Free Radical Research, December 2010; 44(12): 1407–1415

informa
healthcare

Increased nitration and diminished activity of copper/zinc superoxide dismutase in placentas from diabetic rats

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(Received date: 4 March 2010; In revised form date: 21 July 2010)

Abstract

Nitration-induced protein damage in the placenta leads to impaired blood flow and deficient feto–placental exchange in diabetic pregnancies. This work studied the effect of nitric oxide and peroxynitrite on Cu/Zn SOD activity in rat placentas and evaluated whether Cu/Zn SOD is nitrated in the placenta from diabetic rats at mid-gestation. Protein nitration was evaluated by EIA, Cu/Zn SOD activity by inhibition of the epinephrine auto-oxidation, Cu/Zn SOD expression by western blot and specific nitration by immunoprecipitation. This study found higher levels of protein nitration ($p < 0.001$), diminished Cu/Zn SOD activity and enhanced protein expression ($p < 0.01$) in placentas from diabetic rats. Placental Cu/Zn SOD activity was inhibited by peroxynitrite ($p < 0.01$). Besides, nitration of Cu/Zn SOD was elevated in placentas from diabetic rats ($p < 0.01$). These results show that rat Cu/Zn SOD can be nitrated, a modification that could lead to the depressed activity of this enzyme found in placentas from diabetic rats.

Keywords: *Placenta, Cu/Zn SOD, peroxymitrite, diabetes, rat*

Introduction

Diabetes is a metabolic disorder characterized by impaired insulin levels and/or bioavailability, which lead to high glycemia levels and over-production of reactive oxygen species (ROS). These anomalies have been shown to cause severe impairments in reproduction [1–4]. Several diabetic women present impaired ovulation, implantation failure or embryo loss [5,6]. Also, during pregnancy, diabetes leads to embryo anomalies, congenital malformation, intrauterine growth restriction and pre-eclampsia, among other anomalies [7,8]. The diabetic progeny can suffer from respiratory distress, neonatal hypoglycemia and the induction of metabolic diseases later in life due to an abnormal intrauterine programming [9–11]. Moreover, the placental function and morphology are altered in diabetic pregnancies and these alterations might be closely related to many anomalies described for the diabetic gestation [12–16].

Oxidative stress, caused by the increased placental blood flow and mitochondrial metabolic activity, is a common feature of all pregnancies. Commonly, during diabetic gestation, there is an over-production of ROS. In this pro-oxidant environment there are many biological alterations as peroxidated lipids, oxidized proteins, impaired protein function and elevated levels of superoxide anion, which challenge the regular feto–placental development and growth [17–20].

Placentas from diabetic pregnant women and experimental models of diabetes show high concentrations of ROS and oxidative-related damage [21–24].

Oxidative stress is not only caused by an over-production of ROS, but also by a disturbance of the balance between ROS production and their counterpart antioxidant defences. The natural defense against an excess of pro-oxidant species involves the antioxidant-detoxifying enzymes and molecules such as superoxide dismutase (SOD), catalase, vitamins C

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK, Ltd.
DOI: 10.3109/10715762.2010.512039

and E and glutathione. SOD catalyses the dismutation of the superoxide radical to hydrogen peroxide and then catalase converts hydrogen peroxide to water and molecular oxygen. The two tissular SOD isoforms, copper-zinc-containing SOD (Cu/ZnSOD) and manganese-containing SOD (MnSOD), are key players in the antioxidant defence mechanisms of the placenta. MnSOD is a mitochondrial enzyme which has been localized in the villous endothelium in human placenta. Cu/ZnSOD is a 16 KDa-cytosolic enzyme which forms dimers and is localized in the villous stroma in human placenta [18]. The expression of Cu/Zn SOD, although constitutive, is stimulated by heavy metals, hydrogen peroxide and nitric oxide, among others [25]. Previous research has shown an increase in MnSOD protein levels from villous endothelium in human placentas from diabetic patients at term [18]. Differently, Cu/ZnSOD activity has been found depressed in term placentas from diabetic women [17]. Also, we have shown decreased Cu/ZnSOD activity in placentas from diabetic rats at mid and term gestation, an alteration associated with high lipid peroxidation [21,22].

Nitric oxide (.^NO) is a vasoactive agent, which plays an important role in vasculature and smooth muscle relaxation [26,27]. However, .^NO can also combine with superoxide, giving rise to peroxynitrite, a powerful oxidative and nitrating molecule. Peroxynitrite is more reactive than .^NO and can induce severe damage to DNA bases, leading to apoptosis and necrosis [28]. Likewise, peroxynitrite can modify tyrosine residues from several proteins, impairing signalling transduction pathways and enzymatic activity [29–34]. Indeed, many authors have demonstrated a direct relation between protein nitration and peroxynitrite-induced damage [31,33,35]. It has been shown that human MnSOD can be nitrated in tyrosine 34, a post-translational modification that diminishes its activity [36]. On the other hand, as human Cu/ZnSOD does not have tyrosine residues in its structure it is not a target for nitration. Interestingly, the introduction of a tyrosine in a mutant human Cu/ZnSOD in position 133 or 141 makes it available for nitration by peroxynitrite, a reaction facilitated by the availability of the copper atom, needed for the catalysis of nitration [33,37]. Bovine Cu/ZnSOD has a tyrosine in position 108; nitration of this residue by peroxynitrite *in vitro* does not change the activity of the enzyme [33]. Differently, rat Cu/ZnSOD has a tyrosine in position 51, near the hydrophobic pocket of the active site and within the dimer interface (Scheme 1).

Placentas from women with oxidative stress-associated pathologies present an increase in nitrotyrosine-modified-proteins [38]. Particularly, placentas from diabetic patients have increased levels of tyrosine-nitrated proteins and vascular damage caused by peroxynitrite [39,40]. Similarly, our previous studies have shown increased .^NO production and peroxynitrite-induced damage in placentas from diabetic rats throughout gestation [41,42]. Although the ability of peroxynitrite to modify tyrosine residues from human MnSOD and bovine Cu/ZnSOD has been studied *in vivo* and *in vitro* [31,33,37,43], there is yet no evidence of rat placental Cu/ZnSOD nitration. In this work, we aimed to investigate the effect of NO and peroxynitrite on Cu/ZnSOD activity in the rat placenta and analyse whether placental Cu/ZnSOD is nitrated in the placentas from diabetic rats at mid-gestation.

Materials and methods

Reagents

Streptozotocin, citrate buffer, SOD, glycerol, epinephrine hydrochloride N^G-nitro-L-arginine methyl esther (L-NAME), sodium nitroprusside (NP),

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Bovine: MATKAVCVLKGDGPVQGTIHFEAKGDTVVVTGSITGLTEGDHGFHVHQFGDNTQGCTS  (1-58)

Human: MATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTAGCTS (1-60)

Rat:    MAMKAVCVLKGDGPVQGVIHFEQKASGEPVVVSGQITGLTEGEHGFHVHQYGDNTQGCTT (1-60)


Bovine:AGPHFNPLSKKHGGPKDEERHVGDLGNVTADKNGVAIVDIVDPLISLSGEYSIIGRTMVV(59-118)

Human: AGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVV (61-120)

Rat:   AGPHFNPHSKKHGGPADEERHVGDLGNVAAGKDGVANVSIEDRVISLSGEHSIIGRTMVV (61-120)


Bovine: HEKPDDLGRGGNEESTKTGNAGSRLACGVIGIAK (119-152)

Human:  HEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ (121-154)

Rat:    HEKQDDLGKGGNEESTKTGNAGSRLACGVIGIAQ (121-154)
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Scheme 1. Sequence alignment between bovine, human and rat Cu/Zn SOD, showing no tyrosine in human sequence, only one tyrosine in the bovine sequence in position 108 and only one tyrosine residue in the rat sequence in position 51 (a tyrosine involved in the E-class dimer inter-phase).

sodium dodecyl sulphate, tetramethylethylenediamine (TEMED), IGEPAL®, Trizma, sacarose, 2-mercaptoethanol, serum bovine albumin (BSA) and rabbit antibody against actin were purchased from Sigma-Aldrich (St. Louis, MO). Bromophenol blue, Glycine and Bradford reagent were obtained from Bio-Rad (Hercules, CA). Acrylamide, N'N'Bis-methylene n-acrylamide and ammonium persulphate were purchased from Promega (BA, Argentina). Magic Mark molecular weight standard (20–220 KDa) was from InVitrogen (Carlsbad, CA) and peroxynitrite from Cayman Chemical Company (Ann Arbor, MI). Sheep antibody against Cu/Zn SOD, normal rabbit IgG (obtained from non-immunized rabbit) and rabbit antibody against nitrotyrosine were obtained from Calbiochem (San Diego, CA). Horseradish peroxidase conjugated antibody against sheep, alkaline phosphatase conjugated antibody against rabbit and A/G protein sepharose conjugated were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugated antibody against rabbit was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). ECL Western Blotting Analysis System, Rainbow molecular weight standard 12–225 KDa and Amersham Hyperfilm ECL were obtained from G. E. Healthcare (Buckinghamshire, UK). The nitrotyrosine Assay Kit was from Millipore (Billerica, MA).

Animals

Albino Wistar rats were bred in our laboratory with free access to commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina) and water. The animals were kept in a room at a controlled temperature of 20°C, with 14 h light and 10 h dark lighting cycles. To induce diabetes, either streptozotocin (90 mg/Kg) diluted in citrate buffer (pH: 4.5, 0.05 M) or citrate buffer alone were injected to 2-day old female rats [44,45]. Glycemia values from adult diabetic rats were 215 ± 20 mg/dL (mean ± SEM), while those from control rats were 100 ± 10 mg/dL. Adult female control and diabetic rats were mated with healthy males. Pregnancy was confirmed by the presence of sperm cells in vaginal smears and day 0.5 of gestation was designated.

Tissue preparations

Animals were sacrificed by cervical dislocation on day 13.5 of pregnancy and their uteri were removed and placed in Petri dishes with ice cold phosphate saline buffer. The uteri were opened and placentas were separated from the embryos. For western blotting and immunoprecipitation assays, placentas were stored at -80°C. For Cu/ZnSOD activity assays, placentas were cut into thin slices (1 mm thick) and incubated for 30 min in Krebs-Ringer-Carbonate solution (KRB, 5.5 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-}), pH 7.2 at 37°C, either alone or in the presence of NP 600 μM (.^NO donors), L-NAME 600 μM (inhibitor of .^NO synthase (NOS)) or peroxynitrite 100 or 200 μM. Taking into account that NP and peroxynitrite are very reactive short half-life molecules, control experiments were performed assessing Cu/ZnSOD activity after incubation of placental tissue also in the presence of NP decomposed for 15 min or peroxynitrite decomposed for 15 min in the same KRBS medium. Tissues were kept at −80°C until assays were performed. 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.

Evaluation of peroxynitrite

Peroxynitrite is a very reactive molecule which is easily and rapidly decomposed in solution at pH 7. Thus, the peroxynitrite concentration was measured each time the incubations were performed. Serial dilutions were performed from peroxynitrite original solution in KOH 0.3 N. Then, the concentrations were determined in a spectrophotometer Nanodrop from Thermo Scientific (Worcester, MA) at 302 nm, (extinction coefficient: 1,670 M^{-1} cm^{-1}). Concentrations were determined (100 and 200 μM) before each incubation and also after 15 min in the medium used for the control experiments, ensuring that there was no peroxynitrite left in the negative control medium.

Evaluation of Cu/ZnSOD activity

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 and supplemented with 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 −80°C. The activity of Cu/ZnSOD was assayed by the method of Misra and Fridovich [46], as previously [21]. This method is based on the ability of SOD to inhibit auto-oxidation 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. This technique, due to the high pH used, measures the activity of Cu/ZnSOD [46]. The absorbance change was monitored at 480 nm. One unit of Cu/ZnSOD is defined as the amount of enzyme required to cause 50% inhibition of epinephrine auto-oxidation. Enzymatic activity was reported as units/mg protein in the homogenate.

Evaluation of protein nitration

Placental tissues from control and diabetic rats were homogenized in Tris-HCl 0.1 M buffer and then subjected to a Nitrotyrosine Assay Kit (Millipore). Briefly, samples or standards together with an antibody against nitrotyrosine were added to nitrated BSA-coated plates to carry out a competitive ELISA. After incubation and washing a secondary horseradish peroxidase-conjugated antibody was added to the wells. The excess of antibody was washed and LumiGLO® Chemiluminiscent Substrate added and allowed to develop luminescence for 10 min. Relative light units (RLU) were measured in a Luminoskan Ascent Luminescence Microplate Reader (Lab System, Israel). The RLU values were compared with those from the standard curve. Data are reported as μg nitro-BSA equivalents/mg of protein in the homogenate.

Immunoprecipitation of nitrotyrosine-containing proteins

Placental tissues from control and diabetic rats were homogenized in immunoprecipitation buffer (KP 30 mM, ClNa 150 mM, glycerol 10%, IGEPAL 1%, SDS 1% and protease inhibitor cocktail (Sigma)). The technique for immunodetection of nitrated proteins was followed [47]. Briefly, lysates were pre-cleared with 30 μl of sepharose bound protein A/G. The pre-cleared extract was quickly recovered by centrifugation at 10 000 rpm for 10 s. An equivalent volume of 1 mg protein from each of the placental lysates was incubated with an anti-nitrotyrosine antibody (3 μg) for 18 h at 4°C. The nitrotyrosine-antibody complexes were re-incubated with 30 μl of sepharose protein A/G and allowed to interact for 1 h at 4°C. The immunocomplexes were centrifuged at 10 000 rpm for 10 min, at 4°C, saving the supernatant, as a control of the experiment. The beads containing the nitrated proteins were washed with buffer plus protease inhibitors. The sample loading buffer (62.5 mM Tris, 2% SDS, 0.7 M 2-Mercaptoethanol, 10% glycerol, 0.12% bromophenol blue) was then added to the beads and they were heated for 5 min at 95°C. After centrifugation at 10 000 rpm for 1 min the supernatant containing the nitrated proteins was loaded to an SDS gel for western blotting analysis. An immunoprecipitation negative control was performed in parallel with the samples, as follows: 1 mg diabetic placenta lysate was subjected to the same immunoprecipitation protocol with a commercially available normal rabbit IgG (Calbiochem) instead of the anti-nitrotyrosine antibody (also made in rabbit and purchased from Calbiochem). The control was then washed and loaded into a gel with two positive immunoprecipitations performed with anti-nitrotyrosine antibody in control and diabetic placenta lysates. A western-blot analysis was then performed to detect nitrotyrosine-modified proteins.

Western blotting to detect protein expression of nitrotyrosine and Cu/ZnSOD

The samples (supernatants, precipitates or whole lysates) were subjected to western blots to evaluate Cu/ZnSOD or nitrotyrosine levels. Tissues were homogenized in 500 μl of ice-cold lysis buffer (20 mM HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 5 μL protease inhibitor cocktail), incubated on ice for 2 h, centrifuged at 10 000 rpm for 10 min and the supernatant removed. Total proteins (100 μg) were separated using 17% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were then blocked with 2% of blocking reagent (GE Healthcare, NJ) and incubated overnight at 4°C, with either a rabbit anti-Cu/ZnSOD polyclonal antibody (1:500) or a rabbit anti-nitrotyrosine polyclonal antibody (1:1000). The membranes were then probed for 1 h at room temperature with horseradish peroxidase-labelled secondary antibody. The signal was revealed using the ECL enhanced chemiluminescence system. As an internal control, actin was detected with rabbit polyclonal antibody, followed by an alkaline phosphatase conjugated antibody against rabbit and detected with SIGMA fast 5-bromo 4-chloro-3-indoyl phosphate/nitro blue tetrazolium. The bands corresponding to Cu/ZnSOD and actin were identified with the use of two molecular weight standards: pre-stained Full Range Rainbow (12–225 KDa) and chemiluminiscent-detected Magic Mark XP (20–220 KDa). The relative intensity of protein signals was quantified by densitometric analysis using the Image J Software.

Statistical analysis

Results are expressed as means ± SEM. Comparisons between groups were performed using either one way ANOVA in conjunction with Bonferroni's test or Student's *t*-test, where appropriate. The statistical level of significance was defined as $p < 0.05$.

Results

Nitric oxide regulates Cu/ZnSOD activity in placentas from diabetic rats

Cu/ZnSOD activity was found reduced in the placentas from diabetic rats, $p < 0.01$ (Figure 1). In order to determine the influence of .^NO on placental SOD activity we evaluated the enzymatic activity in the presence or absence of a .^NO donor (NP 600 μM) or a NOS inhibitor (NAME 600 μM). Neither the .^NO donor nor the NOS inhibitor modified the placental Cu/ZnSOD activity in control animals (Figure 1), whereas the NO donor inhibited Cu/ZnSOD activity ($p < 0.05$) and NAME increased Cu/ZnSOD

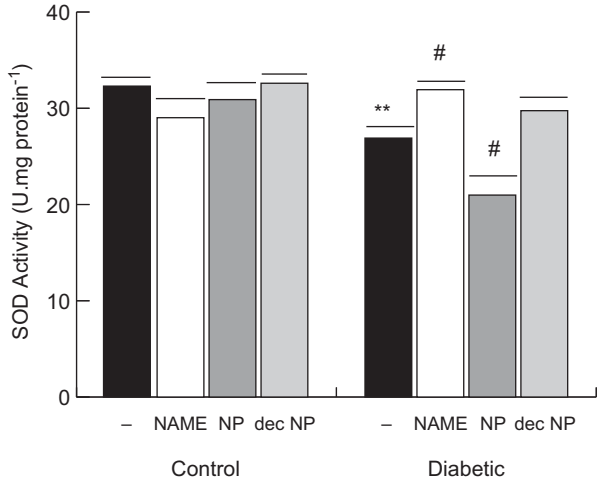

Figure 1. Placental Cu/Zn SOD activity in control and diabetic rats with or without the addition of a NO donor (NP 600 μM), a NOS inhibitor (NAME 600 μM) or 600 μM NP decomposed for 15 min at pH 7.2 (dec NP). Data are expressed as means ± SEM; **represent $p < 0.01$ vs control without additions and # $p < 0.05$ vs diabetic without additions. Eight placentas from different rats were included in each group ($n = 8$ in each group).

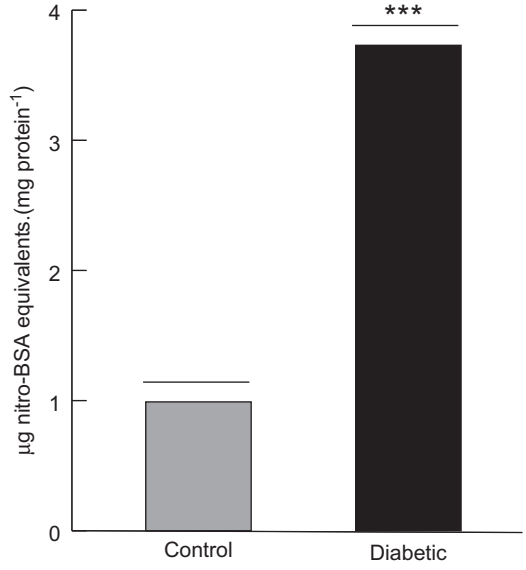

Figure 2. Nitrotyrosine levels in placental tissues from control and diabetic rats. Data are expressed as means ± SEM; *** represent $p < 0.001$ vs corresponding control. Eight placentas from different rats were included in each group ($n = 8$ in each group).

activity ($p < 0.05$) in placentas from diabetic rats (Figure 1). NP previously decomposed for 15 min in the incubation medium at pH 7.2 prevented the NP-induced inhibition of Cu/ZnSOD activity in placentas from diabetic rats and did not affect Cu/ZnSOD activity in placentas from control rats (Figure 1).

Placental protein nitration and inhibition of placental Cu/ZnSOD activity by peroxynitrite

Placental Cu/ZnSOD activity was affected by .^NO only in tissues from diabetic animals; this could indicate that the diabetic environment can modify .^NO, which in turn decreases Cu/ZnSOD activity. Figure 2 shows that protein nitration was highly increased in the placentas from diabetic rats when compared to controls ($p < 0.001$). Considering this, we looked at the capacity of peroxynitrite to modify Cu/ZnSOD activity in rat placenta. The addition of peroxynitrite (100 or 200 μM) to the incubation medium inhibited Cu/ZnSOD activity in rat placental tissue ($p < 0.01$), whereas decomposed peroxynitrite did not affect Cu/ZnSOD activity (Figure 3).

Altered Cu/ZnSOD protein expression in placentas from diabetic rats

In order to determine whether the observed reduction in Cu/ZnSOD activity in the placentas from diabetic rats was related to changes in Cu/ZnSOD protein expression, we evaluated Cu/ZnSOD expression in placentas from control and diabetic rats. Western blotting from placental lysates was performed to identify and quantify Cu/ZnSOD protein levels and normalized to actin levels. Figure 4A shows a representative blot from placental precipitates, with increased intensity of the bands corresponding to the diabetic placentas in the region of 16 KDa corresponding to Cu/ZnSOD. The densitometry analysis corresponding to the Cu/ZnSOD bands normalized to the actin ones (Figure 4B) revealed an increase ($p < 0.01$) in Cu/ZnSOD protein levels in placentas from diabetic rats when compared to controls.

Enhanced nitration of Cu/ZnSOD in placentas from diabetic rats

As Cu/ZnSOD activity was reduced in placental tissues from diabetic rats and Cu/ZnSOD protein expression was increased in diabetic placentas, we hypothesized that Cu/ZnSOD was nitrated in diabetic placentas. To test this, we then investigated the degree of nitration in rat placentas at mid-gestation. An immunoprecipitation of placental lysates was carried out against nitrotyrosine. All the precipitated nitrated proteins were subjected to an electrophoretical separation in SDS-PAGE. The proteins were transferred to a membrane and immunoblotted against nitrotyrosine. The non-nitrated proteins that remained in the supernatant after the immunoprecipitation were also separated in another SDS-PAGE and blotted against nitrotyrosine as a control. The absence of signal indicated the specificity of the immunoprecipitation (data not shown).

Both control and diabetic immunoprecipitates showed bands ranging from 15–120 KDa. Figure 5A shows the blot from representative placental precipitates, with increased intensity of the bands corresponding to the diabetic placentas, especially in the region of 16 KDa, supporting the hypothesis that Cu/ZnSOD

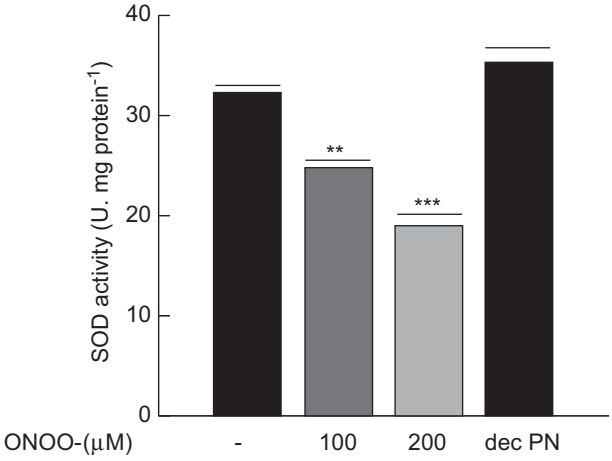

Figure 3. Placental Cu/Zn SOD activity from control rats with or without the addition of peroxynitrite 100, 200 μM or 200 μM peroxynitrite decomposed for 15 min at pH 7.2 (dec PN). Data are expressed as means ± SEM; ** represent $p < 0.01$ and *** $p < 0.001$ vs incubation without additions. Eight placentas from different rats were included in each group ($n = 8$ in each group).

can be nitrated in the diabetic placentas. To test this, we washed and re-probed the membrane with an antibody against Cu/ZnSOD. Figure 5C shows a representative blot of the placental precipitates and Figure 5D the optical density analysis of the 16 KDa band corresponding to Cu/ZnSOD. We found an increase in the amount of nitrated Cu/ZnSOD in diabetic placental precipitates ($p < 0.01$). This can provide an explanation for the enhanced Cu/ZnSOD protein levels and the diminished Cu/ZnSOD activity in the placentas from diabetic rats compared to controls.

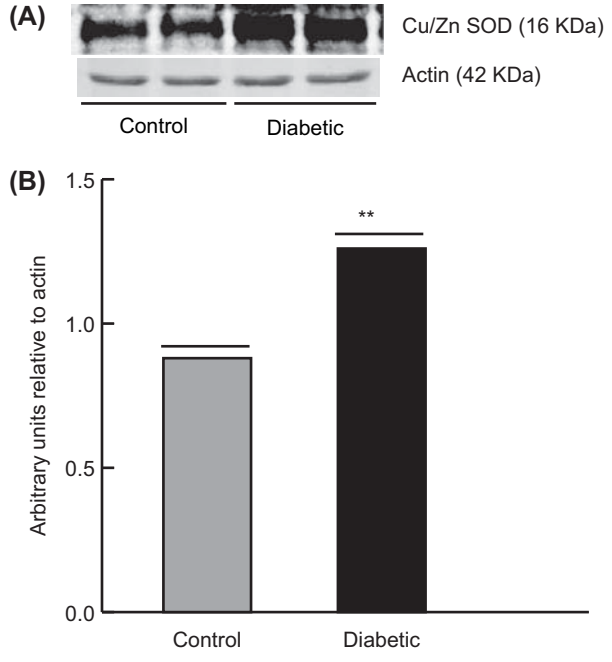

Figure 4. Cu/Zn SOD protein expression in placentas from control and diabetic rats. (A) Representative immunoblot showing the bands corresponding to the 16 KDa protein Cu/Zn SOD. The same membrane was re-probed with an anti-actin antibody. (B) Densitometric analysis of placental Cu/Zn SOD protein expression from control and diabetic rats. Data are expressed as means ± SEM; ** represent $p < 0.01$ vs corresponding controls. Eight placentas from different rats were included in each group ($n = 8$ in each group).

Discussion

During pregnancy, oxidative stress plays an important role in the modulation of invasion and angiogenesis, processes extremely important in placental development and function. Oxygen levels can regulate cell fate, inducing proliferation, invasion or differentiation [18]. We had previously established that placentas from diabetic rats at mid-gestation are subjected to oxidative and nitrative stress, with high levels of nitrotyrosine, increased .^NO, lipid peroxidation and diminished SOD activity [21,41,48]. Impaired SOD activity was also found in placentas from diabetic rats and diabetic patients at term gestation [17,22]. Aiming to find the cause of the diminished SOD activity in diabetic placentas, we assessed the activity of this enzyme in the presence of a .^NO donor and a NOS inhibitor. We found that Cu/ZnSOD activity was not modulated by .^NO in healthy placentas. Interestingly, Cu/ZnSOD activity was inhibited by .^NO only in the diabetic placentas, suggesting that .^NO might be modified in the diabetic tissue, giving rise to a new SOD inhibitor. We then evaluated whether peroxynitrite was able to inhibit Cu/ZnSOD activity in the rat placenta. The use of peroxynitrite to generate nitration of tyrosine residues mimics what happens in tissues subjected to nitrative damage [35]. The fact that in this work peroxynitrite inhibited placental Cu/ZnSOD activity dose-dependently is in agreement with the modulation of Cu/ZnSOD activity by .^NO only in diabetic rat placentas subjected to oxidative stress.

In spite of the reduced enzymatic activity, we found increased levels of Cu/ZnSOD protein expression in placentas from diabetic rats at mid-pregnancy. The paradox of high levels of protein with lower activity can be explained by high levels of protein nitration. We had already immunolocalized peroxynitrite-induced damage in the placentas from diabetic rats on day 14 and 21 of gestation [41,42]. In this work we detected a 4-fold increase in protein nitration in the placentas from diabetic rats as compared to controls. Moreover, we immunoprecipitated the nitrotyrosine-modified proteins from whole lysates of control and diabetic placentas, and found an intense immunostaining profile for nitrotyrosine in placental proteins from diabetic animals. As expected, we observed a 16 KDa band that was more intense in the immunoprecipitates from diabetic placentas than in the control ones. We were able to confirm that this band corresponded to Cu/ZnSOD protein by re-probing the membrane with an antibody against Cu/ZnSOD. Therefore, an increase in Cu/ZnSOD nitration was

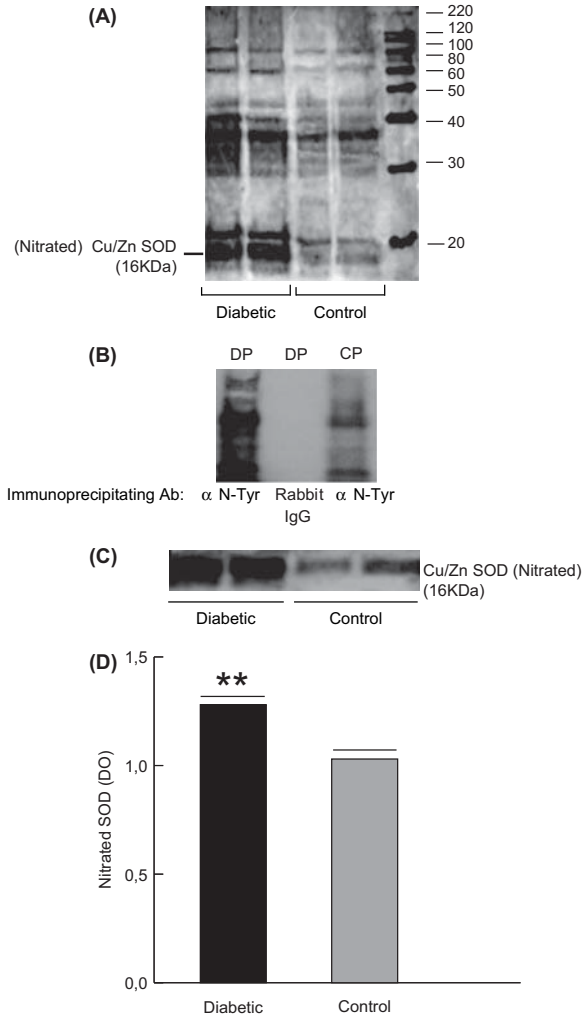

Figure 5. Nitration of Cu/Zn SOD protein in the rat placenta. (A) An immunoprecipitation of lysates corresponding to different placentas from eight control and eight diabetic rats was performed against nitrotyrosine. The immunoprecipitates were loaded on a 15% polyacrylamide SDS-PAGE gel and immunoblotted against nitrotyrosine. Representative immunoblot showing the bands ranging from 12–220 KDa. (B) A negative control from the immunoprecipitation was performed using an irrelevant antibody (rabbit IgG). Briefly, in parallel with the nitrotyrosine immunoprecipitations of two placental lysates, one from control (CP) and the other from diabetic placenta (DP), which were carried out with 3 μg of a primary anti nitrotyrosine antibody (α N-Tyr), 1 mg of diabetic placenta lysate was subjected to the same immunoprecipitation protocol with 3 μg of normal rabbit IgG (Rabbit IgG). After the precipitation procedure, the precipitates were washed and loaded into a gel. A western-blot against nitrotyrosine was carried out, showing the nitrated proteins from control and diabetic placentas and no sign for the negative control (Rabbit IgG). (C) The membrane shown in (A) was re-probed with an anti-Cu/Zn SOD antibody, showing the Cu/Zn SOD band at 16 KDa. (D) Densitometric analysis of placental nitrated Cu/Zn SOD protein from control and diabetic rats. Data are expressed as the means ± SEM. ** represent $p < 0.01$ vs controls. Eight placentas from different rats were included in each group ($n = 8$ in each group).

established in placental tissues from diabetic rats. Nevertheless, modifications of other residues by peroxynitrite cannot be discarded. It has been found that peroxynitrite can oxidize a histidine residue from the active site of recombinant human Cu/ZnSOD [49].

MnSOD activity inhibition by peroxynitrite-mediated tyrosine nitration has been previously described *in vitro* [32,43,50] and *in vivo* in human chronic renal rejection [31]. Moreover, bovine Cu/ZnSOD nitration has also been demonstrated, a modification that does not affect the activity of the enzyme [33]. The difference between bovine and rat Cu/ZnSOD is the position of the only tyrosine residue. In the bovine enzyme, the tyrosine is located in position 108, far from the active site, whereas in the rat enzyme the tyrosine is in position 51, which is near the active site, within the dimer interface domain. In addition, in the rat enzyme, a glutamate residue is localized in position 52, making the tyrosine more susceptible to nitration [34]. These observations suggest that nitration of tyrosine 51 in the rat placenta might lead to the dissociation of the dimers with the consequent loss in activity found in this work.

Among other antioxidant enzymes, placental SOD increases together with oxygen tension through gestation [51–53], providing a defence system that ensures placental function and embryo protection. Indeed, the over-expression of Cu/ZnSOD diminishes the malformation rate of 10.5-day old embryos from diabetic rats [54,55]. Diabetic placentas show enhanced SOD expression [18], suggesting that placental tissue is able to enhance SOD expression in response to oxidative stress. Indeed, the transcription of this cytosolic enzyme is under the control of stress, injury or high concentrations of NO, hydrogen peroxide and heavy metals [25]. Therefore, the nitration and inhibition of Cu/ZnSOD detected in this work is likely to be highly relevant in feto–placental development.

In different tissues, several proteins, such as PARP and metalloproteinase 2, are activated by nitration, whereas others such as p38 MAP kinase, SOD, α-actinin and carnitine palmitoyl transferase are inhibited [29,30,38,56,57]. It has been proposed that nitration, like phosphorylation, should be a cell signalling mechanism, which can either activate or inhibit protein function [28]. However, our results, as well as those from other researchers [30,40,58], suggest that, in pathological circumstances, where nitration is enhanced, severe damage to key events in reproduction is induced. Further research is needed to find and characterize a putative de-nitrating enzyme that can be a target for therapeutic strategies to avoid nitrative stress-induced damage in placental tissue and finally ameliorate foetal and neonatal disturbances.

In summary, our results demonstrate that placental Cu/ZnSOD is nitrated, a peroxynitrite-induced modification likely to be involved in the inhibition of this antioxidant enzyme in diabetic rats. This suggests a feedback mechanism by which diabetes-induced maternal oxidative stress induces placental Cu/ZnSOD expression and nitrative stress inhibits placental Cu/

ZnSOD activity, leaving the tissue with a diminished antioxidant defense and leading to more oxidative stress.

Declaration of interest: This work was supported by grants from Agencia de Promoción Científica y Tecnológica de Argentina (PICT 07-01958, V.W and PICT 05-32268, A.J.) and from Ministerio de Salud Argentina (Ramón Carrillo-Arturo Oñativia).

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This paper was first published online on Early Online on 6 September 2010.