Training-Induced Pressure Fall in Spontaneously Hypertensive Rats Is Associated With Reduced Angiotensinogen mRNA Expression Within the Nucleus Tractus Solitarii

Jorge Vinicius Cestari Felix, Lisete Compagno Michelini

Abstract—Knowing that exercise training reduces arterial pressure in hypertensive individuals and that pressure fall is accompanied by blockade of brain renin-angiotensin system, we sought to investigate whether training (T) affects central renin-angiotensin system. Spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto controls (WKYs) were submitted to training or kept sedentary (S) for 3 months. After functional recordings, brain was removed and processed for autoradiography (brain stem sequential slices hybridized with 35S-oligodeoxynucleotide probes for angiotensinogen [Aogen] and angiotensin II type 1 [AT1A] receptors). Resting arterial pressure and heart rate were higher in SHRs (177±2 mm Hg, 357±12 bpm versus 121±1 mm Hg, 320±9 bpm in WKYs; P<0.05). Training was equally effective to enhance treadmill performance and to cause resting bradycardia (−10%) in both groups. Training-induced blood pressure fall (−6.3%) was observed only in SHR-T. In SHR-S (versus WKYs) AT1A and Aogen mRNA expression were significantly increased within the NTS and area postrema (average of +67% and +41% for AT1A and Aogen, respectively; P<0.05) but unchanged in the gracilis nucleus. Training did not change AT1A expression but reduced NTS and area postrema Aogen mRNA densities specifically in SHR-T (P<0.05 versus SHR-S, with values within the range of WKY groups). In SHRs, NTS Aogen mRNA expression was correlated with resting pressure (y=5.95x+41; r=0.55; P<0.05), with no significant correlation in the WKY group. Concurrent training-induced reductions of both Aogen mRNA expression in brain stem cardiovascular-controlling areas and mean arterial pressure only in SHRs suggest that training is as efficient as the renin-angiotensin blockers to reduce brain renin-angiotensin system overactivity and to decrease arterial pressure. (Hypertension. 2007;50:780-785.)

Key Words: angiotensin II ■ angiotensin receptors ■ blood pressure ■ heart rate ■ hypertension ■ experimental ■ rats

The renin-angiotensin system (RAS) is a widely distributed regulatory system with hormonal, paracrine, and intracrine functions in many tissues.1-4 Brain RAS has been implicated in the pathogenesis (development/maintenance) of several forms of hypertension.2,4-6 All of the components of the RAS (precursor, enzymes, peptides, and receptors) are present in brain areas involved in cardiovascular control as the hypothalamic paraventricular nucleus and dorsal brain stem areas including the nucleus tractus solitarii (NTS), the dorsal motor nucleus of the vagus nerve, and the area postrema.4,7-11 It is important to notice that angiotensinogen (Aogen), angiotensin II (Ang II), and Ang II type 1 (AT1A) receptors are densely expressed within the NTS, indicating the importance of local RAS on cardiovascular control.4,8,10-14 In previous studies we showed both the close relationship between increased Aogen and Ang II AT1A receptor mRNA expression in brain stem areas and elevated blood pressure14 and the permissive role of Ang II to orchestrate, via AT1 receptors, the detrimental cardiovascular responses during development of hypertension.14-18 Interestingly, all deficits of cardiovascular control in hypertensive individuals (increased pressure, higher heart rate [HR], impaired aortic nerve activity, increased sympathetic activity, and depressed baroreflex control15-18), as well as the increased Aogen expression in brain stem areas,14 were reduced/normализed after chronic treatment with losartan, a specific AT1 receptor blocker.14,16-18

Recent clinical trials have also indicated the efficacy of low-intensity exercise training (a nonpharmacological tool) to reduce blood pressure and cardiovascular risk.19 In fact, training has been associated with a variety of peripheral adjustments in hypertensive individuals (remodeling of the heart with simultaneous stroke volume increase and HR decrease, predominance of endothelium relaxing over con-
trictile factors, hypertrophic remodeling of arterioles, capillary angiogenesis, and venule neoformation in exercised muscles, thus increasing flow and O\textsubscript{2} extraction\textsuperscript{20–23} as well as improvement of autonomic control of circulation\textsuperscript{24–26}.

Although pressure fall in hypertension has been associated with blockade of central/peripheral RAS, it is not known whether training-induced benefits on cardiovascular control are dependent or not on changes in the expression/activity of central RAS components. Therefore, we sought now to investigate in spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto controls (WKY) the effects of exercise training on Aogen and AT\textsubscript{1A} mRNA expression in central brain stem areas involved in cardiovascular control. It was also our objective to correlate Aogen and AT\textsubscript{1A} mRNA expression with the functional changes on arterial pressure and HR induced by hypertension and exercise training.

Methods

Animal Protocols

Male SHR and WKY rats, aged 2 months, were housed in Plexiglas cages (4 rats per cage) on a 12/12-hour light/dark schedule and allowed free access to food and water. Rats were initially selected according to their ability to walk/run on a treadmill (Inbramed, KT-300; 8 to 10 sessions at 0.4 up to 0.6 km/h; 0% grade; 10 minutes per day; during 1 to 2 weeks) and then submitted to maximal exercise tests. The test consisted of graded exercise on the treadmill, with increments of 0.3 km/h every 3 minutes, starting at 0.3 km/h up to the maximal intensity attained for each rat. Tests were useful to assign rats with equal performance to trained (T) or sedentary (S) groups and to establish the intensity of training protocol, corresponding to 50% to 60% of maximal exercise capacity (low-intensity training). Exercise training was similar to that used previously.\textsuperscript{23,25} Briefly, it was performed 5 days per week over 12 weeks with intensity increasing progressively by a combination of velocity and time, attaining the maximal duration (1 hour per day) by week 3. Maximal exercise tests were repeated by weeks 6 and 12 to adjust training intensity and to quantifying training effects in both groups, respectively. Rats allocated to sedentary groups were handled every day and submitted once per week to a short period of mild exercise (5 to 10 minutes; 0.4 to 0.8 km/h; 0% grade). At the end of protocols, under ketamine/xylazine/acepromazine (0.7/0.2/0.1 vol/vol/vol; 0.04 mL/100 g body weight, IM) anesthesia, a catheter was introduced into the femoral artery. Basal values of arterial pressure and HR were recorded continuously (40 to 60 minutes; P23Db transducer + carrier amplifier on a 3400 Gould Recorder) 24 to 30 hours later in conscious rats at rest.

All of the surgical procedures and protocols used agreed with the Brazilian College of Animal Experimentation Ethical Principles in Animal Research and were approved by the Institutional Animal Care and Use Committee of the University of São Paulo.

Tissue Preparation

Rats were deeply anesthetized (ketamine/xylazine/acepromazine 0.7/0.2/0.1 vol/vol/vol; 0.08 mL/100 g body weight, IM) and submitted to thoracotomy immediately after the respiratory arrest. Brain was perfused with 0.01 mol/L of PBS (100 to 150 mL; Daiger pump; 20 mL/min) followed by fixative (4% paraformaldehyde in 0.1 mol/L PBS; pH 7.2) for 60 minutes, removed, and stored for 4 hours in the same fixative. It was then immersed in fixative solutions with increments of 0.3 km/h up to 100% of maximal exercise capacity (low-intensity training). For each rat, sequential wells were hybridized with antisense and sense probes for Aogen and AT\textsubscript{1A} according to the technique described by Key et al.\textsuperscript{27} In brief, oligodeoxynucleotide probes complementary to rat nucleotide sequences 126 to 135 for Aogen (NM_134432) and 147 to 170 for AT\textsubscript{1A} (NM_030985) were labeled with \textsuperscript{35}S-dATP (Amersham) and with terminal deoxynucleotidyl transferase (Life Technologies). After no incorporated radioactivity was removed, the probes were diluted in hybridization buffer to yield \(\sim 10^9\) counts per minute per microliter and stored (~20°C). For hybridization reactions (free-floating method), the glass tubes were washed at room temperature (0.01 mol/L of PBS for 15 minutes, followed by 2× standard saline citrate for 30 minutes), and the probes were diluted further to a final concentration of 0.3 to 0.5× 10^6 cpm/100 μL. The probe (400 to 500 μL per glass tube) was added to glass tubes containing the slices and the vials incubated for 18 to 20 hours at 37°C. Posthybridization treatment included high-stringency washes (1× standard saline citrate for 2 hours at room temperature followed by 1× standard saline citrate for 30 minutes at 50°C). Slices from each well were mounted on each slide, covered with polylysine (Sigma), and dried overnight.

Preparation of Oligodeoxynucleotide Probes and In Situ Hybridization

For each rat, sequential wells were hybridized with antisense and sense probes for Aogen and AT\textsubscript{1A} according to the technique described by Key et al.\textsuperscript{27} In brief, oligodeoxynucleotide probes complementary to rat nucleotide sequences 126 to 135 for Aogen (NM_134432) and 147 to 170 for AT\textsubscript{1A} (NM_030985) were labeled with \textsuperscript{35}S-dATP (Amersham) and with terminal deoxynucleotidyl transferase (Life Technologies). After no incorporated radioactivity was removed, the probes were diluted in hybridization buffer to yield \(\sim 10^9\) counts per minute per microliter and stored (~20°C). For hybridization reactions (free-floating method), the glass tubes were washed at room temperature (0.01 mol/L of PBS for 15 minutes, followed by 2× standard saline citrate for 30 minutes), and the probes were diluted further to a final concentration of 0.3 to 0.5× 10^6 cpm/100 μL. The probe (400 to 500 μL per glass tube) was added to glass tubes containing the slices and the vials incubated for 18 to 20 hours at 37°C. Posthybridization treatment included high-stringency washes (1× standard saline citrate for 2 hours at room temperature followed by 1× standard saline citrate for 30 minutes at 50°C). Slices from each well were mounted on each slide, covered with polylysine (Sigma), and dried overnight.

Autoradiography and Signal Quantification

Autoradiography was used to identify the probe in different brain areas and to quantify the amount of radioactivity following training and sedentary protocols in SHRs and WKY rats. In a dark room, slides were dipped in NTB-2 liquid emulsion (Kodak), dried, stored in appropriate black boxes, and kept in a refrigerator for 20 to 30 days. After development (D-19 Kodak developer; F-24 fixative), brain sections were counterstained with cresyl violet. Brain stem areas were identified by microscopic examination (Leica DM LB) of the stained sections (bright field, ×200 magnification). After selecting the area to be quantified, bright- and dark-field photographs (72 337 ×m\textsuperscript{2} window) were obtained. Density measurements of the in situ hybridization signal (pixels per window) were made on the acquired dark-field images (Image Pro Plus, Media Cybernetics) in the NTS, area postrrema, and gracilis nucleus. NTS values are the average of measurements in 3 windows per slice (left + right + central areas) within the commissural NTS. Background measurements were taken from similar adjacent areas showing no labeling.

Statistical Analysis

Results are expressed as mean±SEM. Differences between groups (WKY and SHR) and protocols (sedentary and training) were analyzed by 2-way ANOVA, followed by the Student-Newman-Keuls multiple-comparisons test. Correlation analyses were performed by Pearson statistics. Differences were considered significant at \(P<0.05\).

Results

Efficacy of Training Protocol

Other than the better performance on treadmill presented by the SHR group since the beginning of protocols (data on the Table), low-intensity training was equally effective to improve the performance in SHRs and WKY rats. In both groups, significant increases on attained velocity (as measured by maximal exercise tests on treadmill) were already observed at week 6 of training; at week 12, maximal velocity was, on average, 2-fold higher than that of week 0 \(P<0.05; n=10\) in each group). On the other hand, sedentary groups presented no change (WKY) or slight decrease (SHR) on treadmill performance. It should be noted that, at the end of protocols, the gain on performance (difference in velocity between weeks 12 and 0) was similar for both trained groups.
Aogen mRNA density in the WKY group (Figure 1D). Training did not change completely normalized (not different from mRNA values in the control groups). Training did not change the expression of the Aogen within the commissural NTS was observed in the area postrema but not in the gracilis nucleus (data on Table).

Quantitative data (Table and Figure 1C and 1D) showed higher AT1A mRNA expression and higher Aogen mRNA expression were observed in the area postrema as well as a reduction in mean arterial pressure (MAP) and HR when compared with WKY rats (Table). Trained groups showed significant HR reductions (−9% and −11% versus sedentary controls, WKY and SHR, respectively), but MAP fall was only present in the trained SHR group (−6.3% versus SHR; P<0.05).

Effects of Hypertension and Training on Brain RAS

As illustrated in Figure 1A and 1B, SHRs had dense expression of AT1A and Aogen mRNA within the commissural NTS. Quantitative data (Table and Figure 1C and 1D) showed higher AT1A mRNA expression and higher Aogen mRNA density in sedentary hypertensive rats (+60% and +30%, SHR versus WKY, respectively). Exercise training did not change AT1A receptor expression in either group (Figure 1C) but caused a significant decrease of Aogen mRNA expression in the NTS of the SHR group (Figure 1D). In the SHR group, the expression of the Aogen within the commissural NTS was completely normalized (not different from mRNA values observed in the control groups). Training did not change Aogen mRNA density in the WKY group (Figure 1D). Similar hypertension- and training-induced changes in AT1A and Aogen mRNA expression were observed in the area postrema but not in the gracilis nucleus (data on Table).

Interestingly, Aogen mRNA levels in the commissural NTS were significantly correlated with basal values of MAP only in the SHR group: training-induced reduction in Aogen mRNA expression was positively correlated with MAP fall (Figure 2A). No correlation was observed in the WKY group. In both groups there was a tendency for lower HR values being accompanied by lower Aogen mRNA expression, but values did not attain significance (Figure 2C). Because training had no effect on AT1A receptor expression, there were no correlations between receptor density and cardiovascular changes after training in the SHR and WKY groups (Figure 2B and 2D). When the effects of hypertension on Aogen expression were compared in sedentary and trained groups, significant positive correlations between Aogen mRNA reduction and MAP fall and between Aogen mRNA decrease and HR reduction were observed only in sedentary rats (Figure 3A and 3C). Trained-induced normalization of SHR Aogen mRNA levels precluded any significant correlation with MAP and HR in trained rats. On the other hand, the high density of AT1A mRNA in SHRs, simultaneously with the low expression in the WKY rats, independent of the condition, justified the positive correlation between increased AT1A mRNA expression and increased pressure levels in both sedentary and trained groups (Figure 3B).

Discussion

Original observations, regarding the effects of exercise training on brain RAS, arise from this study. We described that exercise training does not change NTS AT1A mRNA receptor expression in hypertensive or normotensive rats but it com-
pletely normalizes the increased Aogen expression within this area in the SHR group. In addition, we showed in hypertensive rats that reduced Aogen mRNA density after training is correlated with arterial pressure fall and that training completely blocks the positive correlation between Aogen mRNA levels and MAP reduction or HR decrease observed in sedentary groups. We also confirmed that blunting effect of training on brain RAS overactivity (increased Aogen mRNA levels in the SHR group) is specific for dorsal brain stem areas involved in cardiovascular control as the NTS and area postrema.

The present results showing overactivity of the brain RAS in sedentary SHR (increased both Aogen and AT\textsubscript{1A} mRNA expression) confirmed previous observations on increased RAS activity within the brain of hypertensive animals.\textsuperscript{8,11,14,28–30} Interestingly, exercise training was effective to blunt the increased expression of Aogen mRNA without changing that of AT\textsubscript{1A} within the NTS and area postrema. AT\textsubscript{1B} receptor expression was not measured in this study, because central Ang II effects are mainly mediated through AT\textsubscript{1A} receptor subtype,\textsuperscript{13} and, as shown previously, AT\textsubscript{1B} density within the rat NTS is very low (18-fold smaller than that of AT\textsubscript{1A} and close to the sensitivity threshold of the assay) and not affected by hypertension or losartan treatment.\textsuperscript{14} Absence of training-induced change on AT\textsubscript{1A} receptors does not imply the absence of effect, because reduced expression of Aogen decreases both Ang II synthesis and agonist-receptor interaction, thus blunting the activity of the brain RAS. Indeed, the availability of Aogen has been shown to be a main limiting factor for brain angiotensin production, for the central regulation of blood pressure, and for the pathogenesis of hypertension.\textsuperscript{31} In this regard we also observed, in the normotensive group, lower expression of both Aogen and AT\textsubscript{1A} mRNA and that training did not alter the expression of RAS in cardiovascular-controlling areas and did not change baseline pressure. Together these observations reinforce the importance of an overactive brain RAS to condition elevated blood pressure in hypertension and the proposition that blockade of brain and/or plasma Aogen is essential for pressure fall in hypertensive individuals.\textsuperscript{30,32}

The present experimental evidence, showing positive significant correlations between NTS Aogen mRNA expression and MAP levels in the SHR (but not in the WKY group) and in the sedentary rats (not trained; Figure 2A and 3A, respectively), reinforces the association between blood pressure and brain Aogen content, suggesting additionally an important effect of training on them. Previous studies have already shown that hypertension coexists with high Ang II levels in brain areas involved in cardiovascular control.\textsuperscript{14,28,29} It was also shown that Ang II acts centrally to depress reflex control of circulation and to increase volemia and sympathetic outflow, thus contributing to increase blood pres-
sure. Results of the present study indicating overactivity of RAS within the NTS and area postrema (but not in the gracilis nucleus) in the SHR group, simultaneously with blood pressure and HR increase, are in accordance with the observations above. On the other hand, the demonstration that exercise training reduces arterial pressure and HR while blocking the expression of Aogen in the NTS and area postrema is the major finding of the present study, indicating a possible mechanism by which repetitive physical activity reduces pressure load in hypertensive individuals. Although the present set of data did not allow the identification of the effects caused by reduced brain RAS activity on neural/hormonal responses conditioning blood pressure fall, it is possible that baroreflexes and sympathetic outflow (in between other factors) are involved. The NTS (an important integrative site for afferent signaling and efferent autonomic modulation) and area postrema (an area devoid of blood-brain barrier that monitors plasma and cerebrospinal fluid levels of different hormones and other active substances) are putative areas for integration of reflex control of the circulation. Indeed, chronic blockade of AT$_1$ receptors in coarcted hypertensive rats caused both a marked depression on Aogen expression within the NTS and area postrema and a significant blood pressure fall, which occurred simultaneously with increased gain of arterial baroreceptors (high sensitivity and reduced variability of aortic nerve activity), reduced sympathovagal balance to the heart, normalization of baroreceptor reflex control of HR, and sympathetic nerve discharge, thus correcting the deleterious changes induced by hypertension.

It is important to note that chronic treatment with losartan caused exactly the same effects of training on NTS Aogen expression: Aogen mRNA expression was markedly reduced in hypertensive rats pretreated with losartan without any significant change on AT$_{1A}$ receptor density. In addition, in the vehicle-treated groups (similar to the present observation in sedentary versus trained groups; Figure 3) there was a positive correlation between Aogen mRNA expression and basal MAP, which was significantly reduced in the presence of chronic AT$_1$ receptor blockade with losartan. Klett et al. also showed that administration of antisense oligodeoxynucleotide against mRNA-stabilizing protein in SHRs reduced both tissue and plasma Aogen expression and caused significant blood pressure fall. Importantly, antihypertensive effects after blockade of Aogen mRNA synthesis were similar to those observed after inhibition of the RAS by converting enzyme inhibitor or AT$_1$ receptor blockade. These observations, together with those of the present study, showed clearly that training caused a similar antihypertensive effect as the pharmacological therapies and strongly suggest that reduced Aogen expression within brain stem areas controlling cardiovascular function is involved in the mediation of the response. If a similar deactivation also occurs in other brain areas, such as the hypothalamic integrative centers, it remains to be determined.

In summary, our data demonstrate that hypertension and exercise training affect in opposite directions the expression/activity of brain RAS. Increased arterial pressure and HR in SHRs are accompanied by overactivity of brain RAS, whereas exercise training blunts RAS activity (mainly the expression of the Aogen) in cardiovascular-controlling areas, thus contributing to decrease blood pressure and HR in hypertensive individuals. In addition, the strong positive correlation between NTS Aogen mRNA levels and basal values of arterial pressure and their parallel reductions after training reinforce the proposition that low intensity training is...
effective to cause arterial pressure fall and to block Aogen expression, thus decreasing central RAS activity.

**Perspectives**

The present set of data clearly showing the dependence of training-induced pressure fall on the blockade of Aogen expression within brain stem areas involved in cardiovascular control and the similarity of effects of both low-intensity exercise training and pharmaceutical compounds to block the expression/activity of the RAS precursor within the brain, thus reducing pressure levels, reinforces the adoption of exercise training as initiation and/or coadjuvant therapy for prevention/management of hypertension to reduce cardiovascular morbidity and mortality.\(^9\)

**Sources of Funding**

This study was supported by Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (06/50548-9) and Conselho Nacional de Desenvolvimento Cientifico (to J.V.C.F.). L.C.M. is a research fellow from Conselho Nacional de Desenvolvimento Cientifico.

**Disclosures**

None.

**References**

Training-Induced Pressure Fall in Spontaneously Hypertensive Rats Is Associated With Reduced Angiotensinogen mRNA Expression Within the Nucleus Tractus Solitarii
Jorge Vinicius Cestari Felix and Lisete Compagno Michelini

Hypertension. 2007;50:780-785; originally published online July 23, 2007;
doi: 10.1161/HYPERTENSIONAHA.107.094474

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/50/4/780

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/