REPRODUCTION
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The role of nitric oxide on matrix metalloproteinase 2 (MMP2) and MMP9 in placenta and fetus from diabetic rats

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Abstract

Matrix metalloproteinases (MMPs) play an important role in tissue remodeling that accompanies the rapid growth, differentiation, and structural changes of the placenta and several fetal organs. In the present study, we investigated whether the diabetic maternal environment may alter the regulatory homeostasis exerted by nitric oxide (NO) on MMPs activity in the feto-placental unit from rats at midgestation. We found that NADPH-diaphorase activity, which reflects the distribution and activity of NO synthases (NOS), was increased in both placenta and fetuses from diabetic rats when compared with controls. In addition, while a NO donor enhanced MMP2 and MMP9 activities, a NOS inhibitor reduced these activities in the maternal side of the placenta from control rats. This regulatory effect of NO was only observed on MMP9 in the diabetic group. On the other hand, the NO donor did not modify MMP2 and MMP9 activities, while the NOS inhibitor reduced MMP9 activity in the fetal side of both control and diabetic placentas. In the fetuses, MMP2 was enhanced by the NO donor and reduced by the NO inhibitor in both fetuses from control and diabetic rats. Overall, this study demonstrates that NO is able to modulate the activation of MMPs in the feto-placental unit, and provides supportive evidence that increased NOS activity leads to NO overproduction in the feto-placental unit from diabetic rats, an alteration closely related to the observed MMPs dysregulation that may have profound implications in the formation and function of the placenta and the fetal organs.

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Introduction

During pregnancy, the area of the feto-maternal interface increases to allow greater transfer of oxygen and metabolic substrates. These changes involve a large group of dynamic processes that involve cell proliferation, migration, and changes in the extracellular matrix. MMPs are a family of zinc- and calcium-dependent proteolytic enzymes that have the ability to degrade components of the extracellular matrix and the basement membrane. Most MMPs are secreted into the extracellular matrix as inactive precursors, where they are transformed into the active form by the proteolytic removal of the amino-terminal domain (Visse & Nagase 2003). Due to their potential as inducers of tissue damage, MMPs are strictly regulated in multiple steps, including the transcription level, the activation of zymogens, and the inhibition of their active enzymes by a family of four different tissue inhibitors of metalloproteinases (Brew *et al.* 2000).

On the other hand, nitric oxide (NO) is a gaseous messenger produced from L-arginine through a reaction catalyzed by the three isoforms of NO synthases (NOS): calmodulin- and Ca_2^{++}-dependent endothelial (eNOS) and neuronal synthases (nNOS), and calmodulin- and Ca_2^{++}-independent NOS (iNOS). NO is implicated in reproductive events such as ovulation, decidualization, and implantation (Jablonka-Shariff & Olson 1997, Novaro *et al.* 1997, Zhang *et al.* 2005). During late pregnancy, NO is involved in the maintenance of a low vascular resistance, attenuating the action of vasoconstrictors (Baylis *et al.* 1998). Due to its unpaired e^-, NO is able to react with amino acid residues or thiol groups in protein molecules, leading to enzyme activation or inhibition. Regarding matrix metalloproteinases (MMP2) and (MMP9), NO is capable of modulating its activation through the disruption of the union between an atom of Zn and a residue of cysteine located in the catalytic site of the proenzymes (Gu *et al.* 2002, Novaro *et al.* 2002, Pustovrh *et al.* 2002). In the diabetic pathology, NO imbalance is involved in the development of cardiovascular impairment (Mungrue *et al.* 2002), pancreatic damage (Gonzalez *et al.* 1999, 2001), nephropathy

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(Levine 2006), and retinopathy (Park *et al.* 2006). Our previous reports have shown that NO is overproduced in placental tissue and embryos from diabetic experimental models (Jawerbaum *et al.* 2005, Pustovrh *et al.* 2005). This increase is likely to be associated with both placental dysfunction and diabetic embryopathy (Jawerbaum & Gonzalez 2005, Jawerbaum & González 2006). On the other hand, hyperglycemia also induces an excessive production of uterine, placental, and fetal MMPs (Pustovrh *et al.* 2000, 2002, 2005).

Alterations in MMPs expression may be the result of NO overexpression, since NO is able to increase MMPs expression and to activate their zymogens in several tissues and cell types (Novaro *et al.* 2001, Pustovrh *et al.* 2002, Gursoy-Ozdemir *et al.* 2004, Yu *et al.* 2005).

In the present study, we hypothesize that NO may regulate MMPs activity in the feto-placental unit. Moreover, we propose that the diabetic maternal environment may alter the regulatory homeostasis exerted by NO on tissue remodeling at midgestation. In order to verify our hypothesis, we evaluated (i) the expression and localization of fetal and placental NADPH-diaphorase (which reflects the distribution and activity of NOS), (ii) the protein expression of iNOS and eNOS, and (iii) the influence of both NO donors and NOS inhibitors on MMP2 and MMP9 activities in fetuses and placentas from control and diabetic rats.

Results

NO synthase activity localized through NADPH-diaphorase evaluation in placenta and fetus

NO synthase (NOS) activity was evidenced and localized by the histochemical staining evaluation of NADPH-diaphorase. The analysis of placental NADPH-diaphorase from control animals showed that the enzyme is present in the decidua (maternal placental side), and in the junction and labyrinth zones (fetal placental side; Fig. 1A). The optical density analysis showed a higher NADPH-diaphorase activity in the diabetic placenta (0.133 ± 0.009; mean $\pm$ S.E.M.) when compared with controls (0.0790 ± 0.006; $P < 0.001$); this activity was localized in the same zones as those of controls (Fig. 1B).

Similarly, NADPH-diaphorase evaluation in the thoracic region of fetuses from control animals showed enzymatic activity in the spinal cord, spinal ganglions, and ventricular tissue (Fig. 2A and B). In the diabetic group, the NADPH-diaphorase detection showed a significant increase in the enzyme activity when compared with controls in both the thoracic region (control: 0.073 ± 0.04, diabetic: 0.165 ± 0.007; $P < 0.001$) and the cardiac region of diabetic fetuses (control: 0.079 ± 0.005, diabetic: 0.207 ± 0.006; $P < 0.001$) This increase was higher in the cardiac than in the thoracic region of diabetic fetuses ($P < 0.001$; Fig. 2C and D).

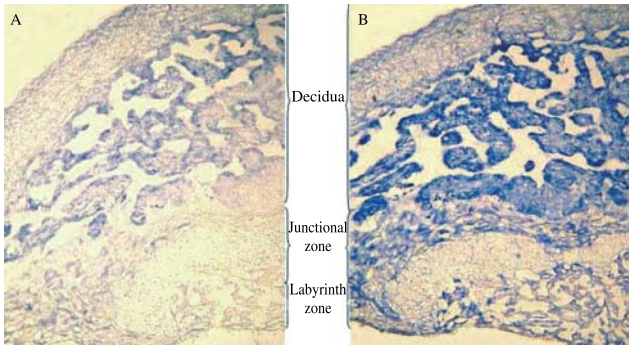

Figure 1 Photomicrographs of NADPH-diaphorase localization in sections of placenta from control (A) and placenta from diabetic rats (B). Enzyme activity was assayed by staining with nitroblue tetrazolium. Magnification: 50×.

Effects of NO on MMP2 and MMP9 activities in placenta and fetus

In order to analyze the influence of NO on placental MMPs activation, both maternal and fetal placental sides obtained from control and diabetic rats were incubated for 1 h in either the presence or absence of sodium nitroprusside (NP, 600 μM), a NO donor, and either in the presence or absence of N^G-nitro-L-arginine methyl ester (L-NAME, 600 μM), an inhibitor of NOS. In agreement with our previous reports (Pustovrh *et al.* 2005), zymography analysis showed that both MMP2 and MMP9 activities were increased in the maternal and fetal placental sides from diabetic rats when compared with controls (Figs 3 and 4).

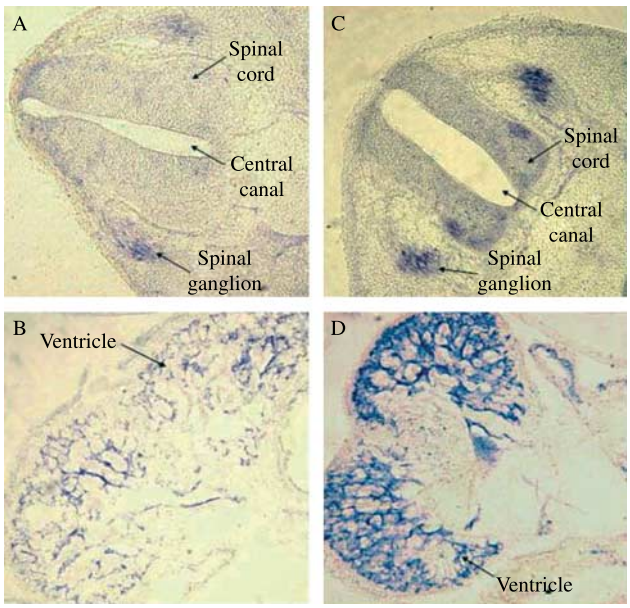

Figure 2 Photomicrographs of NADPH-diaphorase localization in sections of (A) spinal cord and (B) heart from control fetuses; and (C) spinal cord and (D) heart from diabetic fetuses. Enzyme activity was assayed by staining with nitroblue tetrazolium. Magnification: 200×.

The analysis of gelatinolytic activity in the maternal placental side showed that the addition of NP increased the activity of both MMP2 and MMP9 ($P<0.05$) in control tissues when compared with those without additions. In the diabetic group, the activating effect of NP was only observed on MMP9 ($P<0.05$). On the other hand, L-NAME reduced MMP2 and MMP9 activities in the maternal placental side from controls ($P<0.05$ and $P<0.01$ respectively), while it reduced only MMP9 activity in the diabetic group ($P<0.05$; Fig. 3).

When MMPs were analyzed in the fetal placental side from both controls and diabetic rats, we found that neither MMP2 nor MMP9 activities were affected by NP. Differently, the presence of L-NAME significantly decreased MMP9 activity ($P<0.05$) in both experimental groups. On the other hand, MMP2 activity, which was absent in the fetal side of the control placenta, was not modified by L-NAME in the diabetic group (Fig. 4).

Both pro-MMP2 and pro-MMP9 have also been detected and quantified in both maternal and fetal placental sides. As previously stated (Pustovrh *et al.* 2005), these proenzymes were increased in diabetic tissues when related to controls ($P<0.05$). However, pro-MMP2 and pro-MMP9 levels were not modified in the presence of either NP or L-NAME, both in the maternal and fetal placental sides obtained from both control and diabetic rats (Figs 3 and 4).

On the other hand, MMP9 activity could not be detected in the fetuses by zymography at this developmental stage. MMP2 activity was enhanced in diabetic fetuses when compared with controls, and further increased in the presence of NP, in fetuses from both control ($P<0.01$) and diabetic ($P<0.05$) animals when compared with fetuses incubated without additions. Moreover, blockade of NOS activity promoted a decrease of MMP2 activity in fetuses from control ($P<0.01$) and diabetic ($P<0.01$) rats when compared with their respective basal activities (Fig. 5).

As previously observed (Pustovrh *et al.* 2005), pro-MMP2 was found increased in diabetic fetuses when related to controls ($P<0.01$). However, the levels of this proenzyme were not modified in the presence of either NP or L-NAME in the fetuses obtained from both control and diabetic rats (Fig. 5).

Concentrations of inducible and endothelial NOS in placenta and fetus

The evaluation of NO synthase isoenzymes (eNOS and iNOS) by Western blot showed that there were no changes in the concentrations of these NOS isoenzymes - in the placenta from diabetic animals when compared with controls (Fig. 6). Similar concentrations of eNOS and iNOS were also found when the fetuses from control and diabetic animals were evaluated (Fig. 7).

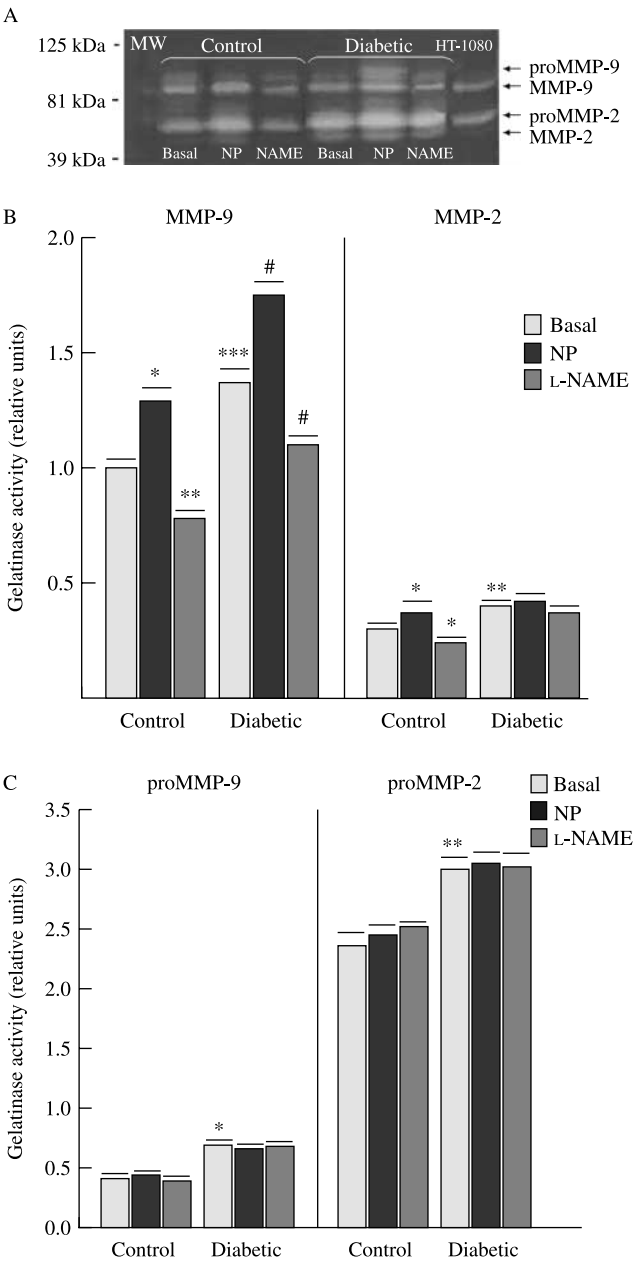

Figure 3 (A) Representative zymogram of MMP2 and MMP9 activities in the maternal placental side from control and diabetic rats on day 13.5 of gestation (i) without any addition (Basal), (ii) with the addition of sodium nitroprusside (NP, 600 μM), and (iii) with the addition of N_{ω}-nitro-L-arginine methyl ester hydrochloride (NAME, 600 μM). Molecular weights derived from the markers in the first lane are shown on the left. Conditioned medium from HT-1080 human fibrosarcoma cells was used as activity standard for MMP2 and MMP9. (B and C) Relative densitometric analysis of the activities of MMP2 and MMP9 (B) and their proenzymes (C) in the maternal placental side from control and diabetic rats on day 13.5 of gestation (i) without any addition (Basal), (ii) with the addition of sodium nitroprusside (NP, 600 μM), and (iii) with the addition of N_{ω}-nitro-L-arginine methyl ester hydrochloride (NAME, 600 μM). Data are the means±S.E.M. ($n=8$ in each group). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control without addition; $^{\#}P<0.05$ versus diabetic without addition.

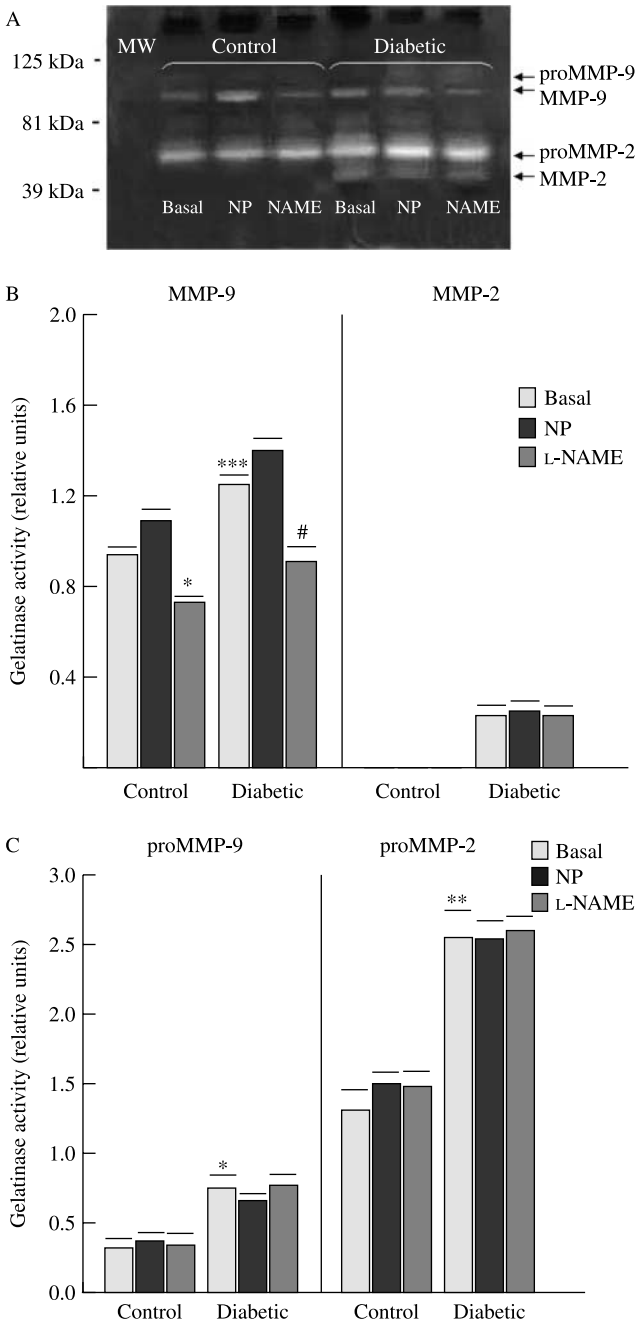

Figure 4 (A) Representative zymogram of MMP2 and MMP9 activities in fetal placental side from control and diabetic rats on day 13.5 of gestation (i) without any addition (basal), (ii) with the addition of sodium nitroprusside (NP, 600 μm), and (iii) with the addition of N_{ω}-nitro-L-arginine methyl ester hydrochloride (NAME, 600 μm). Molecular weights derived from the markers in the first lane are shown on the left. (B and C) Relative densitometric analysis of the activities of MMP2 and MMP9 (B) and their proenzymes (C) in fetal placental side from control and diabetic rats on day 13.5 of gestation (i) without any addition (basal), (ii) with the addition of sodium nitroprusside (NP, 600 μm), and (iii) with the addition of N_{ω}-nitro-L-arginine methyl ester hydrochloride (NAME, 600 μm). Data are the means±S.E.M. ($n=8$ in each group). *$P<0.05$, **$P<0.01$, ***$P<0.005$ versus control without addition; $^{\#}P<0.05$ versus diabetic without addition.

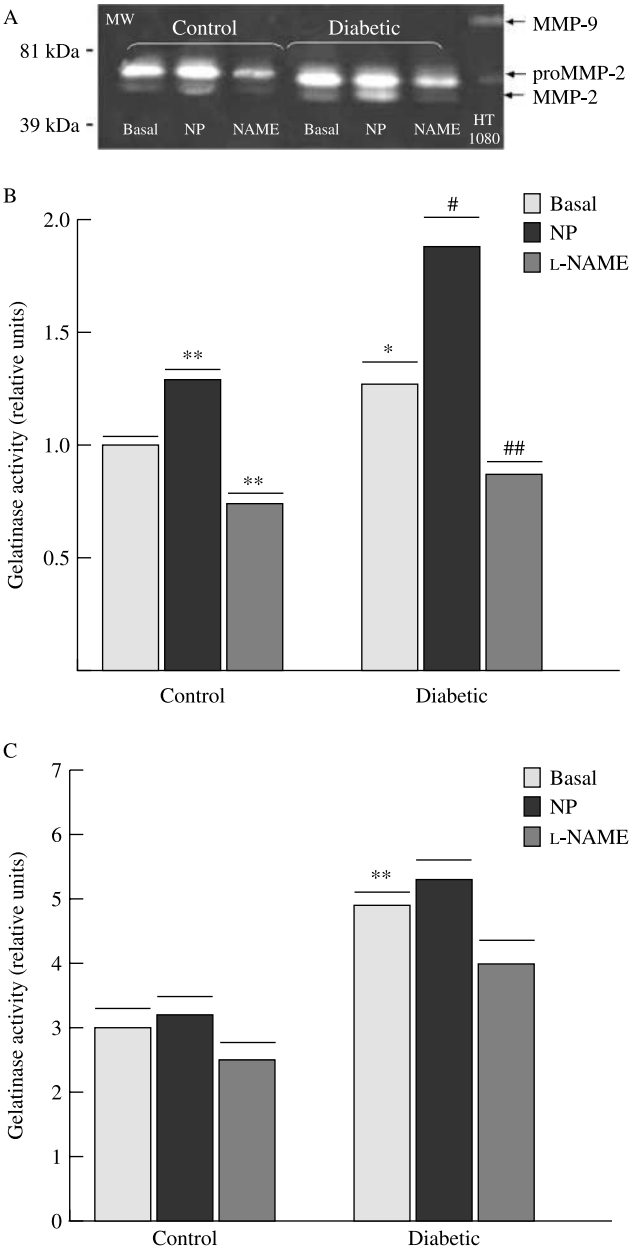

Figure 5 (A) Representative zymogram of MMP2 activity in fetuses from control and diabetic rats on day 13.5 of gestation (i) without any addition (Basal), (ii) with the addition of sodium nitroprusside (NP, 600 μM), and (iii) with the addition of N_{ω}-nitro-L-arginine methyl ester hydrochloride (NAME, 600 μM). Molecular weights derived from the markers in the first lane are shown on the left. Conditioned medium from HT-1080 human fibrosarcoma cells was used as activity standard for MMP2 and MMP9. (B and C) Relative densitometric analysis of the activity of MMP2 (B) and its proenzyme (C) in fetuses from control and diabetic rats on day 13.5 of gestation (i) without any addition (Basal), (ii) with the addition of sodium nitroprusside (NP, 600 μM), and (iii) with the addition of N_{ω}-nitro-L-arginine methyl ester hydrochloride (NAME, 600 μM). Data are the means±S.E.M. ($n=8$ in each group). *$P<0.05$ and **$P<0.01$ versus control without addition; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus diabetic without addition.

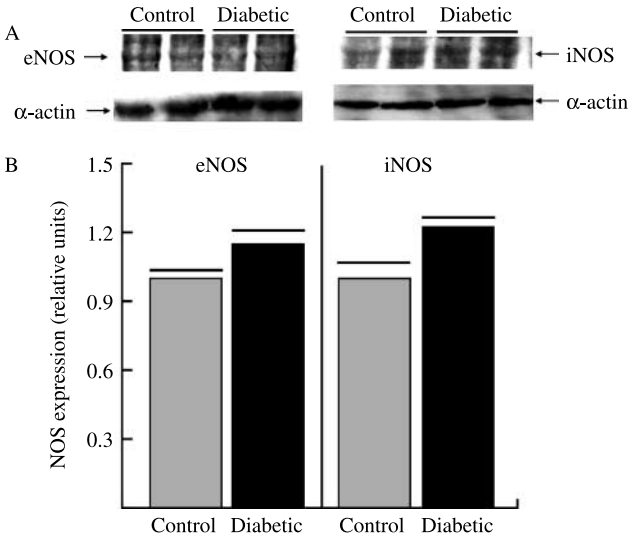

Figure 6 (A) Representative immunoblotting of eNOS and iNOS in homogenates of placenta from control and diabetic rats. (B) Relative densitometric analysis of the eNOS and iNOS expression in placenta from control and diabetic rats on day 13.5 of gestation. Data are the means±S.E.M. (n=8 in each group).

Discussion

Placental NO maintains the myometrial relaxation and the vascular tone in the feto-placental circulation (Kublickiene *et al.* 1997, Magness *et al.* 1997, Pustovrh *et al.* 2002). However, abnormal levels of NO are associated with diabetic-induced abnormalities such as pancreas damage, embryo, and fetal dysmorphogenesis, and diabetic vascular complications (Gonzalez *et al.* 1999, 2001, Jawerbaum & Gonzalez 2005, Jawerbaum & González 2006, Nakagawa *et al.* 2006). NO levels are increased in the placenta from diabetic rats at midgestation and in term placental tissues from diabetic women (Schonfelder *et al.* 1996, Pustovrh *et al.* 2000, 2005).

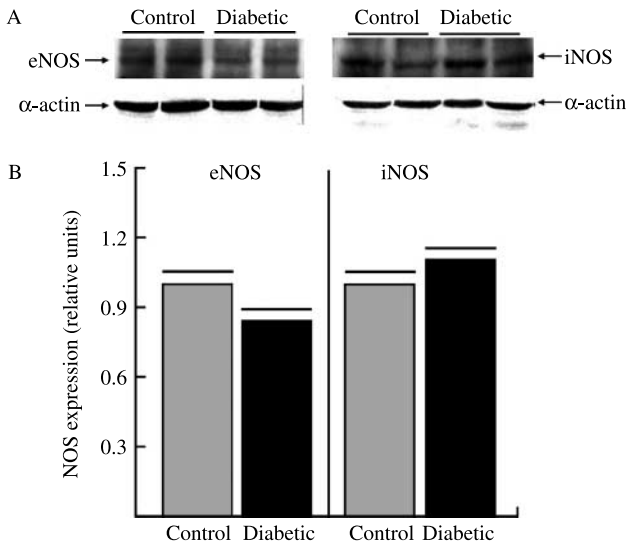

Figure 7 (A) Representative immunoblotting of eNOS and iNOS in homogenates of fetuses from control and diabetic rats. (B) Relative densitometric analysis of the eNOS and iNOS expression in fetuses from control and diabetic rats on day 13.5 of gestation. Data are the means±S.E.M. (n=8 in each group).

In the present work, we found that NADPH-diaphorase was increased in both the maternal and fetal sides of the diabetic placenta when compared with control rats. As the NADPH-diaphorase activity detected by the employed methodology is coupled with NOS, NADPH-diaphorase activity can be used as a marker for NOS activity (Gabbott & Bacon 1993, Loesch *et al.*1993).

This NOS activity increase may be a main factor in the induction of placental nitrosative stress due to maternal diabetes, and may be related to the induction of other reactive nitrogen species. Indeed, our previous findings (Pustovrh *et al.* 2005) showed a nitrotyrosine localization pattern similar to that of MMP2 and MMP9 in the diabetic placenta at midgestation that may be involved in the etiology of morphological disarrangements in the diabetic placenta.

Despite the clear increase in NOS activity in both fetuses and placenta from diabetic rats, we did not find changes in the levels of either iNOS or eNOS, suggesting that post-transcriptional mechanisms are involved in diabetes-induced upregulation of NOS in the feto-placental unit. Indeed, in addition to the regulation of NOS isoforms at the transcriptional level, cumulative evidence show different mechanisms of post-translational regulation of NOS isoforms in several cell types. These mechanisms seem to act mainly through the phosphorylation of tyrosine and depend on the activation of MAPK or Akt signaling systems (Rolle *et al.* 2002, Namkoong *et al.* 2005, Hausel *et al.* 2006)

During invasive events, MMPs are frequently expressed by both the invasive cells and the adjacent stroma. Particularly in implantation, MMPs are secreted by the invasive trophoblasts, as well as by the decidual stromal cells (Salamonsen 1999). MMPs are associated with the erosion of maternal structures during embryo implantation, including trophoblastic invasion of maternal vessels, neoangiogenesis, and the development of the labyrinth layer (Blankenship & King 1994, Solberg *et al.* 2003, Walter & Schonkypl 2006). In experimental models of diabetes, we have previously reported an increase in uterine MMPs activities during the implantation period (Pustovrh *et al.* 2002). We have also found that MMPs are highly activated in the placenta from rats at midgestation and from pregestational diabetic women at term (Pustovrh *et al.* 2000, 2005). Our evidence suggests that an early disturbance of placental development due to maternal hyperglycemia may be persistent through out the whole placental life. Moreover, these alterations are likely to be related to an abnormal proliferation and growth of peripheral villi and with the increased vascularization and functional insufficiency of the placenta from diabetic women. Additional studies are necessary to determine the

link between an abnormal remodeling and the structural and functional alterations found in the diabetic placenta.

Previous works have demonstrated that NO is able to increase MMP2 and MMP9 activities in the uterus from both control and diabetic rats (Novaro *et al.* 2002, Pustovrh *et al.* 2002) and in term human placenta and trophoblast cells in culture (Pustovrh *et al.* 2000, Novaro *et al.* 2001). Several pathways have been proposed for the mechanisms of action of NO on MMPs expression and activity. Interestingly, NO can down-regulate the expression of MMPs genes either through the activation of both the nuclear factor-κB (NF-κB) and the activated protein-1 or by affecting the stability of their mRNA (Eberhardt *et al.* 2000, 2002, Akool el *et al.* 2003). At the post-translational level, NO is able to modulate the activity of the pro-MMPs through the disruption of the union between an atom of Zn and a residue of cysteine located in the catalytic site of the proenzymes (Gu *et al.* 2002). In this work, we found that NO did not induce changes in the pro-MMPs levels in the feto-placental unit, but clearly upregulates the activity of these enzymes. Indeed, in the maternal placental side, NO modulates MMP2 and MMP9 activities in the control group and MMP9 activity in diabetic animals. However, in the fetal placental side, NO did not influence the MMP2 activity, and, although L-NAME inhibited MMP9 activity in both experimental groups, NP was not able to alter this parameter. These findings suggest that the fetal placental tissue, originated from extraembryonic cells, is more resistant to NO than the placental tissue from maternal origin, a characteristic that may imply a protection from a putative damage by an abnormal maternal environment against the fetus.

On the other hand, NO plays an important role in the post-implantation embryo development. Different works have shown the influence of NO on neural development and differentiation (Peunova & Enikolopov 1995, Wu *et al.* 2000), lung branching morphogenesis and angiogenesis (Galambos *et al.* 2002, Young *et al.* 2002), and cardiomyogenesis and myocardial angiogenesis (Bloch *et al.* 1999, Zhao *et al.* 2002). Specifically, eNOS-deficient mice show cardiac valves and ventricular septal defects (Feng *et al.* 2002, Lee *et al.* 2000). Furthermore, murine embryonic heart has been found to express iNOS and eNOS in the tubular heart stage, suggesting an important role for the enzyme throughout heart development (Bloch *et al.* 1999). We have previously found that NO levels are enhanced in embryos from diabetic rats in both early organogenesis and fetal periods (Jawerbaum *et al.* 2001, 2005, Pustovrh *et al.* 2005). In the present study, we found an important increase in NOS activity in both the spinal cord and ventricular heart from diabetic fetuses. In addition, we evidenced that NO was able to increase MMP2 activity in fetuses from both control and diabetic rats. Our results suggest that MMPs respond to NO signals in order to regulate the tissue remodeling that accompanies the rapid growth, differentiation, and structural changes of the fetal organs. Studies that involve MMP2 knockouts or MMP2 overexpression show that the altered activity of this MMP produces changes in the morphology and function of the lung, heart, and pancreas of the developing fetus (Miettinen *et al.* 2000, Kheradmand *et al.* 2002, Ratajska & Cleutjens 2002). Interestingly, diabetes produces alterations in the embryonic development mostly associated with increased risks of neonatal respiratory distress, cardiovascular anomalies, and glucose intolerance in adulthood (Piper & Langer 1993, Van Assche *et al.* 2001). Further research is needed to address whether an abnormal intrauterine environment induces changes in the fetal balance of MMPs that could lead to alterations throughout adult life.

In conclusion, this study demonstrates that NO, overproduced as a result of increased NOS activity in the diabetic feto-placental unit, is able to modulate the activation of MMP2 and MMP9 in these tissues. These results provide further insight into the involvement of NO and MMPs in EMC remodeling during this period of major structural and functional changes in the placenta, and organ development in the fetus, and thus suggest that a dysregulation in MMPs activities may have profound implications in the formation and function of the placenta and fetal organs.

Materials and Methods

Animals

Albino Wistar rats were bred in the laboratory with free access to Purina rat chow and water, under a 14 h light:10 h darkness cycle. At 2 days of age, they were injected either with streptozotocin (90 mg/kg s.c.; Sigma) in citrate buffer (0.05 M, pH 4.5) or with buffer alone (controls). Four days after birth, neonates exhibiting glycosuria higher than 500 mg/dl were considered diabetic. The spontaneous evolution of this treatment leads to a diabetic state (Portha *et al.* 1979) characterized by glycemia values between 150 and 230 mg/dl and marked glucose intolerance, while control rat glycemia levels were below 100 mg/dl. In the evening of proestrus, control, and diabetic females weighing between 200 and 300 g were caged overnight with control males. The following day was designated as day 0.5 of pregnancy if sperm cells were found in the vaginal smear. The experimental procedures were performed in accordance with the guidelines by the local institution, based on 'Principles of Laboratory Animals Care' (NIH publication no. 85-23, revised 1985).

Tissue preparation

On day 13.5 of gestation, animals were killed by cervical dislocation and their placentas and fetuses were removed and placed in Petri dishes containing Krebs–Ringer bicarbonate solution (ionic composition: 11 mM glucose, 145 mM Na^+, 5.9 mM K^+, 2.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 127 mM Cl^-, 25 mM $HCO3^-$, 1.2 mM SO_4^{2-}, and 1.2 mM PO_4^{3-}). Placentas and fetuses were divided into two random groups.

One of them was prepared for further histochemical analyses. The placental explants from the other group were incubated as described below: placental tissues were separated into fetal and maternal side under microscope. At this stage, no infiltration of fetal trophoblasts into maternal decidua has taken place, and placenta structures are easily distinguishable. All tissues were incubated for 1 h in a metabolic shaker under an atmosphere of 5% CO_2 and 95% O_2 at 37 °C, either with or without sodium nitroprusside (NP 600 μM; Sigma) or N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME 600 μM; Sigma). Aliquots of incubation medium were frozen at −70 °C for further determination of MMPs activities.

NADPH-diaphorase evaluation

Placental and fetal tissues from control and diabetic rats were fixed by immersion in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH: 7.4) for 24 h. Tissues were immersed in a solution containing 30% sucrose in 0.1 M phosphate buffer and then embedded in optical cutting temperature medium (Sakura Finetek USA, Torrance, CA, USA). Cryostat sections were processed with the NADPH-diaphorase (NADPH-d) histochemical method, which shows the enzyme localization and reflects the degree of NOS activation (Vincent & Kimura 1992, Dellacorte *et al.* 1995, Morris *et al.* 1997). Briefly, sections from diabetic and control animals were incubated for 1 h at 37 °C in a solution containing 0.1% β-NADPH (1 mg/ml), 0.02% (0.2 mg/ml) nitroblue tetrazolium chloride diluted in 0.1 M phosphate buffer (pH 7.4) and 0.3% Triton X-100 (all reagents from Sigma). Negative control sections were performed omitting the addition of NADPH in the incubation mixture. Sections were mounted on gelatin-coated glass slides and cover slipped with a PBS: glycerol mixture (1:3). Sections were observed and photographed using a Zeiss Axiophot light microscope. The images were digitalized using an image analysis system (Vidas-Kontron, Eching, Germany) and the data were statistically analyzed.

Image analysis

Optical densitometry of sections processed by NADPH-diaphorase was determined in the placentas and fetuses from control and diabetic rats in a Axiophot Zeiss light microscope coupled to a Vidas-Kontron image analyzer. For each experimental condition, the analysis was performed in six sections from different rats.

Zymography

Zymography was performed to evaluate the presence of gelatinase activity as previously described (Woessner & Taplin 1988). In addition, pro-MMPs were also analyzed by zymography, since the exposure to SDS induces changes in pro-MMPs conformation that are associated with their activation. Briefly, 25 μg protein from fetal and placental tissues (maternal and fetal sides) were subjected to a 7.5% SDS-PAGE, in which 1 mg/ml gelatin (type A from porcine skin) had been incorporated. Following electrophoresis, gels were washed in 30% Triton X-100 for 60 min to remove SDS. Then, the gels were incubated in 50 mM Tris buffer pH 7.4, containing 0.15 mM NaCl and 30 mM $CaCl_2$, for 18 h at 37 °C. Gels were stained with Coomassie blue and then destained with 10% acetic acid and 30% methanol in water. The areas of proteolytic activity appeared as negative-stained bands in the dark background.

The identities of MMPs were based on their molecular weights and a positive internal control (HT-1080 conditioned medium) that was run in each gel to allow the standardization of the results obtained in the different zymograms.

The enzymatic activity was evaluated using an image analysis program (Sigmagel, Sigma), and expressed as arbitrary densitometric units, which were normalized to the internal control. Data are shown as relative to the value 1 assigned to the mean values for MMP9 in control placenta, and for MMP2 in control fetuses.

Western blotting analysis

Tissues were homogenized in 500 μl ice-cold lysis buffer (20 mM HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 5 μl protease inhibitor cocktail) and incubated on ice for 2 h. Tissue homogenates were centrifuged at 8000 ***g*** (10 000 r.p.m.) for 10 min and the supernatant was removed. Protein concentration was determined by the Bradford assay. Equal amounts of tissue protein extract were subjected to SDS gel electrophoresis on 10% (w/v) polyacrylamide gel under reducing conditions. Proteins were transferred onto nitrocellulose membranes, blocked with 1% BSA (w/v) in Tris-buffered saline Tween-20 (TBST; 0.01 M Tris–HCl, 0.15 M NaCl, and 0.05% (v/v) Tween-20, pH 7.6) for 1.5 h and subsequently incubated with a polyclonal rabbit antibody to either iNOS (1:500) or eNOS (1:500) overnight at 4 °C. After incubation, the membranes were washed four times with TBST (each time for 10 min), followed by a 1-h incubation with a second goat anti-rabbit antibody conjugated with peroxidase with the ECL Western blotting detection kit (Calbiochem, Darmstadt, Germany) to visualize the iNOS or nNOS bands. The relative intensity of protein signals was quantified by densitometric analysis using the Sigma Gel Program.

Statistical analysis

Results were expressed as means ± S.E.M. Comparisons between groups were performed employing either one-way ANOVA in conjunction with Tukey's test or Student's *t*-test where appropriate. The statistical level of significance was defined as $P<0.05$.

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