Angiotensin-(3–4) (Val-Tyr) Normalizes the Elevated Arterial Blood Pressure and Abnormal Na^+/Energy Handling Associated With Chronic Undernutrition by Counteracting the Effects Mediated by Type 1 Angiotensin II Receptors

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Research Article

Keywords: Regional Basic Diet, Chronic undernutrition, Arterial hypertension, Na+ intake/energy intake ratio, Na+ balance, Ang-(3–4)

Posted Date: December 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1149879/v1

Abstract

Purpose To investigate the mechanisms by which chronic administration of a multideficient diet after weaning alters bodily Na^+ handling, and culminates in high systolic blood pressure (SBP) at a juvenile age.

Methods From 28 to 93 days of age, weaned male Wistar rats were given a diet with low content and poor-quality protein, low lipid, without vitamin supplementation, which mimics the diets consumed in impoverished regions worldwide. We measured food, energy and Na^+ ingestion, together with urinary Na^+ excretion, Na^+ density (Na^+ intake/energy intake), plasma Na^+ concentration, SBP), and renal proximal tubule Na^+-transporting ATPases.

Results Undernourished rats aged 93 days had only one-third of the control body mass, lower plasma albumin, higher SBP, higher energy intake, and higher positive Na^+ balance accompanied by decreased plasma Na^+ concentration. SBP was normalized with Losartan and with Ang-(3–4), and the combination of the 2 substances induced an accentuated negative Na^+ balance as a result of strong inhibition of Na^+ ingestion. Na^+ density in undernourished rats was higher than in control, irrespective of the treatment, and they had downregulated (Na^++K^+)ATPase and upregulated Na^+-ATPase in proximal tubule cells, which returned to control levels after Losartan or Ang-(3–4).

Conclusions Na^+ density, not only Na^+ ingestion, plays a central role in the pathophysiology of elevated SBP in chronically undernourished rats. The observations that Losartan and Ang-(3–4) normalized SBP together with Na^+ distribution and handling give support to the proposal that Ang IIÞAT_1R and Ang IIÞAT_2R axes have opposite roles within the renin-angiotensin-aldosterone system of undernourished juvenile rats.

Introduction

Undernutrition is a condition characterized by a lack of adequate nutrition whose cause is the ingestion of insufficient food containing the necessary substances in both quantity and quality for growth and health, or by an inability to absorb properly or convert nutrients [1]. Nutritional imbalances can be associated with an upregulated renin-angiotensin-aldosterone system (RAAS) leading to renal and cardiovascular pathologies, including arterial hypertension [2], in which altered mechanisms of Na^+ handling in the kidney and the heart seems to be important, as well as modifications of type 1 and type 2 angiotensin II receptor (AT_1R and AT_2R) signaling [3]. Although there is growing information regarding the association between post-weaning undernutrition, RAAS and, especially, altered cardiovascular regulation (reviewed in [4]), the interactions between the two main axes of RAAS (Ang IIÞAT_1R and Ang IIÞAT_2R) in chronic undernutrition remain to be studied. Knowledge concerning (i) the influence of these axes on specific nutritional parameters in undernutrition, and (ii) the chronic undernutrition-induced modifications in renal Na^+-transporting ATPases and renal and bodily Na^+ balance is particularly lacking.

In the present study we induced chronic undernutrition with a multideficient diet that was administered to male Wistar rats for 64 days after weaning. This diet mimics those used in vast impoverished regions of undeveloped countries, and also in pockets of poverty in big cities worldwide. Teodósio *et al*. [5] formulated this diet 30 years ago after observing the dietary habits of families inhabiting rural areas of Pernambuco State (Brazil), and it has served for many years as a model for the study of many more pathologies in different organs and tissues, including kidney, cardiovascular system and central nervous systems [6]. In this diet, named Regional Basic Diet due to its origin, the protein content is low and of poor quality; lipids are also low, as well as vitamins and mineral content.

The rats were chronically exposed to this diet after weaning until 92 days of age because we aimed to cover a period that, in humans, corresponds to the nursing period, the prepubescent period, the adolescent period and the beginning of adulthood [7]. As pointed out 5 years ago by Chianca *et al*. [4], this model reflects the situation encountered in underdeveloped countries, where undernutrition starts after an appropriate nutrition period by breast-feeding. The first pharmacological intervention – to address the objective of shed light on the role of RAAS in undernutrition-induced cardiovascular and renal alterations – was designed to inhibit the Ang IIÞAT_1R axis for the entire period of dietary deficiency. This approach was considered appropriate to investigate the influence of AT_1R signaling blockade in the evolution of arterial pressure and, at the end of the period, *i.e.* at the beginning of adulthood, to have a picture of the influence of RAAS on Na^+ handling and fluid balance in chronically undernourished rats. Blockade of the Ang IIÞAT_1R axis was achieved by administration of Losartan, an antagonist of AT_1R [8]. The second pharmacological intervention determined whether acute activation of the Ang IIÞAT_2R axis at the young adult age modifies arterial pressure and influences Na^+ handling.

Ang IIÞAT_2R axis was stimulated by oral administration of Ang-(3–4) (Val-Tyr), the shortest Angiotensins-derived peptide, which antagonizes most of the Ang II actions in physiological and pathological conditions [9–11] by acting as an allosteric enhancer of Ang II binding to AT_2R [12]. The choice was based on an early observation regarding antihypertensive effects of Ang-(3–4) in humans [9] and, more recently, in spontaneously hypertensive rats (SHR); in SHR, Ang-(3–4) also stimulates urinary excretion of Na^+ [10]. We previously demonstrated that Ang-(3–4) only acts in pro-hypertensive tissular microenvironments of overweight rats [13], *i.e.* in tissues – *e.g.*, renal tissue – in which the activity of local RAAS is high [14].

Materials And Methods

Ethical considerations

All experimental procedures were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Rio de Janeiro (protocols 007/16 and 012/19), and were performed in accordance with the Committee's guidelines, which follow the Uniform Requirements for Manuscripts Submitted to Biomedical Journals. The animal study is reported in accordance with ARRIVE guidelines.

Diets

The Regional Basic Diet (RBD) is a model of multideficient diet [5], its composition being given in Table 1. RBD was prepared in a homemade process using the facilities of the Laboratory of Food Analysis and Processing of the Josué de Castro Institute of Nutrition at the Federal University of Rio de Janeiro. The ingredients were cooked, dehydrated at 60°C in a food greenhouse and then ground before being mixed. Water was added to form a wet mass that was cut in small pieces as the standard commercial control diet (CTRL), and finally dehydrated for 1 day at 60°C. The composition of the CTRL diet (Neovia Nutrição e Saúde Animal, Descalvado, Brazil) follows the recommendations of the American Institute of Nutrition for rodents (AIN-93G) [15]. The dietary Na^+ content was determined by flame photometry after acid extraction with 1 N HNO_3. K^+, Ca^{2+}, Fe^{2+} and vitamin content were as previously determined [5].

Animals and experimental groups

Female Wistar rats were kept and mated in the Vivarium of Neglected Diseases and Undernutrition from the Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro. Male offspring were weaned at 28 days of age. The animals were randomly divided into 2 groups: the first group received the CTRL diet, and the other group received the RBD until the end of the study. At the same day, the 2 groups were randomly subdivided in other 2 subgroups, also originating groups that received Losartan (Los: 30 mg/kg body mass diluted in the drink water; Biosintética, Jurubatuba, Brazil), daily from weaning to 91 days of age, thus starting the additional CTRL+Los and RBD+Los groups. At this age, a sub-group of each 4 groups received vehicle (water) or one single oral dose of Ang-(3–4) (80 mg/kg body mass; EZBiolab, Carmel, IN, USA) by gavage. Thus, the 4 new groups were now: CTRL+Ang-(3–4) (CTRL rats treated with Ang-(3–4)); CTRL+Los+Ang-(3–4) (CTRL+Los rats treated with Ang-(3–4)); RBD+Ang-(3–4) (RBD rats treated with Ang-(3–4)) and RBD+Los+Ang-(3–4) (RBD+Los rats treated with Ang-(3–4)).

Table 1 Composition of diets

	CTRL[1]	RBD[2]
Protein % (w/w)	23	8
Carbohydrate % (w/w)	41	78
Lipids % (w/w)	2.5	1.7
Na % (w/w)	0.3[3]	0.2[3]
Fe % (w/w)	0.018	0.007
Ca % (w/w)	1.8	0.04
K % (w/w)	0.9	0.3
Energy supply kcal/100 g dry weight	278	356
Vitamin supplement	Yes	No

CTRL, Control diet; RBD, Regional Basic Diet.

[1]As indicated by the manufacturer (Neovia Nutrição e Saúde Animal, Descalvado, Brazil).

[2]According to the Laboratory of Experimental and Analysis of Food (LEEAL), Nutrition Department, Federal University of Pernambuco.

[3]According to Muzi-Filho *et al*. (2020) [21].

Before and after 24 h of Ang-(3-4) administration, the rats were placed in metabolic cages

to measure water and food intake, and for recording urinary volume. Before and after the metabolic cage period, the blood pressure of the rats from the 8 groups was measured. The rats were decapitated for plasma collection and kidney dissection to obtain plasma membrane preparations from proximal tubules for the *in vitro* experiments (see below). During the whole period of the experimental protocol, food and filtered water were available *ad libitum*. Note: the experimental period of the 4 groups of animals treated with Ang-(3–4) lasted one extra day because they derived from the other 4 groups.

Blood pressure measurements

Blood pressure was measured by a non-invasive method [16] in conscious rats at day 91 by using a tail-cuff plethysmograph (Insight, model V2.01, Bonther, Ribeirão Preto, Brazil). An additional record at day 92 was carried out in the groups that received Ang-(3–4). Digital signals were recorded and processed by using the appropriate software (viz., Pressure Gauge 1.0, Insight). On the day before the procedure, the rats were acclimated in a heated chamber (30–32ºC) for 10 to 15 min, and the recordings were only taken from the rats without sudden movements. Five determinations were made for each animal and the average of the 5 values was used.

Preparation of plasma membrane-enriched fraction from kidney proximal tubule cells

Membrane preparations were obtained by homogenization and differential centrifugations from the outermost region of the renal cortex (*cortex corticis*) [17], where the cell population corresponds to >95% of proximal tubules, as previously described [18]. Thin transverse slices of the *cortex corticis* (0.5 mm) were separated with a Stadie-Riggs microtome (Thomas Scientific, Swedesboro, NJ, USA), immersed in an isotonic solution containing 10 mM Hepes-Tris (pH 7.4), 250 mM sucrose, 2 mM EDTA, 1 mM PMSF and 0.15 mg/ml trypsin inhibitor type II-S (T1021; Sigma-Aldrich, St. Louis, MO, USA), and dissected using a small ocular scissor. The fragments were homogenized at 4ºC in the same isotonic solution (1 g tissue/4 ml solution) using a Potter Elvejhem homogenizer fitted with a Teflon pestle (5 cycles of 1 min at 1,700 rpm). The resulting homogenate went through 3 successive differential centrifugations: (1) 10,000 *g* for 15 min at 4ºC (JA-20 rotor, Beckman Avanti J-E centrifuge; Beckman Coulter, Fullerton, CA, USA), (2) 15,000 *g* for 20 min at 4ºC (JA-20 rotor, Beckman Avanti J-E centrifuge), and (3) 35,000 *g* for 44 min at 4ºC (70 Ti rotor, Beckman Optimal L-90K ultracentrifuge). The pellets were resuspended in 250 mM sucrose to ~15 mg/ml protein, quantified by the Folin phenol method, using bovine serum albumin as standard [19], aliquoted into tubes and stored at -80ºC. The samples were used to measure the activity of the 2 Na^+-transporting ATPases, the ouabain-sensitive (Na^++K^+)ATPase and the ouabain-resistant Na^+-ATPase, as described below.

Albumin and Na^+ determinations

Plasma albumin was measured with a commercial kit (catalog number: K040, Quibasa-Bioclin, Belo Horizonte, Brazil). Na^+ concentrations in urine and plasma samples were determined by flame photometry (Analyzer, São Paulo, Brazil) using a standard solution containing 140 mequiv Na^+/l (Analyzer).

Determination of the activities of Na^+-transporting ATPases from kidney proximal tubules

The ouabain-sensitive (Na^++K^+)ATPase and the ouabain-resistant, furosemide-sensitive Na^+-ATPase activities were determined by quantifying the inorganic phosphate (P_i) released during ATP hydrolysis [20]. (Na^++K^+)ATPase activity was measured in plasma membranes from proximal tubules (0.025 mg/ml, final concentration), which were preincubated in the absence or presence of 2 mM ouabain (Sigma-Aldrich) for 10 min at 37ºC in a medium (0.5 ml) containing 50 mM Bis-Tris-Propane (pH 7.4), 0.2 mM EDTA, 5 mM $MgCl_2$ and 120 mM NaCl. The reaction was started by simultaneous addition of 24 mM KCl and 5 mM ATP (final concentrations), and stopped 10 min later by adding 0.5 ml of 0.1 M HCl-activated charcoal. The suspension was centrifuged (13,300 *g* for 10 min) and part of the supernatant was diluted with the same volume of a solution containing 0.2 N H_2SO_4, 10 mM ammonium molybdate and 0.3 M $FeSO_4$; absorbance was recorded at 660 nm 20 min.

Ouabain-resistant Na^+-ATPase activity was measured in the same membrane preparations (0.05 mg/ml, final concentration), which were preincubated in the presence of 2 mM ouabain, in the presence or

absence of 2 mM furosemide (Sigma-Aldrich) for 10 min at 37ºC in 20 mM Hepes-Tris (pH 7.0), 10 mM $MgCl_2$ and 120 mM NaCl. Assays started by adding 5 mM ATP in a final volume of 0.5 ml and stopped with 0.5 ml 0.1 M HCl-activated charcoal before being processed as described for $(Na^+ + K^+)$ATPase. The activities were calculated by: (i) the differences between the values obtained in the absence and presence of 2 mM ouabain for $(Na^+ + K^+)$ATPase; (ii) the difference between the values obtained in the absence and presence of 2 mM furosemide for Na^+-ATPase [13,14,21]. Determinations were carried out in triplicate.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6 software (version 6.01, GraphPad Software, Inc., San Diego, CA, USA). Results are expressed as means ± SEM. When comparing 2 groups, the analysis used unpaired or paired student's *t*-test. For the comparisons of 4 groups, the analysis used was one-way ANOVA followed by Bonferroni's test for selected pairs, as indicated in the corresponding table and figure legends. Significant differences were set at $P<0.05$.

Results

Chronical blockade of type 1 Ang II receptors (AT_1R) differentially modified evolution of body mass in CTRL and RBD rats

Table 1 presents the composition of the multideficient RBD that was given to male rats for 64 days after weaning (i.e., from 28 to 92 days of age). The body mass (BM) evolution of normonourished CTRL (empty circles in Fig. 1A) and undernourished RBD rats (empty squares in Fig. 1B) was followed using the equation:

$$BM_t = BM_{28} + BM_{max} \cdot (1 - e^{-kt}) \quad \text{(Eq. 1)}$$

where BM_t corresponds to BM at different times t (days), BM_{28} corresponds to BM at weaning (64 ± 2 g, considering all rats used), BM_{max} is the theoretical value of body mass gain attained at time $\rightarrow \infty$ from the departure value BM_{28}, k is the rate constant of growth and e has the usual meaning. The average k values were different (0.0346 ± 0.0020 *vs* 0.0203 ± 0.0010 $days^{-1}$ for RBD and CTRL rats, respectively; $t = 6.95$, $P<0.0001$, unpaired Student's t test), indicating: (i) that the undernourished rats grew daily at a more significant fraction of their reduced BM_{max} (48 g in RBD *vs* 327 g in CTRL rats); (ii) that the time required to attain 50% of their reduced BM_{max} ($t_{1/2}$) was lower (20 and 34 days, respectively).

Blocking AT_1R by chronic administration of Losartan from weaning differentially modified the profile of growth in CTRL and RBD groups: though the body mass gain was lower in both groups, the corresponding profiles were different (compare the evolution of filled symbols in Fig. 1A & B). In the CTRL animals, 2 different phases of growth – and, therefore, 2 different time-dependencies – were clearly seen when Losartan was given to CTRL rats. AT_1R blockade changed the kinetics of growth over the first period of 28 days of diet administration, following the exponential function:

$BM_t = BM_{28} + e^{kt}$ (Eq. 2)

where the factors have the same meaning as above, and k = 0.1545 ± 0.0027 $days^{-1}$, an apparently faster rate constant. However, in real nutritional terms, this kinetic behavior meant an accentuated decrease in growth at early ages (compare the empty and filled circles in Fig. 1A). Interestingly, the profile rapidly changed from the initial period and onwards, recovering that described by Eq. 1, with a faster k (0.0574 ± 0.0054 $days^{-1}$) ($t_{1/2}$ = 12 days) but always at reduced BM compared to untreated rats (compare the empty and filled circles in Fig. 1A), attaining a theoretical additional mass gain BM_{max} = 110 g. In Losartan-treated RBD rats, the growth curve followed the same single function seen in untreated rats (filled squares in Fig. 1B), attaining a lower body mass gain (BM_{max} = 19 g) with a faster k (0.1079 ± 0.0058 $days^{-1}$) and a very low $t_{1/2}$ (6 days). Table 2 gives a simpler comparison of the kinetic parameters of growth corresponding to the 4 groups.

Body mass of CTRL and RBD rats at 92 days of age treated or untreated with Losartan: effects of an additional acute administration of Ang-(3–4)

Table 2
Kinetic parameters of BM evolution from weaning at 28 days to 90 days of age

	Additional body mass gain BM_{max} (g)	**Rate constant of growing k ($days^{-1}$)**	**Time to 50% of BM_{max} $t_{1/2}$ (days)**
CTRL	327	0.0203	34
CTRL + Los			
1st phase	*	0.1545	**
2nd phase	110	0.0574	12
RBD	48	0.0346	20
RBD + Los	19	0.1079	6
*The theoretical BM_{max} of this phase does not have biological/nutritional meaning because $BM_{max} \rightarrow \infty$ when $t \rightarrow \infty$			
**The same reasoning applies for the calculated $t_{1/2}$ = 4 days in the 1st phase			

Chronic administration of RBD culminated in a 70% decrease in body mass (BM; 97 ± 3 g) at 92 days compared with CTRL rats (306 ± 5 g) given commercial chow (Fig. 2A). This panel gives a better comparison at the end of the experiment of the accentuated difference in BM between the groups CTRL and RBD that did not receive any pharmacological treatment. A proportion of rats from the 4 groups whose growth was described in the subsection above received a single oral dose of Ang-(3–4) (80 mg/kg

BM) on day 91. Figure 2 also presents and compare the effects on BM of the chronic administration of Losartan and acute administration of Ang-(3–4) to CTRL and RBD rats one day later. In Fig. 2B, it can be seen that Losartan provoked a small, though significant, decrease in BM, which was 15% in CTRL and 10% in RBD rats, a repeated qualitative picture in the case of Ang-(3–4) (Fig. 2C). When Ang-(3–4) was given in combination with Losartan, the small decrease in BM remains in CTRL rats, but disappeared in the RBD ones (Fig. 2D). The combination of treatments decreased BM by 20 and 10% in normonourished and undernourished rats, respectively (Fig. 2E & F).

The undernutrition status of rats given RBD is also reflected in the plasma albumin ($[albumin]_{pls}$), with a 40% decrease with respect to CTRL, without an effect of Losartan in both groups (Fig. 3A & B). Acute administration of Ang-(3–4) alone provoked an accentuated hypoalbuminemia in CTRL and RBD rats (Fig. 3C); however, when the 2 drugs were given together to the CTRL group, the trend was attenuated and reversed in the case of RBD rats to the CTRL+Los+Ang-(3–4) values (Fig. 3C & D, respectively) (P=0.7775).

Food and energy intake in CTRL and RBD rats that received Losartan and Ang-(3–4)

The next figures show the data regarding food and energy intake. Food intake in 24 h per 100 g BM was 25% higher in RBD rats at day 92, 24 h after they had been acclimated in individual cages (Fig. 4A). Losartan decreased food intake in RBD, but not in the CTRL group (Fig. 4B), and a fall in food ingestion was provoked by Ang-(3–4) in both groups, being quite remarkable in undernourished rats (Fig. 4C). Combined administration of the drugs (Fig. 4D) decreased feeding of CTRL, but not of RBD rats. Both treatments recovered the CTRL food intake in the undernourished rats (t=0.99, P=0.3466 for RBD+Losartan *vs* CTRL; t=1.05, P=0.3168 for RBD+Ang-(3–4) *vs* CTRL) (comparisons within panels 4B & C, respectively). When the effects of the combined treatments were compared with the respective untreated CTRL and RBD groups, a significant and accentuated anorexic effect was seen in both groups (Fig. 4E & F, respectively).

The energy intake of the RBD rats was 100% higher than in CTRL (Fig. 5A). It was decreased by Losartan (30%) and Ang-(3–4) (35%) in the RBD group with a small, but significant, influence of Ang-(3–4) in CTRL (Fig. 5B & C). In contrast to that found with dietary intake, the drugs did not help in recovering the CTRL energy intake, certainly because the decrease in food did not suffice to compensate for the higher caloric content of the deficient diet (t=7.04, P<0.0001 for RBD+Losartan *vs* CTRL; t=3.42, P=0.0057 for RBD+Ang-(3–4) *vs* CTRL) (comparisons between panels 5B & C, respectively). Combination of treatments accentuated the decrease in energy intake by CTRL rats without further influence in the RBD group (Fig. 5D); they also resulted in pronounced diminution of the caloric ingestion compared with the untreated CTRL and RBD groups (Fig. 5 & F, respectively).

Na^+ intake, Na^+ density and Na^+ balance in CTRL and RBD rats: Losartan- and Ang-(3–4)-induced modifications

To assess the influence of chronic undernutrition and the response to drug treatments in bodily Na^+ handling, we measured Na^+ intake, urinary Na^+ concentration ($[Na^+]_{ur}$), daily urinary volume and Na^+ excretion in 24 h ($U_{Na}V$) at 92 days of age. RBD rats ingested a significantly higher, though small, amount of Na^+ per 100 g BM compared with CTRL animals (Fig. 6A) as a result of the increased intake of food (Fig. 3A). Losartan and Ang-(3–4) had different influences depending on the nutritional condition, and also when given alone or in combination. When the drugs were given alone: (i) no effect of Losartan was seen in Na^+ ingestion by the CTRL group, whereas in contrast there was a strong inhibition of ~30% in the RBD group; (ii) Ang-(3–4) alone inhibited Na^+ ingestion in both groups, which was more accentuated in RBD (35%) than in CTRL rats (10%); (iii) combined treatment potentiated the inhibition in CTRL, but showed that Ang-(3–4) did not modify the effect of Losartan in RBD rats (Fig. 6B & D) (t=1.16, P=0.2696; comparison RBD+Ang-(3–4) *vs* RBD+Los+Ang-(3–4). Comparison of untreated groups with respect to those receiving the combined treatment led to a similar 30% inhibition of Na^+ intake by the drugs (Fig. 6E & F).

Na^+ density is emerging as a concept defined as the ratio between Na^+ and energy in a diet [22] and, therefore, between Na^+ and energy intake. This correlation is presented in Fig. 7 for the 8 experimental groups at day 92 of age, after the administration of vehicle or Ang-(3–4) to CTRL and RBD rats previously treated either with or without Losartan. Two straight lines were seen when the individual data of energy intake were plotted as a function of the corresponding Na^+ intake: one of steepest slope corresponds to the 4 groups of RBD rats, and one of lesser slope includes the 4 groups of CTRL rats. Moreover, 3 clusters can be identified: the frame □ shows that all untreated RBD rats were clustered above the cut-off levels of 35 kcal in 24 h per 100 g BM (horizontal dashed line) and 0.60 mequiv Na^+ ingested in 24 h per 100 g BM (vertical dashed line); the frame □ includes all the CTRL rats that received Ang-(3–4) and Losartan+Ang-(3–4) within a cut-off delimited by 20 kcal in 24 h/100 g BM (horizontal dashed line) and 0.55 mequiv Na^+ in 24 h/100 g BM (vertical dashed line); the frame □ corresponds to the remaining panel, including (i) the RBD rats treated with Losartan, Ang-(3–4) or its combination, and (ii) the CTRL rats treated or untreated with Losartan.

An important characteristic of the RBD phenotype is hypertension. The following results demonstrate that alterations in energy and Na^+ intake encountered in RBD rats are associated with important cardiovascular alterations. The systolic blood pressure (SBP) reached 150 mmHg in contrast with the 120 mmHg value for CTRL rats at 92 days of age, and the pressoric values were reversed by Losartan (totally) or partially by (Ang-(3–4) (Fig. 8A–C). Interestingly, combined treatment resulted in similar SBP (t=0.57, P=0.5765) when the groups RBD+Ang-(3–4) and RBD+Los+Ang-(3–4) were compared (Fig. 8C & D, respectively).

To calculate the $U_{Na}V$ (and then the Na^+ balance besides Na^+ intake) at day 92 of age, we measured urinary volume over 24 h and $[Na^+]_{ur}$. The urinary flux of RBD was higher than in CTRL rats and increased further in the animals that received Losartan, whereas the opposite was encountered in the CTRL group

(Supplementary Fig. 1A & B). Ang-(3–4) alone decreased urinary flux of RBD rats to the levels of CTRL, which have not been modified by the peptide, whereas it decreased the urine volume in CTRL and RBD rats previously treated with Losartan (Supplementary Fig. 1C & D). Comparison of combined treatments with their respective untreated CTRL and RBD shows the opposite, viz. a decrease and an increase, respectively (Supplementary Fig. 1E & F).

$[Na^+]_{ur}$ was lower in RBD rats compared to the CTRL, and Losartan accentuated this diminution, an effect that was the reverse in the CTRL group (Supplementary Fig. 2A & B). Administration of Ang-(3–4), alone or in combination with Losartan, increased $[Na^+]_{ur}$ (Supplementary Fig. 2C & D). Comparison of the combined treatments with the respective untreated groups shows an increase in the CTRL group without any difference in the undernourished group (Supplementary Fig. 2E & F), because the effects of Losartan and Ang-(3–4) are probably the reverse in these rats when assayed alone (Supplementary Fig. 2B & C, right part of both panels).

$U_{Na}V$ values are given in Fig. 9, first demonstrating a small, but significant, decrease in undernourished rats (Fig. 9A), despite an increased Na^+ intake seen in Fig. 6A; the effect of Losartan was the reverse depending on the nutritional status: increased $U_{Na}V$ in CTRL but decreased in RBD rats, with a similar profile in the case of Ang-(3–4) administration, which had no effect on rats given Losartan (Fig. 9B–D). In relation to the respective untreated groups, combined treatment did not modify $U_{Na}V$ in the CTRL group and was inhibitory when the RBD rats were checked (Fig. 9E & F). With these $U_{Na}V$ values and those of Na^+ intake above (Fig. 6), we calculated the Na^+ balance, which showed that the positive Na^+ balance increased by ~100% in RBD rats compared with the CTRL group (Fig. 10A) and indicates the following characteristics: whereas the positive balance strongly decreased in Losartan-treated CTRL rats (Fig. 10B & E), that of RBD rats was insensitive to the drug (Fig. 10F), and consequently the difference between the 2 groups increased to 400% (Fig. 10B). The positive Na^+ balance approached zero in CTRL and was strongly depressed in RBD rats after Ang-(3–4) administration (Fig. 10C). Na^+ balance became negative in the CTRL with the combined treatment and remained similar to that with Ang-(3–4) alone in RBD rats (Fig. 10D & E). The comparison of Fig. 10E and Fig. 10F allowed us to see how the nutritional status modifies the response of Na^+ balance to Losartan and Ang-(3–4).

Figure 11A shows that RBD rats had a lower $[Na^+]_{pls}$, which fell further with the chronic administration of Losartan, Ang-(3–4), and by their combined treatment (Fig. 11B–D). Comparison between the groups RBD+Ang-(3–4) and RBD+Los+Ang-(3–4) showed that their $[Na^+]_{pls}$ were similar (t=1.59; P=0.1413). The treatments modified the $[Na^+]_{pls}$ of CTRL rats following the same profile: the groups CTRL+Ang-(3–4) and CTRL+Los+Ang-(3–4) had similar $[Na^+]_{pls}$ (t=1.36; P=0.2113). A diet-dependent difference in response to pharmacological treatments is seen when one compares the effect of the combination Los+Ang-(3–4) with the untreated CTRL and RBD groups. In the case of CTRL rats, the combination provoked the same effect that Ang-(3–4) alone (compare Fig. 11E with Fig. 11C, left panel). In contrast whereas in the case

of RBD rats, the effect of the combination was similar to that encountered with Los alone (compare Fig. 11F with Fig. 11B, right panel).

Since body Na^+ balance is critically dependent on reabsorption of Na^+ filtered by the renal glomeruli, a process which mostly (~75%) occurs in renal proximal tubules in an ATP-dependent manner [23, 24], we studied the influence of chronic undernutrition on the 2 Na^+-transporting ATPases of proximal tubule cells: the ouabain-sensitive (Na^++K^+)ATPase and the ouabain-resistant Na^+-ATPase [21, 25, 26]. In RBD rats, (Na^++K^+)ATPase was 60% lower than in CTRL and insensitive to both Losartan and Ang-(3–4) (Fig. 12A–C). In CTRL rats, Losartan and Ang-(3–4), alone or in combination inhibited by 25–30% (Fig. 12B–D), to a level that was similar to that found in the RBD group that had received the drugs in combination (compare the 2nd and 4th bars in Fig. 12D). In contrast, RBD-induced undernutrion resulted in a huge upregulation of the ouabain-resistant Na^+-ATPase, which was inhibited by Losartan and Ang-(3–4), recovering the activity of the CTRL with the combined treatment (Fig. 13A–D). Na^+-ATPase of the CTRL rats was completely insenstive to the drugs, alone or combined (Fig. 13B–D).

Discussion

The central results of this study deal with the chronic ingestion of a diet (RBD) [5] that mimics those widely used in vast rural regions and impoverished peripheries of large cities worldwide [27, 28], which has led the development of high blood pressure – the consequence of activation of the Ang II⇒AT_1R axis of RAAS – along with an accentuated decrease in BM and changes in body Na^+ handling. These alterations associated with undernutrition, which were totally or partially reversed by blockade of this axis with Losartan, or by activation of the counteracting Ang II⇒AT_2R axis by Ang-(3–4) [12], occurred despite an increased caloric intake and a dietary Na^+ content 20% lower than in the CTRL diet. Two initial points deserve special consideration regarding the dietary model chosen here: first, its administration was given immediately after weaning, a life period when occurs rapid and definitive developmental events [29, 30]; second, the extremely poor quality of the reduced protein content [5], considered to be the leading cause of morbidity and mortality in today's world [31, 32].

For years, the animal models investigating the mechanisms and processes affected by chronic undernutrition were those based in diets with only dietary low protein content [33, 34], but which do not reflect the situation of multi-deficiency found in the diets used in regions with endemic undernutrition [35]. Besides the quantitative deficiency in proteins (8%), their sources in RBD – 90% from beans and 10% from jerked meat [5] – do not contain the quality required to preserve the normal pools of free amino acids in the subcellular, cytosolic and extracellular compartments, as previously demonstrated [36]. The normal pools of amino acids depend of the total protein ingested in relation to lipids and carbohydrates, amino acids composition [37, 38], and adequate ingestion of pyridoxal phosphate (vitamin B6) for a proper synthesis of non-essential amino acids [39]. The multi-deficiency in RBD and the resulting amino acid disequilibrium [40] seem to provoke accentuated diminution in growth, as seen in Fig. 1B, despite a higher food (Fig. 4A) and energy intake (Fig. 5A) by the undernourished rats.

In terms of associated mechanisms for the later onset of pathologies is the compensatory RBD-induced upregulation of RAAS, which also has a significant role in BM growth in early age under physiological conditions, as demonstrated by the growth profile curve when the AT_1R is blocked by Losartan (Fig. 1A). The development of high systolic blood pressure at a juvenile age of 92 days, which is equivalent to 13–14 years in human lifespan [7], gives support to this hypothesis, which integrates both quantitative and qualitative dietary deficiencies with marked growth retardation in childhood, RAAS upregulation and the genesis of hypertension.

In terms of mechanisms, the importance of both RAAS axes – and in several cases their selectivity – emerged from a better insight into the influence of Losartan and Ang-(3–4) in the different parameters we investigated. When BM is analyzed at the above-proposed juvenile age (Fig. 2), together with food intake (Fig. 4) and energy intake (Fig. 5), the roles of the Ang II⇒AT_1R and Ang II⇒AT_2R axes clearly emerge. Apart from the influence of Losartan in the BM evolution curves discussed above, which indicates the requirement of a functional Ang II⇒AT_1R axis for a proper growth, the data from Figs. 2C, 4C and 5C show that the Ang II⇒AT_2R axis is also involved. BM, food intake and energy intake are inhibited by a single administration of Ang-(3–4), which is a powerful antagonist of different Ang II effects in physiological and pathological conditions [9–12, 41], acting as an allosteric enhancer of AT_2R [12]. Perhaps the effects of Ang-(3–4) rely on circuits of the central nervous system that control hungry and, therefore, food intake [42, 43] in a way that seems to be dependent on the degree of upregulation of the Ang II⇒AT_1R axis towards a pro-hypertensive status [10, 13], as it seems that the effects of combined Losartan and Ang-(3–4) in BM, food ingestion and energy intake disappeared in RBD rats (right panels in Figs. 2D, 4D and 5D).

Analysis of the ensemble of Na^+ density data (Fig. 7) first shows that the RBD rats ingest 59 kcal per mequiv Na^+ against 37 kcal per mequiv Na^+ of the CTRL, as it arises from the slopes of the 2 straight lines or, in other words, that all undernourished groups incorporate more calories per Na^+ independent of the treatment. Since the RBD rats have the highest energy and Na^+ intake (cluster ; see also Fig. 4A) and have high blood pressure (Fig. 8A), we propose that Na^+ density rather than intake of salt alone is a determinant for the onset of hypertension in chronic undernutrition. These observations indicate that compensation for eating food containing small amounts of low-quality proteins become a key hypertensive mechanism. In humans, the relationship between Na^+ and blood pressure varies when energy needs vary [22]. Additionally, analysis of the groups that received Ang-(3–4) alone or in combination with Losartan in clusters and leads to the conclusion that simultaneous activation of the Ang II⇒AT_2R axis by Ang-(3–4) [12] decreases Na^+ and energy intake. However, since (i) the data from CTRL rats fell in the frame below the RBD data (clustered in) in a region of high Na^+ intake, and (ii) the normalization of blood pressure of the RBD rats is complete with Losartan (Fig. 8C), but not with Ang-(3–4) (Fig. 8D), it is proposed that the rapid response to Ang-(3–4) of the Ang II⇒AT_2R axis is compromised in chronic undernutrition, or, at least, is incomplete with a single dose of the peptide.

At this point, the normotensive CTRL, CTRL+Los and RBD+Los rats deserve special consideration (Fig. 8A & B), which have their Na^+ density values in cluster (Fig. 7) and a comparable energy intake ranging 20–25 kcal in 24 h per 100 g BM, being a Na^+ intake much higher in CTRL and CTRL+Los rats than in RBD+Los rats. These observations show that blockade of the Ang II⇒AT_1R axis suffices for the normalization of SBP in undernourished animals, and also reinforce the proposal regarding the role of Ang II-regulated energy intake in the pathogenesis of undernutrition-associated hypertension.

Concerning possible interactions between the Ang II⇒AT_1R and Ang II⇒AT_2R axes in the pathogenesis of hypertension in RBD rats, the contrast between the total normalization of SBP with Losartan alone and the diminished effect of this drug after a single administration of Ang-(3–4) (Fig. 8B–D) also deserves special consideration. Three possibilities arise. First, since the effectiveness of chronic AT_1R blockade disappears when a single dose of Ang-(3–4) activates the Ang II⇒AT_2R axis [12], it could be that the Ang-(3–4)-induced dissociation [10] of AT_1R/AT_2R dimers [44] results in Ang II⇒AT_1R-linked PKC-catalyzed abnormal phosphorylations of the contractile machinery [45] from the heart and aorta in a way that is resistant to Losartan. Second, a non-exclusive possibility is that increased Ang II⇒AT_2R-stimulated PKA upregulates PKC, a central component of Ang II⇒AT_1R signaling. This idea receives support regarding the tight connection between these two pathways [46, 47] in the heart of undernourished rats [36]. The third possibility would be that Ang-(3–4)-stimulated PKA activity switches G-coupling, leading, *e.g.*, to Gi-dependent Losartan-insensitive activation to MAPK, as proposed 2 decades ago for several cardiovascular diseases [48]. MAPK is another central kinase in the heart and kidney from undernourished rats [36]. Once in CTRL rats, since the SBP remained unmodified by Losartan, Ang-(3–4) or combined treatment, it seems clear that they only act in tissues with pro-hypertensive microenvironments [10], *i.e.*, in tissues with increased local activity of RAAS, as in the kidney of RBD rats [14].

The slightly increased Na^+ intake (Fig. 6A) and the positive Na^+ balance in RBD rats, which is ~100% higher than in CTRL rats (Fig. 10A), are indicative of Na^+ accumulation that possibly occurred together with – or perhaps before – the onset of hypertension. Even though the RBD rats had expanded intravascular compartment (8 ml/100 g BM against 6 ml/100 g BM in CTRL [36]), they are hyponatremic (Fig. 11A), a condition that could be seen as similar in the 35 ml of extracellular compartment [49] as a whole. Thus, it may be that the positive $U_{Na}V$ of ~0.1 mequiv in 24 h/100 g BM above the level encountered in CTRL rats reflects Na^+ accumulation occurring in an osmotically silent compartment, such as the dermis. Here, Na^+ bound possibly colocalizes with the glycosaminoglycan scaffold as recently demonstrated in humans [50], and before in rodents [51]. The responses of Na^+ balance to the treatments by Losartan and Ang-(3–4) in CTRL and RBD rats (Fig. 10B–F) – especially the accentuated negative Na^+ balance with the combined treatment (Fig. 10D) – lead us to conclude that they rely on the inhibition of the Ang II⇒AT_1R axis and the counteracting stimulation by Ang-(3–4) of the Ang II⇒AT_2R axis (Fig. 6B & C). This is another evidence that Ang-(3–4) acts as an antagonist of Ang II effects in physiological and pathological conditions [9–12, 41] in a way that is modulated by the activity of local RAAS [10, 11].

Moreover, combined analysis of Fig. 6B & C and Fig. 8B & C reveals an intriguing feature: reduction in Na^+ balance occurs as the result of decreased intake rather than from increased $U_{Na}V$, giving further support to the hypothesis that mechanisms at the level of CNS predominate over those related with renal Na^+ transport, as discussed below.

Besides the RAAS-associated systemic influence of undernutrition in Na^+ balance leading to its progressive accumulation, one of the main tissue-based abnormal mechanisms of Na^+ handling in RBD rats seems to rely on the functioning and regulation of renal Na^+-transporting ATPases, where again RAAS has a central role [52, 53]. In RBD rats, the huge inhibition of proximal tubules (Na^++K^+)ATPase (Fig. 12A), which is responsible for the bulk reabsorption of filtered Na^+ [23], possibly represents the smaller amount of filtered salt-load that needs to be recovered in the tubules of rats with very reduced body mass and, therefore, with reduced Na^+-containing liquid compartments. In contrast, upregulated ouabain-resistant Na^+-ATPase (Fig. 13A) responsible for the fine-tuning of proximal Na^+ reabsorption [25, 26] is likely to be an essential mechanism involved in the increased positive Na^+ balance depicted in Fig. 9A. Since there is no increase in $U_{Na}V$ by treatments with Losartan and Ang-(3–4) (Fig. 8B–D), normalization of the proximal tubule Na^+-ATPase by the 2 compounds may be ascribed to restoration of the fine-tune reabsorption at the level of proximal tubules rather than a contribution for an overall recovery of the normal and bulk Na^+ balance.

In conclusion, this study provides evidence that the chronic administration of a multideficient diet with a low content of protein of very poor quality is the primary cause – rather than excess Na^+ – in the pathogenesis of hypertension in undernourished rats, by simultaneously targeting the Ang II⇒AT_1R axis of local RAAS in kidney, the central nervous system (especially centers of Na^+ hungry control and cardiovascular regulation), and possibly bodily Na^+ distribution and structural modifications of the cardiovascular system itself. Furthermore, these results give support to the view that the antagonism of the Ang II⇒AT_1R axis by the Ang II⇒AT_2R axis within the RAAS is mediated, at least in part, by central and peripheral actions of Ang-(3–4), the potent allosteric enhancer of AT_2R [12].

Declarations

Acknowledgements This work was supported by grants from the Carlos Chagas Filho Rio de Janeiro State Foundation/FAPERJ, the Brazilian National Research Council/CNPq and the Brazilian Federal Agency for Support and Evaluation of Graduate Education/CAPES. The funders were not involved in the data collection, analysis, interpretation and report of the results. The authors express special thanks to the Laboratory of Food Analysis and Processing, Josué de Castro Institute of Nutrition at Federal University of Rio de Janeiro. The finally submitted version of the manuscript was prepared by BioMedES Ltd., UK (www.biomedes.biz).

Author contributions Research idea and study design: AP-A, and AV. Data acquisition: AP-A, JPMV-S, GC-S, and LFN. Data analysis and interpretation: AP-A, JPMV-S, GC-S, HM-F, and AV. Manuscript writing and

review: AP-A, HM-F, and AV. All authors had been read and approved submission.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this manuscript.

References

1. World Health Organization. *Malnutrition*. https://www.who.int/news-room/fact-sheets/detail/malnutrition. (2021).
2. Benabe, J.E., Fernández-Repollet, E., Tapia, E., Luo, C., Martinez-Maldonado, M. Angiotensin II and catecholamines interaction in short-term low protein feeding. *Kidney Int.* **44**, 285–293 (1993). https://doi.org/10.1038/ki.1993.243
3. Sawaya, A.L., Martins, P.A., Grillo, L.P., Florêncio, T.T. Long-term effects of early malnutrition on body weight regulation. *Nutr Rev.* **62**, S127–S133 (2004). https://doi.org/10.1111/j.1753-4887.2004.tb00082.x
4. Silva, F.C., de Menezes, R.C., Chianca, D.A. Jr. The implication of protein malnutrition on cardiovascular control systems in rats. *Front Physiol.* **6**, 246 (2015). http://doi.org/10.3389/fphys.2015.00246
5. Teodósio, N.R., Lago, E.S., Romani, S.A., Guedes, R.C. A regional basic diet from northeast Brazil as a dietary model of experimental malnutrition. *Arch Latinoam Nutr.* **40**, 533–547(1990).
6. Jannuzzi, L.B. *et al*. Undernutrition - thirty years of the Regional Basic Diet: the legacy of Naíde Teodósio in different fields of knowledge. *Nutr Neurosci*. (2021) https://doi.org/10.1080/1028415X.2021.1915631
7. Agoston, D.V. How to translate time? The temporal aspect of human and rodent biology. *Front Neurol.* **8**, 92 (2017) https://doi.org/10.3389/fneur.2017.00092
8. Timmermans, P.B. *et al.* Discovery of losartan, the first angiotensin II receptor antagonist. *J. Hum. Hypertens* **9**:S3–S18 (1995).
9. Matsui, T. *et al.* Val-Tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clin. Exp. Pharmacol. Physiol.* **29**, 204–208 (2002). https://doi.org/10.1046/j.1440-1681.2002.03628.x
10. Dias, J. *et al*. ANG-(3–4) inhibits renal Na^+-ATPase in hypertensive rats through a mechanism that involves dissociation of ANG II receptors, heterodimers, and PKA. *Am. J.Physiol Renal Physiol.* **306**, F855–F863 (2014). https://doi.org/10.1152/ajprenal.00488.2013
11. Dias, J., Axelband, F., Lara, L.S., Muzi-Filho, H., Vieyra, A. Is angiotensin-(3–4) (Val-Tyr), the shortest angiotensin II-derived peptide, opening new vistas on the renin-angiotensin system? *J. Renin Angiotensin Aldosterone Syst.* **18**, 1470320316689338 (2017). https://doi.org/10.1177/1470320316689338

12. Axelband, F.*et al*. Angiotensin-(3–4) counteracts the Angiotensin II inhibitory action on renal Ca^{2+}-ATPase through a cAMP/PKA pathway. *Regul. Pept.* **177**, 27–34 (2012). https://doi.org/10.1016/j.regpep.2012.04.004
13. Luzes, R. *et al.* Angiotensin-(3-4) normalizes blood pressure, decreases Na^+ and energy intake, but preserves urinary Na^+ excretion in overweight hypertensive rats. *Biochim Biophys Acta Mol Basis Dis.* **1867**, 166012 (2021). https://doi.org//10.1016/j.bbadis.2020.166012
14. Silva, P.A. *et al*. Altered signaling pathways linked to angiotensin II underpin the upregulation of renal Na^+-ATPase in chronically undernourished rats. *Biochim Biophys Acta* **1842**, 2357–2366 (2014). https://doi.org/10.1016/j.bbadis.2014.09.017
15. Reeves, P.G., Nielsen, F.H., Fahey, G.C. Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**,1939–1951 (1993). https://doi.org/10.1093/jn/123.11.1939
16. Feng, M. *et al*. Validation of volume-pressure recording tail-cuff blood pressure measurements. *Am. J. Hypertens.* **21**, 1288–1291 (2008). https://doi.org/10.1038/ajh.2008.301
17. Vieyra, A. *et al.* Comparison between calcium transport and adenosine triphosphatase activity in membrane vesicles derived from rabbit kidney proximal tubules. *J. Biol. Chem.* **261**, 4247–4255 (1986). https://doi.org/10.1016/S0021-9258(17)35654-5
18. Whittembury, G., Proverbio, F. Two modes of Na extrusion in cells from guinea pig kidney cortex slices. *Pflügers Arch.* **316**, 1–25 (1970). https://doi.org/10.1007/BF00587893
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951). https://doi.org/10.1016/S0021-9258(19)52451-6
20. Taussky, H.H., Shorr, E. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **202**, 675–685 (1953). https://doi.org/10.1016/S0021-9258(18)66180-0
21. Muzi-Filho, H.*et al*. Histone deacetylase activity and the renin-angiotensin-aldosterone system: key elements in cardiorenal alterations provoked by chronic malnutrition in male adult rats. *Cell Physiol Biochem* **54**:1143–1162 (2020). https://doi.org/10.33594/000000306
22. Murtaugh, M.A. *et al*. Relationship of sodium intake and blood pressure varies with energy intake: secondary analysis of the DASH (Dietary Approaches to Stop Hypertension)-sodium trial. *Hypertension.* **71**:858–865 (2018). https://doi.org/10.1161/HYPERTENSIONAHA.117.10602
23. Féraille, E., Doucet, A. Sodium-potassium-adenosinetriphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiol. Rev.* **81**, 345–418 (2001). https://doi.org/10.1152/physrev.2001.81.1.345
24. Shrimanker, I., Bhattarai, S. Electrolytes. In: StatPearls [Internet]. Treasure Island (FL): *StatPearls Publishing*. (2021) Updated 26 July 2021
25. Bełtowski, J., Borkowska, E., Wójcicka, G., Marciniak, A. Regulation of renal ouabain-resistant Na^+-ATPase by leptin, nitric oxide, reactive oxygen species, and cyclic nucleotides: implications for

obesity-associated hypertension. *Clin. Exp. Hypertens.* **29**, 189–207 (2007). https://doi.org/10.1080/10641960701361585

26. Vieyra, A. *et al*. The role of the second Na^+ pump in mammals and parasites. In: Chakraborti S, Dhalla N (eds) Regulation of membrane Na^++K^+-ATPase. *Advances in Biochemistry in Health and Disease*, (Springer) Cham, pp 93–112 (2016). https://doi.org/10.1007/978-3-319-24750-2_6
27. McLaren, D.S., Pellett, P.L. Nutrition in the Middle East. *World Rev. Nutr. Diet.* **12**, 43–127 (1970). https://doi.org/10.1159/000387584
28. Ramos-Aliaga, R. Biochemical and nutritional aspects in growing rats receiving proteins of 2 dietary regimens of the Peruvian Andes. *Arch. Latinoam. Nutr.* **28**, 378–400 (1978).
29. Tshala-Katumbay, D., Mwanza, J.C., Rohlman, D.S., Maestre, G., Oriá, R.B. A global perspective on the influence of environmental exposures on the nervous system. *Nature.* **527**, S187–S192 (2015). https://doi.org/10.1038/nature16034
30. Santos, M.J.S. *et al*. A Brazilian regional basic diet-induced chronic malnutrition drives liver inflammation with higher ApoA-I activity in C57BL6J mice. *Braz. J. Med. Biol. Res.* **53**, e9031 (2020). https://doi.org/10.1590/1414-431x20209031
31. Forouzanfar, M.H. *et al*. Evaluating causes of death and morbidity in Iran, global burden of diseases, injuries, and risk factors study 2010. *Arch. Iran. Med.* **17**, 304–320 (2014).
32. Hirvonen, K., Bai, Y., Headey, D., Masters, W.A. Affordability of the EAT-Lancet reference diet: a global analysis. *Lancet Glob Health.* **8**, e59–e66 (2020). https://doi.org/10.1016/S2214-109X(19)30447-4
33. Stanier, M.W. The effect of urea loading on volume and concentration of urine in rabbits. *J. Physiol.* **205**, 367–376 (1969). https://doi.org/10.1113/jphysiol.1969.sp008971
34. Claeyssens, S. *et al*. Metabolic changes in rats fed a low protein diet during post-weaning growth. *Metabolism.* **39**, 676–681 (1990). https://doi.org/10.1016/0026-0495(90)90100-q
35. Nwagwu, M.O., Cook, A., Langley-Evans, S.C. Evidence of progressive deterioration of renal function in rats exposed to a maternal low-protein diet in utero. *Br. J. Nutr.* **83**, 79–85 (2000). https://doi.org/10.1017/S0007114500000118
36. Silva, P.A. *et al*. Mechanisms involving Ang II and MAPK/ERK1/2 signaling pathways underlie cardiac and renal alterations during chronic undernutrition. *PLoS One.* **9**, e100410 (2014). https://doi.org/10.1371/journal.pone.0100410
37. McLaughlan, J.M., Illman, W.I. Use of free plasma amino acid levels for estimating amino acid requirements of the growing rat. *J. Nutr.* **93**, 21–24 (1967). https://doi.org/10.1093/jn/93.1.21
38. Munro, H.N. Metabolic regulation in relation to cell development. *Fed. Proc.* **29**, 1490–1496 (1970).
39. Cheney, M.C., Sabry, Z.I., Beaton, G.H. Blood transaminase activities in vitamin B6 deficiency: effect of depletion and repletion on the erythrocyte enzymes. *Can. J. Physiol. Pharmacol.* **45**, 343–351 (1967). https://doi.org/10.1139/y67-038
40. Harper, A.E., Benevenga, N.J., Wohlhueter, R.M. Effects of ingestion of disproportionate amounts of amino acids. *Physiol. Rev.* **50**, 428–558 (1970). https://doi.org/10.1152/physrev.1970.50.3.428

41. Axelband, F. *et al*. Ang-(3–4) suppresses inhibition of renal plasma membrane calcium pump by Ang II. *Regul. Pept.* **155**, 81–90 (2009). https://doi.org/10.1016/j.regpep.2009.03.014
42. Bruce, E.B., de Kloet, A.D. The intricacies of the renin-angiotensin-system in metabolic regulation. *Physiol. Behav.* **178**, 157–165 (2017). https://doi.org/10.1016/j.physbeh.2016.11.020
43. de Araújo, T.M., Velloso, L.A. Hypothalamic IRX3: a new player in the development of obesity. *Trends Endocrinol. Metab.* **31**, 368–377 (2020). https://doi.org/10.1016/j.tem.2020.01.002
44. AbdAlla, S., Lother, H., Abdel-tawab, A.M., Quitterer, U. The angiotensin II AT_2 receptor is an AT_1 receptor antagonist. *J. Biol. Chem.* **276**, 39721–39726 (2001). https://doi.org/10.1074/jbc.M105253200
45. Boguslavskyi, A. *et al*. Phospholemman phosphorylation regulates vascular tone, blood pressure, and hypertension in mice and humans. *Circulation.* **143**, 1123–1138 (2021). https://doi.org/10.1161/CIRCULATIONAHA.119.040557
46. Gomes, P., Soares-da-Silva, P. Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na^+-K^+-ATPase activity. *Am. J. Physio.l Renal Physiol.* **282**, F1084–F1096 (2002). https://doi.org/10.1152/ajprenal.00318.2001
47. Yu, P. *et al*., Dopamine D_1 receptor-mediated inhibition of NADPH oxidase activity in human kidney cells occurs via protein kinase A-protein kinase C cross talk. *Free Radic. Biol. Med.* **50**, 832–840 (2011). https://doi.org/10.1016/j.freeradbiomed.2010.12.027
48. Smith, N.J., Luttrell, L.M. Signal switching, crosstalk, and arrestin scaffolds: novel G protein-coupled receptor signaling in cardiovascular disease. *Hypertension.* **48**, 173–179 (2006). https://doi.org/10.1161/01.HYP.0000232641.84521.92
49. Cornish, B.H., Thomas, B.J., Ward, L.C. Improved prediction of extracellular and total body water using impedance loci generated by multiple frequency bioelectrical impedance analysis. *Phys. Med. Biol.* **38**, 337–346 (1993). https://doi.org/10.1088/0031-9155/38/3/001
50. Hanson, P. *et al*. Sodium in the dermis colocates to glycosaminoglycan scaffold, with diminishment in type 2 diabetes mellitus. *JCI Insight.* **6**, e145470 (2021). https://doi.org/10.1172/jci.insight.145470
51. Titze, J. *et al*. Spooky sodium balance. *Kidney Int.* **85**, 759–767 (2014). https://doi.org/10.1038/ki.2013.367
52. Aperia, A. *et al*. Activation/deactivation of renal Na^+,K^+-ATPase: a final common pathway for regulation of natriuresis. *FASEB J.* **8**, 436–439 (1994). https://doi.org/10.1096/fasebj.8.6.8168694
53. Rangel, L.B. *et al*. PI-PLCβ is involved in the modulation of the proximal tubule Na^+-ATPase by angiotensin II. *Regul. Pept.* **127**, 177–182 (2005). https://doi.org/10.1016/j.regpep.2004.12.007

Figures

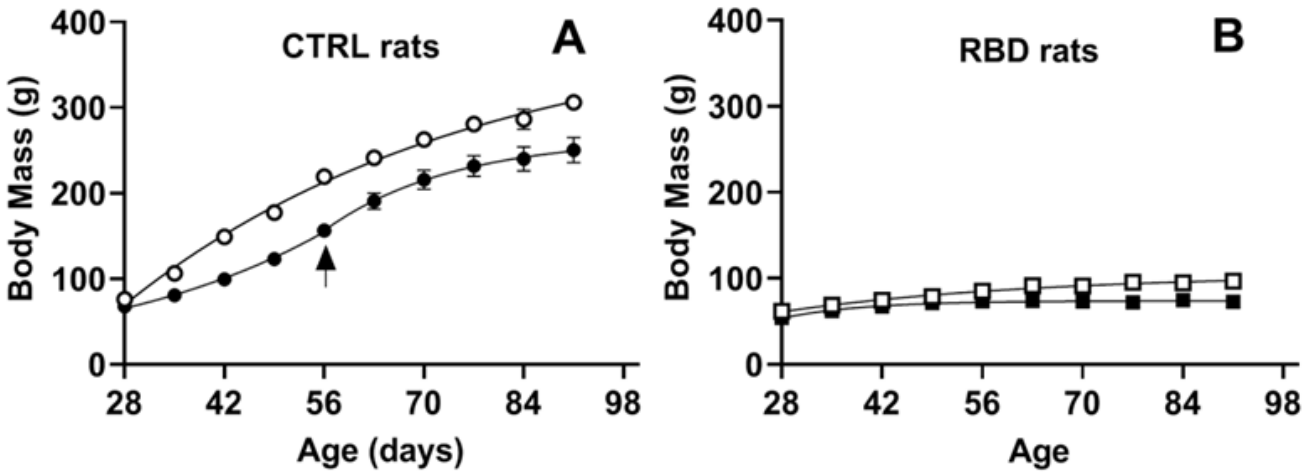

Figure 1

Body mass (BM) development of rats that received the control diet (CTRL) (A) or the multideficient diet (RBD) (B) for 64 days from weaning at 28 days until 92 days of age: effect of Losartan administration for the same period indicated on the *abscissa*. Dietary composition is described in Table 1. Empty symbols: untreated rats; filled symbols: Losartan-treated rats. Data are means ± SEM (n=8–16). In several cases error bars are smaller than the symbol size. Equations 1 and 2 were adjusted to the experimental points, the kinetic parameters of growth being described in the text and summarized in Table 2. The arrow in A (filled circles) indicates the transition between the 2 different phases of growth in Losartan-treated CTRL rats.

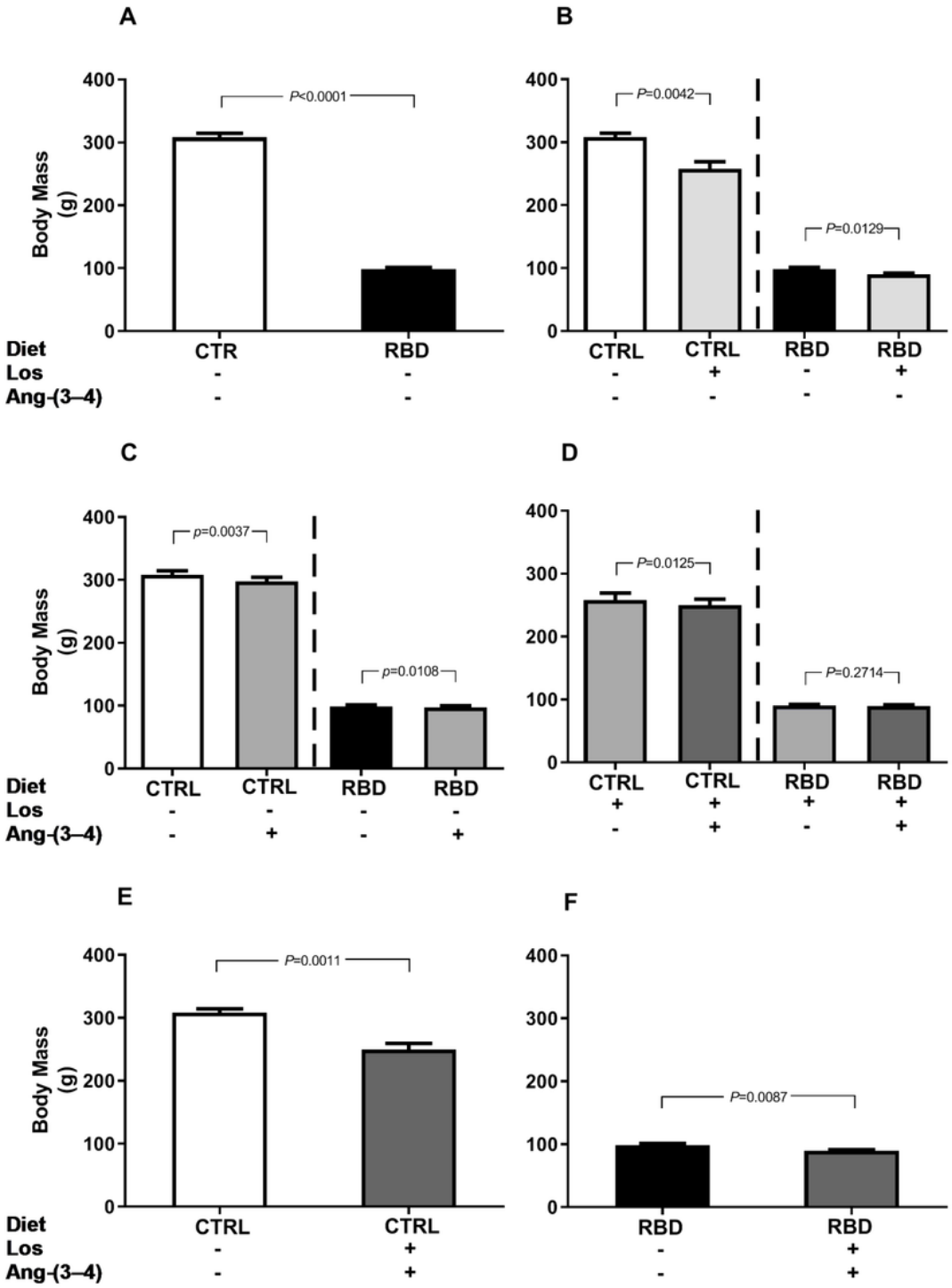

Figure 2

Accentuated diminution of BM due to RBD is slightly intensified by chronic treatment with Losartan and acute administration of a single dose of Ang-(3–4). Bars indicate means ± SEM. Part of CTRL and RBD rats received (i) Losartan in drinking water from weaning to 92 days of age, (ii) vehicle or a single dose (80 mg/kg BM) of Ang-(3–4) by gavage at 92 days of age. (A) Body mass from control (CTRL) and undernourished (RBD). (B) Effects of Losartan. (C) Effects of Ang-(3–4). (D) Effects of the combined

treatments. (E) and (F) Combined treatment (Losartan+ Ang-(3–4)) in comparison with untreated CTRL and RBD, respectively. Diets and treatments are indicated on the *abscissae*. Vertical dashed lines separate CTRL and RBD groups for better visualization of the effect of treatments in normonourished and undernourished rats. Differences were assessed by using unpaired Student's *t*-test (A, B, E and F) or paired *t*-test (the same rats before and after a single Ang-(3–4) administration in C and D) (n=4–8). *P* values are indicated within the panels.

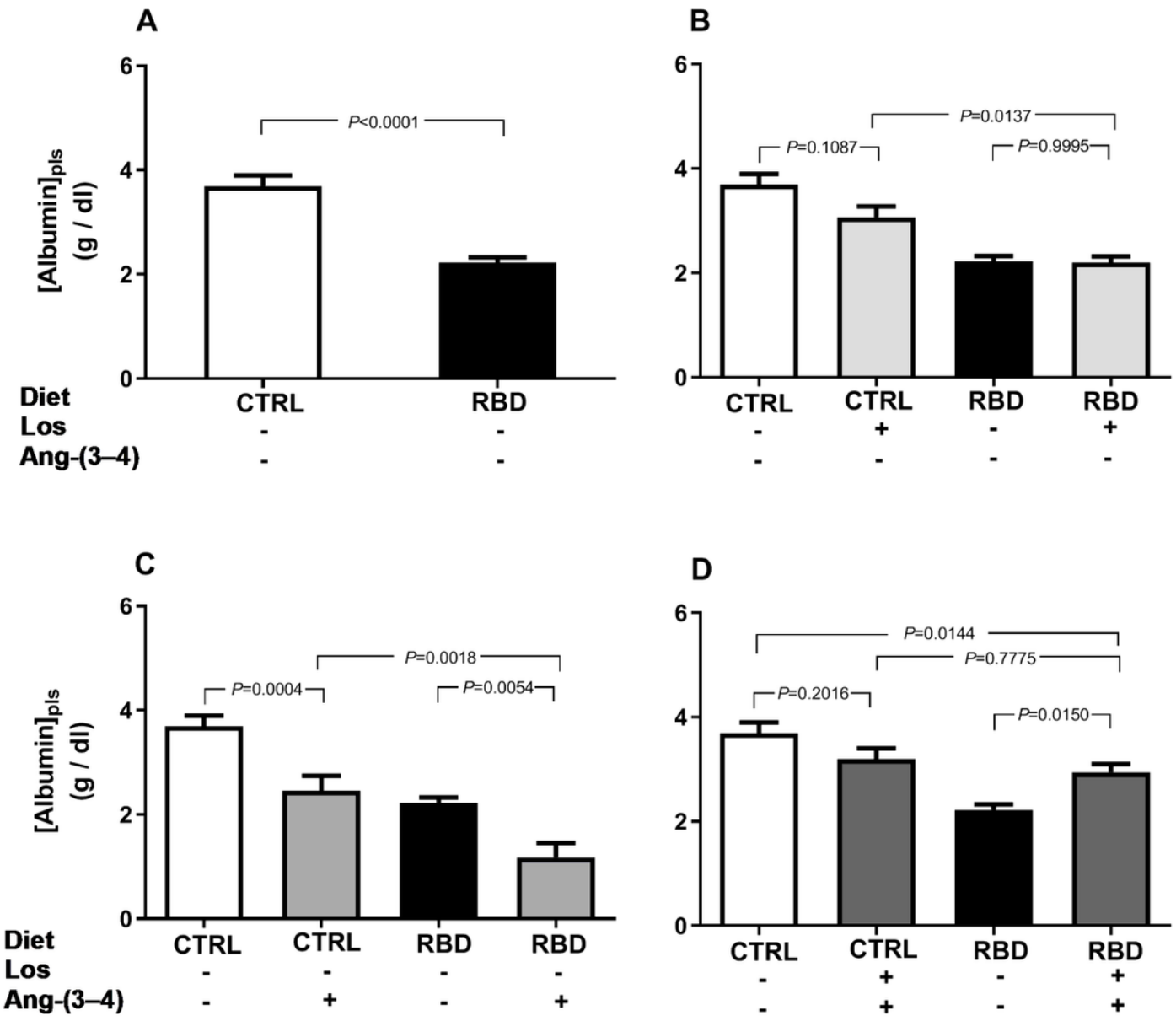

Figure 3

Responses of plasma albumin concentration to Losartan and Ang-(3–4) in normonourished and undernourished rats. Samples were collected at 91 days, except for Ang-(3–4)-treated rats, which were

collected at 92 days. Rats received the diets and treatments in the combinations shown on the *abscissae*. Bars indicate means ± SEM (n= 10–28). Differences were assessed by using unpaired Student's *t*-test (A) or one-way ANOVA followed by Tukey's test (B, C, D). *P* values are given within the panels.

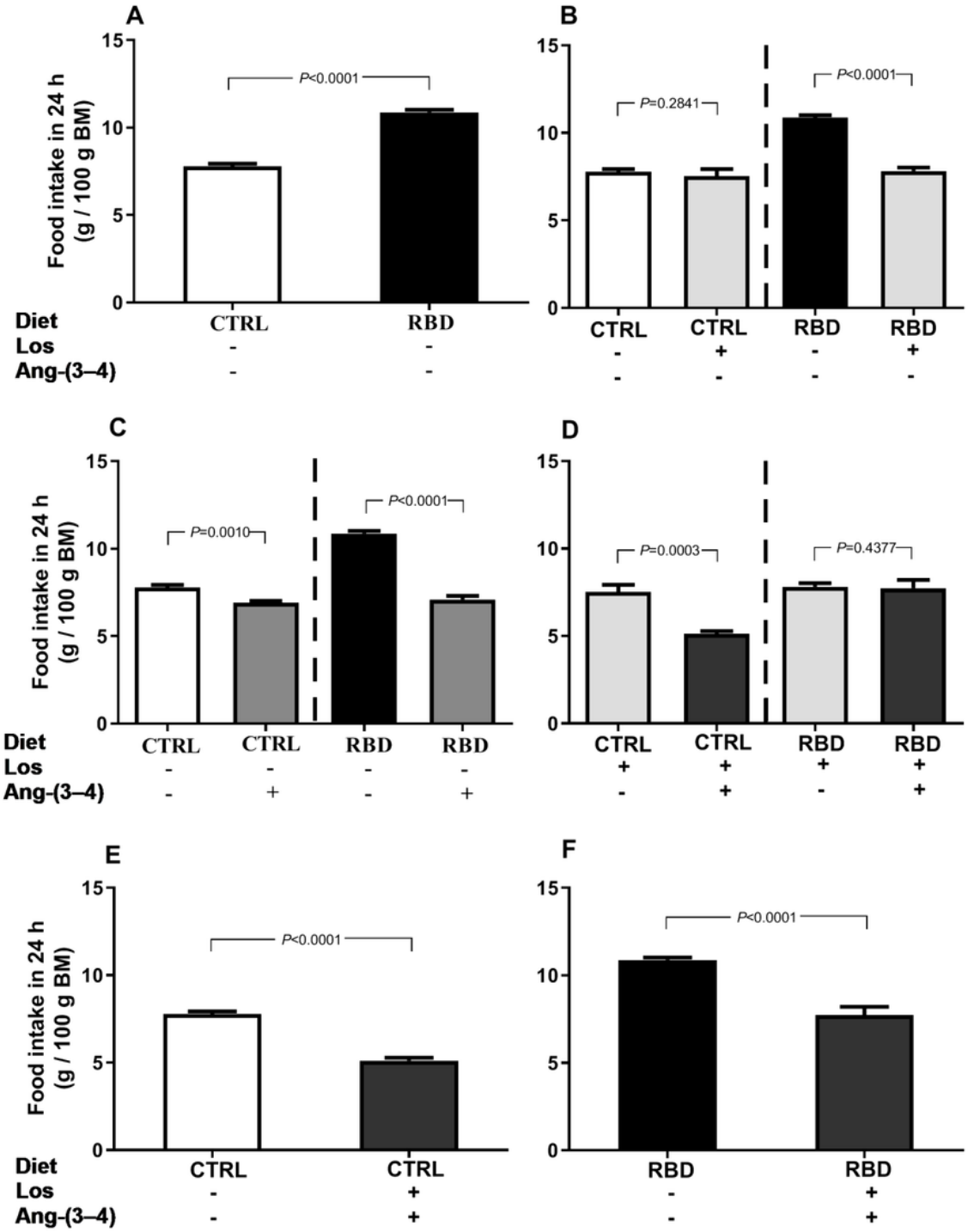

Figure 4

Food intake measured between 90 and 91 days, except for Ang-(3–4)-treated rats, for which determinations were carried out between 91 and 92 days. Bars are means ± SEM (data expressed per 100 g BM) (n=5–8). Diets and treatments were as indicated on the *abscissae*. Diets and treatments were as indicated on the abscissae. Vertical dashed lines separate CTRL and RBD groups for better comparison of the drugs effects in each dietary condition. Differences were assessed using unpaired Student's *t*-test. *P* values are indicated within the panels.

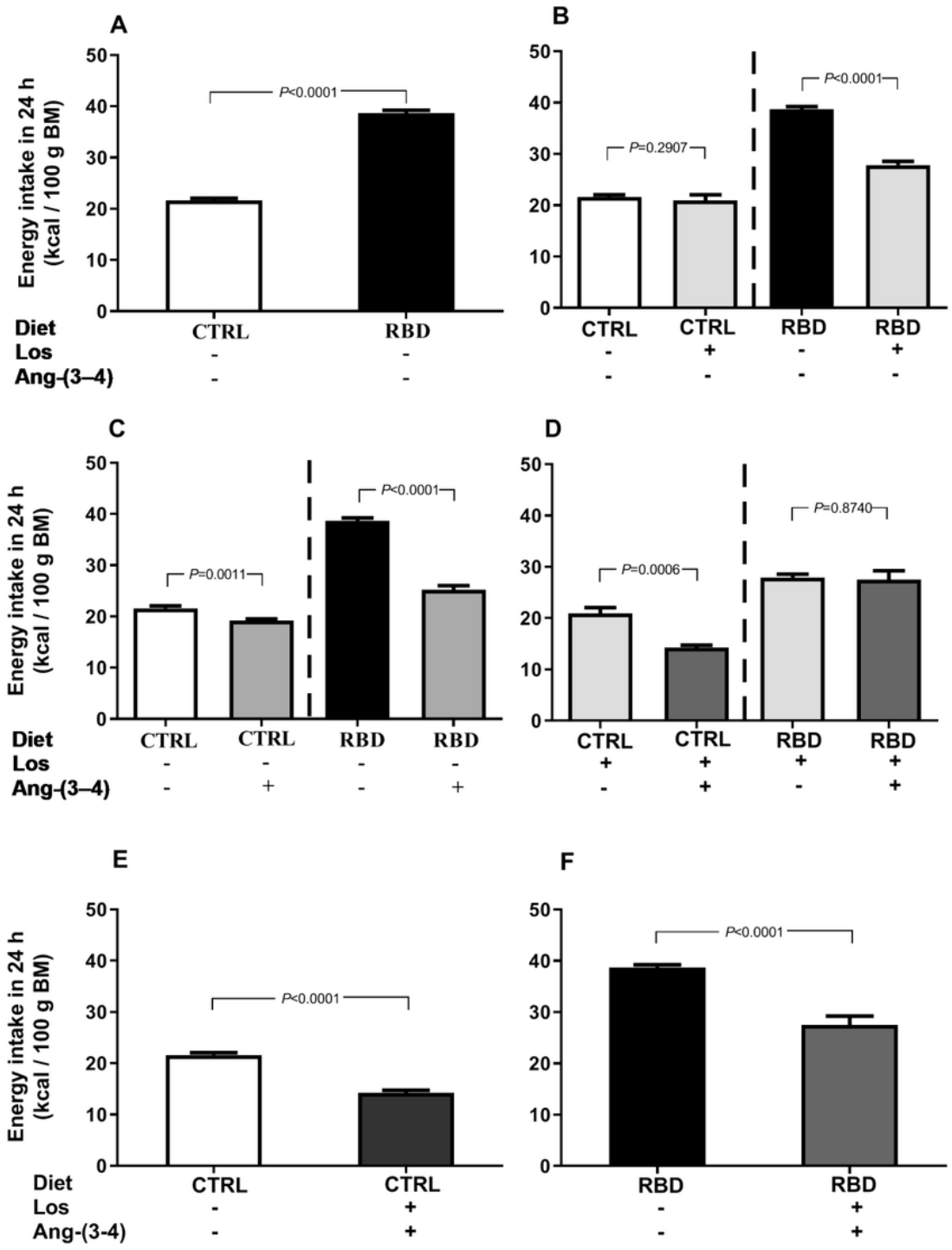

Figure 5

Energy intake calculated from food intake and the diet compositions described in Table 1. Bars are means ± SEM (data expressed per 100 g BM) (n=5–8). Diets and treatments were as indicated on the *abscissae*. Vertical dashed lines separate CTRL and RBD groups for better comparison of the drug effects under each dietary condition. Differences were assessed using unpaired Student's *t*-test. *P* values are indicated within the panels.

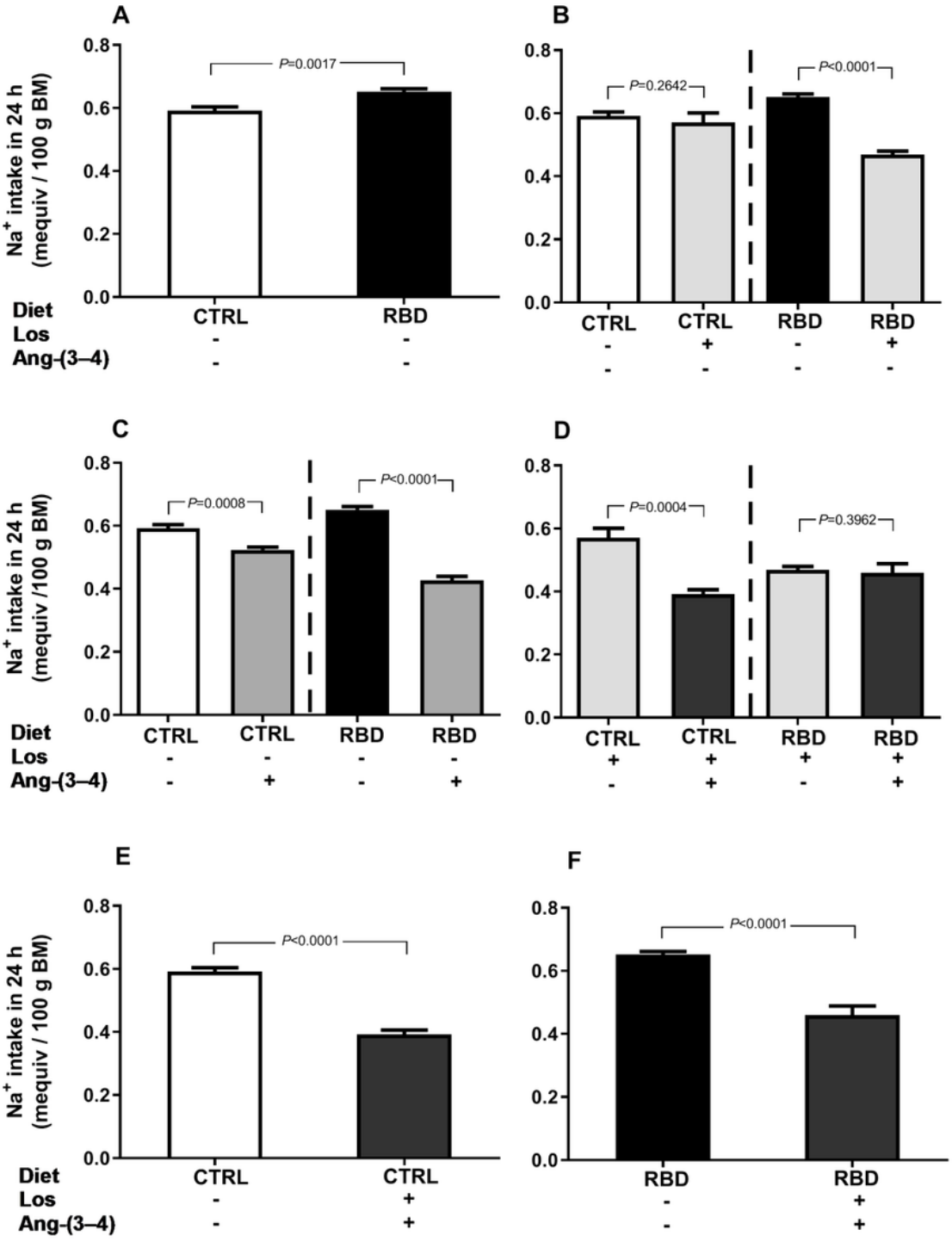

Figure 6

Na^+ intake in 24 h calculated from the dietary Na^+ content and the food intake, measured between 90 and 91 days, except for Ang-(3–4)-treated rats which was at 91 and 92 days. Bars are means ± SEM (data expressed per 100 g BM) (n=4–8). Diets and treatments were as indicated on the *abscissae*. Vertical

dashed lines separate CTRL and RBD groups for better comparison of the drugs effects in each dietary condition. Differences were assessed using unpaired Student's *t*-test. *P* values are indicated within the panels.

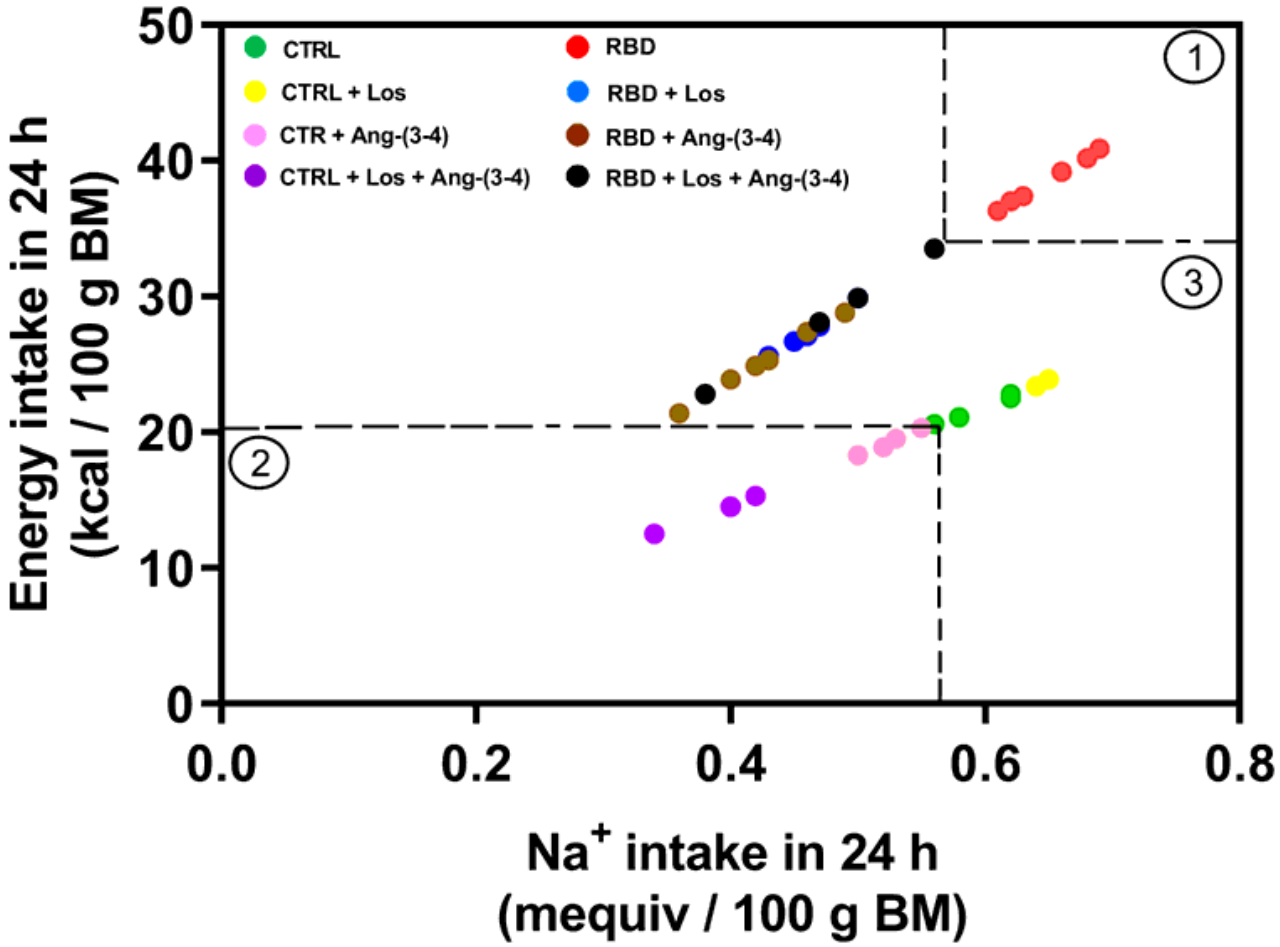

Figure 7

Na^+ density. Correlation between Na^+ intake and energy intake. Data points were calculated from the corresponding values given in Figs. 5 and 6. The dashed lines delimit the 3 different clusters of Na^+ densities (circles) described in the text. Upper line: RBD (red), RBD+Los (blue), RBD+Ang-(3–4) (brown), and RBD+Los+Ang-(3–4) (black). Bottom line: CTRL (green), CTRL+Los (yellow), CTRL+Ang-(3–4) (pink), and CTRL+Los+Ang-(3–4) (lilac). The function Energy intake = slope ´ Na^+ intake was adjusted to the points by the least squares method. Upper line: slope = 59.2 kcal/mequiv of Na^+; r = 0.9996. Bottom line: slope = 36.9 kcal/mequiv of Na^+; r = 0.9994.

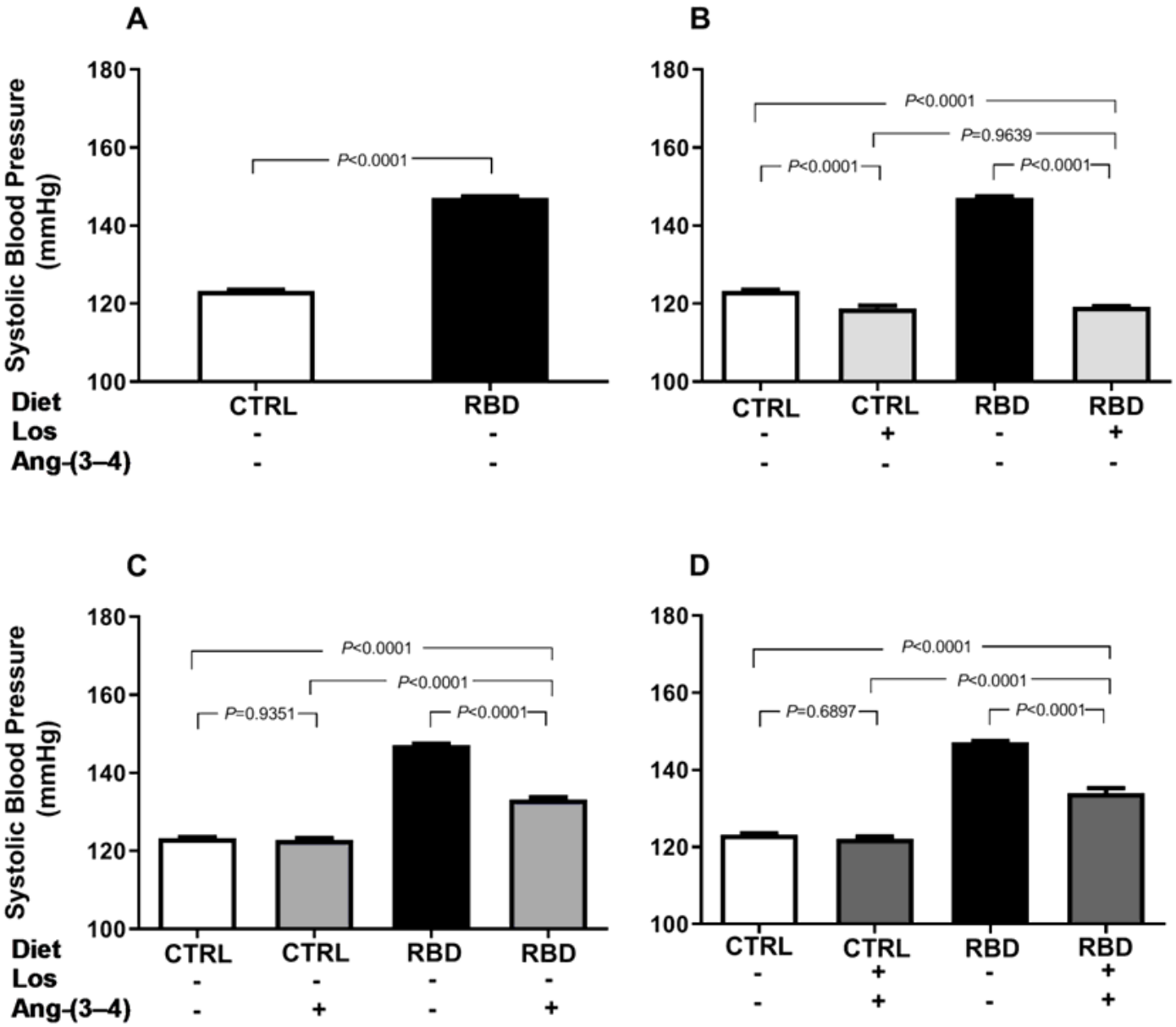

Figure 8

Losartan- and Ang-(3–4)-sensitive elevated systolic blood pressure (SBP) in undernourished rats. SBP was recorded in animals aged 91 or 92 days (in the case of Ang-(3–4)-treated rats) fed on CTRL or RBD diets. Combinations of diets and treatments are indicated on the *abscissae*. Bars are means ± SEM (n = 9–24). Differences between means were analyzed using unpaired Student's *t*-test (A) or one-way ANOVA followed by Tukey's test (B, C, D). *P* values are given within the panels.

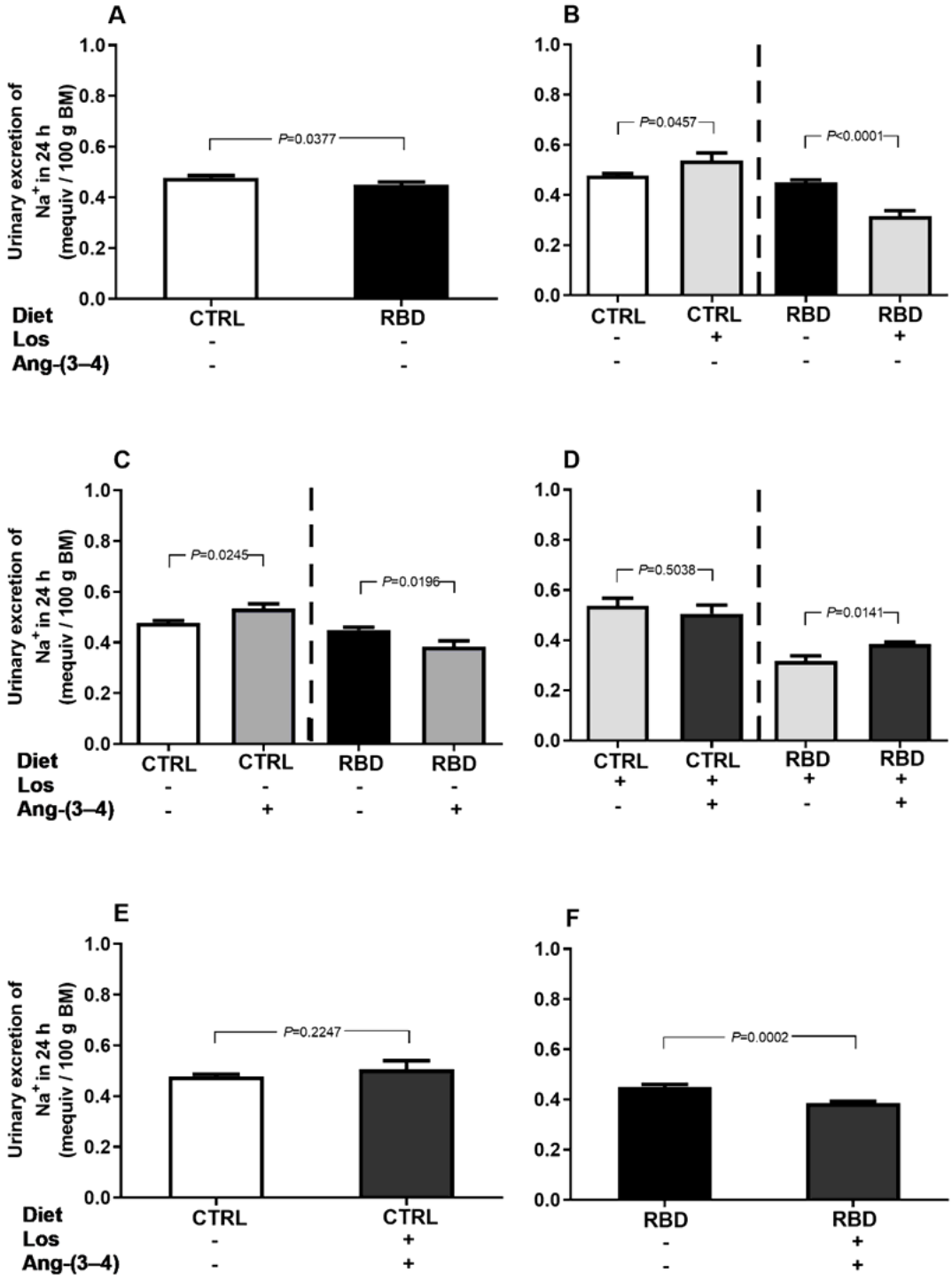

Figure 9

Urinary Na^+ excretion ($U_{Na}V$). Urinary Na^+ excretion in 24 h was calculated from V_{ur} in 24 h/100 g BM and $[Na^+]_{ur}$ (Supplementary Figs. 1 and 2, respectively). Combinations of diets and treatments are indicated on the *abscissae*. Vertical dashed lines in A–D separate panels for comparisons within CTRL and RBD

groups given different treatments. Differences between means were analyzed using unpaired Student's *t*-test. *P* values are given within the panels.

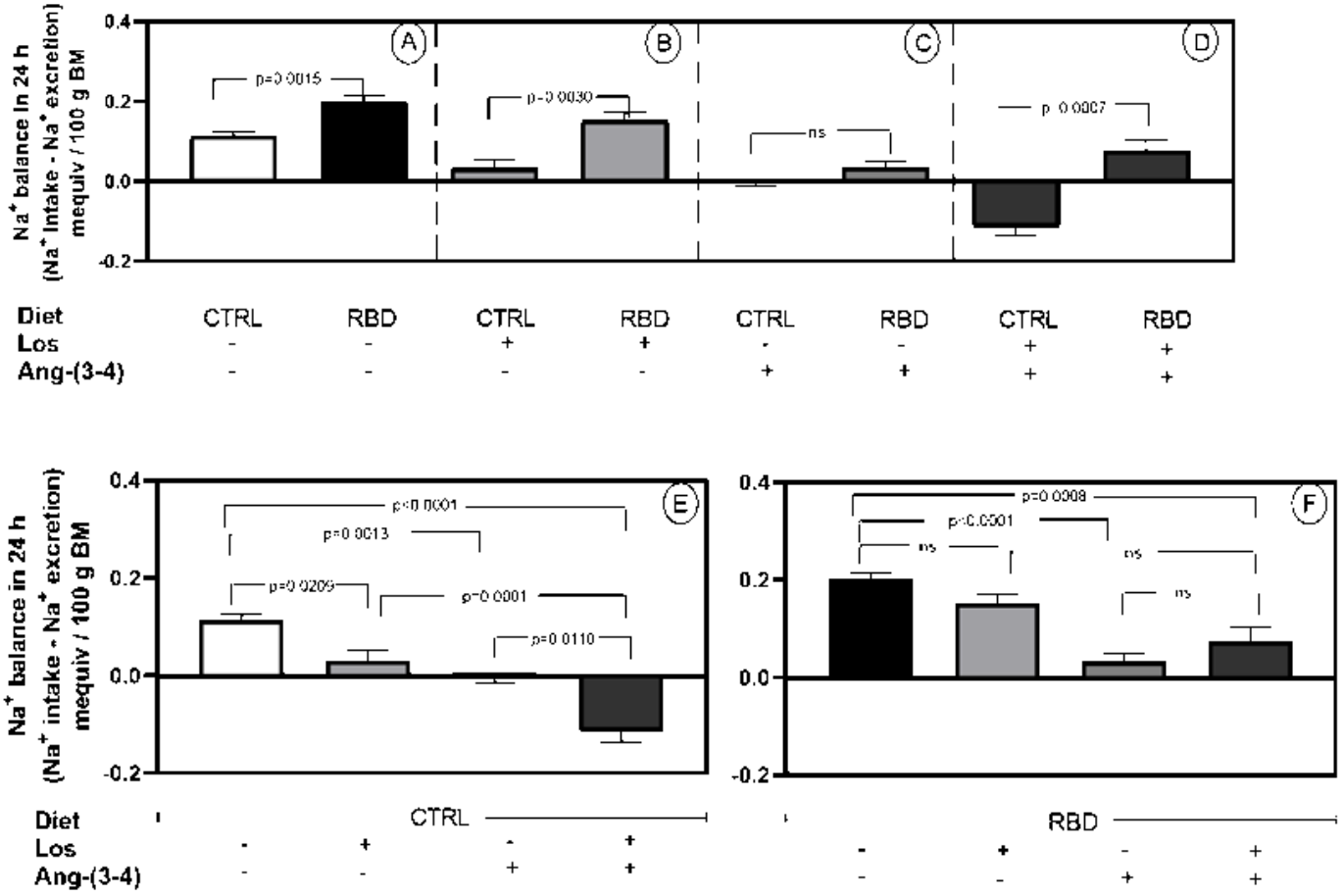

Figure 10

Na^+ balance. Na^+ balance (mequiv in 24 h/100 g BM) was calculated as the difference between Na^+ intake and urinary Na^+ excretion (Figs. 6 and 9, respectively). The combinations of diets and treatments are indicated on the *abscissae*. Bars are means ± SEM. Vertical dashed lines in A–D separate panels allowing comparisons within CTRL and RBD groups subjected to different treatments. Differences between means were analyzed using unpaired Student's *t*-test (A–D) or one-way ANOVA followed by Tukey's test (E, F). *P* values are given within the panels.

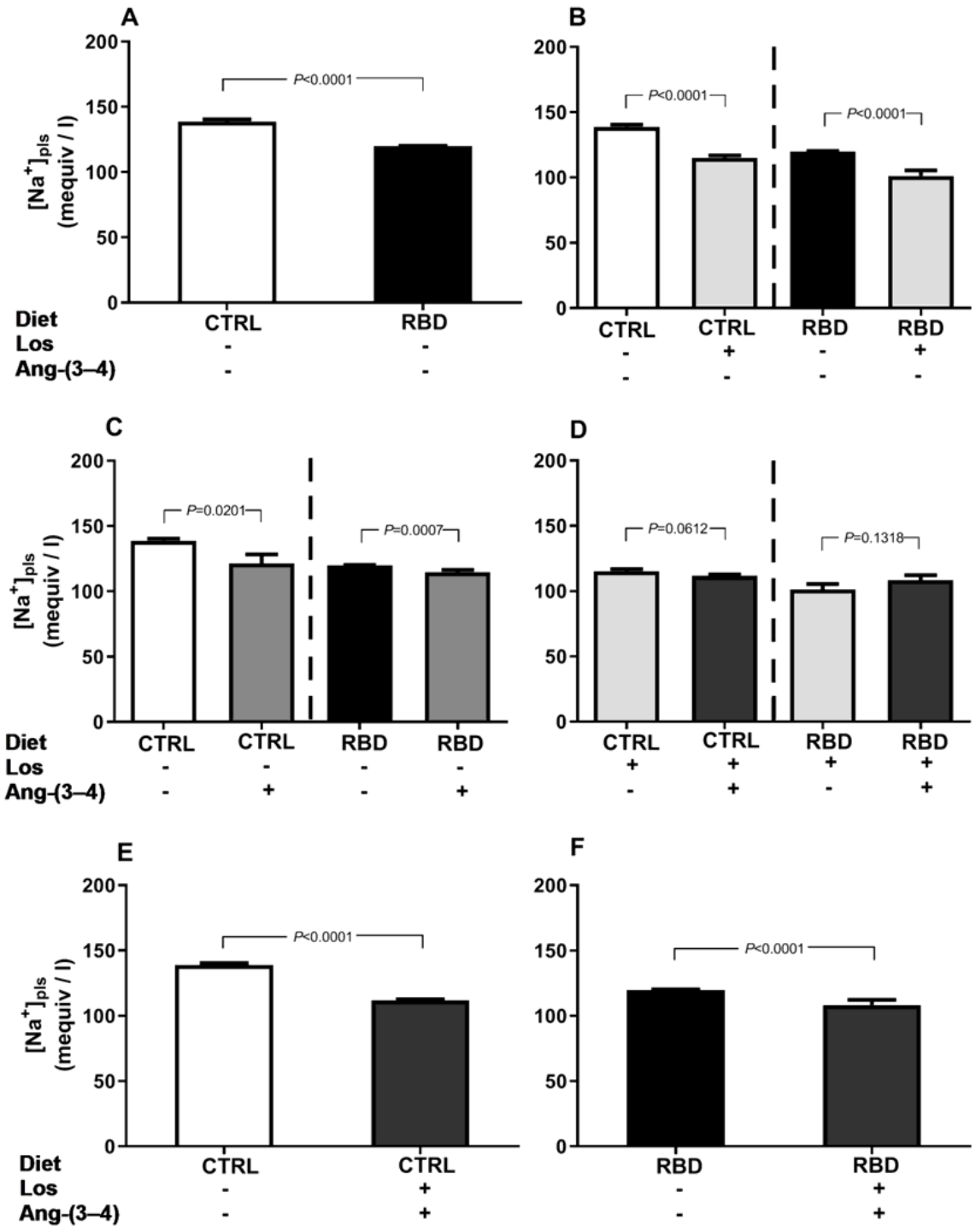

Figure 11

Plasma Na^+ concentration ($[Na+]_{pls}$), determined in samples collected at 91 or 92 days (in the case of Ang-(3–4)-treated rats). Combinations of diets and treatments are indicated on the *abscissae*. Bars are means ± SEM (n = 5–15). Vertical dashed lines in B–D separate panels give the comparisons within CTRL and RBD groups subjected to different treatments. Differences between means were analyzed using unpaired Student's *t*-test. *P* values are given within the panels.

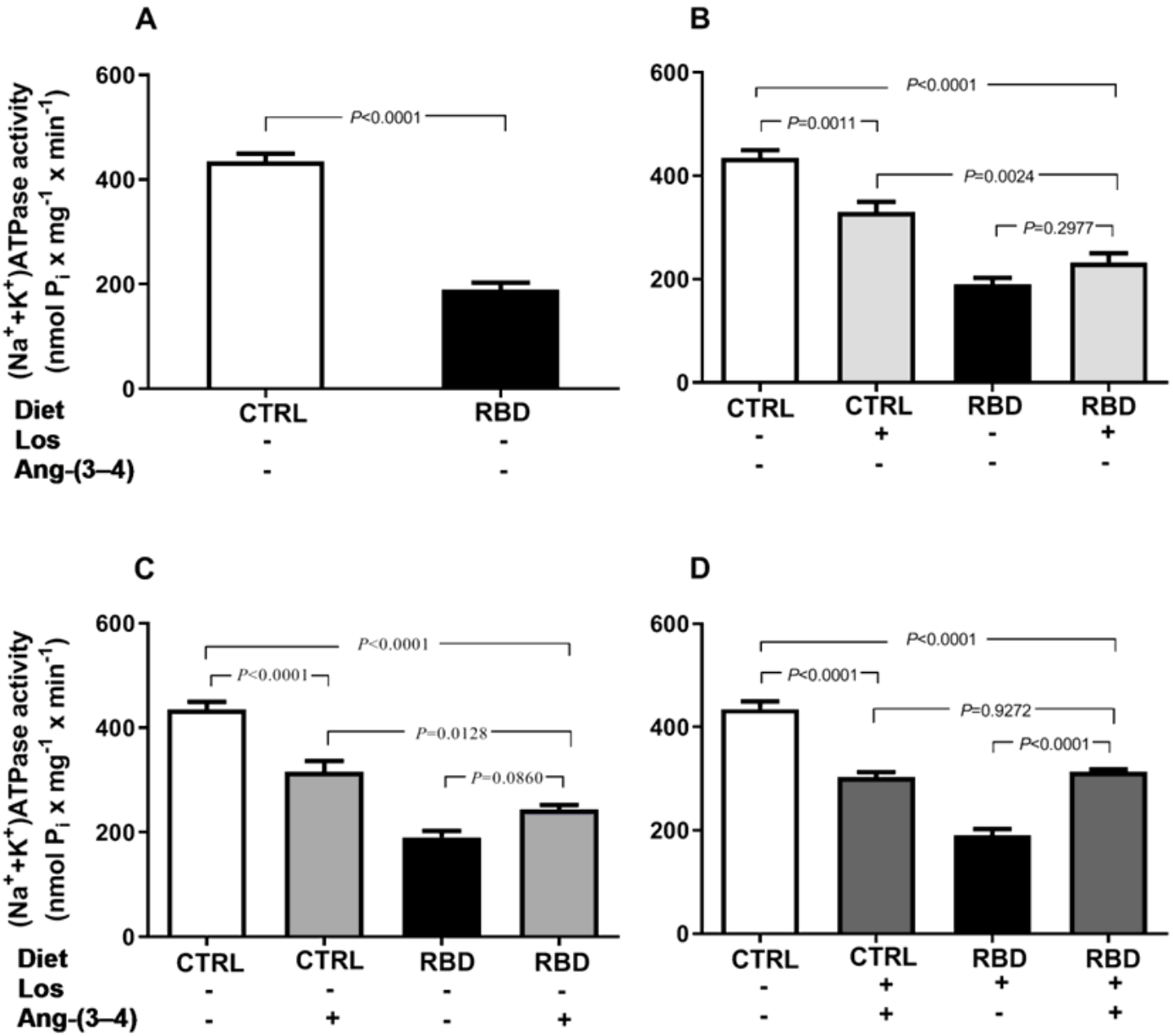

Figure 12

Downregulation of the ouabain-sensitive (Na^++K^+)ATPase from renal proximal tubule cells of chronically undernourished rats. Determinations were carried out in plasma membrane-enriched preparations isolated from the outermost region of the renal cortex (*cortex corticis*) at day 92. Combinations of diets and treatments are indicated on the abscissae. Bars are means ± SEM (n=5–6). Differences were assayed using one-way ANOVA followed by Tukey's test. *P* values are given within the panels.

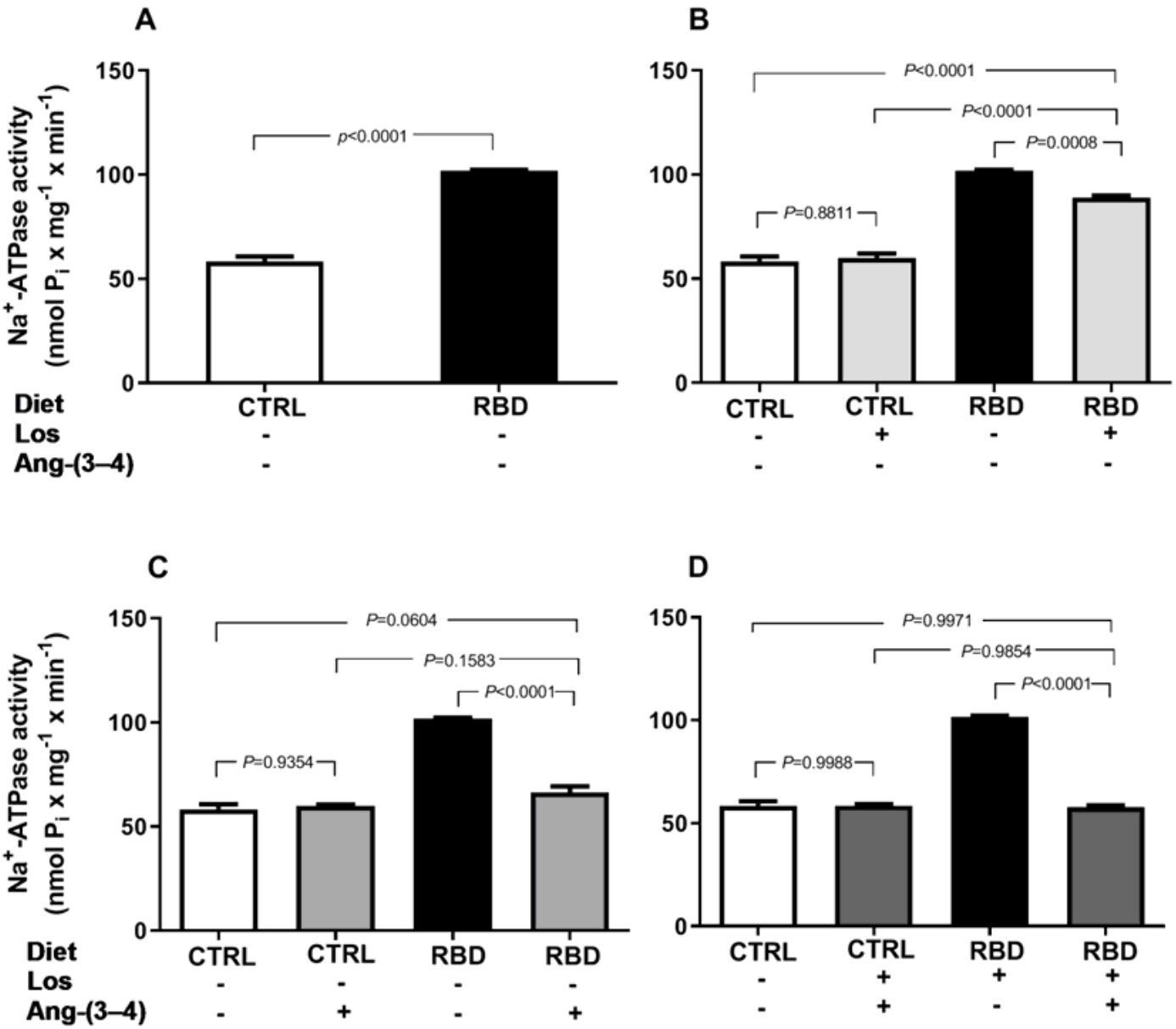

Figure 13

Upregulation of the ouabain-resistant Na^+-ATPase from renal proximal tubule cells of chronically undernourished rats. Determinations were carried out in plasma membrane-enriched preparations isolated from the outermost region of the renal cortex (*cortex corticis*) at day 92. Combinations of diets and treatments are indicated on the *abscissae*. Bars are means ± SEM (n=4). Means were compared using one-way ANOVA followed by Tukey's test. *P* values are given within the panels.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures1e2.pdf