**Thyroid Hormone Activation in Human Vascular Smooth Muscle Cells**

**Expression of Type II Iodothyronine Deiodinase**

Haruo Mizuma, Masami Murakami, Masatomo Mori

**Abstract**—Thyroid hormone has been reported to have significant effects on the peripheral vascular system, including relaxation of vascular smooth muscle cells and antiatherosclerotic effects. To exert its biological activity, thyroxine, which is a major secretory product of thyroid gland, needs to be converted to 3,5,3'-triiodothyronine (T3) by iodothyronine deiodinase. Type I iodothyronine deiodinase (DI) is widely distributed and maintains circulating T3 level, whereas type II iodothyronine deiodinase (DII) is present in a limited number of tissues to provide local intracellular T3. In the present study, we have identified iodothyronine deiodinase in cultured human coronary artery smooth muscle cells (hCASMCs) and human aortic smooth muscle cells (hASMCs). All of the characteristics of the deiodinating activity in hCASMCs and hASMCs were compatible with DII. Northern analysis demonstrated that DII mRNA was expressed in both hCASMCs and hASMCs, and DII mRNA levels as well as DII activities were rapidly increased by dibutyryl-cAMP or forskolin. These data demonstrate, for the first time, the expression of DII in human vascular smooth muscle cells, which is regulated by a cAMP-mediated mechanism. The present results suggest a previously unrecognized role of local T3 production by DII in the pathophysiology of human vascular smooth muscle cells. (*Circ Res. 2001;88:313-318.*)

**Key Words:** coronary artery smooth muscle ■ aortic smooth muscle ■ thyroid hormone receptor ■ cAMP

Thyroid hormone has profound effects on the peripheral vascular system. It is known as a vasodilator that acts directly on vascular smooth muscle cells (SMCs) to cause a relaxation of coronary arteries.1,2 Hypothyroidism is known to be a risk factor for atherosclerosis, and thyroid hormone replacement in hypothyroidism has been reported to protect from atherosclerosis.3,4

To exert its biological activity, thyroxine (T4), which is a major secretory product of the thyroid gland, needs to be converted to 3,5,3'-triiodothyronine (T3) by iodothyronine deiodinase.5,6 There are two types, type I iodothyronine deiodinase (DI) and type II iodothyronine deiodinase (DII), which catalyze conversion of T4 to T3. DI is present in thyroid gland, liver, kidney, and many other tissues, whereas DII is present in a limited number of tissues, including central nervous system, anterior pituitary tissue, and brown fat in the rat.5,6 

The source of T3 mainly depends on circulating T3 in most tissues, local intracellular conversion of T4 to T3 is an important source of T3 in certain tissues where DII exists.5,6

A cDNA encoding DII was cloned from *Rana catesbeiana* tissues,7 and its mammalian counterpart was subsequently isolated from rat brown fat.8 In human, DII mRNA was unexpectedly detected in thyroid gland and other tissues, suggesting previously unrecognized roles of DII in those tissues.9,10 Considering that not only T3, but also T4 has been reported to have effects on peripheral vascular function,11–13 DII may exist in vascular SMCs and may contribute to the pathophysiology of human vascular systems by providing intracellular T3.

In the present study, we have characterized iodothyronine deiodinating activity and identified DII expression in cultured human coronary artery SMCs (hCASMCs) and human aortic SMCs (hASMCs).

**Materials and Methods**

**Materials**

[α32P]UTP, [125I]-labeled 3,3',5'-triiodothyronine (reverse T3 [rT3]), and [124I]T4 were purchased from New England Nuclear Corp, AG 50W-X2 resin and protein assay kit were from Bio-Rad Laboratories, Inc. All other chemicals were obtained from Sigma Chemical Company or Wako Pure Chemical Industries, Ltd, unless otherwise indicated.
Cell Culture

hCASMCs were obtained from Clonetics, and hASMCs were from Cascade Biologics. Both cells were cultured to express α-smooth muscle actin isoform but not factor VIII, indicating the nature of vascular SMCs. These cells were inoculated to 6-well plastic culture plates for the measurement of deiodinase activity or to 60-mm plastic culture dishes for Northern analysis, and the cells were cultured in modified MCDB 131 medium (HuMedia-SG2, KURABO, Osaka, Japan) as previously described. After the cells became confluent, the medium was displaced with serum-free medium to arrest the proliferation of cells for 48 hours. The cells were then incubated in the medium containing compounds to be tested for indicated hours.

Measurement of Deiodinase Activity

Iodothyronine deiodinase activity was measured as previously described with minor modifications. Briefly, SMCs per each well (~2×10^5 cells per well) were washed twice with the washing buffer (100 mmol/L potassium phosphate, pH 7.0), scraped off, and transferred into 1000 μL of ice-cold buffer (100 mmol/L potassium phosphate, pH 7.0, containing 20 mmol/L DTT). After centrifugation at 3000 rpm for 15 minutes at 4°C, the supernatant was discarded. Pellets were sonicated in 100 μL of the assay buffer (in mmol/L, potassium phosphate [pH 7.0] 100, containing EDTA 1 and DTT 20) per tube and were incubated with indicated amounts of [125I]T4 or [125I]rT3, which was purified on the day of experiment, in the presence or absence of 1 mmol/L 6-propyl-2-thiouracil (PTU) or 1 mmol/L iopanoic acid. The reaction was terminated by adding 100 μL of 2% BSA and 800 μL of 10% trichloroacetic acid. The released 125I was separated by column chromatography using AG 50W-X2 resin as previously described and counted. The protein concentration was separated by column chromatography using AG 50W-X2 resin as previously described (KURABO, Osaka, Japan) as previously described. After the cells were cultured in modified MCDB 131 medium (HuMedia-SG2, KURABO, Osaka, Japan) as previously described. After the cells became confluent, the medium was displaced with serum-free medium to arrest the proliferation of cells for 48 hours. The cells were then incubated in the medium containing compounds to be tested for indicated hours.

Results

Characteristics of Iodothyronine Deiodinase in hCASMCs and hASMCs

The deiodinating activity in hCASMCs was measured by the release of I− from 2 nmol/L [125I]T4 or [125I]rT3 in the presence of 20 mmol/L DTT and 1 mmol/L PTU. T3 deiodination was dependent on an incubation period up to 2 hours, as shown in Figure 1A and protein concentration of hCASMCs, as shown in Figure 1B. Optimal pH for the deiodination was ≈7.0. Incubation at 4°C or preheating the cell sonicate at 56°C for 30 minutes completely abolished the deiodination. Both the T4 and rT3, deiodinating activities were not influenced by 1 mmol/L PTU, but were completely inhibited by 1 mmol/L iopanoic acid. Basically identical results were obtained for hASMCs. From the double reciprocal plot, kinetic constants for T4 were calculated to be K_m=2.3 nmol/L and V_max=333.3 fmol I− released/mg protein per hour in hCASMCs, as shown in Figure 1C. V_max for rT3 in hCASMCs was lower than that for T4 (Table). In hASMCs, kinetic constants for T4 and rT3 were comparable with those in hCASMCs (Table). When we fractionated the hCASMC preparation, the highest deiodinating activity was observed in microsomal fraction (755.4 versus 295.3 [total cell homogenate] fmol I− released/mg protein per hour). Taken together, these results indicate that the characteristics of the deiodinating activity in hCASMCs and hASMCs are compatible with DII.

Identification of DII mRNA in Cultured hCASMCs and hASMCs

Northern analysis using human DII cRNA probe was performed to examine whether DII mRNA is expressed in human vascular SMCs. As shown in Figure 2, hybridization signals of DII mRNA ~7.5 kb in size were clearly demonstrated in hCASMCs and hASMCs. The size of DII mRNA in hCASMCs and hASMCs was indistinguishable from that in human thyroid gland, although the amount of DII mRNA in hCASMCs or hASMCs was less than that in human thyroid gland. These results indicate that DII mRNA is expressed in both hCASMCs and hASMCs.

Regulation of DII Expression in Cultured hCASMCs and hASMCs by Thyroid Hormones

One of the important characteristics of DII is the negative regulation of its activity by thyroid hormones. To study the effects of thyroid hormones on deiodinating activity in human vascular SMCs, thyroid hormones were added to the culture.

Kinetic Constants of Iodothyronine Deiodinase in hCASMCs or hASMCs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m, nmol/L</th>
<th>V_max, fmol I− released/mg protein per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCASMCs</td>
<td>T4</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>rT3</td>
<td>7.0</td>
</tr>
<tr>
<td>hASMCs</td>
<td>T4</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>rT3</td>
<td>4.9</td>
</tr>
</tbody>
</table>

*fmol I− released per milligram protein per hour.
medium for 6 hours before the harvest of cultured SMCs. As shown in Figure 3A, the deiodinating activity was decreased by thyroid hormones, and the potency of the inhibitory effect was T₃ > rT₃ > T₄ in hCASMCs. Although the deiodinating activity was measured by the release of I₂ from [¹²⁵I]T₄, control studies demonstrated that the effects of T₄ or rT₃ on the deiodinating activity were time dependent and are thus not explained by dilution of the substrate with T₄ or rT₃ carried over into the assay of deiodinase activity. More importantly, 10⁻⁷ mol/L rT₃ added to the assay buffer of deiodinase activity did not affect the release of I₂ from [¹²⁵I]T₄. DII mRNA in hCASMCs was inhibited by thyroid hormones; the potency of the inhibitory effect was T₃ > T₄ > rT₃, as shown in Figure 3B. Basically identical inhibitory effects of thyroid hormones on the deiodinating activity and DII mRNA expression were observed in hASMCs. Inhibition of the deiodinating activity in hCASMCs and hASMCs by thyroid hormones further indicates the presence of authentic DII activity in cultured hCASMCs and hASMCs. These results suggest that T₃ suppresses DII activity mainly at the pretranslational level, whereas T₄ or rT₃ suppresses DII activity largely at the post-translational level in hCASMCs and hASMCs, in agreement with previous observations for DII in other tissues and cells.¹⁴,²⁰–²²

Stimulation of DII Expression in Cultured hCASMCs and hASMCs by Forskolin or Dibutyl cAMP ([Bu]₂cAMP)  
Because DII activity has been known to be regulated by a cAMP-dependent mechanism,⁵,⁶ the effects of cAMP-elevating agents on DII expression in human vascular SMCs were studied. As shown in Figures 4A and 4B, DII activity and DII mRNA in cultured hCASMCs were clearly increased...
by treatment with forskolin (10^{-5} mol/L) or (Bu)2cAMP (10^{-3} mol/L) for 6 hours. In the time-course study, both DII activity and DII mRNA in cultured hCASMCs were increased by forskolin within 3 hours, as shown in Figure 5. When actinomycin D (5 μg/mL) was added to the culture medium 30 minutes before the incubation with forskolin (10^{-3} mol/L) for 6 hours, the stimulation of DII activity was completely abolished, indicating the requirement of mRNA synthesis for the stimulation of DII activity by a cAMP-mediated mechanism. Forskolin stimulated DII activity and DII mRNA in hASMCs in the same manner, as shown in Figure 6.

Identification of Thyroid Hormone Receptor (TR) Isoforms in Cultured hCASMCs and hASMCs by RT-PCR

To investigate whether TRs are expressed in hCASMCs and hASMCs, we have performed RT-PCR analyses of TR isoforms in those cells. In control human cerebral cortical tissue, all of the TR isoforms were clearly demonstrated by RT-PCR, as shown in Figure 7. Although all of the TR isoforms were also detected in both hCASMCs and hASMCs by RT-PCR, strong expression of mRNA for TRα1 and TRα2 isoforms and relatively weak expression of mRNA for TRβ1 and TRβ2 were observed in both hCASMCs and hASMCs, as shown in Figure 7.

Discussion

The present results have clearly demonstrated that iodothyronine deiodinating activities are present in cultured hCASMCs and hASMCs. The deiodinating activities in hCASMCs and hASMCs were dependent on the protein concentrations, incubation period, pH, temperature, and substrate concentrations. These characteristics clearly indicate the enzymatic nature of the deiodinating activities. The deiodinating activities in hCASMCs and hASMCs were not inhibited by 1 mmol/L PTU and were demonstrated to have low Km for T4 and rT3. Furthermore, the deiodinating activities were decreased by the addition of thyroid hormones to the culture medium. Iodothyronine deiodinating activities in hCASMCs and hASMCs, therefore, have characteristics compatible with DII. Northern analysis using human DII cRNA probe clearly demonstrated the hybridization signals with ~7.5 kb in size in hCASMCs and hASMCs. The size of DII mRNA in hCASMCs and hASMCs was indistinguishable from that in human thyroid gland. The expression of DII in both hCASMCs and hASMCs suggests the ubiquitous expression of DII in human vascular SMCs. Although the expression of DII mRNA in human heart and skeletal muscle has been observed after the molecular cloning of human DII cDNA, the present results appear as the first demonstration of the expression of DII in human vascular SMCs.

DII activity is controlled by thyroid hormones at two levels. T4 and rT3 suppress DII activity mainly at the post-translational level through acceleration of the degradation rate of DII protein. Both T4 and rT3 are more potent in producing this effect than T3, suggesting that this effect does not require TRs. Recently, proteasomal degradation has been demonstrated to be involved in the post-translational regulation of DII activity by thyroid hormones. In contrast, T3 suppresses DII activity by decreasing DII mRNA without affecting its half-life, indicating that this effect is due to suppression of transcription of DII gene through TRs. Because the half-life of DII mRNA is 2 hours and that of DII enzyme is 40 minutes, the suppression of DII by thyroid hormones is very rapid whether the decrease in activity is induced by transcriptional or post-translational effects. In the present study, thyroid hormones inhibited DII mRNA expression, and the potency of the inhibitory effect was T4>T3>rT3. Although the deiodinating activity was also decreased by thyroid hormones, the potency of the inhibitory effect was T4>T3>rT3. These results suggest that T3 sup-
presses DII activity mainly at the pretranslational level, whereas T₄ or rT₃ suppresses DII activity largely at the post-translational level in hCASMCs and hASMCs, in agreement with previous observations for DII in other tissues and cells.¹⁴,²⁰–²² The possible transcriptional regulation of DII by T₄ is further supported by the presence of TR isoforms in hCASMCs and hASMCs demonstrated in the present study. Because DII expression in hCASMCs and hASMCs could be increased in the hypothyroid state, DII in human vascular SMCs might play a role in the protection of human vessels from local T₄ deficiency in hypothyroidism.

In the present study, both DII activities and DII mRNA levels were rapidly stimulated by forskolin or (Bu)²cAMP in hCASMCs and hASMCs, suggesting the pretranslational regulation of DII expression by a cAMP-dependent mechanism. The rapid stimulation of DII mRNA and DII activity was also observed in human skeletal muscle cells, rat astrocytes, and pineal gland.¹⁴,²⁴,²⁵ Recently, a functional cAMP response element has been reported to be present in the human DII promotor region.²⁶ Taken together, it is suggested that DII expression in hCASMCs and hASMCs is regulated by a cAMP-dependent mechanism at the transcriptional level. It is generally accepted that calcium-dependent phosphorylation of myosin light chains initiates the contraction of vascular smooth muscle.²⁷ Although the precise mechanisms underlying the action of endogenous vasodilator remain to be identified, it is postulated that cAMP-dependent protein kinase A inhibits calcium-dependent myosin light chain kinase.²⁸,²⁹ In the present study, it was demonstrated that intracellular accumulation of cAMP significantly stimulated DII expression in hCASMCs and hASMCs. Given that thyroid hormones have been reported to relax vascular SMCs directly,¹,² local production of T₃ by DII might be another vasodilative mechanism mediated by cAMP regulatory cascade.

It has been reported that low serum selenium concentration increases the risk of ischemic heart disease.³⁰ However, the mechanisms involved in the increased frequency of coronary heart disease in selenium deficiency are not known. Human DII cDNA contains in-frame TGA triplets that are not for termination codons but for the rare amino acid selenocysteine, which contains selenium.⁶,¹⁰ Because selenium is required to exert the full DII activity,²⁴ one might speculate that local T₄ production by DII in human vascular SMCs could be decreased by low serum selenium, which may be related to the increased risk of ischemic heart disease in selenium deficiency. Further studies are required to elucidate the pathophysiological roles of DII and selenium deficiency in atherosclerosis.

The physiological importance of intracellular thyroid hormone activation by DII has been clearly demonstrated in certain tissues. Adenohypophysial T₃ production by DII plays a role in feedback regulation of thyrotropin secretion by thyroid hormones.⁵,⁶ In the rat brown adipose tissue, the expression of uncoupling protein is regulated by locally generated T₃, which is provided by DII.³⁰ In the present study, TRs were demonstrated in hCASMCs and hASMCs by RT-PCR, suggesting that locally produced T₃ by DII might play a role through TRs in vascular SMCs. Although the target genes induced by thyroid hormones in vascular SMCs are not known, it is of interest to study the possible role of locally produced T₃ by DII in the regulation of vascular SMC–specific gene expression, which may be associated with SMC dedifferentiation that relates to atherosclerosis and ischemic heart disease.³¹,³²

In summary, the present results demonstrate the expression of functional DII in human vascular SMCs, which may open novel perspectives on the roles of thyroid hormone metabolism in the pathophysiology of human vascular SMCs.

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References


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