Endogenous Angiotensin and Pressure Modulate Brain Angiotensinogen and AT$_{1A}$ mRNA Expression

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Abstract—In the coarctation hypertension model, we showed both dissociation of plasma renin activity from cardiovascular-induced effects and the reversal of hypertension-induced responses by losartan. In this study, we investigated the effects of hypertension on the expression of brain renin-angiotensin system components and the simultaneous functional responses and effects of long-term angiotensin II (AT) receptor blockade on these responses. Rats were given vehicle or losartan for 9 days and subjected to subdiaphragmatic aortic constriction or sham surgery after 4 days of treatment. On the fifth postsurgical day, pressure and heart rate were measured in the conscious state; the brain was perfused and removed afterward. Sequential slices of brainstem were hybridized with $^{35}$S-oligodeoxynucleotide probes for angiotensinogen, AT$_{1A}$, and AT$_{1B}$ receptors and processed for autoradiography and densitometry. In vehicle-treated rats, hypertension was accompanied by tachycardia and marked increments in angiotensinogen and AT$_{1A}$ mRNA expression in the cardiovascular system—controlling brainstem areas. In the nucleus tractus solitarius, AT$_{1A}$ density was correlated with both pressure and heart rate values ($P<0.01$), whereas angiotensinogen levels were correlated with pressure only ($P<0.05$). Losartan did not change the pressure of hypertensive rats (142±4 versus 146±2 mm Hg, losartan versus vehicle) and the hypertension-induced angiotensinogen mRNA expression but did block both tachycardic response and hypertension-induced AT$_{1A}$ mRNA expression. Hypertension and losartan did not change AT$_{1B}$ mRNA expression. The hypertension-induced positive feedback on angiotensinogen and AT$_{1A}$ mRNA expression supports the concept of a permissive role for brain angiotensin II in orchestrating circulatory responses during the development of hypertension.

These data also explain the efficacy of long-term AT$_1$ receptor blockade to reverse hypertension-induced effects. (Hypertension. 2004;43[part 2]:317-323.)

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

It is well known that plasma and the tissue renin-angiotensin system (RAS) are involved in cardiovascular regulation as well as in the development and maintenance of hypertension. The presence of an endogenous RAS in brain areas involved in cardiovascular regulation has been confirmed by several techniques. Stimulation of high-affinity angiotensin II (Ang II) receptors leads to a set of coordinated autonomic and endocrine responses yielding to pressure increase and hypertension; accordingly, blockade of the RAS is highly effective in reversing the angiotensin-induced effects simultaneously with a significant pressure reduction. It is not well established whether the various autonomic and endocrine changes observed in hypertension are caused by the pressure increase, by the concomitant activation of endogenous Ang II, or by a combination of both.

Subdiaphragmatic aortic constriction resulting in coarctation hypertension (CH) is an interesting model to study the relative contribution of pressure and the RAS to the cardiovascular changes leading to hypertension because of the mechanical factor interposed into the circulation. It increases peripheral resistance, the main determinant of pressure elevation. In this model, both converting-enzyme inhibition and Ang II type 1 (AT$_1$) receptor blockade cause small pressure reductions but do not impair the development and magnitude of hypertension. Previous studies by us have shown that CH causes marked activation of plasma renin activity (present from the first hours up to 5 to 7 days), which is accompanied by several cardiovascular responses in the chronic phase, such as increased basal heart rate, depressed afferent signaling by aortic baroreceptors, altered sympathovagal balance to the heart associated with the increased sympathetic outflow to the periphery, and impaired baroreceptor reflex control of the circulation. These responses are blocked by long-term treatment with losartan (LOS), suggesting a dominant role for Ang II. Notwithstanding from 5 to 7 up to 10 days of CH, pressure was similarly increased, and baroreceptor reflex control of heart rate was still markedly depressed, but plasma renin activity...
and heart rate declined to control levels.\textsuperscript{11} Although there is evidence for the participation of Ang II receptors in the nucleus tractus solitarii (NTS) in the maintenance of CH,\textsuperscript{10} it is not clear whether the RAS or pressure (or both) is the main determinant of cardiovascular homeostasis during hypertension.

To uncover the relative role of both in CH-induced cardiovascular changes, the objectives of the present study were as follows: (1) to determine the simultaneous effects of CH on functional changes and mRNA expression of the different RAS components in the brain and (2) to compare in CH and sham-operated control rats the effects of long-term AT\textsubscript{1} receptor blockade on functional responses and on the changes in expression of precursor and Ang II receptors in the brain. Changes in the expression of angiotensinogen (Aogen) and both Ang II type 1 receptors (AT\textsubscript{1A} and AT\textsubscript{1B}) were quantified by in situ hybridization into the NTS and other brainstem areas involved in the modulation of baroreflex control by endogenous Ang II.\textsuperscript{15,16} Heart rate response was used in this study as a quantitative index of the functional effects elicited by CH.\textsuperscript{11–14}

Methods

Functional Experiments

Male Wistar rats (3 to 4 months old, 200 to 300 g) from University of Sao Paulo, Biomedical Sciences Institute Animal Facility, were housed in individual cages on a 12-hour light/dark schedule and allowed free access to standard laboratory chow and water during the whole experimental period (up to 2 weeks). All surgical procedures and protocols used were in accordance with Ethical Principles in Animal Research and were approved by the University of Sao Paulo Ethical Committee for Animal Research. Rats were treated orally for 9 days with vehicle ([VEH] distilled water, 1 mL/kg per day) or LOS (10 mg/kg per day). After a treatment period of 4 days, half of the rats in both VEH and LOS groups were subjected to partial subdiaphragmatic aortic CH to induce hypertension of the upper part of the systemic circulation, according to a technique described previously.\textsuperscript{11,17} Sham-operated rats served as controls. In brief, under ketamine/xylazine/acepromazine anesthesia (0.7:0.2:0.1, vol/vol/0.4 mL/kg), rats were subjected to median laparotomy to dissect the superior mesenteric artery. The extent of aortic narrowing was determined by tail pressure (103.2 versus 119 ± 2 mm Hg). Immediately after respiratory arrest, thoracotomy was performed to allow for transcardiac perfusion (Daiger pump, 20 mL/min) with 0.01 mol/L phosphate-buffered saline (100 to 150 mL), followed by fixative (4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.2, for 60 minutes). The brain was removed, stored for 4 hours in the same fixative, and then immersed in fixative solutions with sucrose gradients (24 hours in fixative with 20% sucrose; 24 hours in fixative with 30% sucrose). Brainstems were blocked, identified, and stored at −80°C until processing. Serial coronal slices (20 μm; Leica CM3050 cryostat) including a long extension of the brainstem (1000 to 1200 μm caudal up to 1200 to 1400 μm rostral to the calamus scriptorium) were cut and collected in 0.01 mol/L phosphate-buffered saline in 12-well culture-tissue wells at 4°C. Slices from each well (~12) were mounted on each slide, covered with poly-ly-sine (Sigma), and dried overnight.

Preparation of Oligodeoxynucleotide Probes and In Situ Hybridization

For each rat, sequential slides (corresponding to sequential wells) were hybridized with antisense probes for Aogen, AT\textsubscript{1A}, and AT\textsubscript{1B} and with sense probes for Aogen, AT\textsubscript{1A}, and AT\textsubscript{1B} according to the technique described by Key et al.\textsuperscript{18} In brief, oligodeoxynucleotide probes complementary to rat nucleotide sequences 126 to 135, 147 to 170, and 6 to 29 for Aogen, AT\textsubscript{1A}, and AT\textsubscript{1B}, respectively, were labeled with \textsuperscript{35}S-dATP (NEN) with terminal deoxynucleotidyl transferase (Life Technologies). After nonincorporated radioactivity was removed, the probes were diluted in hybridization buffer to yield ~10\textsuperscript{6} counts per minute/μL and stored (~20°C). For the hybridization reaction, the slides were washed (0.01 mol/L phosphate-buffered saline) with 2× standard saline citrate at room temperature plus 1°C. After selection of the area to be quantified, bright- and dark-field photographs (91 000-μm\textsuperscript{2} window) were obtained at higher magnification (400×). Density measurements of the in situ hybridization signal (pixels per window) were made on the acquired dark-field images (Image Pro Plus, Media Cybernetics). NTS values are the mean of 2 (left and right sides in the medial NTS [NTS\textsubscript{M}]) or 3 (left and right sides plus central area in the commissural NTS [NTS\textsubscript{Com}]) windows per slice. Background measurements were taken from similar adjacent areas showing no labeling.

Statistical Analysis

Results are expressed as mean±SEM. Differences between groups (CH and sham) and treatments (LOS and VEH) were analyzed by 2-way ANOVA, followed by the Student-Newman-Keuls multiple-comparisons test. Regression analyses were performed by Pearson statistics. Differences were considered significant at \(P<0.05\).

Results

Functional Data

Before induction of CH or sham (control period), LOS treatment caused a significant pressure reduction, as indicated by tail pressure (103±2 versus 119±2 mm Hg in the VEH group). On the fifth experimental day, direct measurements in the conscious state confirmed that both CH\textsubscript{VEH} and CH\textsubscript{LOS} groups were hypertensive (146±2 and 142±4 mm Hg; the
Effects of Hypertension and LOS Treatment on Functional Data, Aogen mRNA, and AT1α mRNA Expression in Different Bulbar Areas

Table 1) when compared with their respective controls, but tachycardia was observed only in the CHVEH group. (343 ± 16 beats per minute, a value 16% higher than shamVEH and 30% elevated over the basal heart rate of CHLOS; the Table).

### Brainstem Aogen mRNA Expression

Figure 1 illustrates hypertension and long-term LOS treatment effects on Aogen mRNA expression in the brainstem. CH was accompanied by a marked increase in Aogen expression in several dorsal brainstem areas, such as the NTS, dorosmotor nucleus of the vagus (DMNV), and hypoglossus nucleus, among others. Interestingly, LOS caused an intense decrease in Aogen message but did not block the CH-induced increment. Quantitative data confirmed in the NTSCOMM (150 to 200 μm rostral to the calamus scriptorium) the efficacy of both CH and LOS to change Aogen mRNA expression (ANOVA P<0.001 for group and treatment effects; P=0.26 for interaction). There was a large CH-induced increase (4841 ± 464 pixels per window, 2.8-fold higher than shamVEH; Figure 1 and the Table) and a significant decrease of its expression in the presence of long-term AT1 blockade (543 ± 68 versus 1729 ± 218 pixels per window, shamLOS vs shamVEH; P<0.05). Figure 1 and the Table also show maintenance of the CH-induced effect on Aogen mRNA expression even after LOS treatment (CHLOS = 2916 ± 245 pixels per window), although the density attained was 40% smaller than the CHVEH value. Interestingly, Aogen mRNA levels in the NTSCOMM were positively correlated with mean arterial pressure values observed in VEH- and LOS-treated groups (Figure 1), with a downward displacement of the mean arterial pressure×density relation in the LOS-treated group. On the other hand, Aogen mRNA expression was not correlated with the heart rate levels exhibited by sham and CH rats treated with VEH or LOS (Figure 1).

The Table also shows that Aogen message exhibited differential expression in the rostrocaudal extension of the NTS, with smaller densities being detected rostrally: at 800 to 900 μm rostral to the calamus scriptorium, corresponding to NTSComm. Aogen mRNA was, on average, 16-fold less than that observed in the NTSCOMM, but similar effects of CH and long-term AT1 blockade on Aogen expression were still observed (ANOVA P<0.01 for group and treatment effects; P=0.08 for interaction). Aogen mRNA expression changes in the DMNV and hypoglossus nucleus behaved similarly to the NTS, although densities in the CHLOS group were similar to those of CHVEH (the Table). In the spinal trigeminal tract, LOS treatment was without effect, but CH still augmented Aogen mRNA expression in both groups. CH and LOS had no effects on Aogen message in the gracilis, cuneatus, or inferior olivary nuclei (the Table).

### Brainstem Aogen Receptors mRNA Expression

Effects of hypertension and long-term LOS treatment on AT1α and AT1β receptor densities were analyzed in the same brainstem areas. There was a similar pattern of distribution of receptor subtypes in the NTSCOMM (present mainly on neu-
rons), but the density differed markedly (Figure 2): expression of AT1A mRNA was, on average, 18-fold greater than that of AT1B (4144 ± 900 vs 236 ± 9 pixels per window, respectively). In addition, AT1B mRNA density in the NTS was not affected by CH and/or LOS treatment (Figure 2).

Besides the NTS, brainstem AT1B mRNA was only observed in the inferior olivary nucleus (very small density), being unaffected by CH and/or LOS (data not shown).

On the other hand, AT1A mRNA expression was detected in several brainstem areas. In the VEH group, establishment of CH was accompanied by a huge increment in the NTS comm AT1A density (showed in bright and dark fields, Figure 3), whereas the AT1B message increase was not changed in the LOS-treated group. In this area, ANOVA confirmed both group (P<0.05) and treatment (P<0.001) effects, with a significant interaction (P<0.05). In the VEH group, hypertension induced a 2-fold increase of AT1A mRNA expression (from 2108 ± 187 to 4144 ± 357 pixels per window, Figure 3 and the Table). LOS treatment did not change AT1A mRNA density in sham rats, but the CH-induced effect was absent even in the presence of a similar pressure load. AT1A mRNA levels in the NTS comm were positively correlated with both mean arterial pressure and heart rate values in the VEH-treated group. Accordingly, no significant correlations were observed after long-term AT1 receptor blockade (Figure 3).

Similar to Aogen mRNA distribution, AT1A message also changed along the NTS, with low expression being observed in the inferior olivary nucleus (very small density), being unaffected by CH and/or LOS (data not shown).

Discussion
This new set of data demonstrates that: (1) CH is accompanied by hyperactivity of the brain RAS, as indicated by the
increased Aogen and AT$_{1A}$ mRNA expression in bulbar areas known to participate in cardiovascular control; (2) long-term AT$_1$ receptor blockade reduces basal brain Aogen mRNA content without blocking pressure load and the CH-induced effect on precursor synthesis; and (3) pretreatment with LOS does not change basal AT$_{1A}$ mRNA expression but completely blocks its CH-induced increment, as it does block the tachycardic effect. Our data also show a topographic distribution of AT$_{1A}$ receptor mRNA along the rostrocaudal extension of the NTS and exclude the participation of AT$_{1B}$ receptors in this area in the mediation of cardiovascular effects.

Ang II has been linked to altered autonomic control in different models of hypertension.4,19–22 Previous studies have documented elevated pressure on the fifth day of CH accompanied by (1) increased plasma renin activity and higher baseline heart rate11; (2) a marked deficit in afferent signaling of pressure levels, as indicated by increased variability and reduced gain of aortic nerve activity13,23; (3) an altered sympathovagal balance, with high sympathetic outflow to the heart14; (4) significant depression of both reflex bradycardia and tachycardia11,12; and (5) changes in splanchnic sympathetic activity during loading and unloading of baroreceptors.14 In CH, it was also shown that long-term treatment with LOS did not change pressure load but caused a significant reduction in baseline heart rate,12 complete normalization of sympathovagal balance to the heart,14 baroreflex control of heart rate,12 or sympathetic activity during stimulation of baroreceptors,14 and partial correction of the aortic nerve activity×pressure relation.13

We have now shown that CH-induced cardiovascular responses are associated with brain RAS overactivity,

![Figure 2. Left, AT$_{1A}$ and AT$_{1B}$ expression in NTS$_{COM}$ of CH rats treated with VEH. Bright- (A and B) and dark- (A’ and B’) field photomicrographs of emulsion-coated tissue (400× magnification) correspond to the window drawn in the diagram at the top of this figure. Black bar=100 μm. Right, Comparison of AT$_{1A}$ and AT$_{1B}$ densities in the NTS$_{COM}$ of CH rats (upper panel) and absence of effects of hypertension and LOS on AT$_{1A}$ mRNA expression (lower panel). *P<0.05, AT$_{1A}$ vs AT$_{1B}$. SH indicates sham-operated rats; AP, area postrema; Sol, NTS$_{COM}$; sol, solitarii tract; 10, molar nucleus of the vagus; Gr, gracilis nucleus; Cu, cuneatus nucleus; Sp5, spinal trigeminal tract. Other abbreviations are as defined in text.](http://hyper.ahajournals.org/)

![Figure 3. Left, Comparison of AT$_{1A}$ mRNA expression in the NTS$_{COM}$ of sham-operated (SH; A and C, A’ and C’) and CH (B and D, B’ and D’) rats treated with VEH (A and B, A’ and B’) or LOS (C and D, C’ and D’). Bright- (A through D) and dark- (A’ through D’) field photomicrographs of emulsion-coated tissue (400× magnification). Right, Effects of hypertension and LOS on AT$_{1A}$ mRNA density in the NTS$_{COM}$ (upper panel). Correlations between AT$_{1A}$ density×mean arterial pressure (MAP) are $y_{VEH}=97x-9006$, $r=0.87$, $P<0.01$; $y_{LOS}=2x+1308$, $r=0.10$, $P>0.05$, and between AT$_{1A}$ density×heart rate (HR) are $y_{VEH}=51x-12610$, $r=0.84$, $P<0.01$; $y_{LOS}=-7x+3609$, $r=0.55$, $P<0.05$ in VEH- and LOS-treated groups, respectively, and are shown in the middle and lower panels, respectively. *P<0.05 vs sham; +P<0.05 vs VEH. #Denotes the significant correlation. b indicates beats. Other abbreviations are as defined in text.](http://hyper.ahajournals.org/)
namely, the increased expression of Aogen and AT\textsubscript{1A} receptor mRNAs in bulbar cardiovascular system–controlling areas, as NTS\textsubscript{COMM} and NTS\textsubscript{M}. In coarcted-hypertensive, VEH-treated animals, NTS\textsubscript{COMM} Aogen mRNA was positively correlated with pressure levels and was not correlated with functional response to the heart, whereas AT\textsubscript{1A} mRNA expression was positively correlated with both pressure and heart rate response. On the other hand, LOS–induced depression of basal Aogen mRNA (without interfering with the CH–induced increment) and the complete blockade of AT\textsubscript{1A} mRNA expression and heart rate response suggest that pressure load overactivates endogenous Ang II, which, via AT\textsubscript{1A}, upregulates expression of its precursor and AT\textsubscript{1A} receptor gene expression. Upregulation of RAS genes by Ang II has been shown in hepatocytes.\textsuperscript{24} In the NTS, Ang II–binding sites have also been shown to be upregulated after CH.\textsuperscript{15} Although the mechanisms responsible for these effects are not completely understood, Klett at al\textsuperscript{24} reported that Ang II stabilizes Aogen mRNA and inhibits its degradation, thus increasing its endogenous levels. The present results suggest that this observation could be extended to the brain RAS. Of interest is the observation that long-term AT\textsubscript{1A} receptor blockade reduced, but did not suppress, the CH–induced Aogen mRNA expression. Therefore, besides Ang II, hypertension per se is able to stimulate brain Aogen synthesis. Our results also suggest a differential control of RAS in the brain, the precursor expression being controlled by both pressure load and Ang II levels, whereas AT\textsubscript{1A} mRNA expression seems to be independent of pressure but is driven by endogenous Ang II. Differential regulation of Aogen and AT\textsubscript{1A} messages has already been described in the subfornical organ during dehydration.\textsuperscript{25}

It is not difficult to reconcile the differential effects of hypertension and LOS on the main components of the brain RAS. Maintenance of elevated pressure has been shown to cause hyperreactivity of the RAS with high Ang II levels.\textsuperscript{2,4,9,11,12,20–22} The peptide acts on the expressed AT\textsubscript{1A} receptors to produce tachycardia but also stimulates Aogen and AT\textsubscript{1A} expression and synthesis\textsuperscript{25,26} (and the present data) through an incompletely understood “positive” feedback mechanism. On the other hand, long-term treatment with LOS only blocks CH–induced AT\textsubscript{1A} receptor mRNA (and its functional effect), without interfering with CH–induced Aogen mRNA expression. Concerning the distribution of precursor, receptors, renin, converting enzyme, and angiotensin fragments in the brain, there are some matches (NTS, DMNV, and spinal trigeminal tract, for example) as well as several topological mismatches.\textsuperscript{3,27} In addition, Aogen message is mainly found in glial cells, whereas receptor messages are observed on neurons.\textsuperscript{3,27} These distribution patterns might offer an anatomic basis for the differential effects on Aogen and AT\textsubscript{1A} messages observed herein. According to the in situ hybridization data, we showed before in CH rats that normalization of either baroreflex responses, aortic nerve activity, and sympathetic discharge by LOS were not dependent on pressure level.\textsuperscript{12–14}

CH and LOS were effective in changing Aogen and AT\textsubscript{1A} mRNA expression not only in the NTS but also in the DMNV and spinal trigeminal tract, other areas involved in cardiovascular homeostasis. Although it is recognized that components of the RAS are not restricted to areas related to cardiovascular control but are widely distributed throughout the brain,\textsuperscript{3,27} it is important to note that no effects were observed in the gracilis, cuneatus, and inferior olivary nuclei after CH and/or LOS treatment. It should be stressed that although we have not measured AT\textsubscript{1A} and Aogen protein expressions, the strict parallel changes between density and functional effects observed after CH and/or LOS, i.e., heart rate\textsuperscript{12,13} (and the present work), afferent discharge, splanchnic sympathetic activity, sympathovagal balance, and baroreflex sensitivity changes,\textsuperscript{11–14} do support the proposition that AT\textsubscript{1A} and Aogen mRNA expressions in the brain were converted to function.

In situ hybridization studies have revealed the dominance of AT\textsubscript{1A} over AT\textsubscript{1B} receptors in numerous cardiovascular and neuroendocrine system–controlling areas.\textsuperscript{27} In a recent study, Chen et al\textsuperscript{28} showed an identical expression pattern of AT\textsubscript{1A} and AT\textsubscript{1B} messages in the rostral forebrain, hypothalamus, and brainstem, although a functional compensatory change with salt loading was only observed for the AT\textsubscript{1A} message. These data support our observation on reduced AT\textsubscript{1A} mRNA density in the NTS and the lack of effect after CH and/or LOS. In addition, we showed an interesting topographic distribution of AT\textsubscript{1A} receptor mRNA along the rostrocaudal extension of the NTS, with higher expression in the NTS\textsubscript{COMM}. The functional significance of this finding is not clear, but it agrees with previous results by us showing a larger depression of reflex bradycardia when Ang II was administered into the NTS\textsubscript{COMM} (vs the NTS\textsubscript{M}) of normotensive rats.\textsuperscript{16} Taken together, these observations strongly suggest that Ang II might exert its central effect on cardiovascular control via AT\textsubscript{1A} receptors.

In summary, our data demonstrate the importance of brain Ang II and that of AT\textsubscript{1A} receptors in specific brainstem cardiovascular system–controlling areas (NTS and DMNV) to control homeostatic processes taking place in hypertension. Multiple effects of Ang II include tachycardia and a positive feedback control on brain RAS activity, as indicated by stimulation of both Aogen and AT\textsubscript{1A} mRNA expression. Activation of brain RAS could explain, in the chronic phase of CH, the maintenance of depressed baroreceptor reflex control of heart rate, even when plasma renin activity had returned to control levels.

**Perspectives**

The present study advances our understanding of cardiovascular control by the brain RAS. It permits us to dissociate the modulatory effects driven by hypertension per se from those conditioned by Ang II and shows the pivotal role of both Ang II and AT\textsubscript{1A} receptors in controlling cardiovascular homeostasis. The observed positive feedback control on brain RAS driven by elevated pressure (mechanically induced) could explain both the frequent involvement of the RAS and the efficacy of its blockade in the treatment of hypertension of different etiologies. These new observations were possible because of the association of a very sensitive, highly discriminative, and quantitative method (in situ hybridization) to evaluate RAS activity in specific brain areas with functional data collection in the CH model that permits the establish-
ment of similar pressure load (caused by a mechanical factor), even in the presence of long-term AT₁ receptor blockade.

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