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Maternal high-sodium intake affects the offspring' vascular renin-angiotensin system promoting endothelial dysfunction in rats

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ABSTRACT

Perinatal sodium overload induces endothelial dysfunction in adult offspring, but the underlying mechanisms are not fully known. The involvement of tissue renin-angiotensin system on high sodium-programmed endothelial dysfunction was examined.

Acetylcholine and angiotensin I and II responses were analyzed in aorta and mesenteric resistance arteries from 24-week-old male offspring of normal-salt (O-NS, 1.3% NaCl) and high-salt (O-HS, 8% NaCl) fed dams. COX-2 expression, O_2^- production and angiotensin converting enzyme (ACE) activity were determined. A separated O-HS was treated with losartan (15 mg kg^{-1}/day) for eight weeks.

Compared to O-NS, O-HS were normotensive. Acetylcholine-induced relaxation was impaired in O-HS arteries, which was improved by tempol, apocynin or indomethacin. The angiotensin I-induced contraction was greater in O-HS arteries, whereas the angiotensin II responses were unchanged. ACE activity, O_2^- production and COX-2 expression were increased in O-HS arteries. In this group, the increased O_2^- production was inhibited by apocynin or losartan. Chronic losartan decreased COX-2 expression and restored the endothelium-dependent vasodilation in O-HS.

Our findings reiterate that perinatal sodium overload programs endothelial dysfunction in adult offspring through a blood pressure-independent mechanism. Our results also suggest that vascular angiotensin II is the main mediator of high sodium-programmed endothelial dysfunction, promoting COX-2 expression and oxidative stress.

1. Introduction

Sodium is an essential micronutrient that plays an important role in many body processes, such as nerve conduction, muscle contraction, fluid balance and blood pressure. However, sodium consumption has been increasing worldwide and has raised the cardiovascular disorders incidence, including arterial hypertension, myocardial infarction or stroke [1–3]. Currently, daily sodium intake is in average tenfold higher than it was the past and has exceeded the estimated physiological needs [4]. For a long time, the associated high-salt consumption deleterious actions had been related merely to the sodium effect on blood pressure. Currently, several other effects have been described; in some cases, they occur independently of the other common risk factors. For example, in adult animals, regardless of changes in blood pressure, high-salt intake induces myocardial fibrosis, left ventricular hypertrophy and vascular dysfunction and remodeling [1,5–7].

It is widely accepted that maternal dietary intake and nutritional environment during fetal development have long-term implications for offspring health [8]. Developmental programming of several diseases has been observed in several studies using animal models of maternal micro and macronutrient restriction and excess. For instance, dietary sodium overload during the pregnancy perturbs placental function, alters fetal development, and predisposes offspring to cardiovascular and renal alterations in adult life. In the experiments of Contreras et al. [9] and Gray et al. [3], offspring of high-salt diet-fed dams exhibited higher blood pressure compared with offspring of control diet-fed dams. Excessive salt intake during the pregnancy also impairs the nephrogenesis, reducing the nephron number [10], produces glomerulosclerosis [11], proteinuria and oxidative stress [12] in adult offspring. Additionally, prenatal salt-exposed offspring exhibited left ventricular hypertrophy, cardiac dysfunction, increased arterial wall thickness and endothelial dysfunction [3,13–15].

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Endothelial dysfunction plays an important role in the development/ maintenance of cardiovascular diseases and has been considered a predictor of vascular events such as atherosclerosis, stroke and myocardial infarction. It is well established that in utero disturbances, such as undernutrition, hypoxia or hyperglycemia can program endothelial dysfunction in adulthood [16–18]. In rats and humans, increased salt intake also compromises endothelial functions by a blood pressure-independent mechanism [5,19,20]. It occurs by impaired endothelial nitric oxide (NO) production/ bioavailability and/or releasing endothelium vasoconstrictor compounds in response to vasoactive stimuli that normally relax the blood vessels [19,21]. In utero sodium overload may also influence endothelial function in adult offspring. Gray et al. [3] showed impaired endothelium-dependent relaxation in femoral and mesenteric arteries from offspring of high-salt diet-fed dams, with reductions in NO- and endothelium-dependent hyperpolarizing factor (EDHF)-mediated responses. Similarly, among spontaneously hypertensive rats (SHR), Maruyama et al. [15] have reported a decreased endothelial NO-mediated vasodilation in aorta of high-salt diet-fed dams' offspring.

The renin-angiotensin system (RAS), specially angiotensin II, plays a significant role in the pathogenesis of endothelial dysfunction, inflammation and vascular fibrosis [22]. The increased sodium intake would be expected to suppress the circulating RAS. Conversely, it has been demonstrated that salt loading causes paradoxical stimulation of local RAS in the kidney, heart and vessels [5,23]. During the pregnancy, decreased plasma angiotensin II levels in mothers and their fetuses have been reported when mothers were fed a high-sodium diet [24,25]. In contrast, an increased renal and cardiac angiotensin II levels have been reported in the adult offspring [24,25]. Moreover, the renal dysfunction observed in these rats was prevented by ACE inhibition [26]. Taken together, these results indicate that local RAS may be involved in cardiac and renal changes in the offspring of mothers fed a high-salt diet. However, it is still unclear whether perinatal exposure to a high-salt diet could affect the local vascular RAS of adult rats and whether it might contribute to endothelial dysfunction among these animals.

Therefore, the current study evaluated the effects of a high-salt diet (8% NaCl) during the pregnancy and lactation on the vascular function of adult male offspring, focusing on the role of local RAS. The responses to constrictor agonists and to the endothelium-dependent vasodilator acetylcholine in conductance and resistance arteries were measured by the wire myograph method and used for vascular function evaluation.

2. Material and methods

All experimental procedures were approved by the Committee for Ethics in Animal Experimentation of the *Universidade Federal de Pernambuco* (UFPE). (Protocol number: 23076.038874/2014-65). The animals were taken care of and used according to the ethical principles for animal experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA) and conform to the US National Institutes of Health (NIH publication, 2011).

2.1. Animals

Male and female Wistar rats were obtained from colonies maintained at the Animal Quarters of the *Departamento de Fisiologia e Farmacologia* of the UFPE and were housed in individually ventilated cages (425 mm × 266 mm × 185 mm; Tecniplast, Buguggiate, Italy). The room where they were kept had a 12 h–12 h light-dark cycle, and the temperature was regulated within the range of 22–24 °C. Rats had free access to tap water and standard rat chow ad libitum.

Three female rats (200–250 g) (during oestrous stage of the estrous cycle) were placed in cages with one male rat during the dark period of the cycle to overnight mate. In the morning, males and females were separated and vaginal smears of each female were examined for the presence of sperm. The day when sperm was found in the vaginal smear was considered day one of gestation. Each pregnant female was transferred into a separate cage and the weight gain was followed up throughout the pregnancy. Dams were fed either (1) a control diet (NS, 1.3% NaCl, from Rhoster, Araçoiaba da Serra, SP, Brazil) or (2) a high sodium diet (HS, 8% NaCl, from Rhoster, Araçoiaba da Serra, SP, Brazil) ad libitum throughout pregnancy and lactation. Dams were allowed to deliver spontaneously. Rats that did not deliver were anesthetized with ketamine and xylazine mixture (80 mg kg^{-1} and 5.0 mg kg^{-1}, respectively, i.p.) and killed by decapitation. After birth, each rat litter was reduced to eight pups to ensure standardized nutrition until weaning. In this study, we used only male offspring. When the male number was not enough to complete eight, females were used but sacrificed at weaning. The unwanted pups were killed by CO_2 inhalation followed by cervical dislocation. At weaning, male offspring was housed three per cage and fed a standard chow diet (Purina Agribands, Paulínia, SP, Brazil) ad libitum until week 24. The offspring from dams fed NS diet were termed O-NS (n = 38), whereas those from dams fed HS diet were termed O-HS (*n* = 41). No more than two rats per litter were used for each experiment.

A part of the O-NS (n = 16) and O-HS (n = 16) groups was treated daily with losartan (15 mg kg^{-1} by gavage) for eight weeks and were termed O-NS-Los and O-HS-Los, respectively. Treatment with losartan was initiated in rats with 16 weeks of age.

2.2. Arterial blood pressure measurement in conscious rats

For arterial pressure measurements, offspring were anesthetized with ketamine and xylazine mixture (80 mg kg^{-1} and 5.0 mg kg^{-1}, respectively, i.p.). The depth of anesthesia was checked by the lack of reflex response to frequent tail pinching, and a supplementary dose of anesthetic was administered when required. A polyethylene catheter (PE-50, Clay Adams, Parsippany, NJ, USA) was inserted into the right carotid artery for pulsatile arterial pressure (PAP) measurements. The distal end of the catheter was tunneled subcutaneously, exteriorized in the mid scapular region and sutured to the skin, which allows the rat freedom of movement. At the end of the surgery, intramuscular injection of the analgesic and anti-inflammatory ketoprofen (5.0 mg kg^{-1}, i.m., Venco Laboratory, Londrina, PR, Brazil) was performed. Animals were allowed free access to food and water. After a recovery period of 24 h, the arterial catheter was connected to a pressure transducer (MLT0380; ADInstruments Pty Ltd., Castle Hill, New South Wales, Australia) connected to an amplifier (Bridge Amp, ML224; ADInstruments) and an acquisition system (PowerLab 4/30, ML866; ADInstruments), and the signals were digitized with appropriate software (Chart Pro; ADInstruments). Mean arterial pressure (MAP) and heart rate (HR) were processed from pulsatile arterial pressure by a data acquisition system. The cardiovascular parameters were recorded once for 60 min, but only the last 30 min were considered for data analysis. The measurements were performed between 2:00 and 5:00 p.m. To avoid the influence of the surgical process on the vascular reactivity, these animals were not used for additional experiments. After blood pressure measurement, all animals were anesthetized (ketamine 80 mg kg^{-1} and xylazine 5 mg kg^{-1}, i.p.) and killed by decapitation.

2.3. Wire myography

Ex vivo experiments were performed in aorta and mesenteric resistance arteries from 24-week-old male offspring. Rats were anesthetized with ketamine and xylazine mixture (80 mg kg^{-1} and 5.0 mg kg^{-1}, respectively, i.p.) and killed by exsanguination. The mesenteric vascular bed and thoracic aorta were removed and placed in Petri dishes with cold (4 °C) Krebs Henseleit solution (KHS; in mmol L^{-1}: 115 NaCl, 2.5 $CaCl_2$, 4.6 KCl, 1.2 KH_2PO4, 1.2, $MgSO_4.7H_2O$, 25 $NaHCO_3$, 11.1 glucose, and 0.03 EDTA).

2.3.1. Thoracic aorta

Segments of the thoracic aorta (~3 mm in length), free of fat and connective tissue were mounted at a resting tension of 1.0 g in an organ chamber containing KHS continuously gassed with 95% O_2, 5% CO_2 (pH 7.4; 37 °C). Isometric tension was recorded by using an isometric force displacement transducer (Letica TRI 201, Panlab, S.L., Barcelona, Spain) connected to an amplifier (Bridge Amp, ML119; ADInstruments) and to an acquisition system (PowerLab 8/35, ML870/P ADinstruments).

2.3.2. Mesenteric resistance arteries (MRA)

The third order branch of the mesenteric arcade was dissected and cut in segments of ~2 mm in length. To measure isometric tension, segments of MRA were mounted between two tungsten wires (40 μm diameter) in a small vessel chamber myograph (Danish Myo Technology A/S, Aarhus, Denmark) connected to an acquisition system (PowerLab 8/35, ADInstruments). After a 15-min equilibration period in oxygenated KHS, at 37 °C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference/wall tension ratio of the segments by setting their internal circumference, L_0, to 90% of what the vessels would have been if they were exposed to a passive tension that was equivalent to the tension produced by a transmural pressure of 100 mmHg. The internal diameter (I_1) was determined according to the equation $I_1 = L_1/\pi$, using specific software for normalization of resistance arteries (DMT Normalization Module, ADInstruments).

2.3.3. Experimental protocols

After a 45-min equilibration period, aortic and MRA segments were initially exposed twice to 75 and 120 mmol L^{-1} KCl, respectively; the first to check their functional integrity and the second to assess the maximum contractility. Then, the concentration-response curves to acetylcholine (0.1 nmol L^{-1} to 30 μmol L^{-1}, Sigma Aldrich, St. Louis, MO, USA) or sodium nitroprusside (0.01 nmol L^{-1} to 100 μmol L^{-1}, Sigma Aldrich) were performed in MRA and aorta previously contracted with noradrenaline or phenylephrine, respectively, at a concentration that produced approximately 50–70% of the contraction induced by KCl in each case. After 60 min, concentration-response curves to phenylephrine (1 nmol L^{-1} to 100 μmol L^{-1}, Sigma Aldrich) were constructed in thoracic aorta. In MRA, noradrenaline (10 nmol L^{-1} to 100 μmol L^{-1}, Sigma Aldrich) was used to study the vasoconstriction induced by alpha-adrenergic receptor activation because it produced responses that were more stable than those produced by phenylephrine. In some aortic segments, the contractile response induced by the cumulative addition of angiotensin I (0.1 nmol L^{-1}–3 μmol L^{-1}, Sigma Aldrich), whose contraction is mediated by angiotensin II converted from angiotensin I by ACE, or angiotensin II (0.1 nmol L^{-1}–10 μmol L^{-1}, Sigma Aldrich) were also analyzed. In MRA, it was not possible to construct stable concentration-response curves to angiotensin I or II, for this reason the contractile response to these peptides was evaluated through the addition of a single concentration (10 μmol L^{-1}) of angiotensin I or angiotensin II. The responses to angiotensin I were determined before and after the addition of the ACE inhibitor captopril (100 μmol L^{-1}, Sigma Aldrich).

To investigate the participation of reactive oxygen species (ROS) or cyclooxygenase (COX) pathway on the acetylcholine and phenylephrine/noradrenaline responses, some arteries were preincubated with the permeable superoxide mimetic tempol (10 μmol L^{-1}, Sigma Aldrich), with the NADPH oxidase inhibitor/antioxidant apocynin (100 μmol L^{-1}, Sigma Aldrich), with the COX inhibitor indomethacin (10 μmol L^{-1}, Sigma Aldrich) or the thromboxane A_2 (TxA_2) receptor (TP) antagonist SQ29548 (1 μmol L^{-1}, Cayman Chemical Company, Ann Arbor, MI, USA). To analyze the role of local RAS on acetylcholine and phenylephrine/noradrenaline responses we performed experiments where the arteries were preincubated with losartan (an angiotensin II receptor blocker acting on the AT_1 receptor subtype, 10 μmol L^{-1}, Sigma Aldrich) or captopril (100 μmol L^{-1}). Additionally, acetylcholine and phenylephrine/noradrenaline responses were also analyzed in aorta and MRA from chronically losartan-treated O-NS and O-HS groups.

2.4. Superoxide anions generation by dihydroethidium (DHE) fluorescence

After isolation and dissection, aortic and MRA segments were immersed in a freezing medium (OCT, Sakura Finetek, Torrance, CA, USA) and stored at −80 °C until the day of experiments. OCT-embedded segments were cut into 10-μm-thick sections and placed on a glass adhesive slide (Star Frost®, Germany). Serial sections were equilibrated under identical conditions for 30 min at 37 °C in Krebs–HEPES buffer (in mmol L^{-1}: 130 NaCl, 5.6 KCl, 2$CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, and 11 glucose, pH 7.4). A fresh buffer containing DHE (5 μmol L^{-1}, Invitrogen, Carlsbad, CA, USA) was applied topically to each tissue section, covered with a cover slip, incubated for 30 min in a light-protected humidified chamber at 37 °C, and then viewed with an optical microscope (Eclipse 80i, Nikon, Tokyo, Japan) equipped with a rhodamine filter and camera (DS-U3, Nikon) using a 20× objective and the same imaging settings in each case. As a negative control, parallel sections were pre-incubated with the superoxide dismutase mimetic manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP, 1 μmol L^{-1}, Merck, Darmstadt, Germany). O_2^- production was quantitatively analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and expressed as total fluorescence (arbitrary units). Fluorescence was measured at least in three sites in each image and the mean of the measurements was taken. DHE-emitted fluorescence was also measured in apocynin- (10 μmol L^{-1}) or losartan (10 μmol L^{-1})-treated arteries.

2.5. Plasma and tissue ACE activity

ACE activity was determined by a fluorimetric assay. The blood was centrifuged (800 *g* for 15 min) for plasma separation. Aorta, MRA and plasma samples were maintained at −80 °C until the day of the experiments. Arteries (aorta and MRA) were homogenized in 50 mmol L^{-1} Tris-HCl buffer, pH 7.4. Protein content was determined with BCA protein assay reagent (Sigma Aldrich), using bovine serum albumin (BSA, Sigma-Aldrich) as standard. Briefly, plasma and supernatants from homogenized arteries (in a volume corresponding to 30 μg of protein) were incubated with 40 μL of assay buffer containing 5.0 mmol L^{-1} Hipuryl-Histidyl-Leucine (Sigma Aldrich) in 0.4 mmol L^{-1} sodium borate buffer with 0.9 mmol L^{-1} NaCl, pH 8.3 for 15 min at 37 °C. The reaction was stopped by the addition of 190 μL of 0.34 N NaOH. The product Histidyl-Leucine was detected by adding 17 μL of 2% o-phthaldialdhyde (OPTA) in methanol. Fluorescence was determined 10 min after OPTA addition, at an excitation wavelength 365-nm and an emission wavelength of 495-nm, in a multimode microplate reader (Varioskan, Thermo Fisher Scientific, Waltham, MA, USA). To correct for the intrinsic fluorescence of the tissues, time zero blanks were prepared by adding samples after NaOH. All assays were performed in triplicate. Results were expressed by nmol mL min mg $protein^{-1}$.

2.6. Western blot

Arteries were homogenized in lysis buffer containing Tris (10 mmol L^{-1}, pH 7.4), sodium lauryl sulfate (SDS, 1%) and sodium metavanadate (1 mmol L^{-1}). Protein content was determined with BCA protein assay reagent (Sigma Aldrich), using bovine serum albumin (BSA, Sigma-Aldrich) as standard. Lysates were electrophoretically separated by 10% SDS-PAGE (30 μg per lane) and transferred to polyvinyl difluoride membranes (GE Healthcare, Chicago, IL, USA) and incubated overnight with monoclonal primary antibodies against COX-1 (1:500, Cayman Chemical Company), COX-2 (1:200, Cayman Chemical

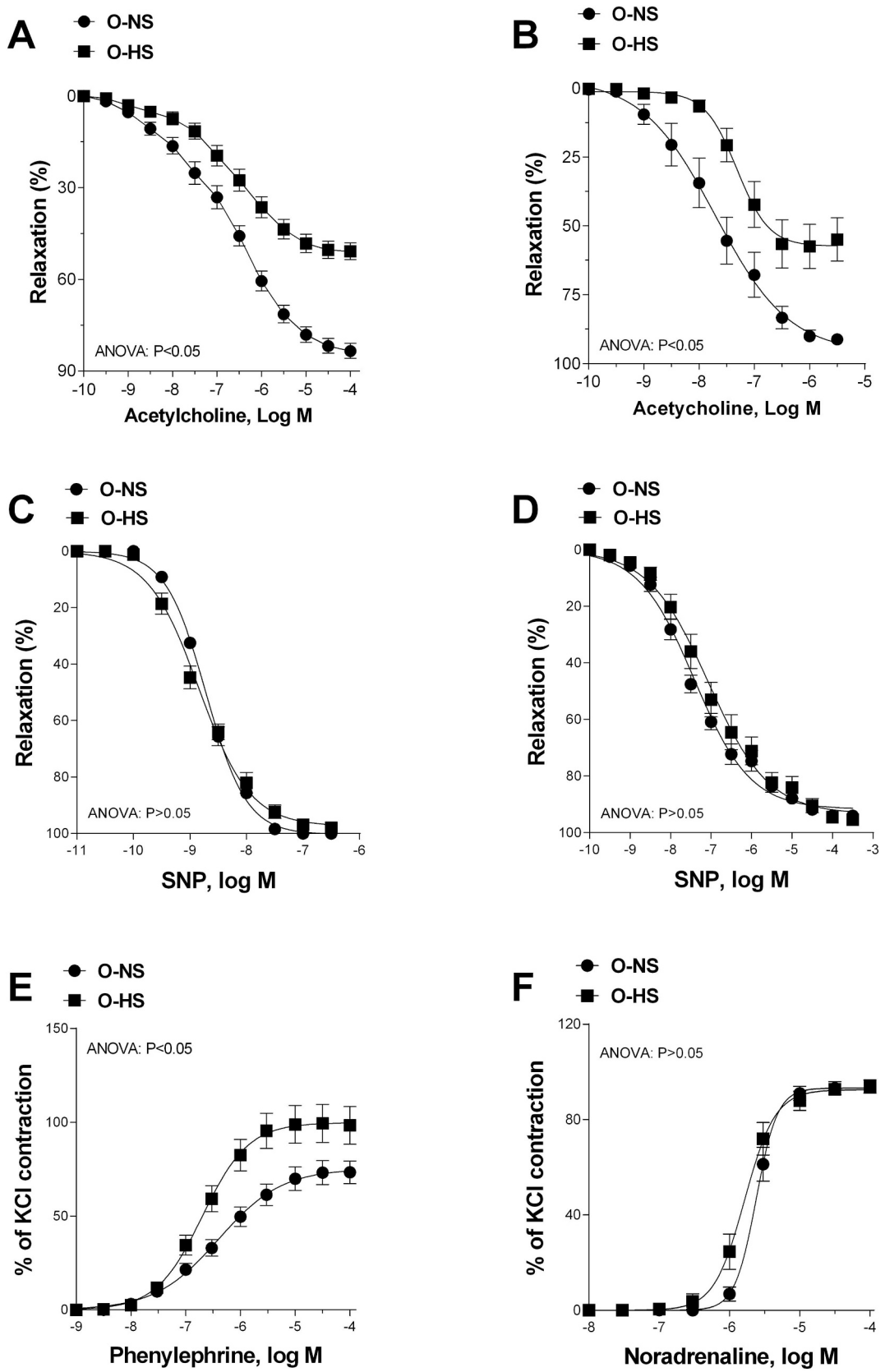

Fig. 1. Endothelium-dependent (A and B) and -independent relaxation (C and D) to acetylcholine and sodium nitroprusside (SNP), respectively, and vasoconstriction to phenylephrine (E) or noradrenaline (F) in aorta (A, C and E) and mesenteric resistance arteries (B, D and F) from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. Results are expressed as mean ± SEM and were analyzed by two-way ANOVA, n = 7 or 8 rats.

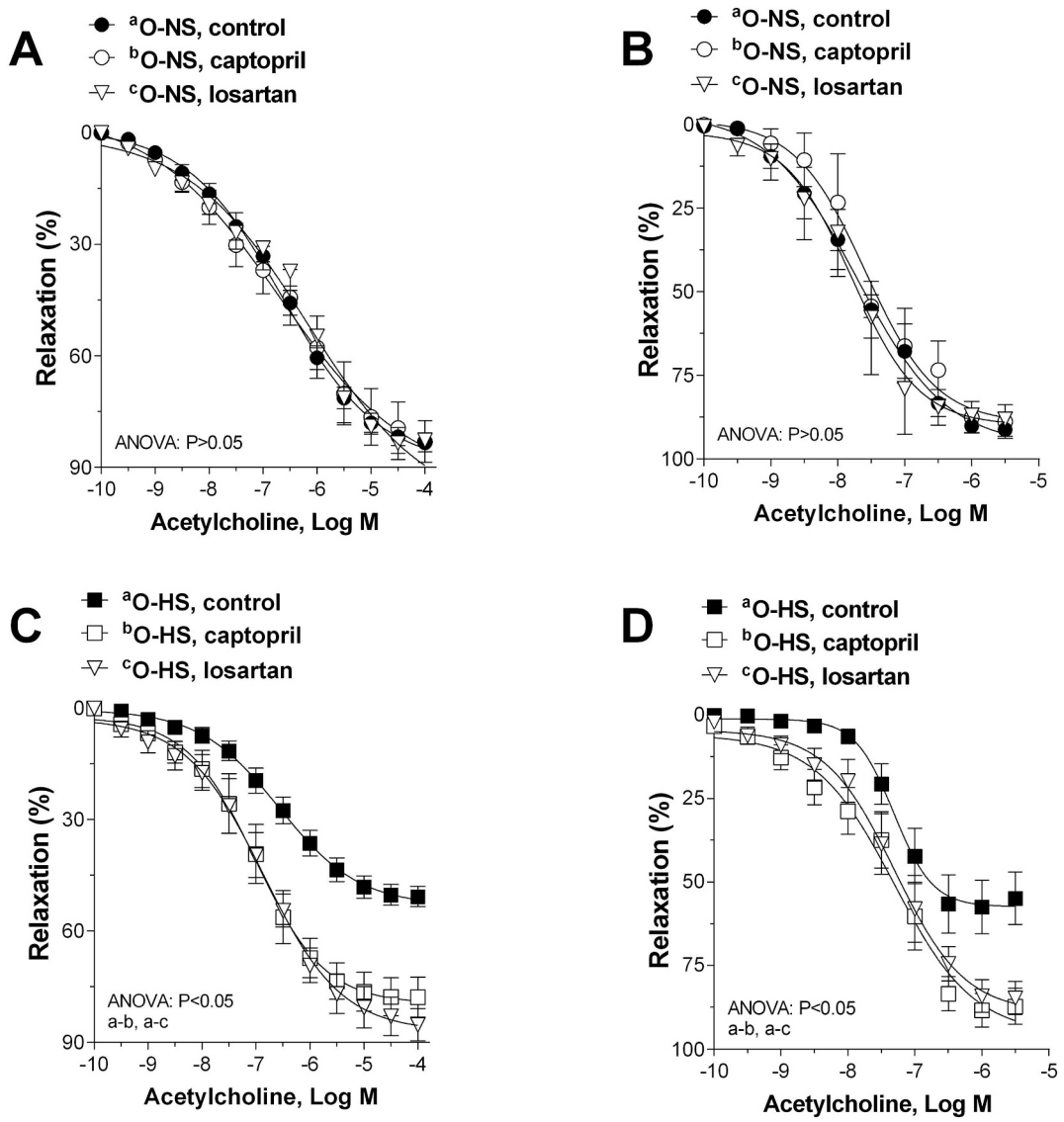

Fig. 2. Effects of the angiotensin converting enzyme inhibitor (captopril) or the AT_1 receptor antagonist (losartan) on the acetylcholine-induced relaxation in aorta (A and C) and mesenteric resistance arteries (B and D) from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. The term 'control' indicates non-preincubated arteries. Results are expressed as mean ± SEM and were analyzed by two-way ANOVA, n = 7 or 8 rats.

Company), AT_1 receptor (1:500, Abcam, Cambridge, MA USA), eNOS (1:500, BD Biosciences, Franklin Lakes, NJ, USA), or NOX 4 (1:500, Abcam). Anti-α-actin (1:3000, Sigma Aldrich) was used to normalize the expression of the evaluated proteins in each sample. Appropriate horseradish peroxidase-labelled anti-mouse or anti-rabbit secondary antibodies (GE Healthcare) were subsequently used for 1 h at room temperature. Immunoreactive proteins were detected by chemiluminescence with ECL Plus (GE Healthcare). The chemiluminescent signals were captured using ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA) and was quantified with Image Lab SoftwareTM version 5.2 (Bio-Rad Laboratories).

2.7. Statistical analysis

Relaxation responses to acetylcholine and sodium nitroprusside were expressed as the percentage of relaxation of the maximum contractile response induced by phenylephrine or noradrenaline. Phenylephrine, noradrenaline angiotensin I and angiotensin II contractile responses were expressed as a percentage of the maximum response produced by KCl.

All values are expressed as mean ± standard error of the mean (S.E.M.) of the number of rats used in each experiment. Results were analyzed using Student's *t*-test, one way or two-way ANOVA. When ANOVA showed a significant treatment effect, the Bonferroni's post hoc test was used to compare individual means (GraphPad Prism Software, San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

Dams fed a diet containing 8% NaCl presented increased water intake (Pregnancy: NS, 15.4 ± 0.56 vs. HS, 38.8 ± 0.23 mL, *t*-test, $P < 0.05$; Weaning: NS, 43.1 ± 3.67 vs. HS, 67.9 ± 9.31 mL, t-test, $P < 0.05$) and diuresis (results not shown) compared to control diet-fed dams. Daily sodium intake was increased in dams fed a diet containing 8% NaCl during the pregnancy and lactation periods (Pregnancy: NS, 39.1 ± 3.59 vs. HS, 261 ± 9.07 mg, *t*-test, $P < 0.05$; Weaning: NS, 72.4 ± 16.5 vs. HS, 481 ± 70.1 mg, $P < 0.05$). Gestation occurred normally, and the rats delivered spontaneously at term (21 days of gestation). Dams fed a high-sodium diet gave birth to similar pups than control diet-fed dams (results not shown).

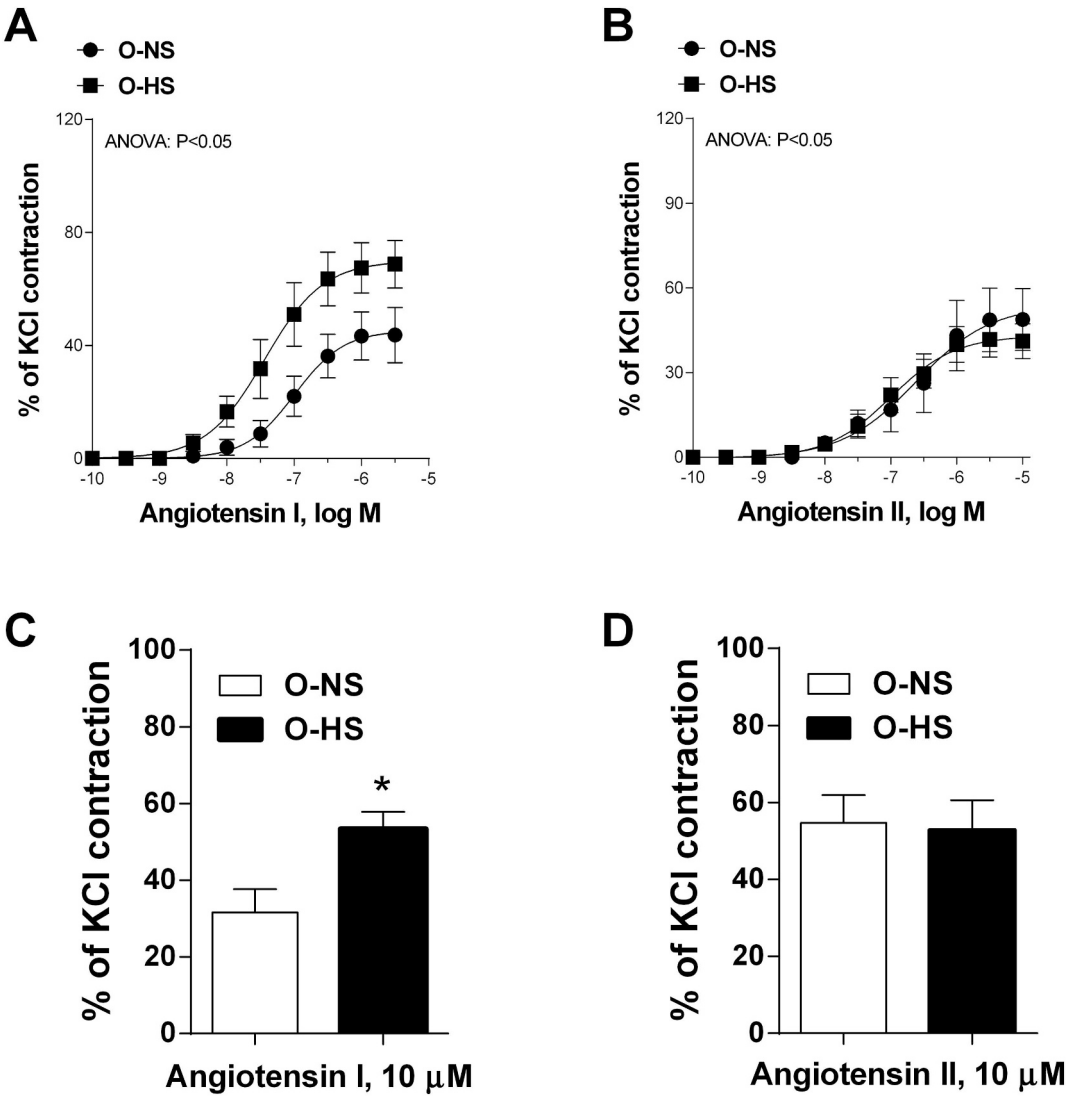

Fig. 3. Contractile responses to angiotensin I (A and C) and to angiotensin II (B and D) in aorta (A and C) and mesenteric resistance arteries (B and D) from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. Results are expressed as mean ± SEM and were analyzed by two-way ANOVA (A and B) or Student's unpaired *t*-test (C and D), n = 5 or 7 rats. *P < 0.05 versus O-NS.

Maternal high-salt diet had no effect on offspring birth weight (NS: 5.70 ± 0.10 vs. HS: 5.90 ± 0.09 g, *t*-test, P > 0.05). However, at 24 weeks of age, the O-HS group presented increased body weight compared to O-NS group (O-NS: 372 ± 22.0 vs. O-HS: 415 ± 14.0 g, *t*-test, P < 0.05).

In this study, there were no significant differences in systolic (SAP), diastolic (DAP) or mean (MAP) arterial pressure between O-NS and O-HS groups (SAP: O-NS, 133 ± 0.62 vs. O-HS, 137 ± 1.90 mmHg, *t*-test, P > 0.05; DAP: O-NS, 86.0 ± 1.50 vs. O-HS, 95.0 ± 3.80 mmHg, *t*-test, P > 0.05; MAP: O-NS, 101 ± 1.16 vs. O-HS, 109 ± 2.82 mmHg, *t*-test, P > 0,05). Heart rate was not different between O-NS and O-HS groups (results not shown).

3.1. Vascular contractility and endothelium-dependent and -independent relaxations

KCl evoked similar contractions in both aorta and MRA from O-NS and O-HS rats (Aorta: O-NS, 4.91 ± 0.06 mN mm^{-1} vs. O-HS, 5.20 ± 0.10 mN mm^{-1}, P > 0.05; MRA: O-NS: 3.22 ± 0.02 mN mm^{-1} vs. HS, 3.19 ± 0.05 mN mm^{-1}, P > 0.05).

Acetylcholine induced endothelium-dependent vasodilator responses that were impaired in both aorta and MRA from O-HS than O-NS (Fig. 1A and B). In arteries from both groups, relaxation evoked by the NO donor sodium nitroprusside was comparable (Fig. 1C and D). Endothelium-intact aortic rings from O-HS group showed an increase of the contractile response to phenylephrine compared with the O-NS group (Fig. 1E). In MRA, the response to noradrenaline was similar in both O-HS and O-NS groups (Fig. 1F).

Involvement of RAS in the changes observed in acetylcholine-induced relaxation was also investigated. In arteries from O-NS group, relaxation to acetylcholine remained unchanged in the presence of captopril or losartan (Fig. 2A and B). However, in both aorta and MRA from O-HS, the response to acetylcholine was similarly increased by captopril or losartan (Fig. 2C and D). The increases in vascular tone elicited by angiotensin I and angiotensin II in aorta and MRA from O-NS and O-HS groups are shown in Fig. 3. The contractile responses induced by angiotensin I in the O-HS aorta and MRA were greater when compared with O-NS group (Fig. 3A and C). No difference was found between the contractile response to angiotensin II obtained from O-HS and O-NS arteries (Fig. 3B and D).

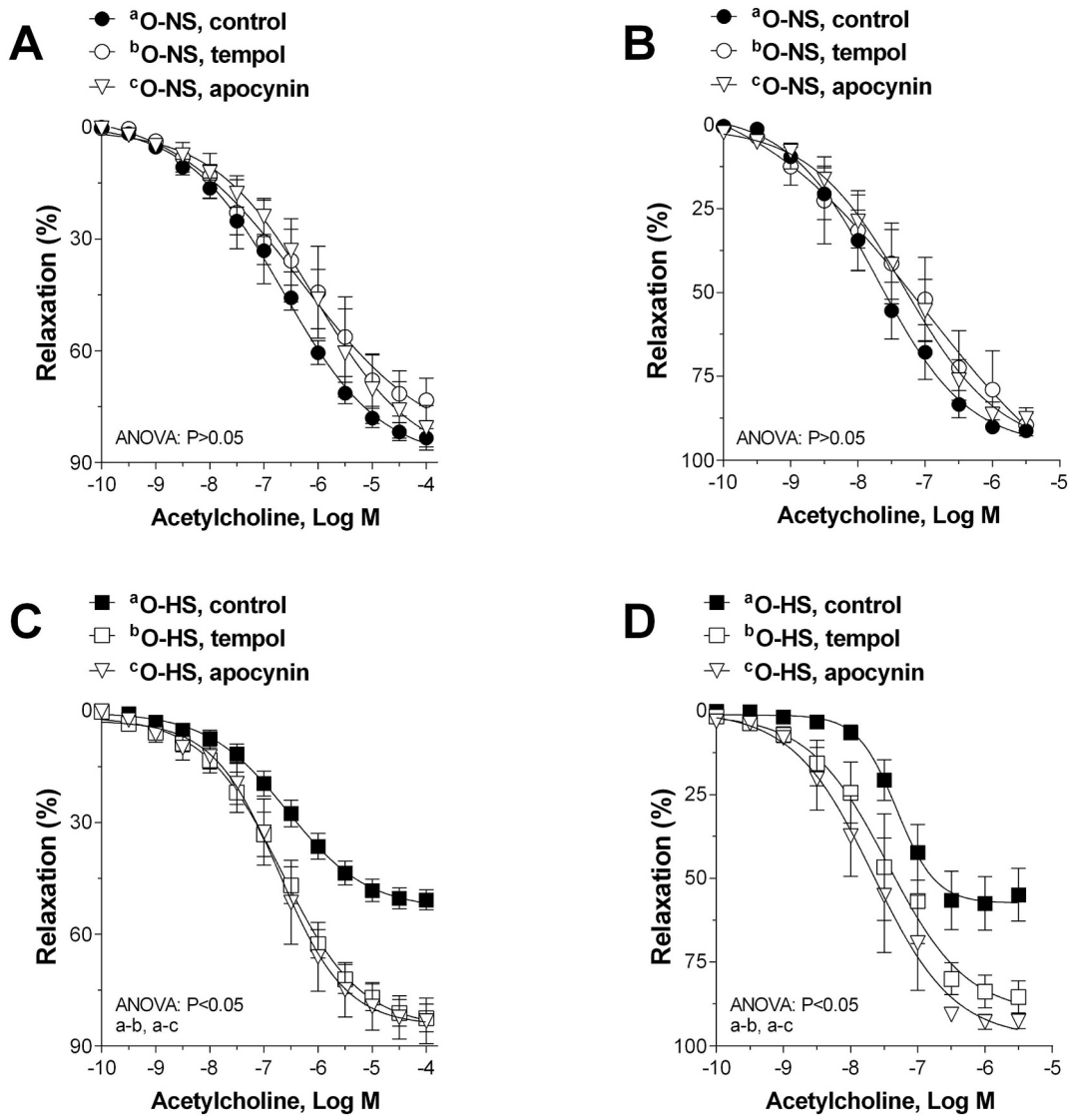

Fig. 4. Effects of tempol or apocynin on the concentration-dependent relaxation to acetylcholine in aorta (A and C) and mesenteric resistance arteries (B and D) from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. The term 'control' indicates non-preincubated arteries. Results are expressed as mean ± SEM and were analyzed by two-way ANOVA, n = 7 or 8 rats.

Preincubation with tempol or apocynin similarly increased relaxation to acetylcholine in aorta and MRA from O-HS group (Fig. 4C and D). In arteries from O-NS, this response remained unaltered in presence of tempol or apocynin (Fig. 4A and B). In O-NS aorta and MRA, acetylcholine-induced vasodilatation was not modified by indomethacin (Fig. 5A and B). By contrast, the altered response to acetylcholine was normalized by indomethacin in aorta and MRA from O-HS group (Fig. 5C and D). To analyze the contribution of TP receptors on acetylcholine response, both aorta and MRA from O-HS group were incubated with SQ29548. Similar to indomethacin, SQ29548 increased relaxation to acetylcholine in both aorta and MRA from O-HS group (Fig. 5C and D).

3.2. Plasma and tissue ACE activity

ACE activity determined in plasma of O-HS was similar to that of O-NS group (Fig. 6A). However, the ACE activity determined in the aorta and MRA homogenates was greater in O-HS than O-NS (Fig. 6B and C).

3.3. Superoxide anions production

As seen in Fig. 7, the intensity of fluorescence emitted by DHE was increased in O-HS aorta (Fig. 7A) and MRA (Fig. 7B) compared with O-NS, indicating increased superoxide anion generation. In addition, incubation with apocynin or losartan was able to decrease the DHE-emitted fluorescence in aorta and MRA from O-HS group to levels similar to those observed in the O-NS group (Fig. 7).

3.4. Western blot analysis

COX-1, AT_1, eNOS, and NOX-4 protein expressions were similar in the aorta (Fig. 8A, C, G and H) and MRA (Fig. 8D, F, I and 7J) from O-NS and O-HS. However, in both arteries from O-HS group, the expression of COX-2 was greater when compared to O-NS group (Fig. 8B and E).

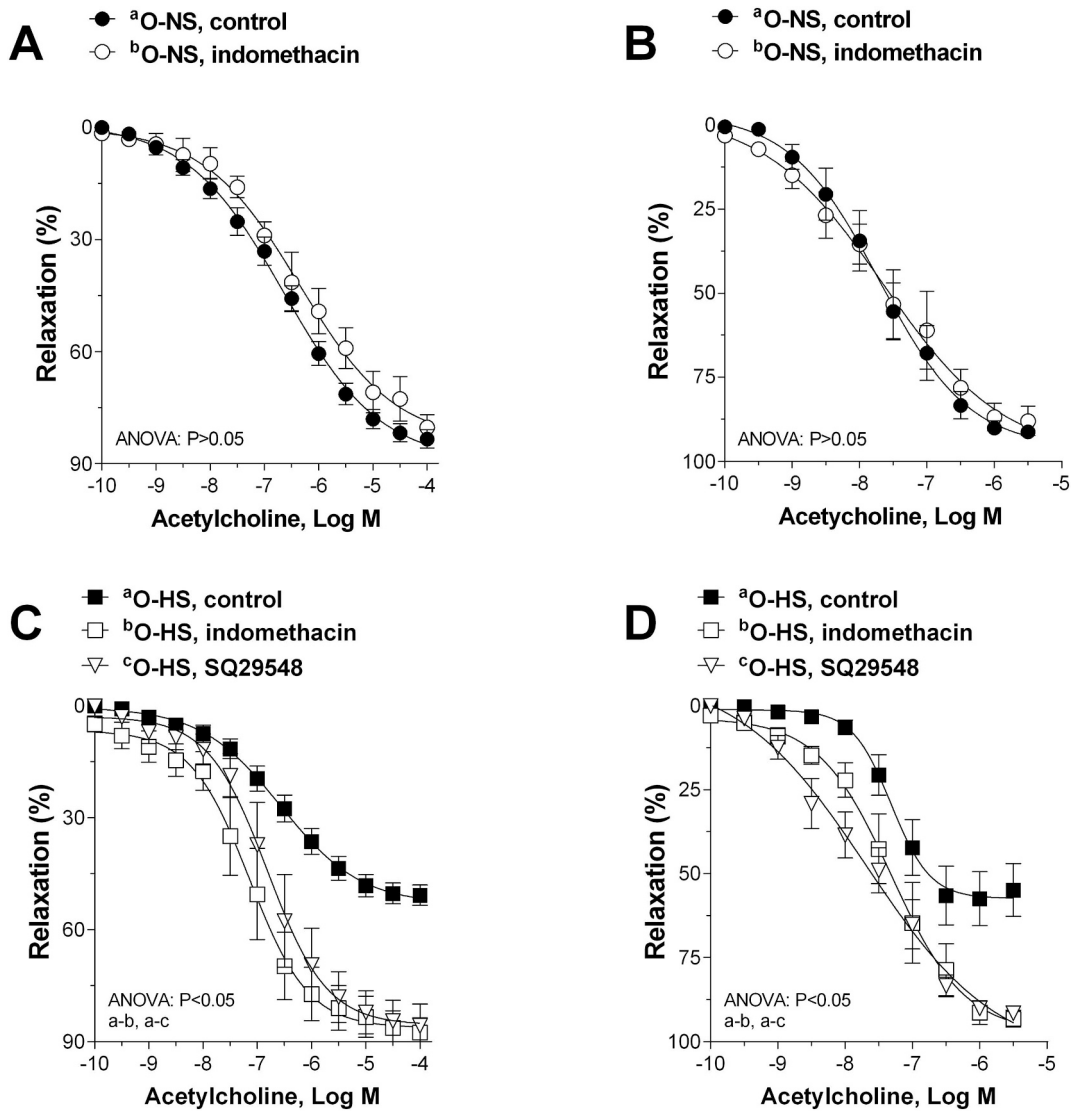

Fig. 5. Effects of cyclooxygenase inhibition (indomethacin) or TP receptor blockade (SQ29548) on the concentration-dependent relaxation to acetylcholine in aorta (A and C) and mesenteric resistance arteries (B and D) from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. The term 'control' indicates non-preincubated arteries. Results are expressed as mean ± SEM and were analyzed by two-way ANOVA. n = 7–8 rats in each group.

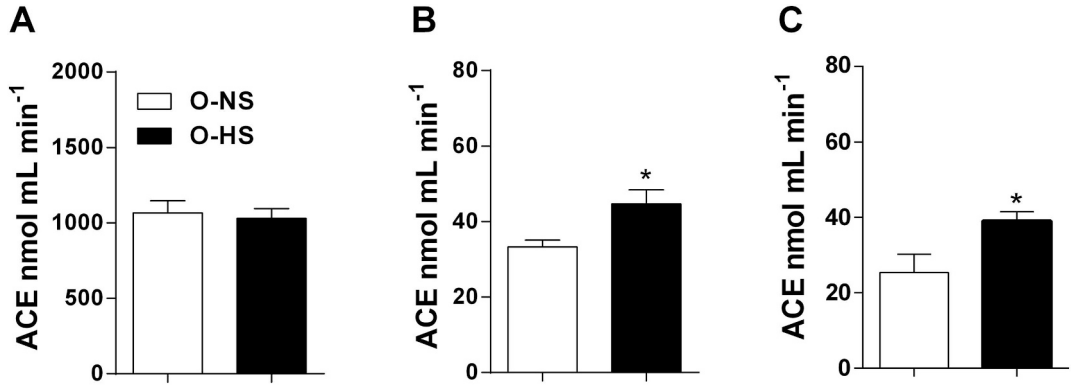

Fig. 6. Bar graphs show plasma (A), aorta (B), and mesenteric resistance arteries (C) angiotensin converting enzyme (ACE) activity of offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. Results are expressed as mean ± SEM and were analyzed by Student's unpaired *t*-test. n = 5 rats in each group. *P < 0.05 versus O-NS.

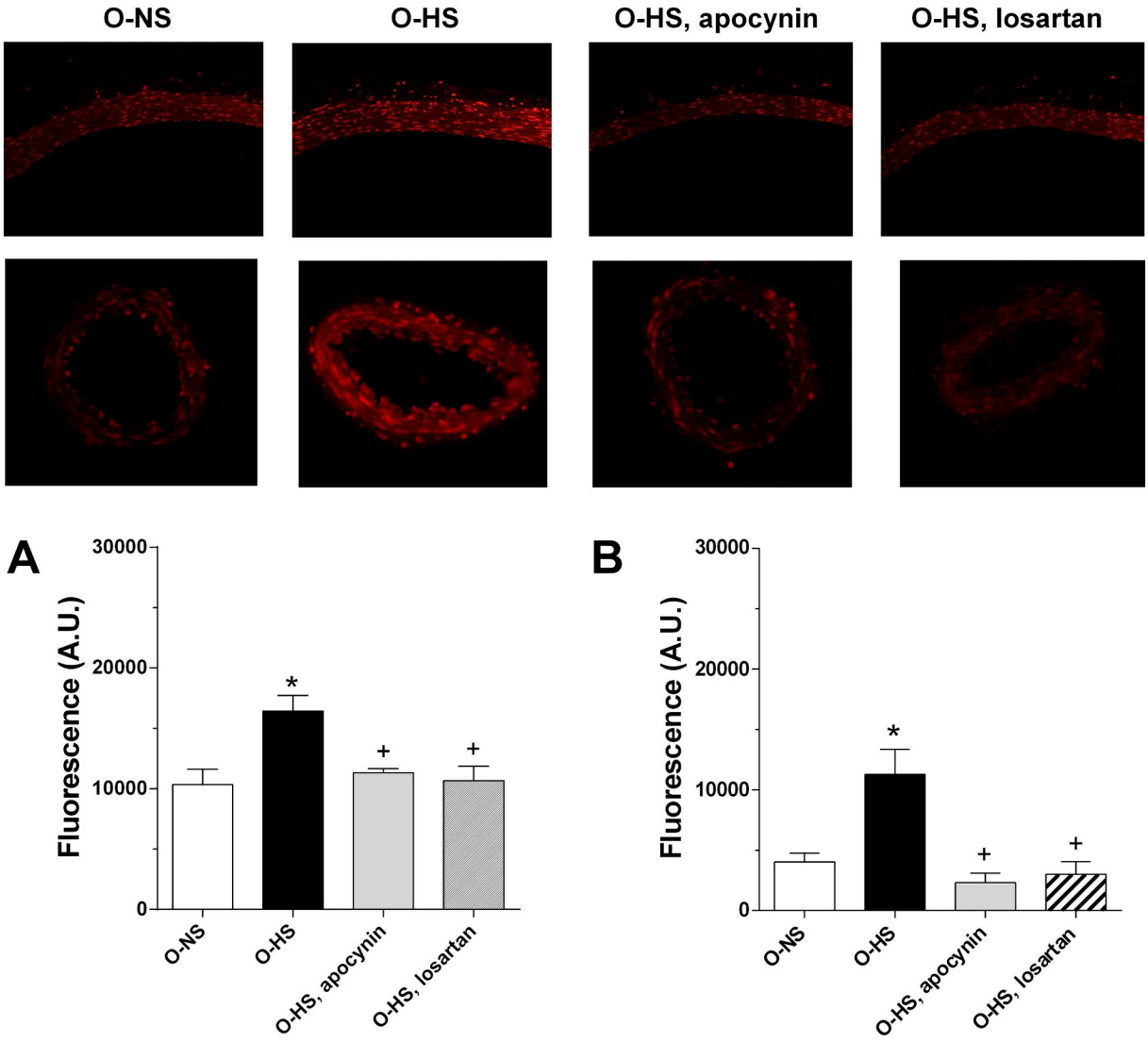

Fig. 7. Increased superoxide anions (O_2^-) generation in aorta and mesenteric resistance arteries (MRA) from offspring of high-sodium fed dams (O-HS). Top panels, representative fluorescence microphotographs of aortic (first row) and MRA (second row) sections labelled with the O_2^- reactive agent dihydroethidine (DHE; original magnification 20×) from O-NS and O-HS incubated with or without apocynin or losartan. Bar graph A and B, densitometric analysis of fluorescence intensity of O-NS and O-HS arteries in absence or presence of apocynin or losartan. The DHE fluorescence signal was evaluated in basal conditions or after incubation with MnTMPyP. Data represent means ± SEM from five animals in each group. One-way ANOVA, *P < 0.05 vs. O-NS, +P < 0.05 versus O-HS.

3.5. Effect of chronic treatment with losartan on vascular reactivity and COX-2 expression

Losartan treatment did not affect vasodilation to acetylcholine in O-NS aorta and MRA, but increased it in arteries from O-HS (Fig. 9A and B). The vasodilation to the NO donor sodium nitroprusside was similar in both losartan-treated O-NS and O-HS (results not shown). Different from that observed in untreated O-HS, in arteries from losartan-treated O-HS, both indomethacin and apocynin failed to produce any detectable effect on vasodilation to acetylcholine (Fig. 9C and D). After losartan treatment, there was no difference in COX-2 expression in arteries from O-NS and O-HS (Fig. 9E and F). In both groups, the blood pressure and heart rate remained unmodified after losartan treatment (results not shown).

4. Discussion

Increasing experimental evidence has been indicating that in utero exposure to sodium overload may predispose to cardiovascular disorders in adulthood [3,9,14,15]. The present study investigated the long-term vascular consequences of perinatal exposure to maternal high-salt diet in male Wistar rats. Our findings indicate that local vascular angiotensin II may be the mediator of high sodium-programmed endothelial dysfunction, associated with increased COX-2 expression and oxidative stress.

It has been demonstrated that perinatal exposure to sodium overload constitute a risk factor for hypertension development in adulthood. However, a body of evidence about maternal high-sodium diet effect on offspring blood pressure contains conflicting data. Although some studies have shown elevated blood pressure in high-salt diet-fed dams' offspring [3,9], others reported no alterations [13,25,27,28,29]. The reason for these conflicting results is unknown, but could be partially explained by different degrees of salt loading, duration of salt exposure or offspring' age. In our study, the O-HS and O-NS groups exhibited similar blood pressure. However, the absence of differences in blood pressure between groups may be related to age. Using a similar rat model (8% NaCl from the first day of pregnancy until weaning) Koleganova et al. [10] supports this hypothesis when observed no difference in mean arterial pressure (MAP) between offspring of salt-fed dams compared with control offspring until postnatal month 4. However, the

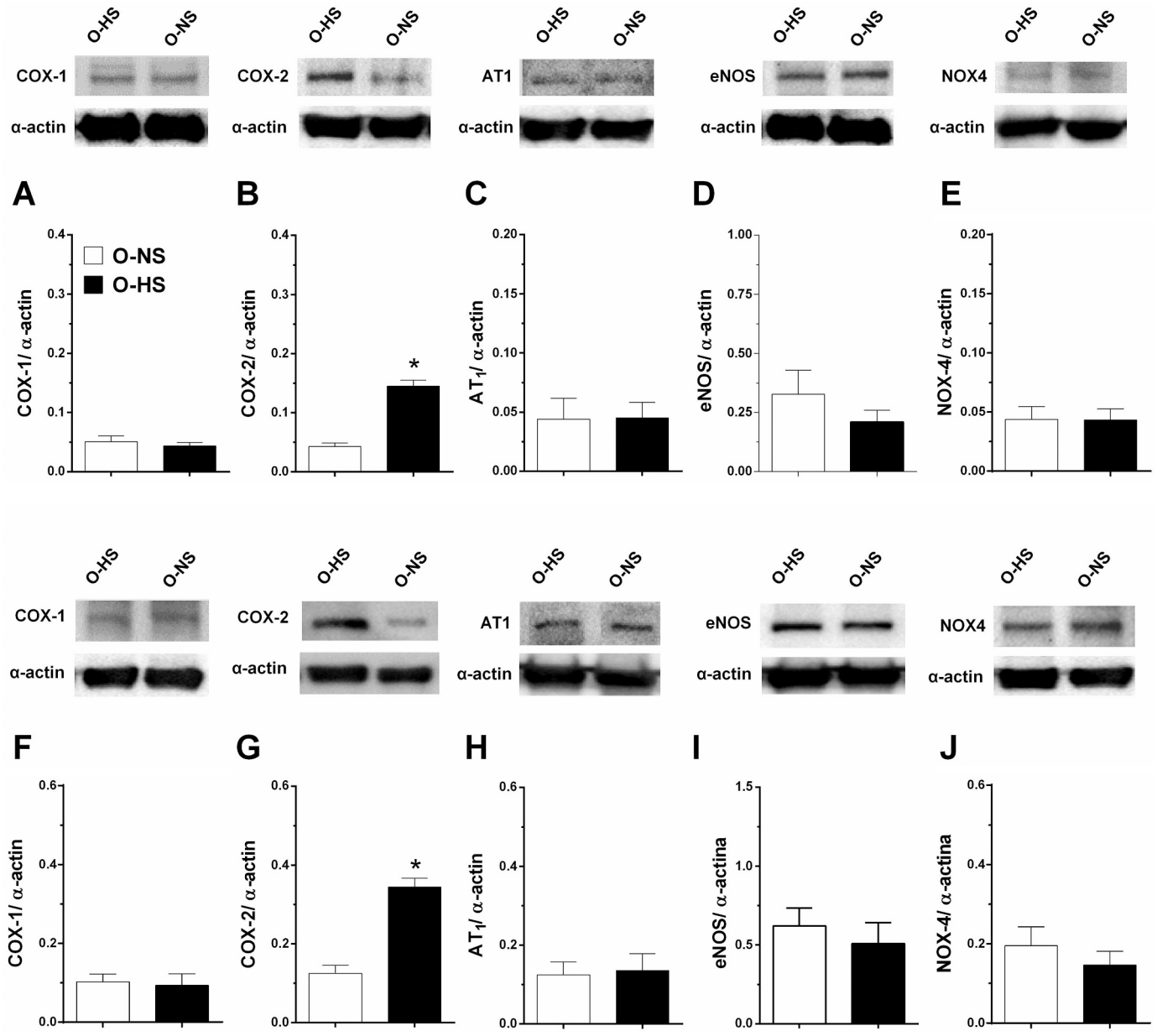

Fig. 8. Effect of perinatal sodium overload on COX-1, COX-2, AT_1 receptor, eNOS and NOX4 protein expression in aorta (upper panel) and MRA (lower panel) of Wistar rats. Bar graphs shows densitometric analysis of the Western blot for COX-1 (A and F), COX-2 (B and G), AT_1 receptor (C and H), eNOS (D and I) and NOX4 (E and J) protein expression in aorta and MRA from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. Results (means ± SEM) are expressed as the ratio between the signal for COX-1, COX-2, AT_1 receptor, eNOS and NOX4 and the signal for their corresponding α-actin. N = 5 in each. Group. Student's unpaired *t*-test, *P < 0.05 versus O-NS.

salt-exposed offspring exhibited higher MAP relative to control group from the age of five months, which was progressively increasing toward the month 9 (the end of the observation period). At postnatal month 6, their high-salt-fed dams' offspring exhibited a MAP that was an average of 7.0 mmHg higher than normal salt control group [10]. In our study, the average difference of MAP between the six-month-old O-HS and age-matched O-NS was 8.0 mmHg, but it did not reach statistical significance (P > 0.05). Therefore, it is possible to hypothesize that, similar to other fetal programming models [18], the phenotype of the sodium-exposed offspring tends to vary according to the age in the moment of analysis.

It is well established that hypertension has a complex association with endothelial dysfunction. However, it is still controversial whether endothelial dysfunction precedes the development of hypertension or whether it is a consequence of increased blood pressure [30]. The results obtained here reveal that O-HS group, although normotensive, presented impaired endothelium-dependent vasodilation and increased vasoconstriction induced by alpha adrenergic stimulation, indicating that maternal high-salt intake promotes derangements of vascular function as a result of blood pressure independent mechanisms. Considering these results, and the fact that the offspring of dams fed high-salt diet present elevated blood pressure with advancing age [10], it is possible to hypothesize that endothelial dysfunction may be involved in hypertension initiation in these animals.

Perinatal exposure to a high-sodium diet can reprogram the offspring's renin-angiotensin system (RAS). As expected, during the pregnancy, circulating RAS from mothers and their fetuses is suppressed when mothers are fed a high-sodium diet [31]. However, it has been demonstrated that the renin activity of adult salt-exposed offspring is unresponsive to a high-salt diet, i.e., high sodium intake does not

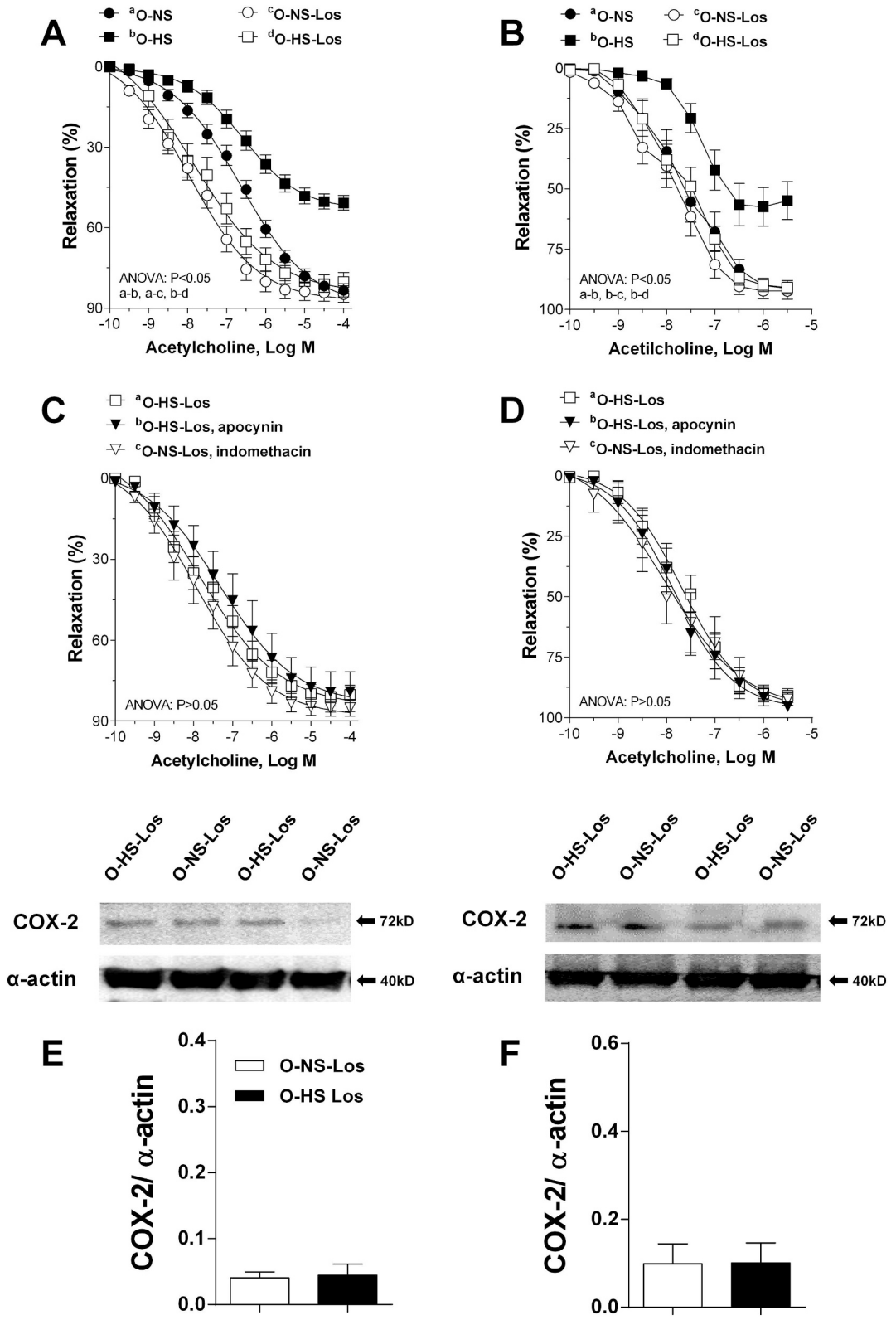

Fig. 9. A and B, effect of chronic losartan treatment (15 mg kg day^{-1}) on the endothelium-dependent relaxation to acetylcholine in aorta (A) and mesenteric resistance arteries (MRA, B) from offspring of normal-sodium (O-NS) and high-sodium (O-HS) fed dams. C and D, effects of indomethacin or apocynin on the concentration-dependent relaxation to acetylcholine in aorta (C) and MRA (D) from losartan (Los)-treated O-NS (O-NS-Los) and O-HS (O-HS-Los) groups. Results are expressed as mean ± SEM and were analyzed by two-way ANOVA. n = 7–8 rats in each group. E and F, COX-2 protein expression in aorta (E) and MRA (F) from O-NS-Los and O-HS-Los. Upper panels show representative blot for COX-2 protein expression in aorta and MRA. Lower panel shows densitometric analysis of the Western blot for COX-2 protein expression. Results (means ± SEM) are expressed as the ratio between the signal for COX-2 and the signal for their corresponding α-actin. N = 5 in each group.

suppress renin secretion nor angiotensin II production [32]. Therefore, perinatal sodium overload may lead to RAS hyperactivity during adulthood. Increased tissue angiotensin II levels have been reported in kidney and heart from the offspring of dams fed high-salt diet [24,25]. In these rats, the structural and functional renal alterations were partially prevented by ACE inhibition [25,26]. Moreover, Lv et al. [33] demonstrated that perinatal sodium exposure increases the expression of cardiac angiotensin II receptors (AT_1 and AT_2) in adult offspring, which were involved in the myocardial apoptotic process in these animals. In several conditions, the occurrence of increased RAS activity and endothelial dysfunction is well known. Therefore, the present study investigated the possible participation of tissue RAS in the altered endothelial function in O-HS group. For this, aorta and MRA from both groups were preincubated with the ACE inhibitor captopril or with the AT_1 receptor antagonist losartan.

In both O-HS aorta and MRA, but not in O-NS arteries, captopril or losartan similarly improved the acetylcholine-induced endothelium-dependent relaxation. In these arteries, although the vasoconstrictor responses elicited by angiotensin II and the AT_1 receptor protein expression were similar between O-HS and O-NS groups, the angiotensin I-induced contraction was significantly potentiated in both aorta and MRA from O-HS, suggesting an increase in vascular formation of angiotensin II. In support of these results, vascular (aorta and MRA), but not plasma, ACE activity were increased in O-HS group. Moreover, chronic treatment with losartan reversed the impairment of acetylcholine-induced relaxation in arteries from O-HS, without affecting this response in O-NS group. Taken together, these findings provide support for the hypothesis that angiotensin II plays a significant role as an endothelial dysfunction mediator in offspring of dams fed high-salt diet.

It has been largely reported that the deleterious effects of angiotensin II in the vasculature, apart from its well-known vasoconstrictor action, occur through its significant pro-inflammatory and pro-oxidative actions. This effect is due, at least in part, to the ability of angiotensin II to bind the AT_1 receptor, increasing NADPH oxidase activity, O_2^- production and COX-2 activity and expression, leading to impairment of endothelium-dependent vasodilation and to increase in vasoconstriction [34–36]. Together with our findings discussed above, these observations led us to hypothesize that COX-2 and NADPH oxidase pathways could be involved in salt-programmed vascular dysfunction in adult offspring.

Indeed, our results indicate that both COX-2 and O_2^- were involved on impaired endothelium-dependent vasodilation seen in O-HS arteries. This is based on several observations. In O-HS aorta and MRA, 1) the permeable SOD mimetic tempol or the NADPH oxidase inhibitor/ antioxidant apocynin similarly increased the acetylcholine-induced relaxation; 2) an enhanced DHE-emitted fluorescence was observed; 3) incubation with apocynin or losartan reduced the DHE fluorescence to similar levels as observed in the O-NS group; 4) COX inhibition (indomethacin) or TP receptor blockade (SQ29548) improved the relaxation elicited by acetylcholine and 5) COX-2, but not COX-1, was overexpressed. The fact that eNOS protein expression was similar in O-HS and O-NS groups, reinforces our conclusion that the blunted endothelium-dependent relaxation in O-HS arteries is related to O_2^--dependent NO degradation and increased COX-2-derived contractile prostanoids release. Our results also demonstrate that chronic treatment with losartan reversed the COX-2 protein overexpression in O-HS aorta and MRA and abolished the potentiating effects of indomethacin and apocynin on the vasodilator response to acetylcholine. Taken together, these findings provide support for the hypothesis that increased COX-2-derived contractile prostanoids release and O_2^- production might be responsible, at least in part, for the angiotensin II-mediated endothelial dysfunction in perinatal sodium overloaded adult offspring.

Several reports using distinct experimental models support the above findings. For example, a) angiotensin II stimulates O_2^- production by increasing NADPH oxidase activity [37]; b) in vascular fibroblasts, smooth muscle and endothelial cells, angiotensin II upregulates COX-2 expression through AT_1R activation [38–40]; c) losartan treatment inhibited COX-2 expression and abolished the increased COX-2-derived contractile prostanoids release and NADPH oxidase-derived O_2^- production observed in aorta from SHR [41] and d) in resistance arteries from hyperglycemia-programmed offspring, AT_1 receptor activation by angiotensin II increased COX-2 expression and contractile prostanoids release and impaired the endothelium-dependent vasodilation [36].

Some studies have revealed a reciprocal interaction between vascular ROS and COX-2 in some situations of cardiovascular damage [42]. COX-2-derived prostanoids generate ROS in various circumstances. On the other hand, increased ROS production stimulates COX-2 expression and activity and increases the contractile prostanoids release that promotes vasoconstriction and impairs endothelium-dependent relaxation through TP receptor activation. In this sense, evidence by Martínez-Revelles et al. [43] indicate a vicious circle between ROS and COX-2-derived prostanoids as a potential mechanism to induce endothelial damage in hypertension. These authors demonstrated that COX-2-derived prostanoids, acting on TP receptor, activate NADPH oxidase activity and ROS generation in arteries from SHR and angiotensin II-infused mice. In addition, ROS derived from mitochondria or NADPH oxidase activate COX-2 and stimulates contractile prostanoids release, resulting in increased vascular contraction. Therefore, based on these observations, we speculate that the increased angiotensin II-mediated O_2^- production and COX-2/TP receptor pathway may act in concert to induce endothelial dysfunction in O-HS arteries.

There are some limitations of the present study that should be mentioned. This study was performed in rats and, although animal studies have contributed much to our understanding of pathophysiology of many diseases, they do not predict with sufficient certainty what will occur in humans. Furthermore, it is debatable whether animal models of sodium overload are representative of the effects of high salt intake in humans. On the other hand, in humans, the studies relating the consumption of sodium to cardiovascular events also have significant limitations related to difficulty in assessment of sodium intake [44]. Another limitation of this study arises from difficulty in determining how perinatal sodium exposure increases vascular ACE activity in adult offspring and why the observed vascular changes did not increase the blood pressure. Therefore, further investigation and experimentation is warranted to identify potential mechanisms beyond the scope of the present study.

In conclusion, the results from the present work confirm previous studies showing that high-sodium consumption during the pregnancy and lactation is able to program the endothelial dysfunction development in adult offspring, independently of changes in blood pressure. These vascular abnormalities are probably mediated by an NADPH oxidase-dependent oxidative stress and by an enhanced COX-2-derived vasoconstrictor prostanoids formation, which by decreasing NO bioavailability and stimulating TP receptors impair the endothelium-dependent vasodilation. These mechanisms are stimulated by angiotensin II from vascular tissue RAS, whose production seems to be increased in O-HS group. If these observations can be extrapolated to humans, the high-salt intake during the pregnancy and lactation may impact the endothelial function of adult offspring, resulting in a greater tendency to vasoconstriction, inflammation, fibrosis, and oxidative stress, potentially increasing the cardiovascular risk.

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Conflict of interest

None.

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