Myometrial constitutive nitric oxide synthase expression is increased during human pregnancy

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Nitric oxide (NO), derived from L-arginine by the action of nitric oxide synthase (NOS), is a mediator of many diverse biological activities, including vasodilatation, neurotransmission and inhibition of platelet adhesion. A role for NO in the maintenance of rat and rabbit pregnancy is supported by a variety of studies. A recent study in women demonstrated that myometrial inducible NOS (iNOS) expression was greater in the early third trimester than either the late third trimester or in the non-pregnant condition, suggesting that increased iNOS expression is involved in the maintenance of human pregnancy. Constitutive NOS (cNOS) expression was not determined. The aim of this study was to compare constitutive NOS (both eNOS and bNOS) expression in the human non-pregnant uterus, preterm pregnant uterus (25–34 weeks gestation) and term pregnant uterus (>37 weeks gestation) using immunohistochemistry and Western blotting. Preterm pregnant samples were taken from women with a variety of pathologies necessitating early delivery. We found that eNOS and bNOS protein concentrations were greater in the preterm pregnant myometrium than non-pregnant myometrium. eNOS, but not bNOS, protein concentration was lower in myometrial samples obtained at term compared with those obtained preterm. We conclude that the constitutive isoforms of NOS are also up-regulated in human pregnancy and may play a role in the maintenance of myometrial quiescence.

Key words: myometrial contraction/nitric oxide/parturition/uterus

Introduction

The understanding and prevention of preterm labour remains one of the primary goals of obstetric research. Of preterm births, 30% are due to idiopathic preterm labour, and prematurity is now the single biggest cause of neonatal death (Office of Population Censuses and Surveys, 1993). At present, the factors maintaining myometrial quiescence during pregnancy, and those which stimulate the onset of parturition at term remain obscure. Until these factors are elucidated, it seems unlikely that effective strategies for the treatment of preterm labour will be found. Much interest has focused recently on the potential role of nitric oxide.

Nitric oxide, derived from L-arginine by the action of nitric oxide synthase (NOS), was originally described as a vascular endothelial product which maintains blood vessels under ‘dilator’ tone (Palmer et al., 1987). The gas exerts its smooth muscle relaxant effects by stimulating soluble guanylate cyclase, thereby increasing cyclic GMP (cGMP), which in turn causes dephosphorylation of myosin light chains (Norman, 1996). However, nitric oxide is also crucially involved in a variety of other physiological processes, including neurotransmission, host defence and smooth muscle relaxation (Anggard, 1994). Three isoforms of nitric oxide synthase have been described. Endothelial NOS (eNOS) and brain NOS (bNOS) are named after the tissue in which they were originally described. Both are calcium dependent enzymes which are constitutively expressed. Inducible NOS (iNOS) is a calcium-independent enzyme whose expression can be induced in diverse cells by various cytokines.

Rat and rabbit studies have demonstrated a role for nitric oxide in the maintenance of uterine quiescence in pregnancy. NOS activity in uterine tissues is high throughout gestation, but decreases prior to the onset of parturition (Norman, 1996). We and others have postulated that nitric oxide may also be responsible for the maintenance of uterine quiescence during pregnancy in humans. Two lines of evidence support this hypothesis. Firstly, nitric oxide relaxes human myometrium both in vitro (Buhimschi et al., 1995; Lee and Chang, 1995; Norman et al., 1997) and in vivo (Lees et al., 1994; Rowlands et al., 1996), and secondly, products of the NO–cGMP pathway are present within both the non-pregnant (Yallampalli et al., 1994; Buhimschi et al., 1995; Telfer et al., 1997) and pregnant human myometria (Buhimschi et al., 1995; Thomson et al., 1997a). Taken together, these data suggest that nitric oxide is produced in the myometrium during pregnancy, and could maintain uterine quiescence via a relaxant effect on the myometrium.

Previous studies, comparing tissues obtained immediately before and after the onset of labour at term, have found no change in nitric oxide synthesis in myometrium, placenta, membranes or decidua (Gude et al., 1994; DiIulio et al., 1996; Ramsay et al., 1996; Thomson et al., 1997a). In support of a role for nitric oxide in the maintenance of pregnancy, Bansal et al. (1997) reported that myometrial iNOS expression, assessed by immunohistochemistry and Western blotting, was...
greater in the early third trimester (26–34 weeks gestation) than either the late third trimester (37–41 weeks gestation) or in the non-pregnant state. None of the women was in labour at the time of sampling. These data suggest that an increase in myometrial iNOS expression might contribute to the maintenance of uterine quiescence during pregnancy. The role of the constitutive isoforms of NOS (eNOS and bNOS) has not been determined. We postulate that the constitutive isoforms of NOS might also be involved in the regulation of uterine activity during pregnancy. The aim of the studies described here was to test this hypothesis, by localising and quantifying eNOS and bNOS isoforms in non-pregnant, preterm pregnant and term pregnant myometrium. We also assessed NOS expression in other tissues within the pregnant uterus (decidua, placenta and membranes).

Materials and methods

Tissue collection

Three groups of women were recruited to the study: (i) 13 non-pregnant, premenopausal women with regular menstrual cycles undergoing hysterectomy for benign disease; (ii) 11 pregnant women who were delivered preterm (25–34 weeks gestation) prior to the onset of labour; and (iii) 11 pregnant women who were delivered at term (>37 weeks gestation) prior to the onset of labour. Informed consent was obtained from each woman prior to recruitment and the study was approved by the local research ethics committee. In the non-pregnant group, four of the women were in the proliferative phase of the cycle, six were in the secretory phase and three were in the menstrual phase. In the pregnant group, women were excluded from the study if they had a multiple pregnancy or evidence of active infection. The indications for delivery in the pregnant women delivered preterm were: pre-eclampsia (n = 4), intrauterine growth restriction (n = 2), haemolisys, elevated liver enzymes and low platelets (HELLP) syndrome (n = 2), placenta praevia (n = 2) and fetal distress of unknown aetiology (n = 1).

In the non-pregnant uteri, myometrial biopsies were taken from the anterior wall of the lower uterine body immediately after hysterectomy. In the pregnant women myometrial biopsies were taken from the upper margin of the lower uterine segment incision during Caesarean section. In all groups, myometrium was separated from surrounding structures by sharp dissection. Biopsies of placenta and fetal membranes with attached decidua capsularis were also obtained from non-pregnant women, five from preterm pregnant women and six from pregnant women at term. The preterm women whose samples were analysed by Western blotting were delivered because of the following indications: placenta praevia (n = 2), pre-eclampsia (n = 1), HELLP syndrome (n = 1) and intrauterine growth restriction (n = 1). Myometrial extracts were prepared by homogenizing frozen tissue in 10 mM Tris–HCl (pH 7.4), 1% sodium dodecyl sulphate (SDS) containing protease inhibitors (BDH). Cell debris was removed by centrifuging at 12 000 g for 5 min. Protein content of the samples was determined by using the BCA protein reagent (Pierce, Chester, UK) and bovine serum albumin reference standards. Electrophoresis was performed using 75 µg aliquots of protein extract in 1x loading buffer (Bio-Rad, Hemel Hempstead, UK) in 7.5% SDS–polyacrylamide gel. Resolved proteins were transferred onto Hybond-ECL membrane using electrophoresis (Amersham, Little Chalfont, UK). The membranes were allowed to air dry and then placed in blocking buffer (10% non-fat milk, 100 mM Tris, 0.1% NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature. The following primary antibodies were used: eNOS, monoclonal antibody raised against a 20.4 kDa protein fragment corresponding to amino acids 1030–1209 of human eNOS (Affiniti, Nottingham, UK) at a dilution of 1/1500; bNOS, a polyclonal antibody raised against amino acids 724–739 of rat brain NOS (Serotec, Oxford, UK) at a dilution of 1/7500. Each primary antibody was diluted in 10 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH 7.5 (TBS-T). The blots were washed for 2 X 2 min, 1 X 5 min and 2 X 5 min in TBS-T and then incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Ig)G (1/10 000) for eNOS, or anti-rabbit IgG (1/30 000) for bNOS in TBS-T for 1 h. The membranes were washed as before then incubated with Amersham ECL detection reagent for 1 min. Thereafter, the membranes were exposed to autoradiographic film (Hyperfilm ECL; Amersham, UK) and the intensity of specific immunoreactive bands was quantified using densitometric scanning. In each blot, one lane was loaded with protein from a positive control (human endothelial cells for eNOS and lysates of rat brain for bNOS). Density of immunostaining was corrected for transfer differences by comparison with intensity of positive control bands. Data were analysed statistically using a Mann–Whitney U test. P < 0.05 was considered to be statistically significant.

Immunohistochemistry

Immunohistochemistry for eNOS and bNOS was performed on each of the biopsies as previously described (Thomson et al., 1997a). Paraffin embedded sections of 8 µm thickness were pretreated with protease digestion (0.01% w/v) protease type XXIV in a 0.01% (w/v) solution of calcium chloride (Sigma, Poole, UK) for 10 min at 37°C prior to staining for eNOS or microwaving (4 X 5 min at full power in citrate buffer) prior to staining for bNOS. The same antibodies were used as for Western blotting, at the following dilutions: eNOS 1/100 and bNOS 1/1750 for placenta and fetal membranes, and 1/2000 for myometrium. Negative controls for eNOS included sections incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 Aspergillus niger glucose oxidase (Dako Ltd, High Wycombe, UK), an enzyme which is not expressed in mammalian cell systems. A section of human umbilical cord was used as a positive control. Immunostaining for eNOS was confined to the umbilical cord endothelium. Negative controls for bNOS included slides incubated without the primary antibody and slides incubated with non-immune rabbit serum (SAPU, Carluke, UK), in place of the primary antibody. In addition, liquid-phase absorption controls were performed by incubating the primary antibody with increasing concentrations of the bNOS immunogen (0.01–100 nmol/ml), (Serotec, Oxford, UK) for 60 min at 37°C prior to incubation with the sections. A section of rat brain was used as a positive control. The localization of each of the constitutive isoforms of NOS and intensity of staining were recorded by two investigators independently (AJT and JEN), who were blind to the source of the biopsy. Intensity and consistency of staining were scored on a scale of 0–6 as previously described, with a score of 0 indicating no staining and 6 indicating strong, consistent staining (Thomson et al., 1997a). Contingency table analysis was performed to determine whether there was a difference.
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![Figure 1. Western blots of (A) non-pregnant (n = 6), (B) preterm (n = 5) and (C) term (n = 6) myometrium using an antibody directed against eNOS. The putative eNOS band is seen at 135 kDa.](image)

![Figure 3. Western blots of (A) non-pregnant (n = 6), (B) preterm (n = 5) and (C) term (n = 6) myometrium using an antibody directed against bNOS. The putative bNOS band is identified at 160 kDa.](image)

Figure 2. Graph showing mean (SE) relative optical density of bands of eNOS protein in non pregnant (n = 6), preterm pregnant (n = 5) and term pregnant (n = 6) myometrium after Western blotting. Optical density was greater in the preterm pregnant than the non-pregnant group (*P < 0.01); and in the preterm pregnant compared with the term pregnant group (**P < 0.05).

Figure 4. Graph showing mean (SE) relative optical density of bands of bNOS protein in non-pregnant (n = 6), preterm pregnant (n = 5) and term pregnant (n = 6) myometrium after Western blotting. Optical density was greater in the preterm pregnant than the non-pregnant group (*P < 0.05).

Results

Western blotting

eNOS

The antibody against eNOS reacted with a band at 135 kDa in the lysate of human aortic endothelial cells. Similar bands were detected in samples of human myometrium (Figure 1). Densitometric analysis of bands indicated that eNOS protein levels were significantly higher in preterm myometrial biopsies compared with either term (P < 0.05) or non-pregnant myometrial biopsies (P < 0.01) (Figure 2).

bNOS

The bNOS antibody reacted with a 160 kDa protein in the rat brain lysate corresponding to bNOS. Similar bands were detected in samples of human myometrium (Figure 3). Densitometric analysis of bands indicated that bNOS protein levels were significantly higher preterm (P < 0.05) compared with non-pregnant, but that there were no significant differences in levels in term and preterm myometrium (Figure 4).

Immunohistochemistry

eNOS

Within sections of myometrium, the eNOS protein was localized to myometrial smooth muscle cells (myocytes) and endothelial cells lining blood vessels. The intensity of staining within myocytes was greater in preterm myometrium compared to both non-pregnant (P < 0.001) and term myometrium (P < 0.005) (Figure 5). Similar differences were seen in endothelial cells within the myometrium (P < 0.05 and P < 0.01 respectively). Myocytes in sections obtained at term had a significantly greater staining intensity to those in non-pregnant samples (P < 0.05). No such differences were observed in endothelial cells.

Within the placenta, eNOS staining was of greatest intensity in the syncytiotrophoblast, of moderate intensity in endothelial cells and was weakly present in villous stromal cells. eNOS immunostaining was significantly more intense within syncytiotrophoblast and endothelial cells in preterm sections than those at term (P < 0.02) (Figure 6). There was no difference in the staining intensity within stromal cells in the two groups.
Immunohistochemical localization of endothelial NOS (eNOS) in (a) non-pregnant, (b) preterm pregnant and (c) term pregnant myometrium. eNOS localized to the myometrial smooth muscle cells and the endothelium of blood vessels. Expression of eNOS was greater in preterm myometrium compared to both non-pregnant and term pregnant myometrium. The negative controls (see Materials and methods) exhibited no reactivity. M = myocytes; E = endothelium. Bars = 50 µm.

Expression of endothelial NOS (eNOS) in (a) preterm and (b) term placenta. Immunostaining for eNOS was more intense in syncytiotrophoblast and endothelial cells in preterm sections than those at term. The negative controls (see Materials and methods) exhibited no reactivity. E = endothelium; ST = syncytiotrophoblast. Bars = 50 µm.

In fetal membranes, eNOS immunoreactivity was localized to amniotic epithelial cells, extravillous trophoblast (the remains of the cytotrophoblastic cells of the trophoblastic shell) and decidual stromal cells. Staining was absent from chorial connective tissue. There was no difference in the intensity of staining in any of the above cell types when comparing preterm to term sections. There was no staining in the negative control slides.

bNOS
In myometrium, bNOS protein was detected within myocytes and endothelial cells (Figure 7). The intensity of staining within myocytes was greater in preterm sections than either that of non-pregnant (P < 0.001) or term (P < 0.05) sections. In addition, bNOS staining in myocytes in sections obtained at term had a significantly greater staining intensity to those in non-pregnant samples (P < 0.05). The immunostaining
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intensity in myometrial vessel endothelial cells was greater in the preterm pregnant compared with the non-pregnant group ($P < 0.05$). There were no other differences in the groups in endothelial cell staining.

bNOS protein was detected in syncytiotrophoblast cells, villous stromal cells and endothelial cells in placenta and within amniotic epithelial cells, chorion, trophoblast and decidual cells of the fetal membranes. There was no significant difference in the intensity of staining within any of these cell types when comparing the two groups of pregnant women. Staining was absent when the primary antibody was omitted. In liquid phase absorption controls, the staining disappeared when 10 nmol/ml of the bNOS immunogen was added to the primary antibody prior to incubation with the sections.

Discussion

These studies demonstrate that the myometrial expression of constitutive NOS (eNOS and bNOS), determined using Western blotting and immunohistochemistry, has been up-regulated by the early third trimester of pregnancy compared to the non-pregnant state. Furthermore, there is a decline in myometrial cNOS (assessed by immunohistochemistry and Western blotting), and in myometrial bNOS (assessed by immunohistochemistry) from the early third trimester to term. Taken together with the results of Bansal et al. (1997), these data indicate that the myometrial expression of each of the three isoforms of NOS is increased during human pregnancy and declines during the third trimester. Since NO is a myometrial relaxant, we propose that such an increase in NOS will contribute to the quiescence of the myometrium during pregnancy.

The timing of parturition is a crucial event in the survival of any species, and it seems unlikely therefore to be controlled by a single biochemical pathway. In human pregnancy, numerous factors, including prostaglandin production, cytokines, gap-junction formation, corticotrophin releasing factor, G protein and thiol protein disulphide isomerase appear to play a role in the regulation of uterine contractility throughout gestation and at the onset of labour (Europe Finner et al., 1994; Hirst et al., 1995; McLean et al., 1995; Morrison et al., 1996; Schwartz, 1997). It seems clear that NO withdrawal does not play a part in the acute stimulus to labour at term since our study suggests that NOS expression decreases prior to the onset of labour. Furthermore there is no change in NOS activity or expression comparing uterine tissues taken immediately before and after the onset of labour (Thomson et al., 1997a). However, it is recognized that the myometrium from women in late gestation or in active labour, is less sensitive to tocolytic agents, and more sensitive to agents which stimulate uterine contractions than myometrium from women in mid-pregnancy (Soloff et al., 1979; Yallampalli and Garfield, 1994; Kim et al., 1995). A decline in myometrial NOS activity prior to the end of the third trimester might be one of the mechanisms by which the uterus is sensitized to contractile agents, and thus prepared for the onset of labour.

We have shown that each of the isoforms of constitutive NOS is up-regulated in the myometrium during pregnancy. Although NOS is increased during rat pregnancy, the isoforms responsible are not known. Data from gene knock-out animals suggest that an absence of any single isoform of NOS has no effect on the normal gestational period (Huang et al., 1993, 1995; Wei et al., 1995). Taken together, these data imply that each of the isoforms of NOS contributes to the maintenance of uterine quiescence during pregnancy, but that no single isoform is by itself crucial.

Factors controlling the observed up-regulation in myometrial constitutive NOS expression during parturition remain obscure. In animals both oestrogen and progesterone have been proposed

Figure 7. Immunohistochemical localization of brain NOS (bNOS) in (a) non-pregnant, (b) preterm pregnant and (c) term pregnant myometrium. bNOS localized to the myometrial smooth muscle cells and the endothelium of blood vessels. Expression of bNOS was greater in preterm myometrium compared to both non-pregnant and term pregnant myometrium. The negative controls (see Materials and methods) exhibited no reactivity. M = myocytes. Bars = 50 µm.
as regulators of NOS in vivo. Oestrogen up-regulates guinea-pig constitutive NOS expression (Weiner et al., 1994). In sheep uterine artery, oestrogen enhanced endothelial NOS activity, and in rat hypothalamus, oestrogen increased bNOS expression (Cecatelli et al., 1996; Veille et al., 1996). In studies of women treated with a gonadotrophin-releasing hormone (GnRH) analogue together with either oestrogen replacement or placebo, fasting plasma nitrate concentrations were higher in the oestrogen treatment group. This suggests that oestrogen increased 'total body' nitric oxide synthesis (Ramsay et al., 1995). Since human pregnancy is associated with an increase in circulating oestriadiol concentration (Turnbull et al., 1974), it is tempting to speculate that oestrogen is responsible for the observed increase in myometrial constitutive NOS expression. However, other mechanisms may also be involved, since oestrogen levels are still high at the end of the third trimester, when a fall in NOS expression was observed. Additional studies are required to investigate this further.

A potential concern with our study is that the 'preterm' group were made up of women with abnormal pregnancies. The majority of biopsies from women in the preterm Caesarean section group were collected from patients delivered either because of pre-eclampsia or intrauterine growth restriction. It is possible that the observed differences between the groups reflect the indications for delivery rather than the differences in gestation. However, there is no evidence that eNOS expression in myocytes is altered by pre-eclampsia or intrauterine growth retardation. Indeed, in our study, when histological slides of preterm myometrium obtained from women with pre-eclampsia or intrauterine growth retardation were compared with preterm myometrium obtained from women delivered for other reasons, no differences were observed in eNOS or bNOS staining in myocytes. A formal statistical analysis was not performed because of the small size of the group. We believe therefore that the myometrial changes in eNOS and bNOS are dependent upon the stage of gestation rather than the indications for Caesarean section.

It is more likely that the observed differences in placental eNOS are due to the indications for delivery. We assessed the expression of eNOS and bNOS in placenta and fetal membranes, collected from women at term and preterm. Whilst the sites of localization of CNOS were similar at each gestation, we found that the intensity of staining for eNOS (but not bNOS) was greater in preterm placental syncytiotrophoblast and preterm placental endothelium than in tissues obtained at term. We did not attempt to perform Western blotting on placenta to confirm this finding because of the multiple cell types present therein, each of which has variable expression of NOS (Thomson et al., 1997a). Again, the majority of histochemically examined biopsies from women in the preterm Caesarean section group were collected from patients delivered either because of pre-eclampsia or intrauterine growth restriction. Each of these conditions is associated with an increase in placental eNOS expression (Myatt et al., 1997). It has been postulated that the greater intensity of eNOS staining in the placenta in the preterm group is a compensatory mechanism attempting to increase feto-placental blood flow in this condition. We propose that differences we observed in placental eNOS expression when term and preterm tissues were compared may reflect the indications for preterm Caesarean section in these patients, rather than a true gestational effect.

Endothelial NOS (but not bNOS) expression was also greater in myometrial vessels from preterm compared with term women. Previous studies have shown that the myometrial vessels in women with pre-eclampsia are different from those in normal pregnant women (Brocklesby et al., 1998). The difference in eNOS expression between term and preterm myometrial vessels may be due to the high proportion of women with pre-eclampsia or intrauterine growth restriction in the preterm group. Further work is required to confirm this hypothesis.

We have demonstrated that the myometrial expression of each of the constitutive isoforms of NOS is increased during pregnancy, but declines during the third trimester. Bansal et al. (1997), showed that preterm labour was associated with lower concentrations of myometrial iNOS. These data suggest that the timing of parturition might be manipulated by the use of agents which will increase or decrease myometrial NOS expression. We suggest that the use of therapeutic agents which either liberate NO or alter myometrial NOS activity, should be targeted specifically to the myometrium. We have previously shown that nitric oxide donors induce cervical ripening in the first trimester of human pregnancy (Thomson et al., 1997b). If such a mechanism also operates in late pregnancy, then the systemic administration of nitric oxide donors, whilst inducing myometrial relaxation, would stimulate cervical ripening and therefore potentially promote preterm delivery. If, however, agents could be devised which would stimulate myometrial NOS expression specifically, these agents may be useful in the treatment of preterm labour, and reduce the substantial morbidity and mortality associated with this condition.

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References


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