Effects of chronic inhibition of inducible nitric oxide synthase in hyperthyroid rats

Isabel Rodríguez-Gómez,1 Rosemary Wangelsteen,1 Juan Manuel Moreno,2 Virginia Chamorro,1 Antonio Osuna,2 and Félix Vargas1

1Facultad de Medicina, Departamento de Fisiología, Granada; and 2Servicio de Nefrología, Unidad Experimental, Hospital Virgen de las Nieves, Granada, Spain

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Rodríguez-Gómez, Isabel, Rosemary Wangelsteen, Juan Manuel Moreno, Virginia Chamorro, Antonio Osuna, and Félix Vargas. Effects of chronic inhibition of inducible nitric oxide synthase in hyperthyroid rats. Am J Physiol Endocrinol Metab 288: E1252–E1257, 2005.—We hypothesized that nitric oxide generated by inducible nitric oxide synthase (iNOS) may contribute to the homeostatic role of this agent in hyperthyroidism and may, therefore, participate in long-term control of blood pressure (BP). The effects of chronic inactivation of iNOS by oral aminoguanidine (AG) administration on BP and morphological and renal variables in hyperthyroid rats were analyzed. The following four groups (n = 8 each) of male Wistar rats were used: control group and groups treated with AG (50 mg·kg−1·day−1, via drinking water), thyroxine (T4, 50 μg·rat−1·day−1), or AG + T4. All treatments were maintained for 3 wk. Tail systolic BP and heart rate (HR) were recorded weekly. Finally, we measured BP (mmHg) and HR in conscious rats and morphological, plasma, and renal variables. T4 administration produced a small BP (125 ± 2, P < 0.05) increase vs. control (115 ± 2) rats. AG administration to normal rats did not modify BP (109 ± 3) or any other hemodynamic variable. However, coadministration of T4 and AG produced a marked increase in BP (140 ± 3, P < 0.01 vs. T4). Pulse pressure and HR were increased in both T4- and T4 + AG-treated groups without differences between them. Plasma NOx (μmol/l) were increased in the T4 group (10.02 ± 0.15, P < 0.05 vs. controls 6.1 ± 0.10), and AG reduced this variable in T4-treated rats (6.81 ± 0.14, P < 0.05 vs. T4) but not in normal rats (5.78 ± 0.20). Renal and ventricular hypertrophy and proteinuria of hyperthyroid rats were unaffected by AG treatment. In conclusion, the results of the present paper indicate that iNOS activity may counterbalance the prohypertensive effects of T4.

HYPERTHYROIDISM MANIFESTS IMPORTANT CHANGES in hemodynamic, renal, and cardiac function (9, 18, 19). It is hemodynamically characterized by a hyperdynamic circulation with increased cardiac output, increased heart rate, and decreased peripheral resistance (4, 17, 18). In animal studies, thyroxine (T4) produces a dose- and time-related rise in arterial blood pressure (BP; see Refs. 36 and 37), increases cardiac and renal weight, and reduces renal sodium excretion (4, 36, 37).

NO is known to play a major role in the regulation of vascular tone (12) and renal sodium excretion (6, 28) and, consequently, of BP (23). NO can be generated by the activity of neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) nitric oxide synthase isoforms, which are all widely distributed in organs related to BP control and present in normal rat kidney (34).

Aminoguanidine (AG) is a selective iNOS inhibitor in vitro (14, 22) and in vivo (21, 25, 32). Several authors have supported a role for iNOS in BP control (21, 25, 32). Thus recent studies showed that NO produced by iNOS may play a significant role in preventing salt-sensitive hypertension in rats with normal salt sensitivity (21, 32) and that acute iNOS inhibition markedly increases BP in cirrhotic hypotensive rats (25).

Fernández et al. (9) demonstrated that hyperthyroidism leads to a significant and reversible enhancement in rat liver NO activity, and our group reported (26) that NOS activity is upregulated in tissues primarily related to BP control in hyperthyroid rats. More recently, we observed that the simultaneous administration of T4 and a suppressor dose of l-NAME produced a marked BP increase, indicating that NO may have a counterregulatory homeostatic role against the prohypertensive effects of thyroid hormone (27).

At the present time, the role of iNOS in hyperthyroidism is not clear, and the mechanisms responsible for the elevated NO activity of hyperthyroidism are not completely established. Moreover, no data have been reported on the contribution of iNOS to the antihypertensive effect of NO in this endocrine disease. With this background, we hypothesized that increased activity of iNOS, an isoform that plays an important role in renal function and BP regulation in various pathophysiological situations, might increase NO production and contribute to the homeostatic role of this factor in the hyperthyroid state. Therefore, the present study was designed to assess the role of iNOS to the long-term control of BP and other variables in the hyperthyroid state. To this end, we studied the effects of the chronic blockade of iNOS with AG in hyperthyroid rats.

METHODS

Animals. Thirty-two male Wistar rats born and raised in the experimental animal service of the University of Granada were used. The experiment was performed according to European Union guidelines for the ethical care of animals. Rats initially weighing 200–250 g were randomly assigned to one of four groups. The groups were as follows: control, AG, T4, and AG + T4 rats. Each experimental group comprised eight animals. All rats had free access to food and tap water except where stated. AG (50 mg/day; ~50 mg·kg−1·day−1) was given in the drinking water. The AG concentration in the drinking fluid was adjusted every 2 days according to the fluid intake of the animals to ensure that a similar dose of iNOS inhibitor was administrated to the T4-treated and control groups.

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EFFECTS OF INOS BLOCKADE ON HYPERTHYROIDISM

Hyperthyroidism was induced by injecting T4 subcutaneously (Merck) at 50 μg·rat⁻¹·day⁻¹ dissolved in 0.5 N NaOH isotonic saline. This dose was chosen because it produces a slight BP increase of <15 mmHg (27, 37).

Experimental protocol. The treatments were administered for 3 wk. Body weight and tail systolic blood pressure (SBP) were measured once a week. Tail SBP was measured with the use of tail-cuff plethysmography in unanesthetized rats. When the experimental period was completed, all rats were housed in metabolic cages with free access to food and their respective drinking fluids and treatments for 4 days (2 days for adaptation + 2 experimental days) to measure food and fluid intake and collect urine samples. Twenty-four-hour urine volume, proteinuria, creatinine, and total excretion of sodium and potassium were measured. The mean values of all intake and urinary variables obtained during the two experimental days were used for statistical analyses among the groups.

After completion of the metabolic study, the rats were anesthetized with ethyl ether. A polyethylene catheter (PE-50) containing 100 units of heparin in isotonic sterile NaCl solution was inserted in the femoral artery for intra-arterial BP, heart rate, and pulse pressure (pulse pressure = SBP − diastolic BP) measurements in conscious rats and for extraction of blood samples. The catheter was tunneled subcutaneously, brought out through the skin at the dorsal side of the neck, and protected with a silver spring. After implantation of the femoral catheter (24 h), intra-arterial BP was measured by a TRA-021 transducer connected to a two-channel Letigraph 2000 recorder (Letica, Barcelona, Spain). After 30 min of stabilization, values from the last 5 min recorded were averaged and used for comparisons among groups. Subsequently, blood samples taken with the femoral catheter were used to determine total protein, electrolytes, and creatinine concentration. The kidneys and ventricles were then removed and weighed. The heart was divided into right ventricle and left ventricle plus septum.

Analytic procedures. Proteinuria was measured by the method of Bradford (3). Plasma and urinary electrolytes and creatinine were measured in an autoanalyzer (Beckman CX4). Plasma NOx (nitrates + nitrates) concentration was measured using nitrate reductase and the Griess reaction (13).

Statistical analysis. The evolution of SBP with time was compared by use of a nested design, with groups and days as fixed factors and rat as random factor. When the overall difference was significant, Bonferroni’s method with an appropriate error was used. Comparisons of each variable at the end of the experiments were done by performing a one-way ANOVA. When the overall ANOVA was significant, we performed pairwise comparisons with Bonferroni’s and Newman-Keuls methods.

RESULTS

BP and heart rate. Figure 1 shows the time course of tail SBP. T4 administration produced a mild increase in SBP at week 2 of treatment vs. control rats. AG administration to normal rats at the dose used in this experiment did not modify the time course evolution of SBP or any other hemodynamic variable. However, the simultaneous administration of T4 and AG produced a marked increase in SBP, which reached significance at week 1 of treatment. These data were confirmed by the final mean arterial pressure measurement in the conscious animals at the end of the experimental period (Fig. 1). Final heart rate and pulse pressure were significantly and similarly increased in both T4- and T4 + AG-treated groups (Fig. 2).

Morphological variables. T4-treated groups showed a significant reduction in body weight gain during the course of the experiment compared with the control groups. AG treatment did not significantly affect the body weight increase in control or T4-treated rats (Table 1). Absolute kidney weight and absolute left ventricular weight were similar in all groups. However, absolute right ventricular weight was increased in both T4-treated groups (Table 1). The kidney-to-body weight ratio and left ventricular-to-body weight ratio were significantly increased in both T4-treated groups compared with their respective controls and were unaffected by AG treatment (Table 1). The left ventricular-to-right ventricular weight ratio was reduced in all T4-treated groups with respect to their controls (Table 1), indicating that the cardiac hypertrophy of the hyperthyroid state affects the right ventricle more than the left one.

Plasma, metabolic, and urinary variables. Plasma NOx concentration was unaffected by AG treatment in control rats. Plasma NOx was significantly increased in the T4 group. AG treatment reduced plasma NOx in T4-treated rats, suggesting that iNOS is partly responsible for the increased NO production induced by thyroid hormones. There were no significant differences in plasma sodium, potassium, or creatinine levels among the groups. Total plasma protein values were significantly decreased in the T4 and T4 + AG groups vs. controls (Table 2).

![Graph showing SBP measurements over time](http://ajpendo.physiology.org/Downloaded/from)
Metabolic studies at the end of treatment showed increased food and fluid intake (g/100 g body wt) in all T4-treated groups compared with their corresponding controls (data not shown).

Urinary and creatinine clearance values are summarized in Table 3. Urine volume was significantly higher in the T4-treated groups. Total sodium and potassium excretion was not significantly modified by T4 or AG treatments. Proteinuria was significantly increased in all T4-treated groups and was unaffected by AG treatment in control and T4-treated rats. Creatinine clearance, normalized per gram kidney weight, was similar in all experimental groups.

**DISCUSSION**

One of the main findings of this study was that AG increased BP in the hyperthyroid rats at doses that had no pressor effect in the control animals. This suggests that these hyperthyroid animals have an elevated sensitivity to AG. At the same time, the elevated plasma levels of nitrates plus nitrites (an index of NO production) in the hyperthyroid animals decreased with the administration of the drug, indicating that this important pressor effect is related to inhibition of NO production. Because AG has been reported to inhibit iNOS (14, 21, 22, 25, 32), these results indicate that the inducible isoform is activated in hyperthyroid animals. Interestingly, the BP increase induced by AG administration in hyperthyroid rats is approximately one-half that produced by l-NAME (27), the nonspecific NOS inhibitor. This suggests that NO derived from eNOS also contributes to counterbalancing the pressor response to T4 administration, since nNOS blockade with 7-nitroindazole did not modify BP in hyperthyroid rats (unpublished observations).

At the concentration used in this study, AG did not significantly affect BP in normal rats, suggesting that only the upregulated NO production was blocked by AG. This proposition is supported by findings that hyperthyroidism causes an upregulation of NO activity (26) and NO production (27 and Table 3) and that AG decreases plasma NOx only in hyperthyroid rats. The absence of effect of AG on BP in normal rats is in agreement with previous observations (7, 31).

This study and several reports (9, 26, 27) provide evidence that the hyperdynamic circulation of hyperthyroidism is accompanied by increased NO production. Cirrhosis of the liver (25) is also associated with hyperdynamic circulation and increased NO production, and cirrhotic rats show an increased pressor responsiveness to iNOS blockade (25). Taken together, these observations indicate that iNOS is activated in rats with hyperdynamic circulation, which may have an important homeostatic role in these animals.

Various mechanisms may participate in the increased BP sensitivity to iNOS blockade in hyperthyroid rats. First, the reduction in plasma NOx induced by AG in hyperthyroid rats may be an index of a reduced NO availability that might produce an imbalance in the NO-ANG II interaction, facilitating prohypertensive vascular and renal actions of ANG II. This phenomenon may be active in hyperthyroidism that produces stimulation of the renin-angiotensin system (11). This possibility is supported by our group’s previous finding in hyperthyroid rats that ANG II type 1 receptor blockade suppressed the BP increase produced by partial NO inhibition with the nonspecific NOS inhibitor L-NAME (27). Second, renal iNOS activity may decrease because of AG administration, potentially contributing to enhanced sodium reabsorption in hyperthyroid rats. Finally, changes in renal iNOS activity can produce important effects on renal sodium excretion (21, 32). iNOS mRNA has been identified in tubular and vascular sections of the kidney. The greatest amount of iNOS mRNA transcribes in sections of the kidney. The greatest amount of iNOS mRNA
was observed in the medullary thick ascending limb and the inner medullary collecting duct (1, 30), the major sites of sodium reabsorption. Mattson et al. (21) reported that chronic intravenous infusion of AG to uninephrectomized Sprague-Dawley rats maintained on a high-salt diet produced a decrease in renal medullary iNOS activity and in urinary sodium excretion and caused hypertension. Moreover, studies in hypertensive rats provide evidence that iNOS is connected with salt sensitivity and BP regulation (8, 32). Therefore, it is reasonable to assume that, in the present study, the AG-induced inhibition of medullary iNOS in the hyperthyroid rat may have increased sodium retention in the distal part of the nephron and aggravated the antinatriuretic effects of thyroid hormones (4, 36, 37), thereby increasing the blood volume and BP, which in turn elevated sodium excretion but at the expense of an increased BP. Indeed, our hyperthyroid rats required an increased BP to achieve a normal sodium excretion during AG administration.

Previous studies have shown that AG is a selective iNOS inhibitor (14, 21, 22, 25, 32). In vitro, the inhibition constant value of AG is 32- to 52-fold lower for iNOS than for eNOS (22). In vivo, AG is 40-fold less potent than L-NMMA to acutely increase BP in rats (5). AG had no effect on ACh-induced relaxation in intact vessels of sham-treated rats but competitively inhibited relaxation by L-arginine of artery rings from endotoxin-treated rats (14). A 6-day intravenous infusion of AG at 10 mg·kg⁻¹·h⁻¹ to normal rats on high sodium intake decreased renal medullary Ca-independent NOS activity without effect on Ca-dependent activity (21). Prevention of the long-term effects of AG on BP in sodium-loaded rats by administration of excess NOS substrate (2% L-arginine in drinking water) argues against nonspecific effects of this drug (21). All of these studies indicate that AG can be used as a selective inhibitor of iNOS in vivo. In fact, AG has been used as a reference compound to analyze the activity of new iNOS inhibitors in vivo and in vitro (16).

Moreover, several groups have shown that oral administration of AG at similar doses to those in the present study can selectively inhibit iNOS activity in different experimental settings. Chronic AG treatment (15 mg·kg⁻¹·day⁻¹ po) significantly suppressed the development of hypertension in spontaneously hypertensive rats and inhibited the increase in aortic iNOS expression, NO production, and superoxide anion formation of these rats (15). Sarthy and Kern (29) observed that retinal homogenates from diabetic rats produced greater amounts of NO and iNOS that were inhibited by oral AG administration.

Cardiac hypertrophy (17, 18, 27) is associated with hyperthyroidism. The ventricular-to-body weight ratio, a measure of relative ventricular hypertrophy, was increased in T₄-treated rats. However, the left-to-right ventricular weight ratio was reduced by T₄ treatment, indicating that the trophic effect of thyroid hormones affects both ventricles and predominates in the right ventricle. These results agree in part with previous observations (27), although the left ventricular-to-right ventricular weight ratio did not reach a statistically significant difference with that of controls in that study. Both ratios were unaffected by AG treatment in the present study. These data suggest that ventricular hypertrophy in hyperthyroidism is unrelated to the BP and agree with previous observations by our group (27) that increases or reductions in BP, induced by L-NAME or losartan, respectively, did not modify ventricular hypertrophy in hyperthyroid rats. Therefore, the present data add further support to our previous suggestion that a direct trophic effect of thyroid hormones on the heart may be responsible for cardiac hypertrophy in hyperthyroidism (27). This proposal is in agreement with the observations of Bedotto et al. (2), who reported that cardiac hypertrophy in hyperthyroid rats is independent of loading conditions, and with studies of cultured cardiomyocytes in which the thyroid hormone promoted cell growth (24).

All T₄-treated groups showed increased proteinuria, as previously reported in hyperthyroid rats by our group (27) and as observed in patients with Graves’ disease (38). The proteinuria is not related to the BP, because it was similar in all T₄-treated rats, as found in the previous study (27), providing further evidence that proteinuria in the hyperthyroid state may be produced by a direct action of thyroid hormones, increasing the permeability of the glomerular barrier. These observations agree with clinical reports (33) of a nephrotic syndrome in thyrotoxic patients.

Several authors have reported that long-term oral administration of AG attenuates renal injury and reduces proteinuria in

### Table 2. Plasma variables in the experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Na, meq/l</th>
<th>K, meq/l</th>
<th>Creatinine, mg/dl</th>
<th>Total Protein, g/dl</th>
<th>NOx, μmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>142.4±0.55</td>
<td>4.34±0.13</td>
<td>0.60±0.02</td>
<td>6.06±0.06</td>
<td>6.10±0.10</td>
</tr>
<tr>
<td>AG</td>
<td>144.1±0.35</td>
<td>4.03±0.16</td>
<td>0.65±0.01</td>
<td>6.00±0.11</td>
<td>5.78±0.20</td>
</tr>
<tr>
<td>T₄</td>
<td>145.1±0.85</td>
<td>4.47±0.07</td>
<td>0.62±0.02</td>
<td>5.56±0.09*</td>
<td>10.02±0.15*</td>
</tr>
<tr>
<td>T₄ + AG</td>
<td>144.9±0.70</td>
<td>4.51±0.09</td>
<td>0.64±0.02</td>
<td>5.53±0.08*</td>
<td>6.81±0.14</td>
</tr>
</tbody>
</table>

Data expressed as means ± SE. *P < 0.05 vs. control group.

### Table 3. Urine variables in the experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urinary Flow Rate, ml/100 g⁻¹·24 h⁻¹</th>
<th>Sodium Excretion Rate, μmol/100 g⁻¹·24 h⁻¹</th>
<th>Potassium Excretion Rate, μmol/100 g⁻¹·24 h⁻¹</th>
<th>Protein Excretion Rate, μmol/100 g⁻¹·24 h⁻¹</th>
<th>Creatinine Cl (100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.70±0.43</td>
<td>310.5±27.1</td>
<td>417.9±51.1</td>
<td>3.65±0.43</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>AG</td>
<td>3.82±0.19</td>
<td>290.1±12.8</td>
<td>377.1±23.0</td>
<td>4.68±0.37</td>
<td>0.31±0.013</td>
</tr>
<tr>
<td>T₄</td>
<td>4.34±1.12*</td>
<td>352.0±45.0</td>
<td>572.0±68.0</td>
<td>8.65±0.93*</td>
<td>0.305±0.021</td>
</tr>
<tr>
<td>T₄ + AG</td>
<td>4.65±0.39*</td>
<td>381.0±31.6</td>
<td>540.1±54.8</td>
<td>7.91±0.71*</td>
<td>0.323±0.017</td>
</tr>
</tbody>
</table>

Data expressed as means ± SE. *P < 0.05 vs. control group.
several experimental chronic renal diseases, including lupus (39), diabetes (31), 5/6 nephrectomy (10), and aging-related nephropathies (20). The mechanism underlying these protective effects is unclear, although some authors reported that the beneficial effects of AG are associated with an inhibition of iNOS (10, 31, 39). However, our results show that AG was unable to reduce proteinuria in hyperthyroid rats. The inability of AG to reduce the proteinuria in hyperthyroid rats may be due to the short duration of our experiment, since the protective effects of AG on proteinuria have been observed after months of treatment (20, 31, 39). Therefore, our data do not rule out that a longer period of AG treatment can reduce proteinuria in hyperthyroid rats.

Creatinine clearance normalized per gram kidney weight was similar in all experimental groups, as observed in a previous study using the same dose and duration of T4 treatment (27). However, this finding contrasts with the reduction in glomerular filtration rate in T4-treated rats previously reported by our group (11, 36), when a larger T4 dose (75 μg·rat⁻¹, day⁻¹) and a longer treatment period (6 wk) were used. In conclusion, the present study shows that iNOS plays a role in long-term control of BP of hyperthyroid rats, indicating that NO generated by iNOS contributes to the homeostatic role of this factor in hyperthyroidism. Moreover, AG treatment did not modify the cardiac hypertrophy or proteinuria of hyperthyroid rats.

iNOS has been implicated in the control of sodium excretion and consequently in BP regulation. The present study analyzed the role of iNOS in the long-term BP control of hyperthyroid rats. The data reported herein provide evidence that iNOS may counterbalance the prohypertensive effects of T4. The present study is, to our knowledge, the first to assess the effects of the blockade of iNOS on hemodynamic and renal abnormalities in the hyperthyroid state and, therefore, opens up new perspectives for the assessment of cardiovascular abnormalities in hyperthyroid disorders.

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