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THE INTER-RELATIONSHIP BETWEEN POLYAMINES AND THE L-ARGININE NITRIC OXIDE PATHWAY IN THE HUMAN PLACENTA

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Summary. Nitric oxide (NO) and polyamines are both products of L-arginine metabolism. In placental villous tissue NO and polyamines have been shown to be synthesized although the physiological significance is not known. We have measured polyamine (putrescine, spermidine and spermine) concentrations and nitric oxide synthase activities (NOS) in first trimester and term placentae from normal and abnormal pregnancies, but no difference was observed in polyamine concentrations between normal term and placentae from growth-retarded and pre-eclamptic pregnancies. Significantly higher levels of polyamines were found in first trimester when compared to normal term placentae and there was a significant correlation between NOS activity and the cellular polyamine levels. Cultures of a trophoblast cell line, BeWo, have been used to study the interaction of added polyamines on NOS activity. Although there was a general tendency for all the polyamines to inhibit NOS activity only putrescine was able to significantly inhibit NO production by these cells. It is thought that the L-arginine-NO-polyamine pathway may have a physiological role during pregnancy.

Polyamines are intracellular regulators of proliferation, differentiation, functional activation and macromolecular biosynthesis in mammalian cells (1,2). Elevated levels of polyamines in amniotic fluid, plasma and urine have been reported in pregnancy with the highest levels of putrescine, spermidine and spermine measured at 11 - 14 weeks gestation (3). High levels of polyamines are also known to occur in neoplastic tissues (4). The role of these substances are as yet unknown although immunosuppression is frequently seen in tissues or biological fluids containing high levels of polyamines. An immunosuppressive role for polyamines during early pregnancy has been proposed and their presence may be important for a successful pregnancy.

Nitric oxide (NO) is a cytostatic and cytotoxic free radical which is produced from L-arginine by the enzyme NO synthase (NOS). L-Arginine is also the precursor of polyamnines. In view of the central role of the L-arginine-nitric oxide pathway in normal

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and abnormal pregnancies (5), the present study was undertaken to determine polyamine levels in placental villous tissues. A choriocarcinoma cell line characteristic of trophoblasts (6-8) was used to determine the interaction of polyamines with NOS activity *in vitro*.

Materials and Methods

Lipopolysaccharides from *E.coli* (LPS; serotype 026: B6), gamma-interferon (IFN-γ), N^G-monomethyl-L-arginine (L-NMMA), beta-NADPH, dithiothreitol, phenylmethylsulphonyl fluoride, soybean trypsin inhibitor, aprotinin and cell culture reagents were obtained from Sigma Chemical Co Ltd., Poole, Dorset. The hydrocholoride salts of putrescine, cadeverine, spermidine and spermine were also from Sigma. L-[U-^{14}C]arginine was from Amersham Life Sciences. All other laboratory reagents were of the purest grade available from BDH Chemicals Ltd., Poole, Dorset.

Placental villi from first trimester voluntary terminations and from term elective Caesarian sections were collected and rapidly frozen in liquid nitrogen. The gestational age of first trimester pregnancies were determined by the crown-rump length of fetuses. The diagnosis of pre-eclampsia was based on a blood pressure of at least 140/90 mm of mercury recorded on 2 separate occasions 4 hours apart, and proteinuria of at least 500 mg of total protein in 24 hours. Growth retardation was diagnosed when the abdominal circumference of the fetus was less than the 2.5th centile and when Doppler measurements showed raised umbilical artery pulsatility index and fetal blood flow redistribution (9).

Placental villous tissues from 7 first trimester (9.9 ± 1.1 weeks), 8 normal (37.8 ± 1.6 weeks), 6 pre-eclamptic (PET; 34.5 ± 4.5 weeks) and 8 growth retarded (IUGR; 33.9 ± 1.5 weeks) term pregnancies were briefly washed in PBS at 4°C, blotted, weighed and rapidly frozen in liquid in nitrogen. All subsequent procedures are carried out at 0 - 4°C. 5 volumes of 0.25M sucrose containing 50mM Tris-HCl, pH 7.0, 1mM dithiothreitol, 1mM EDTA, 100μg/mL phenylmethylsulphonyl fluoride, 10μg/mL of soybean trypsin inhibitor and 2μg/mL of aprotinin were added to tissue samples and homogenized using an Ultra-Turrax homogenizer (top speed) in 4 bursts of 15 seconds over 2 minutes at 4°C. After centrifugation for 5 minutes at 11 000*g*, the supernatant was removed. Samples were kept for protein determination by the method of (10).

Determination of polyamine concentration

An aliquot of the supernatant was deproteinized with 0.5M perchloric acid. After neutralization to pH 8.0 with potassium carbonate, the precipitated potassium perchlorate was removed by centrifugation and the supernatant was dabsylated. Polyamines were separated and analysed by reverse-phase high-performance liquid chromatography of their dimethylaminoazobenzene sulphonyl chloride derivatives (11). Reference standards localized individual polyamine peaks. Polyamines concentrations are presented as nmoles per mg of protein.

NOS activity

The calcium-dependent (caNOS) and calcium-independent NOS (ciNOS) activities in the supernantants were determined by the L-[^{14}C]arginine to L-[^{14}C] citrulline assay (12). Assays were performed at 37°C in a total of 0.1mL consisting of 12.5mM HEPES, pH 7.3 with 1.2mM $MgCl_2$, 0.96mM $CaCl_2$, 60mM L-valine, 1.2mM L-citrulline, 0.024mM L-arginine, 150 000dpm L-[U-^{14}C]arginine, 0.12mM beta-NADPH, and 1.5mM EGTA and/or 1mM L-NMMA (Salter *et al.* 1990). The activity of caNOS was determined from the difference between the [^{14}C]citrulline produced from control samples and samples containing 1mM L-NMMA. The activity of ciNOS was determined from the difference between the [14C]citrulline produced in the presence of 1.5mM EGTA and samples containing 1.5mM

EGTA and 1mM L-NMMA. NOS activities are presented as pmoles of L-citrulline formed per min per mg of protein.

Culture of cells

BeWo cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 containing 100units/mL penicillin, 100μg/mL streptomycin and 10% fetal calf serum (FCS). Cells were cultured in 24-well plates. When cells were near to confluent they were incubated with 1mL of culture medium containing different reagents for 24h and nitrite was measured in the culture media by chemiluminescence (13). Samples containing these metabolites were reduced to NO by refluxing with 1% potassium iodide in glacial acetic acid. The released NO was quantified by a chemiluminescence detector (Sivers Instrument Inc, Boulder, Colorado) after reaction with ozone. HiPerSolv water (BDH Laboratories Supplies, Poole) was used to make sodium nitrite standards.

Statistical assessment

Data was analyzed using the unpaired Student's t test.

Results

Measurement of polyamines in placental villous tissues shows a significantly higher concentration of spermine, spermidine and putrescine in first trimester when compared to normal term placentae (Table 1). Cadeverine was present only at very low concentrations with no difference seen between the two gestational ages. In placental villi from pre-eclamptic or growth retarded pregnancies there were no difference in polyamine concentrations except for a significantly higher amount of cadeverine seem in IUGR.

Table 1. Polyamine levels in placental villous tissues from normal and abnormal pregnancies. *, ** and *** represent $P < 0.05$, 0.01 and 0.001 when compared to normal placenta. Polyamine values are expressed as nmoles per mg of protein and are the means ± SEM of the number placentae shown in parentheses

	Gestational Age (mean ± SD)	Putrescine	Cadeverine	Spemidine	Spermine	Total polyamines
First trimester (7)	9.9 ± 1.1	0.27 ± 0.08*	0.01 ± 0.01	0.62 ± 0.13**	1.31 ± 0.14***	2.25 ± 0.18***
Normal term(8)	37.8 ± 1.6	0.07 ± 0.03	0.02 ± 0.01	0.16 ± 0.04	0.74 ± 0.10	0.99 ± 0.15
Pre-eclamptic (6)	36.0 ± 1.3	0.09 ± 0.05	0.02 ± 0.01	0.11 ± 0.02	0.57 ± 0.08	0.78 ± 0.08
Growth retarded(8)	33.9 ± 1.4	0.17 ± 0.06	0.09 ± 0.03*	0.10 ± 0.02	0.50 ± 0.10	0.83 ± 0.13

Fig. 1 shows the relationship between NOS activity and the total polyamine concentration in placental villous tissues under various conditions. A significant positive correlation was observed between both caNOS and ciNOS activity and the concentrations of polyamines ($r = 0.693$; $p = 0.000031$ for caNOS and $r = 0.747$; $p = 0.0000033$ for ciNOS activity respectively).

When BeWo cells were cultured there was measurable NO produced which was inhibitable by NMMA (Table 2). However, incubation with bacterial LPS was found to have no effect on the activity of NOS. Similarly no effect of spermidine and spermine were seen but 10μ putrescine significantly inhibited NO production (Table 2). BeWo cells were additionally found to synthesize basal levels of polyamines. 1.11, 0.59, 1.77 and 2.46 nmoles/mg of protein of cadeverine, putrescine, spermidine and spermine were found in these cells.

Discussion

Polyamines are known to be regulators of cell growth and differentiation. The higher levels of putrescine, spermidine and spermine seen at 10 - 14 weeks gestation in fetal and maternal body fluids suggest a role for normal fetal well-being (3). The precursor of polyamines, L-arginine, is also the substrate for NO. The ability of normal and abnormal placentae to produce NO and polyamines may provide clues to the function of these substances in normal development.

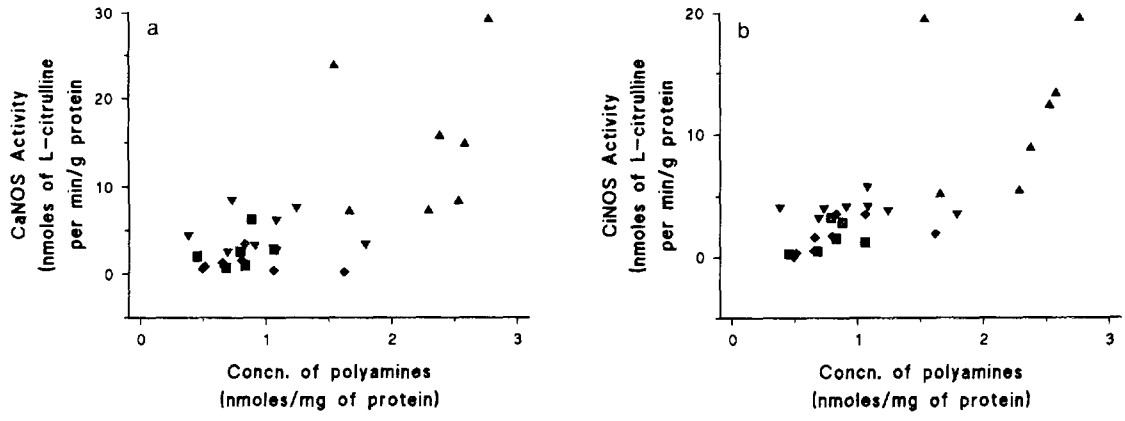

Figure 1. The relationship between (a) caNOS and (b) ciNOS activity and polyamine concentration in placental villous tissue from 7 first trimester (▲), 8 normal (▼), 6 pre-eclamptic (■) and 8 growth-retarded (◆) third trimester pregnancies.

Table 2. BeWo cells were incubated with various reagents for 24h and then nitrite concentrations were measured in the culture medium.* and ** represent $P < 0.001$ and 0.05 when compared to no additions

Culture conditions	pmoles of nitrite formed/24h	n
None	65 ± 12	30
LPS	71 ± 21	21
3mM L-NMMA	17 ± 11*	6
10μ putrescine	35 ± 8**	15
10μ spermidine	55 ± 14	11
10μ spermine	53 ± 20	11

Our data shows no difference of polyamines production in placentae from pre-eclamptic and growth-retarded pregnancies. Decreases in NOS activities were however seen in these pathophysiological cconditions (5). Polyamines were higher in early placental tissues mimicking the higher NOS activities found (14). Arginine concentrations is also known to be significantly higher in first trimester placental villous tissues compared to term (15).

The inhibitory effect of putrescine, but not spermidine and spermine upon NOS activity in is probably a feed-back mechanism to regulate production of NO (Figure 1). *In vivo* this feed-back mechanism is probably controlled in a more subtle manner as a linear correlation was seen between NOS activity and polyamine concentration under many diferrent conditions. The high production of polyamines by the BeWo cell line reflects its malignant natue.

Polyamines are produced by several bacteria (1). In the placenta bacterial-derived polyamines may potentiate the host immune responses resulting in an increase in ciNOS activity. In that respect it is possible to speculate that ciNOS activity in the placenta is different to that present in the macrophage where polyamines attenuates the host immune responses (16).

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