Free Radical Research, December 2005; 39(12): 1285–1293

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Oxidative stress promotes the increase of matrix metalloproteinases-2 and -9 activities in the feto-placental unit of diabetic rats

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Accepted by Professor J. Vina

(Received 16 March 2005; in revised form 26 May 2005)

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Abstract

Maternal diabetes increases the risk of congenital malformations, placental dysfunction and diseases in both the neonate and the offspring's later life. Oxidative stress has been involved in the etiology of these abnormalities. Matrix metalloproteases (MMPs), involved in multiple developmental pathways, are increased in the fetus and placenta from diabetic experimental models. As oxidants could be involved in the activation of latent MMPs, we investigated a putative relationship between MMPs activities and oxidative stress in the feto-placental unit of diabetic rats at midgestation. We found that H_2O_2 enhanced and that superoxide dismutase (SOD) reduced MMPs activities in the maternal side of the placenta and in the fetuses from control and diabetic rats. MMPs were not modified by oxidative status in the fetal side of the placenta. Lipid peroxidation was enhanced in the maternal and fetal sides of the placenta and in the fetus from diabetic rats when compared to controls, and gradually decreased from the maternal placental side to the fetus in diabetic animals. The activities of the antioxidant enzymes SOD and catalase were decreased in the maternal placental side, catalase activity was enhanced in the fetal placental side and both enzymes were increased in the fetuses from diabetic rats when compared to controls. Our data demonstrate changes in the oxidative balance and capability of oxidants to upregulate MMPs activity in the feto-placental unit from diabetic rats, a basis to elucidate links between oxidative stress and alterations in the developmental pathways in which MMPs are involved.

Keywords: *Diabetes, metalloproteinases (MMPs), reactive oxygen species, placenta, fetus, pregnancy*

Introduction

Diabetes mellitus is a disorder that affects the normal development of pregnancy, thus resulting in increased spontaneous abortion, congenital abnormalities and neonatal morbility and mortality rates [1]. In addition, the diabetic pathology leads to structural and functional dearrangements in the placenta [2], that can, in turn, lead to deleterious consequences to the embryo development.

There is now definite evidence that oxygen radicals contribute to the development of the diabetic embryopathy [3]. Reactive oxygen species (ROS) threaten developmental processes due to their ability to induce lipid, protein and DNA damage [4]. The main source of oxidative stress in diabetes appears to be hyperglycaemia-induced process of over-production of superoxide by the mitochondrial electron-transport chain [5]. Indeed, in endothelial cells and in adipocytes, normalization of mitochondrial ROS formation by a number of different approaches prevents hyperglycemia-induced activation of protein kinase C isoforms, the formation of glucose-derived advanced glycation end products, and the increased polyol pathway flux [5,6]. Both hyperglycemia and ketonemia can increase lipid peroxidation as detected

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis
DOI: 10.1080/10715760500188796

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by TBARS and other methods in different tissues and cell types [7–10].

In embryos from diabetic experimental models, increases in lipid peroxidation, oxidative DNA damage and protein carbonylation have been demonstrated [11,12]. There are also evidences of ROS-induced damage in term placental tissues from diabetic women [13,14]. ROS toxicity is limited due to the presence of ROS scavenging systems. Superoxide dismutase (SOD) is one of the main antioxidant enzymes and catalyzes the dismutation of superoxide to H_2O_2, which is subsequently detoxified by catalase or glutathione peroxidase [4]. Oxidative stress promotes embryonic and placental alterations in the antioxidant enzymes in embryos and placentas from experimental diabetic models and in placenta from diabetic women [10,13,15–17]. Accordingly, numerous studies have shown that antioxidants can revert the developmental effects caused by maternal diabetes in experimental animals [10,12,18,19].

Although direct injury to cells is one mechanism by which oxidants promote inflammatory damage, oxidants may also enhance the tissue-destructive effects of proteolytic enzymes [20]. Indeed, oxidants are capable of converting matrix metalloproteinases (MMPs) from latent to active forms [21]. The zymogen conformation of these zinc-containing proteases is maintained due to thiol interactions between cysteine residues in the prodomain and the zinc atom present in their catalytic site [22]. ROS undergo reactions with thiol groups and activate several different MMPs, including the gelatinases MMP-2 and MMP-9 [23,24]. This activation has been found in many circumstances blocked by antioxidants [25,26]. Enhanced MMPs activities plays a substantial role in the pathophysiology of several diseases, including atheriosclerosis rheumatoid arthritis chronic respiratory distress syndrome and gestational trophoblastic disease [27,28].

MMPs are capable of cleaving most components of basement membrane and extracellular matrix, such as collagen, fibrin, laminin and proteoglycans, thus leading to remodelling and also to the release of cryptic information and growth factors contained within [29,22].

Accurately controlled remodelling of the extracellular matrix is an essential aspect of the female reproductive system [30]. The extracellular matrix contains instructive information for the embryo development, and MMPs are involved in the coordination of morphogenesis of multiple organs including lungs, pancreas, heart, skeleton and neural crest derived organs [31]. In addition, MMPs have been clearly involved in implantation and placental developmental events [31–33]. In most of these processes the activities of the gelatinases MMP-2 and MMP-9 have been directly involved [34,35].

We have previously characterized several embryonic and placental alterations in a mild experimental rat model of diabetes induced by neonatal administration of streptozotocin [36]. In this experimental model we found increased MMPs activities in uterine, fetal and placental tissues [37,38], abnormalities probably related to the developmental alterations that lead to feto-placental dearrangements induced by this pathology.

In the present study, we have assessed the changes in MMP-2 and MMP-9 activities under the influence of oxidative stress and we evaluated the oxidative status in the feto-placental unit from control and diabetic rats at midgestation. These studies provide evidence that ROS positively regulate MMPs in the feto-placental unit, and characterize both the maternal and fetal sides of the placenta as a barrier to maternal ROS and the fetus as highly susceptible to ROS-induced changes in MMP activity.

Materials and methods

Materials

Streptozotocin, citrate buffer, hydrogen peroxide (H_2O_2) superoxide dismutase (SOD), (±) epinephrine hydrochloride, ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid and malondialdehyde were purchased from Sigma (St. Louis, MO, USA). Bradford reagent, Trizma base, Glycine, Acrylamide, N′N′ Bis-methylene-acrylamide, amonium persulfate, Coomasie blue R-250 and molecular weight standards were obtained from Bio-Rad (Hercules, CA, USA). Glucostix reagent strips were purchased from Roche Diagnostics (Buenos Aires, Argentina). HT-1080 conditioned medium was obtained from the Cancer Research Laboratory, Center for Pharmacological and Botanical Studies (Buenos Aires, Argentina).

Animals

Albino Wistar rats were bred in the laboratory with free access to Purina rat chow and water, in a lighting cycle of 14 h light: 10 h dark. At 2 days of age, they were injected with either streptozotocin (90 mg/kg s.c.) in citrate buffer (0.05 M, pH 4.5) or buffer alone (controls). Four days after birth, neonates exhibiting glycosuria higher than 500 mg dl^{-1} were considered diabetic. The spontaneous evolution of this treatment leads to a diabetic state as previously described [39]. Adult diabetic rats presented glycemia levels higher than 200 mg dl^{-1} and a marked glucose intolerance, while control rats showed glycemia levels below 110 mg dl^{-1}. In the evening of proestrus, control and diabetic females, weighing between 200–300 g, were caged overnight with control males. The following day was designated as day 0.5 of gestation if sperm cells were found in the vaginal smear.

Tissue processing

Animals were killed by cervical dislocation on day 13.5 of gestation and their placentas and fetuses were

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removed and placed in Petri dishes containing Krebs-Ringer-Bicarbonate solution (KRB, 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 HCO_3^-, 1.2 mM SO_4^{2-}, 1.2 mM PO_4^{3-}). Placental tissues were separated into fetal and maternal side under microscope. At this stage, there is no infiltration of fetal trophoblasts into maternal decidua, and placental structures are easily distinguishable. For lipid peroxidation and antioxidant activity determinations the tissues were stored at −70°C. For zymography analysis the tissues were incubated for an hour in a metabolic shaker, either with or without H_2O_2 (50 μM) or SOD (1000 U/ml). After incubation, aliquots of the incubation medium were frozen at −70°C for further determination of MMPs activities. The guidelines for the care and use of animals approved by our Institution were followed, according to ILAR guide for the Care and Use of laboratory animals (1996), http://www.nih.gov/sigs/bioethics/animals.html.

Zymography

Zymography was performed to evaluate the presence of gelatinase activity as previously described [40]. Fetal and placental samples (25 μg of protein) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis, in which 1 mg/ml gelatin (type A from porcine skin) had been incorporated. Following electrophoresis the 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. Negative staining is indicative of the localization of active proteolytic bands. Enzymatic activities in the gel slabs were quantified by using image analysis (image analysis programme Sigmagel), which evaluates equal areas and quantifies the surface and the intensity of lysis bands. The identities of MMPs were based on their molecular weights. The enzymatic activity is expressed as arbitrary densitometric units relative to value 1 assigned to the control. These bands of enzymatic degradation are specific since they disappear in the presence of EDTA 0.01 M in the incubation buffer.

Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring the levels of thiobarbituric acid reactive substances (TBARS), a simple widely used method employed to assess peroxidation of fatty acids [41,4]. Briefly, approximately 100 mg of tissue was homogenized in 100 mM Tris–HCl buffer pH 7.6. The homogenate was added with trichloroacetic acid (40% w/v) and was centrifuged at 3000 rpm for 10 min. The supernatant was added with an equal volume of thiobarbituric acid (46 mM), and the solution was heated at 95°C for 15 min. Then, the samples were cooled and quantified spectrophotometrically at 530 nm. Malondialdehyde (MDA), submitted to the same conditions as the tissue homogenates, was used as a standard. TBARS were expressed as nmol per mg protein.

Evaluation of catalase and superoxide dismutase activities

Tissues were homogenized in 500 μl of homogenization buffer pH 7.6 (Tris-base 20 mM, EDTA 1 mM, KCl 150 mM, β Mercaptoethanol 1 mM, sacarose 500 mM). Then the homogenates were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatants were removed and stored at −70°C. The activity of catalase was evaluated following Aebi's method [42]. Briefly, the reaction mixture contained 50 mM potassium phosphate (pH 7.2), 3 mM H_2O_2 and the sample. Activity was determined by following the decomposition of H_2O_2 spectrophotometrically at 240 nm. Rates were calculated as the change in optical density of the assay mixture per minute per mg of protein. The activity of SOD was assayed by the method of Misra and Fridovich [43]. This method is based on the ability of SOD to inhibit autoxidation of epinephrine under specific conditions. The prepared sample was added to 3 ml of 50 mM glycine buffer (pH 10.2 30°C) containing 1 mM epinephrine. The absorbance changes were monitored at 480 nm. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of epinephrine auto oxidation. Enzyme activity was reported as units per mg protein in the homogenate.

Statistical analysis

All values presented in this study represent means ± SEM. Comparisons between groups were performed by employing either one-way analysis of variance in conjunction with the Tukey's test or Student's *t*-test, where appropriate. Differences between groups were considered significant when $P = 0.05$ or less.

Results

Effect of reactive oxygen species on MMP-2 and MMP-9 activities in the feto-placental unit from control and diabetic rats

In order to determine whether oxidants affect MMPs activities in the feto-placental unit, both maternal and fetal placental sides and fetuses obtained from control and diabetic rats on day 13.5 of gestation were incubated for one hour in the presence or absence of H_2O_2 (50 μM), an oxidant species, and of SOD (1000 U/ml), a scavenger of free oxygen radicals. In agreement with our previous works [38], we found that both MMP-2 and MMP-9 activities were elevated in the maternal and fetal placental

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sides from diabetic rats when compared to controls (Figures 1 and 2). In addition, the zymography analysis reflected that in the maternal placental side from control and diabetic rats, H_2O_2 increased MMP-2 and MMP-9 activities when compared to control ($p < 0.01$) and diabetic tissues ($p < 0.05$) without additions (Figure 1). On the other hand, SOD addition reduced MMP-2 and MMP-9 activities in the maternal placental side from control ($p < 0.05$ and $p < 0.01$, respectively) and diabetic ($p < 0.01$) rats when compared with the tissues incubated without additions (Figure 1).

Differently, neither the addition of H_2O_2 nor the addition of SOD produced changes in MMPs activities in the fetal placental side from both control and diabetic rats (Figure 2). Similarly to previous reports [38], we found that MMP2 levels were very low and not detectable by the densitometric analysis in the fetal side of the placenta from control rats while MMP2 levels are increased in the fetal side of the placenta from diabetic rats (Figure 2).

On the other hand, when fetuses were evaluated, we observed that at the evaluated developmental stage, all of them lacked MMP-9 activity and fetuses from diabetic rats showed enhanced MMP-2 activity when compared to controls (Figure 3), as previously determined [38]. Interestingly, when the effect of H_2O_2 and SOD was evaluated, we found that the addition of H_2O_2 significantly increased MMP-2 activity in the fetuses from both control ($p < 0.01$) and diabetic ($p < 0.01$) rats when compared to the fetuses without additions (Figure 3). On the other hand, the addition of SOD resulted in a decrease of MMP-2 activity in fetuses from both control ($p < 0.05$) and diabetic ($p < 0.05$) rats when compared to those incubated without additions (Figure 3).

Oxidative balance in the feto-placental unit from control and diabetic rats

Despite the moderate hyperglycemia found in diabetic mothers (glycemia values in diabetic rats

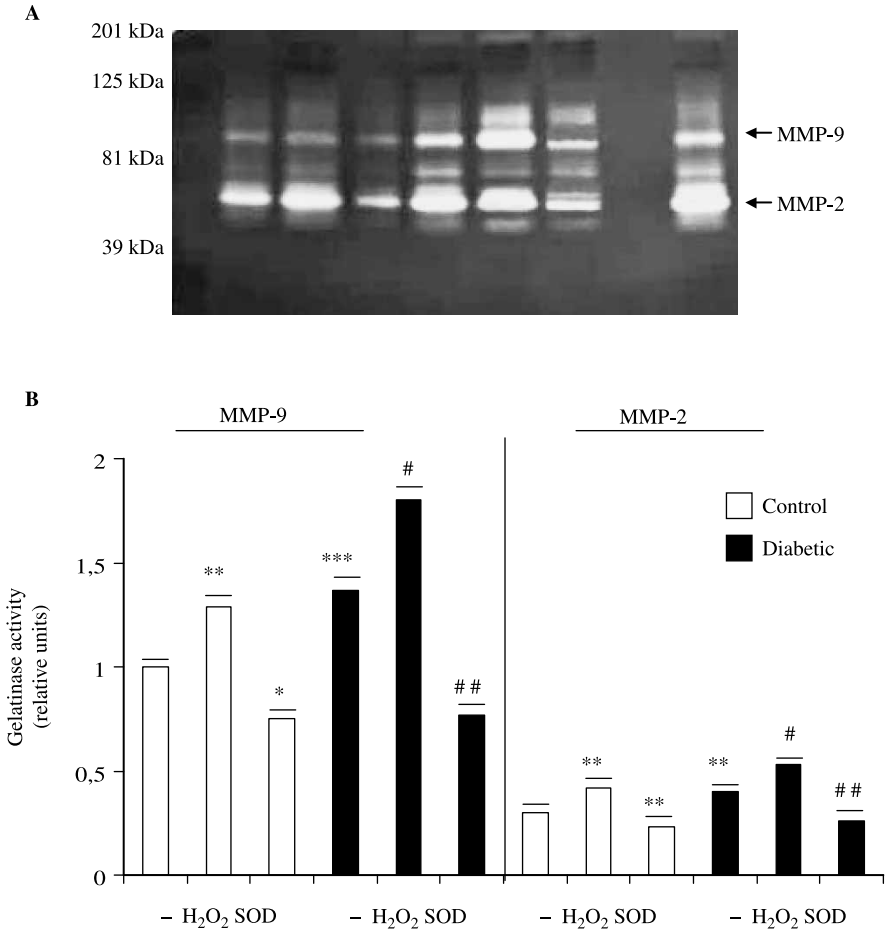

Figure 1. (A) Representative zymogram showing gelatinase activity in maternal placental side from control and diabetic rats on day 13.5 of pregnancy: (i) without any addition; (ii) with H_2O_2 (50 μM) and (iii) with SOD (1000 U/ml). Molecular weights derived from the markers are shown in the fist line on the left. Conditioned medium from HT-1080 human fibrosarcoma cells was used as activity standard for MMP-2 and MMP-9. (B) Densitometric analysis of MMPs activities in maternal placental side from control and diabetic rats: (i) without any addition; (ii) with H_2O_2 (50 μM) and (iii) with SOD (1000 U/ml) by computer-aided densitometry. Data are the mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$ compared to control without any additions $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to diabetic without any additions.

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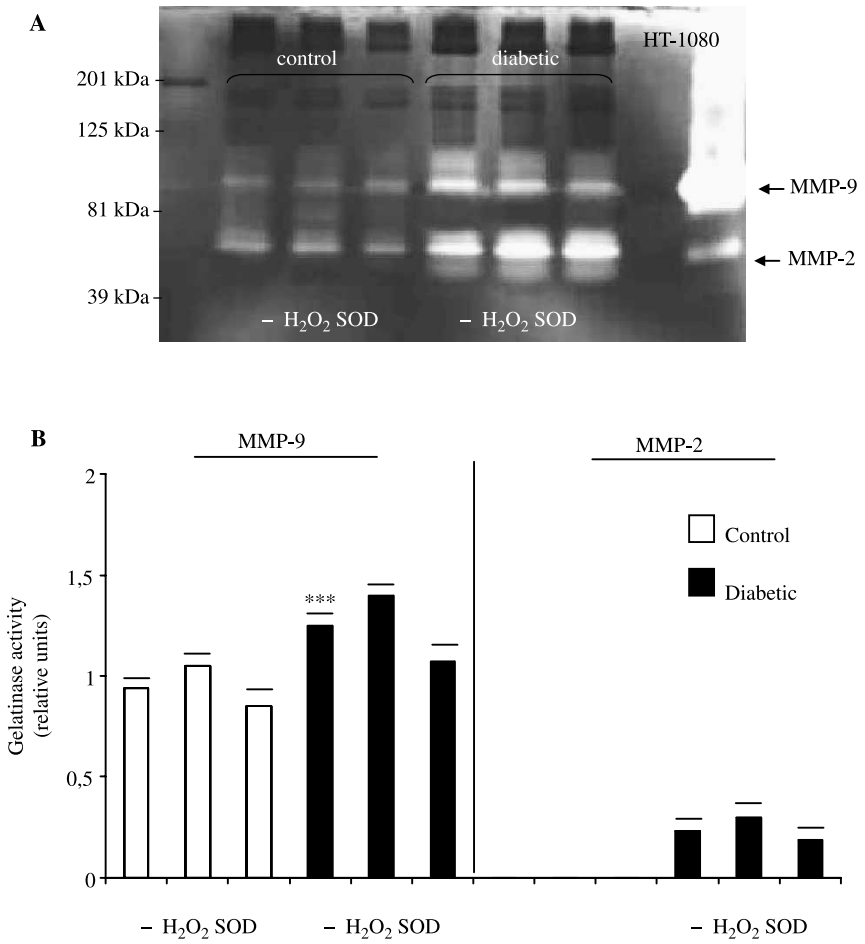

Figure 2. (A) Representative zymogram showing gelatinase activity in fetal placental side from control and diabetic rats on day 13.5 of gestation: (i) without any addition; (ii) with H_2O_2 (50 μM) and (iii) with SOD (1000 U/ml). Molecular weights derived from the markers are shown in the fist line on the left. Conditioned medium from HT-1080 human fibrosarcoma cells was used as activity standard for MMP-2 and MMP-9. (B) Densitometric analysis of MMPs activities in fetal placental side from control and diabetic rats: (i) without any addition; (ii) with H_2O_2 (50 μM) and (iii) with SOD (1000 U/ml) by computer-aided densitometry. Data are the mean ± SEM ($n = 8$ in each group). ***$p < 0.005$ compared to control without any additions.

were 215 ± 21 mg/dl while in control rats were 101 ± 10 mg/dl, $p < 0.001$), evidence of lipid peroxidation was found in the feto-placental unit of diabetic rats on day 13.5 of gestation. Indeed, increased TBARS concentrations were found in the maternal ($p < 0.005$) and fetal ($p < 0.02$) placental sides and in the fetuses ($p < 0.02$) from diabetic rats when related to controls (figure 4).

Activities of catalase and SOD antioxidant enzymes in the feto-placental unit from diabetic rats on day 13.5 of gestation are reported in Table I. The maternal placental side of diabetic rats showed a dramatic reduction of levels of both catalase ($p < 0.02$) and SOD ($p < 0.001$) when compared to controls. In contrast, the fetal placental side from diabetic rats showed increased catalase activity when compared to controls ($p < 0.001$) and no differences in SOD activity. Moreover, fetuses from diabetic rats showed an increase in both catalase ($p < 0.02$) and SOD ($p < 0.001$) activities when compared to controls.

Table I. Activities of catalase and SOD in maternal and fetal sides of the placenta and in fetuses from control and diabetic rats.

	Catalase (nmol/min mg prot)		SOD (U/mg prot)	
	Control	Diabetic	Control	Diabetic
Maternal placental side	131.0 ± 14.2	62.9 ± 10.5**	10.60 ± 0.73	5.08 ± 0.89***
Fetal placental side	90.8 ± 9.1	174.8 ± 17.2***	17.80 ± 1.09	16.60 ± 2.09
Fetuses	120.6 ± 16.5	196.4 ± 20.2*	12.13 ± 0.67	18.53 ± 1.73***

Data are means ± SEM ($n = 8$ in each group). *$p < 0.02$;**$p < 0.005$; ***$p < 0.001$ compared to controls.

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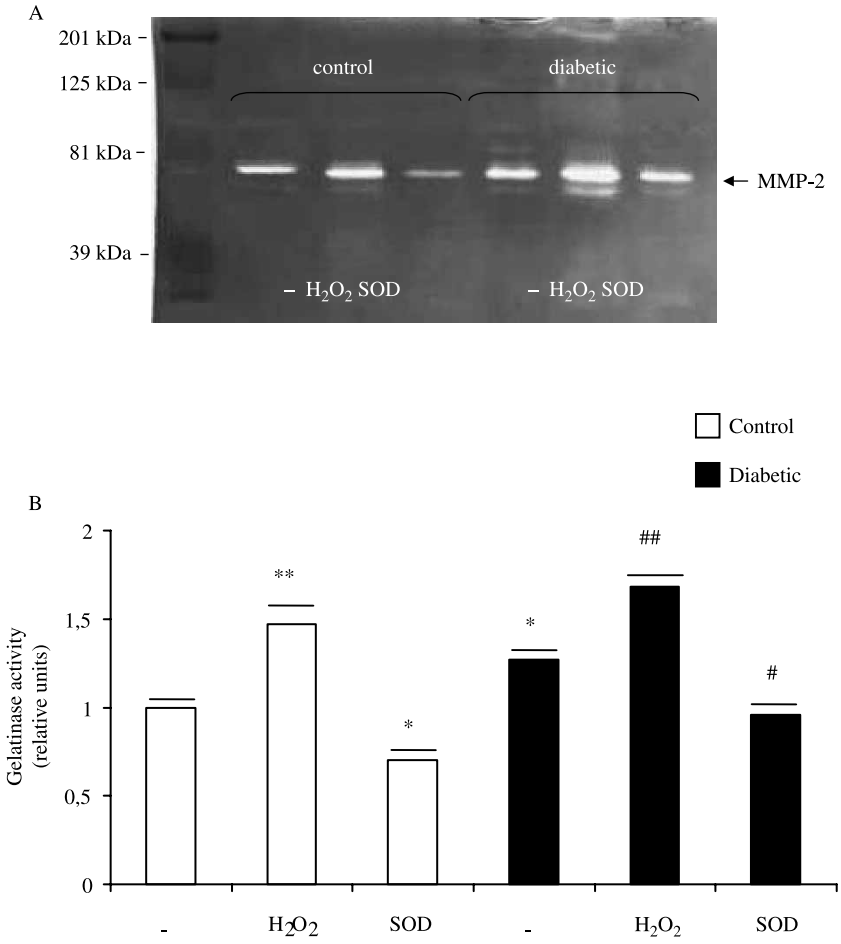

Figure 3. Representative zymogram showing gelatinase activity in fetuses from control and diabetic rats on day 13.5 of gestation: (i) without any addition; (ii) with H_2O_2 (50 μM) and (iii) with SOD (1000 U/ml). Molecular weights derived from the markers are shown in the fist line on the left. (B) Densitometric analysis of MMPs activities in fetuses from control and diabetic rats: (i) without any addition; (ii) with H_2O_2 (50 μM) and (iii) with SOD (1000 U/ml) by computer-aided densitometry. Data are the mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control without any additions $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to diabetic without additions.

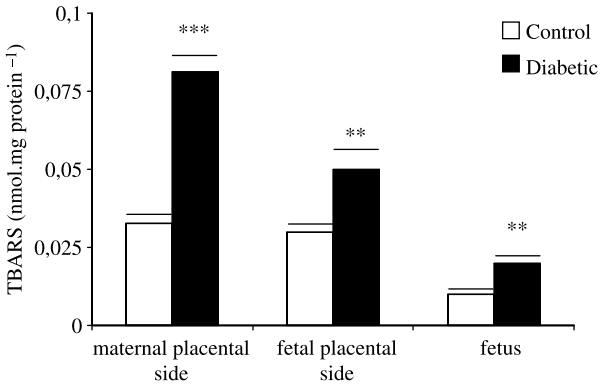

Figure 4. Levels of thiobarbituric acid reactive substances (TBARS) in fetal and maternal sides of the placenta and in fetuses isolated from control and diabetic rats (day 13.5 of gestation). Data are means ± SEM ($n = 8$ in each group). $^{**}p < 0.02$ and $^{***}p < 0.005$ compared to controls.

Discussion

We have previously demonstrated elevated MMPs activities in the feto-placental unit from diabetic rats at midgestation [38]. In the present study, we investigated ROS capacity to regulate MMPs activities in the maternal and placental sides of the placenta and in the fetuses from control and diabetic rats. Although ROS have been previously found to be positive regulators of MMPs activities in different tissues including human term placenta [13,20,26], this work is the first demonstrating that ROS enhance MMPs activity in the feto-placental unit at midgestation. Moreover, the evaluation of this regulatory pathway, together with the evaluation of lipid peroxidation and antioxidant enzymes, led us to estimate both the effectiveness of the placenta as a barrier to maternal ROS and the susceptibility of the different tissues evaluated to ROS-induced changes in MMPs activities.

MMPs are involved in the constant remodelling of the extracellular matrix during feto-placental development [29,31]. These proteases are clearly involved

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in migration and differentiation of trophoblast cells, trophoblast invasion of the decidua, erosion of maternal capillary, angiogenesis, vasculogenesis and development of the laberinth layer [32,33,44,45].

Elevated MMPs in the uterus from diabetic rats during implantation have been previously found [37], indicating that MMPs are affected by maternal diabetes from the initial step of the placental development. Moreover increases in MMPs activities in placental tissues from diabetic rats and patients [13,38,46] are probably involved in the anomalies such as enhanced fibrotic responses disruption of the extracellular matrix components and vascular architecture, enhanced angiogenesis and villous proliferation, and villous immaturity, frequently found in the diabetic placenta [2,47,48].

On the other hand, increased ROS and signs of ROS-induced damage have been previously found in term placenta from diabetic animals and patients [10,13,14,49]. In these tissues, the activities of antioxidant enzymes have been found increased [17,49,50] and decreased [10,13,50].

In the present work, the evaluation of the maternal and fetal sides of the diabetic placenta at midgestation allowed us to find that lipid peroxidation was increased in both sides when compared to controls, and that, differently, the activities of catalase and SOD were decreased in the maternal side while these activities were respectively increased and unchanged in the fetal side when compared to controls. Interestingly, in the diabetic placenta, lipid peroxidation was higher, and the activities of catalase and SOD were lower in the maternal side than in the fetal side. These results suggest a greater impact of maternal diabetes-induced oxidative stress in the maternal side of the placenta when compared to the fetal side. In addition, the oxidants and antioxidants that regulate MMPs activities in the maternal side of the placenta did not affect MMPs activities in the fetal side of the placenta, thus suggesting that the placental tissue of fetal origin is more resistant to oxidative stress than that from maternal origin. Comparative studies have previously shown that in diabetic pregnancies, the embryos appear to be under less oxidative stress than their mothers [12]. In the present work, we found a gradual decrease of lipid peroxidation from the maternal side of the placenta to the fetus, corroborating that the placenta plays an important role as a barrier against maternal-induced oxidative stress in the diabetic pathology.

Oxidants are thought to be responsible for much of the embryonic damage that occurs under hyperglycemic conditions [3]. Indeed, lipid peroxidation and other signs of ROS-induced damage such as protein carbonylation, DNA oxidative damage and increased apoptotic rate have been previously found elevated in the embryos from diabetic rats [11,12,51]. In addition, there is a relative immaturity of the free radical scavenging system in the fetus when compared to the neonate [52]. Nevertheless, our results indicate that the embryo has the capacity of increasing the activity of the antioxidant enzymes SOD and catalase in response to mild hyperglycemic conditions. Both increases and decreases in the activities of antioxidant enzymes have been found in embryos under hyperglycemic conditions, and those alterations seem to be related to both the degree of hyperglycemia and the susceptibility of the animal strain [15,16,53]. Accordingly, reduced malformation rates have been found as a response to various antioxidant treatments in experimental models of diabetes [18,19]. In this work, we found that even though the activities of SOD and catalase are increased in fetuses from diabetic rats, both ROS and antioxidants regulate fetal MMP-2 activity. Therefore, the fetus seems to be exquisitively sensitive to oxidants, and maternal diabetes-induced oxidative stress is likely to be involved in the increase of fetal MMP-2 activity. This increase probably interferes with the developmental processes, such as closure of the neural tube [51], remodelling during lung branching morphogenesis and alveolization [34], formation and vascularization of the four-chambered heart [54] and migration and differentiation of pancreatic islet cells [55], that are occurring at the evaluated embryonic stage in which MMP-2 is involved.

In addition to congenital malformations, maternal diabetes leads to increases in the risks of neonatal respiratory distress and in the development of cardiovascular anomalies and glucose intolerance in the offspring later life [56,57]. We think that diabetes-induced increases of MMPs during fetal development may lead to response patterns in target organs that may persist throughout life and thus explain links between early life events and later development of serious disease, such as respiratory distress, ischaemic heart disease and diabetes.

In summary, our data demonstrate important differences in the oxidant state and the ROS capability to upregulate MMPs in the maternal and fetal sides of the placenta and in the fetuses from diabetic rats: (1) a gradual decrease in lipid peroxidation and an increase in antioxidant enzyme activities from the maternal side of the placenta to the developing fetus; (2) a marked resistance to oxidant-induced MMPs regulation in the fetal side of the placenta, and a particular vulnerability to this pathway in the fetus. The overall result is ROS-induced upregulation of MMPs in the feto-placental unit from diabetic rats, thus suggesting that antioxidant-based therapeutics may prove useful for prevention of both, the diabetes-induced increases of MMPs and the alterations in developmental processes in which MMPs are involved.

Acknowledgements

This work was supported by grants from Agencia de Promoción Científica y Tecnológica de Argentina

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(PICT 05-10652) (E.G, A.J) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2529/99) (A.J.) We also acknowledge the technical assistance of María Esther Castro.

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