Downregulation of Vascular Angiotensin II Type 1 Receptor by Thyroid Hormone

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Abstract—Thyroid hormone has a broad effect on cardiovascular system. 3,3',5-triiodo-L-thyronine (T3), a biologically active form of thyroid hormone, increases cardiac contractility. T3 causes arterial relaxation and reduction of systemic vascular resistance, resulting in an increase in cardiac output. However, the molecular mechanisms of vascular relaxation by T3 are incompletely characterized. We studied the effect of T3 on the angiotensin (Ang) II type 1 receptor (AT,R) expression in vascular smooth muscle cells. T3 dose-dependently decreased expression levels of AT,R mRNA, with a peak at 6 hours of stimulation. Binding assay using [125I]Sar1-Ile8-Ang II revealed that AT,R number was decreased by stimulation with T3 without changing the affinity to Ang II. T3 reduced calcium response of vascular smooth muscle cells to Ang II by 26%. AT,R promoter activity measured by luciferase assay was reduced by 50% after 9 hours of T3 administration. mRNA stability was also decreased by T3. Real-time quantitative reverse transcription–polymerase chain reaction and Western blot analysis revealed that AT,R mRNA and protein were downregulated in the aorta of T3-treated rats. These results suggest that T3 downregulates AT,R expression both at transcriptional and posttranscriptional levels, and attenuates biological function of Ang II. Our results suggest that downregulation of AT,R gene expression may play an important role for T3-induced vascular relaxation.

(Hypertension. 2003;41:598-603.)

Key Words: hormones □ receptors, angiotensin II □ gene expression □ mRNA stability □ vascular relaxation

Thyroid hormone has various effects on the cardiovascular system. L-Thyroxine (T4), the major secretory product of the thyroid gland is inactive. Thyroxine 5’-deiodinase converts T4 to an active hormone, 3,3',5-triiodo-L-thyronine (T3). T3 binds to thyroid hormone receptor that belongs to the nuclear receptor family. The activated thyroid hormone receptor induces gene expression through binding to thyroid hormone response element (TRE) in the promoter region of target genes.

An increase in left ventricular contractility, tachycardia, and reduction of systemic vascular resistance induce high cardiac output state in hyperthyroidism. The positive inotropic and chronotropic action of T3 are direct effects of T3 on cardiac myocytes, and T3 activates many gene expressions such as myosin heavy chain and calcium transport/regulatory proteins in myocytes. Reduction of systemic vascular resistance is also a direct effect of T3 on vascular smooth muscle cells (VSMCs). A recent report showed that T3 caused rapid relaxation of VSMCs, suggesting the presence of rapid nongenomic effect of T3. It was also shown that T3-induced relaxation of VSMCs was not mediated by cAMP or NO. The target genes for T3 action in VSMCs, which are involved in vascular relaxation, have not been determined.

Two isoforms for angiotensin (Ang) II receptor designated type 1 receptor (AT,R)10 and type 2 receptor (AT,R)11 are present. AT,R is a G protein–coupled receptor expressed in various tissues, including blood vessel, kidney, adrenal gland, liver, and reproductive organs. AT,R mediates most of the biological effects of Ang II such as vasoconstriction, water and sodium retention, and cell growth and proliferation, which are traditionally ascribed to the action of Ang II. Activation of AT,R has been also implicated in the pathogenesis of cardiovascular diseases. To date, the role of vascular AT,R in hyperthyroid state has not been reported. We examined whether T3 modulates expression level of AT,R.

Methods

Materials

Dulbecco’s modified Eagle’s medium was purchased from GIBCO BRL. Fetal bovine serum was purchased from BioWittaker. T3, T4, BSA, ionomycin, and actinomycin D were purchased from Sigma Chemical Co. Rabbit polyclonal antibodies against AT,R and actin were purchased from Santa Cruz Biotechnology. Horseradish peroxidase–conjugated second antibodies (antirabbit or antimouse IgG) were purchased from Vector Laboratories Inc. [α-32P]dCTP and [125I]Sar1-Ile8-Ang II were purchased from PerkinElmer Life Sci-
ences. FURA-2/AM (an acetoxyethyl ester form of FURA-2) was purchased from Dojindo. Other chemical reagents were purchased from Wako Pure chemicals unless mentioned specifically.

Cell Culture
VSMCs were isolated, maintained, and used for the experiments as described previously.  

Animals
All procedures and care were approved by the Committee on Ethics of Animal Experiments, Kyushu University, and were conducted according to animal care guidelines of the American Physiological Society. Adult male, 9- to 10-week-old Sprague-Dawley rats (300 to 400 g) were acclimated to a cycle of 12-hour light and 12-hour dark, and temperature was controlled. Rats received intraperitoneal injection of T3 (200 μg/100 g body weight suspended in 0.02 N NaOH) every other day for 10 days (hyperthyroid group). The control group received an injection of 0.02 N NaOH. Systolic blood pressure and heart rate were measured using a tail-cuff method (UR-5000, UEDA). Plasma concentrations of T3, T4, and thyroid stimulating hormone (TSH) were measured by radioimmunoassay. Rats were killed under pentobarbital anesthesia. Aortas were quickly removed and stored at −80°C.

Northern Blot Analysis
Preparation of total RNA and Northern blot analysis for AT1R and 18S rRNA were performed as described previously.  

Estimation of AT1R Number
The number of AT1R binding sites was estimated by binding of [125I]-Sar1-Ile8-Ang II as described previously.  

Measurement of AT1R Gene Promoter Activity
Promoter activity of AT1R gene was measured by luciferase assay and normalized by β-galactosidase activity as described previously.  

Measurement of Intracellular Calcium Response
To examine the fast effect, VSMCs were incubated in Dulbecco’s modified Eagle’s medium containing Fura-2/AM (5 μmol/L) at 37°C for 1 hour and then treated with vehicle or T3 for 10 minutes. Then VSMCs were stimulated with Ang II and [Ca2+]i, was measured with a fluorescence spectrophotometer (CAM-230, Japan Spectroscopy) as previously described.  

Quantitative Reverse Transcription–Polymerase Chain Reaction
The isolated aortas were homogenized, and total RNA was prepared according to an acid guanidinium thiocyanate–phenol-chloroform extraction method. Total RNA was treated with DNase I and subjected to phenol-chloroform extraction and ethanol precipitation. Then, the total RNA (1 μg) was reverse-transcribed using ReverTra Ace-α kit (TOYOBO). Real-time quantitative polymerase chain reaction (PCR) was performed with an ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer’s instruction. The sequences for sense and antisense of AT1R primers were 5′-CACAGTTGCGCGTTCATT-3′ and 5′-TTGGTAAGGCCCAGCCCTAT-3′, respectively. Specific fluorescent probe 5′-TGAGTCTCGGAATTCGACGCTCCC-3′ was labeled at the 5′ end with the reporter VIC and at the 3′ end with the quencher TAMRA. We used GAPDH primers and TaqMan Probe kit (Applied Biosystems). GAPDH probe was labeled at the 5′ end with the reporter VIC and at the 3′ end with the quencher TAMRA.

Statistical Analysis
Statistical analysis was performed with 1-way ANOVA and Fisher test, if appropriate. Mann-Whitney U test compared the difference of Kd and AT1R site (Bmax). Degradation of AT1R mRNA was analyzed by 2-way ANOVA. Data are shown as mean±SEM. *P<0.05 was considered to be statistically significant.

Results
Downregulation of AT1R mRNA Expression by T3
VSMCs were incubated with T3 (1 μmol/L) for varying time periods, and expression level of AT1R mRNA was determined by Northern blot analysis. The expression level of AT1R mRNA was significantly reduced by T3 after 6 hours of stimulation (Figure 1A). Incubation with varying concentra-
tions of T3 for 6 hours resulted in a dose-dependent decrease in AT1 R mRNA expression level (Figure 1B).

To examine whether T3-induced downregulation of AT1 R mRNA requires de novo protein synthesis, we next examined the effect of cycloheximide (10 μg/mL). Incubation with cycloheximide alone for 6 hours slightly upregulated AT1 R mRNA expression. T3 reduced AT1 R mRNA level in the presence of cycloheximide (Figure 2A), suggesting that de novo protein synthesis is not required for T3-induced AT1 R downregulation. T4 also downregulated AT1 R mRNA expression (Figure 2B), suggesting that T4 is locally converted to T3 by iodothyronine deiodinase in VSMCs.

T3-Induced AT1 R Downregulation Is Not Mediated by NO

We previously reported that NO donor inhibited AT1 R expression in VSMCs.15 To examine whether NO mediates downregulation of AT1 R by T3, we examined the effect of cycloheximide (10 μg/mL). Incubation with cycloheximide alone for 6 hours slightly upregulated AT1 R mRNA expression. T3 reduced AT1 R mRNA level in the presence of cycloheximide (Figure 2A), suggesting that de novo protein synthesis is not required for T3-induced AT1 R downregulation. T4 also downregulated AT1 R mRNA expression (Figure 2B), suggesting that T4 is locally converted to T3 by iodothyronine deiodinase in VSMCs.

Corepressor Complex Is Not Involved in T3-Induced AT1 R Downregulation

Thyroid hormone receptor forms a corepressor complex, which includes nuclear corepressor (N-CoR) or its functional homologue, silencing mediator for retinoid and thyroid receptors (SMRT), Sin3, and histone deacetylase (HDAC). Recently, the N-CoR/SMRT corepressor complexes were shown to interact with various transcription factors and suppress target gene expression.19,20 We examined the effect of trichostatin A, an inhibitor for HDAC. T3 downregulated AT1 R mRNA in the presence of trichostatin A, suggesting that the corepressor complex including HDAC is not involved in T3-induced AT1 R downregulation in VSMCs (data not shown).

T3 Decreases the AT1 R Density

To examine whether T3 downregulates AT1 R number, radioligand-binding assays were conducted (Figure 3). We examined binding activity, AT1 R gene promoter activity, and calcium response to Ang II after 6, 9, 12, and 24 hours of T3 stimulation. We found that peak effect of T3 was at 9 hours of stimulation (data not shown). Binding to the vehicle treated VSMCs revealed a $K_d$ value of 5.68 nmol/L and a $B_{max}$ value of 0.36 pmol/mg protein. Binding to VSMCs stimulated with T3 for 9 hours showed a decrease in the $B_{max}$ value to 0.20 pmol/mg protein without a change in the $K_d$ value (5.85 nmol/L). These data suggest that T3 decreased AT1 R protein expression without changing the affinity of the receptor to Ang II.

Suppression of AT1 R Expression by T3 Through Transcriptional and Posttranscriptional Mechanisms

To examine whether T3 suppresses AT1 R promoter activity, AT1 R promoter-luciferase fusion DNA construct was introduced into VSMCs. Then the VSMCs were stimulated with T3 (1 μmol/L) for 9 hours. AT1 R promoter activity was measured by luciferase assay after 6, 9, 12, and 24 hours of T3 stimulation. We found that peak effect of T3 was at 9 hours of stimulation (data not shown). Binding to the vehicle treated VSMCs revealed a $K_d$ value of 5.68 nmol/L and a $B_{max}$ value of 0.36 pmol/mg protein. Binding to VSMCs stimulated with T3 for 9 hours showed a decrease in the $B_{max}$ value to 0.20 pmol/mg protein without a change in the $K_d$ value (5.85 nmol/L). These data suggest that T3 decreased AT1 R protein expression without changing the affinity of the receptor to Ang II.

Figure 2. Effects of cycloheximide and T4 on AT1-R mRNA expression. A, VSMCs were pretreated with or without cycloheximide (10 μg/mL) for 30 minutes and stimulated with T3 (1 μmol/L) for 6 hours (n=6). B, VSMCs were incubated with T4 (1 mmol/L) for varying periods (n=4). The results were analyzed as described in Figure 1. The values (mean±SEM) are expressed as a percent of control culture (100%). **P<0.01 vs control; #P<0.01 vs CHX.

Figure 3. Effect of T3 on AT1-R number in VSMCs. VSMCs were incubated with vehicle or T3 (1 μmol/L, #) for 9 hours. Binding assay using $[^{125}]$-Sar1-Ile8-AngII was performed. Unlabeled Sar1-Ile8-AngII (10 μmol/L) was used to determine nonspecific binding. Specific binding was calculated by subtracting nonspecific binding from total binding. Saturation curve (A) and Scatchard plot analysis (B) are shown. $B_{max}$ and $K_d$ values are described in the text. T3 significantly reduced AT1-R number ($P<0.01$) without affecting its affinity (n=3).

Figure 4A. AT1 R promoter activity measured by luciferase assay was reduced by 50% (Figure 4A), suggesting that T3 suppresses AT1 R gene expression at the transcriptional level. We next examined whether T3 affects AT1 R mRNA stability. In vehicle-treated cells, AT1 R mRNA levels were reduced by 50% after 12 hours (Figure 4B). T3 destabilized AT1 R mRNA, resulting in an AT1 R mRNA
half-life of 6 hours. These data suggest that T3 downregulates AT1R mRNA expression at both transcriptional and posttranscriptional levels.

T3 Decreases Functional Response of VSMCs to Angiotensin II

We examined whether downregulation of AT1 receptor gene expression leads to a reduction of functional response of VSMCs to Ang II stimulation. We examined Ang II–induced elevations of [Ca2+]i. VSMCs were pretreated with either T3 (1 μmol/L) or vehicle for 10 minutes or 9 hours. Then, VSMCs were stimulated with Ang II (100 nmol/L), and [Ca2+]i was measured (Figure 5). Ang II induced a maximal [Ca2+]i increase of 41.1 ± 0.4% in vehicle-treated VSMCs, whereas a maximal [Ca2+]i increase of VSMCs pretreated with T3 was 41.1 ± 2.4% (given in percentage of maximum fluorescence induced by ionomycin treatment) after 10 minutes of incubation. However, treatment with T3 for 9 hours significantly reduced the calcium response of VSMCs to Ang II. Ang II induced a maximal [Ca2+]i increase of 46.4 ± 4.1% in vehicle-treated VSMCs, whereas a maximal [Ca2+]i in VSMCs pretreated with T3 was 34.5 ± 2.2% (P < 0.05), which indicated 26% reduction of calcium response compared with control.

T3 Downregulates AT1R Expression in Rat Aorta

The physiological characteristics of control and hyperthyroid rats are shown in the Table. Heart rate of T3-treated rats was significantly increased (P < 0.01). Body weight of T3-treated rats was not increased during the treatment and was signifi-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control 0 Day</th>
<th>Control 10 Days</th>
<th>T3-Treated 0 Day</th>
<th>T3-Treated 10 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>295 ± 9.3</td>
<td>324 ± 13.2*</td>
<td>302 ± 5.9</td>
<td>302 ± 8.8</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>124 ± 3.7</td>
<td>138 ± 2.8</td>
<td>138 ± 5.7</td>
<td>137 ± 7.7</td>
</tr>
<tr>
<td>Heart rate, beat/min</td>
<td>360 ± 16.3</td>
<td>334 ± 8.4</td>
<td>327 ± 9.6</td>
<td>497 ± 23.31†§</td>
</tr>
<tr>
<td>TSH, ng/mL</td>
<td>8.3 ± 1.0</td>
<td>4.0 ± 0.3§</td>
<td>4.0 ± 0.3§</td>
<td>4.0 ± 0.3§</td>
</tr>
<tr>
<td>Free T3, pg/mL</td>
<td>2.3 ± 0.1</td>
<td>7.2 ± 2.2‡</td>
<td>7.2 ± 2.2‡</td>
<td>7.2 ± 2.2‡</td>
</tr>
<tr>
<td>Free T4, ng/dL</td>
<td>1.7 ± 0.2</td>
<td>0.1 ± 0.02§</td>
<td>0.1 ± 0.02§</td>
<td>0.1 ± 0.02§</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n = 6. BP indicates blood pressure; TSH, thyroid stimulating hormone; T3, triiodothyronine; T4, thyroxine. *P < 0.05, †P < 0.01 vs 0 day; ‡P < 0.05, §P < 0.01 vs control.
In the present study, we demonstrated that T3 downregulates AT1R mRNA expression at both transcriptional and posttranscriptional levels (Figure 4). Downregulation of AT1R by T3 was demonstrated both in cultured VSMCs and rat aorta (Figures 1 and 6). Binding assay showed a decrease in AT1R density without an alteration of the affinity to Ang II (Figure 3). Downregulation of AT1R attenuated Ang II–induced [Ca\(^{2+}\)] response (Figure 5). Although regulation of cardiac Ang II receptor in hyperthyroid state has been reported,\(^{21,22}\) to our knowledge this is the first report showing the downregulation of vascular AT1R by T3.

Mizuma et al\(^8\) have shown the presence of an iodothyronine deiodinase in human VSMCs. This suggests that VSMCs are able to convert T4 to T3. We showed that T4 suppressed AT1R mRNA expression. This suggests that rat VSMCs may have deiodinase activity as reported in human VSMCs. Ojamaa et al\(^9\) reported that T3 caused rapid relaxation of VSMCs, suggesting the presence of rapid nongenomic mechanisms for the action of T3. T3-induced relaxation of VSMCs was not mediated by cAMP or NO.\(^9\) It was also shown that phosphorylation of myosin light chain was not altered by T3.\(^9\) Downregulation of AT1R occurred after several hours of T3-stimulation, indicating that the suppression is a genomic effect of T3. Because 10-minute incubation with T3 did not affect calcium response, it is unlikely that T3 directly inhibits calcium response to Ang II. The attenuated calcium response to Ang II after 9-hour treatment with T3 may reflect the reduced AT1R number. Therefore, both genomic and nongenomic effect of T3 may be involved in the reduction of vascular resistance in hyperthyroid state. Although the mechanism of T3-induced vascular relaxation is still elusive, thyroid hormone affect gene expression in blood vessel.

Four types of thyroid hormone receptor isoform are expressed in human VSMCs, as well as in rat VSMCs.\(^{1,8,23}\) Although the target genes for T3 in VSMCs are incompletely characterized, it is generally accepted that T3-activated thyroid hormone receptor binds to TRE site of the promoter region of target genes and enhances gene transcription.\(^2\) However, there are several genes that are suppressed by T3. The downregulation of gene expression by T3 is mediated by N-CoR and its functional homologue SMRT. In the absence of T3, thyroid hormone receptor recruits corepressor complex including N-CoR/SMRT, Sin3, and HDAC, and suppresses the transcription of target genes of T3. When T3 binds to thyroid hormone receptor, the corepressor complex inhibiting target gene expression is released. The released corepressor complex binds to other transcription factors such as Octamer transcription factor-1 (Oct-1)\(^{19}\) and nuclear factor-κB,\(^{20}\) and inhibits the Oct-1- or nuclear factor-κB–dependent gene transcription. Trichostatin A, an inhibitor of HDAC, is reported to restore SMRT-induced suppression of interleukin-2 gene expression, which is activated by nuclear factor-κB. They also demonstrated that SMRT suppresses thymidine kinase gene expression, which is mediated by AP-1. In our result, however, T3 downregulated AT1R mRNA in the presence of trichostatin A, suggesting that corepressor complex including HDAC is not involved in T3-induced AT1R downregulation. Further study is necessary to determine the mechanisms for T3-induced AT1R downregulation.

Although there is a contradicting result,\(^9\) a recent report showed that vascular endothelium is a target of thyroid hormone, and an excessive endothelial NO production plays a role in vasodilation in hyperthyroidism.\(^{24}\) We previously reported that NO donor inhibited AT1R expression in VSMCs.\(^{15}\) Therefore, it may be possible that T3 indirectly inhibits vascular AT1R expression through production of NO in addition to direct downregulation of AT1R. However, direct activation of NO synthase of VSMCs by T3 is unlikely because L-NAME, an inhibitor of NO synthase, did not affect T3-induced AT1R downregulation in VSMCs.

Recent reports examined the relationship between progression of atherosclerosis and thyroid function in patients with stable angina.\(^{25,26}\) It was shown that more angiographic progression of coronary atherosclerosis was observed in patients with lower serum T3 level after 2 years. This suggests that thyroid hormone may be protective against atherosclerosis. It is also reported that Ang II plays a critical
role in atherosclerotic vascular disease.27–29 Our results suggest that antiatherosclerotic effect of T3 may involve downregulation of AT1R.

Perspectives

In summary, we showed in the present study that thyroid hormone downregulates AT1R expression at both transcriptional and posttranscriptional levels, and attenuates biological function of Ang II. Although further study is necessary to determine whether AT1R downregulation is involved in the renin-angiotensin system in future. Our data suggest that thyroid hormone may be a negative regulator of the renin-angiotensin system. The effect of thyroid hormone on cardiovascular system may be reevaluated in relation to the renin-angiotensin system in future.

Acknowledgments

This study was supported in part by a grant from Yamanouchi Foundation for Research on Metabolic Disorders, Tsukuba, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, to T.I. (14570673).

References

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Hypertension. 2003;41:598-603; originally published online February 17, 2003; doi: 10.1161/01.HYP.0000056524.35294.80
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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