Biomedicine & Pharmacotherapy 69 (2015) 380–387

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

Involvement of nitric oxide and caveolins in the age-associated functional and structural changes in a heart under osmotic stress

Patricia Arza [a], Vanina Netti [a], Francisco Perosi [d], Gustavo Cernadas [a,d], Federico Ochoa [b], Natalia Magnani [c], Pablo Evelson [c], Elsa Zotta [b], Andrea Fellet [a], Ana M. Balaszczuk [a,*]

[a] Department of Physiology, University of Buenos Aires, Buenos Aires, Argentina
[b] Laboratory of Pathophysiology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina
[c] Laboratory of Free Radical Biology (IBIMOL), School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
[d] Department of Cardiology and Internal Medicine, Air Force Hospital, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 21 November 2014
Accepted 11 December 2014

Keywords:
Aging
Water restriction
Heart nitric oxide
Caveolins
Cardiac morphology

ABSTRACT

Previous work done in our laboratory showed that water restriction during 24 and 72 h induced changes in cardiovascular NOS activity without altering NOS protein levels in young and adult animals. These findings indicate that the involvement of NO in the regulatory mechanisms during dehydration depends on the magnitude of the water restriction and on age. Our aim was to study whether a controlled water restriction of 1 month affects cardiac function, NO synthase (NOS) activity and NOS, and cav-1 and -3 protein levels in rats during aging. Male Sprague-Dawley rats aged 2 and 16 months were divided into 2 groups: (CR) control restriction (WR) water restriction. Measurements of arterial blood pressure, heart rate, oxidative stress, NOS activity and NOS/cav-1 and -3 protein levels were performed. Cardiac function was evaluated by echocardiography. The results showed that adult rats have greater ESV, EDV and SV than young rats with similar SBP. Decreased atria NOS activity was caused by a reduction in NOS protein levels. Adult animals showed increased cav-1. Water restriction decreased NOS activity in young and adult rats associated to an increased cav-1. TBARS levels increased in adult animals. Higher ventricular NOS activity in adulthood would be caused by a reduction in both cav. Water restriction reduced NOS activity and increased cav in both age groups. In conclusion, our results indicated that dehydration modifies cardiac NO system activity and its regulatory proteins cav in order to maintain physiological cardiac function. Functional alterations are induced by the aging process as well as hypovolemic state.

1. Introduction

Healthy humans and animals maintain homeostatic control of the balance of body fluids by physiologic and behavioral adaptations [1]. When fluids are limited, such as osmotic stress induced by water restriction, this fluid balance is disturbed, leading to activation of a number of physiological mechanisms to promote the reestablishment of vascular volume and blood pressure, including activation of several neurohormonal factors, such as nitric oxide (NO), catecholamines, endothelins, arginine vasopressin, and renin–angiotensin system, among others [2,3]. Aging is more vulnerable to water balance disorders that could result in a progressive functional and structural decline in multiple organs and, in particular, it has profound effects on heart that is associated with significant mechanical remodeling that includes fibrosis, or accumulation of collagen and other extracellular matrix proteins [4,5].

It is known that NO is one of the major regulators of water and electrolyte homeostasis, with multiple functions influencing cardiovascular system [6]. It was reported that the endothelial isoform of NO synthase (eNOS) is expressed in vascular endothelium and cardiac myocytes, the cellular regulation of eNOS being an important determinant of cardiovascular homeostasis [7]. This isoform is targeted to specialized invaginations of the plasmalemma termed caveolae, which serve as sites for sequestration of signaling proteins, being characterized by the presence of the intrinsic membrane proteins called caveolins (cav) [8]. As cardiovascular function is closely linked to NO system, changes in NO production may affect cardiovascular system regulation during aging process. Synthesis and release of NO decline with age in endothelial cells and this has been implicated in the development

* Corresponding author at: Department of Physiology, School of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956 Piso 7, C1113AAD Buenos Aires, Argentina. Tel.: +54 11 4964 8280; fax: +54 11 4964 8280.
E-mail address: abalasz@ffyb.uba.ar (A.M. Balaszczuk).

http://dx.doi.org/10.1016/j.biopha.2014.12.026
0753-3322/

of several age-dependent cardiovascular diseases [9]. Interestingly, a few studies suggest that changes in cav expression could alter cell function with aging [10]. We provided evidence using an experimental model of hypovolemic state that specific expression patterns of ventricular NOS isoforms, alterations for interaction, are involved in age-related adjustment to acute blood loss [11]. Considering that older adults are a high-risk population for hypovolemic state induced by a dehydration, we hypothesize that the NO pathways are involved in cardiac function regulation after long controlled water restriction, this response being different with aging. Thus, our objective was to evaluate the effect of water restriction on hemodynamics parameters, NOS activity and NOS/cav interaction as well as the structural and functional changes in the heart of young (2 months old) and adult (16 months old) rats.

2. Methods and materials

Male Sprague-Dawley rats obtained through the breeding laboratories of the School of Veterinary (University of Buenos Aires) were received at ages of 2 (200–220 g body weight) and 16 months (500–550 g body weight). Ethical approval for animal experimentation was approved by the ethics committee of the School of Pharmacy and Biochemistry (CICUAL, No. 0031028/2014), University of Buenos Aires. Animals were allowed food and deionized water ad libitum. All the laboratory material was previously washed with nitric acid (20%) and water. Rats were housed separately in plastic cages in a humidity- and temperature-controlled environment, illuminated with a 12:12 light–dark cycle.

2.1. Experimental Protocols (see Fig. 1 for more details)

The rats at 2 and 16 months rats were randomly assigned to:

(1) *Group WR (water restriction)*: Animals were subjected to dehydration, which consisted on depriving them of water for 3 out of 4 days (1 cycle), repeating this for 8 successive cycles (32 days).

This model has been adapted from Gharbi et al., as it represents a true state of dehydration [2]. Rats had continuous access to food.

(2) *Group CR (control restriction)*: Rats had continuous access to both food and water, during 32 days, representing a normal hydration status.

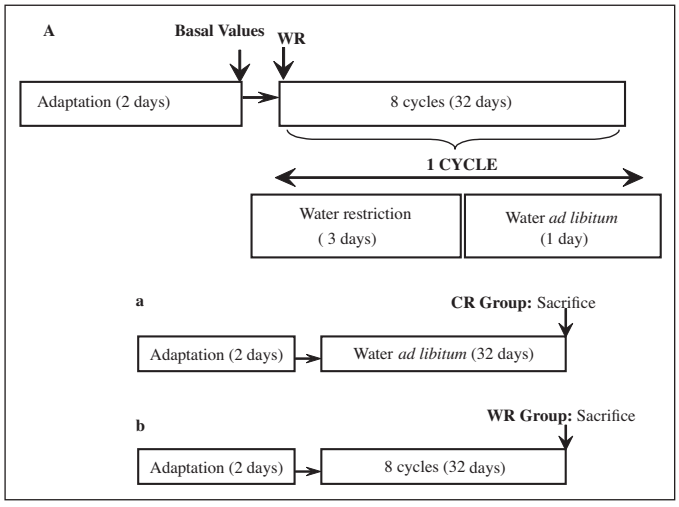

Fig. 1. (A) General design adapted from Gharbi et al. (B) Representation of the 2 groups for young and adult rats in (a) the control groups and (b) experimental groups.

The animals were placed in the metabolic cages for the adaptation to the environment for two days before the beginning of the experiments. Body weight and food intake as well as biochemical, urinary and hemodynamic parameters were evaluated after the adaptation period and at the end of the experimental time.

2.2. Biochemical and urinary parameters

Blood collections from caudal artery were made to determinate hematocrit, plasma osmolarity and urinary collections for urinary osmolarity. Serum Na^+ (mEq) was measured using ion selective analyzer (Tecno-lab t-140). Plasma and urinary osmolarity (mOSM) were measured by microosmometer (μosmetteTM Micro Osmometer). Hematocrit (%) was determinated from duplicate blood-filled hematocrit tubes. Urine volume (ml/min 100 g body weight) was determined gravimetrically.

2.3. Hemodynamic parameters

Systolic blood pressure (SBP) was indirectly measured in the awaken animals by the tail-cuff method using a PowerLab data acquisition system device and LabChart software (AD Instruments). Prior to 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.

2.4. Echocardiography

At the end of the experimental protocol, another group of animals from each experimental group from both, 2-month-old and 16-month-old rats, were anesthetized with urethane (1.0 g/kg, intraperitoneally), their chests were shaved under aseptic conditions and echocardiographic measurements were performed in the left lateral decubitus position. Two-dimensional directed M-mode images were obtained using a Sonoscape (A6 Vet) system with a 9–4 MHz transducer. Measurements were taken in the right parasternal short axis plane at the level of the mitral valve leaflets. End-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF) and systolic volume (SV) were measured from left ventricular by the echocardiography system. All determinations were made according to the guidelines of the American Society of Echocardiography.

2.5. NOS activity

Capacity for left ventricular and right atria NO production was assessed determining NOS activity from different groups of animals by measuring the conversion of [^{14}C (U)]-L-arginine to [^{14}C (U)]-L-citrulline. Tissue homogenates (50 μg protein) were incubated in Tris–HCl buffer (pH 7.4) containing 1 μg/ml L-arginine, [^{14}C (U)]-L-arginine (346 μCi/ml), L-valine (67 mM), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and $CaCl_2$ (2 mM) for 60 min at room temperature. At the end of the incubation period, NOS reaction was arrested by addition of a buffer solution in ice containing 20 mM HEPES buffer and 20 mM EDTA, pH 5.5. Reaction mixtures were loaded onto cation exchange columns (Dowex AG 50W-X8, Na^+ form; Bio-Rad) and [^{14}C (U)]-L-citrulline was eluted from columns with 0–50 ml ddH_2O. The amount of [^{14}C (U)]-L-citrulline eluted was quantified using a liquid scintillation counter (Wallac 1414 WinSpectral; EG&G Company, Turku, Finland). All compounds, except [^{14}C]-L-arginine monohydrochloride (346 mCi/mmol, Amersham Life Science), were purchased from Sigma Chemicals. Protein determination

was made using the Lowry method, using bovine serum albumin as a standard.

2.6. Western blot analysis

Left ventricular and right atria samples were homogenized on ice with a Tissue Homogenizer (Biospec Products Inc.) in homogenization buffer (50 mmol/l Tris, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1% Triton, 1 mmol/l PMSF, 1 μmol/l pepstatin, 2 μmol/l leupeptin, 1× protease inhibitor cocktail from Roche Diagnostics). Protein concentration in the Triton-soluble supernatant was determined using the Lowry assay. Equal amounts of protein (100 μg protein/lane) were separated by electrophoresis in 7.5% SDS-polyacrylamide gels (Bio-Rad, Munich, Germany), transferred to a nitrocellulose membrane (Bio-Rad) and then incubated with rabbit polyclonal anti-NOS or anti-cav isoform antibodies, both diluted at 1:500. The primary antibodies were polyclonal rabbit anti-inducible NOS (iNOS) (epitope at the carboxyl terminus), anti-endothelial NOS (eNOS) (epitope at the amino terminus), anti-neuronal NOS (nNOS) (epitope at the amino terminus), anti-cav-1 (H-97, sc-7875) and anti-cav-3 (H-100, sc-28828). Finally, a secondary immunoreaction with a goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:5000) was performed. Samples were revealed by chemiluminescence using ECL reagent for 2–4 min. Density of the respective bands was quantified by densitometry scanning of Western blots using a Hewlett-Packard scanner and Total lab analyzer software (Biodynamics Corp., Seattle, WA, USA) and protein amounts were calculated by comparison to the densitometry values of the corresponding standard [15]. Protein levels were expressed as a ratio of the optical densities of NOS and cav isoforms and β-actin band (using anti-beta actin, clone EP1123Y, rabbit monoclonal antibody) to check for any inaccuracies in protein loading. The antibodies for the three NOS isoforms (iNOS (610333), eNOS (610298) and nNOS (610311)) were supplied by BD Biosciences and anti β-actin by Millipore (04-1116). Antibodies anti-cav-1 (sc-7875) and -3 (sc-28828) were supplied by Santa Cruz Biotechnology, Inc. and the secondary antibody (170-6515) by Bio-Rad Laboratories. The Western Blot Detection System and Hybond-ECL membranes were supplied by Amersham Pharmacia Biotech. Biochemicals were supplied by Sigma Chemical Co. (Saint Louis, MO, USA). L-[^{14}C (U)] arginine substrate was supplied by Perkin Elmer Life and Analytical Sciences, Boston, MA, USA. A Wallac 1414 WinSpectral (EG&G Company, Turku, Finland) liquid scintillation counter was used. AG 50W-X8 cation exchange resin was supplied by BIO-RAD Laboratories.

2.7. Thiobarbituric acid reactive substances

Heart samples from all groups of animals were homogenized with a glass-Teflon homogenizer in a medium consisting of 120 mM KCl and 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 [12]. Oxidative damage to phospholipids was evaluated in supernatant as thiobarbituric acid reactive substances (TBARS) by a fluorometric assay. Tissue homogenate (100 μL) was 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 1000 × *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.

2.8. Morphologic analysis

At the end of the experimental protocol, another group of animals from each experimental group, from both, 2-month-old and 16-month-old rats, were anesthetized with urethane (1.0 g/kg, intraperitoneally) and were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cardiac tissue was removed and tissues were fixed in formol buffer 10% in PBS 0.1 M (pH 7.4). The tissue sections were dehydrated and included in paraffin. Sagittal cuts (5 μm) were made with a microtome (Leica RM2125, Wetzlar, Germany) and mounted on 2% silane-coated slides. The slides were stained with Mallory's trichrome and they were observed by light microscopy (Nikon Eclipse 200, NY, USA).

2.9. Statistical analysis

Data in tables and figures are expressed as mean ± 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 the Shapiro–Wilk test, and the Levene's test was used to evaluate the homogeneity of variances. When data distribution was not normal, nonparametric Kolmogorov–Smirnov test was applied. When SD presented statistically significant differences, Tamhane's T2 test was used for post hoc comparisons. The Statistical Product and Service Solutions for Windows 9.0 (SPSS, Inc., Chicago, IL) were used for statistical analysis.

3. Results

3.1. General data and plasma parameters of young and adult rats

Basal values reported in Table 1 are from the WR group at the beginning of the experiments, before water restriction cycles. Basal parameters were determined in all animals from each experimental group of both ages. Adult animals had a higher body weight and a lower food intake than young rats. In both groups of animals, body weight was decreased (young: 36%, adult: 32%), despite the fact that food consumption did not vary significantly during this experimental period in both groups of animals (Table 1). The decreased body weight was accompanied by other observable changes, such as dry skin and mucosa (skinfold positive sign), cold extremities, weakness and increased thirst (which was evaluated on the hydration day of the last cycle). This last parameter was significantly higher in young rats (young basal: 12.5 ± 1.2 ml/24 h 100 g, young WR: 31.0 ± 1.6 ml/24 h 100 g and adult basal: 7.74 ± 0.4† ml/24 h 100 g, adult WR: 23.5 ± 1.7 ml/24 h 100 g; $^{*}P < 0.05$ versus basal, $^{\dagger}P < 0.05$ versus young). Dehydration cycles caused hemoconcentration, which was evidenced by increased hematocrit, plasma osmolarity and serum sodium.

Table 1
Body weight, food intake and plasma parameters.

Parameter	Young		Adult	
	BV	WR	BV	WR
Body weight (g)	303 ± 11	194 ± 7*	611 ± 14‡	414 ± 16*,‡
Food intake (g/24 h 100 g)	8.91 ± 0.51	8.54 ± 0.39	4.76 ± 0.70†	6.71 ± 0.49
Hematocrit (%)	47 ± 1	56 ± 1*	49 ± 1	58 ± 2*
Plasma osmolarity (mOSM)	310 ± 2	355 ± 7*	308 ± 2	337 ± 4*
Serum Na^{+} (mEq/l)	133 ± 1	138 ± 1*		140 ± 1*

Changes in body weight, food intake and plasma parameters of basal values (BV) and water restriction (WR) groups in young and adult animals. Data are expressed as mean ± SD ($n = 15$ per group).
* $p < 0.05$ versus respective BV.
† $p < 0.05$ versus respective WR.
‡ $p < 0.05$ versus respective young rats (BV, WR young rats).

Table 2
Urinary parameters.

Parameter	Young		Adult	
	BV	WR	BV	WR
Urine volume (ml/24 h)	12.0 ± 0.7	3.6 ± 0.5*	17.1 ± 0.7‡	4.9 ± 0.6*
Urine osmolarity (mOSM)	2533 ± 62	3198 ± 64*	1773 ± 59‡	2701 ± 99*,‡
COSM (μl/min)	33.0 ± 1.2	9.7 ± 1.1*	50.5 ± 1.7‡	25.6 ± 2.0*,‡

The effect of aging and water restriction on urinary parameters of basal values (BV) and water restriction (WR) groups in young and adult rats. Data are expressed as mean ± SD ($n = 15$ per group).
* $p < 0.05$ versus respective BV.
† $p < 0.05$ versus respective WR.
‡ $p < 0.05$ versus respective young rats (BV, WR young rats).

Table 3
Echocardiographic data.

Parameter	Young		Adult	
	CR	WR	CR	WR
EDV (ml)	0.11 ± 0.01	0.09 ± 0.01	0.26 ± 0.02†	0.27 ± 0.01†
EVS (ml)	0.020 ± 0.001	0.013 ± 0.002	0.070 ± 0.001†	0.067 ± 0.002†
SV (ml)	0.090 ± 0.003	0.080 ± 0.011	0.193 ± 0.021†	0.220 ± 0.015
EF (%)	81 ± 3	82 ± 2	73 ± 4	76 ± 2

End-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF) and systolic volume (SV) of control restriction (CR) and water restriction (WR) groups in young and adult animals. Data are expressed as mean ± SD ($n = 15$ per group).
* $P < 0.05$ versus respective CR.
† $P < 0.05$ versus respective young rats.

3.2. Urinary parameters

The urinary parameters are shown in Table 2. Adult animals had a greater urine volume, urine osmolarity and osmolar clearance than young rats. Urine volume was reduced after dehydration in 2- and 16-month-old rats. Urine osmolarity was increased after water restriction, this parameter being higher in the younger animals. These osmolarity changes were correlated with osmolar clearance results.

3.3. Hemodynamic and echocardiographic parameters

Adult animals had a similar SBP and a greater HR than young rats (Fig. 2). After water restriction, young and adult animals exhibited reduced SBP in comparison to age-matched control animals. HR shows a different pattern in dehydration state with aging; whereas there is an increase of this parameter in the young group, the aged animals showed reduced HR. Table 3 shows echocardiographic data for all groups. We observed a significant increase in EDV, ESV and SV volumes with the advance of age, without changes after water restriction. Moreover, EF is maintained with age and water restriction.

3.4. Nitric oxide synthase in young and adult rats

Fig. 3A illustrates total NOS activity from right atria in young and adult rats. The results show that control adult rats exhibited lower NOS activity than control young rats. After water restriction, atrial NOS activity was reduced in both young and adult rats compared with age-matched control animals. Fig. 3B–D illustrates representative Western blot analysis in this tissue from all groups of animals. The accompanying histograms illustrate the ratio between the mean values of NOS protein levels and β-actin marker for the different groups. Atrial eNOS, iNOS and nNOS protein levels were lower in control adult animals compared with young ones. No changes were observed after water restriction. Fig. 3E shows that cav-1 protein levels were higher in control adult than in control young rats, and they were increased in both groups submitted to dehydration. Regarding cav-3, no differences were observed between young and adult control animals. Young and adult animals showed similar cav-3 protein levels compared with age-matched animals after controlled water restriction (Fig. 3F). Fig. 4A illustrates that adult animals exhibited enhanced ventricular NOS activity in comparison to young rats. Additionally, in the water restricted groups, activity of the enzyme was lower than in control age-matched rats. Fig. 4B shows that ventricular eNOS protein levels were lower in adult animals compared with young animals. iNOS and nNOS protein levels were similar between young and adult rats (Fig. 4B and C). Fig. 4B–D shows that water restriction did not change NOS protein levels in both groups of animals compared to control groups. Adult animals had a lower cav protein levels than young ones (Fig. 4E and F). Water restriction increases both cav-1 and -3 protein levels in young and adult animals (Fig. 4E and F).

3.5. TBARS levels

Adult animals had greater TBARS levels in heart than young rats. Water restriction protocol did not change these levels in young nor in adult rats (Fig. 5).

3.6. Morphologic cardiac examination

Cardiac morphology was evaluated by Mallory's trichrome to detect areas of fibrosis. Adult animals had a similar staining than young rats (Fig. 6a and c). Fig. 6b and d showed the presence of fibrosis after water restriction in both age groups of animals, this increase being greater in adult rats (CR 32%; WR 116*% young rats and CR 36%; WR 208*,†% adult rats *$P < 0.05$ versus respective WR; †$P < 0.05$ versus respective young rats).

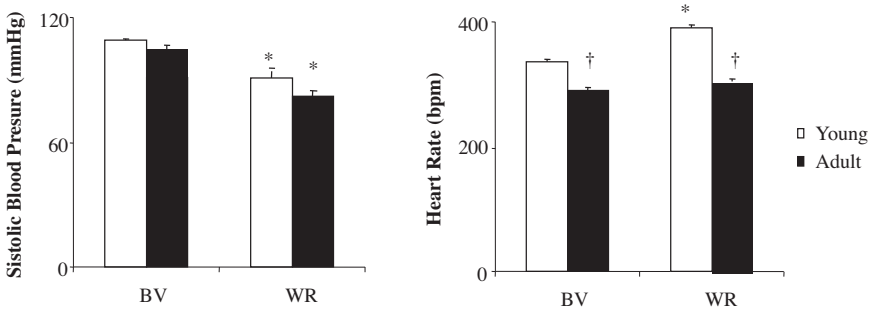

Fig. 2. Systolic blood pressure and heart rate of basal values (BV) and water restriction (WR) groups in young and adult rats. Data are expressed as mean ± SD ($n = 15$ per group), *$P < 0.05$ versus respective WR; †$P < 0.05$ versus respective young rats.

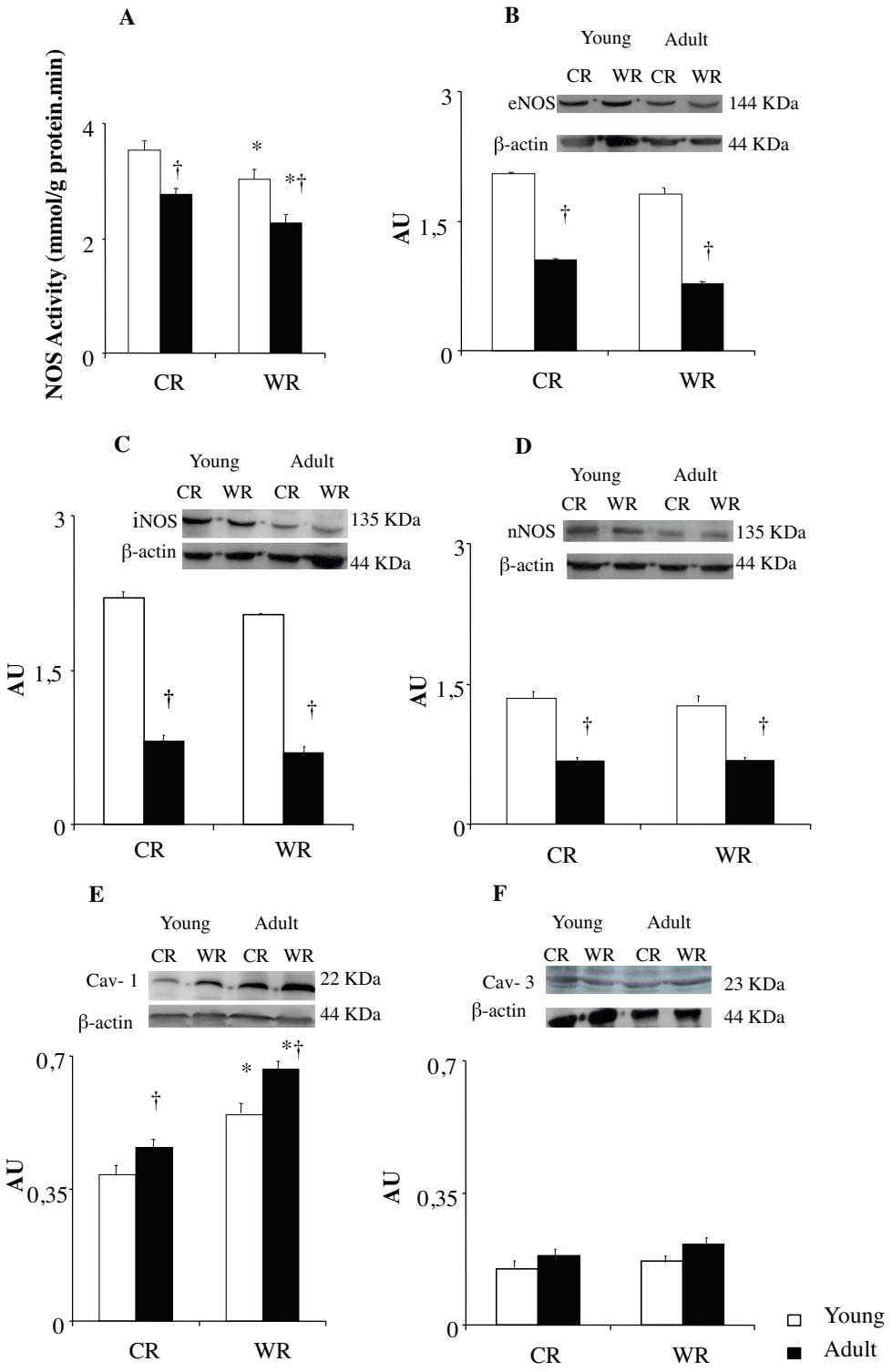

Fig. 3. Total nitric oxide synthase (NOS) activity in atria (A) from control restriction (CR) and water restriction (WR) in young and adult rats. All values are mean ± SEM; $n = 7$; $^{*}P < 0.05$ versus age-matched CR rats; $^{\dagger}P < 0.05$ versus respective young rats. Representative Western blots of eNOS (B), iNOS (C), nNOS (D), caveolin-1 (E) and caveolin-3 (F) carried out on proteins from atria. Histograms illustrate mean NOS protein values for each group. All experiments were performed in triplicate. Each blot was normalized with the expression of the β-actin from the same gels. Data are mean ± SEM; $n = 7$; $^{*}P < 0.05$ versus age-matched CR rats; $^{\dagger}P < 0.05$ versus young rats.

4. Discussion

It is known that NO has been involved in several physiological and pathophysiological processes controlling myocardial function, but still little is known about NO impact on myocardium during hypovolemic state induced by a controlled dehydration during aging. Previous work done in our laboratory showed that water restriction during 24 and 72 h induced changes in cardiovascular NOS activity without altering NOS protein levels in young and adult animals. These findings indicate that the involvement of NO in the regulatory mechanisms during dehydration depends on the magnitude of the water restriction and on age [13].

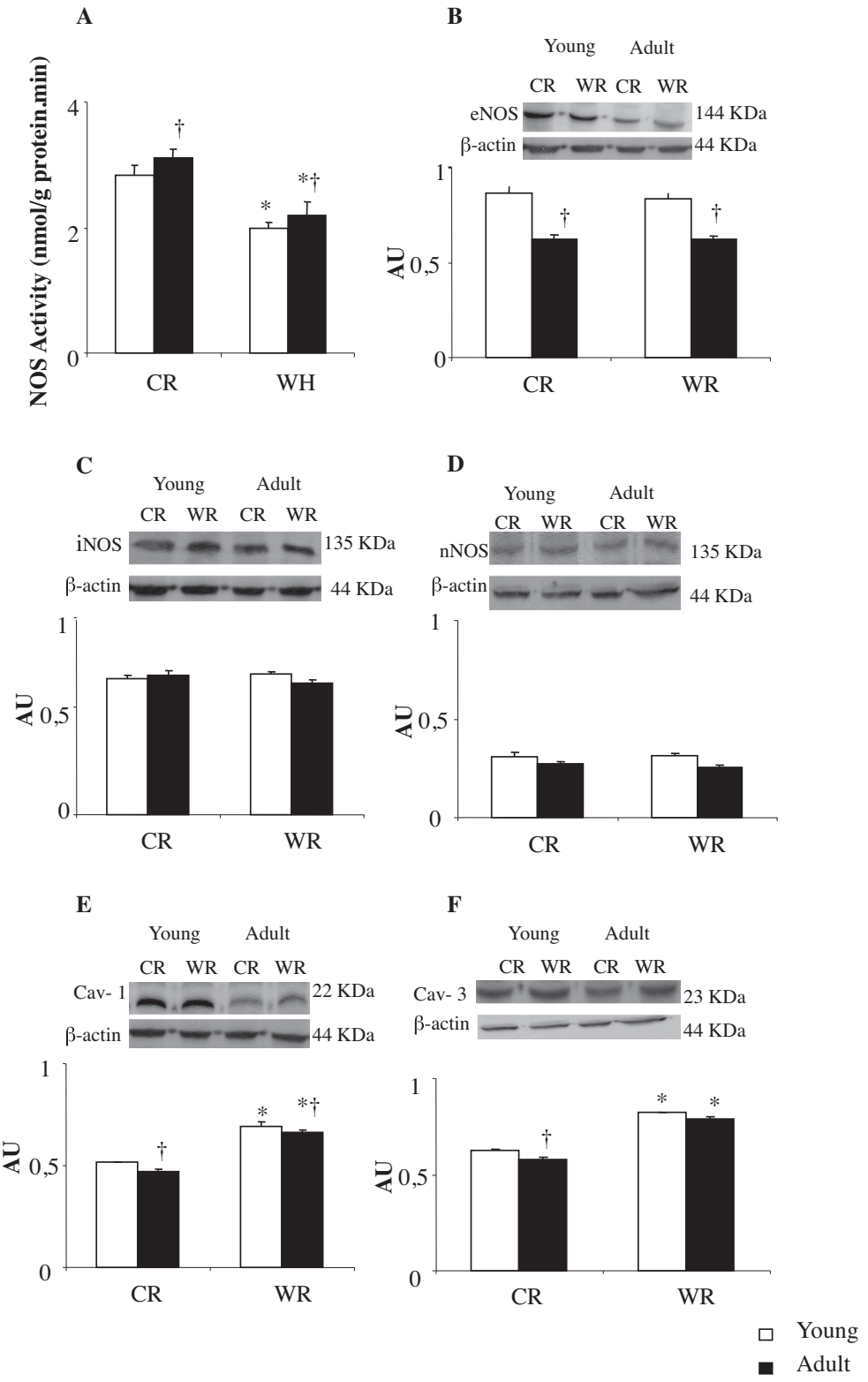

Fig. 4. Total nitric oxide synthase (NOS) activity in left ventricle (A) from control restriction (CR) and water restriction (WR) in young and adult rats. All values are mean ± SEM; $n = 7$; $^{*}P < 0.05$ versus age-matched CR rats; $^{\dagger}P < 0.05$ versus respective young rats. Representative Western blots of eNOS (B), iNOS (C), nNOS (D), caveolin-1 (E) and caveolin-3 (F) carried out on proteins from left ventricle. Histograms illustrate mean NOS protein values for each group. All experiments were performed in triplicate. Each blot was normalized with the expression of the β-actin from the same gels. Data are mean ± SEM; $n = 7$; $^{*}P < 0.05$ versus age-matched CR rats; $^{\dagger}P < 0.05$ versus young rats.

Since there is little information about the degree of dehydration that can be caused by a long-term liquid restriction, we decided to first evaluate different parameters to confirm that our experimental model induced a hypovolemic state. Dehydration is classified as severe when body weight loss is superior to 5% [14]. Our results showed that dehydration state was severe in both cases: there was a 32% and 29% loss of body weight in young and adult rats, respectively. Hematocrit, plasma osmolarity and serum

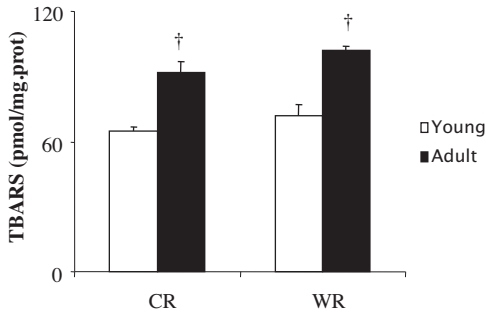

Fig. 5. TBARS levels in heart of control restriction (CR) and water restriction (WR) groups in young and adult rats. Data are expressed as mean ± SD (n = 15 per group), $^{*}P < 0.05$ versus respective WR; $^{\dagger}P < 0.05$ versus respective young rats.

sodium increased confirming fluid loss and dehydration in both young and adult rats. Our findings suggest that adult rats had a lower ability to concentrate urine than young ones, reflecting that the observed changes depend on age and not on water restriction magnitude.

Getting into focus the involvement of NO in the modulation of the cardiovascular adaptation to the hypovolemic state, our results show that there is a reduction in atrial NOS activity associated to the aging process, accompanied by decreased HR in the control adult group. Regarding HR, chronotropic changes with aging are still controversial; some reports suggest no changes in HR [15], while others showed that this parameter decreased in aged rats [16]. So, it is probable that the effect of NO on pacemaker activity would be different with the advance of age. Additionally, we cannot disregard that other neurohumoral factors and/or basal sympathetic tone are also involved in the chronotropic changes [17,18]. Echocardiographic data showed that adult rats have larger ESV, EDV and SV; meanwhile SBP was similar in control young and adult rats. Therefore, NO acting as a chronotropic modulator facilitates the increase in diastolic time and in consequence increasing ESV, EDV and VS to maintain EF in adult rats, which may contribute to maintain SBP during the aging process. When atria NOS isoform protein levels of adult animals were analyzed, it was determined that the decrease in NOS activity was caused by a reduction of three NOS isoform protein levels. Moreover, adult animals showed increased cav-1 protein levels in this tissue. These results may explain the decreased enzymatic activity in the oldest group. Ostrom et al. [19] suggest that cav abundance may alter cellular function with advancing age, considering that NO generated by eNOS has a significant physiological role in maintaining an appropriate microvascular tone and blood flow and exhibits protective effects. Cardiac alterations observed with the advance of age may be also associated with a reduction in NO bioavailability as a consequence of increased oxidative stress, as lipid peroxidation was increased in comparison to the youngest control group (Fig. 5). Additionally, our results showed that cardiac TBARS levels were exacerbated in adult animals compared with young rats, independent of when they were submitted to water restriction. In response to water restriction, the decreased NOS activity observed in the atria of young and adult rats was associated to an increased cav-1. Ratajczak et al. showed that cav dissociation from caveolae is associated to aging and heart dysfunction [10]. The obtained results correlate with HR changes during water restriction in the youngest group. However, in adult rats, HR remained unchanged, which may be explained by an age-related decreased response during hypovolemic state. Moreover, there is increasing data that indicate that NO also modulates several components of excitation-contraction coupling. Therefore, increased NO production in adult ventricular tissue of control animals may be compensating the age-related contractile dysfunction. Higher ventricular NOS activity in adult animals would be caused by a reduction in both cav protein levels. Kawabe and coworkers observed that cav are down-regulated during the aging process [20]. We suggest that age-related changes in ventricular NOS activity are mainly a consequence of altered modulation of enzymatic activity by cav in adulthood. Some pathways for down-regulating caveolin-1 expression (MAP kinase pathways, Src family kinases) and post-translational modifications such as ubiquitination and phosphorylation have emerged as important regulators of protein stability and function [21]. Supporting this fact, during water restriction, in both age groups, the reduced NOS activity is associated to increased levels of cav-1 and cav-3. The reduction in enzyme activity impact on ventricular function to preserve the EF during the osmotic stress is induced in this experimental model.

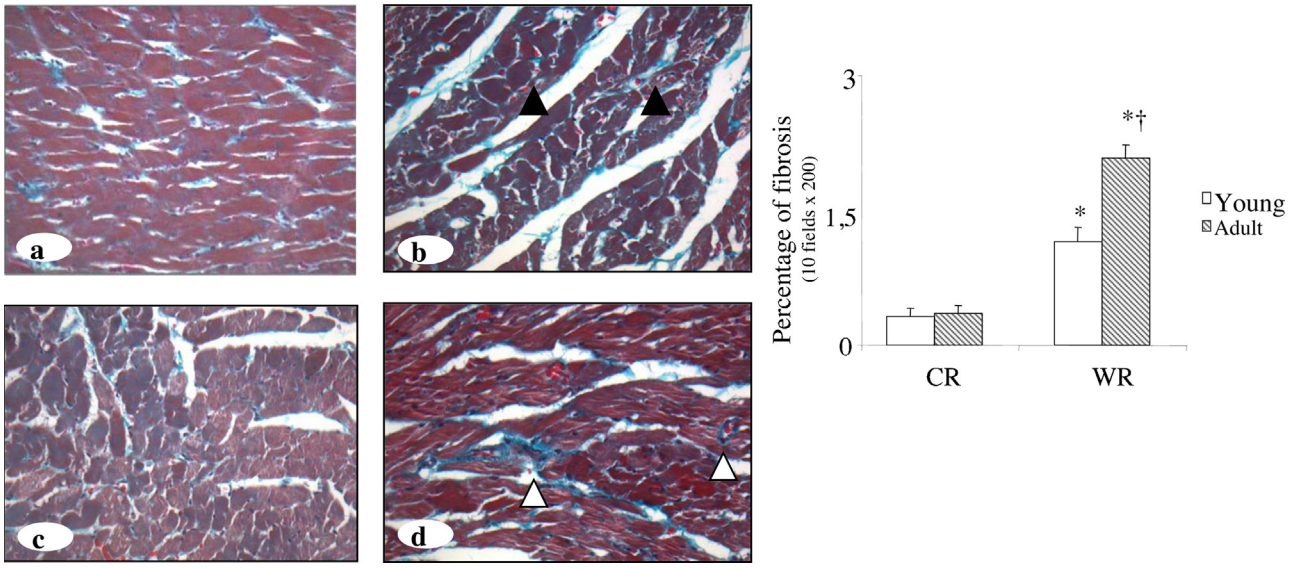

Fig. 6. Fibrosis detection. Areas of fibrosis stained in blue were observed in water restriction young rats (b, black arrowheads) as compared to controls (a), and in water restriction adult rats (d, white arrowheads) compared to control (c). Mallory's trichrome, 200×. Using a grid superimposed on the image, the number of points overlapping the blue collagen staining was counted, and the percentage of blue collagen area in the examined area was measured. Data are expressed as mean ± SD (n = 15 per group), $^{*}P < 0.05$ versus respective WR; $^{\dagger}P < 0.05$ versus respective young rats.

Considering that fibrosis is measured primarily by histological methods, we evaluated Mallory's trichrome stain as biomarker of aging in cardiac tissue to assess how modulation of the advance of age influences the rate and degree of cardiac cellular level. Surprisingly, adult animals had higher lipid peroxidation than young rats; however, Mallory's trichrome stain revealed no changes between young and adult control animals. These findings could suggest that the age of adult rats was not sufficiently senescent to alter myocardial cytoarchitecture. Water restriction caused more severe structural alterations in heart tissue, older animals being more susceptible to osmotic stress. Thus, NO pathways among other mechanisms (natriuretic peptides, bradykinin, and angiotensin II, among others) changes in order to adapt cardiac chambers to maintain function in an adult dehydrated heart.

In conclusion, the novel data of the present work demonstrate that dehydration state induced by water restriction triggers different regulatory mechanisms that involve cardiac NO system and caveolins in order to preserve cardiac function. Modifications in NO bioavailability could be compensating the age-associated functional and structural changes in a heart under osmotic stress.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by the Universidad de Buenos Aires grants B010 and B605 and Instituto de Química y Metabolismo del Fármaco-Consejo Nacional de Investigaciones Científicas y Tecnológicas (IQUIMEFA-CONICET).

References

[1] Horswill CA, Janas LM. Hydration and health. Am J Lifestyle Med 2011;5:304–15.
[2] Gharbi N, Mornagui B, El-Fazaa S, Kamoun, Gharib C. Effect of dehydration on nitric oxide, corticotropic and vasopressinergic axis in rat. C R Biol 2004;327:12–20.
[3] Knight WD, Ji LL, Little JT, Cunningham J. Dehydration followed by sham rehydration contributes to reduced neuronal activation in vasopressinergic supraoptic neurons after water deprivation. Am J Physiol Regul Integr Comp Physiol 2010;299:R1232–40.
[4] Goldspink DF, Burniston JG, Tan LB. Cardiomyocyte death and the ageing and failing heart. Exp Physiol 2003;88:447–58.
[5] Centurione DC, Giulio C, Cacchio M, Rapino M, Bosco D, Grifone G, et al. Correlations between protein kinase c (zeta) signaling and morphological modifications during rat heart development and aging. Mech Ageing Dev 2005;124:957–66.
[6] Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, et al. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature 2002;416:337–9.
[7] Sase K, Michel T. Expression and regulation of endothelial nitric oxide synthase. Trends Cardiovasc Med 1997;7:28–37.
[8] Chidlow JH, Sessa WC. Caveolae, caveolins, and cavins: complex control of cellular signalling and inflammation. Cardiovasc Res 2010;86:219–25.
[9] Hirai DM, Copp SW, Hageman KS, Poole DC, Musch TI. Aging alters the contribution of NO to regional muscle hemodynamic control at rest and during exercise in rats. J Appl Physiol 2011;111:989–98.
[10] Ratajczak P, Damy T, Heymes C, Oliviéro P, Marotte F, Robidel E, et al. Caveolin-1 and -3 dissociations from caveolae to cytosol in the heart during aging and after myocardial infarction in rat. Cardiovasc Res 2003;57:358–69.
[11] Arreche ND, Sarati LI, Martinez CR, Fellet AL, Balaszczuk AM. Contribution of caveolin-1 to ventricular nitric oxide in age-related adaptation to hypovolemic state. Regul Pept 2012;179(1–3):43–9.
[12] Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi EA. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch Biochem Biophys 2001;388:261–6.
[13] Fellet AL, Arza PR, Nunez N, Arranz CT, Balaszczuk AM. Hypovolemic state: age-related influence of water restriction on cardiac nitric oxide synthase in rats. Eur J Nutr 2011;50(8):657–64.
[14] Wathen JE, MacKenzie T, Bothner JP. Usefulness of the serum electrolyte panel in the management of pediatric dehydration treated with intravenously administered fluids. Pediatrics 2004;114:1227–34.
[15] Pestana M. Hypertension in the elderly. Int Urol Nephrol 2001;33:563–9.
[16] Irigoyen MC, Moreira ED, Werner A, Ida F, Pires MD, Cestari IA, et al. Aging and baroreflex control of RSNA and heart rate in rats. Am J Physiol Regul Integr Comp Physiol 2000;279:R1865–71.
[17] Sears C, Ashley E, Casadei B. Nitric oxide control of cardiac function: is neuronal nitric oxide synthase a key component? Phil Trans R Soc B 2004;359:1021–44.
[18] Moreno C, Lopez A, Llinas MT, Rodriguez F, Lopez-Farre A, Nava E. Changes in NOS activity and protein expression during acute and prolonged Ang II administration. Am J Physiol 2002;28:31–7.
[19] Ostrom SR, Liu X, Head BP, Gregorian C, Seasholtz TM, Insel PA. Localization of adenylyl cyclase isoforms and G protein-coupled receptors in vascular smooth muscle cells: expression in caveolin-rich and noncaveolin domains. Mol Pharmacol 2002;62:983–92.
[20] Kawabe JI, Grant BS, Yamamoto M, Schwencke C, Okumura S, Ishikawa Y. Changes in caveolin subtype protein expression in aging rat organs. Mol Cell Endocrinol 2001;176(1–2):91–5.
[21] Engelman JA, Zhang XL, Razani B, Pestell RG, Lisanti MP. p42/44 MAP kinase-dependent and -independent signaling pathways regulate caveolin-1 gene expression. J Biol Chem 1999;274:32333–41.