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Abstract

Purpose:
During the postnatal stage, cardiovascular nitric oxide (NO) system and caveolins (cav) may be regulated differentially in response to hypovolemic state induced by water restriction. Our aim was to examine the effects of water restriction on NO synthases (NOS) and cav in the atria, ventricle and aorta of growing rats.
Methods:
Male Sprague–Dawley rats aged 25 and 50 days were divided into (n = 15): WR: water restriction 3 days; WAL: water ad libitum 3 days. Systolic blood pressure, NOS activity and NOS/cav protein levels were measured.
Results:
Dehydration induced a larger increase in SBP in WR25 group. Ventricular NOS activity, eNOS and nNOS of WR25 pups were increased, and both cav were decreased. In the WR50 group, NOS activity remained unchanged. In the atria, NOS activity, eNOS and nNOS decreased in WR25 associated with increased cav-1; in the WR50 group, NOS activity was increased without changes in NOS isoforms. In the aorta of WR25, NOS activity and iNOS were decreased; NOS activity was unchanged in WR50, despite the decreased levels of eNOS and increased iNOS, cav-1 and cav-3.

Conclusions:
NO system adjustments in cardiovascular system under osmotic stress in vivo depend on postnatal age, being eNOS and nNOS, the isoforms that determine NOS activity in cardiac tissue in 25-day-old pups. Changes in cav abundance during hypovolemic state may contribute to age-related NO production.

Keywords (separated by '-')	Nitric oxide - Caveolins - Water restriction - Cardiovascular system - Postnatal growth
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ORIGINAL CONTRIBUTION

Dehydration affects cardiovascular nitric oxide synthases and caveolins in growing rats

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Keywords Nitric oxide · Caveolins · Water restriction · Cardiovascular system · Postnatal growth

Introduction

Dehydration is a significant problem of the newborn infant, and it is currently considered one of the main causes of mortality in children [1]. An inadequate hydration during infancy may have cardiovascular consequences in adulthood, such as sodium retention and higher blood pressure [2]. Cardiovascular response to dehydration consists of the activation of several neurohumoral systems to maintain blood pressure and perfuse tissues appropriately, despite the contraction of blood volume. Many studies have shown increased activity of the renin-angiotensin system, levels of vasopressin and sympathoadrenal activity in water-deprived rats [3, 4]. Another well-known regulator of cardiovascular function is nitric oxide (NO), synthesized by NO synthases (NOS) in cardiomyocytes and endothelial cells [5]. In cardiovascular system, eNOS expressed in vascular endothelium and cardiomyocytes has paracrine effects on myocardial contraction, such as the modulation of cardiac muscle relaxation, oxygen consumption

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and β-adrenergic response [5]. Neuronal isoform is present in parasympathetic ganglia and cardiac myocytes, and it is the most relevant isoform involved in the regulation of cardiac excitation–contraction coupling by autocrine mechanisms [5]. On the other hand, iNOS expression has been reported in inflammatory cells, fibroblasts, myocytes and coronary vascular smooth muscle, being its expression associated with septic shock, myocarditis, transplant rejection and isquemia [6, 7]. NO has been involved in fluid homeostasis, considering its diuretic and natriuretic actions on the kidney [8] and its neuromodulatory effects on the hypothalamic–pituitary–adrenal axis and vasopressinergic axis in water-deprived rats [9]. Despite the fact that NO system has also been implicated in cardiac development during early stages of life [10, 11], cardiovascular NO system response to hypovolemia has not been fully studied during this period.

Caveolins (cav), the structural proteins of caveolae, are well-known negative regulators of the activity of the endothelial isoform of NOS (eNOS). In cardiomyocytes, eNOS is associated with caveolin-3 (cav-3) and, to a lesser extent, to caveolin-1 (cav-1). In vascular tissue, eNOS is also targeted to caveolae interacting with cav-1 in endothelial cells [12]. Cav-3 is also found in the vasculature, but it is expressed in vascular smooth muscle [13]. Within caveolae, cav control aspects of the activity of signaling pathways involved in cardiac development, modulating the angiogenic process, cell proliferation and differentiation and T-tubule biogenesis [14]. Moreover, cav have also been implicated in the regulation of enzymes associated with signaling pathways that determine cellular redox status [15], and some authors hypothesize that oxidative stress may be involved in osmosensor signaling [16]. Therefore, changes in cav abundance are likely to modulate the cardiac growth process as well as the adjustment to osmotic stress.

In previous work done in our laboratory, we showed that changes in cardiovascular NO system in response to water deprivation were dependent on the age of the animals, as young and aging rats were studied [17]. Moreover, cav are also involved in cardiac NOS regulation in an age-dependent way during hypovolemic state [18]. The mentioned data suggest that during the postnatal stage, cardiovascular NO system and cav may be regulated differentially during hypovolemic state to support the hemodynamic alterations. The involvement of NO pathways in adaptation to hypovolemic state and their age-related differences are necessary to provide an appropriate treatment in order to prevent the consequences of this nutritional deficiency on growth and development or later in the adult life. Thus, the objective was to evaluate the effects of osmotic stress triggered by water restriction on NOS activity and NOS/cav protein levels in the right atria, left ventricle and thoracic aorta in growing rats.

Materials and methods

Animals

Male Sprague–Dawley rats were obtained from the breeding laboratories of the School of Pharmacy and Biochemistry (University of Buenos Aires, Argentina). Newborn rats were maintained with their dam until day 21 (weaning age). When they reached the age of 25 and 50 days, the animals were housed individually in metabolic cages with an automatic light/dark cycle of 12 h/12 h and fed with standard rat chow from nutriments Purina (Buenos Aires, Argentina) and tap water ad libitum until the beginning of the experimental period. Animals were cared for in accordance with guidelines of the 'Guide for the Care and Use of Laboratory Animals' (National Academy Press, 1996, USA). All study protocols were reviewed and approved by the National Administration of Medicine, Food and Medical Technology, Department of Health and Environment of the Nation, Argentina (No. 6344-96).

Experimental protocol

Male Sprague–Dawley rats aged 25 and 50 days were randomly assigned as follows ($n = 15$ each group):

WAL group animals had continuous access to both food and water during the 3-day experimental period, representing a normohydrated group of rats.

WR group rats were deprived of water for 72 h but had continuous access to food.

Animals of both experimental groups were placed in metabolic cages 2 days before the beginning of the experiments in order to adapt to the new environment. At the end of each experimental period and with the purpose of validating that water restriction established a dehydration status, we determined body weight and hematocrit, as previously described [19]. At the end of each experimental period, animals from each group were killed by decapitation in order to isolate the right atria, the left ventricle and thoracic aorta.

Histological evaluation of cardiac tissue

Cardiac tissue from all groups of animals were fixed in phosphate-buffered 10 % formaldehyde (pH 7.2) and embedded in paraffin using conventional histological techniques. Paraffin sections were cut at 4 μm with a microtome (Leica RM 2125, Wetzlar, Germany), deparaffined and rehydrated. The slides were stained with Mallory's trichrome. Sections were analyzed using an Olympus BX51 light microscope equipped with a digital camera (Qcolor 3 Olympus America Inc., Canada) and connected to the Image-Pro Plus software. The major and minor

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diameters of cardiomyocytes were measured using Q Capture Pro software in 4-μm-thick cross sections of the heart stained with hematoxylin and eosin at 400× magnification. Cardiomyocytes chosen to be measured had central and nearly round shape nuclei. Afterward, major and minor diameters were averaged to obtain mean cardiomyocyte diameter.

Hemodynamic parameters

Systolic blood pressure (SBP) was indirectly measured in awaken animals by the tail-cuff method using a PowerLab data acquisition system device and LabChart software (AD Instruments). Prior to the measuring SBP, rats were warmed in a thermostated and silent room for 30 min. The SBP value for each rat was calculated as the average of five separate measurements at each session. Heart rate (HR) was also calculated from the pulse pressure signals by using LabChart software.

Total NOS activity

NO system activity in cardiac tissue was assessed by measuring the conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline. 50 μg of protein of homogenized tissues were incubated in Tris-HCl buffer (pH 7.4) containing 1 mg/mL of L-arginine, [^{14}C] L-arginine (346 mCi/mL), L-valine (67 mM), NADPH (1 mM), calmodulin (30 nM), tetrahidrobiopterin (5 μM) and $CaCl_2$ (2 mM) for 1 h at room temperature. Negative controls were performed by adding 10 mM L-NAME (NOS unspecific inhibitor) to reaction medium. At the end of the incubation period, reaction was arrested by adding HEPES-EDTA solution (pH 5.5) at 0 °C. Reaction mixtures were loaded into cation exchange columns (Dowex AG 50 W-X8, Na + form; Bio-Rad), and [^{14}C]-L-citrulline was eluted from columns with 1 mL of ddH2O. The amount of [^{14}C]-L-citrulline eluted was quantified using a liquid scintillation counter (Wallac 1414 WinSpectral; EG&G, Finland). All compounds, except [U^{14}C]-L-arginine monohydrochloride (346 mCi/mmol, Amersham Life Science), were purchased from Sigma Chemicals. Total protein levels were determined by the Lowry method, using bovine serum albumin as a standard.

Protein levels of NOS and caveolin isoforms

NOS protein levels were determined by Western blotting. Tissues were disrupted on ice using a tissue homogenizer (Omni International) in buffer containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM EGTA, 1 % Triton, 1 mM PMSF, 1 μM pepstatin, 2 μM leupeptin, 1× protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined by Lowry assay. Equal amounts of protein (50–75 μg protein/lane) of pooled samples were separated by electrophoresis in 8 % SDS-polyacrylamide gels (Bio-Rad), transferred to a nitrocellulose membrane (BioRad) and blocked with 5 % nonfat milk. Membranes were incubated with rabbit antibodies against each NOS and cav isoform (dilution 1:1,000) and an anti-rabbit antibody conjugated with HSP (dilution 1:10,000). Samples were revealed by chemiluminescence using ECL reagent for 2–4 min. Bands were quantified by densitometry scanning using a Hewlett-Packard scanner and gel analyzer tools of Image J software (NIH). Each Western blot was made by triplicate. Protein levels were expressed as a ratio of the optical densities of each NOS/cav isoform and β-actin band to detect inaccuracies in protein loading. Western blot detection system and Hybond-ECL membranes were from Amersham Pharmacia Biotech.

The antibodies for NOS isoforms detection [iNOS (610333), eNOS (610298) and nNOS (610311)] were supplied by BD Biosciences, anti β-actin antibody by Millipore (04-1116) and antibodies anti-cav-1 (sc-7875) and -3 (sc-28828) were supplied by Santa Cruz Biotechnology and the secondary antibody (170-6515) by Bio-Rad laboratories.

Thiobarbituric acid reactive substances (TBARS)

Heart samples (left ventricle and right atria) and thoracic aorta tissue from all groups of animals were homogenized in a medium consisting of 120 mM KCl 30 mM phosphate buffer (pH 7.4) (1:5) at 0–4 °C. The suspension was centrifuged at 600*g* for 10 min at 0–4 °C to remove nuclei and cell debris. Oxidative damage to phospholipids was evaluated in the supernatant as thiobarbituric acid reactive substances (TBARS) by a fluorometric assay. Tissue homogenates (100 μL) were added to 200 μL 0.1 N HCl, 30 μL 10 % (w/v) phosphotungstic acid and 100 μL 0.7 % (w/v) 2-thiobarbituric acid. The mixture was heated in boiling water for 60 min. TBARS were extracted in 1 mL of *n*-butanol. After a centrifugation at 1,000*g* for 10 min, the fluorescence of the butanolic layer was measured in a Perkin Elmer LS 55 luminescence spectrometer at 515 nm (excitation) and 553 nm (emission). A calibration curve was prepared using 1,1,3,3-tetramethoxypropane as standard. Results were expressed as pmol TBARS/mg protein.

Statistical analysis

Data in tables and figures are expressed as mean values ± SD. Data were evaluated with one-way analysis of variance (ANOVA), and Tukey's post hoc test for multiple comparisons was used. Normal distribution was assessed by using Shapiro–Wilk test. The Levene's test of equality of error variance was used to evaluate the homogeneity of variances. When SD presented statistically significant

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Table 1 General features of 25- and 50-day-old rats

	25-day-old rat		50-day-old rats	
	WAL	WR	WAL	WR
Body weight (g)	83 ± 10	52 ± 6*	240 ± 12†	179 ± 10*
Hematocrit (%)	42 ± 1	56 ± 1*	48 ± 1†	63 ± 1*
Heart weight (g)	0.46 ± 0.07	0.34 ± 0.08*	0.83 ± 0.13†	0.73 ± 0.09
Heart weight/body weight (g/100 g)	0.49 ± 0.04	0.50 ± 0.06	0.35 ± 0.04†	0.38 ± 0.05
Cardiomyocyte diameter (μm)	15.4 ± 0.3	12.1 ± 0.2*	17.5 ± 0.4†	16.3 ± 0.3

Results are expressed as mean ± SD ($n = 10$ each group)

* $p < 0.001$ versus respective C,
# $p < 0.001$ versus respective R,
† $p < 0.001$ versus C25

differences, Tamhane's T2 test was used for post hoc comparisons. All statistical procedures were performed using SPSS statistical software package release 16.0 version.

Results

Features of dehydrated animals

In order to confirm that our experimental model induced a hypovolemic state, we determined body weight and hematocrit. Results are shown in Table 1. Animals from both age groups presented decreased body weight and increased hematocrit as expected. Moreover, heart weight and mean cardiomyocyte diameter were larger for the 50-day-old group. However, the ratio of heart weight/body weight (HW/BW) was smaller for this age group compared to the 25-day-old pups. Both heart weight and cardiomyocyte diameter were decreased in dehydrated animals from the youngest group. However, when comparing the ratio HW/BW, it did not show any significant differences when compared to control animals. In contrast, animals from the oldest group who were submitted to water restriction did not present any differences in these parameters, with exception of body weight, as previously described. Cardiac tissue stained with Masson's trichrome did not show increased fibrosis in neither of the experimental groups, as it can be observed in Fig. 1.

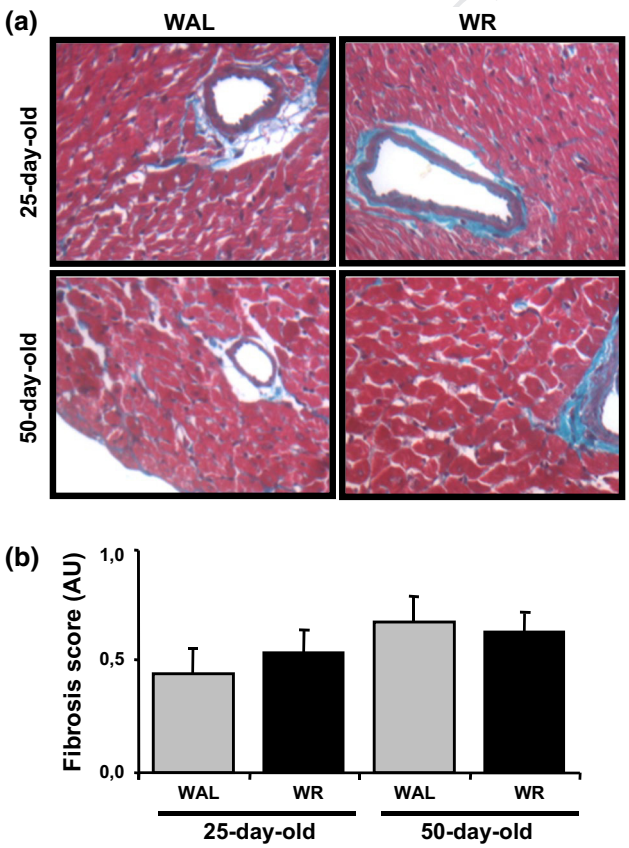

Fig. 1 Trichrome staining of the heart of 25- and 50-day-old rats submitted to water restriction (WR) or water ad libitum (WAL) ($n = 4$ each group). **a** Representative photographs of cardiac tissue (×400). **b** Fibrosis quantification shows no significant differences among the experimental groups (AU: arbitrary units). Data are expressed as mean ± SD

Hemodynamic parameters

Control animals aged 50 days presented higher SBP and lower HR values than control 25-day-old rats. Water restriction caused a significant rise in SBP in both age groups, larger for the youngest group. However, HR decreased in the 25-day-old pups but was increased in 50-day-old rats. Results are shown in Fig. 2.

Total NOS activity

Control pups presented higher levels of cardiac NOS activity when compared to the 50-day-old group. In the left ventricle (Fig. 3a), no changes were observed in response to water restriction in the oldest group; however, NOS activity was increased in the 25-day-old pups submitted to dehydration. In the right atrium, NOS activity was decreased in dehydrated pups but was increased in the 50-day-old rats (Fig. 4a). In aorta tissue, NOS activity was also higher in the youngest group as shown in Fig. 5a. In response to water restriction, 25-day-old pups presented decreased NOS activity in the thoracic aorta; however, the oldest group did not show changes in NOS activity.

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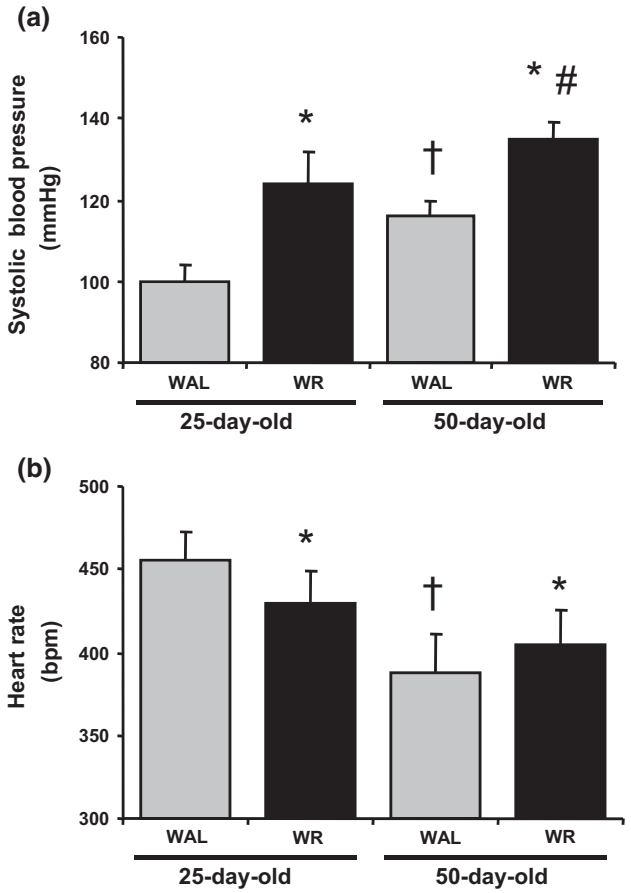

Fig. 2 Hemodynamic parameters of 25- and 50-day-old rats submitted to water restriction (WR) or water ad libitum (WAL) ($n = 10$ each group). **a** Systolic blood pressure. **b** Heart rate. Data are expressed as mean ± SD. $^{*}p < 0.01$ versus respective WAL; $^{\#}p < 0.01$ versus respective WR; $^{\dagger}p < 0.01$ versus 25-day-old rats

NOS isoforms protein levels

As it can be observed in Fig. 3b–d, in the left ventricle, NOS protein levels were higher in the 50-day-old control group compared to 25-day-old WAL pups. NOS isoforms protein levels were increased in response to dehydration in both age groups, except for iNOS in the youngest group. In the right atrium, eNOS and iNOS protein levels were similar between the two age groups, and nNOS protein levels were higher in the youngest group (Fig. 4b–d). In response to dehydration, eNOS and nNOS were decreased in the 25-day-old pups, without changes in the inducible isoform. In the oldest group, NOS isoforms protein levels did not change. In the thoracic aorta, eNOS and iNOS protein levels were lower in the 50-day-old group, as shown in Fig. 5b, c. No positive reactivity was found for neuronal isoform in both age groups. Dehydrated pups presented decreased levels of iNOS without changes in eNOS, whereas the oldest group had increased levels of iNOS but decreased eNOS.

Caveolin 1 and 3 protein levels

Ventricular cav-1 and 3 protein levels were higher in control 25-day-old pups when compared to the oldest group (Fig. 3e, f). After dehydration, in the youngest group, both cav isoforms were decreased. However, in the 50-day-old group, cav-1 was increased but cav-3 decreased after dehydration. In Fig. 4e, f, in the right atria, cav-1 protein levels were increased in response to dehydration in the youngest group, without changes in the oldest group. In the thoracic aorta, both cav protein levels were lower in the oldest group compared to the 25-day-old group. Water restriction increased both cav levels in the 50-day-old group, whereas in the youngest group, cav-1 did not change, and cav-3 was increased (Fig. 5d, e).

TBARS

Control animals of 25 days of age presented higher TBARS levels in comparison with the 50-day-old group in the studied tissues. In the left ventricle, after water restriction, lipid peroxidation was increased in both age groups, being this rise larger in the youngest group (Fig. 6a). Results in the right atria (Fig. 6b) indicated that lipid peroxidation was decreased in the youngest group, but it was increased in the oldest one. In the aorta tissue, rats of 25 days of age presented decreased TBARS levels in response to dehydration. The oldest group did not show changes in this parameter after water restriction. Results are shown in Fig. 6c.

Discussion

The present work was designed to study the effects of dehydration induced by water restriction on cardiovascular NO system and cav during postnatal growth, studying 25- and 50-day-old rats. In order to validate that water restriction protocol was efficient to establish a hypovolemic state, body weight and hematocrit were determined in both age groups. The decreased body weight and increased hematocrit in both age groups allowed us to confirm that, as previously described [19]. There are very few reports focused on the heart of dehydrated individuals, especially during postnatal growth. We observed that in the 25-day-old pups, the decreased heart weight was accompanied by a decreased mean cardiomyocyte diameter; meanwhile, the oldest group did not show changes in either of these parameters (Table 1), confirming that the youngest group suffered a more severe osmotic stress as shown in previous work done in our laboratory [19]. When studying fibrosis development in the heart, no differences were observed in both age groups submitted to water restriction. The present data in 25-day-old animals coincide with microcardia, a

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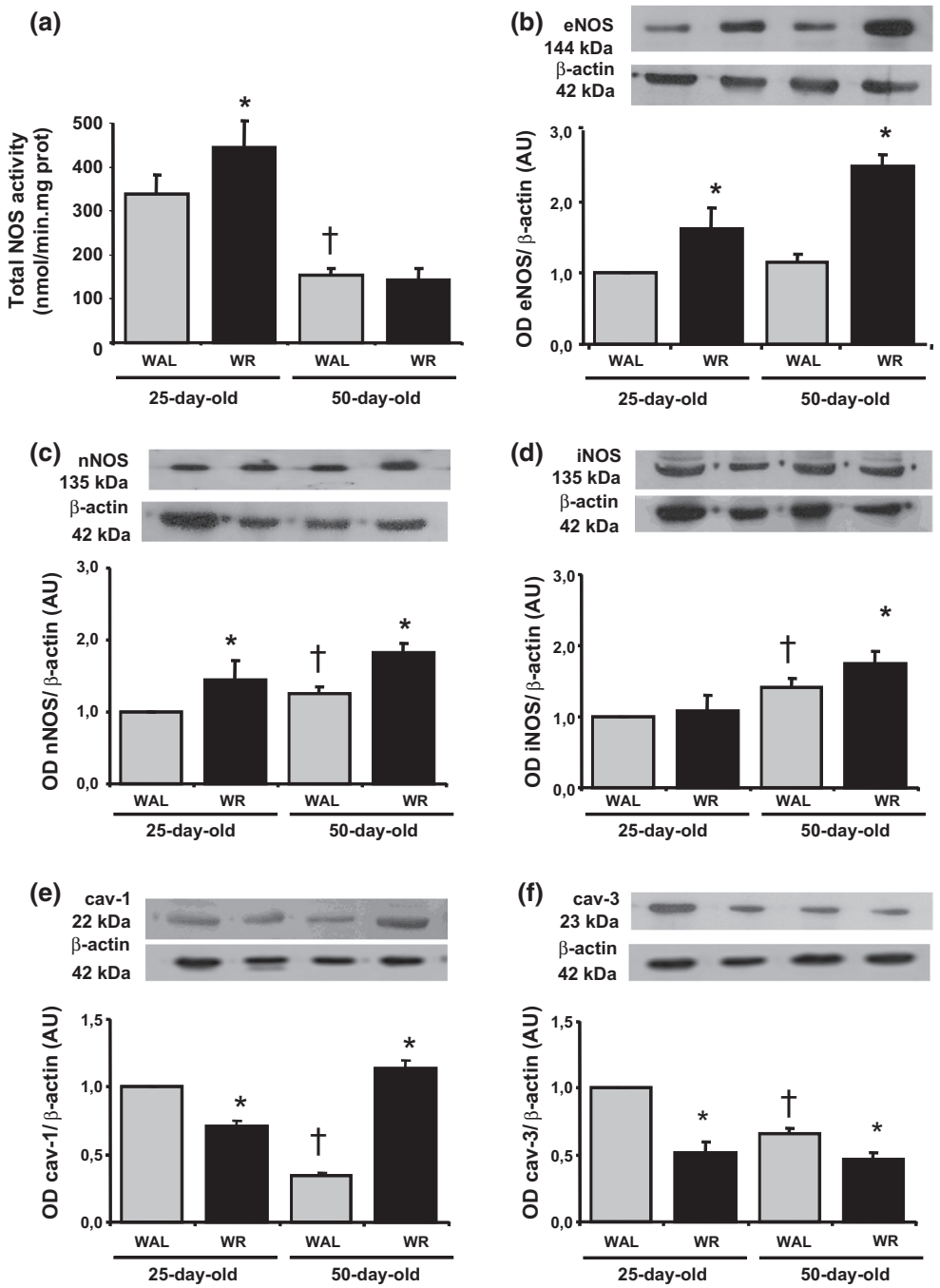

Fig. 3 NO system and caveolins in the left ventricle of water-restricted rats (WR) and control animals (WAL): Total NOS activity (**a**), representative Western blots of NOS isoforms: endothelial (**b**), neuronal (**b**) and inducible (**c**) NOS and cav isoforms: caveolin-1 (**e**) and caveolin-3 (**f**) of 25- and 50-day-old rats ($n = 6$ each group). Histograms illustrate the ratio between mean total protein and β-actin protein levels. Data are expressed as mean ± SD. *$p < 0.01$ versus respective WAL; †$p < 0.01$ versus 25-day-old rats

condition that is often observed in young children, secondary to severe fluid loss [20]. Thus, even though it is difficult to compare experimental models to humans, we suggest that a 3-day water restriction protocol in infant rats may be a useful animal model to study this pathological condition during postnatal life.

In order to study if water restriction induced age-related hemodynamic changes, we determined SBP and HR in control and dehydrated animals of both age groups. It is well known that cardiac function is enhanced from weaning age to adult life [21], accompanied by functional and anatomical changes in the cardiovascular system and autonomic

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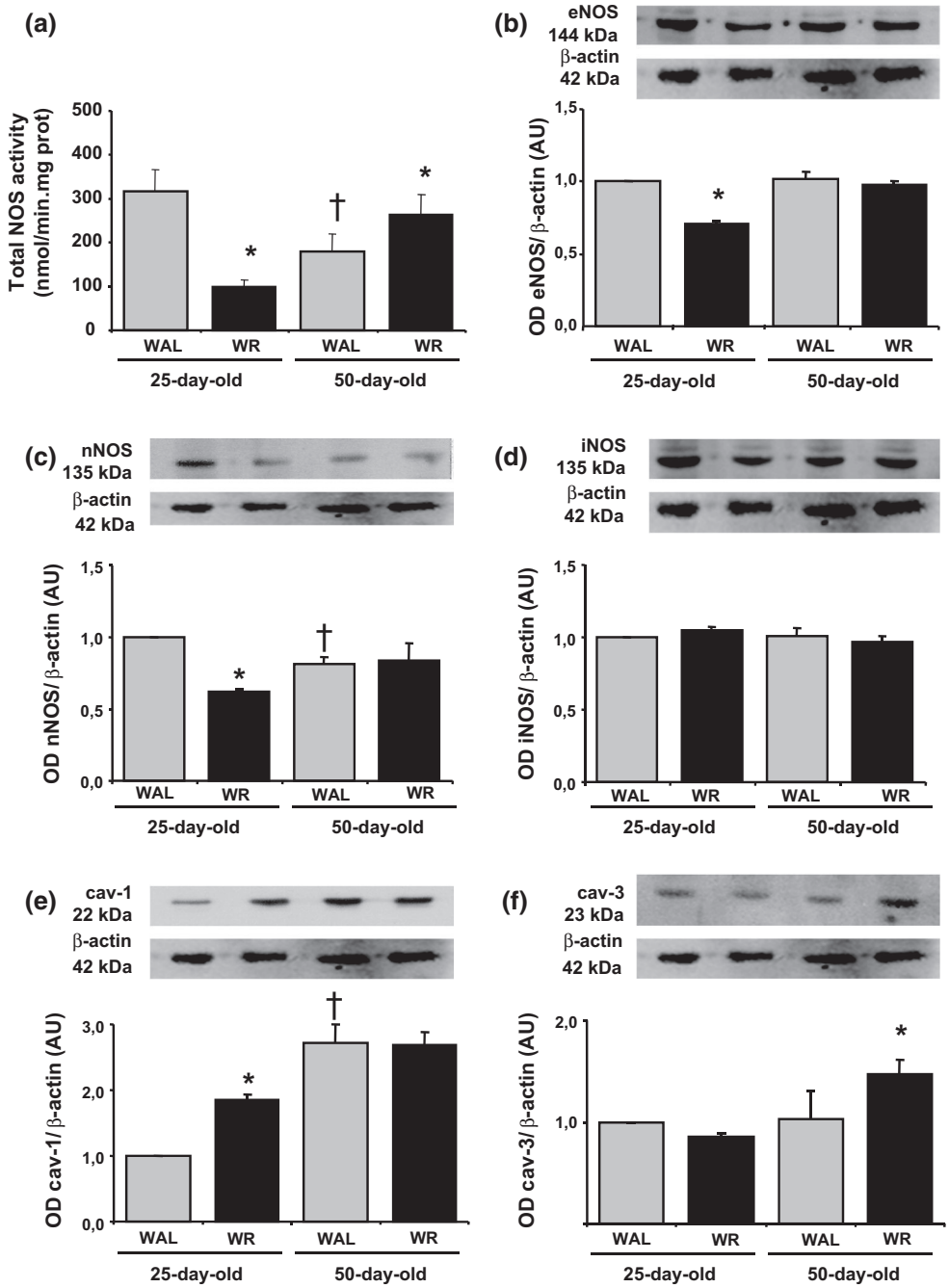

Fig. 4 NO system and caveolins in the right atria of water-restricted rats (WR) and control animals (WAL): Total NOS activity (**a**), representative Western blots of NOS isoforms: endothelial (**b**), neuronal (**b**) and inducible (**c**) NOS and cav isoforms: caveolin-1 (**e**) and caveolin-3 (**f**) of 25- and 50-day-old rats ($n = 6$ each group). Histograms illustrate the ratio between mean total protein and β-actin protein levels. Data are expressed as mean ± SD. *$p < 0.01$ versus respective WAL; †$p < 0.01$ versus 25-day-old rats

nervous system maturation [22]. This may account for the differences in basal SBP and HR values in both age groups. Response to osmotic stress was also different with postnatal age: In the youngest group, we observed a higher increase in SPB in comparison with the 50-day-old group (24 and 16 %, respectively). On the other hand, HR was decreased by 6 % in the youngest group and increased by 5 % in the older rats. Different neurohumoral factors and sensitivity to their action may be responsible for this distinct hemodynamic response to dehydration, such as NO system and angiotensin II levels, which are elevated during postnatal development [23]. In agreement with this, when analyzing NO system activity, our results indicated that in cardiac tissue of control animals, NOS activity was higher

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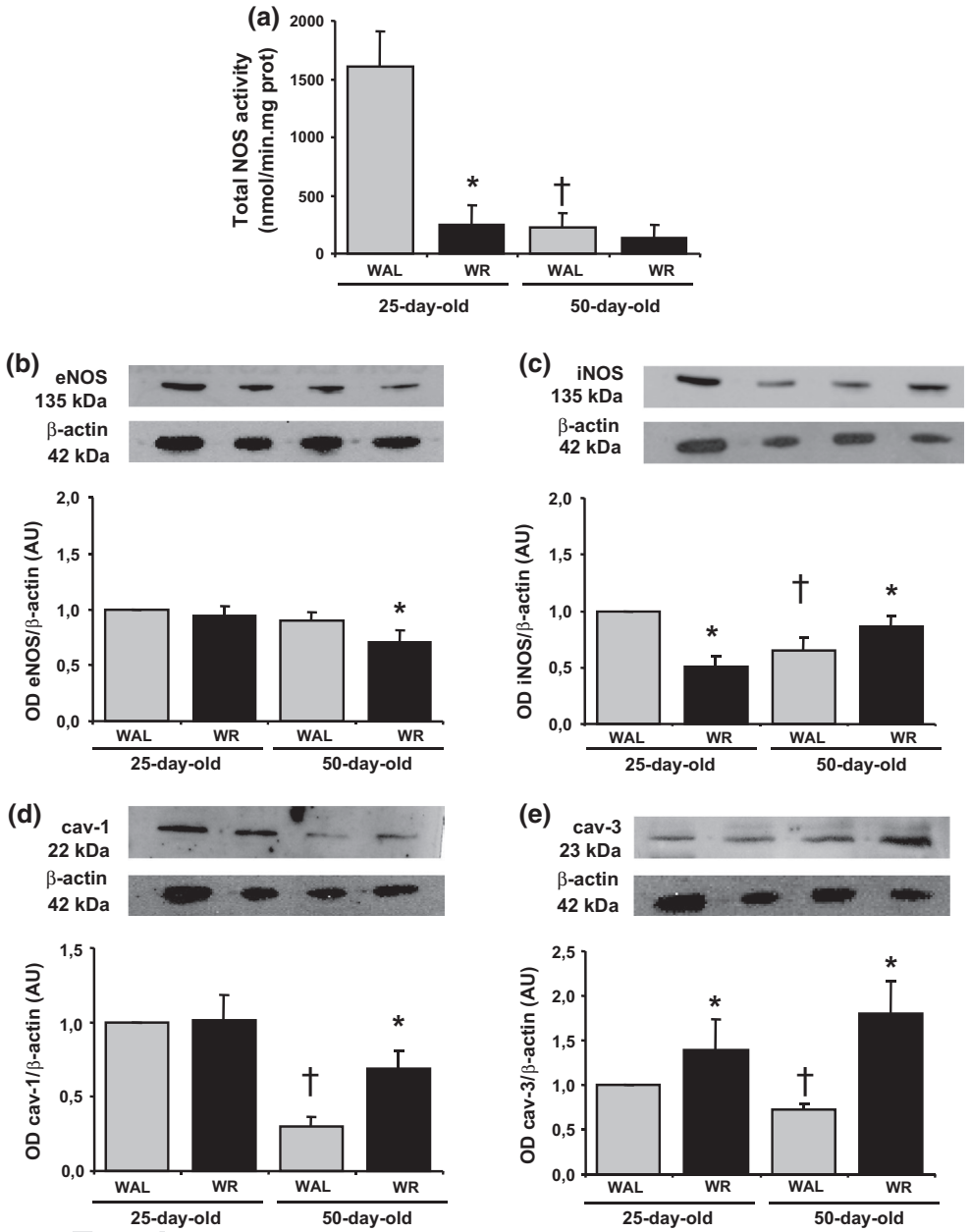

Fig. 5 NO system and caveolins in the thoracic aorta of water-restricted rats (WR) and control animals (WAL): Total NOS activity (**a**), representative Western blots of NOS isoforms: endothelial (**b**) and inducible (**c**) NOS and cav isoforms: caveolin-1 (**d**) and caveolin-3 (**e**) of 25- and 50-day-old rats ($n = 6$ each group). Histograms illustrate the ratio between mean total protein and β-actin protein levels. Data are expressed as mean ± SD. *$p < 0.01$ versus respective WAL; †$p < 0.01$ versus 25-day-old rats

in 25-day-old pups compared to 50-day-old rats, as well as in aorta tissue. Even though higher cav levels would be expected in the oldest group, it was observed that these proteins were decreased in ventricular and aorta tissues. Cav downregulation in the left ventricle and aorta tissue associated with postnatal growth may influence not only NO production but also other signaling pathways that contribute to cardiovascular system maturation. In line with this, Doyle et al. suggest that cav changes in aorta smooth muscle cells during development participate in tissue differentiation [13].

Continuing with the study of NO system, we observed that the effects of water restriction on cardiovascular NO system were age and tissue specific. In the left ventricle, during osmotic stress, it was observed that NOS activity was increased in the 25-day-old pups, due to an increase in eNOS and nNOS and reduced cav-1 and cav-3 protein levels in this age group. In contrast, in the older rats, no

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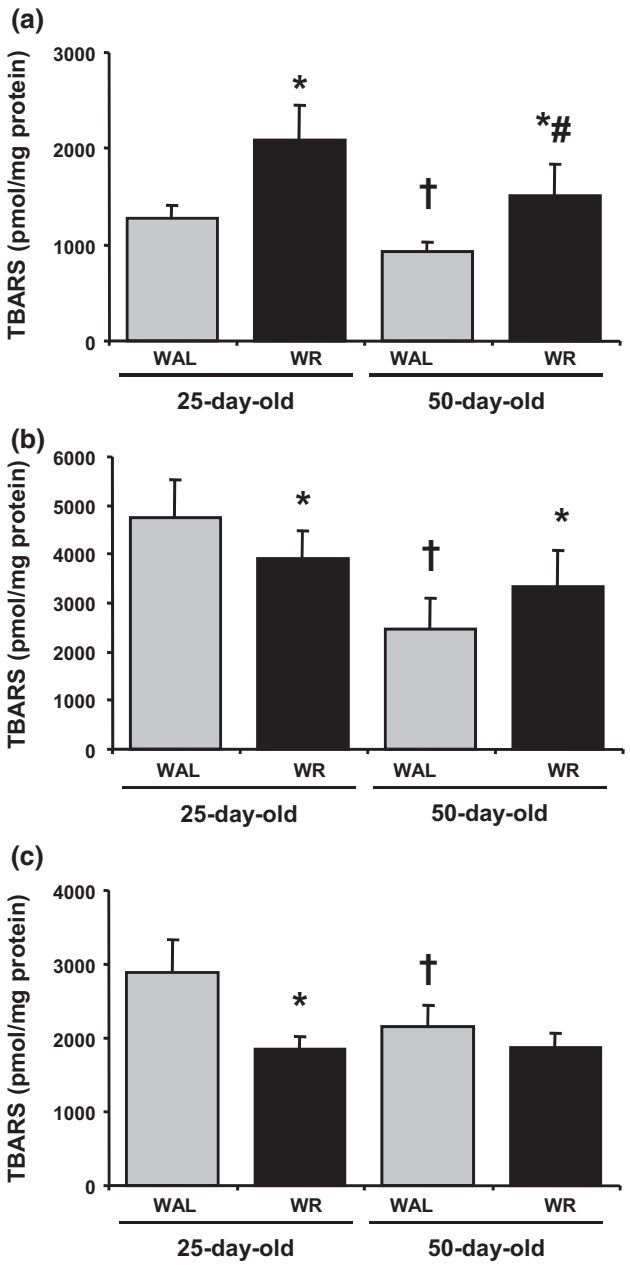

Fig. 6 Thiobarbituric acid reactive substances (TBARS) levels in the left ventricle (**a**), right atria (**b**) and thoracic aorta (**c**) of 25- and 50-day-old rats submitted to water restriction (WR) or water ad libitum (WAL) ($n = 5$ each group). Data are expressed as mean ± SD. $^{*}p < 0.01$ versus respective WAL; $^{\#}p < 0.01$ versus respective WR; $^{\dagger}p < 0.01$ versus 25-day-old rats

changes were observed in enzyme activity despite the increased protein levels of all NOS isoforms. This may be explained, at least in part, by increased protein levels of cav-1 in this age group which may reduce eNOS activity. Considering that NO system in the heart has been involved in the modulation of myocardial contraction and relaxation, oxygen consumption and the modulation of beta adrenergic response [5], increased ventricular NO production may participate in adaptation to osmotic stress in young pups, since they are more sensitive to dehydration, as mentioned above, and since they also show a higher increase in lipid peroxidation in comparison with the WR 50-day-old group. Some authors have reported a link between oxidative stress and downstream modulation of protective pathways via cav [24]. Moreover, it has been reported that cav expression is regulated by both osmotic and oxidative stress [15, 25], which may be increased in our experimental conditions as TBARS levels increased in both age groups. Volonte et al. [26] showed that cellular osmotic or oxidative stress can induce cav-1 tyrosine phosphorylation which represents an important downstream element in the signal transduction cascades. Therefore, the observed age-related changes in cav abundance during osmotic stress in vivo not only have an impact on NOS activity but also on other molecules regulated by caveolae sequestration. Apart from changes in cav abundance, we cannot disregard that alterations in subcellular localization of cav would also influence NOS activity.

Findings in atria tissue of dehydrated rats indicate a reduction in NOS activity in the 25-day-old pups, probably caused by a reduction in constitutive NOS isoforms, as described in the left ventricle. Moreover, there was an increase in cav-1 protein levels in this tissue that may also contribute to the decreased NO production by eNOS. In contrast, in the 50-day-old group, NOS activity was increased without changes in NOS isoforms protein levels. However, cav-1 protein levels remained unchanged, and cav-3 was increased. Thus, it is likely that other NOS regulators or posttranslational modifications of the enzyme may be involved in the observed changes of NO production. Additionally, it is interesting to notice that HR and atrial NOS activity presented a similar pattern, suggesting that atrial NO production in vivo may contribute to HR regulation. NO's chronotropic effects are controversial. It has been reported that NO has a positive chronotropic effect which can be blocked by L-NAME, however, at higher concentrations, and it has a bradycardic effect, implicating that its effects on HR depend on the concentration of NO [27, 28]. Previous results from our laboratory indicate that NO has a tachycardic effect, in agreement with the results obtained in the present study [29]. We cannot disregard that other neurohumoral factors are also involved in the observed chronotropic changes. Furthermore, in this tissue, the reduction in NOS activity observed in the youngest WR group may be a cause for reduced lipid peroxidation, considering that NO is a free radical capable of oxidizing lipids and other macromolecules by itself or it may combine with superoxide anion to form peroxynitrite, which may also decompose to form a strong oxidant hydroxyl radical, resulting in tissue injury [30].

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In aorta tissue, we also observed an age-dependent effect of osmotic stress on NO system. NOS activity was greatly decreased in response to dehydration in 25-day-old pups, which was accompanied by decreased levels of iNOS, without changes in eNOS. We suggest that if such changes were to occur in resistance blood vessels, they may contribute to the larger increase in blood pressure in response to osmotic stress in this age group. An imbalance of reduced production of NO or increased production of reactive oxygen species (ROS) may promote endothelial dysfunction [31]. It was reported that caloric restriction increased antioxidant defenses and decreased TBARS levels in the cardiovascular system [32]. However, oxidative stress has not been fully studied in models of volume depletion. Because ROS can interact and inactivate NO, vascular oxidative stress can lead to decrease NO bioavailability [33]. The decreased lipid peroxidation observed in response to dehydration may be protective in order to prevent endothelial dysfunction in this age group. In contrast, in the oldest group, NOS activity remained unchanged in spite of the decreased levels of eNOS and increased iNOS. As well as in cardiac tissue, other mechanisms may determine NOS activity in the 50-day-old group. Interestingly, both cav were upregulated in response to osmotic stress, with the exception of cav-1 in the youngest group, probably contributing not to further decrease endothelial NO production.

In conclusion, the novel finding of the present study is that dehydration state induced by water restriction triggers different regulatory mechanisms during postnatal growth that involve NOS, cav and lipid peroxidation in a tissue specific way, in order to modulate the changes of the hemodynamic parameters. NO production in cardiac and aorta tissues under osmotic stress in vivo depend on postnatal age, being eNOS and nNOS, the isoforms that determine NOS activity in the heart of 25-day-old pups. Changes in cav abundance in vivo during hypovolemic state may contribute to age-related NO production.

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