Expression of the calcium-independent cytokine-inducible (iNOS) isoform of nitric oxide synthase in rat placenta

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The presence of the calcium-independent cytokine-inducible nitric oxide synthase (iNOS) has been investigated in rat placenta from day 19 of gestation till delivery. iNOS has been detected at the mRNA, enzyme activity and protein levels in complete placenta. Immunocytochemical detection of iNOS was heterogeneously distributed in control placenta. Intraperitoneal injection of pregnant rats at 21 days of gestation with lipopoly-

saccharide (LPS) increased the iNOS immunoreactivity in the decidua basalis of the placenta, and, when the mRNA levels and enzyme activity were measured in total tissue, a moderate increase (approx. 160 %) was observed. A constitutive nuclear factor κ B activity was observed in placenta from both control and LPS-treated animals. These results indicate constitutive expression of iNOS in rat placenta.

INTRODUCTION

Nitric oxide (NO) is a messenger molecule playing key roles in multiple physiological processes [1]. Synthesis of NO from L-arginine is catalysed by NO synthase (NOS; EC 1.14.13.39). At least three different NOS isoenzymes have been identified in mammalian tissues, exhibiting an important degree of tissue-specific expression as well as major differences in their regulatory properties [1–3]. Two constitutively expressed Ca^{2+} /calmodulin dependent NOS isoforms are mainly present in brain (nNOS) and endothelia (eNOS), and share 60 % homology at the amino acid sequence level. A third form, cytokine-inducible Ca^{2+} -independent NOS (iNOS), is expressed in cells such as macrophages, neutrophils and hepatocytes, after stimulation with lipopolysaccharide (LPS) and pro-inflammatory cytokines. Three distinct cDNAs encoding neuronal [4], endothelial [5,6] and macrophage forms of NOS [7–9] have been isolated.

It has been suggested that increased NO production plays a role in the haemodynamic accommodation characteristic of pregnancy [10]. Increased NO biosynthesis has been detected in both pregnant rats and humans [10,11]. During pregnancy NOS activity is expressed in the placental villous tree where it may act to regulate placental blood flow, fetal nutrition and growth. This NOS activity correlates with plasma progesterone levels in the uterus and decidua preterm [11,12]. Chronic reduction in NO production in rats during the last third of pregnancy resulted in significant intrauterine growth retardation and haemorrhagic foci in fetal hind limbs [13].

The NOS isoenzymes present in the placenta responsible for continuous NO synthesis have been characterized using various complementary approaches, including measurement of the enzyme activity [11], the use of more or less specific NOS inhibitors [10,13] and immunohistochemistry [10,11]. Most of the research in this field has focused on the distribution and activity of the eNOS isoenzyme [11]. However, the presence of an important Ca^{2+} -independent NOS activity has been reported [14,15].

The placenta has a number of important functions during pregnancy, both biological and immunological. As expected

from the anatomical structure, the presence of constitutive eNOS in human placental villous tissue, endothelium of the umbilical artery and vein, and the trophoblast has been reported [15–17]; however, few data are available describing the presence and function of the iNOS in placental tissue.

The present study was undertaken to investigate the presence of iNOS in complete placenta from pregnant rats and to compare the abundance of this isoenzyme with respect to eNOS. Our results show that iNOS is significantly expressed in the placenta and the possible mechanisms supporting the expression of this isoform are discussed.

MATERIAL AND METHODS

Chemicals

[α -³²P]Deoxycytidine triphosphate (3000 Ci/mmol) and [U-¹⁴C]arginine (292 mCi/mmol) were from Amersham International (Little Chalfont, Bucks., U.K.). Restriction enzymes were from Boehringer (Mannheim, Germany). Other chemicals and biochemicals were from Sigma (St. Louis, MO, U.S.A.) and Merck (Darmstadt, Germany). Chemicals for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.). LPS was from *Salmonella typhimurium* (Sigma).

Animals and preparation of cells

Pregnant albino Wistar rats (300–350 g), fed *ad libitum* on a standard laboratory diet, were used. Pregnancy and gestational age were determined by standard criteria [18], and the animals were killed between 08:30–09:30 h. Placenta were removed by Caesarean section after Nembutal anaesthesia of the dam. Except where stated, the complete placenta without the umbilical vein was used in the preparation of the samples used. The placenta was quickly freeze-clamped in liquid N₂.

Samples for immunohistochemistry were collected as follows: deeply anaesthetized animals (intraperitoneal injection of

Abbreviations used: Ab, antibody; DTE, dithioerythritol; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; NF, nuclear factor; NOS, nitric oxide synthase.

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2.5 ml/kg body weight of Equithesin; Janssen Laboratories) were ventilated through a tracheal cannula and perfused through the left ventricle with 50 ml of 0.9 % saline as a vascular rinse, followed by 500 ml of fixative solution (4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The placenta was removed, cut into 4–5 mm coronal blocks, and post-fixed for 4 h with fixative solution. LPS was injected intraperitoneally at 1 mg/kg body weight for the indicated period of time.

Resident peritoneal macrophages were prepared from rats lightly anaesthetized with ether as described [19]. Rat endothelial cell cultures were prepared as described previously [20]. Animal care was as indicated in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, U.S.A. (N.I.H. 80-23).

iNOS enzyme activity assay

Placenta from control or LPS-treated pregnant rats were homogenized in 4 vol. (w/v) of a medium containing 20 mM Tris/HCl (pH 8), 1 mM dithioerythritol (DTE), 10 μ M 6R-5,6,7,8-tetrahydro-L-biopterin (Dr. B. Schirck's Laboratories, Jona, Switzerland), 10 μ M leupeptin and 0.5 mM PMSF. The homogenate was centrifuged at 105 000 g for 30 min and the supernatant was subjected to 2',5'-ADP-Sepharose chromatography to partially purify NOS [19,21]. NOS activity was followed by the production of [U-¹⁴C]citrulline from [U-¹⁴C]arginine [21]. The reaction mixture contained 50 μ M [U-¹⁴C]arginine (0.3 μ Ci), 20 mM Hepes (pH 7.5), 1 mM EGTA, 0.5 mM NADPH, 0.1 mM DTE and 10 μ M 6R-5,6,7,8-tetrahydro-L-biopterin (200 μ l final volume). After incubation for 10 min at 30 °C, an ice-cold solution of 10 mM EGTA/0.1 mM citrulline/10 mM Pipes, pH 5.5 (1 ml) was added and 1 ml of this mixture was applied to a Dowex AG 50W-X8 (Na⁺ form) column (1 ml vol.). [U-¹⁴C]-Citrulline was eluted with water (3 ml) and the radioactivity in the eluate was estimated by scintillation spectroscopy. The enzyme activity was expressed as the difference in product formation in the absence or presence of 0.25 mM *N*-methyl-L-arginine in the reaction mixture [22].

Western blot analysis of iNOS and eNOS

Placenta, cultured resident peritoneal macrophages from control or LPS-treated rats and cultures of rat vascular endothelial cells were each homogenized in a medium containing 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM β -mercaptoethanol, 200 mM NaCl, 2 μ g/ml leupeptin, 50 μ g/ml tosyl-lysylchloromethane, 100 μ g/ml PMSF and 0.5 % (v/v) Triton X-100 at 4 °C. After centrifugation at 105 000 g for 30 min, the protein content of each supernatant was determined and 1 ml portions, containing the same amount of protein, were each mixed with 100 μ l of an ADP-Sepharose 4B suspension [1:1 (w/v) in the same medium]. After thorough mixing for 30 min at 4 °C, the ADP-Sepharose gel was washed $\times$ 3 with 1 ml of PBS and the gel suspension was centrifuged in an Eppendorf centrifuge for 15 min. The pellets were boiled in Laemmli sample buffer [250 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol and 2 % β -mercaptoethanol] (150 μ l), and proteins were separated by SDS/PAGE (10 % gels) [23]. The proteins were transferred to poly(vinylidene difluoride) membranes (Millipore) (3 mA/cm² for 4 h), hybridized with either the anti-murine iNOS antibody (Ab), as recommended by the supplier (Transduction Laboratories), or with the anti-bovine eNOS Ab (generously given by Dr. S. Lamas, Centro de Investigaciones Biológicas, Madrid), and detection was achieved using enhanced chemiluminescence (Amersham International). The iNOS Ab (used at 1:2500 dilution) recognized a common band in extracts from mouse and

rat peritoneal macrophages, which had been stimulated for 24 h with 1 μ g/ml of LPS, but not in the corresponding unstimulated cells. The eNOS Ab (used at 1:1000 dilution) recognized a 140 kDa band in both bovine and rat cultured endothelial cells, but not in mouse or rat macrophages.

Northern blot analysis

Total RNA was extracted by the guanidinium isothiocyanate method and aliquots of RNA (30 μ g) were separated by electrophoresis in a 0.9 % (w/v) agarose gel containing 2 % (v/v) formaldehyde (20 mA for 15 h) [24]. The RNA was transferred to a Nytran membrane (NY 13-N; Schleicher & Schuell, Dassel, Germany) using 10 $\times$ SSC (1.5 M NaCl/0.3 M sodium citrate, pH 7.4). The membrane was prehybridized and the levels of iNOS and eNOS mRNA were determined using *Eco*RI-*Hind*II and *Eco*RI fragments from the iNOS (murine) and eNOS (bovine) cDNAs, respectively [5,7], labelled with [α -³²P]dCTP using the Rediprime labelling kit (Amersham International) as probes. The membranes were washed with 0.1 $\times$ SSC and 0.1 % (w/v) SDS at room temperature for 10 min and twice at 50 °C for 30 min, before being exposed to X-ray film (Hyperfilm). Quantification of the NOS mRNA was performed by laser densitometry (Molecular Dynamics) using, as a control, a probe specific for 18 S rRNA inserted into a PBR322 plasmid. The specificity of the probes was assessed by comparing the hybridization of LPS-activated murine macrophages (iNOS) and bovine endothelial cells (eNOS) [5,19].

Preparation of nuclear extracts

A modified procedure based on the method of Costa et al. was used [25]. Placenta from control or LPS-treated rats were homogenized in 8 vol. (w/v) of buffer A [10 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTE, 0.5 mM PMSF, 2 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 μ g/ml tosyl-lysylchloromethane, 5 mM NaF, 1 mM NaVO₄, 10 mM Na₂MO₄, 0.15 mM spermine, 0.5 mM spermidine, 0.5 M sucrose, 50 μ g/ml trypsin inhibitor]. After incubation for 10 min at 4 °C, Nonidet P-40 was added [0.5 % (v/v) final concentration]. The tubes were vortexed gently for 15 s and the nuclei were sedimented by centrifugation at 800 g for 15 min at 4 °C. The supernatants were stored at –80 °C (cytosolic extract); the nuclei pellets were resuspended in 250 μ l of buffer A supplemented with 20 % (v/v) glycerol and 0.4 M KCl and incubated for 30 min with gentle vortexing. Nuclear proteins were separated by centrifugation at 13 000 g for 15 min and aliquots of the supernatant were stored at –80 °C. Proteins were measured using the Bio-Rad (Richmond, CA, U.S.A.) protein reagent [26]. All steps of cell fractionation were carried out at 4 °C.

Electrophoretic mobility shift assay (EMSA)

Synthetic oligonucleotides corresponding to the proximal κ B motif of the iNOS promoter were prepared using a Pharmacia oligonucleotide synthesizer [27,28]. The oligonucleotide (5'-CCAACTGGGGACTCTCCCTTTGGGAACA-3') was annealed after incubation for 5 min at 85 °C in 10 mM Tris/HCl (pH 8), 50 mM NaCl, 10 mM MgCl₂ and 1 mM DTE. Aliquots of 100 ng of annealed oligonucleotides were end-labelled with Klenow enzyme fragment in the presence of 50 μ Ci of [α -³²P]dCTP and the other unlabelled dNTPs in a final volume of 20 μ l. The oligonucleotides were precipitated with ethanol and extracted with phenol/chloroform (1:1). The DNA probe (10⁵ d.p.m.) was used for each binding assay as follows: 8 μ g of

protein extract were incubated for 15 min at 4 °C with the DNA and 2 µg/ml of poly(dI:dC), 5 % glycerol, 1 mM EDTA, 100 mM KCl, 5 mM MgCl₂, 1 mM DTE and 10 mM Tris/HCl, pH 7.8, in a final volume of 15 µl. The incubation mixture was applied to a 6 % (w/v) polyacrylamide gel which had been electrophoresed previously for 30 min at 100 V. The gels were run at 0.8 V/cm² in 45 mM Tris/borate, pH 8.0, transferred to 3MM Whatman paper, dried under vacuum at 80 °C and exposed to X-ray film.

RNAse protection assay

An antisense riboprobe was generated using T7 RNA polymerase after subcloning a fragment of 450 bp in a pGEM5ZF vector (Promega) and linearization with *Hind*III [7]. The cRNA contained 450 bp and was complementary to bases 161–614 of the murine iNOS mRNA. Total RNA (5 µg) from macrophages, macrophages treated with LPS or placenta (30 µg) were incubated at 85 °C for 5 min with a molar excess (800 000 counts/min) of the riboprobe and hybridized at 45 °C overnight. A mixture of RNase T (2 µg/ml) and RNase A (40 µg/ml) was used to degrade the single-stranded unhybridized probe for 1 h at 30 °C, and the digested products were analysed on a 6 % (w/v) acrylamide/8 M urea gel. The gel was dried and autoradiographed.

Immunohistochemistry

Fixed placenta from control and LPS-treated animals were frozen and serial 40-µm-thick frontal sections were cut with a Leitz sledge microtome. Free-floating sections were incubated with iNOS affinity-purified polyclonal antiserum (Transduction Laboratories). The sections were processed by the avidin–biotin peroxidase complex (ABC) procedure [29,30] to visualize immunoreactive sites. Control procedures were carried out in two animals, and no immunolabelling was observed when the primary antibody was omitted or replaced with an equivalent concentration of preimmune and normal rabbit serum.

Data analysis

All experiments were carried out by assaying the samples in duplicate or triplicate. Statistical analyses were performed by the Student's *t* test for paired data (parametric test) and by the Wilcoxon test (non-parametric values). *P* < 0.05 was considered significant.

RESULTS

Presence of iNOS in placental tissue

Total RNA was extracted from complete placenta of rats at day 20 and 21 of gestation and was hybridized with iNOS and eNOS probes. As shown in Figure 1(A), the placental tissue exhibited a band (4.4 kb) with the iNOS probe, whereas in cultured rat vascular endothelial cells the levels of iNOS mRNA were undetectable. In a positive control, an intense band of iNOS was observed in mRNA samples from peritoneal rat macrophages treated with LPS. The same samples were used to determine the amount of eNOS and only rat endothelial cells exhibited an intense band when hybridized with the corresponding eNOS probe. However, overexposure of the film revealed, as expected from results from other groups [11], a faint band in placenta but not in macrophages (results not shown). These data suggest that in complete placenta the iNOS mRNA is more abundant than that of the eNOS isoenzyme. This was also observed at the protein level where the amount of immunoreactive iNOS

Figure 1 mRNA and protein levels of iNOS and eNOS in placenta

Total RNA and protein extracts were prepared from complete placenta on days 20 or 21 of gestation, as indicated. Rat peritoneal macrophages (Mφ) were cultured for 18 h in the absence or presence of 0.5 µg/ml of LPS. Rat vascular endothelial cells were cultured until confluent. (A) Total RNA (30 µg) from each preparation was used to determine the content of iNOS and eNOS mRNA by Northern-blot analysis. Normalization of lane charge was carried out by hybridization with an 18 S ribosomal probe. (B) Protein (10 µg) from each preparation was used to determine the iNOS and eNOS protein content by Western-blot analysis. The results shown are representative of four experiments.

correlated with the corresponding mRNA levels (Figure 1B). Moreover, when an eNOS specific antibody was used, the amount of protein detected in extracts from complete placenta was very low when compared with the relative abundance in the cultured rat endothelial cells.

Despite the correlation between the mRNA and protein levels of iNOS it was important to establish that the mRNA detected in placenta with the iNOS probe effectively corresponded to this isoenzyme. To do this, RNA from LPS-stimulated placenta and macrophages, which produced a similar band in Northern blot when hybridized with the iNOS probe, were analysed by the RNase protection assay. As shown in Figure 2, the pattern of bands in samples from LPS-treated macrophages and placenta was identical with two important protected bands of 116 and 111 bp which confirms the presence of iNOS mRNA in the total tissue. The presence of several bands in the gel was due to the use of a mouse riboprobe with minimal differences in the sequence with respect to the rat counterpart.

The relative amount of iNOS mRNA in placenta was studied using animals at different gestational ages. As shown in Figure 3, no significant differences in iNOS levels were observed between day 19 of gestation and delivery. Because pregnant animals suffering septic shock are more likely to abort or suffer other materno/fetal dysfunctions [31] we investigated whether the placental iNOS mRNA levels could be up-regulated in animals with this condition. Treatment of rats of 21 days of gestation with LPS moderately increased (164 %) the amount of placental iNOS mRNA (Figure 3), whereas in other maternal tissues (liver and peritoneal macrophages) a large increase in iNOS mRNA was observed (results not shown).

Because the anatomy of the placenta includes various cell types, fixed tissue sections were analysed for the immuno-

Figure 2 RNase protection assay of iNOS

RNA (5 μ g) from primary cultures of rat macrophages (M ϕ) was incubated for 18 h in the absence or presence of 0.5 μ g/ml of LPS, or placental RNA (30 μ g) from rats at 21 days of gestation was incubated with a riboprobe from the murine iNOS gene. After RNase treatment the samples were analysed by PAGE. DNA from phage NX174 digested with *Hind*III was used as a size marker for the bands. The results shown are representative of three experiments.

localization of iNOS. In control placenta, immunoreactive cells were found heterogeneously distributed throughout the placental villi and specifically near and under the chorionic plate. The cotyledons and villi were non-immunoreactive (Figure 4A). Magnification of the section showed that each villus is covered by a thin layer of syncytio-trophoblasts containing fetal capillaries, mesoderm and multipolar syncytial immunoreactive cells (Figure 4B). A detail of syncytial cells shows the granular nature of the immunoreactive end-product (Figure 4C), similar to that described previously in activated macrophages [32]. Placenta from LPS-treated animals (Figure 5A) showed increased immunoreactivity distributed near the basal plate or maternal septum, decidua basalis and in the periphery of the cotyledons. The reaction product in the immunoreactive cells appeared as immunopositive cytoplasmic granules surrounding clear and large nuclei (Figures 5B and 5C).

NOS activity in placenta

NOS activity was measured in samples of complete placenta following the synthesis of [U- 14 C]citrulline from [U- 14 C]arginine [21]. As shown in Table 1, no statistically significant differences in NOS activity were observed in placenta from day 19 to 22 of gestation. When the placenta from animals of 21 days of gestation

Figure 3 Presence of iNOS mRNA in complete placenta

Total RNA (30 μ g) from placenta was analysed by Northern blot using a probe specific for iNOS. Placenta from animals at 21 days of gestation injected intraperitoneally with LPS were also analysed. The results show the means $\pm$ S.E.M. of three experiments in which a pool of three placentae from different animals was used. *, $P < 0.005$.

treated with LPS was assayed, a 162 % increase of NOS activity was observed after 8 h of treatment.

Nuclear extracts from placenta contain proteins involved in iNOS expression

Since iNOS expression is mainly controlled at the transcriptional level, nuclear factors involved in the transcription of this gene would be expected to be active in placenta. The expression of iNOS has been associated with persistent nuclear factor (NF)- κ B activation as a necessary requirement [27,28]. To address this point we investigated, using the EMSA, whether the sequence corresponding to the proximal NF- κ B motif of the mouse iNOS promoter region binds members of the NF- κ B family. As Figure 6 shows, nuclear proteins prepared from total placental extracts (day 19 to 22 of gestation) specifically bind to the proximal NF κ B sequence of iNOS in the EMSA. Analysis of the proteins by supershift assays and by Western blot using specific antibodies revealed that these bands corresponded to p50-p50 homodimers and to p50-p65 heterodimers respectively (results not shown). When placental samples from animals treated with LPS were analysed, a 5-fold increase in the p50-p65 heterodimers was observed.

DISCUSSION

Flow of nutrients from the maternal circulation towards the fetus constitutes an essential requisite for fetal growth. This process is mediated by the flux of precursors through the placental barrier

Figure 4 Immunohistochemical detection of iNOS in rat placenta

Distribution of iNOS immunoreactivity in placenta from rats at 21 days of gestation. (A) Cross-section of a control placenta. (B) Magnification of the placental villi. (C) Detail of immunoreactive syncytial cells. The arrows indicate the distribution of immunoreactive sites. Abbreviations: ch, chorionic plate; st, stem of villus; uv, umbilical vein; ivp, intervilli space; sy, syncytiotrophoblast; M, myometrium; ps, placental septum; sb, spiral blood vessel; db, decidua basalis. Bars correspond to 0.01 mm.

Figure 5 Detailed iNOS immunoreactivity in placenta from LPS-treated rats

Pregnant rats at 21 days of gestation were treated with LPS for 8 h. (A) Cross-section of placenta from LPS-treated rats. (B) Immunoreactivity of syncytial cells (sy). (C) High-power magnification of (B). Abbreviations: ch, chorionic plate; uv, umbilical vein; sy, syncytiotrophoblast; ps, placental septum; sb, spiral blood vessel; db, decidua basalis. Bars correspond to 0.01 mm. Arrows indicate immunoreactivity.

and umbilical vein [33], and therefore regulation of blood flow is an essential component of fetal development. One characteristic of both umbilical and placental circulation is the absence of innervation, and, for this reason, it is conceivable that the existence of multiple alternative mechanisms may be involved in the regulation of the vascular tone [34]. In support of this, the presence of eNOS as well as the effective synthesis of NO by endothelial cells in the umbilical vessels has been established [35]. Some controversy exists regarding the nature of the NOS activity present in endothelial cells of umbilical vessels; in addition to eNOS, some authors claim the presence of nNOS at the end of pregnancy [36], as well as a calcium-independent NOS activity,

Table 1 NOS activity in placenta

NOS activity was measured in extracts of complete placenta after partial purification by 2',5'-ADP-Sepharose on various days of gestation. Where pregnant rats (21 days of gestation) were injected intraperitoneally with LPS, NOS activity was measured at the indicated times after treatment. The results are the means $\pm$ S.E.M. of four experiments in which a pool of four placenta was used. *, $P < 0.01$, LPS-treated versus control placenta.

Days of gestation	LPS treatment (h)	NOS activity (units per mg of protein)
19	—	27.4 $\pm$ 2.0
20	—	26.6 $\pm$ 2.1
21	—	30.2 $\pm$ 2.6
22	—	26.6 $\pm$ 2.8
21	0	28.1 $\pm$ 2.5
21	2	29.5 $\pm$ 2.7
21	4	38.7 $\pm$ 2.7
21	8	45.0 $\pm$ 3.2*

Figure 6 Binding of nuclear proteins to the κ B motif of the promoter region of iNOS

Nuclei were isolated from complete placenta from either control or from LPS-treated rats after 1 h of treatment, on the indicated days of gestation. Nuclear protein extracts were prepared as described in the Materials and methods section and were incubated with an oligonucleotide sequence corresponding to the proximal κ B motif of the murine promoter region of iNOS. After PAGE, the ratio between the intensity of the bands corresponding to the p50-p65 heterodimers and the p50-p50 homodimers was measured. The results shown are the mean intensity ratio ($\pm$ S.E.M.) of three experiments. *, $P < 0.01$.

which presumably corresponds to iNOS [15,37,38]. Moreover, the physiological role of placental NO synthesis has been related to the mechanism responsible for the vasodilation present in pregnancy and impairment of NO synthesis is a likely candidate for pregnancy-induced hypertension, a common cause of maternal and fetal morbidity and mortality [39]. Indeed, the presence

of iNOS activity has been identified in human placenta in pathological conditions such as diabetes [38].

Several reports show an important increase in NO synthesis during pregnancy both in human and in rat [10–12]. This increased NO synthesis has been detected using various approaches: (1) an enhanced release of urinary nitrates, even in animals fed a low nitrate diet; (2) a reduction in the secretion of nitrate after administration of different NOS inhibitors, and (3) the detection of complexed NO haemoglobin by paramagnetic resonance spectroscopy in the blood of pregnant animals [10,11]. The synthesis of NO may involve the activity of various NOS isoenzymes, since a variety of more or less selective inhibitors of NOS isoforms all affect fetal development. This has been shown for aminoguanidine, a preferred inhibitor of iNOS, which when given to pregnant rats induces serious alterations in fetal development [40]. Several histochemical studies have shown the presence of NOS activity, mostly eNOS and to a lesser extent nNOS, in various structures of the placenta [11,35,36]. Our data showing a significant amount of iNOS mRNA and protein in total rat placenta from healthy animals was unexpected, especially when compared with the moderately low levels of eNOS detected using the same approaches. The samples analysed were from day 19 of gestation to delivery, a period in which the role of NO synthesis seems to be particularly critical [36,39]. Due to the large amount of iNOS mRNA measured, and because this result was contrary to our expectation, appropriate controls were carried out to ensure that the iNOS isoform was effectively expressed in placenta. (1) The iNOS and eNOS probes used for Northern-blot analysis specifically recognized the iNOS and eNOS mRNA in various control tissues (the same blot was used for Northern-blot hybridizations shown in Figure 1). (2) RNase protection analysis of the iNOS from placenta and rat macrophages stimulated with LPS yielded the same band profile when the mouse iNOS probe was used, confirming the presence of iNOS. Moreover, an absence of bands was observed when RNA from endothelial cells was used (results not shown). (3) A polyclonal antibody raised against iNOS recognized the protein in the placenta, but not in endothelial cells or unstimulated macrophages. (4) A high level of calcium-independent activity was measured in extracts from complete placenta. Altogether, these data support the presence of iNOS in placenta under physiological conditions.

Because septic shock is an unfavourable condition during pregnancy [13,31], the possibility of an enhanced iNOS expression in the placenta of rats suffering septic shock was investigated. Our results indicate that the total amount of iNOS mRNA increased moderately under these conditions. At the protein level, a roughly parallel increase in the enzymic activity was observed. However, immunocytochemical localization revealed preferential accumulation in the maternal villi, with only a minimal expression in the fetal region. Moreover, a certain cell-specificity was observed in the expression of iNOS in response to LPS. These results suggest an important role for placenta in protection against the up-regulation of iNOS in fetal tissue. The cytokines released in the course of septic shock may exert a more pronounced effect in the fetus than NO itself. This is the case for tumour necrosis factor- α , which specifically impairs fetal growth [31,41]. Also, it is possible that, in the course of LPS treatment, down-regulation of eNOS occurs as reported previously [1,2].

The activity of iNOS is mainly controlled at the transcriptional level [2,3]. This is because this isoenzyme, unlike the eNOS and nNOS isoenzymes, is not regulated by effectors such as calcium/calmodulin [3]. Therefore, the amount of iNOS protein correlates closely to the activity of this enzyme. Studies on the mechanisms that regulate iNOS expression in rodent cells revealed that activation of the NF- κ B transcription factor is a necessary but

not sufficient requirement for the transactivation of the gene [7–9,27,28]. NF- κ B activity is retained in the cytosol through interaction with an inhibitory subunit such as inhibitory- κ B; degradation of inhibitory- κ B favours the translocation of NF- κ B to the nucleus and the binding of NF- κ B to regulatory sequences [42]. When NF- κ B activation was measured in nuclear extracts from placenta, specific binding to the κ B motif of the iNOS promoter was observed and the presence of p50 homodimers and p50–p65 heterodimers was apparent. Although the cells containing activated NF- κ B remain uncharacterized, the relative abundance of active NF- κ B complexes in nuclei from placental cells supports the possibility of continuous transcription of iNOS in some cell types. Indeed, when pregnant animals were injected with LPS, an increase in NF- κ B was apparent concomitant with a moderate increase in the mRNA levels of iNOS. Taking these results together, we can hypothesize that the cells expressing iNOS under physiological conditions probably reflect the existence of a constitutive or autocrine activation mechanism. One likely candidate is relaxin, which is produced in the uterus and has been described as an inducer of iNOS at the end of pregnancy [43]. However, further work is required to determine more precisely the nature of the cells responsible and the mechanism of triggering iNOS expression in the placenta.

Finally, the presence of physiological and constitutively expressed iNOS in the course of development is not only restricted to the placenta. The physiological presence of iNOS in fetal brain vessels replaced by eNOS in the course of development has been demonstrated recently [44]. Unravelling of whether the results observed in rat placenta parallel the situation in the human counterpart merits further research.

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REFERENCES

- Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) *Pharmacol. Rev.* **43**, 109–142
- Nathan, C. (1992) *FASEB J.* **6**, 3051–3064
- Marletta, M. A. (1993) *J. Biol. Chem.* **268**, 12231–12234
- Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. and Snyder, S. H. (1991) *Nature (London)* **351**, 714–718
- Lamas, S., Marsden, P. A., Li, G. K., Tempst, P. and Michel, T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6348–6352
- Janssens, S. P., Shimouchi, A., Quertermans, T., Bloch, D. B. and Bloch, K. D. (1992) *J. Biol. Chem.* **267**, 15274–15276
- Xie, Q.-W., Cho, H. J., Calacay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. and Nathan, C. (1992) *Science* **256**, 225–228
- Lyons, C. R., Orloff, G. J. and Cunningham, J. M. (1992) *J. Biol. Chem.* **267**, 6370–6374
- Lowenstein, C. J., Glatt, C. E., Bredt, D. S. and Snyder, S. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6711–6715
- Conrad, K. P., Joffe, G. M., Kruszyna, H., Kruszyna, R., Rochelle, L. G., Smith, R. P., Chavez, J. E. and Mosher, M. D. (1993) *FASEB J.* **7**, 566–571
- Conrad, K. P., Vill, M., McGuire, P. G., Dail, W. G. and Davis, A. K. (1993) *FASEB J.* **7**, 1269–1276
- Yallampalli, C., Garfield, R. E. and Byam-Smith, M. (1993) *Endocrinology* **133**, 1899–1902
- Diket, A. L., Pierce, M. R., Munshi, U.K., Voelker, C. A., Eloby-Childress, S., Greenberg, S. S., Zhang, X. J., Clark, D. A. and Miller, M. J. (1994) *Am. J. Obstet. Gynecol.* **171**, 1243–1250
- Sooranna, S. R. and Das, I. (1995) *Biochem. Biophys. Res. Commun.* **212**, 229–234
- Morris, N. H., Sooranna, S. R., Learmont, J. G., Poston, L., Ramsey, B., Pearson, J. D. and Steer, P. J. (1995) *Br. J. Obstet. Gynaecol.* **102**, 711–714
- Eis, A. L., Brockman, D. E., Pollock, J. S. and Myatt, L. (1995) *Placenta* **16**, 113–126
- Garvey, E. P., Tuttle, J. V., Covington, K., Merrill, B. M., Wood, E. R., Baylis, S. A. and Charles, I. G. (1994) *Arch. Biochem. Biophys.* **311**, 235–241
- Di Marco, P. N., Ghisalberti, A. V., Pearce, P. H. and Oliver, I. T. (1976) *Biol. Neonate* **30**, 205–215

- 19 Terenzi, F., Diaz-Guerra, M. J. M., Casado, M., Hortelano, S., Leoni, S. and Bosca, L. (1995) *J. Biol. Chem.* **270**, 6017–6021
- 20 Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J. and Cohen, R. A. (1994) *Nature (London)* **368**, 850–853
- 21 Hortelano, S., Genaro, A. M. and Bosca, L. (1992) *J. Biol. Chem.* **267**, 24937–24940
- 22 Hortelano, S., Dewez, B., Genaro, A. M., Diaz-Guerra, M. J. M. and Bosca, L. (1995) *Hepatology* **21**, 776–786
- 23 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 24 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 25 Costa, R. H., Graysom, D. R., Xanthopoulos, K. G. and Darnell, Jr., J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3840–3844
- 26 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 27 Xie, Q.-W., Whismant, R. and Nathan, C. (1993) *J. Exp. Med.* **177**, 1779–1784
- 28 Xie, Q.-W., Kashiwabara, Y. and Nathan, C. (1994) *J. Biol. Chem.* **269**, 4705–4708
- 29 Hsu, S. M., Raine, L. and Fanfer, H. (1981) *J. Histochem. Cytochem.* **29**, 577–580
- 30 Rodrigo, J., Springall, D. R., Uttenthal, O., Bentura, M. L., Abadia-Molina, F., Riveros-Montero, V., Martinez-Murillo, R., Polak, J. M. and Moncada, S. (1994) *Phil. Trans. R. Soc. Lond. B* **345**, 175–221
- 31 McKay, D. G. and Wong, T. (1963) *Am. J. Pathol.* **42**, 357–377
- 32 Riveros-Moreno, V., Palacios, M., Rodrigo, J., Martinez-Murillo, R. and Moncada, S. (1996) in *The Biology of Nitric Oxide, Part 5* (Moncada, S., Stamler, J., Gross, S. and Higgs, E. A., eds.), p. 160, Portland Press Ltd., London
- 33 Carroll, M. J. and Young, M. (1983) *Biochem. J.* **210**, 99–105
- 34 Fox, S. B. and Khong, T. Y. (1990) *Placenta* **11**, 59–62
- 35 Tsukahara, H., Gordienko, D. V. and Goligorsky, M. S. (1993) *Biochem. Biophys. Res. Commun.* **193**, 722–729
- 36 Sexton, A. J., Loesch, A., Turmaine, M., Miah, S. and Burnstock, G. (1995) *Placenta* **16**, 277–288
- 37 Baylis, S. A., Weiner, C. P., Sandra, A., Russell, R. J., Wei, X.-Q., Moncada, S., Stribos, P. J. L. M. and Charles, I. G. (1996) in *The Biology of Nitric Oxide, Part 5*, (Moncada, S., Stamler, J., Gross, S. and Higgs, E. A., eds.), p. 237, Portland Press Ltd., London
- 38 Schonfelder, G., John, M., Hopp, H., Fuhr, N., van der Giet, M. and Paul, M. (1996) *FASEB J.* **10**, 777–784
- 39 Lopez-Jaramillo, P., Teran, E. and Moncada, S. (1995) *Med. Hypotheses* **45**, 68–72
- 40 Voelker, C. A., Miller, M. J., Zhang, X. J., Eloby-Childress, S., Clark, D. A. and Pierce, M. R. (1995) *Pediatr. Res.* **38**, 768–774
- 41 Heyhorne, K. D., Witkin, S. S. and MacGregor, J. A. (1992) *Am. J. Obstet. Gynecol.* **167**, 920–925
- 42 Thanos, D. and Maniatis, T. (1995) *Cell* **80**, 529–532
- 43 Bani, D., Bigazzi, M., Masini, E., Bani, G. and Sacchi, T. B. (1995) *Lab. Invest.* **73**, 709–716
- 44 Galea, E., Reiss, D. J., Xu, H. and Feinstein, D. L. (1995) *FASEB J.* **9**, 1632–1637