative action of estradiol on endometrium in castrate monkeys. *Fertil. Steril.*, **52**, 1055–1060.

Zancan, V., Santaggati, S., Bolego, C. *et al.* (1999) 17β-estradiol decreases nitric oxide synthase II synthesis in vascular smooth muscle cells. *Endocrinology*, **140**, 2004–2009.

Zelinski-Wooten, M.B., Slayden O.D., Chwalisz, K. *et al.* (1998) Chronic treatment of female cycling rhesus monkeys with low-doses of the antiprogestin ZK 137 316: establishment of a regimen that permits normal menstrual cyclicity. *Hum. Reprod.*, **13**, 259–267.