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Vanina A. Netti, Mariana C. Vatrella, Melina F. Chamorro, María I. Rosón, Elsa Zotta, Andrea L. Fellet & Ana M. Balaszczuk

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ORIGINAL CONTRIBUTION

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Abstract
Purpose Aquaporin-1 (AQP1) is the predominant water channel in the heart, linked to cardiovascular homeostasis. Our aim was to study cardiovascular AQP1 distribution and protein levels during osmotic stress and subsequent hydration during postnatal growth.
Methods Rats aged 25 and 50 days were divided in: 3d-WR: water restriction 3 days; 3d-WAL: water ad libitum 3 days; 6d-WR+ORS: water restriction 3 days + oral rehydration solution (ORS) 3 days; and 6d-WAL: water ad libitum 6 days. AQP1 was evaluated by immunohistochemistry and western blot in left ventricle, right atrium and thoracic aorta.
Results Water restriction induced a hypohydration state in both age groups (40 and 25 % loss of body weight in 25- and 50-day-old rats, respectively), reversible with ORS therapy. Cardiac AQP1 was localized in the endocardium and endothelium in both age groups, being evident in cardiomyocytes membrane only in 50-day-old 3d-WR group, which presented increased protein levels of AQP1; no changes were observed in the ventricle of pups. In vascular tissue, AQP1 was present in the smooth muscle of pups; in the oldest group, it was found in the endothelium, increasing after rehydration in smooth muscle. No differences were observed between control groups 3d-WAL and 6d-WAL of both ages.
Conclusion Our findings suggest that cardiovascular AQP1 can be differentially regulated in response to hydration status in vivo, being this response dependent on postnatal growth. The lack of adaptive mechanisms of mature animals in young pups may indicate an important role of this water channel in maintaining fluid balance during hypovolemic state.

Keywords Aquaporin-1 · Dehydration · Heart · Postnatal growth · Vascular tissue

V. A. Netti (✉) · M. C. Vatrella · M. F. Chamorro · A. L. Fellet · A. M. Balaszczuk
Cátedra de Fisiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, IQUIMEFA, CONICET, Junín 956, C1113AAD Buenos Aires, Argentina
e-mail: vnetti@conicet.gov.ar

M. I. Rosón
Cátedra de Fisiopatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires, Argentina

E. Zotta
Laboratorio de Fisiopatogenia, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Buenos Aires, Argentina

Introduction

During postnatal period, water is the quantitatively most essential nutrient [1]. Dehydration is defined as a state of body water content deficit, due to primary losses or inadequate water intake, resulting in a hypohydration state. At early stages of life, individuals are more susceptible to hypohydration than adults, because of their smaller body weights and different fluid distribution [2, 3]. In previous work done in our laboratory, we demonstrated that cardiovascular response to a 24- and 72-h water restriction period is different in 2- and 12-month-old rats [4]. However, cardiovascular response to hypohydration has not been fully studied in early stages of life. It is well known that cardiac function is enhanced from weaning age to adult life [5], accompanied by functional and anatomical changes in the cardiovascular tissue and autonomic nervous

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system maturation [6]. These age-related changes implicate that osmotic stress may impact differently on the postnatal heart. In addition, many clinical and experimental studies have described that inadequate hydration during postnatal period may have consequences on cardiovascular system in adulthood [7, 8].

It is well known that water handling in the heart relies on a family of membrane proteins called aquaporins [9]. In cardiovascular system, Aquaporin-1 (AQP1) is the quantitatively predominant aquaporin, which is expressed in the heart [10] as well as in blood vessels [11, 12]. Its functional relevance was evidenced in knockout mice, where cardiomyocyte water permeability was significantly reduced only in the absence of AQP1 [10]. Even though this water channel has been linked to cardiovascular homeostasis, its physiological role still remains to be explored [13]. Alterations of AQP1 expression were observed during myocardial edema, suggesting that this protein may be important in water handling during pathological states [9]. In agreement with this, research in animal models demonstrated that this channel participates in promoting angiogenesis in the ischemic myocardium [14] and in the cardiac response to chronic anemia [15]. Additionally, it was reported that AQP1 is upregulated in the hypertonic environment. In vitro, Jenq et al. [16] showed that there is an induction of AQP1 protein after NaCl supplementation. This was also described in the kidney and in the peritoneum [17, 18]. However, changes in cardiovascular AQP1 expression during osmotic stress have not been fully explored in in vivo experiments.

Considering that the first month of life is an attractive period to study the transition from the fetal/neonatal to the adult life, and that during this stage, individuals are at risk of hypohydration, the objective of this study was to validate the hypothesis that cardiovascular AQP1 may be altered during osmotic stress and subsequent hydration, being this response dependent on the stage of postnatal growth studied. With this aim, we evaluated the effects of water restriction and rehydration on AQP1 protein levels and distribution in the left ventricle, right atria and thoracic aorta of 25- and 50-day-old rats.

Materials and methods

Experiments were conducted on male Sprague-Dawley rats from the breeding laboratories of the School of Pharmacy and Biochemistry (University of Buenos Aires). Newborn rats were maintained with their dam until day 21 (weaning age). At the age of 25 and 50 days, the animals were housed individually in metabolic cages specially designed 2 days before the experiments in order to adapt to the new environment and kept with an automatic light/dark cycle of 12 h/12 h, fed with standard rat chow and received tap water ad libitum until the beginning of the experiments. 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 groups (see Fig. 1)

Animals aged 25 and 50 days were randomly assigned as follows:

3d-WR: Rats were deprived of water for 72 h but had continuous access to food ($n = 10$).

3d-WAL: Animals had continuous access to food and water during the 3-day experimental period, representing a normohydrated group ($n = 10$).

6d-WR+ORS: Rats deprived of water for 72 h received oral rehydration solution (ORS) ad libitum for 72 h ($n = 10$).

6d-WAL: Animals had continuous access to food and water for 6 days ($n = 10$). This group was included as a control group in order to compare rehydrated animals

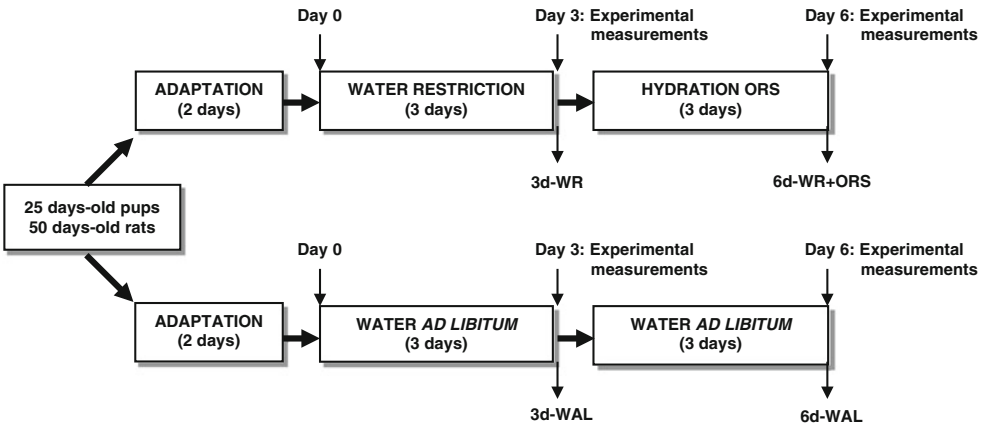

Fig. 1 Experimental protocol (see description in Sect. 2.1)

with animals of their same age. They also represent a normohydrated group.

Commercial ORS formulation was prepared as recommended by WHO [19]: NaCl 2.6 g; KCl 1.5 g; trisodic citrate 2.9 g; and glucose 13.5 g diluted in 1 l of water.

At the end of each experimental period and with the purpose of validating if water restriction established a hypohydration state, we determined in all animals ($n = 10$ per group): body weight, total water body content, weight of adrenal glands and biochemical parameters. All animals from each group were anesthetized with ethyl urethane (1.5 g/kg body weight, i.p.), and a cannula was inserted in the abdominal aorta to perfuse the heart and thoracic aorta with saline to avoid contamination of erythrocytes, since AQP1 is present in red blood cells plasma membrane [20]. Then, right atrium, left ventricle and thoracic aorta segments from all experimental groups were isolated to study AQP1 localization ($n = 5$) and protein levels ($n = 5$).

Body weight, total water body content and weight of adrenal glands

Body weight, food and water consumption (except for 3d-WR group) were determined everyday to assess the health status of the animals. Total body water content was determined by measuring wet and dry weight of each rat after total dissection as previously described [21]. Weight of adrenal glands was measured postmortem as a stress indicator and standardized by body weight.

Biochemical parameters

Blood collections were made to determinate serum Na^+, hematocrit and plasma osmolarity. Serum Na^+ was measured using an ion-selective analyzer t-410 (Tecnolab). Plasma osmolarity was measured by a micro-osmometer (μosmette TM Micro-Osmometer). Hematocrit was determined from duplicate blood-filled hematocrit tubes.

AQP1 immunohistochemistry

Left ventricle, right atrium and thoracic aorta were fixed in phosphate-buffered 10 % formaldehyde (pH 7.2) and embedded in paraffin using conventional histological techniques. Paraffin sections were cut at 3 mm, deparaffined and rehydrated. Endogenous peroxidase activity was blocked by treating with 0.5 % H_2O_2 in methanol for 30 min. AQP1 was detected using a rabbit antibody (1:500). Immunostaining was carried out using a commercially modified avidin–biotin–peroxidase complex technique, Vectastain ABC kit (Universal Elite, Vector Laboratories) and counterstained with hematoxylin. The sections were observed by light microscopy (Nikon Eclipse 200). Images were acquired by using Nikon Digital SIGHT D5-Fi1 and NIS Elements-F 3.0 software. Negative controls were performed by omitting primary antibody.

Western blot

Left ventricle, right atrium and thoracic aorta 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 12 % SDS–polyacrylamide gels (Bio-Rad), transferred to a nitrocellulose membrane (Bio-Rad) and blocked with 5 % nonfat milk. Membranes were incubated with a rabbit antibody against AQP1 (dilution 1:500) and an anti-rabbit antibody conjugated with HSP (dilution 1:6,000). Samples were revealed by chemiluminescence using ECL reagent for 2–4 min. Blots were then stripped and reincubated with a rabbit antibody against β-actin (dilution 1:2,000). 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 the AQP1 and β-actin band to detect inaccuracies in protein loading.

Materials

The antibody against AQP1 (H-55) was a rabbit polyclonal antibody raised against amino acids 215–269 of AQP1 of human origin purchased from Santa Cruz Biotechnology, Inc. Antibody specificity was tested in AQP1 −/− mice [22]. The antibody against β-actin was from BD, Biosciences. Western blot detection system and Hybond-ECL membranes were from Amersham Pharmacia Biotech. Drugs were from Sigma Chemical Co.

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 the Shapiro–Wilk test, and the Levene's test was used to evaluate the homogeneity of variances. When data distribution was not normal, nonparametric Kolmogórov–Smirnov test was applied. When SD presented statistically significant 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

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

Body weight, total body water content and weight of adrenal glands

Results are shown in Table 1. Water restriction caused a significant loss of body weight in both age groups, which was reversible with ORS treatment in both cases. 25-day-old pups from 6d-WAL group continued to grow, as their body weight was increased in comparison to 3d-WAL. Following ORS treatment, rehydrated 25-day-old rats did not reach the weight of animals of their same age (6d-WAL group), meanwhile the 50-day-old did. Additionally, as it is shown in Table 1, in 25-day-old pups, restraint of drinking water decreased total body water content and increased adrenal gland weight, being these changes reversed by ORS treatment. 50-day-old rats also presented decreased corporal water content, which returned to normal after ORS treatment. However, adrenal gland weight was not statistically different in this age group.

Biochemical parameters

Table 1 also shows that hematocrit values were larger for control 50-day-old rats compared to the 25-day-old group. Serum Na^+ and plasma osmolarity were not statistically different between the two 3d-WAL groups. After water restriction period, there was an increase in hematocrit in 25- (30 %) and 50-day-old rats (27 %). Additionally, both 3d-WR groups presented increased both Na^+ and plasma osmolarity. The mentioned alterations were reversed after ORS therapy.

Effects of water restriction and subsequent hydration on cardiovascular AQP1

AQP1 immunohistochemistry

To evaluate AQP1 cellular localization, we performed an immunohistochemical staining of the heart and aortic tissue. Results are shown in Fig. 2. Immunostaining of the left ventricle of 25- (left upper panel) and 50-day-old rats (bottom panel, a and b) showed AQP1 immunoreactivity in the endocardium and endothelial cells of capillaries and venules, but not in small arterioles. No arteriolar profile was found to be positive in all sections examined from both age groups. No modifications in staining density or labeling pattern were observed in the youngest 3d-WR, 6d-WAL or 6d-WR+ORS groups. However, in the 50-day-old rats, AQP1 was observed in the cardiomyocyte sarcolemma only after water restriction (bottom panel, c and d), being this change reversed by treatment with ORS. In the right atrium, AQP1 was also present in the endocardium and endothelial cells (25-day-old: middle upper panel, 50-day-old: bottom panel, e) and no changes were observed in 3d-WR or 6dWR+ORS of both age groups. In control pups, AQP1 was present mainly on vascular smooth muscle of thoracic aorta, being this pattern unchanged during osmotic stress or rehydration (right of upper panel). In the 50-day-old group, this protein was present on the endothelium of control rats and not in vascular smooth muscle (bottom panel, g and h), and we observed its presence on vascular smooth muscle only after ORS therapy (bottom panel, i).

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

	25-day-old rats				50-day-old rats			
	3d-WAL	3d-WR	6d-WAL	6d-WR+ORS	3d-WAL	3d-WR	6d-WAL	6d-WR+ORS
Body weight (g)	86 ± 11	50 ± 5*	107 ± 13*	81 ± 11$^{\#}$	246 ± 15†	177 ± 14*	276 ± 20	238 ± 20$^{\#}$
Body weight change (%)	100 ± 0	60 ± 12*	129 ± 11*	95 ± 10$^{\#}$	100 ± 0	75 ± 8*	109 ± 8	103 ± 9$^{\#}$
H_20 content (ml/100 g)	76.0 ± 3.2	71.2 ± 1.5*	74.1 ± 3.5	75.5 ± 3.6$^{\#}$	80 ± 2.5	74.4 ± 2.5*	79.2 ± 1.7	78.8 ± 1.6$^{\#}$
Adrenal weight (mg/100 g)	12.4 ± 2.3	17.5 ± 4.0*	10.6 ± 2.4	12.5 ± 3.2$^{\#}$	8.6 ± 1.9†	11.1 ± 1.1	7.6 ± 1.3	8.4 ± 0.8
Hematocrit (%)	43 ± 1	56 ± 1*	45 ± 1	45 ± 1$^{\#}$	49 ± 1†	62 ± 1*	46 ± 1	48 ± 1$^{\#}$
Seric Na^+ (mEq/L)	134 ± 1	138 ± 2*	134 ± 2	134 ± 3$^{\#}$	136 ± 3	143 ± 4*	138 ± 1	138 ± 2$^{\#}$
Plasma Osm (mOsm)	317 ± 2	344 ± 2*	323 ± 1	323 ± 1$^{\#}$	321 ± 2	362 ± 2*	327 ± 2	326 ± 1$^{\#}$

Results correspond to the last day of each experimental period and they are expressed as mean ± SD. Percentage of body weight change was calculated comparing each group to 3d-WAL of each age

* $p < 0.001$ versus respective 3d-WAL; $^{\#}$ $p < 0.001$ versus respective 3d-WR; † $p < 0.001$ versus 25-day-old

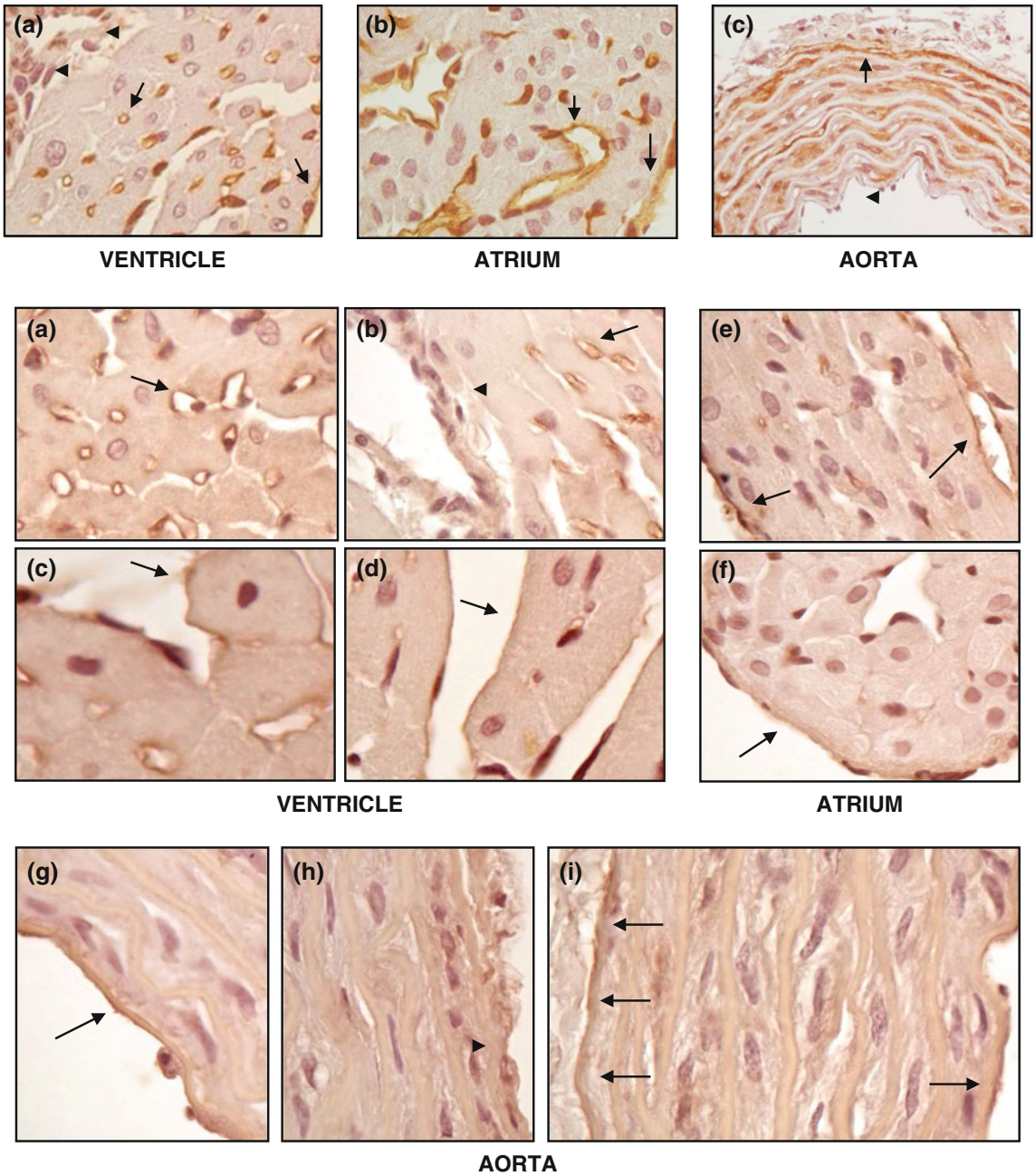

Fig. 2 Immunohistochemical staining of AQP1 in cardiovascular tissue from 25- and 50-day-old rats ($n = 5$). In the *upper panel*, 25-day-old rats: Ventricular and atrial AQP1 (×1,000) in the endocardium and endothelial cells of cardiac capillaries and venues (*arrow*), and not in muscular arterioles (*arrow head*). In the aorta (×400), the endothelium is negative (*arrow head*) and smooth muscle is positive (*arrow*). In the *bottom panel*, 50-day-old rats: In control animals, AQP1 in endothelium and endocardium of left ventricle (*panel* **a** and **b**, *arrows*) and not in larger blood vessels (*panel* **b**, *arrow head*). After water restriction, AQP1 in cardiomyocyte membrane (*panels* **c** and **d**, *arrows*). In the atrium, AQP1 in the endothelium and endocardium (*panel* **e**, *arrows*), after water restriction (*panel* **f**). In the aorta, AQP1 in the endothelium (*panel* **g**, *arrow*) and not in vascular smooth muscle (*panel* **h**, *arrow head*). After rehydration, AQP1 in vascular muscular cells (*panel* **i**, *arrows*). All images were ×1,000

AQP1 protein levels

Immunoblot analysis for AQP1 in the three tissues examined revealed a major band at 28 kD in all samples, as well as more diffuse bands from 35 to 50 kD, corresponding to nonglycosylated and glycosylated isoforms of AQP1, respectively. Fig. 3a shows that in the left ventricle, total AQP1 protein levels were higher in control pups compared

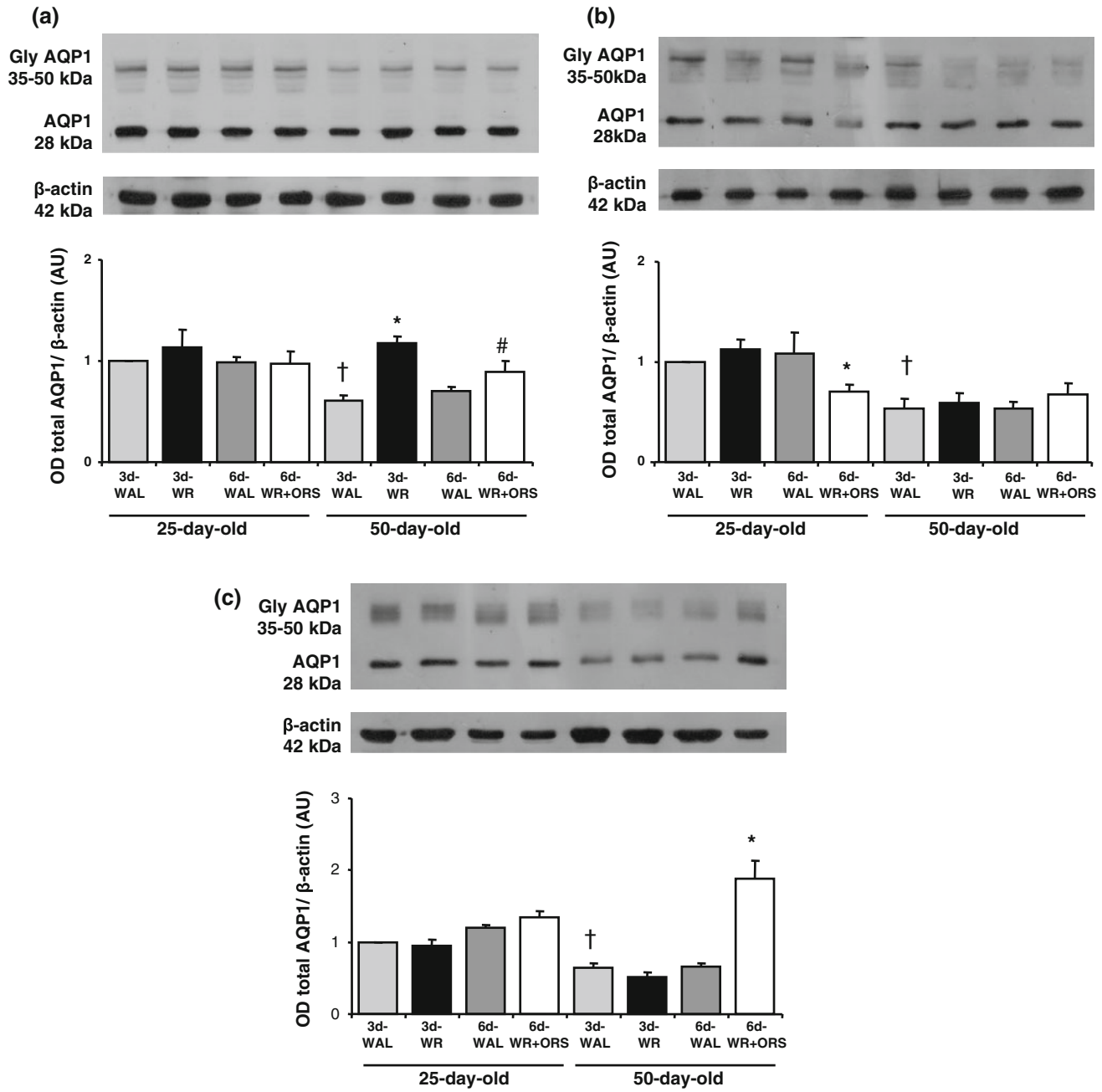

Fig. 3 Representative western blots of AQP1 in left ventricle (*panel* **a**), right atrium (*panel* **b**) and thoracic aorta (*panel* **c**) of 25- and 50-day-old rats ($n = 5$). *Histograms* the ratio between mean total AQP1 and β-actin protein levels. Data are expressed as mean ± SD. $^{*}p < 0.001$ versus respective 3d-WAL; $^{\#}p < 0.001$ versus respective 3d-WR; $^{\dagger}p < 0.001$ versus 25-day-old

to the 50-day-old rats. In 3d-WR, 6d-WAL and 6d-WR+ORS groups, both the pattern and the protein levels of AQP1 in this tissue were unchanged in the youngest group. However, in the oldest rats, AQP1 increased after water restriction, at the expense of its nonglycosylated fraction; hydration with ORS normalized AQP1 protein levels. In the right atrium, control 50-day-old rats also presented lower levels of total AQP1 when compared to 25-day-old pups. Total AQP1 protein levels were not modified by water restriction in the youngest group but in 6d-WR+ORS, both glycosylated and nonglycosylated fractions of AQP1 were significantly decreased. No modifications were observed in the oldest group (Fig. 3b). In the aorta, AQP1 levels did not change in 25-day-old rats

(Fig. 3c). Control 50-day-old rats also presented lower AQP1 protein levels, and vascular AQP1 protein levels were only increased after rehydration with ORS.

Discussion

The present study investigated the effects of water restriction and rehydration on cardiovascular AQP1 protein levels and distribution during postnatal development. Water restriction protocol used as our experimental model was successful to induce a hypohydration state in both age groups, as was evidenced by the decreased body weight and water content and increased hematocrit, plasma osmolarity and serum Na^+ (Table 1). Dehydration is classified as severe when body weight loss is superior to 10 % [23]. Our results showed that hypovolemic state was severe in both cases: there was a 40 and 25 % loss of body weight in 25- and 50-day-old rats, respectively. Even though the water restriction period was similar in both age groups, it was observed that the percent decrease in body weight was different. This may evidence that 25-day-old rats experienced a more severe hypohydration than the oldest group. Further supporting this, we observed an increased adrenal gland weight in pups, but not in the 50-day-old group. ORS therapy was able to reverse the observed changes in both age groups, confirming that animals were again hydrated. However, pups were unable to recover the lost weight after rehydration when compared to animals of their same age (6d-WAL group), indicating again that they suffered a more severe hypovolemic state.

In order to study the localization of AQP1 in cardiovascular system, we first performed an immunohistochemical staining of cardiac and aortic tissues of control animals. Cardiac AQP1 was localized primarily in the endocardium and endothelial cells of ventricular and atrial tissue of both age groups. The presence of this protein was reported in isolated cardiomyocytes by RT-PCR, but in lower quantities compared to endothelial cells [10]. Different technical approaches and their sensitivity may account for these discrepancies. Interestingly, in the present study, we report the novel finding of a very specific pattern of vascular AQP1 in the heart: It is present in endothelial cells of nonmuscular capillaries, but not in larger blood vessels. A similar pattern was described by Devuyst et al. [24] in other tissues, such as the peritoneum. This distribution may be related to the function of each type of blood vessel. It was described that AQP1 allows the passage of small gas molecules, such as CO_2 [25]. Since microvasculature endothelium is involved in blood–tissue exchange of gas molecules and nutrients, AQP1 might be more important in such blood vessels. In the aortic tissue, it was reported that AQP1 is present in endothelial cells as well as in vascular smooth muscle, being much more abundant in muscular cells [26]. Surprisingly, we found out that in control pups, this water channel was nearly absent in the endothelium and very abundant in smooth muscle cells, whereas in the oldest group, this pattern was reversed: AQP1 was detectable mostly in the endothelium and very low signal came from the muscular cells. This distribution may be implicated in maintaining water balance during postnatal stages, as aquaporin-mediated water flow allows a specific regulation of water transport.

Continuing our study of AQP1, we observed that during osmotic stress and rehydration, no changes were observed in the labeling pattern or protein levels of AQP1 in the 25-day-old pups. However, in the oldest group, changes in AQP1 protein levels were associated with the induction of this protein in cell types where it was not evident before by immunohistochemical staining. In the left ventricle, the increase in AQP1 after water restriction was confined to the cardiomyocyte membrane, whereas in aorta, this protein was increased in vascular smooth muscle after rehydration. As pups experienced a more severe hypohydration, alterations in AQP1 would be expected, but it is also possible that in the absence of such changes, the 25-day-old pups were not able to recover as efficiently as the 50-day-old group did.

In hypertonicity conditions, it was described that the induction of AQP1 depends on the activation of MAPK pathways [27] and that under osmotic stress, there is a decreased AQP1 ubiquitination and increased stability [28]. Our results showed that in response to osmotic stress induced by water restriction, there was an increase in ventricular AQP1 protein levels, only in the 50-day-old group. Under this condition, increased AQP1 on the cell membrane may implicate more severe changes in cell volume which may not be beneficial for most cells, leading to cellular dehydration. On the other hand, we cannot disregard that AQP1 would be participating on cellular volume regulatory mechanisms. However, its role in cell volume regulation has not been fully explored. It was described that cells synthesize several solutes to prevent water loss during osmotic stress [29] and aquaporins have been involved in regulatory volume increase mechanisms [30]. Moreover, during rehydration period, increased levels of this water channel may contribute to tissue hydration, as we observed in vascular tissue of 50-day-old rats. Such changes in the microvasculature may implicate more efficient water redistribution.

On the other hand, our results also showed in the three studied tissues of control animals that there is a downregulation of AQP1 with postnatal growth. In the prenatal period, it was described that AQP1 is present in the fetal heart and its mRNA was greatly decreased after birth [31]. Further work by Jonker and co-workers demonstrated that

there are no changes in AQP1 mRNA or protein levels the sheep heart from late gestation to adult life [15]. This difference may be explained in terms of the species and the age of the animals. It was also reported that postnatal changes in aquaporins have been implicated in several physiological changes [3]. Yamamoto et al. [32] showed that these changes could contribute to the maturation of urine concentrating ability. Moreover, other studies described that increased AQP1 during postnatal maturation in the carotid body may be related to osmoreceptor function [33]. Thus, in our experimental model, we suggest that changes in AQP1 might be involved in cardiac function development during postnatal life.

In conclusion, the main finding of this study is that AQP1 in cardiac and vascular tissues can be differentially regulated in response to hydration status in vivo. We also showed that AQP1 is developmentally regulated in the rat heart and such changes may be related to cardiovascular water handling during postnatal growth. The lack of these adaptive mechanisms of mature animals in young pups, who suffered from a more severe hypohydration, may evidence an important role of this water channel in maintaining fluid balance during hypovolemic state.

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Conflict of interest The authors declare no conflict of interest.

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