# Antisense Inhibition of Thyrotropin-Releasing Hormone Reduces Arterial Blood Pressure in Spontaneously Hypertensive Rats

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# Antisense Inhibition of Thyrotropin-Releasing Hormone Reduces Arterial Blood Pressure in Spontaneously Hypertensive Rats

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***Abstract***—Thyrotropin-releasing hormone (TRH) plays an important role in central cardiovascular regulation. Recently, we described that the TRH precursor gene overexpression induces hypertension in the normal rat. In addition, we published that spontaneously hypertensive rats (SHR) have central extrahypothalamic TRH hyperactivity with increased TRH synthesis and release and an elevated TRH receptor number. In the present study, we report that intracerebroventricular antisense (AS) treatment with a phosphorothioate oligonucleotide against the TRH precursor gene significantly diminished up to 72 hours and in a dose-dependent manner the increased diencephalic TRH content, whereas normalized systolic blood pressure (SABP) was present in the SHR compared with Wistar-Kyoto (WKY) rats. Although basal thyrotropin was higher in SHR compared with WKY rats and this difference disappeared after antisense treatment, no differences were observed in plasma T4 or T3 between strains with or without AS treatment, indicating that the effect of the AS on SABP was independent of the thyroid status. Because the encephalic renin-angiotensin system seems to be crucial in the development and/or maintenance of hypertension in SHR, we investigated the effect of antisense inhibition of TRH on that system and found that TRH antisense treatment significantly diminished the elevated diencephalic angiotensin II (Ang II) content in the SHR without any effect in control animals, suggesting that the Ang II system is involved in the TRH cardiovascular effects. To summarize, the central TRH system seems to be involved in the etiopathogenesis of hypertension in this model of essential hypertension. **(*Hypertension*. 2001;37[part 2]:365-370.)**

**Key Words:** angiotensin II ■ antisense ■ blood pressure ■ hypertension ■ SHR ■ thyroid hormones ■ TRH ■ TSH

In addition to its endocrine function, thyrotropin-releasing hormone (TRH; pyro-Glu-His-Pro-amide) also serves as a neurotransmitter in the central nervous system.[1] TRH immunoreactivity is widely distributed throughout the central nervous system, including the brain and spinal cord.[2] Although the lack of antagonists to the TRH receptor has made difficult to determine the physiological role for the extrahypothalamic TRH system, its presence in brain nuclei involved in cardiovascular regulation, such as the periventricular region and the preoptic area, suggests that this tripeptide may modulate the cardiovascular function.[3] In fact, many groups have described that brain microinjections of TRH produce dose-dependent pressor effects.[4]

Recently, we have reported that the overexpression of the TRH precursor in the third ventricle of the central nervous system of normal rats induces a long-lasting elevation of arterial blood pressure along with an increase in the diencephalic TRH content in a dose-dependent manner. These effects were specifically reversed by an antisense treatment, indicating that the extrahypothalamic TRH system effectively participates in cardiovascular regulation in the rat.[5]

Spontaneously hypertensive rats (SHR) have been extensively used as an essential hypertension model. In these rats, an enhanced brain angiotensin system, and a muscarinic cholinergic hyperactivity have been detected that may participate in the development or maintenance of hypertension.[6,7] On the other hand, we have shown that TRH facilitates the pressor response to centrally infused acetylcholine, increasing the number of muscarinic receptors. In turn, in vitro superfusion experiments with preoptic area slices showed that cholinergic muscarinic stimulation evoked a specific TRH release.[8] In addition, it has been described that spontaneously hypertensive rats exhibit a supersensitivity to the hypertensive effects of exogenous TRH.[9] Accordingly, in a previous study, we published that SHR presented an increase of both, TRH content and TRH precursor mRNA abundance in the

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preoptic area with a higher cerebrospinal fluid TRH concentration and an augmented TRH receptor number in this area compared with its control normotensive strain Wistar-Kyoto (WKY). In addition, we also found that a polyclonal antibody raised against TRH infused peripherally or intracerebroventricularly significantly decreased arterial blood pressure.[10] These results pointed out that, in addition to the regulation of the cardiovascular system in a normal rat, extrahypothalamic TRH may participate in the pathogenesis of the hypertension in this genetic model. However, the question as to whether an increased activity of the TRH system is responsible for the hypertension, either by a direct action or through the activation of other mechanisms, in the abnormal biochemical environment of the central nervous system of the SHR, remains to be answered.

Antisense molecules have been used to successfully inhibit protein synthesis in several biological systems. They can bind to a complementary region of the target gene and/or mRNA and block selected gene expression without changing the functions of other genes.[11] Therefore, we conclude that the AS strategy may be a good option for a gene therapy approach to inhibit TRH. Consequently, in the present study, we attempted to reduce the production of TRH in the diencephalic region by administration of antisense phosphorothioate oligonucleotides against the translation initiation codon region of the TRH-precursor gene and analyze whether the TRH antisense treatment is able to normalize the blood pressure in the SHR. We found that a single intracerebroventricular antisense injection decreased both the elevated diencephalic TRH content and the systolic blood pressure (SABP) for up to 72 hours only in the SHR strain in a dose-dependent manner, whereas sense or vehicle treatment failed. Normalization of blood pressure seems to be independent of changes in thyroid status, since, although basal plasma thyroid-stimulating hormone (TSH) was increased in SHR with respect to its control and TRH antisense treatment reduced TSH, there were no changes in plasma thyroid hormone levels.

As we pointed out above, the interactions between systems involved in the cardiovascular regulation are still unknown. Because the brain renin-angiotensin system is considered important for cardiovascular regulation and it has been reported that SHR present an augmented central angiotensin II (Ang II) synthesis and Ang II receptor number, and antisense treatments against angiotensinogen, angiotensin converting enzyme, or the angiotensin type I receptor ($AT_1$) normalizes blood pressure in these animals,[12–16] we also measured diencephalic Ang II content in our experiments. We found that TRH antisense treatment decreases the Ang II diencephalic content to values comparable to those of the WKY control rats

To summarize, these results pointed out that (1) the central TRH system participates in the development or maintenance of hypertension in this rat model, and (2) the TRH cardiovascular effects are independent of its classic endocrine function in the hypothalamus-pituitary axis and seems to involve the encephalic renin-angiotensin system.

## Methods

### Animals

Adults (16-week-old, 250 to 300 g) male SHR of the Okamoto-Aoki strain and age-matched male normotensive WKY rats were housed in a room with a control temperature (23±1°C) under a 12-hour light/dark schedule. Food and water were given ad libitum. Animal experimentation protocols were approved by our Institutional Animal care and Use Committee.

### Intracerebroventricular Oligonucleotide Treatment

SHR and WKY males were anesthetized with pentobarbital (45 mg/kg). A 25-gauge stainless steel cannula was directed to the third ventricle through a burr hole in the skull for injection with the aid of a stereotaxic atlas.[17] Coordinates for injection were 1.3 mm posterior to the bregma on the midline and 4.5 mm below the dura. Oligonucleotides were dissolved in phosphate-buffer-saline (PBS). At the end of each experiment, the position of the cannula was assessed by histologic examination. A total injection volume of 10 μL was used. Control rats received vehicle only.

SABP and heart rate were recorded daily during the experiments by the tail-cuff plethysmographic method. Basal values correspond to the mean of 3 independent measurements during 3 days before the injection. Daily SABP was the average of 3 measurements taken 2 minutes apart. In separate sets of experiments, animals were killed by decapitation at different time points after intracerebroventricular injection. The brain tissue was processed as described below.

Oligonucleotides were made resistant to nucleases by DNA backbone phosphorothioation and were synthesized (Biosynthesis Inc) as 23-mers targeted to bases 20 to 42 (AS: 5′AAC CAA GGT CCC GGC ATC CTG GA 3′) of rat pre-TRH gene encompassing the translation initiation codon (GenBank accession number M23643). As control, we used a sense oligonucleotide (S: 5′ TCC AGG ATG CCG GGA CCT TGG TT 3′). The screening of known rat genes from the genomic database of the National Center for Biological Information (NCBI) using the Blast program indicates specificity of the sequences used in oligonucleotides design and confirms their 100% homology with rat pre-TRH gene. AS and S: high score 115 for pairs with rat TRH sequences M23643, M12138 and M36317. Oligonucleotides were dissolved in PBS and 25, 50, 100 or 200 μg ICV were injected in a total volume of 10 μL.

### Diencephalic TRH Content Determination by Radioimmunoassay

Animals were killed by decapitation and brains were rapidly removed. The diencephalic region of each animal was rapidly dissected with the aid of a stereotaxic atlas. To avoid the degradation of authentic TRH and the formation of TRH-like substances, samples (1 diencephala) were boiled in 2 mol/L acetic acid, 100 mmol/L HCl for 20 minutes, homogenized, and centrifuged at 10 000 g for 10 minutes. The supernatants were lyophilized, and residues were dissolved in radioimmunoassay (RIA) buffer. We confirmed that 90% of TRH-like immunoreactivity correspond to authentic TRH using a previously reported chromatographic method that consists of a SP-Sephadex C25 and high-performance liquid chromatography.[8]

RIA for TRH has been described in detail.[8] In brief, a polyclonal anti-TRH antibody was raised in New Zealand White rabbits immunized with TRH coupled to bovine serum albumin using the bis-diazotized benzidine reaction. Standards or samples were incubated with $^{125}$I- and anti-TRH (1/10 000) at 4°C overnight. Bound hormone was pelleted with rabbit normal serum and a second antibody against rabbit IgG. All samples were assayed in duplicate. The minimum detectable amount was 5 to 10 pg. Intra-assay and interassay coefficients of variation were less than 7.0% and 14.0%, respectively.

### Determination of TSH and Thyroid Hormones in Plasma

Blood samples were collect on EDTA-containing tubes, and plasma was obtained by centrifugation. TSH was carefully determined by Dr

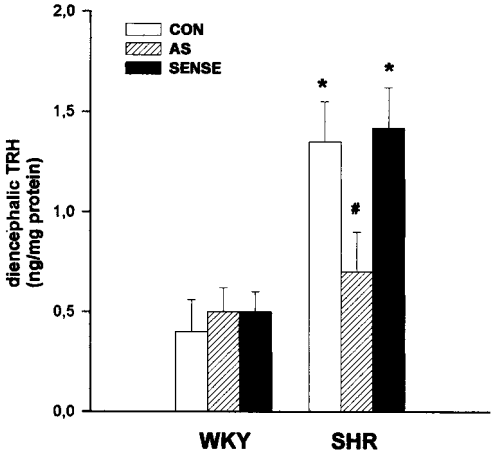


**Figure 1.** TRH antisense treatment on diencephalic TRH content after 24 hours of injection into SHR and WKY rats. A total of 100 $\mu$g of phosphorothioates oligonucleotide sense (SENSE) and antisense (AS) or saline (10 $\mu$L, CON) was injected intracerebroventricularly by stereotaxis. Results are expressed as mean±SD, n=8. *$P$<0.03 with respect to WKY in the same condition. #$P$<0.03 with respect to control SHR.

P. Hacke (BsAs) using a previously published RIA (kit NIDDK) using rTSH RP3 (AFP-5512B) as TSH standard, and anti-rat TSH (RIA 6). Total plasma T4 and T3 concentrations were measured using electrochemiluminescence immunoassay (kits 1010 and 2010, Roche).

### Diencephalic Ang II Content Determination by RIA

For the Ang II determination, samples were processed identically as the samples described for TRH determinations using a RIA developed in our laboratory.[18] In brief, a polyclonal anti–Ang II antibody was raised in New Zealand White rabbits immunized with Ang II coupled to bovine serum albumin using glutaraldehyde (2.5 mg/L). Standards or samples were incubated with $^{125}$I- and anti–Ang II (1/15 000) at 4°C overnight. Bound hormone was pelleted with rabbit normal serum and a second goat antibody against rabbit IgG. All samples were assayed in duplicate. The minimum detectable amount was 5 to 10 pg. Intra-assay and interassay coefficients of variation were less than 9.0% and 16.0%, respectively.

### Statistical Analysis

Results are expressed as mean±SD from independent experiments. Statistical significance between means for the effects of treatments on SABP were determined by 2-way ANOVA with repeated-measurements on one factor. Where pairwise comparisons were made after ANOVA, the Tukey's test for individual differences was used.

## Results

In this study, we confirm our previous observations of the elevated SHR diencephalic TRH content with respect to their age-matched WKY. In addition, we show that a phosphorothioate TRH antisense treatment injected intracerebroventricularly decreases the increased diencephalic TRH content (diencephalic TRH ng/mg protein) with a maximum effect at 24 hours after antisense injection up to 72 hours, only in the SHR, whereas sense and control treatment failed (Figure 1). No differences were observed between antisense or sense treatments compared with vehicle-treated WKY animals at all time points.

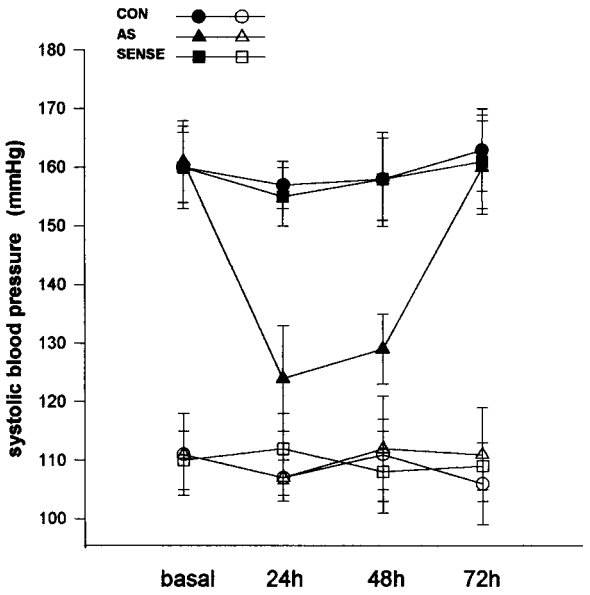


**Figure 2.** TRH antisense treatment on systolic blood pressure (SABP, mm Hg) in SHR and WKY animals. A total of 100 $\mu$g of phosphorothioate oligonucleotides sense (SENSE) and antisense (AS) or saline (10 $\mu$L, CON) was injected intracerebroventricularly by stereotaxis, and SABP was monitored by the tail-cuff plethysmographic method in the basal condition and 24, 48, and 72 hours after injection. Open and filled symbols stand for WKY and SHR, respectively. Results are expressed as mean±SD, n=8. AS treatment significantly ($P$<0.02) decreases SABP at 24 and 48 hours only in SHR.

As shown in Figure 2, basal SABP was significantly higher in the SHR with respect to their controls WKY (SHR: 161±7 versus WKY 111±8 mm Hg, n=8, $P$<0.02). TRH antisense treatment decreased the elevated SABP of SHR with a maximum effect at 24 hours up to 72 hours, whereas sense and control treatments had no effect. No differences were observed in the arterial blood pressure of the control WKY rats among antisense, sense, and vehicle treatments throughout the experiments.

In addition, in SHR, we observed that SABP and diencephalic TRH content reductions were dependent on antisense oligonucleotide doses, reaching a plateau at 100 $\mu$g (Figure 3). No significant changes were observed in heart rate with antisense, sense, or vehicle treatments in both strains (data not shown).

As shown in Figure 4, TSH concentrations in plasma were significantly elevated (more than 2-fold) in SHR compared with WKY control rats. TRH antisense intracerebroventricular injection significantly reduced TSH plasma levels to similar values of those of WKY control rats, 24 hours after treatment. There were no differences between thyroid hormones (T3 ng/mL and T4 $\mu$g/dL) in basal conditions between both strains and antisense; sense and vehicle treatments had no effects on thyroid status in these animals.

At 24 hours after injection, when the hypotensive effect peaked, the TRH antisense treatment also significantly reduced the increased diencephalic Ang II content (pg/mg protein) of the SHR to values comparable with those found in WKY normotensive rats, whereas the sense and vehicle

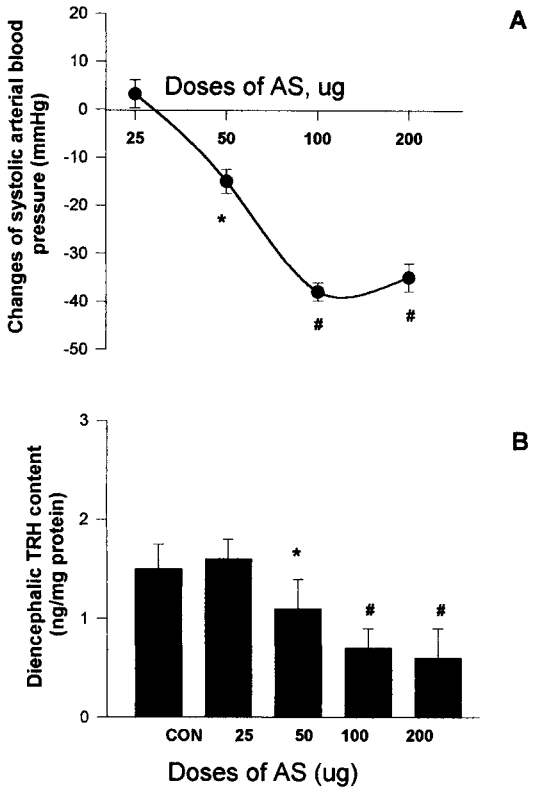


**Figure 3.** Dose-dependent decreases in diencephalic TRH content (A) and systolic blood pressure (B) induced by AS oligonucleotide against the pre-TRH gene in the SHR. CON stands for control vehicle–treated animals. Results are expressed as mean±SD, n=5. *$P<0.04$ with respect to CON. #$P<0.04$ with respect to AS-50 $\mu$g.

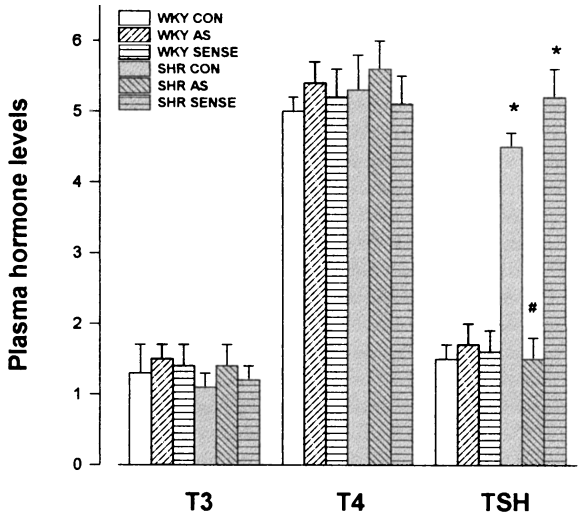


**Figure 4.** TRH antisense treatment on plasma thyrotropin (TSH ng/mL) and thyroid hormones, triiodothyronine (T3, ng/mL) and L-thyroxine (T4, $\mu$g/dL), at 24 hours after injection of 100 $\mu$g of phosphorothioate oligonucleotide sense (SENSE) and antisense (AS) or saline (10 $\mu$L, CON) in SHR (gray bars) and WKY control rats (white bars), n=8. *$P<0.03$ with respect to WKY in the same condition. #$P<0.03$ with respect to control SHR.

treatments failed to produce any changes in both strains (Table).

## Discussion

In physiological studies, intravenous or intracerebroventricular TRH injections increased arterial blood pressure.[4] This effect was blocked by destruction of the sympathetic system, indicating that the pressor effect could be mediated by catecholamines involving the modulations of diverse neurotransmitter system activity.[19]

In 1995, we reported for the first time that SHR show a significant hyperactivity of the extrahypothalamic TRH system with a 2-fold increase of the preoptic hypothalamic area TRH content and TRH receptor number along with a significantly increased TRH concentration in the cerebrospinal fluid. These findings, in addition to the Northern blot analysis indicating a 3-fold augmented TRH precursor mRNA, have pointed out that SHR present increases in the TRH synthesis and release in the central nervous system. A significant hyperactivity of the TRH system was also apparent at the prehypertensive stage of these rats, indicating that alterations in this system may participate in both the development and maintenance of the hypertension in this model.[10] Because until now, no specific antagonist is available for the TRH receptor in that previous work, as a first approach, we have analyzed the effect of a polyclonal antibody against TRH injected peripherally or intracerebroventricularly and found a significant but very short-lasting hypotensive effect, probably because of the presence of neutralizing endogenous TRH and the difficulty of big molecules to reach the synaptic space.[10] However, it remains controversial as to whether the increased expression of the extrahypothalamic TRH system in the spontaneously hypertensive strain is the cause of the elevated arterial blood pressure.

In the present study, we assessed in the SHR the TRH antisense treatment that was proved to be efficient as strategy for antagonising the TRH cardiovascular effects in the normal rat with induced TRH overproduction.[5] Therefore, we report, for the first time that a TRH antisense single intracerebroventricular injection reduced the augmented diencephalic TRH content and normalizes arterial blood pressure of the SHR, without any effect in the WKY normotensives rats. The TRH AS-induced decrease in the SABP of the SHR is related to the reduction in diencephalic TRH content, because we observed no changes in other TRH-containing brain areas such as amygdala or olfactory bulb (data not shown). Even though the explanation for the absence of any effect of the TRH antisense treatment in the normotensive strain is not

**Diencephalic Ang II Content 48 Hours After Injection of 100 $\mu$g of Sense and Antisense Phosphorothioate Oligonucleotides or Vehicle (Control) in SHR and WKY Control Rats**

| Treatment | SHR | WKY |
|---|---|---|
| Control | 13.93±6.47* | 4.67±2.90 |
| Antisense | 6.48±1.99† | 5.92±3.41 |
| Sense | 14.54±2.12* | 5.34±3.12 |

Results are expressed as picograms per milligram of protein; mean±SD, n=5.

*$P<0.02$ with respect to WKY control rats.

†$P<0.02$ with respect to vehicle-treated SHR.

apparent, one possibility is that a very low turnover of the endogenous TRH system would be unaffected by a single TRH antisense injection or that, under normal conditions, the TRH system does not exert a tonic effect on central cardiovascular regulation. Decreases of diencephalic TRH content and systolic blood pressure induced by the TRH antisense treatment show an onset of less than 24 hours and decline at 72 hours. This rapid onset may suggest that there is a high turnover of the periventricular TRH in the SHR. On the other hand, these transitory effects are what can be expected for a single antisense injection of the oligonucleotide, which, even though it has been made resistant to nucleases by phosphorothioation, may still suffer a significant degradation rate.[20] In fact, a similar time course has been observed for other antisense treatments.[21] In any case, using this approach we were able to demonstrate the effectiveness of the antisense oligonucleotide treatment in decreasing arterial blood pressure in the SHR. Accordingly, Suzuki et al[22] reported that antisense inhibition of TRH receptor gene expression induced similar effects on SABP in this hypertensive strain.

The observed effects of the TRH antisense oligonucleotide is sequence specific and seem not due to a nonspecific toxicity, because treatment with sense oligonucleotide, which had a similar percentage base composition, showed no effects. We have no evidence supporting the final mechanisms of action of the antisense, which can act by decreasing the pre-TRH mRNA abundance at a transcriptional or post-transcriptional level and/or inhibiting the pre-TRH mRNA translation machinery, but the decrease of the tripeptide content in the diencephalon means that the treatment was an effective blocker of the precursor TRH production. Although those experiments were not focused on the TRH activity of the hypothalamic-pituitary axis, another possible site of action of the antisense oligonucleotides to TRH is the hypothalamus, where alterations in the TRH synthesis might affect thyroid function indirectly influencing the cardiovascular tone. SHR are characterized by several neuroendocrine abnormalities, including a chronic hypersecretion of TSH of unknown etiology associated with normal plasma T3 and T4 levels.[23] This inappropriately high TSH secretion in euthyroid SHR may be the result of an impaired hormone negative feedback regulation of the central TRH system or a inappropriate pituitary TSH production.[24] Accordingly to other authors and our own previous data, we showed that SHR in basal conditions present a higher (3-fold) plasma TSH concentration compared with control WKY rats.[10,25] In the present study, plasma samples were collected 24 hours after the injection when antisense treatment reached its maximum hypotensive effect and we found that TRH antisense treatment normalize plasma TSH in SHR without affecting TSH levels in control rats. Despite this effect of antisense treatment on TSH level of the SHR, no differences were observed in plasma T4 and T3 between strains with or without antisense treatment. The euthyroid state of the SHR, despite of altered plasma TSH levels, has not yet been explained and deserves further investigation, but either a decrease in thyroid TSH receptors or an abnormal TSH biological activity can be implied.[26] At any rate, our findings indicate that TRH antisense induced hypotensive effect does not seem to be explained by changes in the thyroid status. Additional studies are necessary to delineate the complex interactions that may take place in the effect of the extrahypothalamic TRH system in cardiovascular regulation but TRH cardiovascular effects seem to be rather mediated by the sympathetic system, because TRH injections produce an increase in plasma catecholamine levels, and adrenalectomy avoids its hypertensive effects.[19] Accordingly, it is tempting to speculate that a TRH antisense-induced fall in the sympathetic activity would produce more obvious effects in SHR because of their higher sympathetic tone compared with WKY control animals.[27] Because TRH is a potent prolactin releaser,[28] it can be hypothesized that anti-sense treatment against TRH may decrease SABP by affecting prolactin levels. We cannot reject that possibility because we have not measured prolactin, but this seems unlikely because prolactin has been described not to be involved in the pathogenesis of the hypertension in the SHR.[29] In addition, prolactin may not alter arterial blood pressure directly and may require a week to potentiate the pressor effect of norepinephrine.[30] In any case, the participation of prolactin in the TRH antisense effects remains to be explored.

On the other hand, the vast interactions between neurotransmitters and neuropeptide systems involved in the cardiovascular regulation are still unknown. The brain renin-angiotensin system is considered one of the most important in the blood pressure control either by a direct action or through the activation of other neurohumoral mechanisms. The augmented central Ang II production with an increased Ang II receptor number is a common reported feature of the SHR.[6,31] A growing body of evidence shows that antisense treatments against angiotensinogen, angiotensin converting enzyme, or $AT_1$ receptor normalizes blood pressure in the SHR.[12–14,32,33] Therefore, we measured Ang II diencephalic content in this model. We found that TRH antisense treatment decreases the elevated Ang II diencephalic content to values comparable to the WKY control rats without any effects in the WKY control animals. Taking into account the theoretical high specificity of the TRH antisense treatment (100% homology with precursor TRH gene), the decrease on diencephalic Ang II content suggest that the TRH may exert some regulation over the angiotensin system. A note of caution should be mentioned here, it is not possible to infer from changes in neuropeptide content as to whether those are produced by alterations in neuropeptide synthesis and/or release. In fact, if one assumes that antisense–induced Ang II content decrease is the result of a diminished synthesis, then TRH exerts a positive effect on the Ang II system; conversely, if one assumes that the Ang II content decrease means a higher release rate of Ang II, then this change in Ang II may be interpreted as a compensatory mechanism induced by the fall in arterial blood pressure with no biochemical connection with the TRH system. For that reason, it should be mentioned that we were not able to show either a corresponding increase in diencephalic Ang II content in basal conditions or its decrease after antisense treatment in our TRH overproduction rat model, suggesting that an Ang II-TRH interaction may require the altered neurochemical environment that characterizes the brain of SHR. Consequently, more investi-

gations are necessary to determine the reach of this intriguing interaction between both neuropeptide systems.

To conclude, we believe that these results demonstrate the important role that the extrahypothalamic TRH system plays in the development and/or maintenance of the elevated arterial blood pressure in this genetic hypertensive rat model. TRH cardiovascular effects are not mediated by thyroid hormones and seem to involve the angiotensin system.

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