Human placental nitric oxide synthase activity is not altered in diabetes

J. L. DI IULIO, N. M. GUDE*, R. G. KING†, C. G. LI, M. J. RAND and S. P. BRENNECKE*
Pharmacology Research Unit, RMIT University, G.P.O Box 3476V, Melbourne, Victoria 3000, Australia, *Department of Perinatal Medicine, Royal Women’s Hospital, 132 Grattan Street, Carlton, Victoria 3053, Australia, and †Department of Pharmacology, Monash University, Wellington Road, Clayton, Victoria 3168, Australia

ABSTRACT

Endothelial nitric oxide synthase (NOS) protein and mRNA have been identified and calcium-dependent NOS activity has been measured in human placentae during normal pregnancy. Recently, mRNA and protein for the inducible isoform of NOS have been detected in placentae of women with gestational diabetes. The aim of this study was to determine whether calcium-independent (ciNOS) and/or total (tNOS) NOS activities were increased in placentae obtained after vaginal delivery or Caesarean section from women assigned to the following groups according to standard obstetric criteria: gestational diabetes, diabetes before pregnancy and non-diabetic controls. tNOS and ciNOS were assessed by measuring the conversion of [3H]-L-arginine to [3H]-L-citrulline in the three groups. Michaelis–Menten constants (K_m) and maximum velocities of reaction (V_max) were calculated using Lineweaver–Burk analysis for tNOS. There were no significant differences in either ciNOS, V_max or K_m values between any of the three groups (normal, ciNOS 12.7±1.6%, V_max 16.6±3.3 pmol·min⁻¹·mg⁻¹ protein, K_m 15.30±2.6 μmol/l; gestational diabetes, ciNOS 15.4±1.4%, V_max 14.8±5.2 pmol·min⁻¹·mg⁻¹ protein, K_m 10.5±1.7 μmol/l; diabetes before pregnancy, ciNOS 13.4±1.1%, V_max 14.9±3.4 pmol·min⁻¹·mg⁻¹ protein, K_m 17.7±2.2 μmol/l). The presence of macrosomia did not affect tNOS activity in those with diabetes before pregnancy, and glycosylated haemoglobin levels measured between weeks 27 and 39 were not correlated with ciNOS activity. The results from the present study do not provide evidence for increased placental tNOS or ciNOS activities in pregnancies complicated by gestational diabetes or diabetes present before pregnancy.

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as ‘carbohydrate intolerance of variable severity with onset or first recognition during pregnancy’ [1]. GDM complicates about 5% of pregnancies and placentae obtained from such pregnancies can show signs of plethora, chorangiosis, oedema and poorly developed villous vasculature [2,3], although the severity of these changes may be affected by the severity of diabetes. Together, these placental abnormalities, in addition to high maternal glucose levels, can perturb placental villous blood flow and metabolism leading to fetal morbidity such as macrosomia, oedema, hypoglycaemia and hypocalcaemia [2,3].

Nitric oxide (NO) is a small gaseous molecule generated from L-arginine by the enzyme nitric oxide synthase (NOS). It has emerged as a physiological mediator of human placental vascular function [4]. Three distinct forms of NOS have been identified, namely endothelial

Key words: gestational diabetes, nitric oxide synthase, placenta, Type I diabetes mellitus.

Abbreviations: GDM, gestational diabetes mellitus; NO, nitric oxide; ciNOS, calcium-independent nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; tNOS, total nitric oxide synthase.

Correspondence: Dr J. L. Di Iulio.
NOS (eNOS), neuronal NOS (nNOS) and immunological (inducible) NOS (iNOS) [5]. Studies screening for NOS mRNA species and protein expression in normal-term placentae have demonstrated the presence of eNOS, and NOS activity assays show that the majority of placental NOS activity is calcium-dependent [6–9]. In addition, there is also evidence to suggest that in pathological states such as pre-eclampsia, eNOS is the predominant isoform [10]. In normal placentae, eNOS has been localized to the syncytiotrophoblast cell layer and the villous endothelium [6,8]. Calcium-dependent nNOS has also been reported in the human placenta [11]. Syncytiotrophoblast-derived NO is thought to inhibit platelet and leucocyte aggregation in the intervillous space, as well as adhesion to the syncytiotrophoblast surface, whereas NO generated from the villous endothelium is likely to play a role in the maintenance of low placental vascular resistance.

A recent study by Schonfelder et al. [3] detected iNOS mRNA in term placentae from women with GDM. In addition, iNOS protein expression was detected immunohistochemically, and found to be localized to endothelial cells and trophoblasts. The authors of this report postulated that increased levels of NO generated by iNOS in the fetal-maternal compartment of the placenta may be responsible for the blood flow disturbances and capillary dilation often associated with GDM.

Although there is evidence for the presence of iNOS in placenta of women with GDM, it is not known whether iNOS makes an appreciable contribution to the total placental NO production in GDM and whether total NOS (tNOS) activity is increased. Therefore, the aim of this study was to compare calcium-independent (cNOS) activity and tNOS activity in term placentae obtained from women with GDM and from gestation-matched, non-diabetic controls. In addition, NO activity was measured in placentae obtained from women with diabetes before pregnancy [Type I (insulin-dependent) and Type II (non-insulin-dependent) diabetes mellitus], as NO produced from the activation of iNOS in the pancreas is thought to contribute to cell death associated with the development of Type I diabetes [12]. Furthermore, the level of NOS activity and the nature of NOS present in Type I diabetes are unknown.

**METHODS**

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

**Tissue collection**

Patients were classified into three groups; those with normal pregnancies; those with diabetes before pregnancy, either Type I or Type II diabetes mellitus; and those with GDM. Patients were tested for GDM with a glucose tolerance test performed in the morning after an overnight fast and using a 75-g glucose load. A diagnosis of GDM was made if the fasting plasma glucose level was ≥5 mmol/l and/or the 2-h plasma glucose level after the glucose tolerance test was ≥8.0 mmol/l [13]. Blood glucose levels of patients with GDM were maintained within normoglycaemic limits either by diet alone or with appropriate insulin treatment. Glycosylated haemoglobin levels (which correlate with long-term diabetic control) were recorded on one occasion between weeks 27 and 39 of gestation.

**Placental NOS activity**

NOS activity was assessed by measuring the conversion of [3H]-arginine into [3H]-citrulline by a well-established method [9,10,14–16]. Within 15 min of delivery, samples of villous tissue from beneath the basal plate were taken at random from the placenta (2–5 g), from which amnion and chorion tissue had been removed. Tissues were frozen in liquid nitrogen and stored for up to 1 month. The frozen tissues were thawed and homogenized using an Ultra Turrax (T25) blade homogenizer (Germany) for 20 s in ice-cold buffer (pH 7.8; 1 g of wet tissue in 5 ml of buffer) containing 20 mmol/l HEPES, 0.32 mol/l sucrose, 1 mmol/l EDTA and 1 mmol/l dithiothreitol. The homogenate was centrifuged at 1000 g for 10 min at 4 °C and the supernatant was collected. Placental NOS activity has been shown not to be diminished in supernatants prepared by this technique compared with the corresponding homogenates [9].

A 4 ml sample of placental supernatant was passed through a column containing 2 ml of Dowex (50ZS-200, Na+ form) to remove endogenous l-arginine and methyl-l-arginines.

A 340 μl sample of supernatant was incubated for 30 min at 37 °C with 60 μl of a buffer (pH 7.8) containing

**Drugs and chemicals**

β-NADPH, FAD, calmodulin, l-arginine and Dowex (50ZS-200) ion-exchange resin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EDTA, HEPES, sucrose and (6R)-5,6,7,8-tetrahydro-l-biopterin dihydrochloride were from ICN (U.S.A.). Dithiothreitol and calcium chloride were obtained from British Drug Houses (U.K.). The Bradford protein assay reagent was from Bio-Rad (U.S.A.) and [3H]-l-arginine (specific radioactivity 60–62 Ci/mmole) was obtained from Amersham (Australia).
Table 1  Pregnancy characteristics of the three groups of patients

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy</th>
<th>GDM</th>
<th>Diabetes before pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>11</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>38.5 ± 0.2</td>
<td>38.9 ± 0.3</td>
<td>37.4 ± 0.3</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.8 ± 0.4</td>
<td>3.7 ± 0.8</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Parity</td>
<td>2.5 ± 0.4</td>
<td>2.7 ± 0.6</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Infant weight (g)</td>
<td>3342 ± 77</td>
<td>3395 ± 107</td>
<td>3784 ± 221</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>628 ± 24</td>
<td>629 ± 54</td>
<td>679 ± 39</td>
</tr>
<tr>
<td>Glycosylated Hb (%)</td>
<td>—</td>
<td>5.0 ± 0.2</td>
<td>7.1 ± 0.3*</td>
</tr>
<tr>
<td>GDM receiving insulin (n)</td>
<td>—</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Type I diabetes (n)</td>
<td>—</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>Type II diabetes receiving insulin during pregnancy (n)</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Fetal macrosomia (n)</td>
<td>—</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Glycosylated haemoglobin (Hb) was measured between 27 and 39 weeks of gestation. In rows 2 to 7, the values are means ± S.E.M. *Significantly greater than GDM (P < 0.05).

The mixture was applied to 2-ml Dowex (Na+ form) columns and [3H]-citrulline was eluted with 2 × 2 ml of distilled water. The eluant was collected in 20 ml scintillation vials to which 12 ml of scintillation fluid (Ultima Gold; Packard, U.S.A.) was added. [3H]-Citrulline levels were then measured in a liquid scintillation counter (Minaxi Tri-Carb 4000 Series; United Technologies Packard, U.S.A.).

Inhibition of [3H]-citrulline formation by the specific NOS inhibitor Nω-nitro-l-arginine had previously confirmed the presence of NOS activity in placental tissues [9]. In addition, incubation with l-valine had previously shown that arginases were not making a significant contribution to the enzyme activity being measured [15].

For all experiments, blanks were prepared using the same protocol, but the tissue homogenate was replaced with 340 µl of tissue-free buffer. The blank value was subtracted from the total value obtained with each tissue fraction.

The protein content of the supernatant was determined by the dye-binding assay of Bradford [17]. Aliquots of a placental homogenate were stored at −80 °C and routinely used to determine intra- and inter-assay coefficients of variation, which were 3.6% (n = 5) and 13.7% (n = 12) respectively.

The values for the Michaelis–Menten constant (Km) and maximum velocity of reaction (Vmax) for tNOS activity were calculated by Lineweaver–Burk analysis as previously reported [9]. For enzyme calcium-dependency studies, calcium chloride was omitted from the reaction mixture and endogenous calcium was removed from the homogenate by the addition of 4 mmol/l EDTA.

A power calculation was performed on the data for ciNOS activity for the normal group of patients by the method of Neyman and showed that an n value of 6 for each group was required for a 50% increase in ciNOS activity.

Statistics

Statistical analysis was performed by Statgraphics Plus version 5 (STSC Inc., Rockville, MD, U.S.A.) and GraphPad Prism version 2.01 (GraphPad Software Inc., San Diego, CA, U.S.A.). Data are presented as means ± S.E.M. Data were tested for normal distribution using a Kolmogorov–Smirnov test. Significant differences between means were determined using analysis of variance, followed by the Newman–Keuls test. Where percentage data were presented the Kruskal–Wallis one-way analysis by ranks for non-parametric data, followed by the Wilcoxon test for individual differences, was used. A value of P < 0.05 was considered statistically significant.

RESULTS

Table 1 shows the characteristics of the pregnancies of the three groups of women from whom placental samples were obtained for the study; non-diabetic controls, GDM and diabetes before pregnancy. There were no
significant differences in birth weight between the three groups. The mean glycosylated haemoglobin level was significantly higher for diabetes before pregnancy compared with GDM.

NOS activity measured via the conversion of l-arginine into l-citrulline was detected in all placentae. The omission of calcium and the addition of 4 mmol/l EDTA in the reaction mixture resulted in significant reductions in placental tNOS activity for all three groups. The proportion of ciNOS activity was not significantly different between the three groups. The values for calcium-insensitive activity, expressed as a percentage of the corresponding activity in the presence of calcium, were 12.7 ± 1.6% (n = 6, normal control), 15.4 ± 1.4% (n = 7, GDM) and 13.4 ± 1.1% (n = 5, diabetes before pregnancy).

$V_{max}$ and $K_m$ values for tNOS enzyme activity were calculated via Michaelis–Menten kinetic analysis for all three groups. There were no significant differences in $V_{max}$ values between the three groups (Figure 1). Similarly, the $K_m$ values calculated for each group were not significantly different (Figure 2).

In the group of women with diabetes before pregnancy there were no significant differences in tNOS activity in those with macrosomia ($V_{max}$ 12.1 ± 1.8 pmol·min$^{-1}$·mg$^{-1}$ protein, n = 3) compared with those without macrosomia ($V_{max}$ 17.8 ± 6.9 pmol·min$^{-1}$·mg$^{-1}$ protein, n = 3). In addition, there was no correlation between ciNOS activity and glycosylated haemoglobin levels, measured between weeks 27 and 39 of pregnancy ($r^2 = 0.0304, n = 12$), in the diabetic women.

Schonfelder et al. [3] reported the expression of iNOS protein in both insulin- and diet-treated GDM women at term, and speculated that iNOS activity could increase the overall NO content in the placental milieu. It was suggested that increased NO levels may contribute to placental blood flow changes and capillary dilation in GDM. In addition, since iNOS-generated NO exhibits cytotoxic effects and can contribute to programmed cell death, iNOS may participate in the development of chorangiosis and the relative immature villous structure. The results obtained in the present study, however, provide no evidence that total placental NOS activity (as judged by measurement of $V_{max}$) is altered in GDM, nor that ciNOS activity makes an additional contribution to overall placental NO production in GDM compared with normal pregnancy. The assay used to measure NOS in the present study is well established. It has recently been pointed out, however, that in vitro NOS activity determined using this method (with cofactors in excess) may not reflect in vivo NOS activity if the availability of cofactors is limiting, as has been reported in other tissues [18].

The $K_m$ values obtained for total placental NOS in GDM and non-diabetic controls were similar to those previously reported for placental NOS by our group [9,10,16], but were markedly lower than that reported for iNOS [19]. eNOS has been localized to the syncytiotrophoblast and endothelium of stem villous vessels in normal placentae [6,8] and enzyme activity has been well characterized [9]. The results from the present study indicate that the predominant form of NOS in the...
placentae of women with GDM is calcium-dependent (i.e. eNOS, or possibly nNOS). If iNOS contributed markedly to NO production in the placentae of diabetic patients one would expect a significant change in the ratio of calcium-dependent to calcium-independent NOS activity when compared with non-diabetic controls. However, this was not observed.

A possible explanation for the failure of our study to detect additional iNOS activity in the placentae of women with GDM, despite the appearance of protein, could be that the iNOS protein, although reportedly expressed in significant amounts [3], may not be in a particularly active form. This would account for no significant differences in $K_m$ values and calcium-dependent to calcium-independent enzyme activity ratios between GDM and non-diabetic controls.

It has been well documented in the literature that patients with Type I diabetes have increased mortality and morbidity due to vascular disease. Abnormalities in endothelial cell morphology and function [20] and impairment of the NO/ L-arginine pathway may underlie diabetic vascular disease [21,22]. A study by Sank et al. [23] provided evidence of endothelial dysfunction in cultured umbilical endothelial cells from humans with Type I diabetes. In addition, placenta from women with Type I diabetes have been found to contain more voluminous capillary beds of greater length, diameter and surface area when compared with non-diabetic controls [24]. However, the effect of these placental abnormalities on NOS activity remains unknown. The results of this study revealed that iNOS activity (as judged by measurement of $V_{max}$) was not altered in placentae from patients with diabetes before pregnancy (Type I and Type II diabetes) compared with non-diabetic controls. In addition, $K_m$ values and the proportion of cNOS activity obtained for the normal group and the group with diabetes before pregnancy were not significantly different from each other, suggesting that calcium-dependent NOS is the predominating form of NOS. However, additional studies are required to further elucidate the nature of NOS isoforms present in placentae of patients with Type I diabetes.

In conclusion, our results determined that cNOS activity is not significantly different in placentae obtained from women with GDM or diabetes before pregnancy compared with normal controls, and that total placental NOS activity is not altered in these pathological conditions.

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**REFERENCES**


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