Nitric oxide synthase distribution during implantation in the mouse

T.L. Purcell1, R. Given2, K. Chwalisz3 and R.E. Garfield1,4

1Departments of Obstetrics & Gynecology and 2Anatomy and Neurosciences, The University of Texas Medical Branch, Galveston, TX 77555, USA, and 3Research Laboratories of Schering AG, Berlin, Germany

The peri-implantation period is a critical time during murine development. Although the importance of nitric oxide has been demonstrated during gestation, its role in implantation has not been fully defined. The aim of this study was to quantify (by Western blotting) two prominent nitric oxide synthase (NOS) isoforms, inducible (iNOS) and endothelial (eNOS) and localize all three forms [iNOS, eNOS, and neuronal (nNOS)] by immunohistochemistry in uterine tissue from days 4 through 8 of pregnancy. By day 6, iNOS values were significantly elevated in implantation sites compared with interimplantation regions and continued to rise through day 8. Analysis of eNOS was similar, but implantation site values peaked by days 6 and 7. Labelled iNOS cells were within the decidua, around myometrial vessels, and within the ectoplacental cone. At implantation, eNOS was conspicuous, displaying label adjacent to the embryo in vessels of the primary decidual zone. nNOS was localized mainly in the mesometrium and myometrium and did not appear to change throughout the peri-implantation period. The increased iNOS and eNOS values following implantation in the embryonic site may imply roles in tissue remodelling, immunosuppression and vasoregulation. Nitric oxide may play an important role in the mechanisms of implantation where these factors are keys to successful pregnancy.

Key words: decidua/embryo/implantation/mouse/nitric oxide synthase

Introduction

The peri-implantation period is a critical time between fertilization and early development of the embryo. During implantation, the blastocyst undergoes initial inner cell mass differentiation and development to produce an epiblast and visceral and parietal endoderm, while the trophoblast initiates interactions with the uterus (Enders et al., 1978). The trophoblast–uterine interaction involves several progressive stages as the blastocyst moves into the endometrium. It becomes closely apposed to and then firmly attached to the apical surface of the uterine epithelium. The trophoblast then penetrates the uterine epithelium, underlying basement membrane components, and endometrial stroma and finally approaches the maternal capillaries (Schlaefke and Enders, 1975; Blankenship and Given, 1992). During this time, luteal hormones transform the surrounding uterine stroma into metabolically active, enlarged decidual cells (Abrahamsohn, 1983; Parr et al., 1986) and the uterine glands change their secretory pattern (Given and Enders, 1980, 1981). The decidualized cells form an expanding capsule around the implanting blastocyst (Schlaefke and Enders, 1975) and are also responsible, along with the uterine glands, for producing cytokines and growth factors that help nurture the developing embryo.

However, many steps in the regulation of implantation have not yet been clearly defined. The small but potent molecule, nitric oxide (NO), may be a common factor in several processes related to implantation. Evidence suggests that cytokines, prostaglandins, histamine, matrix metalloproteinases (MMPs), and hormones all play a role, and alone or in combination, may alter NO concentrations. NO is a biological free radical that shows chemical activity within numerous cell systems and is responsible for altering vasoactivity, smooth muscle contraction, neurotransmission (Moncada et al., 1991; Forstermann and Kleinert, 1995) and connective tissue remodelling (Owens et al., 1997). It has been shown to inhibit platelet aggregation, stimulate angiogenesis, reduce blood pressure, and alter the function of the immune system (Sooranna et al., 1995). l-arginine is converted by the enzyme nitric oxide synthase (NOS) into NO and citrulline. Three well-known isoforms of NOS, neuronal (nNOS), endothelial (eNOS), and cytokine-inducible NOS (iNOS), have been identified. The inducible form (iNOS) is calcium-independent and was first detected in macrophages. It may be induced by interferon-γ or lipopolysaccharides (LPS) (Herrero et al., 1996) and produces large quantities of NO for a long duration. The other two constitutive isoforms (nNOS and eNOS) require calcium/calmodulin for activation and produce much smaller quantities of NO for short periods (Berdeaux, 1993).

Since implantation has been compared to an inflammatory reaction, it is possible that the link between leukocytes, the decidual response, and suitable implantation may be NO, which is also known to be involved in inflammation. One cytokine, colony-stimulating factor-1 (CSF-1), has receptors that are highly expressed by trophoblast cells during implantation (Bartocci et al., 1986; Pollard et al., 1987). Several types
of CSF have been shown to up-regulate the production of NO via iNOS by alveolar macrophages (Iorens et al., 1993; Olivares et al., 1995; Blau et al., 1997). Cytokines also induce NO synthesis via iNOS (Herrero et al., 1996) or alter NO production and induce an inflammatory response by also activating the inducible cyclo-oxygenase isoform (COX-2), thus further stimulating prostaglandin production (Salvemini, 1997). Early work done by Shelesnyak suggested that histamine released from mast cells played a key pivotal role in initiating the decidual response (Psychoyos, 1986) and recently it has been shown to assist the implantation process in rodents by inducing macrophage production of NO. MMPs are also thought to play a role in implantation, since heightened expression of several prominent types of MMPs that aid in the invasive process have been observed during the peri-implantation period in mice (Sharkey et al., 1996). The result of this extracellular matrix (ECM) turnover may be the recruitment of degradative cell types such as macrophages and natural killer cells, which are also associated with elevated NO production. Furthermore, several studies indicate that NO directly regulates MMPs through feedback mechanisms (Chwalisz et al., 1996; Tamura et al., 1996; Okamoto et al., 1997), which may be a critical step in implantation. Other evidence suggests that NO may mediate the increased production of cyclic guanosine 3',5'-monophosphate (cGMP) and activate vasorelaxation (Berdeaux, 1993). Cyclic GMP injected intraluminally induced embryonic implantation in rats. Since cGMP is an intermediate in the NO effector pathway, other investigators suggest this enhancement was due to cGMP acting as a second messenger for oestrogen, which is essential for normal implantation in the mouse and rat (Hou and Gorski, 1993). Studies have shown that the increased concentrations of oestrogen later in pregnancy are associated with elevated NO and cGMP activity (Weiner et al., 1994; Rosenfeld et al., 1996). Progesterone in the rat has also been shown to influence NO values during mid- to late gestation (Yallampalli et al., 1994; Buhimschi et al., 1996).

NO is also important in early embryonic survival (Gouge et al., 1998). However, excessive NO values from exogenous NO donors was found to be harmful to preimplanting and implanting mouse embryos (Barroso et al., 1994; Blau et al., 1997). Cytokines also induce NO synthesis via iNOS (Herrero et al., 1996) or alter NO production and induce an inflammatory response by also activating the inducible cyclo-oxygenase isoform (COX-2), thus further stimulating prostaglandin production (Salvemini, 1997). Early work done by Shelesnyak suggested that histamine released from mast cells played a key pivotal role in initiating the decidual response (Psychoyos, 1986) and recently it has been shown to assist the implantation process in rodents by inducing macrophage production of NO. MMPs are also thought to play a role in implantation, since heightened expression of several prominent types of MMPs that aid in the invasive process have been observed during the peri-implantation period in mice (Sharkey et al., 1996). The result of this extracellular matrix (ECM) turnover may be the recruitment of degradative cell types such as macrophages and natural killer cells, which are also associated with elevated NO production. Furthermore, several studies indicate that NO directly regulates MMPs through feedback mechanisms (Chwalisz et al., 1996; Tamura et al., 1996; Okamoto et al., 1997), which may be a critical step in implantation. Other evidence suggests that NO may mediate the increased production of cyclic guanosine 3',5'-monophosphate (cGMP) and activate vasorelaxation (Berdeaux, 1993). Cyclic GMP injected intraluminally induced embryonic implantation in rats. Since cGMP is an intermediate in the NO effector pathway, other investigators suggest this enhancement was due to cGMP acting as a second messenger for oestrogen, which is essential for normal implantation in the mouse and rat (Hou and Gorski, 1993). Studies have shown that the increased concentrations of oestrogen later in pregnancy are associated with elevated NO and cGMP activity (Weiner et al., 1994; Rosenfeld et al., 1996). Progesterone in the rat has also been shown to influence NO values during mid- to late gestation (Yallampalli et al., 1994; Buhimschi et al., 1996).

Materials and methods

Animals

A total of 177 total timed pregnant ICR outbred white mice (25–35 g) were received from Charles River Laboratories (Wilmington, MA, USA) on day 2 of pregnancy. Mice were maintained on a 14 h light:10 h dark cycle and food and water ad libitum. The presence of the sperm plug was designated as day 1 of pregnancy. All procedures were approved by the Animal Care and Use Committee of The University of Texas Medical Branch.

Tissue collection

Mice (n = 20) were anaesthetized with sodium pentobarbital solution (2.5 mg/mouse) at noon on days 4, 5, 6, and 7. Between three and seven mice were collected from each time observed and used in the immunohistochemical and Western blot experiments. On day 4, the uteri were removed and the uterine horns were flushed with buffered saline to confirm pregnancy. Free blastocysts were collected and examined under a dissecting microscope for the numbers present and stage of development. Only uteri containing blastocysts were analysed. Mouse implantation sites were localized at 12:00 noon on days 5–7 by injection of 1% Evan’s blue dye in Hank’s balanced salt solution into the femoral vein. After 5 min, the lower abdomen was opened and the presence of implantation sites was indicated by blue swellings in the uterine horns. Both horns were removed and separated. One horn was fixed in a 4% paraformaldehyde fixative (30 min) followed by 0.1 M phosphate-buffered saline (PBS) with CaCl₂ (pH 7.5). Implantation sites were then separated, dehydrated in ethanol, embedded in paraffin, and later stained for the presence of NOS (see immunohistochemistry). The contralateral uterine horn was immediately separated into site and interimplantation site areas and then frozen in liquid nitrogen for later processing (see Western blot analysis).

Immunohistochemistry

Paraffin-embedded tissue was sectioned to a thickness of 6–7 µm and placed on Fisherbrand Superfrost/Plus microscope slides (Pittsburgh, PA, USA). Sections were then deparaffinized in xylene, rehydrated in a series of ethanol solutions, and stained using standard
immunohistochemistry procedures. Sections were incubated in PBS (pH 7.5) with 0.1% Triton X-100, quenched in 3% hydrogen peroxide, and non-specific labelling was blocked according to Zymed Histomouse SP Kit (Zymed, San Francisco, CA, USA). Sections were then incubated in rabbit polyclonal anti-NOS primary antibody (either iNOS, nNOS, or eNOS; Transduction Laboratories, Lexington, KY, USA) diluted 1:50 or 1:100 in a moist chamber overnight at 4°C, then washed in PBS and incubated in biotinylated secondary antibody according to the Zymed Histomouse SP Kit. Streptavidin–peroxidase was bound to the biotin of the secondary antibody. Peroxidase then converted the chromogen substrate to a red reaction product to localize the appropriate antigen. Sections of aorta, LPS-stimulated spleen, and mesometrium were used for positive controls for eNOS, iNOS, and nNOS respectively. Negative control slides were obtained by replacing the primary antibody with the same concentration of non-immune rabbit serum. Tissues were counterstained lightly with haematoyxlin and photographed under a Nikon microscope (Microphot-FXA; Nikon, Tokyo, Japan).

Western blotting

Tissue samples were immediately frozen in liquid nitrogen and stored at −70°C. Tissues were suspended in five volumes of 50 mM Tris-buffer (pH 7.4) containing 0.1 mM EGTA, 0.14 µl/ml ß-mercaptoethanol and one tablet of complete protease inhibitor (Boehringer Mannheim, Indianapolis, IN, USA) per 25 ml of total homogenizing buffer. Tissue was homogenized with a Polytron (Boehringer Mannheim, Indianapolis, IN, USA) per 25 ml of total β-mercaptoethanol and one tablet of complete protease inhibitor buffer (pH 7.4) containing 0.1 mM EGTA, 0.14 µM NaCl, 0.14 µM MgCl2, and 0.5% Triton X-100. Protein concentrations were measured with a BCA kit (Pierce, Rockford, IL, USA). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on a 7.5% polyacrylamide separating gel and a 4% stacking gel (Bio-Rad, Richmond, CA, USA). Aliquots of 10 µl cytosol, which contained 33 µg of total protein, were diluted 1:2 v/v with electrophoresis sample buffer (250 mM Tris–HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% Bromophenol Blue, and 2% ß-mercaptoethanol) and reduced by boiling for 5 min. Positive control samples (2 µl of mouse macrophage lysate for iNOS and 2 µl of human endothelial extract for eNOS; Transduction Laboratories, Lexington, KY, USA) were also loaded. After electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 100 V for 70 min and blocking with 1% bovine serum albumin (BSA), the blots were incubated with one of the anti-NOS isoforms (iNOS or eNOS). The following dilutions and exposure times were used: 1/1000, overnight at 4°C for iNOS or 1/500 for 1 h at room temperature for the eNOS. Even though at very high concentrations the antibody to iNOS shows a small amount of cross-reactivity to nNOS, each isoform can be separated based on its specific molecular weight: iNOS = 130 kDa; eNOS = 140 kDa; and nNOS = 155 kDa. The blots were examined by enhanced chemiluminescence (ECL) using a Dekmate III scanner and PDI 1D software package (PDI; Lexington, KY, USA) were also loaded. After electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 100 V for 70 min and blocking with 1% bovine serum albumin (BSA), the blots were incubated with one of the anti-NOS isoforms (iNOS or eNOS). The following dilutions and exposure times were used: 1/1000, overnight at 4°C for iNOS or 1/500 for 1 h at room temperature for the eNOS. Even though at very high concentrations the antibody to iNOS shows a small amount of cross-reactivity to nNOS, each isoform can be separated based on its specific molecular weight: iNOS = 130 kDa; eNOS = 140 kDa; and nNOS = 155 kDa. The blots were examined by enhanced chemiluminescence (ECL) using the Western blot detection system (Amersham, Arlington Heights, IL, USA). The enzyme conjugate antihuman immunoglobulin G (IgG: horseradish peroxidase) was used as a secondary antibody. Autoradiography film was exposed to the blot until satisfactory density was achieved. LuminoGraphs were densitometrically scanned and analysed using a Dekmate III scanner and PDI 1D software package (PDI; Huntingstation, NY, USA). The amounts were normalized against the optical density of the positive control band within the same gel to make gel-to-gel comparisons. In order to assess the linearity of the assay, different amounts of total protein (10, 20, 40, 60 µg) were loaded and the optical density readings were expressed as a function of the amount of total protein. The correlation coefficient (r²) for a linear fit was 0.99. Results were expressed as mean ± SEM. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by post-hoc tests using Fisher’s least significant difference. P < 0.05 was considered to be significant.

NOS inhibition by L-NAME

Pregnant mice (n = 123, Charles River Laboratories, Wilmington, MA, USA) were on day 2 of pregnancy. Of these, 80 were divided into three experimental groups (n = 26, n = 26, and n = 28) and given the NO inhibitor, L-NAME (2.5, 10, and 50 mg/mouse/day) that were allowed to develop until term. The number of live fetuses or pups was recorded as well as their weight. Those not delivering were killed on day 22 and uteri were examined for the presence of retained placentae, resorbed embryos or unusual scarring within the uterus. Random samples were collected for Western blot analysis and immunohistochemistry.

Effects at term of peri-implantation administration of L-NAME

The injection schedule described previously was repeated on 34 pregnant mice (12 saline-injected controls and 22 injected with 10 mg/mouse/day of L-NAME) that were allowed to develop until term. The number of live fetuses or pups was recorded as well as their weight. Those not delivering were killed on day 22 and uteri were examined for the presence of retained placentae, resorbed embryos or unusual scarring within the uterus. Random samples were collected for Western blot analysis and immunohistochemistry.

Results

Immunohistochemistry

Prior to implantation (day 4 of pregnancy), stromal staining for eNOS was not apparent. However, prominent staining developed in small capillaries of the primary decidual zone (Figures 1A and B) during and after implantation (days 6–8). Staining was also present in myometrial vessels at all time periods, as well as the basal portion of the luminal epithelial cells prior to implantation. Mouse aortic endothelial tissues were used as positive and negative controls (Figures 1C and D) and demonstrated appropriate labelling. Staining for iNOS was most apparent in leukocytes, typically those expressing iNOS in the uterus (macrophages, mast cells, and natural killer cells; Hunt et al., 1997) and found between the inner and outer muscle layers of the myometrium at all time points examined (Figure 2C). The decidual cells also displayed positive staining scattered throughout the decidual zone. At the time of implantation, distinctive labelling for iNOS was also apparent within cells of the ectoplacental cone (Figure 2A). As implantation progressed, the intensity of this label became more prominent (Figure 2B). There was also a slight increase in iNOS positive cells within the stroma near the embryo (Figures 2A and B). LPS-stimulated mouse spleen tissue demonstrated the presence and absence of iNOS labelling in positive and negative controls, respectively (Figures 2D and E). Specific staining for nNOS was located primarily within the mesometrium (Figure 3A), in the outer myometrial muscle layer (Figure 3B), and no
major differences were observed in the time periods examined. All control tissues showed the appropriate staining (Figure 3C).

**Western blots**

The two most prominent isoforms during peri-implantation of the mouse, iNOS and eNOS, were quantified in uterine tissue, implantation sites, and interimplantation site segments. The band labelled 130 kDa was attributed to iNOS and was aligned with our macrophage lysate positive control (Figure 4A). All samples ($n = 3–7$ per gel) were compared with a macrophage positive control as well as a non-pregnant mouse uterine sample. The control, non-pregnant sample, and separating broad range standard were run in each gel. Six gels (10 lanes per gel) were run comparing sites and interimplantation site segments of the same gestational age. The day 5 site and interimplantation site concentrations of iNOS were statistically

![Figures 1–3. For legend see facing page](https://academic.oup.com/molehr/article-abstract/5/5/467/1013678)
the same, but on days 6, 7, and 8, iNOS values were significantly higher in the implantation sites compared with the interimplantation sites (Figure 5). This difference increased with gestational age (day 8 showed the greatest differences). Values of iNOS in pregnant, day 4 uterine tissue (prior to implantation) were also measured and compared with the number of blastocysts flushed from the uterus. There was no correlation between the amount of iNOS present and the embryo number. However, on average, values of iNOS were higher in pregnant, day 4 uterine tissue than in all interimplantation sites. These results demonstrate that iNOS is present in increasing amounts in the implantation sites, compared with the interimplantation sites, and it is up-regulated throughout the progression of embryonic implantation.

The same tissue and gel protocol was used for Western blot analysis of eNOS. However, human endothelial cell extract was used as the positive control for eNOS and aligned to a band at 140 kDa (Figure 4B). Results from examination of eNOS values were similar to that of the iNOS study: Implantation site tissue contained higher values of eNOS than interimplantation sites at all gestational ages. These differences were statistically significant from days 6–8 (Figure 6). Again, no correlation existed between blastocyst number and eNOS values in day 4 uteri.

An obvious difference in the pattern of iNOS and eNOS isoforms was that iNOS values in implantation sites continued to increase throughout the peri-implantation period, days 4–8 (Figure 5), while eNOS values of the same tissue peaked at

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**Figure 1.** Endothelial nitric oxide synthase (eNOS) labelling following implantation. (A) Day 6 mouse implantation site showing label for eNOS in areas peripheral to the embryo (E) and in the primary decidual zone (D). Staining surrounding small capillaries within this area was only apparent during and after implantation (original magnification ×210). (B) Day 8 implantation site also showing staining for eNOS in the primary decidual zone. Label appears around vessels (arrows), but not within the embryo itself (original magnification ×275). (C) Positive control of mouse abdominal aorta showing strong label for eNOS over the endothelium (original magnification ×550). (D) Negative control abdominal aorta in which non-immune rabbit serum replaced the primary antibody (original magnification ×550).

**Figure 2.** Inducible nitric oxide synthase (iNOS) labelling following implantation. (A) Day 6 implantation site showing distinct cells labelled for iNOS within the ectoplacental cone (EC) (original magnification ×275). E = embryo. (B) Day 8 implantation site showing label for iNOS in the ectoplacental cone (EC) and in the decidua (arrows) surrounding the embryo (E). Note the increased intensity of iNOS staining on day 8 (original magnification ×140). (C) Positive cells stained for iNOS in tissue around blood vessels in the myometrium (non-pregnant uterus). O = outer longitudinal muscle layer (original magnification ×275). (D) Mouse spleen tissue stained as positive control for iNOS. The mouse was injected with lipopolysaccharide (LPS) 6 h prior to tissue harvest. Note the distinctive dark chromogen label (original magnification ×275). (E) The same mouse spleen tissue as above (D) prepared as negative control for iNOS. The primary antibody was replaced with non-immune rabbit serum (original magnification ×275).

**Figure 3.** Neuronal nitric oxide synthase (nNOS) labelling following implantation. (A) Day 6 mouse mesometrium demonstrating concentrated label for nNOS. This localization is apparent throughout the observed early stages of pregnancy and does not appear to change (original magnification ×275). (B) Day 8 mouse myometrium displaying nNOS label in the outer longitudinal muscle (O), which is observed throughout the peri-implantation period. I = inner circular muscle (original magnification ×140). (C) Day 6 mouse mesometrium stained as a negative control. No labelling occurred in areas known to contain nNOS (original magnification ×275).

**Figure 4.** Western blots for (A) inducible nitric oxide synthase (iNOS) and (B) endothelial NOS (eNOS). These Western blots demonstrate the presence of the specific (A) iNOS and (B) eNOS isoforms respectively. Each Western blot represents random samples for each particular day in a given gel. Luminochromes were densitometrically scanned and analysed. NP = non-pregnant uterus; std = broad range standard; d = gestational day; b = between sites (implantation site); I = iNOS control (mouse macrophages); E = eNOS control (human endothelium).

**Figure 5.** Peri-implantation graph of inducible nitric oxide synthase (iNOS). iNOS values on day 4 were higher than non-pregnant and interimplantation tissue, and were comparable with day 5 tissue. On days 6–8, iNOS values were significantly higher in implantation sites compared to interimplantation site regions. This difference increased with gestational age. D = days.
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Figure 6. Peri-implantation graph of endothelial nitric oxide synthase (eNOS). There was no correlation in eNOS values between day 4 uterine tissue and blastocyst number. Implantation site tissue contained higher values of eNOS than interimplantation regions, with differences that were statistically significant on day 6 and later. Values of eNOS within implantation sites peaked at day 6, remained elevated (day 7), then declined on day 8 of pregnancy.

Figure 7. The effect of NG-nitro-L-arginine methyl ester (L-NAME) on implantation. Inhibition of nitric oxide synthase (NOS) activity occurred with the administration of L-NAME. Three different concentrations of L-NAME (2.5, 10, and 50 mg/mouse/day) injected during the peri-implantation period reduced the number of blastocysts implanting in a dose-dependent manner when compared with controls on day 7.

day 6 and 7 (Figure 6). By day 8, eNOS had significantly declined.

NOS inhibition by L-NAME

Saline-injected control mice demonstrated a high incidence of pregnancy (Figure 7). However, when pregnant mice were administered L-NAME (day 3 through day 6) in increasing concentrations (2.5, 10, and 50 mg/mouse/day), the pregnancy rate (numbers of mice that contained implantation sites on day 7) dropped in a dose-dependent fashion. The 2.5 mg dose schedule reduced the implantation percentage slightly but was not statistically significant. Implantation percentage was significantly reduced when 10 mg/mouse/day was administered. The most dramatic results were seen in the mice receiving 50 mg L-NAME dosage where implantation was reduced by 48%, compared with the saline control mice. However, pregnant mice whose embryos progressed past the window of implantation (observed at 12:00 noon on day 7) did not appear to be reduced in size or number of potential implanting offspring.

Effects at term of peri-implantation administration of L-NAME

In the extended study of pregnant females injected with 10 mg of L-NAME/mouse/day on days 3–6, only 16 of the 22 L-NAME-treated mice (10 mg/mouse/24 h) were pregnant (73%), while 11 out of 12 control animals (92%) were pregnant. This represents a 19% reduction from that of the control, comparable with the 21% drop found in the previously mentioned 10 mg dosage study (Figure 7). The average fetal or pup weight on day 19 was similar in both the controls (mean, 1.47 g) and the treated mice (1.53 g). Of the 16 females that were pregnant in the treated group, five pregnant mice had some type of anomaly. Two mice had undelivered placentas (three in one mouse, two in the other, i.e. five in total), two mice had resorbed embryos still present in utero (one in one mouse, three in the other, four uterine total), and one animal had three unusual scars present with a transparent appearance.

Discussion

NOS appears to play an important role in the peri-implantation development of the mouse. All three NOS isoforms are: (i) present within the mouse implantation site, with iNOS and eNOS being the most prominent in the ectoplacental cone as well as the layers of the myometrium and the primary decidual zone respectively; (ii) highly expressed in the implantation site regions when compared with the interimplantation site areas; and (iii) elevated during the peri-implantation period (days 6–8). Furthermore, implantation is inhibited in a dose-dependent manner when a NOS inhibitor (L-NAME) is administered during this time. Western blot and immunohistochemistry demonstrate that NO may be involved in the trophoblast-uterine interaction during implantation and could modulate such processes as the inhibition of uterine contractility, vasodilation, angiogenesis, modulation of the ECM, and suppression of the immunological response to the fetal allograft.

The iNOS label was most apparent in the ectoplacental cone and between the inner and outer muscle layers of the myometrium during the peri-implantation period. Staining for iNOS was also visible throughout the uterine stroma where clusters of labelled cells appeared to increase and relocate adjacent to the embryo as pregnancy continued. Western blots also indicated that the iNOS values were significantly higher in the implantation sites compared to the interimplantation site regions. These differences increased with gestational age, peaking on day 8.

These findings support a role for iNOS in producing large quantities of NO which may be necessary for producing a localized relaxation of the myometrium and to facilitate attachment during implantation. Previous studies have shown
that NO and NOS values are elevated in the rat uterus during late pregnancy which may promote smooth muscle relaxation. These values drop prior to, and during, labour in rats and rabbits which are correlated with low NO values and subsequent uterine contraction in preparation for labour (Natuzzi et al., 1993; Huang et al., 1995; Buhimschi et al., 1996; Sladek and Roberts, 1996). The uterine response at implantation has also been compared with an inflammatory response, and NO produced at this time may play a key role in the inflammatory events and immunosuppression. The expression of the iNOS gene has been shown in the mouse uterine leukocytes during pregnancy, with values most elevated in mid-gestation and down-regulated in later stages of pregnancy (Hunt et al., 1997). The elevated NO values may be responsible for the local oedema and inflammation and may be correlated with an increase of leukocytes (known sources of iNOS) in the area of the primary decidual zone.

The iNOS isoform is calcium/calmodulin-independent and produces large quantities of NO within tissue lasting from several hours to several days when induced by lipopolysaccharides, γ-interferon, or other endotoxins (Moncada et al., 1991; Chwalisz et al., 1996). Other studies indicate that the large production of NO may contribute to the immune response (Hunt et al., 1997) by ridding the host of foreign bacteria and parasites (Forstermann and Kleinert, 1995). Inflammatory cells, including macrophages, natural killer cells, neutrophils and B-cells, were positively expressed at the feto–maternal interface (Duclos et al., 1994). These cells are commonly associated with iNOS and NO production (Hunt et al., 1997).

The most dramatic change observed in our studies was immunohistochemical staining for eNOS in the area surrounding the embryo on day 6. Dark prominent label appeared in the primary decidual zone. Western blot quantification revealed that relative protein values of eNOS were similar to that of iNOS. Values of eNOS were significantly higher in implantation sites by day 6, remained elevated, and were significantly increased when compared to interimplantation site tissue on days 6–8. During this time, crucial changes occur in the maternal vasculature adjacent to the implantation site as maternal capillaries expand in the area adjacent to the embryo (Blankenship et al., 1990; Blankenship and Given, 1992). However, by day 8, the overall values of eNOS decreased in both implantation sites and interimplantation sites. These findings suggest that eNOS, which has been mainly associated with the endothelium of blood vessels, might regulate the formation of newly established capillaries. Significant ECM changes also occur at the implantation site, and the presence of eNOS juxtaposed to the embryo suggests that NO production may be involved in the remodelling of the ECM as well as establishment of the maternal–fetal vasculature. Papapetropoulos et al. (1997) showed when NO production was inhibited by the use of L-NAME, capillary tube formation by rat endothelial cells was also inhibited in vitro and reversed by the addition of a NO donor (L-arginine). Other work also suggests that NO is involved in the remodelling during angiogenesis (RayChaudhury et al., 1996). eNOS has also been implicated in increased blood flow to the embryo, especially in the area adjacent to the implanting embryo (Rogers, 1995). NO is also known for its strong vasodilation affects (Palmer et al., 1987; Moncada et al., 1991). Other evidence supports the role of NO as one of the key players in modulating the perfusion of the placenta (Purcell et al., 1997), while lack of NO may be partially responsible for pre-eclampsia and fetal growth retardation (Yallampalli et al., 1994; Liao et al., 1996).

Labelling of nNOS was not as prominent as the other isoforms and did not appear to fluctuate during the implantation period. Persistent sparse staining for nNOS was found within the mesometrium and outside the outer longitudinal myometrial muscle layer. Since negligible amounts of nNOS were detected in the Western blot analysis as well, these results indicate that nNOS is not a large contributor to the production of uterine NO. This is consistent with previous studies where the presence of nNOS was detected only in the uterine tissue of non-pregnant animals. Magness et al. (1997) characterized the protein expression of the constitutive NOS isoforms in uterine and systemic arteries in sheep. Although eNOS was detected and localized within both arterial systems, nNOS was not detected (Magness et al., 1997). Buhimschi et al. (1996) also found that only the nNOS isoform was lacking in the rat uterus during gestation, although it has been identified in the epithelium of cycling rat uterus utilizing NADPH diaphorase staining and immunohistochemistry (Schmidt et al., 1992).

Inhibition of NO production by L-NAME had a dramatic effect on early pregnancy. The number of pregnant mice with implating blastocysts decreased in a dose-dependent fashion when L-NAME was administered to pregnant mice during gestation days 3–6. The number of pregnant mice with implanting embryos was most significantly reduced after administration of high doses of L-NAME (50 mg); implantation was diminished to 48% of the saline control group.

When pregnant mice which were given L-NAME (10 mg/mouse/day) during the peri-implantation period and the fetuses were allowed to develop to term, the pregnancy rate was reduced from 92% in the saline control animals to 73% of the L-NAME-treated animals. Pregnant mice with implanted embryos that reached term did not appear to have a reduced fetal number and gestation sites were of normal size when compared to controls. However, of the 16 L-NAME-treated females, five mice developed conspicuous complications including undelivered placentas, resorbed embryos, and/or unusual uterine scarring.

Inhibition of NO production during the peri-implantation period appeared to affect the maternal and embryonic interactions in a dose-dependent manner. Fewer females maintained their pregnancy during implantation when NO production was blocked. If the process of implantation was altered critically by low NO values, then the young embryos did not implant. However, if implantation was not affected, L-NAME-treated mice at term showed similar findings to the controls. Inhibition of NO was sometimes expressed as anomalies in the uterus, placenta and pups.

L-NAME is commonly known for its ability to elevate blood pressure in late pregnant rats by the inhibition of NO production (Buhimschi et al., 1995; Nathan, 1995; Yallampalli et al., 1996). L-NAME has been shown to reduce the efficacy
of pregnancy by decreasing the number of pups per litter or the fetal weight, or by increasing uterine growth retardation in the rat (Helmbrecht et al., 1996; Liao et al., 1996). L-NAMe also alters the production of NOS activity and certain prostaglandins (PGE and PGF2) in the rat in early pregnancy, which may also influence uterine receptivity (Novaro et al., 1996). In another study, a reduction was also found in the amount of implanting rat embryos following L-NAMe administration (Novaro et al., 1997). However, intraluminal administration of the drug may have altered implantation due to disruption and mechanical problems.

Our results suggest a significant role for NO in the process of implantation and early pregnancy in the mouse. It may be critical for maintaining a relaxed state of the uterus, facilitating proper attachment and orientation of the blastocyst, and sufficient perfusion of the embryo. Most of the previous studies of NO have focused on the later stages of pregnancy. In one study, NO has been shown to activate guanylate cyclase, increase cGMP, and stimulate smooth muscle relaxation (Weiner et al., 1994). NO has also been shown in the uterus during mid- to late gestation (Buhimschi et al., 1996). NO and NOS values are elevated during pregnancy which promotes vasodilation and smooth muscle relaxation. These values drop dramatically prior to, and during, labour in rats and rabbits; and this implies a correlation between uterine contraction of the smooth muscle, vascular activity, and preparation of pregnancy termination (Huang et al., 1995; Chwalisz et al., 1996). However, in most of the NO studies, the focus has been on the later stages of pregnancy where NO production has already declined, indicating the drop in NO synthesis is important in preparation for parturition (Thomson et al., 1997).

Certain cellular events of implantation are critical for survival and include the breakdown of uterine stroma, penetration through maternal ECM, and formation of fetal–maternal blood vessels. NO may alter uterine function and ECM degradation by stimulating enzyme production and release from adjacent cells as well as recruiting cells (e.g. leukocytes and macrophages, which enhance NO production) into the tissue surrounding the implantation site. Several studies link NO production directly to the regulation of MMP activation and hence ECM remodelling (Chwalisz et al., 1996). Chondrocytes stimulated with interleukin-1 (IL-1) produced large amounts of NO; this, in turn, stimulated the production of two MMPs, MMP-2 and MMP-9, which resulted in ECM breakdown (Tamura et al., 1996). Evidence shows that MMPs, other cytokines, and growth factors including IL-1, CSF-1, leukaemia stimulating factor (LIF), and epidermal growth factor (EGF) are up-regulated during implantation (Bartocci et al., 1986; Pollard et al., 1987; Stewart et al., 1992; Das et al., 1994; Sakkoula et al., 1997) and may increase NO production via increased NOS. This increased production of NO during peri-implantation may help direct the regulation of ECM breakdown necessary for invasion of the embryo into the uterine stroma.

Sex steroids, especially progesterone and oestrogen, may also influence NO production. Inhibition of progesterone and NO production was devastating to embryonic development and implantation in the rat (Chwalisz et al., 1999). Progesterone appears to have the strongest influence over the production of NO in rat uterine tissue and blood vessels during late pregnancy (Buhimschi et al., 1996). At term, when oestrogen concentrations begin to rise, NO values drop along with progesterone concentrations then myometrial activity resumes (Yallampalli et al., 1994). Oestrogen has been shown not only to aid in uterine receptivity of the blastocyst (Stewart and Cullinan, 1997) but also to increase the constitutive forms of NOS (eNOS and nNOS) and their mRNA in both vascular and non-vascular tissue of the guinea pig. Both oestrogen and progesterone may cause changes in the uterus and the embryo that allow for the establishment of successful implantation (Hou and Gorski, 1993; Chwalisz et al., 1995). Steroid hormones also regulate the production and action of certain MMPs and tissue inhibitors of MMPs (TIMPs) during ECM remodelling of implantation (Edwards, 1995). NO production may be the intermediate pathway affecting this tissue turnover as well as affecting vasoactivity.

There is also strong evidence of the interaction between prostaglandins and the success of implantation (Jacobs et al., 1996). Indomethacin, a prostaglandin inhibitor, administered during peri-implantation can block implantation in rodents (Jacobs et al., 1994). L-NAMe, a general NOS inhibitor, was able to prevent the rise in PG, and if embryos were not present, the rise in prostaglandins did not occur (Novaro et al., 1996). NO production, via iNOS, may also be induced by other cytokines, prostaglandins, and other growth factors (Herrero et al., 1996). Since certain cytokines and hormones are present in elevated concentrations during implantation, the interaction of these molecules in concert with the production of NO may be critical in the process of implantation.

References


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NOs distribution during implantation in the mouse

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