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Brain, Heart and Kidney Correlate for the Control of Blood Pressure and Water Balance: Role of Angiotensinases

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Key Words
Neurovisceral integration · Autonomic nervous system · Aminopeptidases · Hypertension · Renin-angiotensin system

Abstract
The renin-angiotensin system (RAS) plays a major role in the control of blood pressure (BP) and water balance by coordinating brain, heart and kidney functions, connected with each other by hormonal and neural mechanisms through the autonomic nervous system (ANS). RAS function may be monitored by the study of the enzymes (angiotensinases) involved in the metabolism of its active peptides. In order to study the relationship between the brain-heart-kidney axis and the control of BP and water balance, we analyzed the correlation of angiotensinase activities, assayed as arylamidase activities, between hypothalamus, left ventricle, renal cortex and renal medulla, collected from Wistar-Kyoto and spontaneously hypertensive rats, treated or not treated with L-NAME [N(G)-nitro-L-arginine methyl ester]. This compound not only inhibits the formation of nitric oxide but also disrupts the normal function of the ANS activating the sympathetic nervous system (SNS) to increase BP. In addition, to assess the influence of the SNS, we studied the effect of its blockade by treatment of both strains with propranolol. The present results support the notion that RAS function of the brain-heart-kidney axis, as reflected by the activities of angiotensinases, is reciprocally connected by afferent and efferent mechanisms between these locations, presumably through the ANS. These results reveal new aspects of neuroendocrine regulation possibly involving the ANS.

Introduction

The neurovisceral integrative model is an old recovered but growing concept that supports the existence of bidirectional regulatory mechanisms involving not only the brain and heart [1–4] but also virtually the entire organism from brain to peripheral systems, including, for example, such an unexpected target as the gut and even its microbiota [5].

Blood pressure is regulated in physiological and pathological conditions by major neurovisceral integrative mechanisms connecting brain, heart and kidney. To maintain 'homeostasis', these mechanisms involve hor-

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monal as well as afferent and efferent neural feedback responses to exogenous and/or endogenous changes [3, 4]. They also involve brain anticipatory responses of need following the concept of 'allostasis', i.e. achieving homeostasis through advanced adjustment, especially of the hypothalamic-pituitary-adrenal axis and the autonomic nervous system (ANS) [6].

The renin-angiotensin system (RAS) plays a major role in the control of blood pressure and water balance by coordinating brain, heart and kidney functions [7], connected with each other by hormonal and neural mechanisms through the ANS [3, 4, 8]. RAS function may be monitored by the study of the enzymes (angiotensinases) involved in the metabolism of their active peptides [9].

We have previously reported a significant relationship, with a high level of correlation, of angiotensinase activities between the hypothalamus (HT) and the left ventricle (VT) in control and L-NAME [N(G)-nitro-L-arginine methyl ester]-treated Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) and suggested the possible existence of a reciprocal neural connection between both locations, presumably involving the ANS [4]. These unexpected results prompted us to investigate the possibility that (1) the kidney, as an organ directly involved in the control of blood pressure, also participates in this neurovisceral interaction and (2) the ANS plays a specific role in this interaction. Therefore, in order to study the relationship between the brain-heart-kidney axis in the control of blood pressure and the role of the ANS in this interaction, we analyzed the correlation of angiotensinase activities in the anterior HT, VT, renal cortex (RC) and renal medulla (RM) collected from WKY and SHR, treated or not treated with L-NAME. Since L-NAME not only inhibits the formation of nitric oxide but also activates the sympathetic nervous system and, therefore, increases blood pressure as it was clearly demonstrated independently of the routes of administration [10–12], we also evaluated the effect of the β-blocker propranolol on both strains. Both systolic blood pressure (SBP) and diuresis levels were measured as physiologic parameters.

The enzymatic activities analyzed [measured in their soluble (Sol) and membrane-bound (MB) forms by fluorimetric methods] were: aspartyl aminopeptidase (AspAP) which metabolizes Ang I to Ang 2–10, glutamyl aminopeptidase (GluAP) which metabolizes Ang II to Ang III and cholecystokinin, alanyl aminopeptidase (AlaAP) which transforms Ang III to Ang IV, an enzyme that also acts as an enkephalinase, and finally cystinyl aminopeptidase (CysAP), identified as insulin-regulated aminopeptidase and AT_4 receptor (in its MB form), which also hydrolyzes vasopressin or oxytocin [9] (fig. 1).

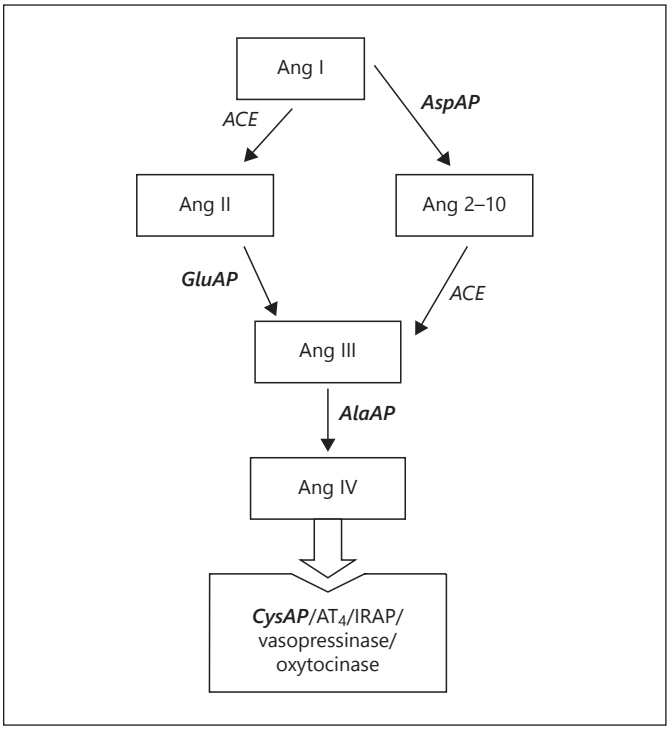

Fig. 1. Partial representation of the RAS pathway in which the angiotensinases studied in the present work are indicated. ACE = Angiotensin-converting enzyme; IRAP = insulin-regulated aminopeptidase.

Materials and Methods

Animals and Treatments

The study was performed in 30 adult male WKY rats, divided into control (n = 10), L-NAME- (n = 10) and propranolol-treated (n = 10) animals, and in 24 adult male SHR, divided into control (n = 8), L-NAME- (n = 8) and propranolol-treated (n = 8) animals, all of them weighing 100–150 g at the beginning of the study. All of the experimental procedures involving animals were performed in accordance with the European Communities Council Directive 86/609/EEC and were approved by the bioethics committee of the University of Jaén. L-NAME (Sigma; 70 mg/kg/day) and propranolol (Sigma; 100 mg/kg/day) were given in drinking water for 4 weeks [4, 13]. Chronic administration of L-NAME and propranolol in drinking water at these dosages has been reported to be an appropriate method to achieve the full effect of the drugs [10, 14]. To avoid the influence of daytime and light-dark conditions or season, the experiments were performed in summer (northern hemisphere) under light conditions (between 9:00 and 12:00 a.m.) [15].

Blood Pressure and Diuresis Measurement

SBP was monitored in nonanesthetized animals by the plethysmographic method throughout the experimental period (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). The rats were placed in plastic holders and warmed to 37°C for each recording

session. At least 15 determinations were made, and the mean of the stable values within a range of 5 mm Hg was recorded as the SBP level. Measurements at the beginning and end of the recording sessions were discarded. To adapt the animals to the procedure, blood pressure was measured on several occasions before the beginning of the study. Twenty-four hours before sacrifice, the animals were housed in metabolic cages, and urine volume was measured in individual animals [4, 16].

Surgical Procedure and Collection of Tissue Samples

At the end of the treatment period, after recording SBP, each rat was perfused with saline through the left cardiac VT under equithensin anesthesia (2 ml/kg body weight; 42.5 g/l chloral hydrate dissolved in 19.76 ml ethanol, 9.72 g/l Nembutal©, 0.396 l/l propylenglycol and 21.3 g/l magnesium sulfate in distilled water), and samples from the HT, VT, RC and RM were obtained as previously described [4, 16]. Shortly after perfusion, the brain was quickly removed (less than 60 s) and cooled in dry ice. At the time of the analysis, brains were individually defrosted, and when they reached the appropriate consistence, they were sliced by hand with a blade. The anterior HT was identified in the slides and dissected according to the stereotaxic atlas by Paxinos and Watson [17]. The selected area (pooled left and right) was between 7.7 and 3.7 mm anterior to the interaural line. In addition, the heart was removed and, immediately, a left ventricular sample was taken. The kidneys were also removed, and samples from the cortex and medulla (pooled left and right) were collected. Both heart and kidney samples were quickly frozen in dry ice.

Procedures for Enzymatic and Protein Assays

To get the Sol fraction, tissue samples were homogenized in a hypoosmolar medium (10 mM HCl-Tris buffer, pH 7.4) and ultracentrifuged at 100,000 *g* for 30 min at 4°C. The supernatants were used as a source for protein and enzyme assays. To get the particulate fraction, the pellets were rehomogenized in HCl-Tris buffer (pH 7.4) plus 1% Triton X-100 to solubilize membrane proteins. After centrifugation (100,000 *g*, 30 min, 4°C), MB activity and proteins were measured in triplicate in the supernatants. To ensure complete recovery of activity, the detergent was removed from the medium by adding adsorbent polymeric Bio-beads SM-2 (Sigma; 100 mg/ml) and shaking the samples for 2 h at 4°C.

Sol and MB angiotensinases, measured as AspAP, GluAP, AlaAP and CysAP activities, were determined fluorometrically using the arylamide derivatives aspartyl-, glutamyl-, alanyl- and cystinyl-β-naphthylamide as substrates as previously described [16, 18]. Briefly, AlaAP and CysAP were measured using Ala- or Cys-β-naphthylamide as substrates; 10 μl of each supernatant and plasma were incubated for 30 min at 25°C with 1 ml of the substrate solution: 2.14 mg/100 ml of Ala-β-naphthylamide or 5.53 mg/100 ml of Cys-β-naphthylamide, 10 mg/100 ml bovine serum albumin (BSA), and 10 mg/100 ml dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4, for AlaAP and 50 mM HCl-Tris buffer, pH 6, for CysAP. AspAP was determined with Asp-β-naphthylamide as substrate: 10 μl of each supernatant and plasma were incubated for 120 min at 37°C with 1 ml of the substrate solution (2.58 mg/100 ml Asp-β-naphthylamide, 10 mg/100 ml BSA, 10 mg/100 ml DTT and 39.4 mg/100 ml $MnCl_2$ in 50 mM HCl-Tris buffer, pH 7.4). GluAP was determined using Glu-β-naphthylamide as substrate: 10 μl of each supernatant was incubated during 120 min at 37°C with 1 ml of the substrate solution (2.72 mg/100 ml Glu-β-naphthylamide, 10 mg/100 ml BSA, 10 mg/100 ml DTT and 0.555 g/100 ml $CaCl_2$ in 50 mM HCl-Tris buffer, pH 7.4).

All the reactions were stopped by adding 1 ml of 0.1 M of acetate buffer, pH 4.2. The amount of β-naphthylamine released as a result of the enzymatic activity was measured fluorometrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified in triplicate by the method of Bradford [19], using BSA as a standard. Specific MB aminopeptidase activities were expressed as pmol of the corresponding substrate hydrolyzed per min per mg of protein. Fluorogenic assays were linear with respect to time of hydrolysis and protein content.

Statistical Analysis

Differences between the groups were evaluated using two-way analysis of variance. Post hoc comparisons were made with least significant difference tests. p values <0.05 were considered significant. To study the association between angiotensinases of the HT, VT, RC and RM, Pearson's coefficient of correlation was computed [20]. Computations were performed using SPSS 13.0 and STATA 9.0. p values <0.05 were considered significant.

Results

SBP and Diuresis

Compared with control animals, SBP increased significantly in WKY ($p < 0.001$) [21] and SHR ($p < 0.001$) [4] after L-NAME treatment. However, SBP was unaffected after propranolol treatment either in WKY or in SHR [13]. SBP was significantly higher in the control ($p < 0.001$), L-NAME- ($p < 0.05$) and propranolol- ($p < 0.001$) treated SHR groups than in the WKY group (fig. 2). Compared with controls, diuresis decreased significantly ($p < 0.001$) in propranolol-treated WKY rats and increased ($p < 0.001$) in L-NAME-treated SHR. Diuresis was also higher ($p < 0.05$) in L-NAME-treated SHR than in similarly treated WKY rats (fig. 2).

Correlational Study

The correlations between hypothalamic angiotensinase activities and VT, RC and RM in control, L-NAME- and propranolol-treated WKY and SHR are presented in tables 1 and 2.

The results clearly indicated that the number, the level of significance and the direction (positive or negative) of the correlations between HT and VT or kidney depended on the strain analyzed, the selected tissue and the type of treatment. Number, level and direction of correlations changed after L-NAME treatment and, in general, the effect of L-NAME was reversed after propranolol treatment in the comparisons of HT with VT as well as of HT with kidney (tables 1, 2).

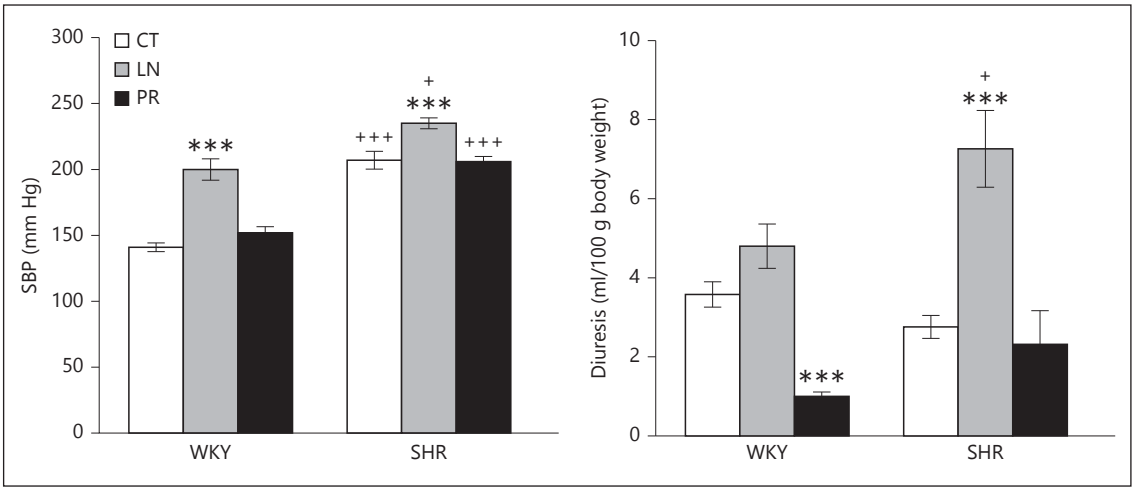

Fig. 2. SBP and diuresis in control versus L-NAME- and propranolol-treated WKY (n = 10) and SHR (n = 8) at the end of 4 weeks of treatment. Values represent means ± SEM. CT = Control; LN = L-NAME; PR = propranolol. *** $p < 0.001$, compared to control rats. $^{+}$ $p < 0.05$, $^{+++}$ $p < 0.001$, compared to WKY rats.

HT versus VT

In WKY rats, the comparison between HT and VT demonstrated 1 positive and 1 negative significant correlation in control and L-NAME-treated groups. The treatment with propranolol increased the level of significance and the number of correlations markedly; there was almost the same number of positive and negative correlations (5 positive/4 negative; table 1).

In SHR, only 1 positive correlation was observed between HT and VT. After L-NAME treatment, the number and level of significance of correlations increased markedly and the direction of correlations changed to exclusively negative. Treatment with propranolol restored the characteristics previously observed in controls; there was a low level of significance and only 2, exclusively positive, correlations (table 2).

HT versus RC

The comparison between HT and RC in WKY rats demonstrated no significant correlations in control animals. After L-NAME treatment, the number of correlations increased and they were mainly positive (3 positive/1 negative). When the animals were treated with propranolol, the level of significance was reduced and the number of correlations changed to 2 which were exclusively negative (table 1).

In SHR, only negative correlations between HT and RC were observed in the control group. After L-NAME treatment, the level of significance and the number of correlations increased radically and the direction of correlations changed to exclusively positive. Propranolol treatment reduced the number of correlations (only 2 significant correlations) and the level of significance and changed the direction of correlations to exclusively negative, similar to what was observed in the control SHR group (table 2).

HT versus RM

The comparison between HT and RM in WKY rats demonstrated a high number of highly significant, exclusively negative, correlations in control animals. Treatment with L-NAME tremendously reduced both the level of significance and the number of correlations, resulting in only 1 positive correlation. In contrast, propranolol treatment increased the number of correlations, which were mainly negative (7 negative/2 positive), substantially and similarly to control animals (table 1).

In SHR, control rats demonstrated only 1 positive correlation. L-NAME treatment increased the number of correlations to 3, but in this case, they were exclusively negative. After propranolol treatment, the direction of significant correlations was mainly positive (3 positive/1 negative; table 2).

No significant correlations were observed when VT, RC and RM were compared with each other.

To summarize, hypothetical positive and negative feedback between HT, VT, RC and RM in control, L-NAME- and propranolol-treated WKY and SHR is represented in figures 3 and 4. The thickness of the line indicates the weight of the correlation.

Table 1. Significant correlations in WKY rats

WKY rats		
Control rats	L-NAME-treated rats	Propranolol-treated rats
HT vs. VT (positive and *negative* correlations) HT MB CysAP vs. VT Sol AlaAP, r = +0.714, p = 0.02 *HT Sol GluAP vs. VT Sol AlaAP, r = –0.722, p = 0.01*	**HT vs. VT (positive and *negative* correlations)** HT MB CysAP vs. VT Sol GluAP, r = +0.718, p = 0.02 *HT MB GluAP vs. VT MB AlaAP, r = –0.738, p = 0.02*	**HT vs. VT (positive and *negative* correlations)** *HT Sol CysAP vs. VT Sol CysAP, r = –0.786, p = 0.006* *HT Sol GluAP vs. VT MB AlaAP, r = –0.776, p = 0.008* *HT Sol GluAP vs. VT MB GluAP, r = –0.762, p = 0.01* *HT Sol GluAP vs. VT MB AspAP, r = –0.685, p = 0.02* HT Sol AspAP vs. VT Sol AspAP, r = +0.676, p = 0.03 HT MB AspAP vs. VT Sol AlaAP, r = +0.664, p = 0.03 HT MB AspAP vs. VT Sol GluAP, r = +0.840, p = 0.002 HT MB AspAP vs. VT Sol AspAP, r = +0.635, p = 0.04 HT Sol GluAP vs. VT Sol AlaAP, r = +0.717, p = 0.01
HT vs. RC No correlations	**HT vs. RC (mainly positive correlations)** HT MB CysAP vs. RC Sol AlaAP, r = +0.720, p = 0.02 HT MB CysAP vs. RC Sol CysAP, r = +0.739, p = 0.02 HT MB CysAP vs. RC Sol GluAP, r = +0.834, p = 0.005 *HT Sol AlaAP vs. RC Sol AspAP, r = –0.731, p = 0.02*	**HT vs. RC (*negative* correlations)** *HT Sol AlaAP vs. RC Sol AspAP, r = –0.672, p = 0.03* *HT MB AspAP vs. RC MB AlaAP, r = –0.652, p = 0.04*
HT vs. RM (*negative* correlations) *HT MB AspAP vs. RM Sol AlaAP, r = –0.781, p = 0.007* *HT MB AspAP vs. RM MB AlaAP, r = –0.847, p = 0.002* *HT MB AspAP vs. RM MB CysAP, r = –0.805, p = 0.004* *HT MB AspAP vs. RM MB GluAP, r = –0.900, p = 0.0004* *HT MB GluAP vs. RM MB AlaAP, r = –0.939, p < 0.0001* *HT MB GluAP vs. RM MB CysAP, r = –0.882, p = 0.0007* *HT MB GluAP vs. RM MB GluAP, r = –0.865, p = 0.001* *HT Sol AspAP vs. RM MB AlaAP, r = –0.737, p = 0.01*	**HT vs. RM (positive correlation)** HT MB AspAP vs. RM MB AspAP, r = +0.758, p = 0.01	**HT vs. RM (mainly *negative* correlations)** *HT Sol AlaAP vs. RM Sol GluAP, r = –0.637, p = 0.04* *HT Sol CysAP vs. RM MB AlaAP, r = –0.698, p = 0.02* *HT Sol CysAP vs. RM MB CysAP, r = –0.643, p = 0.04* *HT MB AlaAP vs. RM Sol AspAP, r = –0.643, p = 0.04* *HT MB GluAP vs. RM Sol GluAP, r = –0.640, p = 0.04* *HT MB AspAP vs. RM Sol GluAP, r = –0.746, p = 0.01* *HT MB AspAP vs. RM Sol AspAP, r = –0.676, p = 0.03* HT MB CysAP vs. RM MB CysAP, r = +0.638, p = 0.04 HT MB CysAP vs. RM MB GluAP, r = +0.671, p = 0.03

Level of correlations between HT Sol or MB angiotensinase activities versus Sol or MB left VT, RC and RM angiotensinase activities in the control, L-NAME- and propranolol-treated WKY rats. Pearson's correlation coefficients (r) and p values are indicated. Negative correlations are indicated in italics.

Table 2. Significant correlations in SHR

SHR		
Control rats	L-NAME-treated rats	Propranolol-treated rats
HT vs. VT (positive correlations)	**HT vs. VT (*negative* correlations)**	**HT vs. VT (positive correlations)**
HT Sol GluAP vs. VT Sol GluAP, r = 0.743, p = 0.03	*HT Sol GluAP vs. VT Sol AlaAP, r = –0.819, p = 0.01* *HT Sol AspAP vs. VT Sol AlaAP, r = –0.820, p = 0.01* *HT Sol AspAP vs. VT Sol CysAP, r = –0.869, p = 0.005* *HT Sol CysAP vs. VT Sol AlaAP, r = –0.838, p = 0.009* *HT Sol CysAP vs. VT Sol CysAP, r = –0.937, p = 0.0006* *HT Sol GluAP vs. VT Sol CysAP, r = –0.916, p = 0.001* *HT MB CysAP vs. VT Sol CysAP, r = –0.712, p = 0.04*	HT Sol GluAP vs. VT Sol CysAP, r = +0.743, p = 0.03 HT MB AspAP vs. VT MB AspAP, r = +0.718, p = 0.04
HT vs. RC (*negative* correlations)	**HT vs. RC (positive correlations)**	**HT vs. RC (*negative* correlations)**
HT Sol AspAP vs. RC Sol AlaAP, r = –0.733, p = 0.03 *HT Sol AspAP vs. RC MB GluAP, r = –0.919, p = 0.001* *HT Sol GluAP vs. RC Sol AlaAP, r = –0.814, p = 0.01* *HT Sol GluAP vs. RC Sol CysAP, r = –0.836, p = 0.009* *HT Sol AlaAP vs. RC MB GluAP, r = –0.723, p = 0.04*	HT Sol AspAP vs. RC Sol AlaAP, r = +0.902, p = 0.002 HT Sol AspAP vs. RC MB GluAP, r = +0.879, p = 0.004 HT Sol GluAP vs. RC Sol AlaAP, r = +0.921, p = 0.001 HT Sol GluAP vs. RC Sol CysAP, r = +0.888, p = 0.003 HT Sol AspAP vs. RC Sol CysAP, r = +0.808, p = 0.01 HT Sol CysAP vs. RC Sol AlaAP, r = +0.888, p = 0.003 HT Sol CysAP vs. RC Sol CysAP, r = +0.783, p = 0.02 HT Sol CysAP vs. RC MB GluAP, r = +0.945, p = 0.0004 HT Sol GluAP vs. RC MB GluAP, r = +0.828, p = 0.01 HT MB AlaAP vs. RC Sol AlaAP, r = +0.739, p = 0.03 HT MB AspAP vs. RC Sol GluAP, r = +0.784, p = 0.01 HT MB CysAP vs. RC Sol AlaAP, r = +0.722, p = 0.04 HT MB CysAP vs. RC MB GluAP, r = +0.749, p = 0.03 HT MB GluAP vs. RC Sol AlaAP, r = +0.723, p = 0.04 HT MB GluAP vs. RC MB GluAP, r = +0.751, p = 0.03	*HT MB AlaAP vs. RC Sol GluAP, r = –0.822, p = 0.01* *HT MB AlaAP vs. RC Sol AspAP, r = –0.727, p = 0.04*
HT vs. RM (positive correlation)	**HT vs. RM (*negative* correlations)**	**HT vs. RM (mainly positive correlations)**
HT MB GluAP vs. RM MB AlaAP, r = 0.712, p = 0.04	*HT Sol CysAP vs. RM Sol AlaAP, r = –0.771, p = 0.02* *HT Sol CysAP vs. RM Sol CysAP, r = –0.736, p = 0.03* *HT Sol GluAP vs. RM Sol AlaAP, r = –0.713, p = 0.04*	HT Sol AlaAP vs. RM MB AlaAP, r = +0.737, p = 0.03 HT Sol AspAP vs. RM Sol AlaAP, r = +0.720, p = 0.04 HT Sol AspAP vs. RM Sol CysAP, r = +0.777, p = 0.02 *HT MB CysAP vs. RM Sol GluAP, r = –0.809, p = 0.01*

Level of correlations between HT Sol or MB angiotensinase activities versus Sol or MB left VT, RC and RM angiotensinase activities in the control, L-NAME- and propranolol-treated SHR. Pearson's correlation coefficients (r) and p values are indicated. Negative correlations are indicated in italics.

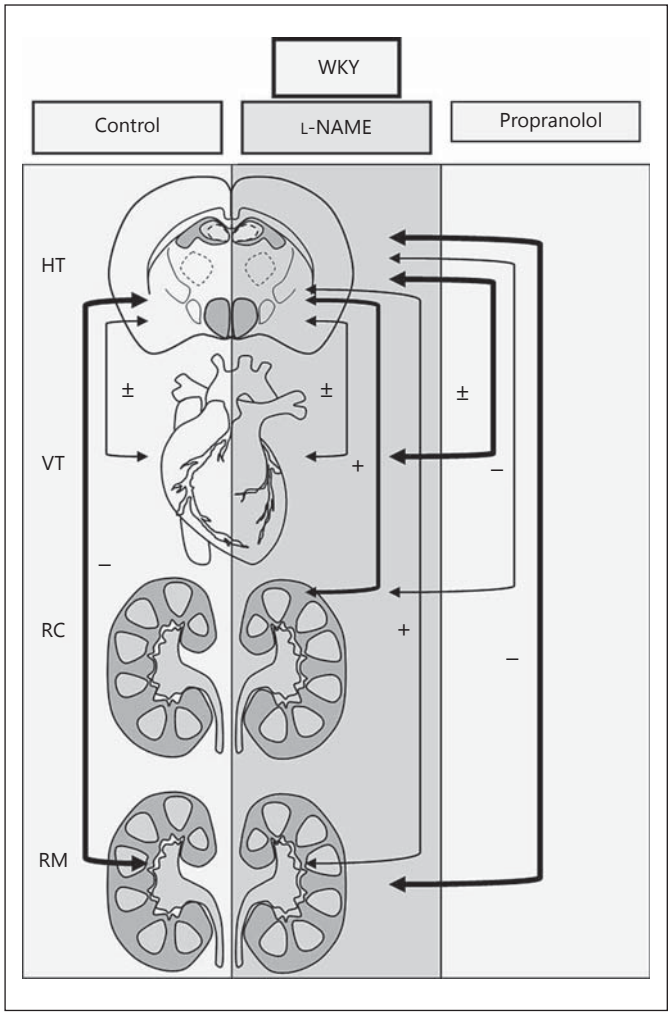

Fig. 3. Hypothetical positive (+) or negative (–) feedback between HT and VT, RC or RM in WKY rats, depending on the main tendency observed in table 1. The higher the level and number of correlations, the thicker the line.

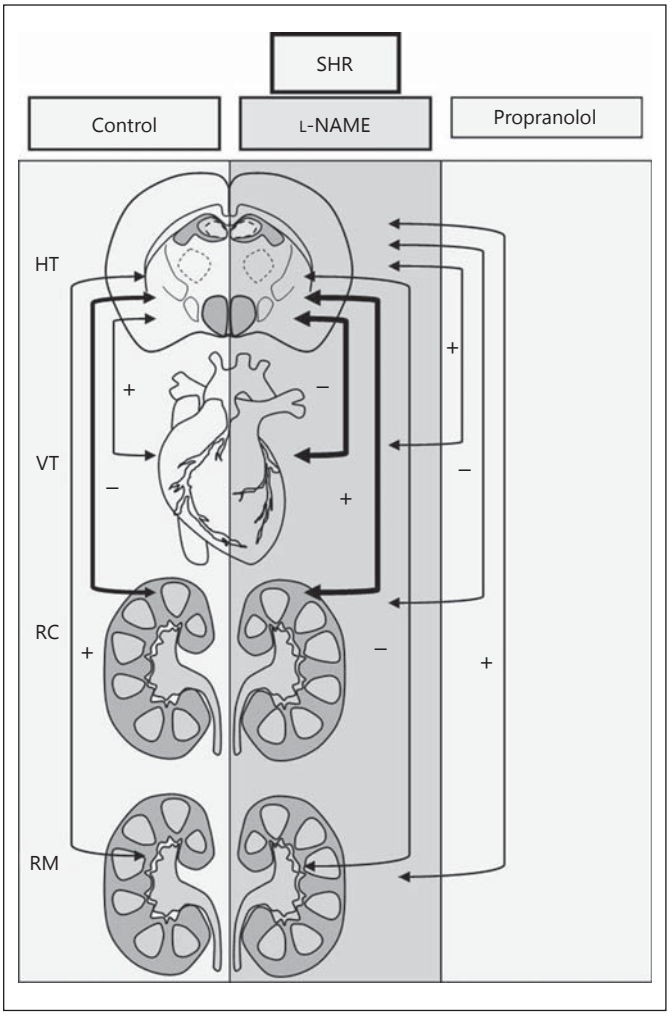

Fig. 4. Hypothetical positive (+) or negative (–) feedback between HT and VT, RC or RM in SHR, depending on the main tendency observed in table 2. The higher the level and number of correlations, the thicker the line.

Discussion

L-NAME significantly increased SBP in WKY and SHR as previously reported [4, 21]. However, propranolol at the dose used did not modify SBP either in hypertensive animals as previously reported [13] or in WKY rats (fig. 2). Other authors have also reported similar results and hypothesized that the influence of β-blockers on SBP may be variable depending on several factors such as the type of β-blocker used, the place of breeding or the stage of development of the rat [22–24]. Diuresis decreased significantly in propranolol-treated WKY rats and increased in L-NAME-treated SHR (fig. 2).

The present results support the notion that the RAS function of the brain-heart-kidney axis, reflected here through the enzymatic activities implied in its cascade, is reciprocally connected by afferent and efferent mechanisms, presumably through the ANS, and may influence the control of blood pressure and water balance. In general, the effects of L-NAME on angiotensinases were reversed to the effects observed in controls after propranolol treatment in the VT as well as in the kidney. This demonstrates the involvement of the ANS. Considering that SHR are animals with increased sympathetic activity [25], treatment with L-NAME will further activate the sympathetic system [26]. Indeed, in this group, we obtained the highest

number of exclusively positive correlations between HT and RC, which were drastically reduced and changed to a negative direction after the sympathetic blockade with propranolol (table 2). Therefore, we hypothesized that there was an involvement of the sympathetic system in the observed connection between the HT and the RC. According to this hypothesis, we noticed that there were no such correlations between the HT and the RC in control WKY rats, which do not have a sympathetic activation. In contrast, after L-NAME treatment, which activates the sympathetic system, a number of correlations, which were mainly positive, appeared in WKY rats. The sympathetic blockade with propranolol in this strain of normotensive animals reduced the number of correlations compared with the group of L-NAME-treated animals and changed their direction to negative (table 1). In the group of control SHR, a strain with activated sympathetic system, a high number of correlations appeared, in this case with a negative direction. However, after L-NAME treatment, the number of correlations increased radically in these animals (the highest in all groups), presumably linked to sympathetic overactivation, and the correlations changed to positive. On the contrary, the sympathetic blockade with propranolol reduced the correlations drastically and returned their direction to negative (table 2). Therefore, we assume that there is a clear involvement of the sympathetic system in the observed relationship of angiotensinases in the HT and the RC: the greater the sympathetic activation, the greater the activated connection between the HT and the RC. Also, the more activity in the HT, the more activity in the RC and vice versa, suggesting a reinforcement between both tissues. However, after sympathetic blockade, there was a clear reduction in the number of correlations, together with a change of the direction of correlations to negative. This supports a retro-inhibitory control between the HT and the RC in these conditions: the greater the angiotensinase activity in one of them, the less in the other.

Comparing HT and VT in WKY rats, blockade of the sympathetic system with propranolol increased the number of significant correlations markedly, without a clear prevalence of positive and negative correlations. This suggests the activation of a neural connection between HT and VT, presumably through the parasympathetic system (table 1). In SHR, the results suggested a clear sympathetic activation of the HT/VT connection in the L-NAME group, but in this case with a negative character, suggesting a reciprocal retro-inhibitory control between the HT and the heart (table 2).

In the comparison of the HT with the RM in WKY rats, there was a drastic reduction in the number of correlations between both tissues from exclusively negative correlations to only 1 positive correlation when the sympathetic system was activated by L-NAME. However, after the sympathetic blockade with propranolol, the number of correlations increased again and they were mainly negative. This suggests that the parasympathetic system mediates a highly significant reciprocal retro-inhibitory control between both tissues in basal conditions of normotensive animals (table 1). However, in SHR, the results suggested an apparent activation of the connection with a positive character when the sympathetic system was blocked by propranolol, being therefore possibly mediated by the parasympathetic branch (table 2).

These correlations suggest the involvement of the ANS which directly regulates the status of the angiotensinases in these tissues by some feedback mechanisms not yet described. Additionally, these correlations may also indicate secretory processes for angiotensinases in the neuroeffector junction from nerve terminals and/or from tissue cells under these circumstances. The enzymes would then be activated or not, depending on whether an appropriate biochemical environment surrounding their peptide substrates was reached [9, 27–29]. We suggested early the functional importance of aminopeptidase activities (including angiotensinases) in nerve terminals and synaptic events [27, 30] exhibiting different functional roles depending on whether the Sol or MB fraction was analyzed [30, 31]. Ang II containing motor and sensory nerve fibers was described in the kidney and heart. They are sources of tissue Ang II acting as a neuropeptide cotransmitter or neuromodulator [32–34]. In addition, anterograde and retrograde axonal transport of some Sol and MB peptidases such as angiotensin-converting enzyme or leucyl aminopeptidase in the rat sciatic nerve was described [28, 29]. These observations allow the postulation of the existence of an axonal anterograde or retrograde cotransport of angiotensinases together with their angiotensin peptide substrates and/or angiotensin receptors, in the same or different vesicles [29, 35, 36]. Therefore, a functional role for angiotensinases in a coordinated regulation involving the HT, heart and kidney could be hypothesized. Compared with the physiological status, a positive or negative feedback under certain drug-induced or pathological conditions may be enhanced or reduced through those neural connections. This modification would be reflected by the status of activity of peptide-hydrolyzing enzymes such as angiotensinases.

The present results reveal new aspects of neuroendocrine regulation whose functional meaning remains to be elucidated. Figures 3 and 4 summarize the main tendency

of the correlations observed between HT, VT, RC and RM in control, L-NAME- and propranolol-treated WKY and SHR. To sum up, we observed that in WKY rats (fig. 3), the correlations between HT and VT were similar in control, L-NAME- or propranolol-treated groups, with no clear positive or negative influence in both locations. This fits with the suggestion that the L-NAME-induced increase in blood pressure is mainly due to an increased peripheral resistance and not to an increased volume output [37]. In the kidney, L-NAME induced a positive correlation mainly between HT and RC, which also suggests an increased sympathetic activation, therefore leading to an increased renin secretion and thus contributing to the increase in peripheral resistance. Propranolol treatment induced an increased inhibitory influence between HT and RM, as observed in controls. In addition, there was also a new inverse correlation between HT and RC, which might be related to the observed reduction of diuresis in this group (fig. 2).

In SHR, the striking response observed after L-NAME treatment in comparison with controls is particularly interesting (fig. 4). There was a strong inverse correlation between HT and VT together with a marked direct positive correlation between HT and RC, which may support, as in WKY rats, a reduction in the volume output together with a sympathetic overactivation of the RC with increased renin secretion leading to increased peripheral resistance. This highly positive correlation between HT and RC may also be related to the remarkable increase in diuresis after L-NAME treatment (fig. 2). Interestingly, the positive correlation between HT and RM observed in control SHR turned into a negative correlation after L-NAME treatment. After propranolol treatment, the responses followed the same pattern as in controls (fig. 2, 4).

The present results support the involvement of the RAS, evaluated through the angiotensinase activities, in a neurovisceral integrative regulation of blood pressure and water balance connecting HT, VT and kidney presumably through the ANS. Furthermore, our results demonstrated that in SHR, after L-NAME treatment, there were negative correlations between HT and VT but positive correlations between HT and RC. Since it has been reported that SHR with nitric oxide synthesis inhibition very closely mimic the cardiac and renal outcomes seen in patients with essential hypertension [38], the highly significant correlations observed in our present report in L-NAME-treated SHR may underlie the pathological reciprocal mechanism linked to the severity of cardiorenal damage.

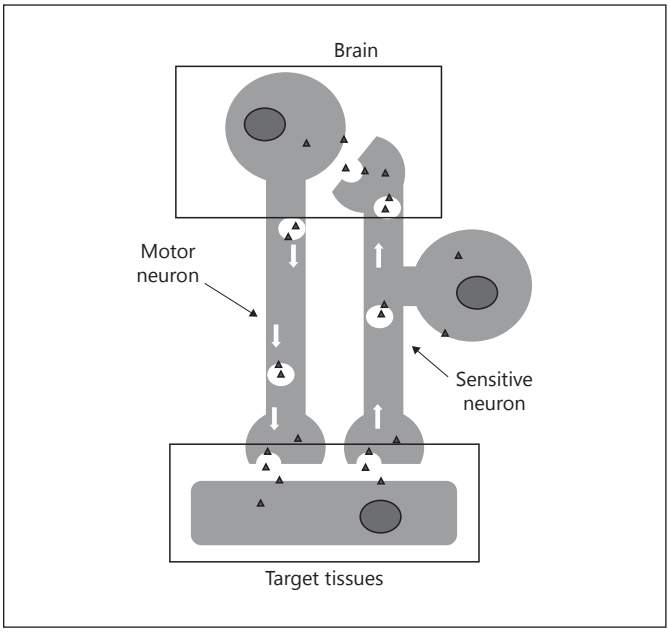

Fig. 5. Schematic illustration of the possible mechanism proposed in the text of neuroendocrine integration involving angiotensinases (black triangles) through the ANS.

To summarize, the present unexpected results suggest a hypothetic general mechanism. This implies a bidirectional flux of Sol and MB angiotensinases in vesicles through anterograde and retrograde axonal transport between nerve terminals and neuronal bodies of motor and sensitive autonomic neurons (fig. 5). The status of activation of autonomic nerves would partly determine the selective action of the enzymes in the analyzed locations. In addition, an explanation for the present results may also in part involve neuroendocrine reflexes between the HT, the heart and kidney, reflexes in which the angiotensinases would participate, regulating selective endogenous substrates, as it is the case with the enzyme oxytocinase (CysAP) controlling oxytocin during the suckling-induced milk ejection reflex [39].

Aminopeptidases (including angiotensinases) present broad substrate specificity and, usually, several neuropeptides and hormones may be hydrolyzed by one or several enzymes in their Sol or MB form. The activities of such enzymes can focus on a specific substrate by various mechanisms including a specific biochemical environment such as a precise pH or Km affinity for the substrate. The presence of endogenous or exogenous activators or inhibitors, such as levels of steroids or drug treatments [9], could also be involved. Furthermore, according to the present results, the status of activation of

the ANS appears to play a major role. However, we cannot discard a direct influence of L-NAME or propranolol on the enzymes in modulating their activities. Sol and MB enzymes may address their activities to the same substrates, but their behavior and mechanisms of regulation are different [4]. They may act in coordination to control the function of one or several substrates. The multiple correlations observed in the present study, involving different types of Sol and MB activities, reveal the heterogeneity and complexity of the mechanisms that regulate enzymatic activities and, as a consequence, their endogenous substrates. These activities appear not only to depend on the expression of the enzymes themselves but also on the local biochemical environmental conditions surrounding the enzymes and their nerve-autonomic control.

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