EXPERIMENTAL STUDY

Nitric oxide synthase activity in hyperthyroid and hypothyroid rats

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Abstract

Objective: Thyroid disorders are accompanied by important changes in haemodynamic and cardiac functions and renal sodium handling. Since nitric oxide (NO) plays a crucial role in regulating vascular tone and renal sodium excretion, the present paper was designed to determine whether changes in the activity of NO synthase (NOS) participate in the cardiovascular and renal manifestations of thyroid disorders.

Methods: We measured NOS activity in the heart (left and right ventricles), vessels (aorta and cava) and kidney (cortex and medulla) of euthyroid, hyperthyroid and hypothyroid rats after 6 weeks of treatment. NOS activity was determined by measuring the conversion of L-[3H]-arginine to L-[3H]-citruline.

Results: NOS activity was higher in all tissues from hyperthyroid rats when compared with controls, except in the right ventricle. In the hypothyroid group, NOS activity showed a more heterogeneous pattern, with significant increases in both ventricles but significant reduction in the aorta, while in the vena cava, renal cortex and medulla the enzyme activity also tended to be higher, but significance was not reached.

Conclusions: These data indicated that NOS activity was upregulated in tissues primarily related to blood pressure control in hyperthyroid rats, suggesting that an increased NO production may contribute to the hyperdynamic circulation in hyperthyroidism and may have a protective homeostatic effect in the target organs of the hypertension that accompanies this endocrine disease. The aortic and renal findings in hypothyroid rats suggested a possible role for NOS in the increased peripheral resistance and the normal pressure–diuresis–natriuresis response of these hypotensive animals, although hypothyroidism produced a heterogeneous tissue response in NOS activity.

Introduction

Thyroid disorders are common endocrine disorders in humans and animals (1) and are accompanied by important changes in haemodynamic, cardiac and renal function (2–4). Disturbances in the regulation of systemic arterial blood pressure are seen in both hypo- and hyperthyroid states in man and other animals (3–5). Hyperthyroidism manifests a hyperdynamic circulation with increased cardiac output, increased heart rate and decreased peripheral resistance, whereas the hypothyroid state is associated with low cardiac output, bradycardia and raised peripheral resistance (2–4). Hyperthyroidism increases the responsiveness of resistance vessels to the endothelium-dependent vasodilator, acetylcholine, whereas methimazole-induced hypothyroidism attenuates the endothelium-dependent response (5). Thyroid dysfunctions also affect cardiac and renal weight as well as renal sodium handling (2, 6, 7). Thus, hyperthyroidism occurs with a reduced sodium excretion after a saline load (7) and a blunted pressure–diuresis–natriuresis (PDN) response (6, 7) whereas, in hypothyroidism, a normal sodium excretion (7) or a tendency to sodium loss (2) have been reported.

Nitric oxide (NO) is produced through the transformation of L-arginine to L-citruline by a family of enzymes known as NO synthases (NOS). It is well known that NO is an important factor regulating vascular tone (8), renal sodium excretion and the PDN response (9, 10) and therefore arterial blood pressure (11). Both acute and chronic administration of NOS inhibitors severely attenuate the PDN response and increase systemic arterial blood pressure (12, 13), and transgenic mice that lack the NOS gene are hypertensive when compared with wild-type controls (14).

Given the importance of NOS activity in cardiovascular and renal homeostasis, the aim of the present paper...
was to analyze whether changes in NOS activity participate in the cardiovascular and renal manifestations of thyroid disorders. To this end, we determined NOS activity by measuring the rate of formation of radio-labelled $L^{-}[3H]$-citruline from $L^{-}[3H]$-arginine in tissues primarily involved in blood pressure control from hyper- and hypothyroid rats. Moreover, the tissues selected for study are also the target organs in hypertensive disease.

**Materials and methods**

**Animals**

Male Wistar rats born and raised in the experimental animal service of the University of Granada were used. The experiments were performed according to European Union guidelines for the ethical care of animals. Rats initially weighing 180–200 g were maintained on standard chow and tap water *ad libitum* except where stated. The animals were divided into three groups: control, hyperthyroid and hypothyroid rats (*n* = 16 in each group). Hyperthyroidism was induced by injecting s.c. thyroxine (T$_4$; 300 µg/kg per day dissolved (1 µg/µl) in isotonic saline (100 ml) plus 1 ml 0.5 M NaOH). Hypothyroidism was induced by the continuous administration of 0.03% methimazole via the drinking water. Control rats were injected with the same solution as the hyperthyroid rats but without T$_4$. These treatments were administered for 6 weeks.

The effectiveness of these treatments was assessed by comparing serum T$_4$, serum tri-iodothyronine (T$_3$), mean arterial pressure, heart rate, pulse pressure and final thyroid, renal, ventricular and body weights of control and treated rats (*n* = 8 in each group). Blood pressure and heart rate were recorded using an TRA-121 transducer connected to a two-channel Letigraph 2000 recorder (Letica SA, Barcelona, Spain) in conscious rats through a polyethylene catheter inserted into the femoral artery and exteriorized at the dorsum of the neck. Blood pressure was measured 24 h after catheter implantation and blood samples were then taken via the arterial catheter to determine serum T$_3$ and T$_4$ levels, which were measured by ELISA (Immunoassay System; Baxter, Miami, FL, USA).

**Measurement of NOS activity**

The tissues chosen to measure NOS activity were: the heart (left and right ventricles), the vessels (aorta and cava) and the kidney (cortex and medulla). The heart was studied because of the important cardiac manifestations of both thyroid disorders (1–4), and left and right ventricles were analyzed to determine if the possible changes in NOS activity affect high and low pressure circulations. The vessels were studied because of the important abnormalities in vascular function in both thyroid diseases (5) and the analysis of arteries and veins was for the reason given above with respect to heart ventricles. NOS activity was measured in the renal cortex and medulla of hyper- and hypothyroid animals to assess the possible role of NO in the abnormalities in renal sodium handling of these animals (6, 7), since NO is essential in renal haemodynamics and renal sodium excretion (9), especially in the renal medulla, a structure that plays an important role in volume and blood pressure control (10).

The tissues were removed and homogenized with the aid of a tissue grinder (Omni International, Warrenton, VA, USA) at 3000 r.p.m. in ice-cold homogenization buffer (25 mM Tris, 0.5 mM D,L-dithiothreitol (DTT), 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.6). The homogenized tissues were placed in 1 ml of the same buffer and sonicated (six times for 10 s). The crude homogenate was centrifuged 5 min at 3000 g and aliquots of the supernatant were either stored at −20°C for total protein determination (15) or used immediately for NOS activity measurement. NOS activity was determined following the method of Bredt & Snyder (16), monitoring the conversion of $L^{-}[3H]$-arginine to $L^{-}[3H]$-citruline. The final incubation volume was 100 µl and consisted of 10 µl crude homogenate added to buffer to give a final concentration of 25 mM Tris, 1 mM DTT, 30 µM 5,6,7,8-tetrahydro-L-biopterin dihydrochloride (H$_4$-biopterin), 10 µM flavin adenine dinucleotide (FAD), 0.5 mM inosine, 0.5 mg/ml BSA, 0.1 mM CaCl$_2$, 10 µM $L^{-}$-arginine and 50 mM $L^{-}[3H]$-arginine, at pH 7.6. The reaction was started by the addition of 10 µl NADPH (0.75 mM final) and maintained for 30 min at 37°C. Control incubations were performed by omitting NADPH. The reaction was stopped by the addition of 400 µl cold 0.1 mM Hepes, 10 mM EGTA and 0.175 mg/ml $l^{-}$-citruline, pH 5.5. The reaction mixture was decanted into a 2 ml column packed with Dowex-50 W (50 × 8 – 200) ion exchange resin (Na$^+$ form) eluted with 1.2 ml water. $L^{-}[3H]$-citruline was quantified by liquid scintillation spectroscopy. The retention of $L^{-}[3H]$-arginine in this process was greater than 98%. Specific enzymatic activity was determined by subtracting the control value, which was usually less than 1% of the radioactivity added. NOS activity is expressed as pM $L^{-}[3H]$-citruline produced/mg protein per min.

**Drugs**

The following drugs were used: heparin from Leo, Madrid, Spain, pentobarbital sodium (Nembutal) from Serva, Heidelberg, Germany, $l^{-}$-thyroxine from Merck, Darmstadt, Germany and methimazole, $l^{-}$-arginine, $l^{-}$-citruline, HEPES, DTT, leupeptin, aprotinin, pepstatin, PMSF, BSA, Dowex-50 W, FAD, NADPH, H$_4$-biopterin from Sigma Quı ´mica, Madrid, Spain. $L^{-}[3H]$-arginine
(58 Ci/ mmol) was obtained from Amersham International plc, Amersham, Bucks, UK. Tris–HCl and calcium chloride were obtained from Merck, Darmstadt, Germany.

**Statistical analysis**

The results for each variable were compared with one-way ANOVA. When the overall ANOVA was significant, pairwise comparisons were performed with Bonferroni’s method.

**Results**

**Biological variables**

The effects of T<sub>4</sub> or methimazole administration on biological variables are shown in Table 1. Animals given T<sub>4</sub> or methimazole for 6 weeks gained significantly less weight than their age-matched controls during this period. Mean arterial pressure, pulse pressure, heart rate, renal weight, ventricular weight, and serum T<sub>3</sub> and T<sub>4</sub> levels were increased and decreased in hyper- and hypothyroid rats respectively. Thyroid weight was decreased in hyperthyroid and increased in hypothyroid rats. Therefore, rats given T<sub>4</sub> for six weeks developed characteristic manifestations of hyperthyroidism, whereas those given methimazole for a similar period developed hypothyroidism.

**NOS activity**

Apart from the right ventricle, increased NOS activity was found in those tissues analysed from hyperthyroid rats (Figs 1–3). In the hypothyroid group, NOS activity showed a more heterogeneous pattern. It was significantly increased in both ventricles (Fig. 1) but significantly reduced in the aorta, while in the vena cava (Fig. 2), renal cortex and medulla (Fig. 3) the enzyme activity also tended to be higher, but significance was not reached. NOS activity was greater in the left than in the right ventricle in hyperthyroid rats (P < 0.01) and no difference between the left and right ventricle was observed in control and hypothyroid groups (Fig. 1). The comparison of NOS activity between arteries and veins showed a higher activity in the aorta (P < 0.05) in the control group, similar levels in the aorta and cava in hyperthyroid rats and a higher activity (P < 0.05) in the veins of hypothyroid rats (Fig. 2). NOS activity was greater in the medulla than in the cortex in the control (P < 0.01) and hypothyroid rats (P < 0.05) and similar in both renal tissues from hyperthyroid rats (Fig. 3).

**Discussion**

The present study provides new evidence that NOS activity is higher in most tissues primarily related to blood pressure control in hyperthyroid rats. The mechanism responsible for the enhancement of NOS activity in hyperthyroid rats is not known and various factors may participate alone or in combination. This could be due to the following: a compensatory response to the high arterial pressure of these animals (17); an increased release of vasoactive substances such as angiotensin II (18) or endothelin (19), which increase NO production and are increased in hyperthyroid rats (20, 21); or to the shear stress mechanism induced by the hyperdynamic circulation of these animals. Shear stress regulates the expression of NOS (22) and a putative shear stress response element has been described in the promoter sequence of the NOS gene (23). An upregulation of constitutive NOS has been reported in the aorta of other diseases that occur with hyperdynamic circulation, such as liver cirrhosis (24) and iron-deficiency anaemia (25). Alternatively, the hyperdynamic circulation of hyperthyroidism may be secondary to a direct effect of thyroid hormones on NOS activity. Stimulation of NOS activity via a non-genomic signal generation (10–30 s) has been observed in synaptosomes prepared from adult cerebral cortex after the addition of T<sub>4</sub> (26). However, T<sub>4</sub> administration was unable to stimulate inducible NOS activity in mesangial cells and tubular epithelial cells (27). These results seem to indicate that the direct effects of thyroid hormones vary depending on the NOS isoform and the tissue studied.

**Table 1 Biological variables.** Body weight (BW), ventricular weight (VW), renal weight (RW), thyroid weight (TW), mean arterial pressure (MAP), heart rate (HR), pulse pressure (PP), T<sub>4</sub> and T<sub>3</sub> plasma levels in control, hyperthyroid (treated with 300 μg T<sub>4</sub>/kg per day, s.c.) and hypothyroid (treated with 0.03% methimazole in the drinking water) rats. Values are means ± s.e.m. (n = 8 in all groups).

<table>
<thead>
<tr>
<th>Groups</th>
<th>BW (g)</th>
<th>VW (mg)</th>
<th>RW (mg)</th>
<th>TW (mg)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
<th>PP (mmHg)</th>
<th>T&lt;sub&gt;4&lt;/sub&gt; (μg/dl)</th>
<th>T&lt;sub&gt;3&lt;/sub&gt; (ng/dl)</th>
</tr>
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<tbody>
<tr>
<td>Hyperthyroid</td>
<td>332±32</td>
<td>1145±30</td>
<td>1620±38</td>
<td>22.3±1.0</td>
<td>147±3</td>
<td>407±10</td>
<td>72±4</td>
<td>40.1±1.5</td>
<td>320±7.1</td>
</tr>
<tr>
<td>Control</td>
<td>408±2</td>
<td>960±32</td>
<td>1200±36</td>
<td>34.8±2.5</td>
<td>118±3</td>
<td>370±5</td>
<td>43±4</td>
<td>4.2±0.3</td>
<td>50±7.2</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>251±3</td>
<td>565±22</td>
<td>860±37</td>
<td>136±8.5</td>
<td>103±2</td>
<td>320±10</td>
<td>28±1</td>
<td>0.4±0.1</td>
<td>4.3±2.1</td>
</tr>
</tbody>
</table>

*P < 0.05. **P < 0.01 compared with control group.
The heterogeneous pattern of NOS activity in the tissues from the hypothyroid group is difficult to reconcile with a common explanation or hypothesis, but it may be the result of changes in the expression of the different isoforms of NOS or even related to changes in the NOS activity of subcellular fractions. In fact, it has recently been reported (28) that liver and skeletal muscle mitochondrial NOS is increased in hypothyroidism and inversely correlated with serum T3, whereas in neural tissues hypothyroidism is associated with a reduced NOS activity (29).

Considering the importance of NO in the control of vascular tone (10), the increased and decreased NOS activities in the aorta (in hyper- and hypothyroid rats respectively) suggests that this alteration may play a role in the changes in total peripheral vascular resistance previously reported in these animals (2–4). These data are consistent with the increased and decreased responsiveness to acetylcholine, the endothelium-dependent vasodilator, in perfused kidneys and isolated aortas from hyperthyroid and hypothyroid rats respectively (5). Our data in the hypothyroid group contrast with the increased staining of endothelial NOS using immunohistochemical techniques in the aortic endothelium of hypothyroid rats (30). However, it is well known that immunohistochemistry is a suitable technique to detect the presence of proteins in a given tissue but it is not a very accurate method to detect differences between groups. In fact, these observations were not accompanied by a quantitative analysis (30).

The upregulation of constitutive NOS activity in hypertension may contribute to forestalling left ventricular and aortic hypertrophy (31) and to maintaining an adequate renal function (32). The increased NOS activity in these same organs of our hypertensive hyperthyroid group supports these roles and is consistent with the hypothesis that increased constitutive NOS activity may have protective homeostatic effect in all target organs of hypertensive disease (32).

Renal NOS activity is usually lower in the cortex than in medulla, as observed in the control group. Medullary NO plays a major role in the renal regulation of sodium and water excretion and therefore in the control of arterial blood pressure (8, 9). The normal values in medullary NOS activity in our hypothyroid rats indicate that the medulla has an adequate capacity to synthesize NO, which is consistent with the normal sodium handling of hypothyroid rats under normal conditions and after several challenges (6, 7). The high NOS activity in the renal cortex of hyperthyroid rats may be secondary to the hyperdynamic circulation of these animals, because cortical NOS activity is mainly produced by constitutive endothelial NOS (33). The similar levels of NOS activity in the renal cortex and medulla of our hyperthyroid rats might produce a defective NO.
generation in renal medulla, which could participate in the blunted PDN response of these animals (6).

NOS activity in the left ventricle of hyperthyroid rats was twofold that in controls. This alteration would be secondary to the high blood pressure of these rats rather than caused by the hyperdynamic circulation or increased heart rate, because NOS activity in the right ventricle was similar to controls. These results agree with previous observations of higher NOS activity in the left heart of adult SHR rats and no differences between the left and right side of hearts from WKY rats (34). The increased NOS activity in both ventricles of the hypothyroid group is in consonance with the increased mitochondrial NOS reported in the skeletal muscle of 131I-thyroidectomized rats (28), which has been implicated in thyroid-dependent regulation of O2 uptake.

The increased NOS activity in the selected tissues of hyperthyroid rats agrees with published findings in other tissues. A study using quantitative in situ hybridization histochemistry with a specific oligodeoxynucleotide probe showed that T3-induced hyperthyroidism more than doubled the prevalence of NOS gene transcript in the paraventricular (PVN) and supraoptic (SON) nuclei, whereas hypothyroidism produced a highly significant reduction in NOS gene transcripts in the PVN and SON (29). Fernandez et al. (35) demonstrated that hyperthyroidism leads to a significant and reversible enhancement in rat liver NOS activity.

In summary, our data have demonstrated that NOS activity is upregulated in tissues primarily related to blood pressure control in hyperthyroid rats. Thus, an increased NO production may participate in cardiovascular manifestations of this disease. NOS activity in the aorta and renal medulla of hypothyroid rats might participate in the increased peripheral resistance and the normal sodium handling of these hypertensive animals. However, the tissue response to NOS activity in hypothyroidism was heterogeneous.

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