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Decreased reactive oxygen species production and NOX1, NOX2, NOX4 expressions contribute to hyporeactivity to phenylephrine in aortas of pregnant SHR

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ABSTRACT

Aims: We determined whether decreased reactive oxygen species (ROS) production in the aorta of pregnant spontaneously hypertensive rats (SHR) resulted in increased nitric oxide (NO) bioavailability and hyporeactivity to phenylephrine (PE).
Main methods: Systemic and aortic oxidative stress were measured in pregnant and non-pregnant Wistar rats and SHR. Furthermore, the hypotensive effects of apocynin (30 mg/kg) and Tempol (30 mg/kg) were analyzed. Intact aortic rings of pregnant and non-pregnant rats were stimulated with PE in the absence of or after incubation (30 min) with apocynin (100 μmol/L). The effect of apocynin on the concentrations of NO and ROS were measured in aortic endothelial cells (AEC) using DAF-2DA (10 mmol/L) and DHE (2.5 mmol/L), respectively. Western blotting was performed to analyze eNOS, NOX1, NOX2, NOX4 and SOD expression. ROS production was analyzed by the lucigenin chemiluminescence method.
Key findings: Aortic oxidative stress and ROS concentration in AEC were reduced in pregnant Wistar rats and SHR, when compared to non-pregnant rats. ROS production and NOX1, NOX2 and NOX4 expression in the aortas were decreased in pregnant SHR, but not in pregnant Wistar rats. Increased eNOS expression in aortas and NO concentration in AEC were observed in pregnant Wistar rats and SHR. Apocynin reduced PE-induced vasoconstriction in the aortas of non-pregnant Wistar rats and SHR, and pregnant Wistar rats, but not in the aortas of pregnant SHR.
Significance: Taken together, these results suggest that ROS production was decreased in the aortas of pregnant SHR and could contribute to higher NO bioavailability and hyporeactivity to PE in the aortas of pregnant SHR.

1. Introduction

Reactive oxygen species (ROS) participate in several physiological process including signaling and transduction pathways, cell growth and differentiation, defense against pathogens, activation of transcription factors, and reproduction [1,2,3]. However, increased ROS levels contribute to pathological conditions like hypertension and vascular diseases, such as atherosclerosis, proliferation and hypertrophy of vascular smooth muscle cells, and endothelial dysfunction [4,5,6].

In the cardiovascular system, an important source of ROS are the NOX1, NOX2, NOX4 and NOX5 subunits of the NOX family that constitute the NAD(P)H oxidase enzymatic complex [7,8] that reduces molecular oxygen and generates unstable species such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Antioxidant enzymes, including superoxide dismutase (SOD), which dismutates O_2^- into H_2O_2 [9], catalase (CAT), and glutathione peroxidase (GPx), play a role in balancing ROS levels.

Increased ROS production by NOX, peroxynitrite degradation of cofactor tetrahydrobiopterin (BH_4), and uncoupled endothelial nitric oxide synthase (eNOS) contribute to endothelial dysfunction in a pregnancy-induced hypertension model, where rats were treated with deoxycorticosterone acetate (DOCA) and 0.9% saline [10] or by reduced uteroplacental perfusion pressure (RUPP) in rats, an animal experimental model pre-eclampsia [11]. However, in spontaneously hypertensive rats (SHR), the reduced blood pressure observed in late pregnancy may be related to a decrease in oxidative stress. Compared to non-pregnant SHR, higher GPx activity was observed in the mesenteric arterial bed of

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pregnant SHR [12]. Recently, we reported a low ROS concentration in aortic endothelial cells (AEC) of pregnant SHR compared to non-pregnant SHR [13].

Pregnancy, in women as well as in normotensive or hypertensive rats, is a physiological process characterized by hemodynamic changes such as increased plasmatic volume [14] and decreased vascular response to the vasoconstrictor, phenylephrine (PE) [15,16,17]. We previously demonstrated that the overproduction of NO, *via* the PI3K-Akt-eNOS pathway, modulates the hyporeactivity to PE in the aortas of pregnant SHR [13]. However, the role of ROS in aortic hyporeactivity to PE in pregnant SHR has not been previously evaluated. We therefore hypothesized that an increase in NO bioavailability is associated with a decrease in ROS production in the aortas of pregnant SHR. Our objective was to analyze the effect of apocynin (a non-selective inhibitor of NOX activity) and Tempol (a SOD mimetic) on blood pressure and the effect of apocynin in aorta reactivity to PE in pregnant SHR. Furthermore, we assessed the ROS production and expression of eNOS, NOX1, NOX2, NOX4, and SOD-1 in the aortas of these animals.

2. Materials and methods

2.1. Animals

Experiments were approved by the Animal Research Ethics Committee of the School of Dentistry of Araçatuba — UNESP (process 00658-2013) and were in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care. Animals used were as follows: non-pregnant or pregnant, 12-week-old, normotensive Wistar rats and SHR (SBP ≥ 150 mm Hg, evaluated by tail plethysmography, PowerLab, ADInstruments, Melbourne, Australia). Animals received standard chow and water *ad libitum* under controlled conditions (22–24 °C, 12 h light/dark cycle). Non-pregnant rats in physiological estrus were utilized as the control group. For mating purposes, males of matched strains, Wistar rats or SHR (SBP ≥ 150 mm Hg), were used. Day 0 of pregnancy was determined by the presence of sperm in vaginal smear [18] and pregnant rats were used during the late pregnancy period (18th–20th days).

2.2. Measurement of blood pressure (BP) and heart rate (HR)

Rats were anesthetized with ketamine (45 mg/kg) and xylazine (5 mg/kg), polyethylene cannulas (PE_{50} coupled $PE_{10,}$ — Intramedic Polyethylene Tubing, BD Company, New Jersey, USA) filled with heparinized saline solution (0.1%), were placed in the femoral artery to record mean arterial pressure (MAP) and HR and in the femoral vein for drug administration. After 24 h, in conscious rats, the intra-arterial cannula was connected to a pressure transducer and amplifier (PowerLab, ADInstruments, Melbourne, Australia) for analysis of MAP and HR at basal conditions and during drug administration. Apocynin (30 mg/kg) and Tempol (30 mg/kg) were intravenously administered and their effects were analyzed. The doses of apocynin and Tempol were chosen based on previous studies of our group [19]. The parameters were calculated using software LabChart 7 (ADInstruments, Melbourne, Australia) and the results were expressed as the difference between MAP values at basal condition and after drug administration (Δ MAP).

2.3. Determination of systemic and aortic oxidative damage

Rats were killed by decapitation, and blood and thoracic aortas were collected. The aortas frozen in liquid nitrogen were crushed and sonicated in phosphate buffer (30 mmol/L) and KCl solution (120 mmol/L), pH 7.4, using a sonicator (Vibra Cell Sonics, Newtown, CT, USA). Homogenate was centrifuged (Centrifuge 5415R, Eppendorf, Hamburg, Germany at 3000 rpm, 10 min, 4 °C) and the supernatant was separated. Blood was collected in heparinized tubes and centrifuged (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 3000 rpm, 15 min, 4 °C. Plasma was removed and erythrocytes were washed with saline (1:1 *w*/*v*), mixed with $MgSO_4$ (4 mmol/L) and acetic acid (1 mmol/L) solution (1:10 *w*/*v*) and the hemolysate were stored at −80 °C.

Hemolysate and supernatant were assessed for thiobarbituric acid reactive substances (TBARS) [20], as an indicator of lipid peroxidation levels. Trichloroacetic acid (10% *w*/*v*) was added to the samples and this was followed by centrifugation (3000 rpm, 3 min, Centrifuge 5810R). The supernatant was collected, added to 2-thiobarbituric acid (0.67% *w*/*v*) and placed in a water bath (100 °C, 15 min). The absorbance was measured with a spectrophotometer at 535 nm and results were expressed in nmol/mg protein using the appropriate molar extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$). The protein concentration was measured by the Lowry method [21].

2.4. Vascular reactivity

Thoracic aorta rings (2 mm) were placed between two stainless steel hooks and connected to an isometric force transducer (DMT, ADInstruments, Melbourne, Australia), maintained in a chamber containing Krebs solution (mmol/L): NaCl 130.0; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 14.9; glucose 5.5; $CaCl_2$ 1.6; pH 7.4, 95% O_2 and 5% CO_2, 37 °C. For stabilization, rings were maintained for 30 min at a baseline tension (30 mN) and vitality was confirmed with KCl solution (120 mmol/L). Endothelium integrity was confirmed by acetylcholine-induced relaxation (ACh 1 mmol/L) in rings contracted with PE (100 nmol/L). After washing, the rings were incubated, or not, with apocynin (100 μmol/L) for 30 min and concentration-effect curves to PE (1 nmol/L to 0.1 μmol/L) were plotted. Data were expressed as the maximum effect (Emax, the maximal amplitude in the concentration—effect curves for contraction to PE) and pD_2 (negative logarithm transformation of EC_{50}, concentration that produced half-maximal contraction amplitude). Response curves were compared for pregnant and non-pregnant Wistar rats and SHR.

2.5. Flow cytometry

AEC were mechanically removed with the aid of a flat plastic rod and maintained in modified Hanks solution (mmol/L): $CaCl_2$ 1.6; $MgCl_2$ 1.0; NaCl 120.0; KCl 5.0; NaH_2PO_4 0.5; glucose 10.0 and HEPES 10.0, pH 7.4. These cell suspensions were centrifuged (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 1000 rpm for 5 min, at 25 °C and a total of 2500 AEC/rat were analyzed by flow cytometer (Attune™ Acoustic Focusing Cytometer, Applied Biosystems, Australia). Specifically, the AEC were incubated for 20 min with DAF-2/DA (10 μmol/L) fluorescent probes to evaluate NO concentration and with DHE (2.5 mmol/L) for ROS concentration; they were then excited with a blue laser at 488 nm with emission at 530/30 nm. Fluorescence intensity emission was detected in basal conditions and in presence of apocynin (100 μmol/L, 30 min). Cytofluorographic tracings generated by a software (Attune Cytometric Software, Applied Biosystem, Australia) were analyzed and the results were presented as the mean of the medians of fluorescence intensity from the cells. The median fluorescence intensity was compared between groups. This methodology is in agreement with the method previously described in Bonaventura et al. [22].

2.6. Lucigenin chemiluminescence measurement

NAD(P)H oxidase-dependent ROS production was quantified in aortic rings from non-pregnant and pregnant Wistar rats and SHR (n = 4–7/group) by lucigenin chemiluminescence method, described previously [23,24]. Luminescence was measured in a luminometer (Orion II luminometer, Berthold detection systems, Pforzheim, Germany). The results were expressed as relative light unit (RLU)/mg protein and compared between groups.

2.7. Western blotting

The aortas were crushed in liquid nitrogen and homogenized in RIPA buffer (154 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.8 mmol/L ethylenediamine tetraacetic acid, 65.2 mmol/L Tris base) and a protease inhibitor cocktail. The homogenates were centrifuged (10,000 rpm, 10 min, 4 °C, Centrifuge 5415R, Eppendorf, Hamburg, Germany) and the supernatant protein was quantified by the Lowry method [21]. Protein (100 μg) was subjected to electrophoresis on polyacrylamide gel (8% or 10%) and transferred to a nitrocellulose membrane. The membranes were blocked (6% nonfat milk in Tris buffer solution for 30 min) and incubated overnight with the primary antibodies against eNOS (1:2500, Santa Cruz Biotechnology, Dallas, USA), SOD-1 (1:1 000, Santa Cruz Biotechnology, Dallas, USA), NOX1 (1:250, Santa Cruz Biotechnology, Dallas, USA), NOX2 (1:500, Abcam, Cambridge, UK) and NOX4 (1:1000, Santa Cruz Biotechnology, Dallas, USA) at 4 °C. Following this, incubation was performed with an anti-rabbit (for eNOS: 1:2000; SOD-1 and NOX4: 1:10,000 and NOX2: 1:3000; Santa Cruz Biotechnology, Dallas, USA) or anti-goat (for NOX1: 1:1000; Santa Cruz Biotechnology, Dallas, USA) secondary antibody for 1 h at room temperature. Bands were detected by chemiluminescence (ECL Plus, GE Healthcare, UK), exposed to a radiographic film (GE Healthcare, UK) for detection of the bands and measured for densitometry. β-Actin (primary 1:4000, Sigma-Aldrich, St. Louis, USA; secondary 1:8000, Santa Cruz Biotechnology, Dallas, USA) was used for normalization of the results. The results were compared between groups.

2.8. Drugs and fluorescent probes

Acetylcholine, apocynin, Tempol, phenylephrine, DAF-2DA and DHE used were purchased from Sigma-Aldrich (St. Louis, MO, USA). For *in vitro* experiments, all these components were dissolved in appropriate solvents (Krebs or Hanks solution). Apocynin and Tempol were diluted in saline (0.15 mol/L) when used for *in vivo* experiments.

2.9. Statistical analysis

Data are expressed as mean ± SEM (standard error of mean) and n represents the number of animals used in experiments. Statistical analysis of the results were performed using two-way analysis of variance, ANOVA (Graph Pad Prism, 3.0 version) followed by Tukey post-hoc test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Measurement of MAP, HR, and evaluation of Δ MAP following apocynin and Tempol administration

The MAP of pregnant Wistar rats (90.12 ± 1.1 mm Hg) and SHR (112.8 ± 2.1 mm Hg) was reduced significantly ($p < 0.05$, $n = 7$ for each group) compared to that of non-pregnant rats (Wistar rats: 105.0 ± 2.5 mm Hg; SHR: 156.7 ± 2.3 mm Hg). Consequently, an increase in HR ($p < 0.05$, $n = 7$ for each group) was observed in pregnant Wistar rats (420.0 ± 5.7 bpm) and pregnant SHR (410.0 ± 4.3 bpm) when compared to non-pregnant Wistar rats (393.6 ± 3.7 bpm) and non-pregnant SHR (387.1 ± 3.0 bpm). When apocynin was administered intravenously, a hypotensive effect was observed in all groups although its magnitude was lower in pregnant SHR ($p < 0.05$, Fig. 1A). Tempol produced a similar hypotensive effect in all groups (Fig. 1B).

3.2. Systemic and aortic oxidative damage

Compared to non-pregnant Wistar rats, the systemic oxidative damage evaluated by the TBARS analysis ($n = 5$ for each group) was higher in non-pregnant SHR, and low in non-pregnant Wistar rats and non-pregnant SHR compared to pregnant Wistar rats and pregnant SHR, respectively ($p < 0.05$, Fig. 2A). However, in the aortas, oxidative damage in pregnant Wistar rats and pregnant SHR ($n = 7$ for each group) was lower than that in non-pregnant Wistar rats and non-pregnant SHR ($p < 0.05$, Fig. 2B).

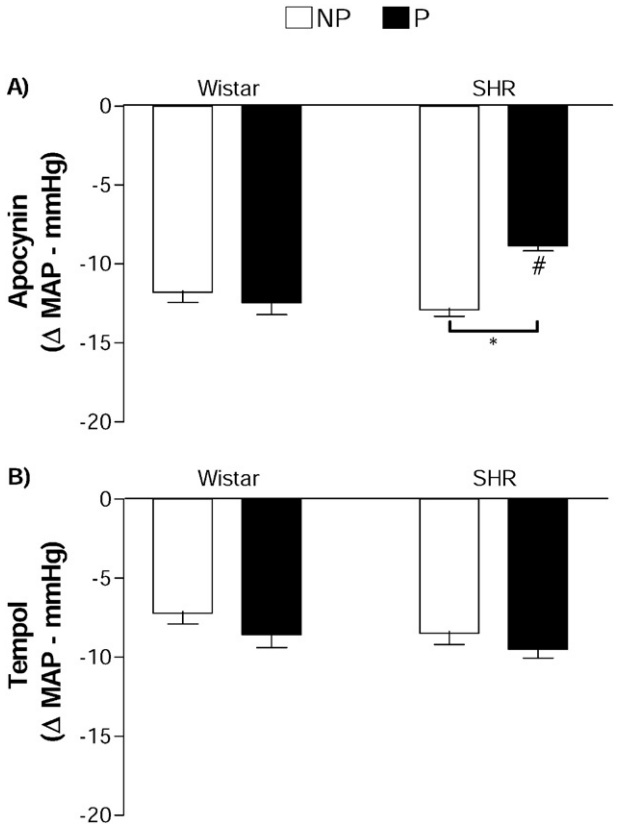

Fig. 1. Hypotensive effect of Apocynin (A) and Tempol (B) in non-pregnant (NP, white bars) and pregnant (P, black bars) Wistar rats and SHR. Data are expressed as variation of mean arterial pressure (ΔMAP, in mm Hg). Bars represent the mean ± SEM of the results ($n = 5$). $^{*}p < 0.05$ NP SHR *versus* P SHR, $^{\#}p < 0.05$ P SHR *versus* other groups.

3.3. Vascular reactivity to PE in intact aortic rings under basal conditions and on incubation with apocynin

In late pregnancy, normotensive and hypertensive rats exhibited hyporeactivity to PE in the aortas compared to non-pregnant rats, as shown in Fig. 3A, B (Emax: non-pregnant Wistar rats: 29.5 ± 1.5; pregnant Wistar rats: 21.3 ± 1.8; non-pregnant SHR: 30.8 ± 1.6; pregnant SHR: 21.2 ± 2.7; mN, $n = 5$). The PE potency was reduced in the aortas of pregnant Wistar rats (pD_2: 6.9 ± 0.07) when compared to the aortas of non-pregnant Wistar rats (pD_2: 7.2 ± 0.08). Alteration in PE potency was not observed in the aortas of pregnant SHR (pD_2: 7.2 ± 0.12) when compared to non-pregnant SHR (pD_2: 7.3 ± 0.04). We observed a reduced ($p < 0.05$) Emax of concentration–response curves to PE in the aortas of non-pregnant Wistar rats (19.1 ± 2.7 mN, $n = 5$ for each group) and pregnant Wistar rats (12.1 ± 1.9 mN; $n = 5$ for each group) incubated with apocynin compared to the aortas that were not incubated (Fig. 3A). However, we observed a reduced Emax in the aortas incubated with apocynin, only in non-pregnant SHR group (21.1 ± 2.9 mN; $n = 5$ for each group, $p < 0.05$, Fig. 3B). In the presence of apocynin, PE potency was not altered in the aortas of pregnant Wistar rats (pD_2: 6.5 ± 0.14), non-pregnant Wistar rats (pD_2: 6.9 ± 0.14), pregnant SHR (pD_2: 7.22 ± 0.12) and non-pregnant SHR (pD_2: 7.37 ± 0.04).

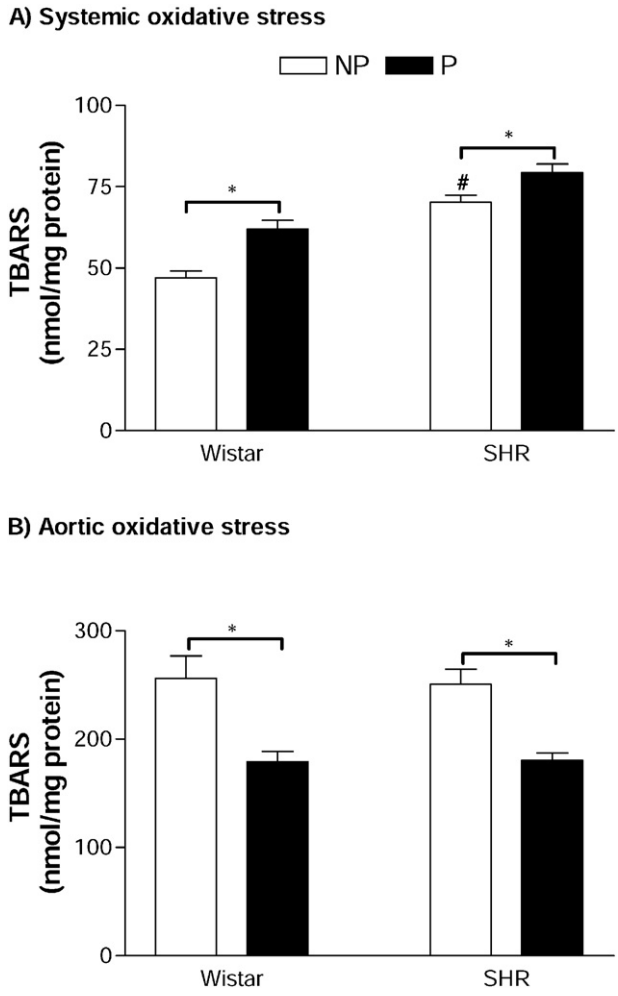

Fig. 2. Determination of the 2-thiobarbituric acid reactive species (TBARS, in nmol/mg protein) in (A) erythrocytes (representing systemic oxidative stress, n = 5) and (B) in aortas (representing aortic oxidative stress, n = 7) of non-pregnant (NP, white bars) and pregnant (P, black bars) Wistar rats and SHR. Bars represent the mean ± SEM of the results. *p < 0.05 NP *versus* P rats in both groups. #p < 0.05 NP SHR *versus* NP Wistar rats.

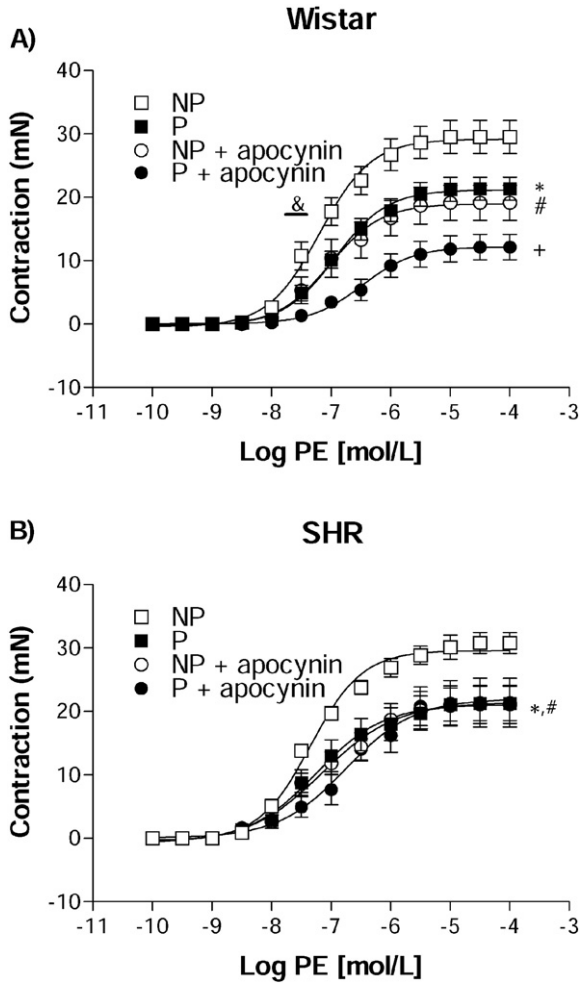

Fig. 3. Concentration–response curves to phenylephrine (PE, 0.1 nmol/L — 0.1 μmol/L) in intact aorta rings of non-pregnant (NP, white symbols) and pregnant (P, black symbols) Wistar rats (A) and SHR (B), in the absence or in the presence of apocynin (+apocynin, 100 μmol/L). Values are mean ± SEM of the results (n = 5). In A and B, *p < 0.05 Emax values in P *versus* NP; #p < 0.05 Emax values in NP + apocynin *versus* NP. In A, +p < 0.05 Emax values in P + apocynin *versus* P; &p < 0.05 pD_2 values in NP *versus* other groups.

3.4. Cytosolic concentration of NO and ROS in aortic endothelial cells

The NO concentration in AEC measured by fluorescence intensity (FI) of DAF-2DA increased in pregnant Wistar rats and SHR when compared to non-pregnant Wistar rats and SHR (p < 0.05, n = 5–6 for each group). Apocynin increased FI in AEC of pregnant and non-pregnant normotensive and hypertensive rats. Differences between pregnant and non-pregnant were maintained after apocynin incubation (p < 0.05, n = 5–6 for each group, Fig. 4A, B).

The ROS concentration measured by FI of DHE decreased in AEC of pregnant Wistar rats and SHR when compared to non-pregnant Wistar rats and SHR (p < 0.05, n = 5–6 for each group). When AEC were incubated with apocynin, we observed a decrease in FI in all groups and the difference between pregnant and non-pregnant Wistar rats and SHR was maintained (p < 0.05, n = 5–6 for each group, Fig. 4C, D).

3.5. NAD(P)H oxidase-dependent ROS production in aorta

NAD(P)H oxidase-dependent ROS production was higher in aortic rings of non-pregnant SHR when compared to other groups (p < 0.05, n = 4–7 for each group, Fig 5). ROS production was not altered between the aortas of pregnant and non-pregnant Wistar rats. However, decreased ROS production was observed in the aortas of pregnant SHR when compared to aortas of non-pregnant SHR.

3.6. eNOS, SOD-1, NOX1, NOX2 and, NOX4 and expressions in aortas

A higher expression of eNOS (p < 0.05, n = 5 for each group) was observed in the aortas of pregnant Wistar rats and SHR than in non-pregnant Wistar rats and SHR (Fig. 6A, B). For SOD-1 expression, no difference between pregnant and non-pregnant groups was observed (n = 5 for each group, Fig. 6A, C).When we evaluated NOX expression, increased NOX2 (Fig. 6A, E) and NOX4 (Fig. 6A, F) expressions in aortas of non-pregnant SHR group were observed when compared to the other groups (p < 0.05, n = 5 for each group). NOX1, NOX2 and NOX4 expressions decreased in the aortas of pregnant SHR than in non-pregnant SHR.

4. Discussion

Here, we found that aortic oxidative damage, ROS concentration in AEC, ROS production and NOX1, NOX2 and NOX4 expression in the aortas decreased in pregnant SHR. Moreover, these factors contribute to aortic hyporeactivity to PE associated with pregnancy in SHR.

During pregnancy, maternal metabolism and metabolic activity of the placenta increase, leading to increased oxidative stress that can be observed through oxidative stress markers (such as lipid peroxidation [25,26]) in blood and urine [27,28]. We observed increased lipid

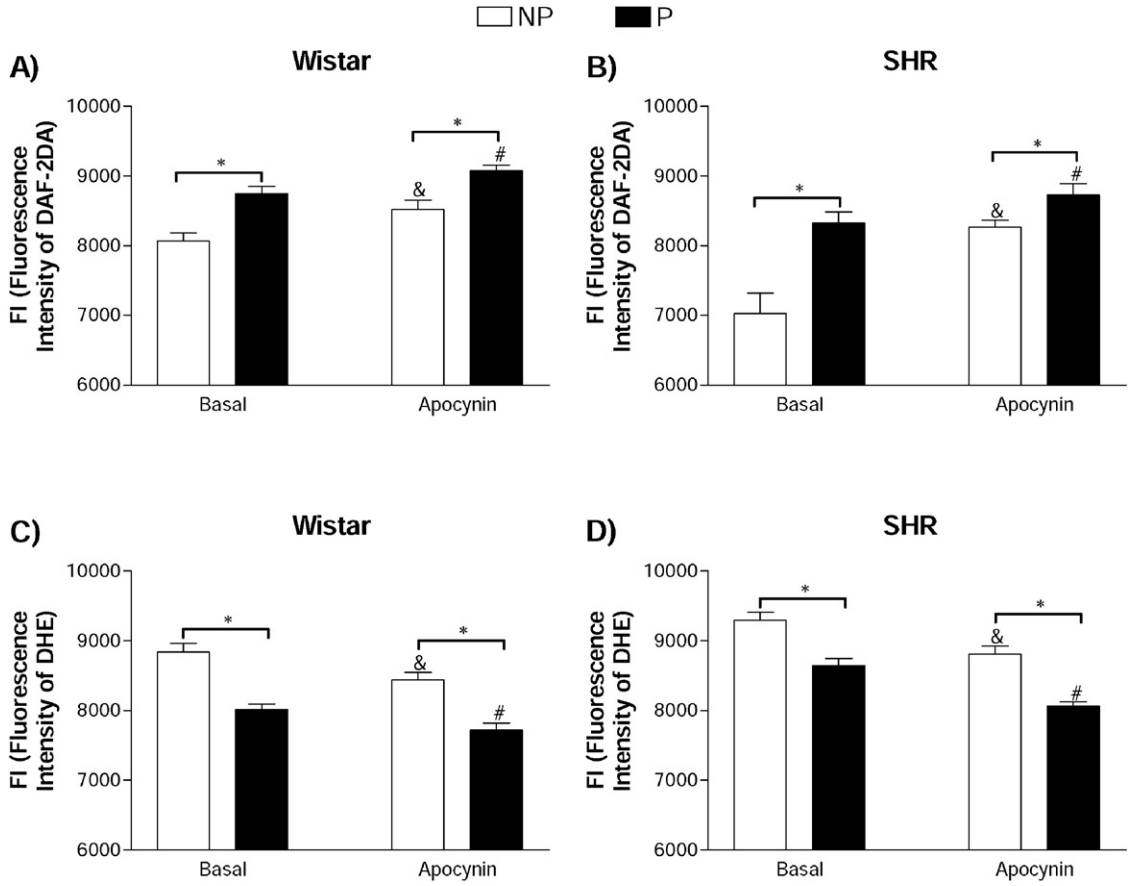

Fig. 4. Fluorescence intensity (FI, in arbitrary units) of 4,5-diaminofluorescein diacetate (DAF-2DA, A and B) and dihydroethidium (DHE, C and D) in aortic endothelial cells of non-pregnant (NP, white bars) and pregnant (P, black bars) Wistar rats and SHR in basal conditions (Basal) or in presence of apocynin. Bars represent the mean ± SEM of the results (n = 5–6). *p < 0.05 P *versus* NP, &p < 0.05, NP in presence of apocynin *versus* NP basal, #p < 0.05 P in presence of apocynin *versus* P basal.

peroxidation in the blood of normotensive or hypertensive pregnant rats (Fig. 2A).

Oxidative stress is influenced differently by pregnancy in different organs [29]. A lower ROS production rate has been reported in the hearts of pregnant mice than in non-pregnant mice [30], and reduced oxidative stress was observed in the mesenteric bed of pregnant Wistar rats and SHR compared to non-pregnant, matched rats [12], despite increased superoxide anion production in mesenteric segments from pregnant normotensive rats [31]. We observed reduced aortic oxidative damage (Fig. 2B) and reduced ROS concentration in endothelial cells (Fig. 4C, D) of normotensive or hypertensive pregnant rats when compared to non-pregnant rats. Moreover, we observed a decreased NAD(P)H oxidase-dependent ROS production in aortas of pregnant SHR (Fig. 5). These findings could be attributed to the altered NOX expression, since we observed reduced NOX1, NOX2 and NOX4 expression in the aortas of pregnant SHR (Fig. 6D, E, F). The NOX1, NOX2, and NOX4 subunits are highly expressed in endothelial and vascular smooth muscle cells in hypertension [8,32,33,34]. NOX1, NOX2 and NOX4 gene and protein expressions are increased in the aortas of SHR [35,36]. Our results suggest that pregnancy reduces NOX expression in vascular cells of SHR; however, pregnancy does not alter SOD-1 expression in these cells (Fig. 6C). Confirming our studies, Cunningham et al. (2013) observed that SOD expression and total antioxidant status in the aorta did not change in late pregnancy [29].

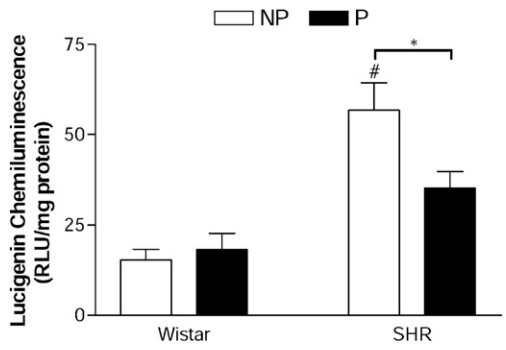

Fig. 5. NAD(P)H oxidase-dependent ROS production in aortic rings of non-pregnant (NP, white bars) and pregnant (P, black bars) Wistar rats and SHR. Results are expressed as mean ± SEM (n = 4–7). *p < 0.05 P *versus* NP SHR; #p < 0.05 NP SHR *versus* Wistar rats.

Apocynin has widely been used as non-selective inhibitor of NOX [37,38]. When apocynin was administered intravenously in the female rats, a hypotensive effect was observed. However, we observed that the hypotensive effect of apocynin was lower in pregnant SHR than in other groups (Fig. 1A). This effect is in accord with the results described above and reinforces the suggestion that NOX activity is reduced in blood vessels of pregnant SHR. Because NOX expression was lower in pregnant SHR than in non-pregnant SHR, the same concentration of apocynin had a lesser effect on blood pressure in pregnant SHR than in the other groups. Tempol, a mimetic of SOD, induced a similar hypotensive effect in pregnant and non-pregnant rats of both groups (Fig. 1B), suggesting that SOD activity is not altered in pregnant rats. In concordance with previous studies [12,16], we also observed a decrease in blood pressure levels, followed by an increase in heart rate, in late pregnancy of both normotensive and hypertensive rats. Studies have associated the blood pressure reduction with an increased bioavailability of NO in vascular cells of pregnant rats [17,39]. Recently, we demonstrated that the PI3K/Akt/eNOS pathway contributes to a higher expression of phosphorylated eNOS in the aortas and increase

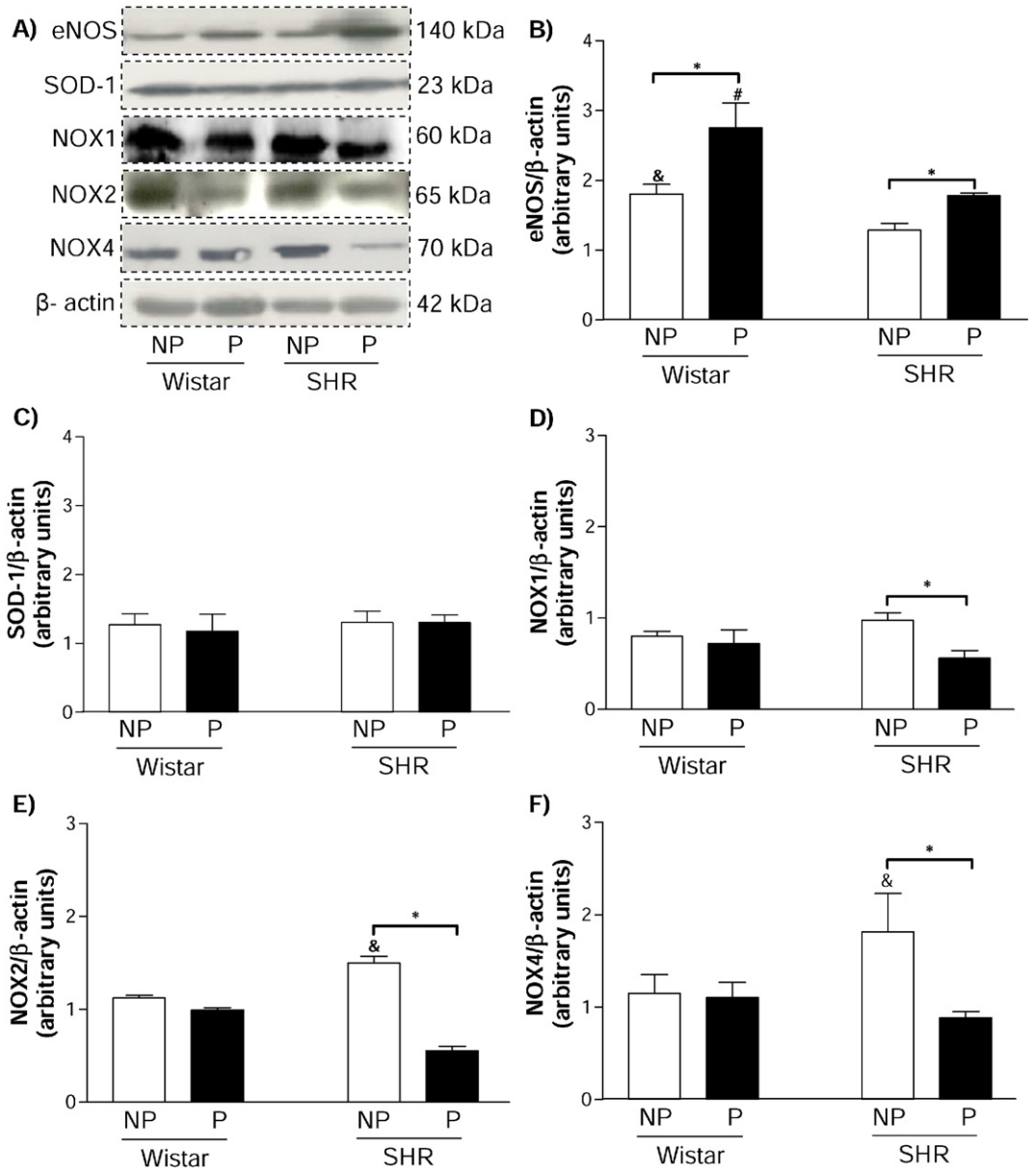

Fig. 6. Western blotting analysis of eNOS (B), SOD-1 (C), NOX1 (D), NOX2 (E) and NOX4 (F) in non-pregnant (NP, white bars) and pregnant (P, black bars) Wistar rats and SHR. Data are shown in typical blots (A) and in the graphics. Data are expressed as ratio to β-actin (arbitrary units). Bars represent the mean ± SEM of the results (n = 5). *p < 0.05 NP *versus* P, #p < 0.05 P Wistar *versus* other groups, &p < 0.05 NP SHR *versus* other groups.

in NO concentration in endothelial cells of pregnant SHR [13]. Corroborating these data, increased basal NO concentration in endothelial cells (Fig. 4A, B) and total eNOS expression in the aortas (Fig. 6B) were also observed in pregnant Wistar rats and pregnant SHR.

Increased NO bioavailability in pregnancy was associated with aortic hyporeactivity to PE [15,16,17]. In this study, we demonstrated that, in pregnancy, aorta hyporeactivity to PE could be more significant owing to the inhibition of ROS production in the aortas of SHR, but not in the aortas of Wistar (Fig. 3A, B). Apocynin lowered the Emax of concentration–response curves to PE in the aortas of pregnant and non-pregnant Wistar rats (Fig. 3A). In presence of apocynin, a blunted aortic response to PE was still observed in the aortas of pregnant Wistar rats. We observed that apocynin diminished ROS concentration (Fig. 4C) and increased NO concentration levels (Fig. 4A) in AEC of non-pregnant and pregnant Wistar rats. However, the effect of apocynin on the aortic reactivity to PE was observed only in non-pregnant SHR, and not in the aortas of pregnant SHR (Fig. 4B). In presence of apocynin, differences in PE-induced contractile responses between the aortas of pregnant and non-pregnant SHR were abolished (Fig. 3B). Apocynin did not change the Emax to PE in pregnant SHR aortas and also the hypotensive effect of apocynin was reduced in pregnant SHR, these results suggest an altered NOX activity only in pregnant SHR, which was confirmed by decreased NOX1, NOX2 and NOX4 expressions in the aortas of pregnant SHR. Therefore, under these conditions, apocynin could not alter hyporeactivity to PE in the aortas of pregnant SHR.

5. Conclusion

Our results demonstrate that the physiological process of pregnancy decreases oxidative damage, ROS production, NOX1, NOX2 and NOX4 expressions in SHR aortas. Taken together, our results suggest that these mechanisms contribute to higher NO bioavailability and aorta hyporeactivity to PE observed in pregnant SHR.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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