Reduction of placental nitric oxide synthase activity in pre-eclampsia

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INTRODUCTION

Although pre-eclampsia is a serious and common complication of human pregnancy, its pathogenesis is unclear. Current knowledge suggests that a genetic predisposition may lead to poor immunobiochemical adaptation of the mother to the conceptus, which manifests in failure of trophoblast invasion of maternal placental-bed spiral arteries and associated impaired placentation blood flow and placental-bed atherosis.

Bioynthesis of the gas nitric oxide (NO) and its second messenger cyclic GMP increase during pregnancy [4–6]. NO is a potent smooth muscle relaxant and it has been postulated that an increase in nitric oxide synthase (NOS) activity contributes to reduced maternal vascular tone and reactivity in various organ beds throughout gestation [7, 8]. NO may also play a role in maintaining low fetal vascular resistance, as well as attenuating the actions of vasoconstrictors, in the human placenta [9–11].

Although it has been postulated that reduced production of NO is an important feature of pre-eclampsia and contributes to the disturbance in levels and reactivity of vasoactive substances, supportive evidence is inconclusive [3]. Plasma measurement of the nitrite and nitrate metabolites of NO have shown both reduction [12] and no change [13] in pre-eclampsia compared with normal pregnancy. In addition, urinary nitrite and nitrate levels, as well as cyclic GMP levels, were found not to change with pre-eclampsia [14–16]. Reduced release of NO as measured by bioassay, in response to stimuli in perfused umbilical vessels from pre-eclamptic pregnancies, has been reported [17, 18]. However, no difference was found between normal and pre-eclamptic tissues when NO metabolites were measured in the supernatant of either placental explants [19] or cultured umbilical-vein endothe-

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Abbreviations: CS, Caesarean section; NO, nitric oxide; NOS, nitric oxide synthase; NVD, vaginal delivery.
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lial cells [20]. On the other hand, NOS inhibition caused increases in perfusion pressure of perfused placentae in vitro [9, 10], as well as hypertension and intrauterine growth retardation in vivo in pregnant rats [21, 22]. Thus, the role of NO in the pathogenesis of pre-eclampsia requires further clarification.

Three NOS isoforms have been cloned and sequenced, and have been shown to have significant sequence homology [23]. Constitutive calcium-dependent NOS activity has been measured in human placental tissue [24–26]. In addition, endothelial cell NOS mRNA and immunoreactivity, but not that of brain NOS, have been localized to the syncytiotrophoblast and villous endothelium of human term placentae [27, 28]. NO from the endothelium of placental villi is likely to influence fetal placental haemodynamics, while syncytiotrophoblast production of NO may prevent both platelet and leucocyte aggregation in the intervillous space, as well as adhesion to the syncytiotrophoblast surface. Thus, the placenta is likely to make a significant contribution to NO production during pregnancy. Recent reports have shown that placental NOS activity was reduced in pregnancies characterized by placental insufficiency, as diagnosed by abnormal umbilical artery waveforms [29], and that NOS expression was altered in terminal villous vessels and syncytiotrophoblast of placentae from pre-eclamptic compared with normotensive pregnancies [30]. Given the variation in established NO metabolite results in pre-eclampsia, the measurement of enzyme activity provides an alternative method of assessing NO production, and thereby clarifying the role of NO in pre-eclampsia. We have tested the hypothesis, therefore, that human placental NO production is decreased during pre-eclampsia by comparing NOS activity from normal and pre-eclamptic placentae.

METHODS

Experimental procedures used in this project were approved by the Royal Women’s Hospital Research and Ethics Committee in accordance with the National Health and Medical Research Council of Australia guidelines. Written, informed consent was obtained from all patients participating in the study.

Drugs and chemicals

\( \beta\)-NADPH, FAD, calmodulin, L-arginine, HEPES and EDTA were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Sucrose and (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride were obtained from ICN, Costa Mesa, CA, U.S.A. Dithiothreitol, CaCl\(_2\)2H\(_2\)O and NaOH were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. The Dowex AG50WX-8 ion-exchange resin and the Bradford protein assay reagent were from Bio-Rad, Hercules, CA, U.S.A. \(^{3}H\)L-Arginine (62 Ci/mmol) was obtained from Amersham, Sydney, New South Wales, Australia.

Tissue collection

Human placentae were obtained after vaginal delivery (NVD) or Caesarean section (CS) from women who had been assigned to the following groups according to standard obstetric criteria: term non-pre-eclamptic control, term pre-eclamptic, preterm non-pre-eclamptic control and preterm pre-eclamptic. Pre-eclampsia was defined as a persistently elevated blood pressure (>140/90 mm Hg) in association with proteinuria (>300 mg/24 h), both appearing for the first time in the latter half of a primigravid pregnancy with resolution of the hypertension and proteinuria during the puerperium.

Placental NOS assay

NOS activity was assessed by measuring the conversion of \(^{3}H\)L-arginine into \(^{3}H\)L-citrulline using a modification of a previously described method [26]. Within 15 min of delivery, samples of villous tissue from beneath the basal plate were taken at random from placenta (3–5 g) from which amnion and chorion tissue had been removed. Tissues were frozen in liquid nitrogen and stored for up to 3 months. Frozen tissue was homogenized using an Ultra Turrax (T25) blade homogenizer in ice-cold 20 mmol/l HEPES buffer, pH 7.8, containing 0.32 mol/l sucrose, 1 mmol/l EDTA and 1 mmol/l dithiothreitol (1 g of wet tissue in 5 ml of buffer). The homogenized tissue was centrifuged at 1000 g for 10 min at 4°C and the supernatant was collected. Placental NOS activity has previously been shown not to be diminished in supernatants prepared by this technique compared with the corresponding homogenates [26]. In order to remove endogenous L-arginine, 4 ml aliquots of the supernatant were passed over columns containing 2 ml of Dowex (AG50WX-8, Na\(^{+}\) form) ion-exchange resin. Fractions (340 \(\mu\)l) of the column eluants were then incubated for 30 min at 37°C with 60 \(\mu\)l of buffer containing (final concentrations) 1 mmol/l NADPH, 1 \(\mu\)Ci/ml \(^{3}H\)L-arginine, 2 \(\mu\)mol/l FAD, 1 mmol/l magnesium acetate, 5 units/ml calmodulin, 1 \(\mu\)mol/l (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride and 0.45 mmol/l CaCl\(_2\)2H\(_2\)O. To placental homogenates, 0.5–32 \(\mu\)mol/l non-radiolabelled L-arginine was added. At the end of the incubation period, 0.75 ml of ice-cold 20 mmol/l HEPES buffer (pH 7.8) containing 5 mmol/l L-arginine and 4 mmol/l EDTA was added to each tube. This mixture was applied to 2 ml Dowex (AG50WX-8, Na\(^{+}\) form) columns and eluted with 2×2 ml of distilled water. \(^{3}H\)L-Citrulline levels in the 4 ml eluate were then quantified by
liquid scintillation spectroscopy. Inhibition of \[^{3}\text{H}]\)-citrulline formation by the specific NOS inhibitor L-nitro-L-arginine had previously confirmed the presence of NOS activity in placental tissues [26]. As a previous study had shown that neither the inclusion of the protease inhibitor PMSF in the homogenization mixture, nor the inclusion of the arginase inhibitor L-valine in the reaction mixture, significantly altered placental NOS activity compared with controls without the addition of either drug [31], these drugs were omitted from this study. For all experiments, blanks containing \[^{3}\text{H}]\)-arginine, but no tissue homogenate, were treated exactly as homogenates. The radioactivity eluted from the columns for blanks was routinely subtracted from the values obtained with tissue fractions. Protein content in the tissue homogenates was determined by the Bradford dye-binding assay [32]. The Michaelis-Menton constant (Km) and maximum velocity of reaction (Vmax) values were calculated for placental samples using Lineweaver-Burk analysis as previously reported [26]. Aliquots of a placental homogenate were stored at -80°C and were routinely used to determine intra-assay and inter-assay coefficients of variation, which were 3.6% (n = 5) and 7.4% (n = 13) respectively.

**Statistics**

Statistical analysis was performed by Statgraphics Plus version 5 (STSC Inc, Rockville, MD, U.S.A.). Data are presented as means ± SEM. Significant differences between means were determined using analysis of variance, followed by Newman-Keuls test. Statistical significance was taken as P < 0.05.

**RESULTS**

The gestational ages of the placentae from each of the four groups were as follows [mean ± SEM weeks (n)]: term non-pre-eclamptic controls, 39.1 ± 0.2 (19); term pre-eclamptic, 38.3 ± 1.0 (7); preterm non-pre-eclamptic controls, 32.5 ± 1.1 (10); preterm pre-eclamptic, 30.3 ± 1.1 (9). The gestational ages of the term and preterm pre-eclamptic placentae were not significantly different from their corresponding gestation-matched controls. As shown in Fig. 1(a), Vmax was significantly reduced in both term and preterm pre-eclamptic placentae compared with placentae from corresponding gestation-matched controls. As shown in Fig. 1(b), Km values for NOS were not significantly altered, there is no evidence for a substantial change in the ratio of the different types of NOS present in pre-eclampsia. For example, term placentae delivered by NVD and by CS were: NVD, 26.0 ± 3.5 pmol min\(^{-1}\) mg of protein\(^{-1}\) and 20.7 ± 2.5 μmol/l (n = 6) respectively; and CS, 30.6 ± 3.9 pmol min\(^{-1}\) mg of protein\(^{-1}\) and 19.2 ± 1.3 μmol/l (n = 6) respectively.

**DISCUSSION**

These results provide evidence that human placental NOS activity (as judged by measurement of Vmax) is significantly reduced in pre-eclampsia. Such a reduction was evident at both term and preterm gestations. However, since Km values were not significantly altered, there is no evidence for a substantial change in the ratio of the different types of NOS present in pre-eclampsia. For example,
inducible NOS has a high $K_m$ [33], whereas constitutive NOS has a low $K_m$. The latter has been shown to be the predominant form in normal term human placentae [24, 26, 27]. Nevertheless, further investigation of the nature of NOS isoforms during normal pregnancy and pre-eclampsia may be warranted. In addition, placental NOS activity was found not to be influenced by mode of delivery, as has been previously reported [31].

In a previous study, our group demonstrated that placental NOS activity is decreased in pregnancies complicated by a relative decrease in Doppler ultrasound measurements of umbilical artery diastolic blood flow velocity as measured by Doppler ultrasound, which is an indicator of placental insufficiency [29]. The present result complements that observation and is similar to a report that described reductions in NOS activity in pregnancies complicated with both fetal growth retardation and hypertension [34]. Two other reports, however, described either a small increase or no change in placental NOS activity with pathological pregnancies [35, 36]. In these later studies, enzyme activity was either only measured at one concentration of the substrate L-arginine and the $V_{max}$ was not determined [35], or the $V_{max}$ was established by measuring enzyme activity at 5 μmol/l L-arginine and applying a correction factor [36]. In contrast, the present study involved (in all cases) a complete kinetic investigation using a range of L-arginine concentrations and calculation of both $K_m$ and $V_{max}$ via Lineweaver–Burk analysis, thus providing a sound basis for $V_{max}$ calculation. The present result was also in contrast to studies that found no difference in NO metabolites in the supernatant of incubated placental tissue from normal and pre-eclamptic pregnancies [19]. Enzyme activity, however, may be a more appropriate measurement of NO production than nitrite and nitrate, which may be subject to confounding influences [34].

The endothelial NOS isoform has been localized to the syncytiotrophoblast and the endothelium of stem villous vessels in normal placentae [27, 28]. In a recent report, differences in endothelial NOS expression were observed between normal and pre-eclamptic placentae [30]. In pre-eclampsia, NOS immunostaining appeared in the small villous vessels where underlying smooth muscle not present in normal tissue had developed, whereas NOS immunostaining in the syncytiotrophoblast of pre-eclamptic placentae appeared more diffuse than that in normal placentae. Endothelial cell production of NO is likely to be involved in maintaining low placental vascular tone [9, 37]. However, the function of NO produced by trophoblast tissue is not well understood. It has been proposed that it may be involved in the prevention of adhesion of both platelets and leukocytes to the syncytiotrophoblast, which forms the lining of the intervillous space, as well as in the prevention of aggregation of these blood cells with each other within the intervillous space [30]. Ultrastructural changes to the syncytiotrophoblast that are known to occur in pre-eclampsia [38] may involve reduced NOS activity. Hypoxia has been shown to reduce NO production [39] as well as expression of endothelial NOS [40]. Reduced uteroplacental blood flow in response to deficient trophoblast invasion and remodelling of spiral arteries is likely to be associated with reduced interstitial oxygen concentrations, which may reduce placental NO production. Reduced production of NO by the syncytiotrophoblast may lead to increased platelet and leukocyte adhesion and aggregation, activation of the coagulation cascade and extensive interstitial thrombus formation. Free haemoglobin formed by haemolysis in severe pre-eclampsia may further reduce trophoblast-derived NO by binding to it [30].

The work of Ghabour et al. [30] suggested that NOS may be upregulated in the endothelium of small terminal villous vessels during pre-eclampsia. Our present study assessed total placental NOS activity independent of its cellular site of origin, and shows that there is an overall decrease in placental NOS activity. Despite this finding in vitro, the enzyme may function normally in vivo. Nevertheless, if placental NOS activity in vivo were reduced during pre-eclampsia it would be expected to have an adverse effect on placental haemodynamic function. Supporting this suggestion, it has been reported that administration of an NO-donating drug resulted in an improvement in abnormal umbilical artery Doppler waveforms and reduction in human fetal umbilical–placental vascular resistance in vivo [41]. In conclusion, our data indicate that pre-eclampsia is associated with reduced placental NOS activity and that reduced placental NO production could therefore be involved in the pathogenesis of this important and common obstetric complication.

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REFERENCES


