Peroxynitrites and impaired modulation of nitric oxide concentrations in embryos from diabetic rats during early organogenesis

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Abstract

Maternal diabetes significantly increases the risk of congenital malformation, a syndrome known as diabetic embryopathy. Nitric oxide (NO), implicated in embryogenesis, has been found elevated in embryos from diabetic rats during organogenesis. The developmental signaling molecules endothelin-1 (ET-1) and 15-deoxy Δ12,14-prostaglandin J2 (15dPGJ₂) downregulate embryonic NO levels. In the presence of NO and superoxide, formation of the potent oxidant peroxynitrite may occur. Therefore, we investigated peroxynitrite-induced damage, ET-1 and 15dPGJ₂ concentrations, and the capability of ET-1, 15dPGJ₂ and prostaglandin E₂ (PGE₂) to regulate NO production in embryos from severely diabetic rats (streptozotocin-induced before pregnancy). We found intense nitrotyrosine immunostaining (an index of peroxynitrite-induced damage) in neural folds, neural tube and developing heart of embryos from diabetic rats ($P < 0.001$ vs controls). We also found reduced ET-1 ($P < 0.001$) and 15dPGJ₂ ($P < 0.001$) concentrations in embryos from diabetic rats when compared with controls. In addition, the inhibitory effect of ET-1, 15dPGJ₂ and PGE₂ on NO production found in control embryos was not observed in embryos from severely diabetic rats. In conclusion, both the demonstrated peroxynitrite-induced damage and the altered levels and function of multiple signaling molecules involved in the regulation of NO production provide supportive evidence of nitrosative stress in diabetic embryopathy.

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Introduction

Diabetes mellitus profoundly alters reproductive function, thus resulting in increased spontaneous abortion, congenital anomalies and neonatal morbidity/mortality rates (Schwartz & Teramo 2000). The frequency of congenital malformations in the offspring of diabetic mothers is estimated to be 5–12%, compared with 2–3% in the non-diabetic population (Kitzmiller et al. 1978, Langer & Conway 2000). The malformations in offspring of diabetic women and also in experimental diabetic models most often occur in the heart and the central nervous system (Eriksson et al. 1991, Martínez-Frias 1994). The precise pathogenesis of the development caused by maternal diabetes is complex, has multiple origins and is not understood in full. Increased oxidative stress, alterations in prostaglandin formation, increased formation of glycated proteins and increased apoptotic rate have been proposed as possible explanations for diabetes-induced congenital defects (Reece et al. 1998, Eriksson et al. 2003, Horal et al. 2004, Jawerbaum & Gonzalez 2005). In addition, we have previously demonstrated that nitric oxide (NO) production is elevated in the embryos from mild and severe diabetic rat models during early organogenesis (Jawerbaum et al. 1998, 2001).

NO is a versatile signal molecule implicated in several physiological and pathological processes (Moncada et al. 1991). NO is produced from l-arginine in almost all cell types by a family of three nitric oxide synthases (NOS) and acts as a diffusible short-distance acting second messenger that can freely pass through lipid membranes (Moncada et al. 1991). Several studies have shown that NO is produced in the oocyte stage and throughout early embryo development and is involved in oocyte maturation, ovulation, fertilization and implantation (overviewed by Thaler & Epel 2003). In addition, studies support an important role for NO in post-implantation embryo development, probably regulating cell survival,
apoptosis and differentiation in a time- and space-dependent manner needed throughout organogenesis. This is suggested by changes in the pattern of NOS expression during organogenesis (Topel et al. 1998, Young et al. 2002), embryonic heart defects and cardiomyocyte apoptosis in endothelial NOS knockout mice (Feng et al. 2002), apoptotic effects of both NOS inhibitors and NO donors in cephalic morphogenesis (Lee & Juchau 1994, Plachta et al. 2003), and differentiation effects of NO during lung branching morphogenesis (Young et al. 2002).

As NO synthesis and function are critical during embryo development, multiple regulatory pathways are likely to control the appropriate NO concentrations required to ensure normal embryo development.

Our previous studies have demonstrated the capability of endothelin-1 (ET-1) and 15-deoxy Δ⁰₁⁰⁻₁₄ prostaglandin J₂ (15dPGJ₂) of regulating NO production in the embryo during organogenesis (Jawerbaum et al. 2002, Sinner et al. 2002). ET-1 is a potent vasoconstrictor peptide originally isolated from cultured endothelial cells (Yanagisawa et al. 1988) that plays an important role in embryo organogenesis, as demonstrated by craniofacial, great vessel, heart, thyroid and thymus congenital defects seen in ET-1 knockout mice (Kurihara et al. 1994) or as a result of ET-1 receptor blockage (Treinen et al. 1999). We have previously found that ET-1 is a negative regulator of NO concentrations in the embryo from control and mild diabetic rats during embryo organogenesis (Sinner et al. 2002). On the other hand, 15dPGJ₂ is a cyclopentenone prostaglandin (PG) synthesized by non-enzymatic dehydration within the cyclopentane ring of PGD₂ (Straus & Glass 2001). In contrast to classical prostaglandins, which bind to cell surface receptors but possesses a reactive α,β-unsaturated carbonyl functional group that binds free sulphhydryl groups of cysteine residues in cellular proteins and regulates the activity of nuclear receptors and nuclear factors such as peroxisome proliferator-activated receptor gamma (PPARγ) and nuclear factor kappa B (NFkB) (Kliwer et al. 1995, Rossi et al. 2000). 15dPGJ₂ has potent anti-inflammatory properties and represses the genes encoding pro-inflammatory cytokines and inducible NOS (Ricote et al. 1998). We have previously found that 15dPGJ₂ is a negative regulator of NO levels in the embryo from control and mild diabetic rats during embryo organogenesis (Jawerbaum et al. 2002). NO concentrations have also been found to be regulated by PGE₂ in different tissues (Paliege et al. 2004, Sakamoto et al. 2004). On the other hand, NO is a positive regulator of PGE₂ concentrations during embryo organogenesis in both control and mild diabetic rats (Jawerbaum et al. 1998). Indeed, impairment of NO positive regulation leads to reduced PGE₂ levels in the embryo from severely diabetic rats, an alteration that has been related to the increased diabetes-induced neural tube defects (Piddinton et al. 1996, Jawerbaum et al. 2001). This impairment of NO biological function leading to low intraembryonic PGE₂ levels seems to be directly related to the increased reactive oxygen species (ROS) generated in the embryo under hyperglycemic conditions (Jawerbaum & Gonzalez 2005). Indeed, NO may turn biologically inactive due to its interaction with ROS, leading to the formation of the potent oxidant peroxynitrite (Beckman et al. 1990). The peroxynitrite anion is highly cytotoxic because it inhibits mitochondrial electron transport, oxidizes sulphhydryl groups in proteins, initiates lipid peroxidation, and nitrates amino acids such as tyrosine, thus affecting many signal transduction pathways (overviewed by Szabó 2003). In the presence of peroxynitrite, formation of nitrotyrosine is particularly favored, and the appearance of this product in biological samples is taken as a diagnosis of exposure of peroxynitrite (Greenacile & Ischiropoulos 2001).

In the present work we investigated evidence of peroxynitrite-induced damage, levels of the embryonic developmental signals ET-1 and 15dPGJ₂, and the capability of ET-1, 15dPGJ₂ and PGE₂ to downregulate the elevated NO concentrations in the embryo from severely diabetic rats. We demonstrated peroxynitrite-induced damage, reduced ET-1 and 15dPGJ₂ concentrations and impairment of multiple NO regulatory pathways in embryos from this experimental diabetic model.

Materials and Methods

Animals

Albino Wistar rats bred in the laboratory were fed Purina rat chow, which was available ad libitum. Female rats weighing 200–230 g were made diabetic with a single i.p. injection of streptozotocin (55 mg kg⁻¹) (Sigma, St Louis, MO, USA) in citrate buffer (0.05 M, pH 4.5), as previously described (Jawerbaum et al. 2001). Control rats were injected with buffer only. Diabetic rat glycemia was measured five days after treatment by glucosestix reagent strips (Bayer Diagnostics, Buenos Aires, Argentina) and a glucometer. Estrous cycles in diabetic rats were observed throughout the two weeks after streptozotocin administration, and during this period they became pregnant if mated. Both normal and diabetic females were mated with control male rats. Mating was confirmed by the presence of sperm in vaginal smears. When a positive pregnancy was identified, this was designated day 0.5 of gestation. The guidelines for the care and use of animals approved by the local institution were followed, and accorded with the guidelines set out in Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985).

Embryo preparations

Animals were killed by cervical dislocation on day 10.5 of pregnancy and the uteri were transferred to Petri dishes with Krebs Ringer bicarbonate (KRB) solution: 11.0 mM glucose, 145 mM Na⁺, 2.2 mM Ca²⁺, 1.2 mM Mg²⁺, 127 mM Cl⁻, 25 mM HCO₃⁻, 1.2 mM SO₄²⁻ and 1.2 mM
PO₄³⁻. By use of a stereomicroscope and watchmaker forceps the balls of decidual tissue were removed from each uterus, and gently opened to free the conceptuses. The embryos were dissected out of the yolk sacs and evaluated morphologically under a stereomicroscope. Viability was established by the presence of a beating heart. The embryos were categorized as morphologically normal or as showing either neural tube defects or other malformations. Embryonic growth was quantified by direct measurement of the protein content (Bradford 1976) with bovine serum albumin as a standard. Embryos in resorption stages received no further analyses. Two embryos from each mother were selected at random for immunohistochemical analysis, fixed in 4% formalin, subsequently dehydrated in graded ethanol, transferred to xylene and finally embedded in paraffin. The remaining embryos were stored at −70°C for measurement of ET-1 and 15dPGJ₂ concentrations or incubated as follows: four embryos were incubated together in a metabolic shaker, under an atmosphere of 5% CO₂ in 95% O₂ at 37°C for 1 h in 1 ml KRB with or without the addition of ET-1 10⁻⁹ M (Sigma), 15dPGJ₂ 2 x 10⁻⁶ M (Cayman Chemical Co., Ann Harbor, MI, USA) or PGE₂ 10⁻⁷ M (Sigma). After incubations, embryos were stored at −70°C until determination of nitrate/nitrite concentrations.

**Nitrotyrosine localization**

Immunostaining of nitrotyrosine, an index of peroxynitrite-induced damage was performed as previously described (Pustovrh et al. 2005). Briefly, paraffinized embryos from six control and six diabetic rats were sliced in 5 μm-thick sections. Thereafter, the slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 0.5% hydrogen peroxide in absolute methanol for 30 min at room temperature. Then, sections were rehydrated through a graded series of ethanol and were processed using the nitrotyrosine mouse monoclonal antibody (Calbiochem, La Jolla, CA, USA) (1:200 dilution) as primary antibody and the peroxidase anti-peroxidase technique (PAP), as follows. Sections were incubated in 10% normal goat serum (Sigma) for 1 h at room temperature, and then incubated in the primary antibody for 48 h at 4°C. Later, sections were incubated in goat anti-mouse secondary antibody (Sigma) (1:50 dilution) for 1 h at room temperature and mouse PAP (Sigma) (1:100 dilution) for 1 h at room temperature. All antibodies were diluted in PBS containing 0.2% Triton X-100. Color development was performed with a solution containing 0.06% 3,3’-diaminobenzidine (DAB) (Sigma) plus 0.01% hydrogen peroxide in Tris saline buffer for 15–30 min. Control sections were performed by omitting the primary antibody. After color development, sections were counterstained with hematoxylin. Sections were dehydrated, mounted with Entellan New (Merck, Darmstadt, Germany) and observed with a Zeiss Axiophot light microscope.

**Image analysis**

Optical density of nitrotyrosine residues in the embryonic slides was measured in an Axiophot Zeiss light microscope equipped with a video camera on line with a Zeiss-Kontron VIDAS image analyzer. The resolution of each pixel was 256 gray levels. For each experimental condition, the analysis was performed in six embryonic sections from six control and six diabetic rats. Optical density was evaluated in an area of 120 μm² ten times per section.

**Nitrate/nitrite determinations**

Embryonic concentrations of nitrates and nitrites, stable metabolites of NO, were quantified by employing an assay kit for nitrate and nitrite determinations (Assay Design Inc., Ann Arbor, MI, USA). The embryos were sonicated in Tris hydrochloride buffer solution pH 7.4, and an aliquot separated for protein determination. Nitrates in the supernatant were reduced to nitrites using nitrate reductase, and total nitrites were measured by the Griess reaction (Green et al. 1982). Optical densities were measured at 540 nm in a microtiter plate using sodium nitrite and sodium nitrate as standards. Results are expressed in nmol mg⁻¹ protein⁻¹.

**Endothelin-1 analysis**

ET-1 was measured in control and severely diabetic embryos by employing an ET-1 enzyme immunoassay (EIA) kit (Cayman Chemical Co.). Four embryos were sonicated in 250 μl 6% acetic acid and extraction of samples was performed on Sep-Pack C18 cartridges pretreated with 5 ml methanol and 5 ml acidified water at pH 3.0. After washing with 10 ml 0.1% trifluoroacetic acid, endothelins were eluted with 3 ml methanol/water/trifluoroacetic acid (90/10/0.1). The cartridge eluates were evaporated and the resulting dried residues were resuspended in phosphate-buffered saline (PBS), pH 7.2 for subsequent EIA measurements. Briefly, the kit uses a monoclonal antibody to ET-1 and is based on a double-antibody technique. Each well of the microtiter plate was coated with the ET-1 antibody, which binds the ET-1 introduced in the well. An acetylcholinesterase:endothelin Fab’ conjugate was also added to the well, allowing the two antibodies to bind on opposite sides of the ET-1 molecule. The concentration of ET-1 was determined by measuring the enzymatic activity of the acetylcholinesterase by the addition of Ellman’s reagent (Caymen Chemical Co.) and measurement of the yellow-colored product on a microplate reader at 412 nm. Results are expressed as pg mg⁻¹ protein.

**15dPGJ₂ determination**

15dPGJ₂ was measured in control and severely diabetic embryos by employing a 15dPGJ₂ EIA kit (Cayman Chemical Co.). The embryos were sonicated in PBS, an aliquot
expressed as pg μg protein⁻¹.

**Statistical analyses**

All data are presented as the mean±S.E.M. Statistical analyses were performed by employing Student’s t-tests, chi-square tests or one-way analysis of variance in conjunction with the Tukey’s test where appropriate. Differences between groups were considered significant when P<0.05.

**Results**

**Glycemia in diabetic rats and morphological characteristics of the embryos from control and severely diabetic rats**

Glycemia in the diabetic rat females was greatly elevated (P<0.001) when compared with controls (Table 1). Embryos obtained from diabetic rats at day 10.5 of gestation showed increased resorption rate (P<0.001), reduced somite number (P<0.05) and diminished protein content (indicating growth delay, P<0.01), when compared with embryos from control rats (Table 1). Embryonic malformations, mainly in the neural tube and developing heart, were increased (P<0.001) when compared with embryos from controls (Table 1).

**Peroxynitrite-induced damage in embryos from control and severely diabetic rats**

In order to characterize peroxynitrite-induced damage, the localization of nitrotyrosine residues was evaluated by immunohistochemistry in embryonic slides from control and diabetic rats on day 10.5 of gestation (Fig. 1). Absence of nitrotyrosine immunostaining was observed in both the cephalic and heart regions of the developing control embryos (Fig. 1A and B). In contrast, a strong nitrotyrosine immunoreactivity was detected in the closing neural folds (cephalic region, Fig. 1C), and in the developing heart, primitive digestive tube and neural tube (heart region, Fig. 1D) of the embryos from diabetic rats. Densitometric analysis of the slides showed that the staining was higher in both the cephalic (P<0.001) and the heart (P<0.001) sections of the embryos from diabetic rats when compared with controls (Table 2). In the absence of primary antibody, no immunostaining was observed in embryonic slides of control and diabetic rats (data not shown).

**ET-1 concentrations and the effect of ET-1 on nitrate/nitrite concentrations in embryos from control and severely diabetic rats**

The concentrations of ET-1 were decreased in the embryos from severely diabetic rats when compared with controls (56%, P<0.001) (Fig. 2A). Embryos obtained from control and severely diabetic rats on day 10.5 of gestation were incubated for 1 h in the presence or absence of ET-1 10⁻⁷ M, and nitrate/nitrite concentrations were then evaluated (Fig. 2B). In embryos from severely diabetic rats, nitrate/nitrite concentrations were increased when compared with controls (146%, P<0.001). The presence of ET-1 diminished the embryonic nitrate/nitrite concentrations in embryos from control rats (29%, P<0.05) but did not modify NO production in embryos from severely diabetic rats (Fig. 2B).

**15dPGJ₂ concentrations and the effect of 15dPGJ₂ on nitrate/nitrite concentrations in embryos from control and severely diabetic rats**

The concentrations of 15dPGJ₂ were dramatically decreased in the embryos from severely diabetic rats when compared with controls (92%, P<0.001) (Fig. 3A). Embryos obtained from control and severely diabetic rats on day 10.5 of gestation were incubated for 1 h in the presence or absence of 15dPGJ₂ 2×10⁻⁶ M, and nitrate/nitrite concentrations were then evaluated (Fig. 3B). The presence of 15dPGJ₂ 2×10⁻⁶ M diminished the embryonic nitrate/nitrite concentrations in embryos from controls (21%, P<0.05), but was not able to reduce the high NO levels in the embryos from severely diabetic rats (Fig. 3B).

**Effect of PGE₂ on nitrate/nitrite concentrations in embryos from control and severely diabetic rats**

The concentrations of PGE₂ have been found to be reduced in the embryos from severely diabetic rats (Jawerbaum et al. 2001). Embryos obtained from control and severely diabetic rats on day 10.5 of gestation were incubated for

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**Table 1** Rat glycemia and embryo morphological characteristics from control and severely diabetic rats on day 10.5 of gestation. Values are percentage of embryos (chi-square statistics) or means±S.E.M. (Student’s t-test) for embryos obtained randomly from 10 control and 12 diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia (mg/dl)</td>
<td>100±12</td>
<td>421±21*</td>
</tr>
<tr>
<td>Resorptions (embryos)</td>
<td>1% (1/138)</td>
<td>14% (17/120)*</td>
</tr>
<tr>
<td>Malformations (embryos)</td>
<td>1% (1/37)</td>
<td>22% (23/103)*</td>
</tr>
<tr>
<td>Somite number</td>
<td>13±1</td>
<td>10±1**</td>
</tr>
<tr>
<td>Protein content (μg)</td>
<td>64±6</td>
<td>40±4***</td>
</tr>
</tbody>
</table>

*P<0.001, **P<0.05, ***P<0.01 vs embryos from control rats.
1 h in the presence or absence of PGE$_2$ $10^{-7}$M, and nitrate/nitrite concentrations were then evaluated (Fig. 4). The presence of PGE$_2$ diminished the embryonic nitrate/nitrite concentrations in embryos from control rats (27%, $P < 0.05$), but did not modify the elevated NO levels found in the embryos from severely diabetic rats (Fig. 4).

**Discussion**

The embryonic malformation rate is clearly dependent on hyperglycemia levels, as found in human diabetic pregnancies, in *in vitro* culture of rodent embryos in hyperglycemic conditions and in diabetic experimental models (Miller et al. 1988, Eriksson et al. 1991, Jawerbaum & Gonzalez 2005).

The present work is the first to show peroxynitrite-induced damage in the embryo from diabetic mothers. Indeed, an intense nitrotyrosine immunostaining was detected in the neural folds, neural tube and developing heart of embryos from severely diabetic rats, whereas no immunostaining for nitrotyrosine was detected in control embryos. Peroxynitrite-induced damage has previously been found in different diabetic tissues, including the placenta from diabetic patients and diabetic rats (Lyall et al. 1998, Pustovrh et al. 2005). Peroxynitrite formation in the embryo from diabetic rats is probably the result of increased NO, increased ROS and diminished antioxidants (Jawerbaum et al. 2001, Eriksson et al. 2003), and is probably related to the increased apoptotic rate, reduced antioxidant capacity and increased malformation rate found in this pathology. Indeed, peroxynitrite, formed by the interaction of NO with superoxide anion, is more cytotoxic than NO and than superoxide in a variety of experimental conditions (overviewed by Szabó 2003). In addition to being a terminal mediator of cell injury, peroxynitrite enhances and triggers a variety of pro-inflammatory processes, and impairs antioxidant systems. For instance, tyrosine nitration leads to dysfunction of nitrated proteins, as has been shown in the case of superoxide dismutase and prostacyclin synthase (Yakamura et al. 1998, Zou et al. 2002). Peroxynitrite mediates the depletion of glutathione, one of the key cellular antioxidants.
Peroxynitrite also oxidizes critical sulphhydryl groups responsible for the inhibition of critical enzymes in the mitochondrial respiratory chain (Hausladen & Fridovich 1994). In addition, peroxynitrite-modified cellular proteins are subject to accelerated degradation via the proteosome (Grune et al. 1998). Peroxynitrite can also activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which can trigger a cellular suicide pathway (Szabó et al. 1997).

Due to the powerful capability of inducing multiple derangements, the intense peroxynitrite-induced damage found in the embryos from severely diabetic rats may suggest that peroxynitrite is one of the central mechanisms through which badly controlled diabetes induces embryo loss and congenital malformations.

Evidence of the effect of the multiple damage induced by peroxynitrite may be the observed lack of regulatory pathways of ET-1, 15dPGJ2 and PGE2 in the embryo from severely diabetic rats. Indeed, these agents down-regulated NO production in the embryos from control and mildly diabetic rats (Jawerbaum et al. 2002, Sinner et al. 2002), but not in the embryos from severely diabetic rats, an alteration probably leading to a sustained NO production and further formation of peroxynitrite. Peroxynitrite formation may also impair NO bioavailability and thus its physiological functions, as suggested by the impaired effect of NO as a regulator of both PGE2 production in oocytes and embryos from severely diabetic rats and murine yolk vasculogenesis under hyperglycemic conditions, even when NO is overproduced (Jawerbaum et al. 1999, 2001, Nath et al. 2004).

Another original finding of this work was the reduced concentrations of ET-1 found in the embryos from severely diabetic rats. In mildly diabetic rats, we previously found increased ET-1 levels (Sinner et al. 2002). As observed with...
Peroxynitrite-induced damage in embryos from diabetic rats

Our previous studies have shown that 15dPGJ2 is generated in the organogenetic embryo and that its levels are reduced in the embryos from mildly diabetic rats (59%, Jawerbaum et al. 2002). A more marked reduction (92%) and an impairment of the regulation of embryonic NO production was found in this work in the embryos from severely diabetic rats. We have previously found reduced 15dPGJ2 levels in pancreatic β cells and placenta from diabetic rats, and in term placenta from diabetic patients (Gonzalez et al. 2001, Jawerbaum et al. 2004, Capobianco et al. 2005). The multiple biological effects of 15dPGJ2, such as its capacity for regulating cell survival, death and differentiation, and its anti-oxidant and anti-inflammatory properties, seem to be the result of its interaction with nuclear factors and receptors, including NFkB, PPARγ, and nuclear factor-E2-related factor 2 (Straus & Glass 2001, Levonen et al. 2004, Lim et al. 2004). As most of these studies have been conducted in isolated cultured cells, a profound study on the role of 15dPGJ2 during embryo development will be needed to address the impact of the diminished 15dPGJ2 concentrations in diabetes-induced congenital malformations.

Our data demonstrated reduced levels of ET-1 and 15dPGJ2, and an aberrant nitridergic homeostasis in embryos from severely diabetic rats. The observation of the presence of peroxynitrite-induced damage in those embryos, combined with that of the impairment of regulation of NO production by different developmental signaling molecules, suggests that peroxynitrite could have a major role in diabetic embryopathy.

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