Thyroid status and nitric oxide in rat arterial vessels

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Abstract

Thyroid disease has profound effects on cardiovascular function. Hypo- and hyperthyroidism, for example, are associated with reduced and increased maximal endothelium-dependent vasodilation respectively. We therefore hypothesized that the capacity for vascular nitric oxide (NO) formation is decreased in hypothyroidism and increased in hyperthyroidism. To test this hypothesis, rats were made hypothyroid (HYPO) with propylthiouracil or hyperthyroid (HYPER) with triiodothyronine over 3–4 months. Compared with euthyroid control rats (EUT), HYPO exhibited blunted growth and lower citrate synthase activity in the soleus muscle; HYPER exhibited left ventricular hypertrophy and higher citrate synthase activity in the soleus muscle (P<0·05 for all effects). The capacity for NO formation was determined in aortic extracts by formation of [³H]L-citrulline from [³H]L-arginine, i.e. NO synthase (NOS) activity. Thyroid status modulated NOS activity (EUT, 36·8 ± 5·5 fmol/h per mg protein; HYPO, 26·0 ± 7·9; HYPER, 64·6 ± 12·7; P<0·05, HYPER vs HYPO). Expression of endothelial and neural isoforms of NOS was modulated by thyroid status in a parallel fashion. Capacity for responding to NO was also determined via measuring cGMP concentration in aortae incubated with sodium nitroprusside. Stimulated cGMP formation was also modulated by thyroid status (EUT, 73·0 ± 20·2 pmol/mg protein; HYPO, 152·4 ± 48·7; HYPER, 10·4 ± 2·6; P<0·05, HYPER vs HYPO). These data indicate that thyroid status alters capacities for both formation of and responding to NO. The former finding may contribute to previous findings concerning vascular function in thyroid disease states.

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Introduction

Thyroid disease is estimated to affect 10% of the population (Tunbridge & Caldwell 1991, Canaris et al. 2000). A hallmark of two common forms of thyroid disease, hypo- and hyperthyroidism, is exercise intolerance. Several studies have examined various mechanisms potentially responsible for exercise intolerance in these disease states. In hypothyroidism, it appears that cardiovascular dysfunction is largely responsible for poor exercise tolerance; in hyperthyroidism, other factors such as hyperthermia and altered patterns of substrate utilization are implicated (cf. McAllister et al. 1995a).

Cardiovascular dysfunction during exercise in hypothyroidism is profound. We have previously reported that skeletal muscle blood flow during treadmill running is reduced ~50% in hypothyroid rats compared with their euthyroid counterparts (McAllister et al. 1995b). Lower blood flow would impair delivery of O₂ and substrates to exercising muscle. When vasomotor responses were determined for euthyroid and hypothyroid rats, endothelium-dependent dilation was also found to be blunted in isolated aortae (Delp et al. 1995, McAllister et al. 2000) and isolated perfused kidneys (Vargas et al. 1995) in the hypothyroid state. Paradoxically, given that exercise tolerance is reduced, hyperthyroidism is associated with enhanced muscle blood flow during exercise (Frey 1967, McAllister et al. 1995c) and augmented endothelium–dependent vasodilation (Scivoletto et al. 1986, Vargas et al. 1995, McAllister et al. 1998, 2000, Honda et al. 2000).

Thus, in both thyroid disease states, alterations in endothelium–dependent vasodilation are consistent with patterns of blood flow to exercising skeletal muscle.

Endothelium–independent vasodilation has generally been reported to be normal in both the hypothyroid (Delp et al. 1995, McAllister et al. 2000) and hyperthyroid states (McAllister et al. 1998, 2000), although exceptions have been reported (Vargas et al. 1995, Honda et al. 2000). Collectively, these findings suggest that thyroid status primarily affects the vascular endothelium. Endothelium–dependent dilation of the rat aorta is primarily mediated by nitric oxide (NO) (Delp et al. 1993). We therefore hypothesized that altered endothelium–dependent vasodilation with changes in thyroid status is due to changes
in the capacity for NO formation, as reflected by NO synthase (NOS) activity. We also hypothesized that the response to NO, as reflected by the capacity of vascular smooth muscle to generate cGMP, is not altered by thyroid status.

Materials and Methods

Animal treatments

Two series of male Sprague–Dawley rats (Charles River, Wilmington, MA, USA), initially weighing 150–175 g, were housed three per cage in a room with controlled temperature (20–21 °C) and light (12 h light:12 h darkness). In each series, rats were randomly assigned to one of three groups: euthyroid control (EUT), hypothyroid (HYPO) and hyperthyroid (HYPER). HYPO rats were made hypothyroid over 3–4 months by the Institutional Animal Care and Use Committee of Kansas State University, and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Treatment efficacy

Efficacy of propylthiouracil and triiodothyronine treatments was assessed by determination of citrate synthase activity in the soleus muscle, gain in body weight during the treatment period and left ventricular weight/body weight ratio. Activity of citrate synthase, a marker enzyme for oxidative capacity, was determined spectrophotometrically (Shimadzu UV-1201, Shimadzu, Kyota, Japan) according to the method of Srere (1969), as done previously (McAllister et al. 1995c, 1998, 2000). All rats were allowed free access to food and water except for EUT, which were slightly food-restricted (~90% of normal food intake) over the final month of the treatment period to match body weights with those of HYPO and HYPER. This mild degree of food restriction would not affect thyroid status in EUT, since only either starvation or severe food restriction has been reported to reduce thyroid hormone levels (cf. Danforth & Burger 1989). All treatments were approved by the Institutional Animal Care and Use Committee of Kansas State University, and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

NOS activity

With the first series of rats (NOS Series), capacity for vascular NO formation was assessed by determining NOS activity in aortic extracts of EUT, HYPO and HYPER. Extracts of aortae were prepared in the following manner:

the entire aorta was excised, quickly cleaned of fat and connective tissue under a dissecting microscope (Nikon, Melville, NY, USA), and cut into rings ~1 mm in axial length. Vascular rings were transferred to microcentrifuge tubes containing 250 µl of a buffer solution containing (in mM unless otherwise indicated): 50 Tris–HCl, 0·10 EDTA, 0·10 EGTA, 0·10 ml/100 ml β-mercaptoethanol, 20 CHAPS, 1·0 phenylmethylsulfonyl fluoride, 0·002 leupeptin and 0·001 pepstatin A, pH 7·8. Vascular rings were sonicated (Fisher Sonic Dismembrator 60, Fisher, Pittsburgh, PA, USA) in this buffer solution (5 s, three times), and then centrifuged at 250 000 g (Beckman TL-100 ultracentrifuge, Beckman, Palo Alto, CA, USA) for 30 min at 4 °C. Aliquots of the resultant supernatant were assayed for NOS activity.

NOS activity was determined according to the method of Pollock et al. (1991), in which the conversion of [3H]-arginine to [3H]-citrulline is measured. Aortic extracts were incubated for 1 h at 27 °C in a buffer solution with (final concentration, in mM unless otherwise indicated): 50 Tris–HCl, 54 l-valine, 1·2 MgCl2, 1·0 NADPH, 0·01 tetrahydrobiopterin, 0·005 l-arginine, 50 U/ml calmodulin and 0·002 FAD, pH 7·8. [3H]-arginine (0·010 mCi; New England Nuclear, Boston, MA, USA) was also included. Aliquots of extracts were incubated in this buffer solution that contained CaCl2 (1·78 mM) and in buffer solution that did not contain CaCl2 to determine Ca21–dependent NOS activity. Appropriate backgrounds (i.e. aliquots of buffer solution rather than aortic extract) were incubated in parallel. At the end of the incubation period, the NOS reaction was arrested with addition of a buffer solution containing 20 mM HEPES and 8·0 mM EDTA, pH 5·5. Reaction mixtures were loaded onto cation exchange columns (Dowex AG 50WX-8, Na+ form; Bio-Rad), and [3H]-citrulline was eluted from columns with 0·50 ml (× 2) ddH2O. Scintillation fluid was added to eluent, and samples were counted in a liquid scintillation counter (Packard, Meriden, CT, USA).

NOS expression

Western blotting was used to determine expression of the endothelial and neural isoforms of NOS (eNOS and nNOS respectively), as well as that of guanylate cyclase (GC), in aortae from EUT, HYPO and HYPER. Aortic extracts prepared for determination of NOS activity (see above) were subsequently used in Western blotting experiments. For each sample (eNOS, nNOS), a volume of extract containing 5·0 µg total protein was used. For GC, 2·5 µg total protein were used. Samples were loaded onto a 4–12% Bis–Tris gel, and electrophoresed at 200 V for 50 min. Transfer to polyvinylidene difluoride membrane was done at 200 mA for 2 h. Blocking was done for 60 min in Tris-buffered saline with 0·10 ml/100 ml Tween 20 and 5·0 g/100 ml non-fat milk (TBST-milk). Following blocking, overnight incubation with a primary antibody
positively-charged slides, and de-parafinized (Microm, Walldorf, Germany), floated onto a cold DMEM–HEPES modification buffer solution with 1·0 mM isobutylmethylxanthine, pH 7·4 (basal) or the same buffer solution with 10−4 M sodium nitroprusside, an NO donor, also included (stimulated). After a 30 min incubation period, the GC reaction was arrested with 70% trichloroacetic acid and then neutralized with 10·0 M KOH. Vessels were homogenized (IKA Labortechnik T25 homogenizer, Labortechnik, Staufen, Germany) using 10 s bursts until complete homogenization was achieved. Homogenates were centrifuged (Damon PR-J centrifuge) at 1500 g for 10 min. The resultant supernatant was assayed for cGMP concentration; the pellet was used for determination of protein concentration (see below). Concentrations of cGMP in supernatants was determined using an RIA kit (Amersham Pharmacia Biotech).

In addition to determining aortic cGMP concentration, experiments were conducted to determine release of cGMP from aortae. Stimulation of cGMP formation in whole aortae (sectioned into 1 mm axial length rings) using an NO donor was done as described above. After centrifugation (1500 g for 10 min) to pellet aortic rings, cGMP concentration in supernatants was determined with an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA).

Protein concentration

Protein concentration was determined on aortic extracts to normalize NOS activities, and on pellets of homogenized vessels to normalize cGMP concentrations. Protein concentration was determined with a kit utilizing the Bradford procedure (Bio-Rad).

Statistical analysis

All data are presented as means ± s.e. Data for EUT, HYPO and HYPER were analyzed using one-way ANOVA (Steel & Torrie 1980). When ANOVA indicated a difference among groups, the Tukey test was used as a post-hoc test to discern differences between pairs of groups (Steel & Torrie 1980). P<0·05 was considered significant for all analyses.

Results

Treatment efficacy

Treatment of rats with propylthiouracil successfully induced the hypothyroid state. Citrate synthase activity was lower in HYPO than EUT in the soleus muscle for both series of rats (Fig. 1). In addition, HYPO failed to increase body weight at a normal rate, reflected by lower final body weights than those of EUT and HYPER, in both series (Table 1). Similarly, two lines of evidence indicated that treatment of rats with triiodothyronine induced the
hyperthyroid state. First, citrate synthase activity was increased in HYPER (vs EUT) soleus muscles in both series of rats (Fig. 1).Secondly, left ventricular weight/body weight ratio was increased in HYPER compared with EUT (Table 1), reflecting left ventricular hypertrophy.

NOS activity and expression

Figure 2 illustrates Ca$^{2+}$-dependent NOS activity for EUT, HYPO and HYPER aortic extracts. Thyroid status significantly modulated NOS activity, with values for HYPER being more than twice those for HYPO ($P<0.05$). Neither HYPO nor HYPER, however, differed significantly from EUT in NOS activity. In two control experiments, aortae from normal rats demonstrated a mean reduction of 81% in Ca$^{2+}$-dependent NOS activity when treated with the competitive NOS inhibitor N$^\omega$-monomethyl-l-arginine (4·29 mM) (Pollock et al. 1991). eNOS and nNOS protein expression differed significantly between HYPER and HYPO in a parallel fashion, as illustrated in Fig. 3. Immunohistochemistry for eNOS (Fig. 4) and nNOS (Fig. 5) demonstrated markedly greater nNOS expression in vascular smooth muscle of HYPER (Fig. 5). Expression of eNOS was confined to endothelium in all groups and did not differ visibly among groups (Fig. 4).

cGMP concentration

Basal levels of cGMP were not different among groups, being $0·214\pm 0·055$, $0·181\pm 0·051$ and $0·101\pm 0·027$ pmol/mg protein for EUT ($n=4$), HYPO ($n=3$) and HYPER ($n=4$) respectively. Figure 6A illustrates data for stimulated levels of cGMP. Stimulation of GC with sodium nitroprusside increased cGMP concentration substantially over basal levels for EUT, HYPO and HYPER. Stimulated cGMP concentration attained was, similar to NOS activity, significantly modulated by thyroid status. Values for HYPO were significantly greater than those for HYPER ($P<0.05$); however, neither HYPO nor HYPER were significantly different from EUT. GC protein expression did not differ significantly among groups (Fig. 6B).

Thyroid status can affect tissue protein concentration; thus, data for cGMP levels were also expressed and analyzed on a per tissue weight basis. Thyroid status did not affect basal cGMP concentration (EUT, $463\pm 81\pm 10$ pmol/g; HYPO, $12·7\pm 3·2$; HYPER, $6·2\pm 1·5$; ns). Stimulated cGMP concentration was, however, modulated by thyroid state (EUT, $463\pm 81\pm 10$ pmol/g; HYPO, $334\pm 56$; HYPER, $82·2\pm 8·8$; $P<0·05$, HYPER vs EUT, HYPO).

**Table 1** Indicators of treatment efficacy. Values are means ± s.e. For NOS Series $n=7, 8$ and $n=7$ for Euthyroid, Hypothyroid and Hyperthyroid respectively; for cGMP Series $n=12, 10$ and $n=12$ for Euthyroid, Hypothyroid and Hyperthyroid respectively.

<table>
<thead>
<tr>
<th></th>
<th>NOS Series</th>
<th>cGMP Series</th>
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<tr>
<td>LV weight/body weight ratio (mg/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthyroid</td>
<td>2·03 ± 0·08</td>
<td>1·93 ± 0·03</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>1·86 ± 0·05</td>
<td>2·10 ± 0·06</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>2·50 ± 0·10†</td>
<td>2·45 ± 0·07†</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthyroid</td>
<td>497 ± 15</td>
<td>516 ± 15</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>284 ± 6*</td>
<td>253 ± 6*</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>478 ± 15†</td>
<td>493 ± 15†</td>
</tr>
</tbody>
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LV, left ventricle. *Different from Euthyroid in same series, $P<0·05$; †different from Hypothyroid in same series, $P<0·05$.
When release of cGMP from vascular smooth muscle was determined, cGMP concentration in the incubation medium was also elevated in HYPO (EUT, 0.057 ± 0.11 pmol/ml per mg wet weight, n=7; HYPO, 0.110 ± 0.017, n=9; HYPER, 0.072 ± 0.010, n=8; P<0.05, HYPO vs EUT).

Discussion

The key findings of this study are that capacities for both formation of and responding to NO in a rat arterial vessel are modulated by thyroid status. The hyperthyroid state was associated with increased capacity for NO formation, but reduced capacity for responding to NO, compared with the hypothyroid state. Capacity for NO formation, assessed by NOS activity, is primarily a property of the vascular endothelium. Capacity for responding to NO, estimated by NO donor-stimulated concentration of cGMP, resides in vascular smooth muscle. These data indicate that thyroid status affects more than the endothelial layer of the vascular wall, contrary to what a majority of previous studies had suggested.

Rat models of hypo- and hyperthyroidism

Treatment of rats with propylthiouracil was successful in inducing the hypothyroid state. Citrate synthase activity,
an index of oxidative capacity, was reduced in the soleus muscle of HYPO. This effect of hypothyroidism on mitochondrial protein content of skeletal muscle has been repeatedly reported for rodent muscle by us (Delp et al. 1995, McAllister et al. 1995b, 2000) and others (e.g. Ianuzzo et al. 1977). Conversely, treatment with triiodothyronine was associated with increased citrate synthase activity, as reported previously by us (McAllister et al. 1995c, 1998, 2000) and others (e.g. Ianuzzo et al. 1977). HYPO also exhibited blunted growth; in addition, HYPER demonstrated left ventricular hypertrophy. These findings, all hallmarks of hypo- and hyperthyroidism, indicate that our rat models of thyroid disease are valid.

Capacity for NO formation

Although neither hypo- nor hyperthyroid status significantly altered NOS activity relative to the euthyroid state, the capacity for NO formation was clearly greater in aortae from HYPER than in those from HYPO. Our findings for aortic NOS activity in different thyroid states resemble those reported by Quesada et al. (2002). Further, these findings are consistent with earlier findings concerning vascular function. We (Delp et al. 1995, McAllister et al. 2000) and others (Vargas et al. 1995) have found endothelium-dependent dilation of rat vasculature to be blunted in hypothyroidism; conversely, we (McAllister et al. 1998, 2000) and others (Scivoletto et al. 1986, Vargas et al. 1995, Honda et al. 2000) have observed augmented endothelium-dependent vasodilation in the hyperthyroid state. Since a primary mediator of endothelium-dependent dilation in vasculature of the rat is NO (Delp et al. 1993), our findings collectively suggest that alterations in endothelium-dependent vasodilation with changes in thyroid status are, in part, accounted for by an altered capacity for NO formation. Vascular function
studies have demonstrated differences in endothelium-dependent dilation among EUT, HYPO and HYPER, whereas NOS activity in the present study only differed significantly between HYPO and HYPER. Changes in formation of vasodilatory prostaglandins and/or endothelium-derived hyperpolarizing factors may also contribute to alterations in endothelium-dependent vasodilation with altered thyroid status.

Interestingly, both eNOS and nNOS protein expression were modulated in HYPO and HYPER in a manner consistent with NOS activity. Our data for NOS activity represent capacity for Ca\(^{2+}\)-dependent NO formation; eNOS and nNOS are Ca\(^{2+}\)-dependent isoforms of this enzyme (cf. Pollock et al. 1991). While the significance of modulation of eNOS expression for endothelium-dependent vasodilation is clear, that of alterations in nNOS expression with changes in thyroid status is less clear. It has been postulated that nNOS, which appears to be expressed only in vascular smooth muscle, may modulate contractile mechanisms (Brophy et al. 2000). Our immunohistochemical findings are in agreement with this earlier study; indeed, there was a marked increase in nNOS expression in vascular smooth muscle of HYPER aorta (Fig. 5). While the functional significance of this finding is uncertain, increased nNOS expression likely contributed to greater Ca\(^{2+}\)-dependent NOS activity in HYPER.

Modulation of eNOS and nNOS expression by thyroid status may involve several stimuli, two of which are thyroid hormone concentration and chronic level of blood flow/shear stress. Hypo- and hyperthyroidism have effects on protein expression in most tissues (Yen 2001); their effects on skeletal muscle metabolic and contractile protein expression are particularly well established (Ianuzzo et al. 1977, Caiozzo & Haddad 1996). It is unknown whether thyroid hormone affects protein expression in the vascular wall. Effects of chronically increased blood flow/shear stress on the vascular endothelium are better understood.

Figure 5 Immunohistochemical analysis of Euthyroid, Hypothyroid and Hyperthyroid aortae for nNOS. Vascular lumen located at top of each panel; non-immune panel represents a section not incubated with antibody to nNOS. Note positive staining for nNOS in smooth muscle of vascular wall, and greater staining for nNOS in the Hyperthyroid panel.
Studies using cultured endothelial cells exposed to increased shear stress (Nishida et al. 1992, Noris et al. 1995, Ranjan et al. 1995, Uematsu et al. 1995), and whole blood vessels studied in vitro after a period of chronically increased blood flow (Miller et al. 1986, Miller & Vanhoutte 1988, Miller & Burnett 1992, Nadaud et al. 1996), have reported increased endothelium-dependent vasodilation, increased NOS activity, and/or increased eNOS expression. It is therefore possible that increased cardiac output, a feature of human hyperthyroidism (Martin et al. 1991) and animal models of the disease (cf. Morkin et al. 1983), led to increased eNOS expression in HYPER via a chronic increase in shear stress on the aortic endothelium.

Response to NO

An unexpected finding in this study was that thyroid status also modulated the response of vascular smooth muscle to NO. While aortae from EUT, HYPO and HYPER all demonstrated large increases in cGMP concentration upon stimulation with an NO donor (i.e. stimulated vs basal cGMP concentrations), the increase in HYPO was markedly greater than that in HYPER (Fig. 6A). Normalizing cGMP concentration to tissue weight, rather than tissue protein concentration, did not change this finding. We were, however, unable to confirm depressed capacity for cGMP formation in HYPER when we determined cGMP release from vascular smooth muscle. Nonetheless, our data uniformly indicate that capacity for cGMP formation is increased in HYPO. This finding was unexpected, since we have previously found that aortae from hypothyroid rats (Delp et al. 1995, McAllister et al. 2000) exhibit normal relaxation responses to sodium nitroprusside at the same concentration used in the present study to stimulate GC. Further, we found that GC protein expression was similar among EUT, HYPO and HYPER, a finding inconsistent with alterations in the capacity for stimulated cGMP formation. Differences in breakdown of cGMP do not underlie our findings, since we included isobutyl-methylxanthine, a phosphodiesterase inhibitor, in our assay system to eliminate cGMP breakdown as a confounding variable. These findings emphasize the complexity of vascular cGMP formation and may indicate that acute regulation of GC (e.g. phosphorylation of GC, cytosolic Ca\(^{2+}\) concentration; cf. Friebe & Koessler 2003), rather than simple modulation of GC protein amount, is most important in determining cGMP formation in different thyroid states.

Perspectives

Exercise intolerance in hypothyroidism appears to be largely accounted for by cardiovascular dysfunction. Skeletal muscle blood flow during exercise is blunted (McAllister et al. 1995b), as is endothelium-dependent vasodilation (Delp et al. 1995, Vargas et al. 1995, McAllister et al. 2000). Conversely, hyperthyroidism is associated with augmented muscle blood flow during exercise (Frey 1967, McAllister et al. 1995c) and with enhanced endothelium-dependent vasodilation (Scivoletto et al. 1986, Vargas et al. 1995, McAllister et al. 1998, 2000, Honda et al. 2000). Our findings for NOS activity, as well as for eNOS and nNOS protein expression, suggest that changes in capacity for NO formation contribute to alterations in endothelium-dependent vasodilation in hypo- and hyperthyroidism. They may, therefore, contribute to the mechanistic basis for altered skeletal muscle blood flow during exercise in thyroid disease states.

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Figure 6 (A) Concentration of cGMP in aortae stimulated with sodium nitroprusside. Values are means ± S.E.; n=8, 8 and 8 for Euthyroid (open bar), Hypothyroid (hatched bar) and Hyperthyroid (stippled bar) respectively. †Different from Hypothyroid, P<0.05. (B) Expression of GC protein. Values are mean ± S.E.; n=6, 5 and 8 for Euthyroid (open bar), Hypothyroid (hatched bar) and Hyperthyroid (stippled bar) respectively. DU, densitometry units, normalized to GAPDH. There are no differences in GC expression among groups. Inset is a representative Western blot for GC. EU, Euthyroid; HO, Hypothyroid; HY, Hyperthyroid.
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