

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

VERÓNICA WHITE<sup>1</sup>, EVANGELINA CAPOBIANCO<sup>1</sup>, ROMINA HIGA<sup>1</sup>,  
NORA MARTÍNEZ<sup>1</sup>, MARÍA SOSA<sup>1</sup>, MARÍA C. PUSTOVRH<sup>1,2</sup> & ALICIA JAWERBAUM<sup>1</sup>

<sup>1</sup>Laboratory of Reproduction and Metabolism, CEFyBO-CONICET, School of Medicine, University of Buenos Aires, and

<sup>2</sup>Laboratory of Biology of Development. IFIBYNE, Department of Biodiversity and Experimental Biology, School of Exact and Natural Sciences, University of Buenos Aires, Paraguay 2155 (C1121ABG), Buenos Aires, Argentina

(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 fetoplacental 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, peroxynitrite, 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 fetoplacental 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

Correspondence: Verónica White, PhD, Laboratory of Reproduction and Metabolism, CEFyBO-CONICET, School of Medicine, University of Buenos Aires, Paraguay 2155 17<sup>th</sup> floor (C1121ABG), Buenos Aires, Argentina. Tel: 0054 011 4508 3680 (119). Fax: 0054 011 4508 3680 (106). Email: vernica73@yahoo.com.ar; vernica73@gmail.com

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 ( $\cdot\text{NO}$ ) is a vasoactive agent, which plays an important role in vasculature and smooth muscle relaxation [26,27]. However,  $\cdot\text{NO}$  can also combine with superoxide, giving rise to peroxynitrite, a powerful oxidative and nitrating molecule. Peroxynitrite is more reactive than  $\cdot\text{NO}$  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  $\cdot\text{NO}$  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  $\text{N}^{\text{G}}$ -nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (NP),

```

Bovine: MATKAVCVLKGDPVQGTIHFQAKGDTVVVTGSITGLTEGDHGFHVHQFGDNTQGCTS (1-58)
Human: MATKAVCVLKGDPVQGIINFEQKESNGPVKVVWGSIKGLTEGLHGFHVHEFGDNTAGCTS (1-60)
Rat: MAMKAVCVLKGDPVQGVIIHFQKASGEPVVVSGQITGLTEGEHGFHVH(Y)EDNTQGCTT (1-60)

Bovine: AGPHFNPLSKKHGGPKDEERHVGDLGNVTADKNGVAIVDIVDPLISLSG(Y)SIIGRTMVV (59-118)
Human: AGPHFNPLSRKHGGPKDEERHVGDLGNVTADKGDVADVSIEDSVISLSGDHCCIIGRTLTVV (61-120)
Rat: AGPHFNPHSKKHGGPADEERHVGDLGNVAAGKGDVANVSIEDRVISLSGEHSIIIGRTMVV (61-120)

Bovine: HEKPDDLGRGGNEESTKTGNAGSRLACGVIGIAK (119-152)
Human: HEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ (121-154)
Rat: HEKQDDLKGGNEESTKTGNAGSRLACGVIGIAQ (121-154)

```

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<sup>®</sup>, Trizma, saccharose, 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 peroxyntirite 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  $215 \pm 20$  mg/dL (mean  $\pm$  SEM), while those from control rats were  $100 \pm 10$  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<sup>+</sup>, 5.9 mM K<sup>+</sup>, 2.2 mM Ca<sup>2+</sup>, 1.2 mM Mg<sup>2+</sup>, 127 mM Cl<sup>-</sup>, 25 mM HCO<sub>3</sub><sup>-</sup>, 1.2 mM SO<sub>4</sub><sup>2-</sup>, 1.2 mM PO<sub>4</sub><sup>3-</sup>), pH 7.2 at 37°C, either alone or in the presence of NP 600  $\mu$ M ( $\cdot$ NO donors), L-NAME 600  $\mu$ M (inhibitor of  $\cdot$ NO synthase (NOS)) or peroxyntirite 100 or 200  $\mu$ M. Taking into account that NP and peroxyntirite 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 peroxyntirite 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/signs/bioethics/animals.html>.

### Evaluation of peroxyntirite

Peroxyntirite is a very reactive molecule which is easily and rapidly decomposed in solution at pH 7. Thus, the peroxyntirite concentration was measured each time the incubations were performed. Serial dilutions were performed from peroxyntirite 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:  $1,670 \text{ M}^{-1} \text{ cm}^{-1}$ ). Concentrations were determined (100 and 200  $\mu$ M) before each incubation and also after 15 min in the medium used for the control experiments, ensuring that there was no peroxyntirite left in the negative control medium.

### Evaluation of Cu/ZnSOD activity

Tissues were homogenized in 500  $\mu$ l of homogenization buffer, pH 7.6 (Tris-base 20 mM, EDTA 1 mM, KCl 150 mM,  $\beta$  Mercaptoethanol 1 mM and supplemented with saccharose 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  $\mu\text{g}$  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  $\mu\text{l}$  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  $\mu\text{g}$ ) for 18 h at 4°C. The nitrotyrosine-antibody complexes were re-incubated with 30  $\mu\text{l}$  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  $\mu\text{l}$  of ice-cold lysis buffer (20 mM HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 5  $\mu\text{l}$  protease inhibitor cocktail), incubated on ice for 2 h, centrifuged at 10 000 rpm for 10 min and the supernatant removed. Total proteins (100  $\mu\text{g}$ ) 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  $\pm$  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  $\cdot\text{NO}$  on placental SOD activity we evaluated the enzymatic activity in the presence or absence of a  $\cdot\text{NO}$  donor (NP 600  $\mu\text{M}$ ) or a NOS inhibitor (NAME 600  $\mu\text{M}$ ). Neither the  $\cdot\text{NO}$  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

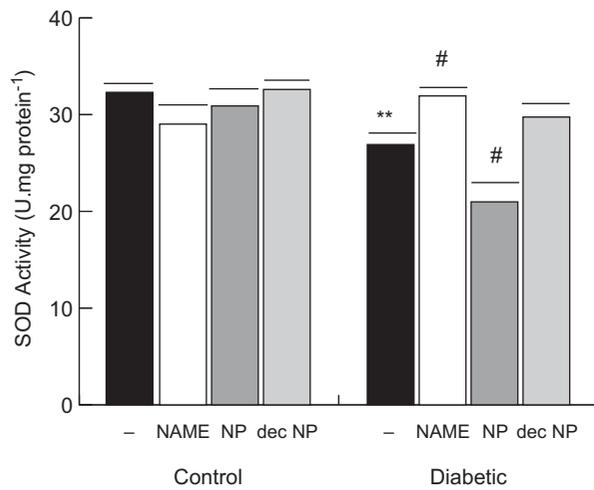


Figure 1. Placental Cu/Zn SOD activity in control and diabetic rats with or without the addition of a NO donor (NP 600  $\mu$ M), a NOS inhibitor (NAME 600  $\mu$ M) or 600  $\mu$ M NP decomposed for 15 min at pH 7.2 (dec NP). Data are expressed as means  $\pm$  SEM; \*\*represent  $p < 0.01$  vs control 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).

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  $\cdot\text{NO}$  only in tissues from diabetic animals; this could indicate that the diabetic environment can modify  $\cdot\text{NO}$ , 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  $\mu$ M) 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

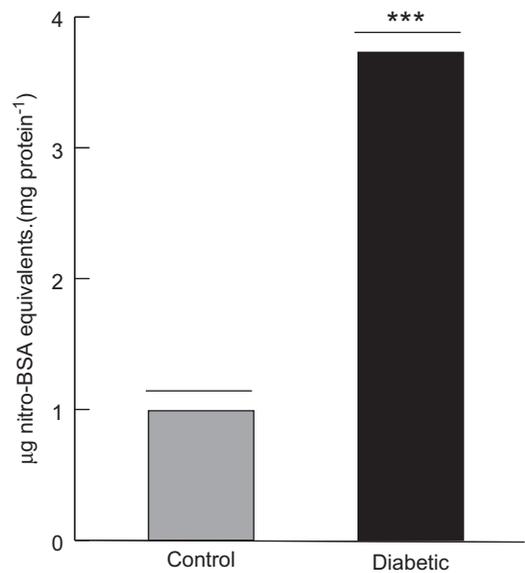


Figure 2. Nitrotyrosine levels in placental tissues from control and diabetic rats. Data are expressed as means  $\pm$  SEM; \*\*\* represent  $p < 0.001$  vs corresponding control. Eight placentas from different rats were included in each group ( $n = 8$  in each group).

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

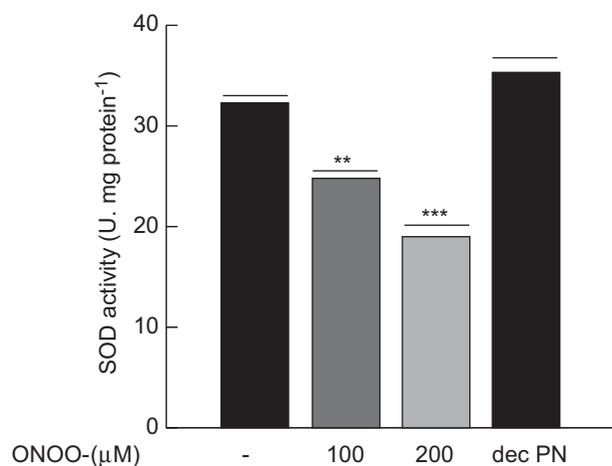


Figure 3. Placental Cu/Zn SOD activity from control rats with or without the addition of peroxynitrite 100, 200  $\mu\text{M}$  or 200  $\mu\text{M}$  peroxynitrite decomposed for 15 min at pH 7.2 (dec PN). Data are expressed as means  $\pm$  SEM; \*\* represent  $p < 0.01$  and \*\*\*  $p < 0.001$  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

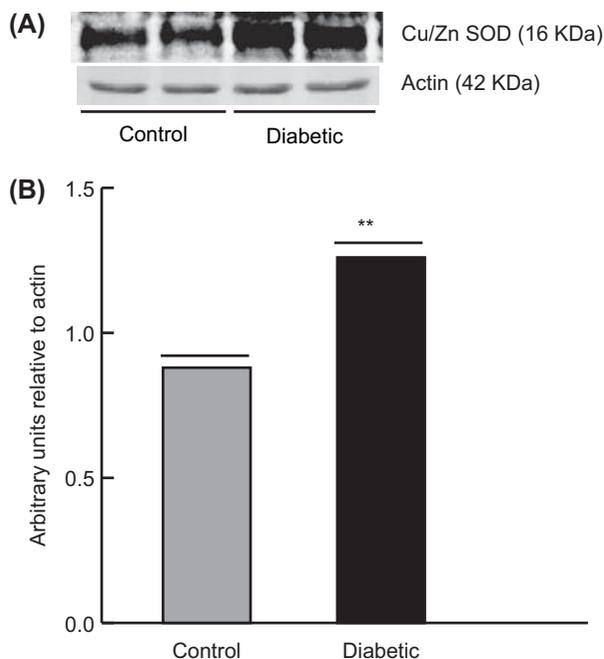


Figure 4. Cu/Zn SOD protein expression in placentas from control and diabetic rats. (A) Representative immunoblot showing the bands corresponding to the 16 KDa protein Cu/Zn SOD. The same membrane was re-probed with an anti-actin antibody. (B) Densitometric analysis of placental Cu/Zn SOD protein expression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

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.

## 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  $\cdot\text{NO}$ , 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  $\cdot\text{NO}$  donor and a NOS inhibitor. We found that Cu/ZnSOD activity was not modulated by  $\cdot\text{NO}$  in healthy placentas. Interestingly, Cu/ZnSOD activity was inhibited by  $\cdot\text{NO}$  only in the diabetic placentas, suggesting that  $\cdot\text{NO}$  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  $\cdot\text{NO}$  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

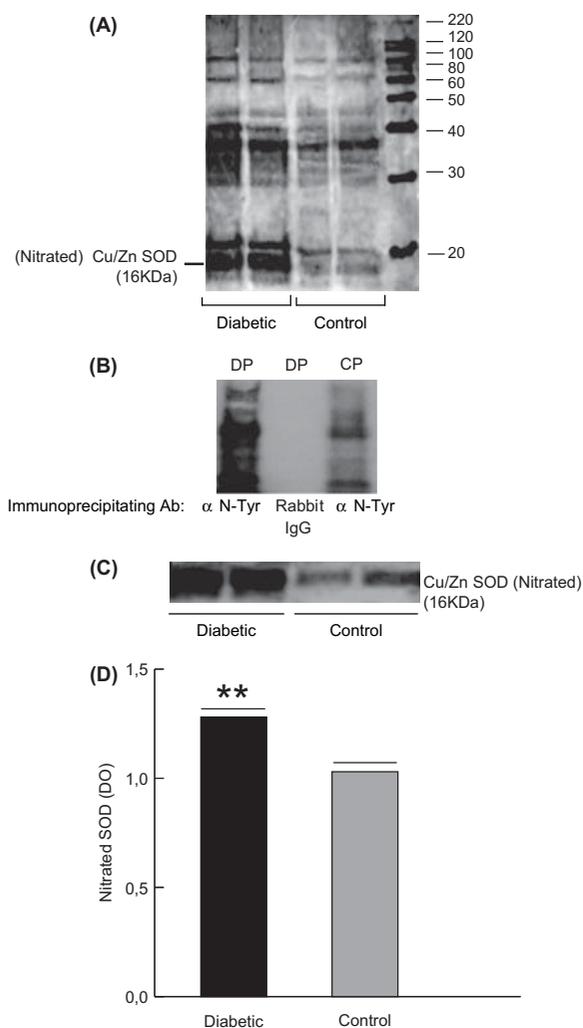


Figure 5. Nitration of Cu/Zn SOD protein in the rat placenta. (A) An immunoprecipitation of lysates corresponding to different placentas from eight control and eight diabetic rats was performed against nitrotyrosine. The immunoprecipitates were loaded on a 15% polyacrylamide SDS-PAGE gel and immunoblotted 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  $\mu$ g of a primary anti nitrotyrosine antibody ( $\alpha$  N-Tyr), 1 mg of diabetic placenta lysate was subjected to the same immunoprecipitation protocol with 3  $\mu$ g 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  $\pm$  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 fetoplacental development.

In different tissues, several proteins, such as PARP and metalloproteinase 2, are activated by nitration, whereas others such as p38 MAP kinase, SOD,  $\alpha$ -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 nitrate 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 nitrate stress inhibits placental Cu/

ZnSOD activity, leaving the tissue with a diminished anti-oxidant 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).

## References

- [1] Foster W, Myllynen P, Winn LM, Ornoy A, Miller RK. Reactive oxygen species, diabetes and toxicity in the placenta - a workshop report. *Placenta* 2008;29(Suppl A):105-107.
- [2] Jawerbaum A, Gonzalez E. Diabetic pregnancies: the challenge of developing in a pro-inflammatory environment. *Curr Med Chem* 2006;13:2127-2138.
- [3] Ornoy A. Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. *Reprod Toxicol* 2007;24:31-41.
- [4] King GL, Loeken MR. Hyperglycemia-induced oxidative stress in diabetic complications. *Histochem Cell Biol* 2004;122:333-338.
- [5] Farrell T, Neale L, Cundy T. Congenital anomalies in the offspring of women with type 1, type 2 and gestational diabetes. *Diabet Med* 2002;19:322-326.
- [6] Kitzmiller JL, Buchanan TA, Kjos S, Combs CA, Ratner RE. Pre-conception care of diabetes, congenital malformations, and spontaneous abortions. *Diabetes Care* 1996;19:514-541.
- [7] Phaloprakarn C, Tangjitgamol S. Risk assessment for preeclampsia in women with gestational diabetes mellitus. *J Perinat Med* 2009;37:6:617-621.
- [8] Eriksson UJ. Congenital anomalies in diabetic pregnancy. *Semin Fetal Neonatal Med* 2009;14:85-93.
- [9] Kitzmiller JL, Cloherty JP, Younger MD, Tabatabaai A, Rothchild SB, Sosenko I, Epstein MF, Singh S, Neff RK. Diabetic pregnancy and perinatal morbidity. *Am J Obstet Gynecol* 1978;131:560-580.
- [10] Michael Weindling A. Offspring of diabetic pregnancy: short-term outcomes. *Semin Fetal Neonatal Med* 2009;14:111-118.
- [11] Simeoni U, Barker DJ. Offspring of diabetic pregnancy: long-term outcomes. *Semin Fetal Neonatal Med* 2009;14:119-124.
- [12] Jansson T, Cetin I, Powell TL, Desoye G, Radaelli T, Ericsson A, Sibley CP. Placental transport and metabolism in fetal overgrowth - a workshop report. *Placenta* 2006;27(Suppl A):109-113.
- [13] Saldeen P, Olofsson P, Laurini RN. Structural, functional and circulatory placental changes associated with impaired glucose metabolism. *Eur J Obstet Gynecol Reprod Biol* 2002;105:136-142.
- [14] Hempstock J, Jauniaux E, Greenwold N, Burton GJ. The contribution of placental oxidative stress to early pregnancy failure. *Hum Pathol* 2003;34:1265-1275.
- [15] Herrera E, Amusquivar E. Lipid metabolism in the fetus and the newborn. *Diabetes Metab Res Rev* 2000;16:202-210.
- [16] Desoye G, Shafir E. The human placenta in diabetic pregnancy. *Diabetic Rev* 1996;4:70-89.
- [17] Kinalski M, Sledziewski A, Telejko B, Kowalska I, Kretowski A, Zarzycki W, Kinalska I. Lipid peroxidation, antioxidant defence and acid-base status in cord blood at birth: the influence of diabetes. *Horm Metab Res* 2001;33:227-231.
- [18] Myatt L, Cui X. Oxidative stress in the placenta. *Histochem Cell Biol* 2004;122:369-382.
- [19] Radaelli T, Varastehpour A, Catalano P, Hauguel-de Mouzon S. Gestational diabetes induces placental genes for chronic stress and inflammatory pathways. *Diabetes* 2003;52:2951-2958.
- [20] Zabih S, Wentzel P, Eriksson UJ. Altered uterine perfusion is involved in fetal outcome of diabetic rats. *Placenta* 2008;29:413-421.
- [21] Pustovrh MC, Jawerbaum A, Capobianco E, White V, Martinez N, Lopez-Costa JJ, Gonzalez E. Oxidative stress promotes the increase of matrix metalloproteinases-2 and -9 activities in the feto-placental unit of diabetic rats. *Free Radic Res* 2005;39:1285-1293.
- [22] White V, Jawerbaum A, Sinner D, Pustovrh C, Capobianco E, Gonzalez E. Oxidative stress and altered prostanoid production in the placenta of streptozotocin-induced diabetic rats. *Reprod Fertil Dev* 2002;14:117-123.
- [23] Coughlan MT, Vervaart PP, Permezel M, Georgiou HM, Rice GE. Altered placental oxidative stress status in gestational diabetes mellitus. *Placenta* 2004;25:78-84.
- [24] Myatt L, Kossenjans W, Sahay R, Eis A, Brockman D. Oxidative stress causes vascular dysfunction in the placenta. *J Matern Fetal Med* 2000;9:79-82.
- [25] Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med* 2002;33:337-349.
- [26] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-142.
- [27] Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic Res* 1999;31:577-596.
- [28] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;87:315-424.
- [29] Fukumoto K, Pierro A, Zammit VA, Spitz L, Eaton S. Tyrosine nitration of carnitine palmitoyl transferase I during endotoxaemia in suckling rats. *Biochim Biophys Acta* 2004;1683:1-6.
- [30] Webster RP, Brockman D, Myatt L. Nitration of p38 MAPK in the placenta: association of nitration with reduced catalytic activity of p38 MAPK in pre-eclampsia. *Mol Hum Reprod* 2006;12:677-685.
- [31] MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* 1996;93:11853-11858.
- [32] MacMillan-Crow LA, Crow JP, Thompson JA. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry* 1998;37:1613-1622.
- [33] Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin JC, Smith CD, Beckman JS. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys* 1992;298:431-437.
- [34] Smith CD, Carson M, van der Woerd M, Chen J, Ischiropoulos H, Beckman JS. Crystal structure of peroxynitrite-modified bovine Cu,Zn superoxide dismutase. *Arch Biochem Biophys* 1992;299:350-355.
- [35] Quijano C, Romero N, Radi R. Tyrosine nitration by superoxide and nitric oxide fluxes in biological systems: modeling the impact of superoxide dismutase and nitric oxide diffusion. *Free Radic Biol Med* 2005;39:728-741.
- [36] Neumann H, Hazen JL, Weinstein J, Mehl RA, Chin JW. Genetically encoding protein oxidative damage. *J Am Chem Soc* 2008;130:4028-4033.
- [37] Macfadyen AJ, Reiter C, Zhuang Y, Beckman JS. A novel superoxide dismutase-based trap for peroxynitrite used to detect entry of peroxynitrite into erythrocyte ghosts. *Chem Res Toxicol* 1999;12:223-229.
- [38] Webster RP, Roberts VH, Myatt L. Protein nitration in placenta - functional significance. *Placenta* 2008;29:985-994.
- [39] Myatt L, Rosenfield RB, Eis AL, Brockman DE, Greer I, Lyall F. Nitrotyrosine residues in placenta. Evidence of peroxynitrite formation and action. *Hypertension* 1996;28:488-493.

- [40] Kossenjans W, Eis A, Sahay R, Brockman D, Myatt L. Role of peroxynitrite in altered fetal-placental vascular reactivity in diabetes or preeclampsia. *Am J Physiol Heart Circ Physiol* 2000;278:1311–1319.
- [41] Pustovrh MC, Jawerbaum A, Capobianco E, White V, Lopez-Costa JJ, Gonzalez E. Increased matrix metalloproteinases 2 and 9 in placenta of diabetic rats at midgestation. *Placenta* 2005;26:339–348.
- [42] Capobianco E, Jawerbaum A, Romanini MC, White V, Pustovrh C, Higa R, Martinez N, Mugnaini MT, Sonez C, Gonzalez E. 15-Deoxy-Delta(12,14)-prostaglandin J2 and peroxisome proliferator-activated receptor gamma (PPARgamma) levels in term placental tissues from control and diabetic rats: modulatory effects of a PPARgamma agonist on nitridergic and lipid placental metabolism. *Reprod Fertil Dev* 2005;17:423–433.
- [43] MacMillan-Crow LA, Thompson JA. Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (Y34F) by peroxynitrite. *Arch Biochem Biophys* 1999;366:82–88.
- [44] White V, Gonzalez E, Pustovrh C, Capobianco E, Martinez N, Do Porto DF, Higa R, Jawerbaum A. Leptin in embryos from control and diabetic rats during organogenesis: a modulator of nitric oxide production and lipid homeostasis. *Diabetes Metab Res Rev* 2007;23:580–588.
- [45] Portha B, Picon L, Rosselin G. Chemical diabetes in the adult rat as the spontaneous evolution of neonatal diabetes. *Diabetologia* 1979;17:371–377.
- [46] Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972;247:3170–3175.
- [47] MacMillan-Crow LA, Thompson JA. Immunoprecipitation of nitrotyrosine-containing proteins. *Methods Enzymol* 1999;301:135–145.
- [48] Pustovrh MC, Jawerbaum A, White V, Capobianco E, Higa R, Martinez N, Lopez-Costa JJ, Gonzalez E. The role of nitric oxide on matrix metalloproteinase 2 (MMP2) and MMP9 in placenta and fetus from diabetic rats. *Reproduction* 2007;134:605–613.
- [49] Alvarez B, Demicheli V, Duran R, Trujillo M, Cervenansky C, Freeman BA, Radi R. Inactivation of human Cu,Zn superoxide dismutase by peroxynitrite and formation of histidinyl radical. *Free Radic Biol Med* 2004;37:813–822.
- [50] Yamakura F, Taka H, Fujimura T, Murayama K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J Biol Chem* 1998;273:14085–14089.
- [51] Watson AL, Palmer ME, Jauniaux E, Burton GJ. Variations in expression of copper/zinc superoxide dismutase in villous trophoblast of the human placenta with gestational age. *Placenta* 1997;18:295–299.
- [52] Qanungo S, Mukherjee M. Ontogenic profile of some anti-oxidants and lipid peroxidation in human placental and fetal tissues. *Mol Cell Biochem* 2000;215:11–19.
- [53] Jauniaux E, Hempstock J, Greenwold N, Burton GJ. Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies. *Am J Pathol* 2003;162:115–125.
- [54] Hagay ZJ, Weiss Y, Zusman I, Peled-Kamar M, Reece EA, Eriksson UJ, Groner Y. Prevention of diabetes-associated embryopathy by overexpression of the free radical scavenger copper zinc superoxide dismutase in transgenic mouse embryos. *Am J Obstet Gynecol* 1995;173:1036–1041.
- [55] Zabihi S, Wentzel P, Eriksson UJ. Maternal blood glucose levels determine the severity of diabetic embryopathy in mice with different expression of copper-zinc superoxide dismutase (CuZnSOD). *Toxicol Sci* 2008;105:166–172.
- [56] Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases *in vitro*. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996;98:2572–2579.
- [57] Borbely A, Toth A, Edes I, Virag L, Papp JG, Varro A, Paulus WJ, van der Velden J, Stienen GJ, Papp Z. Peroxynitrite-induced alpha-actinin nitration and contractile alterations in isolated human myocardial cells. *Cardiovasc Res* 2005;67:225–233.
- [58] Lyall F, Gibson JL, Greer IA, Brockman DE, Eis AL, Myatt L. Increased nitrotyrosine in the diabetic placenta: evidence for oxidative stress. *Diabetes Care* 1998;21:1753–1758.

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