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Involvement of inducible nitric oxide synthase and estrogen receptor ESR2 (ERβ) in the vascular dysfunction in female type 1 diabetic rats

Simone Marcieli Sartoretto[a], Fernanda Fernandes Santos[a], Beatriz Pereira Costa[a], Graziela Scalianti Ceravolo[b], Rosângela Santos-Eichler[a], Maria Helena Catelli Carvalho[a], Zuleica Bruno Fortes[a], Eliana Hiromi Akamine[a,*]

[a] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[b] Department of Physiological Sciences, Biological Sciences Center, State University of Londrina, Paraná, Brazil

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ABSTRACT

Aims: Inflammation is involved in diabetes-related vascular dysfunction. Estrogen receptor ESR2/ERβ induces the expression of inducible nitric oxide (NO) synthase (iNOS) and inflammation. The present study investigated the effect of alloxan-induced type 1 diabetes on the iNOS and ESR2 expression and the effect of the chronic iNOS inhibition on the vascular smooth muscle dysfunction in diabetic female rats. In addition, we evaluated the involvement of ESR2 in iNOS expression.

Main methods: Alloxan-induced diabetic female rats were treated or not with iNOS inhibitor (L-NIL). iNOS and ESR2 immunostaining, S-nitrosylated proteins and IL-1β protein expression in aorta and plasmatic NO levels were analyzed. Contractile response to noradrenaline was analyzed in endothelium-denuded aorta. iNOS mRNA expression was analyzed in isolated aortic smooth muscle cells (ASMCs) of female rats, incubated with 22 mM glucose and an ESR2 antagonist.

Key findings: Aortic iNOS and ESR2 immunostaining, S-nitrosylated proteins, IL-1β protein expression and plasmatic NO levels were all increased, whereas noradrenaline-induced contraction was reduced in aorta of diabetic female rats. With the exception of iNOS and ESR2 immunostaining, all these parameters were corrected by L-NIL treatment. High glucose increased iNOS mRNA expression in ASMCs, which was reduced by an ESR2 antagonist.

Significance: We demonstrated that increased iNOS-NO contributed to the impairment of the contractile response of aortic smooth muscle cells in female type 1 diabetic rats and that increased expression of iNOS may involve the participation of ESR2/ERβ.

1. Introduction

Microvascular and macrovascular disease are the main causes of morbidity and mortality in patients with diabetes types 1 and 2 [1]. There is a low-level inflammation that may lead to macrovascular and microvascular complications in diabetic patients [2,3].

Reduced vasodilation and increased vascular contraction and blood pressure are the most frequent changes observed in diabetes [4–6], but reduction of the blood pressure and vascular contraction have been observed in diabetic patients and experimental models of type 1 diabetes [7–10]. In animal models of type 1 diabetes induced in males, vascular inflammation may be involved in the reduction of mean arterial pressure, heart rate and pressor responses to α-adrenergic agonists [11,12]. However, the participation of inflammation in the vascular dysfunction in diabetic females has not been investigated.

Diabetes abolishes the protection of the estrogen on the cardiovascular system in females [13–15]. The biological actions of estrogens are mediated by activation of the estrogen receptors ESR1 (ERα), ESR2 (ERβ) and GPER. The ESR1, through canonical and non-canonical signaling, and GPER appear to mediate the cardioprotective actions of estrogen, including increased endothelial nitric oxide (NO) synthase expression, beneficial NO production and vasodilation [16–18]. In addition, activation of the ESR1 promotes inhibition of expression of inflammatory factors, such as inducible NO synthase (iNOS) [16,19]. On the other hand, pharmacological activation of the ESR2 increases the expression of cytokine-driven iNOS in rat vascular smooth muscle [20],

* Corresponding author at: Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, 1524 Professor Lineu Prestes Av., 05508-000 São Paulo, SP, Brazil.
E-mail address: eliakamine@usp.br (E.H. Akamine).

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raising the hypothesis that the ESR2 receptor can be induced by injuries and contributes to inflammation.

In a previous paper, we demonstrated that the contractile response induced by noradrenaline is reduced in endothelium-denuded aorta, but in endothelium-intact aorta of 30-day diabetic female rats is similar to that of control female rats. A reduced NO production and an increased endothelin production by endothelium from aorta of diabetic female rats counterbalance the reduction in smooth muscle contraction to noradrenaline [10].

In the present study we sought to test the hypothesis that type 1 diabetes induces vascular smooth muscle dysfunction in female rats by increasing iNOS expression, which may include an involvement of ESR2. Since alterations in vascular reactivity may contribute to aortic stiffness, compromising aorta function to supply adequate blood flow to tissues and organs and to damp pressure and flow oscillations, and also that removal of endothelium is completed with almost no harm to the smooth muscle layer, we used thoracic aorta as an experimental model of arterial blood vessel in order to study the vascular smooth muscle dysfunction.

Therefore, we investigated the effect of alloxan-induced type 1 diabetes on the iNOS expression and the effect of the chronic iNOS inhibition on the vascular smooth muscle dysfunction in aorta of diabetic female rats. In addition, we evaluated the effect of alloxan-induced type 1 diabetes on the estrogen receptor ESR2 and the involvement of this receptor in iNOS expression.

2. Materials and methods

2.1. Animals

The experimental protocols were performed in accordance with the Brazilian National Law and were approved by the Ethics Committee for Animal Use in Experimentation of the Institute of Biomedical Sciences (ICB), University of São Paulo (USP) (protocol numbers 007/04 and 110/2013).

Eight to ten-week-old female Wistar rats (180–200 g) were obtained from the Animal Facility at the ICB/USP and were randomized into four groups: (1) nondiabetic control female rats, (2) diabetic female rats, (3) diabetic female rats treated with insulin and (4) diabetic female rats treated with L-NIL (iNOS inhibitor). The animals were housed in constant room temperature (22 ± 24 °C), 12 h light/dark cycle and 60% humidity, with standard rat chow and water ad libitum.

2.2. Induction of diabetes

After 10 h of food deprivation, diabetes mellitus was induced with an injection of alloxan (Sigma-Aldrich, USA; 40 mg/kg) dissolved in physiological saline through the tail vein. The same volume of the physiological saline was administrated through the tail vein in control female rats.

2.3. Characterization of experimental type 1 diabetes

Thirty days after alloxan administration, female rats were placed in a metabolic cage for 24 h to evaluate food and water consumption and urine volume. Glycosuria was assessed in the urine by using glucose-sensitive strips (Bayer, Brazil). After 4 h of food deprivation, tail vein blood samples were obtained to determine glycaemia by using a blood glucose monitor (Roche, Germany). The serum insulin (Rat Insulin RIA Kit, Linco, USA) and estradiol (Siemens Healthcare Diagnostics Inc., USA) levels were assessed by radioimmunoassay. To characterize the sex hormone status of the female rats, we analyzed the estrous cycle by vaginal smears and the uterus weight.

2.4. Treatment of the diabetic female rats

To verify if all vascular changes were induced by diabetes, a set of diabetic female rats were treated with insulin. Treatment with insulin started 15 days after alloxan administration in diabetic female rats that presented glycaemia above 200 mg/dL and was maintained for 15 days. Six IU of NPH insulin were administrated subcutaneously in 2 daily doses for 15 days: 2 IU in the morning (8:00–10:00 a.m.) and 4 IU in the afternoon (4:00–6:00 p.m.) [21]. All the experiments described below were performed 12–15 h after the last insulin dose.

To inhibit iNOS, diabetic female rats that presented glycaemia above 200 mg/dL were treated with iNOS inhibitor L-NIL (Cayman Chemical, USA) by intragastric gavage, at 3 mg/kg/day [22], starting 3 days after alloxan administration and maintained for 27 days. So, the rats were used 30 days after alloxan injection, similar to that of the insulin treated group.

2.5. Measurement of plasma NO metabolites

Plasma NO metabolites were measured by using the apparatus Sievers NO Analyzer (NOA 280, GE Analytical Instruments, USA). Blood samples were collected in heparinized tubes and centrifuged for 15 min at 2000 ×*g*. Plasma samples (100 μL) were injected in purge vessel containing a reducing agent (0.8% vanadium III in 1 M HCl) at ~90 °C to convert nitrate and nitrite to NO. The NO produced was carried by an inert gas to the NOA, where it reacted with ozone to form excited-state nitrogen dioxide, which emits a photon when relaxing to the basal state. The emitted light is proportional to the amount of NO present in the sample and it was measured using a standard curve.

2.6. Preparation of isolated aorta for contractile response analysis

In the present study we used thoracic aorta as an experimental model of arterial blood vessel more easily handled and experimentally manipulated. Furthermore, the removal of endothelium is completed with almost no harm to the smooth muscle layer. Thirty days after alloxan administration, the animals were anesthetized with thiopental (50 mg/kg, i.p.), the thoracic aorta was excised and cleaned from connective tissue and sectioned in 4 mm rings. The endothelium was removed by gently rubbing the intima surface with a stainless-steel rod. The aorta rings were mounted in an isolated tissue chamber containing Krebs-Henseleit solution (in mM: 130 NaCl; 4.7 KCl; 14.9 $NaHCO_3$; 1.6 $CaCl_2{\cdot}2H_2O$; 1.18 KH_2PO_4; 1.17 $MgSO_4{\cdot}7H_2O$; 0.026 EDTA and 5.5 glucose) and were maintained in 95% O_2 and 5% CO_2 at 37 °C. The segments were submitted to a tension of 1.5 g and were equilibrated for approximately 60 min. Isometric tension was recorded by using an isometric force transducer (TRI201PAD, Panlab, Spain) connected to a data acquisition system (PowerLab 8/30, ADInstruments Pty Ltd., Australia). The integrity of the smooth muscle cells was evaluated by the ability of the aortic rings to contract in response to 90 mM KCl. The effectiveness of the endothelial removal was confirmed by the absence of relaxation to acetylcholine (1 μM). Cumulative concentration-response curves to noradrenaline (0.01 nM – 30 μM) were obtained in the endothelium-denuded aorta rings. In a set of endothelium-denuded aorta rings from control and diabetic rats, iNOS inhibitor L-NIL (10 μM) was added 30 min before the start of concentration-response curves to noradrenaline. Contraction was expressed as increase of tension (g).

2.7. Imunohistochemistry for iNOSand ESR2

To evaluate the density of immunostaining for iNOS and ESR2 in aorta of diabetic female rats, the animals were anesthetized with thiopental (Cristalia, Brazil; 50 mg/kg, i.p.) thirty days after alloxan administration; the thoracic aorta was excised and cleaned from connective tissue. Aortic rings were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) and embedded in paraffin. Non-serial cross-

sections (8 μM) were obtained with a microtome (Leica, Germany) and placed on slides treated with poly-L-lysine. The sections were deparaffinized and hydrated through xylenes and graded alcohol series and antigen recovery was performed with citrate buffer (0.01 M, pH 6.0). Endogenous peroxidase activity was quenched by incubating sections in 0.3% hydrogen peroxide for 30 min and then sections were incubated with normal goat serum (ABC Elite Kit, Vector Laboratories, USA) to avoid non-specific staining. Then, sections were incubated overnight with a rabbit polyclonal iNOS antibody (Abcam, UK) or rabbit polyclonal ESR2 antibody (Millipore,USA), diluted 1:100 in phosphate buffer (0.1 M, pH 7.4) containing 0.3% Tween and 5% normal goat serum. After that, sections were incubated with a 1:1000 dilution of biotinylated goat anti-rabbit antibody (Vector Laboratories, USA), then with avidin-biotin-peroxidase complex (ABC EliteKit, Vector Laboratories, USA). The sections reacted with 0.05% 3,3-diaminobenzidine and 0.06% hydrogen peroxide. Controls for immunostaining were performed by omitting the primary antibody, which completely abolished staining. Digital images were obtained by using a light microscope (Nikon Instruments Inc., USA). Five fields of each section were photographed and 3 sections were analyzed for each animal. The immunostaining densitometry was determined in the tunica media by using the Image J software (NIH, USA).

2.8. Western blotting for S-nitrosylated proteins and IL-1β

Segments of endothelium-denuded aorta were frozen and homogenized in cold lysis buffer RIPA (Millipore, USA; 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.25% deoxycholic acid; 1% NP-40; 1 mM EDTA) containing sodium orthovanadate (1 mM), phenylmethylsulfonylfluoride (1 mM) and a cocktail of protease inhibitors (1 μg/mL). Homogenates were centrifuged at 10,000 ×*g* for 30 min at 4 °C. Total protein content was quantified by BCA method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, USA).

For S-nitrosylated protein detection homogenates were prepared using a commercial kit (Cayman Chemical, USA). Supernatants were incubated with blocking reagent to block free thiol groups on a shaker for 30 min at 4 °C. The supernatants were then incubated for 1 h at room temperature with reducing reagent to reduce S-NO bonds present in the samples and the resulting thiol groups were biotinylated. Ice-cold acetone was added and supernatants were incubated for 1 h at −20 °C and then centrifuged at 3000 ×*g* for 10 min at 4 °C. Acetone was removed and wash buffer was added and total protein content was quantified by BCA method.

Fifty microgram (for IL-1beta) or 30 μg (for S-nitrosylation analysis) of total protein were treated with Laemmli's buffer containing 350 mM dithiothreitol, separated by electrophoresis on a polyacrylamide gel (12%) and transferred to polyvinyl difluoride membrane (Amersham Hybond-P, GE Healthcare Life Sciences, UK). Non-specific binding sites were blocked with 5% non-fat dry milk or 2% bovine serum albumin (for S-nitrosylation analysis) in Tris-buffered saline solution with 1% Tween for 1 h at room temperature. The membranes were then incubated with 1:750 dilution anti-IL-1β antibody (BioLegend, EUA) overnight at 4 °C and, after washing, with anti-hamster IgG antibody conjugated to horseradish peroxidase (1:2000) for 2 h at room temperature. For S-nitrosylation analysis, membranes were incubated with S-nitrosylation detection reagent. Subsequently, the membranes were incubated with enhancer chemiluminescence substrate (Pierce ECL Western Blotting substrate, Thermo Fisher Scientific, USA) and exposed to films. Intensity of chemiluminescence signals was quantified by densitometry using Image J software (NIH, USA). Densitometry for S-nitrosylated proteins was performed for all bands in each lane. This intensity was normalized by densitometry of bands stained with Ponceau stain as described by Romero-Calvo et al. [23]. For S-nitrosylation analysis, the same membranes were used to determine α-actin content using an anti-α-actin antibody (1:10000), which was used to normalize abundance of S-nitrosylated proteins.

2.9. Aortic smooth muscle cell culture

Aortic smooth muscle cell culture was performed as previously described and characterized [24]. In brief, thoracic aorta was cleaned from adipose and connective tissue, cut in little pieces and incubated with enzymatic solution (2 mg/mL type-V collagenase, 0.5 mg/mL elastase, 0.4 mg/mL soybean trypsin inhibitor and 2 mg/mL bovine serum albumin) for 120 min at 37 °C, under agitation. Smooth muscle cells were dissociated by pipetting the digested tissue through a p1000 tip, and then were centrifuged at 600 ×*g* for 10 min at 20 °C. The cell pellet was re-suspended in Dulbecco Minimal Essential Medium (DMEM) containing 20% fetal calf serum, 2 mM glutamine, 20 mM Hepes (pH 7.4) and 0.5% penicillin/streptomycin. Smooth muscle cells were cultured in a six well plate up to reaching 80% confluence and the culture medium was replaced with normal (5 mM) or high glucose (22 mM) serum-free medium for 24 h to render the cells quiescent. A set of cells was incubated with high glucose, serum-free medium and the estrogen receptor ESR2 antagonist PHTPP (20 μM). Each cultured cell line was initially prepared from aorta pooled from 6 female rats. The experiments were repeated with at least four different primary cell cultures used in passages 3–4.

2.10. Real-time PCR for iNOS

Total RNA was isolated from aortic smooth muscle cells by using TRizol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized by using 2 μg of total RNA and M-MLV reverse transcriptase (Promega, USA). cDNA (100 ng) was submitted to real-time PCR amplification using SYBR® Green PCR Master Mix (Applied Biosystems, USA) and iNOS oligonucleotides, with thermal conditions as follows: 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time PCR was performed, recorded, and analyzed using the Corbett Research system (Corbett Life Sciences, Australia). Oligonucleotide sequences were designed using the NCBI Primer Design tool (forward: GGATATCTTCGGTGCGGTCTT; reverse: CTGTAACTCTTCTGGGTGTCAGA). α-actin was used as a housekeeping gene. Cycle threshold (Ct) values obtained for α-actin did not differ among groups. The changes in mRNA expression were calculated using the 2-ΔΔCt method [25].

2.11. Statistical analysis

Data are expressed as means ± SEM. Maximal response and log of concentration that promoted 50% of maximal response (EC50) were determined from individual concentration-response curves using non-linear regression analysis. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple comparisons. Repeated measures two-way ANOVA followed by Tukey post hoc test for multiple comparisons was used for the analysis of each point of the concentration-response curves (GraphPad Prism6, GraphPad Software Inc., USA). Values of probability (P) < 0.05 were considered significant.

3. Results

3.1. Biological parameters and the effect of treatments

Thirty days after alloxan administration, female rats presented lower insulinemia and body weight gain and increased glycaemia, food and water intake and urine volume than female rats that received vehicle (Table 1). Glycosuria was detected only in the diabetic female rats (Table 1). Treatment of diabetic female rats with 6 IU/day (administrated in two doses) of NPH insulin for 15 days corrected the body weight gain, water intake and urine volume and partially corrected the insulinemia and food intake (Table 1). Glucose was still detected in the urine from diabetic female rats treated with insulin (Table 1). Insulin

Table 1
General characteristics of control, diabetic and diabetic rats treated with insulin (6 IU/day, s.c.) or L-NIL (iNOS inhibitor; 3 mg/kg/day, oral gavage).

	Control (10)	Diabetic (12)	Diabetic-insulin (11)	Diabetic-LNIL (11)
Glycaemia (mg/dL)	115.6 ± 2.3	550.5 ± 5.9*	545.7 ± 7.6*	565.7 ± 8.7*
Insulinemia (μU/mL)	11.4 ± 0.4	6.6 ± 0.5*	9.3 ± 0.7*#	6.2 ± 0.4*
Body weight gain (g)	38.8 ± 3.2	−2.1 ± 8.5*	32.5 ± 4.0#	2.6 ± 3.5*
Food intake (g/24 h)	6.7 ± 0.3	18.8 ± 1.1*	13.2 ± 0.6*#	17.5 ± 0.6*
Water intake (mL/24 h)	11.7 ± 0.8	79.7 ± 3.8*	23.2 ± 1.4#	70.2 ± 4.1*
Urine volume (mL/24 h)	4.1 ± 0.4	65.1 ± 4.2*	13.2 ± 1.3#	51.2 ± 3.3*#
Glycosuria (g/dL)	Negative	Positive	Positive	Positive
Estradiol (pg/mL)	21.5 ± 1.8	26.5 ± 4.3	21.8 ± 1.7	23.3 ± 2.4
Uterus weight (mg/100 g body weight)	33.2 ± 2.8	17.0 ± 2.3*	29.5 ± 2.6	19.7 ± 0.5*

Data are mean ± SEM. One-way ANOVA: * $P < 0.05$ versus Control; #$P < 0.05$ versus Diabetic. Levels of estradiol and uterus weight were measured in the diestrous phase. The number of animals evaluated is indicated in parentheses.

administration was not able to maintain low glycaemia levels between doses of insulin. Four hours after the first and second doses of insulin, the glycaemia levels were 94.0 ± 12.0 mg/dL and 146.0 mg/dL, respectively. Then, glycaemia increased and 14 h after the second dose, similar glycaemia levels were found between insulin treated and untreated diabetic female rats (Table 1). The treatment of diabetic rats with L-NIL did not change any of these parameters (Table 1).

Diabetic female rats presented vaginal smear consistent with a continuous diestrous phase (data not shown). To avoid influences of estrous cycle on data analyzed, all experiments were conducted with female rats in the diestrous phase. In this condition, estradiol concentration was similar among the groups (Table 1).

3.2. Analysis of iNOS, NO production and S-nitrosylated proteins

The immunostaining for iNOS was increased in tunica media of aorta sections of diabetic female rats. Treatment with insulin reduced the iNOS immunostaining (Fig. 1). On the other hand, the iNOS immunostaining did not differ between the iNOS inhibitor L-NIL-treated and untreated diabetic female rats (Fig. 1). The NO plasmatic concentration and abundance of S-nitrosylated proteins in aorta were increased in diabetic female rats and treatment with either insulin or iNOS inhibitor L-NIL reduced them (Fig. 2).

3.3. Effect of iNOS inhibition on vascular contraction

The contraction induced by noradrenaline was decreased in aortic rings without endothelium of diabetic female rats when compared with control female rats (Fig. 3). The maximal response, but not EC50, to noradrenaline was reduced in aortic rings of diabetic female rats in relation to those from control female rats (Tables 2; 3). Acute incubation with the iNOS inhibitor L-NIL did not change the contraction induced by noradrenaline in control female rats. On the other hand, L-NIL acutely administered improved the contractile response to noradrenaline in diabetic female rats, but this response was still different from that of control female rats (Fig. 3A; Table 2). In comparison, long-term treatment with L-NIL as well as treatment with insulin completely corrected the reduced contraction induced by noradrenaline in endothelium-denuded aortic rings from diabetic female rats (Fig. 3B; Table 3).

3.4. Analysis of inflammatory marker IL-1β

The protein expression of IL-1β was increased in aorta from diabetic

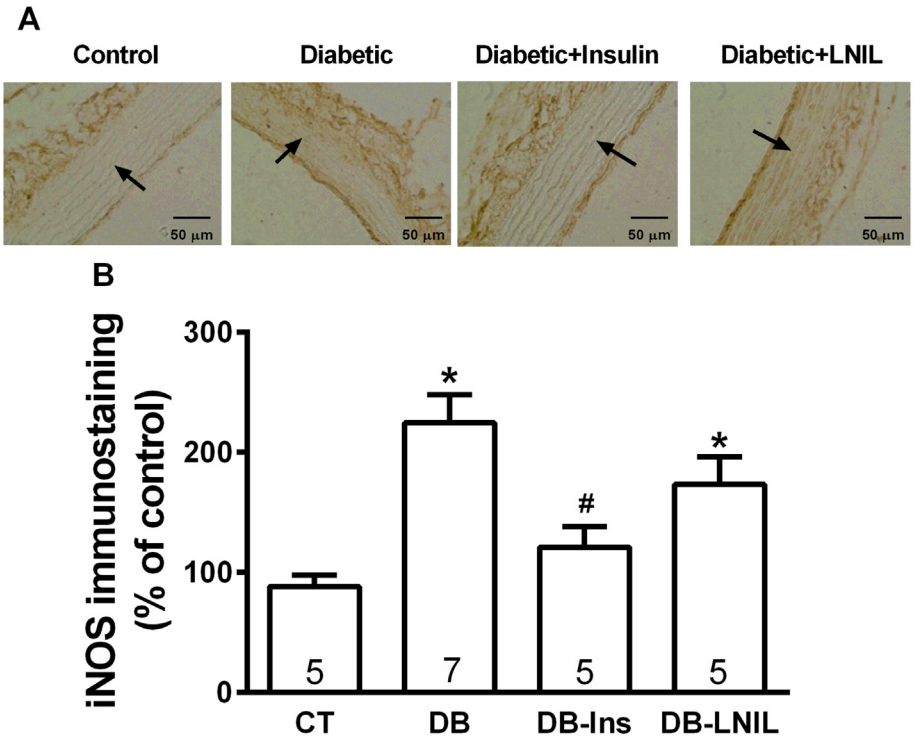


Fig. 1. A: representative digital images showing iNOS immunostaining in aorta sections from control (CT), diabetic (DB) and diabetic rats treated with insulin (DB-Ins; 6 IU/day, s.c.) or iNOS inhibitor L-NIL (DB-LNIL; 3 mg/kg/day, oral gavage). Arrows indicate tunica media. B: bar graphs showing iNOS immunostaining densitometry in tunica media of aorta sections, expressed as percentage increase of control. Data were expressed as mean ± SEM. One-way ANOVA: * $P < 0.05$ versus CT; #$P < 0.05$ versus DB. The number of animals evaluated is indicated in the bars.

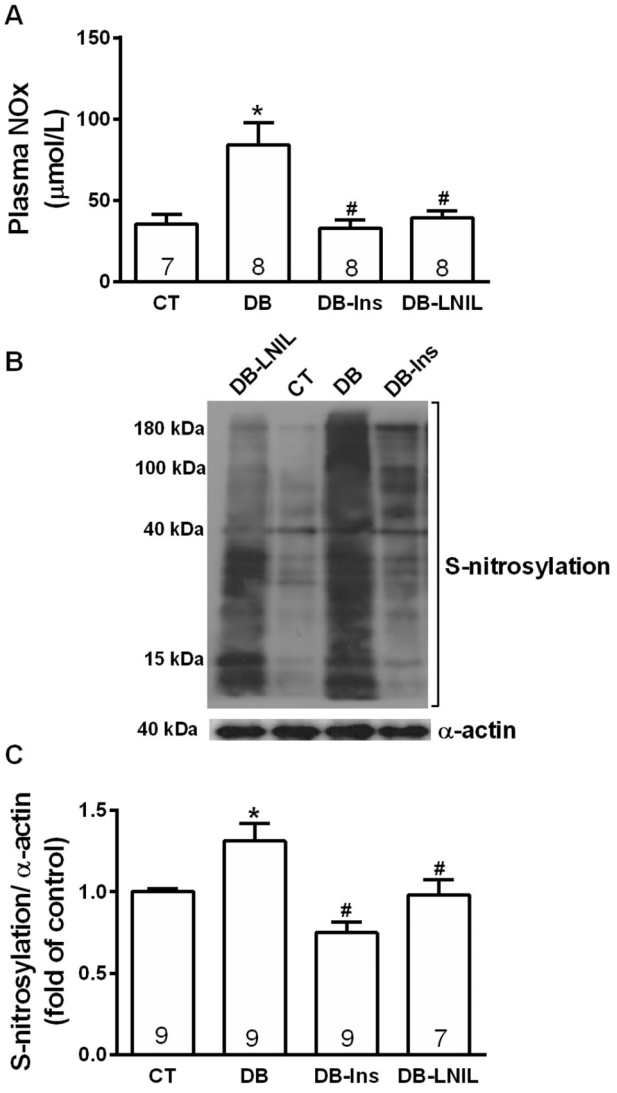


Fig. 2. A: plasma concentration of oxides of nitrogen (NOx) in control (CT), diabetic (DB) and diabetic rats treated with insulin (DB-Ins; 6 IU/day, s.c.) or iNOS inhibitor L-NIL (DB-LNIL; 3 mg/kg/day, oral gavage). B: representative image of immunoblotting for S-nitrosylated proteins in endothelium-denuded aorta homogenate from CT, DB, DB-Ins and DB-LNIL. C: densitometric analysis of S-nitrosylated proteins. Data were expressed as mean ± SEM. One-way ANOVA: * $P < 0.05$ versus CT; $^{\#}P < 0.05$ versus DB. The number of animals evaluated is indicated in the bars.

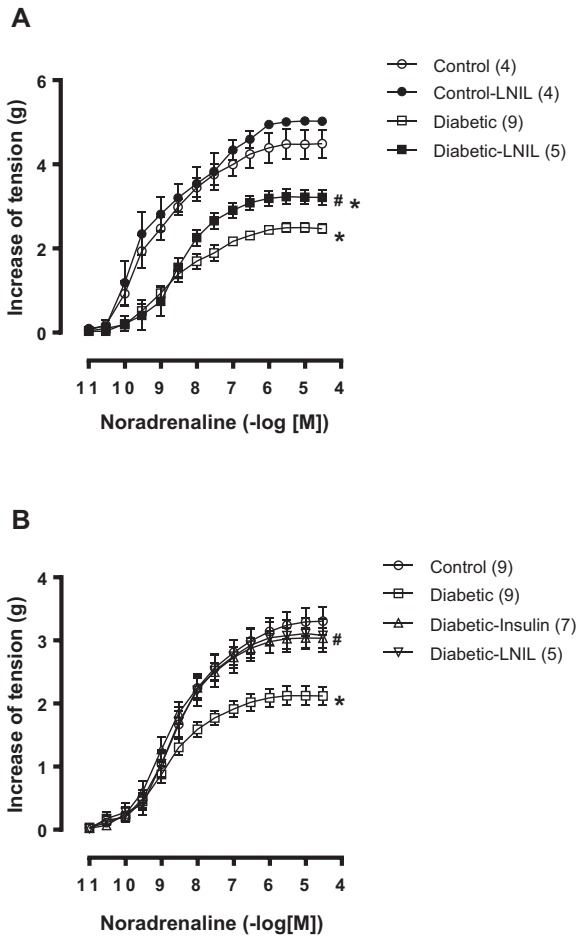


Fig. 3. A: concentration-response curves to noradrenaline in endothelium-denuded aortic rings, incubated with vehicle or iNOS inhibitor (L-NIL, 10 μM), from control and diabetic rats. B: concentration-response curves to noradrenaline in endothelium-denuded aortic rings from control, diabetic and diabetic rats treated with insulin (6 IU/day, s.c.) or iNOS inhibitor L-NIL (3 mg/kg/day, oral gavage). The results are expressed as increase of tension (gram, g). Data were expressed as mean ± SEM. Two-way ANOVA: * $P < 0.05$ versus Control; $^{\#}P < 0.05$ versus Diabetic. The number of animals evaluated is indicated in parentheses.

female rats when compared to control female rats. The treatment with either insulin or iNOS inhibitor L-NIL corrected the increased IL-1βexpression in aorta of diabetic female rats (Fig. 4).

3.5. Analysis of immunostaining for estrogen receptor ESR2

Tunica media of aortic sections of diabetic female rats presented higher immunostaining for ESR2 than aortic sections of control female rats. Treatment with insulin reduced it and treatment with the iNOS inhibitor L-NIL did not change it (Fig. 5).

3.6. Effects of estrogen receptor ESR2 antagonism on the iNOS mRNA

Aortic smooth muscle cells incubated with high glucose concentration for 24 h showed increased levels of iNOS mRNA (Fig. 6). The antagonist of estrogen receptor ESR2 PHTPP prevented the high glucose-induced iNOS mRNA increase in aortic smooth muscle cells (Fig. 6).

4. Discussion

Animal models of diabetes and obesity in males present impaired vascular function, such as reduction in the contraction and dilation, as a result of increased vascular inflammation [12,26,27]. Although female sex-related cardiovascular protection is well documented, in the present study we showed that alloxan-induced type 1 diabetes promoted vascular inflammation in female rats, similar to that observed in diabetic males [9,12,28]. This is supported by the fact that an increased iNOS protein expression, and in consequence increased NO levels, and increased IL-1β protein expression was observed in the aortic wall from diabetic female rats. The fact that all these alterations were corrected by insulin treatment supports the conclusion that they were due to diabetes.

It has been observed that IL-1β stimulates the iNOS expression and the release of a large amount of NO in the vascular smooth muscle cells cultured either in normal glucose [29–31] or in high glucose [28], as well as in vascular smooth cells isolated from Goto-Kakizaki diabetic male rat, a genetic non-obese diabetes model [32]. On the other hand, treatment with an iNOS inhibitor inhibits the lipopolysaccharide-

Table 2
Maximal response and sensibility (EC50) to noradrenaline in endothelium-denuded aortic rings, incubated with vehicle or iNOS inhibitor (L-NIL, 10 μM), from control and diabetic rats.

	Control (4)	Control – LNIL (4)	Diabetic (9)	Diabetic – LNIL (5)
Maximal response (g)	4.42 ± 0.32	4.93 ± 0.11	2.45 ± 0.10*	3.13 ± 0.17*#
Log EC50	9.93 ± 0.59	9.90 ± 0.66	8.79 ± 0.25	8.71 ± 0.31

Data are mean ± SEM. One-way ANOVA: * $P < 0.05$ versus Control; #$P < 0.05$ versus Diabetic. The number of animals evaluated is indicated in parentheses.

induced plasma IL-1β and TNF-α elevation in non-diabetic rats [33]. Furthermore, knockdown of iNOS by siRNA inhibits the high glucose-induced IL-1β and TNF-α expression in vascular smooth muscle cells [28]. All those data show that inflammatory stimulus generate a positive feedback loop to amplify the vascular inflammation. Although it has not been possible to determine the initial stimulus, we showed that interruption of the cycle may inhibit the vascular inflammation because treatment with iNOS inhibitor reduced the IL-1β protein abundance in aorta from diabetic female rats.

Takahashi and co-workers [34] showed that increased iNOS-derived NO stimulated by lipopolysaccharide is involved in the reduced contraction to phenylephrine in the aorta from Goto-Kakizaki diabetic male rats. Since incubation of aorta in high glucose-containing medium did not affect the response, the authors concluded that pro-inflammatory stimulus rather than hyperglycemia contributed to vascular damages in diabetes. In contrast, in female diabetic rats, our results led us to suggest that the alterations are due to type 1 diabetes since the increased iNOS expression and NO levels as well as the reduced noradrenaline-induced contraction were completely corrected by insulin treatment. On the other hand, since the metabolic alterations were not completely corrected by insulin treatment, we suggest that normalization of metabolic parameters is not needed for the correction of the vascular dysfunction, at least in females. In addition, mechanisms independent of metabolic alterations could also be responsible for the alterations found in alloxan-induced diabetic female rats.

In smooth muscle cells, NO binds to the heme iron, activates soluble guanylate cyclase and triggers the GMPc/PKG pathway that, in turn, regulates the sensitivity of contractile proteins to calcium and the intracellular calcium concentration and reduces contraction [35]. Therefore, inhibition of iNOS could increase the contractile response. In the present study, acute incubation with an inhibitor of iNOS partially corrected the reduced contractile response to the adrenergic receptor agonist in the aorta of diabetic female rats, whereas the chronic treatment of diabetic female rats with iNOS inhibitor (for 27 days) completely corrected this attenuated vasoconstriction. These findings led us to suggest that increased iNOS expression may be involved in the reduced noradrenaline-induced contraction in female diabetic rats. In contrast to what was observed in the present study, a depressed pressor responsiveness to α1-adrenergic receptor stimulation in diabetic male rats was completely corrected by acute iNOS inhibition [9] and only partially corrected by 8-week iNOS inhibitor treatment [12]. Therefore, different mechanisms could be involved in the vascular alterations observed in male and female diabetic rats.

In addition to triggering the GMPc/PKG pathway, NO signaling can involve the post-translational regulation by S-nitrosylation, the attachment of a NO moiety to the cysteine thiol, and other NO-based protein modifications [36]. S-nitrosylation of PKC inhibits the

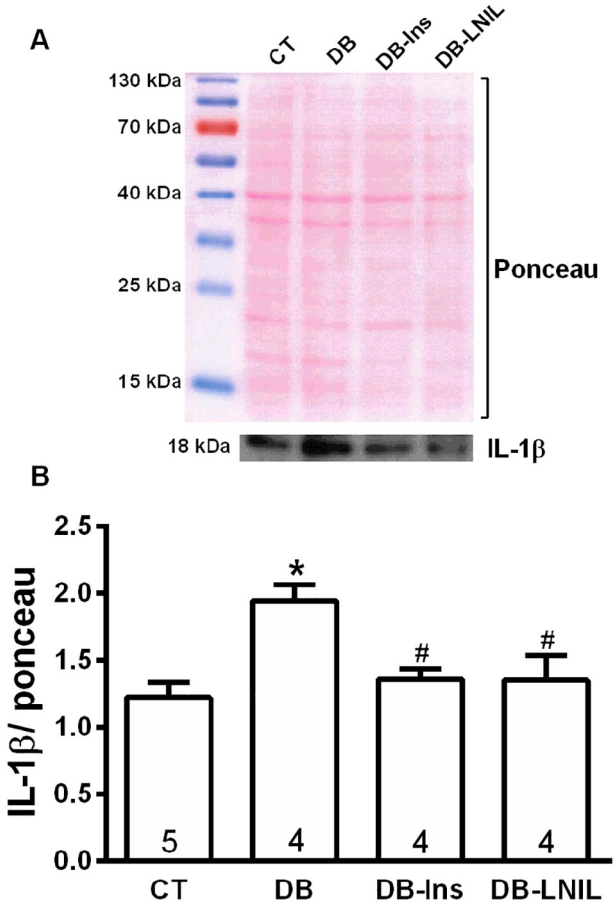


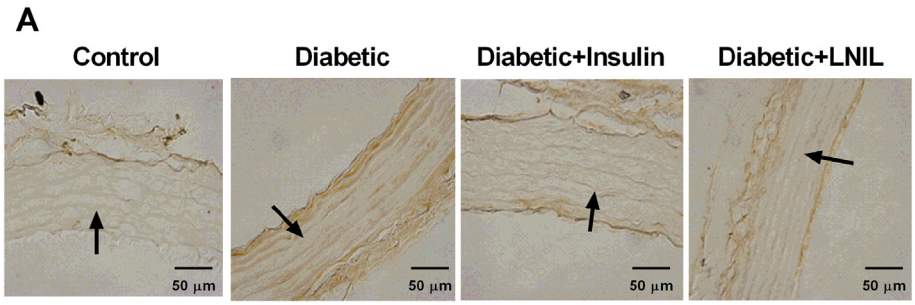
Fig. 4. A: representative image of immunoblotting for IL-1β in endothelium-denuded aorta homogenate and membrane stained with Ponceau from control (CT), diabetic (DB) and diabetic rats treated with insulin (DB-Ins; 6 IU/day, s.c.) or iNOS inhibitor L-NIL (DB-LNIL; 3 mg/kg/day, oral gavage). B: ratio of densitometric analysis of IL-1β and Ponceau staining. Data were expressed as mean ± SEM. One-way ANOVA: * $P < 0.05$ versus CT; #$P < 0.05$ versus DB. The number of animals evaluated is indicated in the bars.

phosphorylation of contractile proteins and the contractile response in mice aorta [37]. Moreover, S-nitrosylation of the α1-adrenergic receptor decreases ligand binding and vasoconstriction induced by α1-adrenergic receptor stimulation in pulmonary artery of male rats [38]. Although we could not determine which proteins were S-nitrosylated, we observed an increase in the expression of S-nitrosylated proteins in

Table 3
Maximal response and sensibility (EC50) to noradrenaline in endothelium-denuded aortic rings control, diabetic and diabetic rats treated with insulin (6 IU/day, s.c.) or iNOS inhibitor L-NIL (3 mg/kg/day, oral gavage).

	Control (9)	Diabetic (9)	Diabetic – Insulin (7)	Diabetic – LNIL (5)
Maximal response (g)	3.27 ± 0.22	2.12 ± 0.14*	3.02 ± 0.17	3.04 ± 0.25
Log EC50	8.56 ± 0.13	8.89 ± 0.14	8.86 ± 0.15	8.67 ± 0.15

Data are mean ± SEM. One-way ANOVA: * $P < 0.05$ versus Control. The number of animals evaluated is indicated in parentheses.



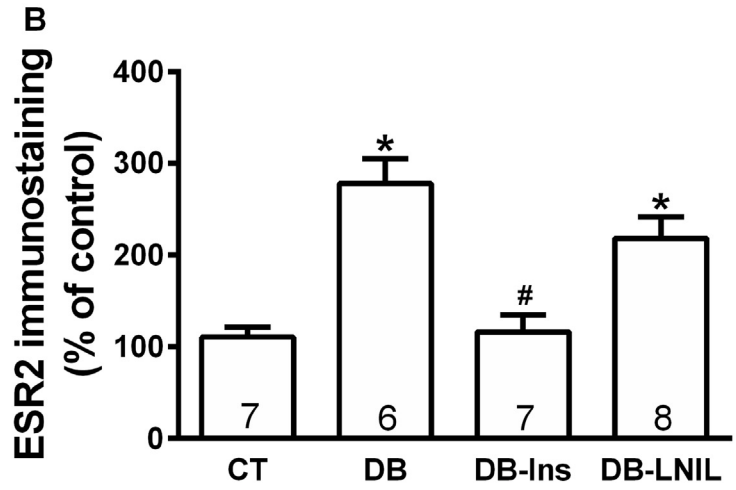


Fig. 5. A: representative digital images showing ESR2 immunostaining in aorta sections from control (CT), diabetic (DB) and diabetic rats treated with insulin (DB-Ins; 6 IU/day, s.c.) or iNOS inhibitor L-NIL (DB-LNIL; 3 mg/kg/day, oral gavage). Arrows indicate tunica media. B: bar graphs showing ESR2 immunostaining densitometry in tunica media of aorta sections, expressed as percentage increase of control. Data were expressed as mean ± SEM. One-way ANOVA: * $P < 0.05$ versus CT; $^{\#}P < 0.05$ versus DB. The number of animals evaluated is indicated in the bars.

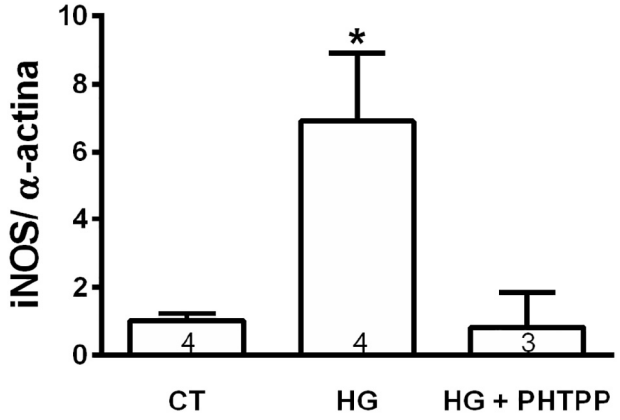


Fig. 6. Inducible nitric oxide synthase (iNOS) mRNA levels in aortic smooth muscle cells incubated with normal (5 mM) (CT) and high glucose (22 mM) (HG) medium and high glucose medium plus estrogen receptor ERS2 antagonist PHTPP (20 μM) (HG + PHTPP). Data were expressed as mean ± SEM. One-way ANOVA: * $P < 0.05$ versus CT and HG + PHTPP. The numbers indicated in the bars represent the numbers of repetitions with different primary cell cultures.

aorta of diabetic female rats, which were reduced by treatment with L-NIL. Since treatment with L-NIL also corrected the reduced vasoconstriction, reduction of S-nitrosylation could be involved in the effects of the iNOS inhibition on the reduced contractile response.

In females, the protective effect exerted by estrogen on the cardiovascular system is lost in high-glucose conditions and in diabetes [39,40]. Changes in the pattern of expression and/or activation of estrogen receptor subtypes may, at least in part, be responsible for the loss. Estrogen regulates the expression of its receptors in endothelial cells, increasing the expression of ESR1 and decreasing ESR2 [41]. However, when endothelial cells are plated on a medium containing high glucose concentration, estrogen induces a different expression pattern of their receptors, with reduced expression of ESR1 and increased ESR2 [42].

In the present study, we found an increased immunostaining for ESR2 in aorta of female diabetic rats. It is known that both ESR2 agonists [20] and activation of the ESR2 by estrogen [43] induce expression of inflammatory factors in vascular smooth muscle cells incubated with a normal glucose concentration. Therefore, an increase in ESR2 could contribute to vascular inflammation and loss of estrogen protection in female diabetic rats. On the other hand, we showed that incubation with high glucose medium increased the abundance of iNOS mRNA in aortic smooth muscle cells from female rats, which was reduced by ESR2 antagonist. Thus, increased expression of the ESR2 could result in an increase in iNOS expression that contributes to the reduction of the contractile response to the adrenergic agonist in female diabetic rats. This may be reinforced by the fact that L-NIL treatment did not change the iNOS and ESR2 immunostaining in aorta of diabetic female rats, indicating that L-NIL treatment blocks downstream rather than upstream mechanisms to iNOS.

The fact that insulin treatment corrected all vascular alterations led us to suggest that those alterations were due to type 1 diabetes. However, since insulin treatment did not totally correct the metabolic alterations, we cannot discard the involvement of mechanisms independent of metabolic effects of insulin. As insulin treatment completely normalized the estrous cycle (data not shown), further studies are necessary to evaluate the influences of the fluctuations in sex hormone levels in the vascular alterations found in alloxan-induced diabetic female rats.

5. Conclusion

In conclusion, we demonstrated that the increased expression of iNOS and NO generation contributed to the impairment of the contractile response of aortic smooth muscle cells in female type 1 diabetic rats and that increased expression of iNOS may include the participation of estrogen receptor ESR2 (ERβ). Our data also show that inflammation plays a major role in vascular dysfunction in female type 1 diabetic rats.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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