Thyroid Hormone Targets Matrix Gla Protein Gene Associated With Vascular Smooth Muscle Calcification

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Abstract—Thyroid hormones have marked cardiovascular effects in vivo. However, their direct effects on vascular smooth muscle cells have been unclear. Because thyroid hormones play critical roles in bone remodeling, we hypothesized that they are also associated with vascular smooth muscle calcification, one of the pathological features of vascular sclerosis. To test this hypothesis, we examined the effects of 3′,3,5-triiodo-L-thyronine (T_3) on the expression of calcification-associated genes in rat aortic smooth muscle cells (RAOSMCs). Quantitative RT-PCRs revealed that a physiological concentration of T_3 (15 pmol/L free T_3) increased mRNA level of matrix Gla protein (MGP), which acts as a potent inhibitor of vascular calcification in vivo, by 3-fold in RAOSMCs, as well as in cultured human coronary artery smooth muscle cells. In RAOSMCs transiently transfected with a luciferase reporter gene driven by the MGP promoter, T_3 significantly stimulated luciferase activity. In addition, RNA interference against thyroid hormone receptor-α gene diminished the effect of T_3 on MGP expression. Aortic smooth muscle tissues from methimazole-induced hypothyroid rats (400 mg/L drinking water; 4 weeks) also showed a 68% decrease in the MGP mRNA level, as well as a 33% increase in calcium content compared with that from the control euthyroid animals, whereas hyperthyroidism (0.2 mg T_3/kg IP; 10 days) upregulated MGP mRNA by 4.5-fold and reduced calcium content by 11%. Our findings suggest that a physiological concentration of thyroid hormone directly facilitates MGP gene expression in smooth muscle cells via thyroid hormone nuclear receptors, leading to prevention of vascular calcification in vivo. (***Circ Res*. 2005;97:550-557.)**

Key Words: calcium ■ gene expression ■ nuclear receptors ■ vascular smooth muscle ■ thyroid hormone

Thyroid hormone has marked effects on differentiation, development, and metabolic balance of virtually every body tissue. The action of thyroid hormone is mediated by high-affinity thyroid hormone nuclear receptors (TRs), which recognize specific response elements in the promoters of target genes and regulate their transcriptional activity in response to the hormone. Alterations in thyroid hormone levels have a profound impact on the cardiovascular system, which include changes in myocardial contractility, heart rate, and resistance of peripheral vasculature. Hyperthyroidism leads to positive inotropic, lusitropic, and chronotropic effects on the heart and low systemic vascular resistance, whereas the opposite is observed in hypothyroidism. In myocardium, the mechanisms for these changes are based on altered expression levels of several key proteins involved in the regulation of intracellular ion homeostasis. The effects of thyroid hormone on cardiac contractility as well as rates of contraction and relaxation are mainly mediated by increases in the levels of the sarcoplasmic reticulum Ca^{2+}-ATPase and decreases in its inhibitor phospholamban in cardiomyocytes.[1] The positive chronotropic effect of thyroid hormone is associated with altered expression levels in plasmalemmal ion channels/transporters in the heart, such as Kv1.5, Kv4.2, minK, hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), HCN4, Na^+-Ca^{2+} exchanger, and Na^+-K^+-ATPase.[2–5] In contrast, although ≈25% of hypothyroid patients have diastolic hypertension,[6] the mechanism for the altered systemic vascular resistance under an abnormal thyroid hormone status is not well understood. To date, a loss of nongenomic vasodilating action of thyroid hormone[7] and atherosclerosis attributable to hypercholesterolemia[8] have been associated with the increased systemic vascular resistance under hypothyroidism.[9] Recently, mRNAs for TR isoforms were identified in aortic and coronary smooth muscle cells, suggesting that a direct genomic action of thyroid hormone may play a significant role in vascular smooth muscle.[10] Although extremely high concentrations of thyroid hormone are known to regulate expression of several genes in vascular smooth muscle cells,[10,11] the physiological and direct target genes of thyroid hormone in vascular smooth muscle cells are not known.[9]

Arterial calcification is a common pathological feature of vascular sclerosis, as well as a variety of metabolic disorders such as diabetes and renal disease. Decades ago, cretinism

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was found to be associated with arterial calcification, especially when patients did not receive sufficient thyroid hormone replacement therapy.[12] However, the mechanism for the calcification in cretins has been to date unclear. A subset of vascular smooth muscle cells, named "calcifying vascular cells," was demonstrated recently to undergo osteogenic and chondrogenic differentiation in culture, indicating that some vascular smooth muscle cells still have the potential for multiple lineages.[13] Because thyroid hormone plays a critical role in bone remodeling,[14] we hypothesized that thyroid hormone is also associated with vascular calcification. To test this hypothesis, we investigated the effect of thyroid hormone on vascular smooth muscle calcification and expression profiles of calcification-associated genes in vitro and in vivo, and identified matrix Gla protein (MGP) gene as a target of thyroid hormone in vascular smooth muscle cells.

Materials and Methods

Cell Culture

α-Actin–positive rat aortic smooth muscle cells (RAOSMCs) were obtained from Cell Applications, Inc. and were cultured on 6-well cell culture plates at 37°C in a humidified atmosphere of 95% air/5% CO_2 in growth medium (GM; Dulbecco's Minimal Essential Medium [DMEM] supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells up to passage 5 were used for the experiments. The culture media were changed every 48 hours. Thyroid hormone–depleted serum was prepared as described previously.[15] To evaluate effects of thyroid hormone on gene expression profiles of the synthetic phenotype of RAOSMCs, the cells at 50% of confluence were cultured in thyroid hormone–depleted medium (TDM; DMEM containing 10% thyroid hormone-depleted serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) for 2 days and stimulated with 3′,3,5-triiodo-L-thyronine (T_3) for another 2 days. The contractile form of RAOSMCs was obtained by culturing confluent cells in serum-free differentiation medium (DM; DMEM supplemented with 1× ITS-X (Invitrogen), 5 mmol/L taurine, 100 U/mL penicillin, and 100 μg/mL streptomycin) for 10 days, followed by T_3 treatment for 2 days. The transition of the cell phenotype in DM was confirmed by immunoblotting for nonmuscle myosin heavy chain (SMemb) and smooth muscle myosin heavy chain-2 (SM2). To examine effects of thyroid hormone on calcium accumulation, confluent RAOSMCs were cultured as described previously[16] in TDM with or without 100 ng/mL recombinant human bone morphogenic protein-2 (rBMP2; R & D Systems) in a cell culture dish with or without collagen type IV (Col4) coating (BD Biosciences). Supplementation with β-glycerophosphate, which facilitates smooth muscle cell calcification,[17] was omitted because of a decrease in the signal-to-background ratio. Cells were subsequently stimulated with T_3 for 5 days. The concentrations of free T_3 (fT_3) and free L-thyroxine (T_4) in the serum-containing medium were determined using chemiluminescent enzyme immunoassay at a clinical diagnostic laboratory. The detection limits for fT_3 and fT_4 were 1.1 pmol/L and 1.7 pmol/L, respectively. Human coronary artery smooth muscle cells (HCASMCs; Cell Applications, Inc.) were maintained and transformed into the contractile phenotype before treatment with T_3 by culturing for 7 days in serum-free HCASMC DM (311D-500; Cell Applications, Inc) according to manufacturer instructions.

Animals

Male Sprague-Dawley rats (Japan SLC; Shizuoka, Japan) were maintained on rodent chow (Certified diet MF; Oriental Yeast, Co) and given water ad libitum. For generation of hypothyroid animals, methimazole (MMI; 400 mg/L) was added to the drinking water for 4 weeks. Hyperthyroid rats were generated by daily injection of T_3 (0.2 mg/kg body weight IP) for 10 days. Plasma concentrations of fT_3 and fT_4 were measured, as described above. After the treatment with MMI or T_3, the thoracic aorta was isolated. Animals were 12 weeks old when killed. Aortic smooth muscle tissue for measurements of calcium accumulation and gene expression was cleared of fat, connective tissue, and an endothelium and stored at −80°C until use. For pathological examination, the aortic tissue was fixed in 10% formaldehyde. Transverse aortic sections were taken from the fixed tissue and stained with hematoxylin and eosin. All animals were treated in accordance with laboratory animal care guidelines of National Institute of Health Sciences at Tokyo.

Calcium Accumulation

Calcium content in RAOSMCs and rat aortic smooth muscle tissues were determined as described previously[16] using *o*-cresolphthalein complexone method. Protein concentration was determined using Bio-Rad protein assay reagent and BSA as a standard.

Real-Time Quantitative RT-PCR

Total RNA was isolated from smooth muscle cells and tissues using Sepasol reagent (Nakalai Tesque) containing 0.1 mg/mL glycogen (Roche Diagnostics) and was treated with DNaseI (Promega) according to the manufacturer protocols. To quantitate specific mRNA levels, the real-time progress of target sequence-specific amplification was monitored during RT-PCR using TaqMan chemistry and PRISM7000 Sequence Detection System (Applied Biosystems). An 18S ribosomal RNA was used as an internal control for each RNA level. Sequences of the primers and the TaqMan probes are listed in supplemental Table I (available online at http://circres.ahajournals.org).

Western Blot Analysis

RAOSMCs and aortic smooth muscle tissues were homogenized in lysis buffer as described previously.[18] After measuring protein concentrations, proteins were separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes, which were incubated with anti-SMemb or anti-SM2 monoclonal antibodies (Yamasa) or an anti-MGP polyclonal antibody (TransGenic) for 1 hour at room temperature. Subsequently, membranes were incubated with the secondary antibody conjugated with horseradish peroxidase for 1 hour. Signals were visualized and quantified using ECL Plus system (Amersham Biosciences) and LAS-3000 Imaging System (FUJIFILM), respectively.

Promoter Activity Assay

Fragments between −1752 and −1 of 5′ flanking sequence of the MGP gene exon 1 and between −1895 and −1 of 5′ flanking sequence of the stanniocalcin-1 (STC1) gene exon 1 were amplified using rat tail genomic DNA as a template. The primers for PCR amplifications were designed as based on the nucleotide sequences (MGP forward: CAAGGGTACCGGTTTGAGAGACCACGAGAC; MGP reverse: CTTGAAGCTTCCTGTGAGTCTGCCTCTGTG; STC1 forward: CAAGCTCGAGCCCCTGATATTTCAGCATGG; STC1 reverse: CTTGAAGCTTAGGTGAGGATTTGAGGAGG). The amplicons were subcloned into the firefly luciferase expression vector pGL3-Basic (Promega). RAOSMCs in the contractile state, grown on a 24-well plate, were transiently cotransfected with 225 ng/well of each promoter luciferase plasmid and 75 ng/well of phRL-TK control plasmid (Promega) using FuGene6 (Roche). Three hours after transfection, cells were incubated with or without T_3. The luciferase activity was defined as a ratio of the firefly luciferase signal to the renilla luciferase signal, which was measured with Dual-Luciferase Reagent (Promega). The transfection efficiency of the plasmids was estimated to be 1% to 10% of the total RAOSMCs, as assessed by transfection experiments with an enhanced green fluorescent protein expression vector pEGFP-N1 (BD Biosciences Clontech; data not shown).

RNA Interference Against TRα

RAOSMCs in the synthetic form were transiently transfected with StealthRNAi (Invitrogen) specific for TRα gene (sense: CCAGAAGAACCUCCAUCCCACCUAU; antisense: AUAGGUGGGAUG-

Free Thyroid Hormone Concentrations in Rat Plasma and Serum-Containing Cell Culture Medium

Plasma/Medium	fT_3 (pmol/L)	fT_4 (pmol/L)	Calculated Range of Thyroid Hormone Activity (pmol fT_3/L)
Plasma from a euthyroid rat	4.2±0.6	27±2	6.9–31
Plasma from a hypothyroid rat (MMI treatment for 1 month)	<1.1	<1.8	<1.3–2.9
GM (DMEM, 10% FCS, P/S)	3.7±0.2	15±0.0	5.2–19
TDM (DMEM, 10% thyroid hormone–depleted FCS, P/S)	<1.1	2.7±0.2	<1.4–3.8
TDM (DMEM, 10% thyroid hormone–depleted FCS, P/S) + 1 nmol/L T_3	15±1*	3.4±0.1*	15–18

The range of thyroid hormone activity was calculated assuming that X% (X=0–100) of T_4 is processed to T_3 at the site of action in vivo and that the affinity of T_4 for TRs is one tenth that of T_3.

P/S indicates penicillin and streptomycin.

Values are means±SD; *P<0.05 vs TDM without T_3 supplementation (Student t test).

GAGGUUCUUCUGG) or StealthRNAi negative control medium GC (Invitrogen) using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions and incubated in TDM for 2 days. Cells were then treated with or without T_3 (15 pmol/L fT_3) for another 2 days. Total RNA was isolated from cells before and after T_3 treatment for mRNA determinations.

Statistics

Data were expressed as means±SEM unless otherwise indicated. Data were analyzed for statistical significance by Student t test or ANOVA with Student-Newman–Keuls test as a post hoc test. Significance was imparted at the P<0.05 level.

Results

Free Thyroid Hormone Concentrations

As shown in the Table, plasma concentrations of fT_3 and fT_4 in euthyroid rats were 4.2±0.6 pmol/L and 27±2 pmol/L (mean±SD), respectively. The MMI treatment decreased fT_3 and fT_4 to <1.1 pmol/L and to <1.8 pmol/L, respectively, indicating that the animals were hypothyroid. The fT_3 and fT_4 levels in GM containing 10% FCS were 3.7±0.2 pmol/L and 15±0.0 pmol/L (mean±SD), respectively, and happened to be similar to those in plasma from euthyroid rats. The free concentrations of thyroid hormones in TDM (<1.1 pmol/L fT_3; 2.7 pmol/L fT_4) were low enough to keep TRs inactivated because the K_d values of T_3 for TRs are known to be ≈10 to 100 pmol/L[19] and because T_4 has ≈10-fold lower affinity for TRs than that of T_3. In vascular smooth muscle cells, T_4 is known to be converted to T_3 by type II iodothyronine deiodinase,[10] although the conversion rate in vivo is not clear. Assuming that X% (X=0 to 100) of T_4 is processed to T_3 at the site of action in vivo and that the affinity of T_4 for TRs is one tenth of that of T_3, the total activity of thyroid hormones in euthyroid rat plasma should be equivalent to that of 6.9 to 31 pmol/L [4.2+27×X/100+27×(1−X/100)/10] of fT_3 alone. The supplementation of T_3 to TDM at 1 nmol/L of total concentration resulted in an increase in fT_3 to 15±1 pmol/L (mean±SD), with a slight increase in fT_4, therefore, it was regarded within a euthyroid range.

T_3-Induced Gene Expression and Calcification in Cultured RAOSMCs

Vascular smooth muscle cells show a high degree of plasticity and are able to interchange between a differentiated, contractile phenotype and a proliferating, synthetic phenotype. Therefore, we first examined the effects of T_3 on expression profiles of calcification-associated genes in both phenotypes. RAOSMCs in GM predominantly expressed a marker of synthetic phenotype: SMemb. The replacement of the medium with DM reduced the protein level of SMemb to 28% and increased SM2 expression by 11.6-fold (online Figure I), indicating the transition of the phenotype. In the synthetic phenotype, T_3 (1 nmol/L total T_3=15 pmol/L fT_3) led to upregulation of mRNAs for MGP and STC1×3.3-fold and 1.3-fold, respectively (Figure 1A), whereas osteopontin

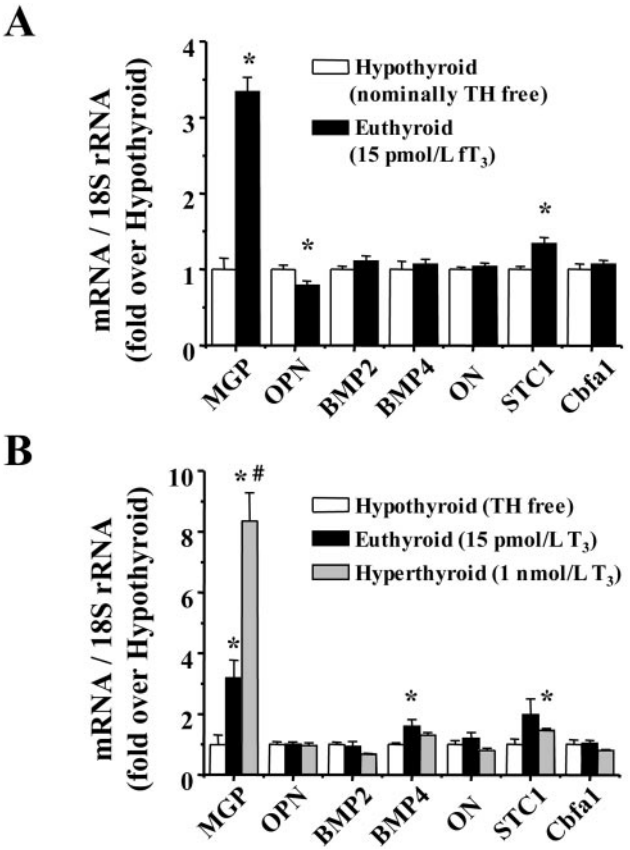

Figure 1. T_3 regulated expression of calcification-associated genes in cultured RAOSMCs in synthetic and contractile forms. A, Effect of T_3 on mRNA expression of calcification-associated genes in the synthetic form of RAOSMCs cultured in TDM. Cells were treated with 15 pmol/L fT_3 for 2 days. B, Effect of T_3 on mRNA expression of calcification-associated genes in the contractile form of RAOSMCs cultured in the DM. Cells were treated with 15 pmol/L or 1 nmol/L T_3 for 2 days. Values are expressed as means±SEM (n=4) TH indicates thyroid hormone. *P<0.05 vs hypothyroid; #P<0.05 vs euthyroid.

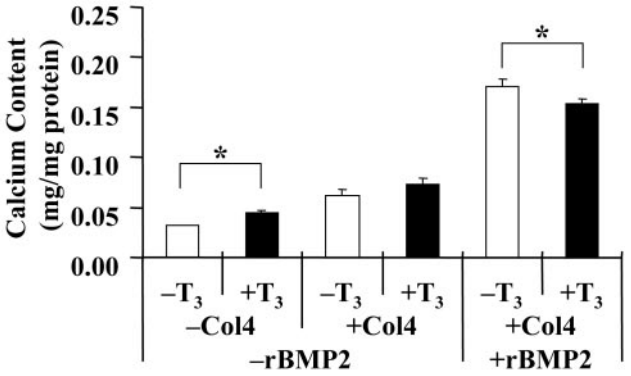

Figure 2. Effects of T_3 on calcium accumulation in cultured RAOSMCs. The confluent cells cultured in TDM were stimulated by 15 pmol/L fT_3. In the absence of rBMP2, T_3 significantly increased calcium content in RAOSMCs cultured in noncoated vessels. Supplementation with rBMP2 increased the basal calcium content in RAOSMCs in Col4-coated vessels and reversed the effect of T_3 on the calcification. Values are expressed as means±SEM (n=6 to 8); *$P<0.05$.

(OPN) was downregulated by 21%. The mRNA levels of BMP2, bone morphogenic protein-4 (BMP4), osteonectin (ON), and core binding factor α1 (Cbfa1; also known as Runx2 or Osf2) were not significantly altered by the T_3 treatment. Specific signals for osteocalcin and bone sialoprotein mRNA were not detected by several independent primer sets in all experiments of the present study, presumably because of their low abundance in vascular smooth muscle cells. In the contractile phenotype in DM, T_3 (15 pmol/L) induced upregulation of MGP and BMP4×3.2-fold and 1.6-fold, respectively (Figure 1B). A higher concentration of T_3 (1 nmol/L) resulted in a further increase in the MGP mRNA level and a significant upregulation of STC1 mRNA, indicating the dose dependency of the effects. Messenger RNA levels of OPN, BMP2, ON, STC1, and Cbfa1 in the contractile phenotype were not significantly altered by the T_3 treatment. Among the calcification-associated genes, MGP and STC1 genes were commonly upregulated by T_3 in both phenotypes, suggesting that these two genes were relatively essential in RAOSMCs as targets of thyroid hormone.

Because the effect of MGP on calcification and osteogenic differentiation of vascular smooth muscle cells depends on availability of BMP2 and Col4,[20,21] cell calcification was determined in the absence or presence of these factors. In the absence of rBMP2 and Col4 coating, treatment with T_3 (1 nmol/L total T_3=15 pmol/L fT_3) for 5 days resulted in an increase in calcium content by 39% in RAOSMCs (Figure 2). The same treatment tended to have the similar effect on cells in a Col4-coated vessel in the absence of rBMP2. In contrast, T_3 led to a significant decrease in cellular calcium by 10% in the presence of rBMP2 and Col4-coating.

Transcriptional Regulation of MGP and STC1 Genes by T_3

To test a hypothesis that the promoters of MGP and STC1 genes were under regulation of thyroid hormone, RAOSMCs were transiently transfected with luciferase reporters under control of the MGP and STC1 promoters. The 5′ flanking sequences of MGP and STC1 genes have been submitted to

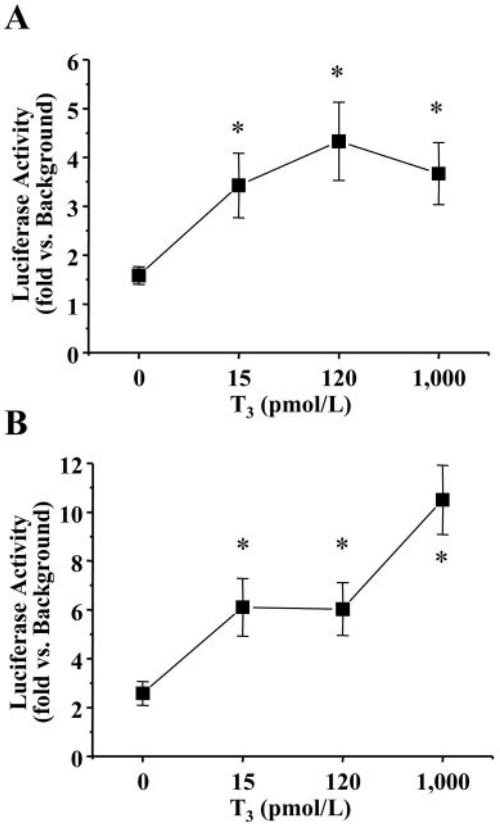

Figure 3. T_3 activated luciferase reporter genes driven by rat MGP (A) and STC1 (B) promoters in the contractile form of RAOSMCs. Cells were transiently cotransfected with a reporter plasmid containing a promoter region of rat MGP or STC1 gene and an internal control plasmid (phRL-tk) and were treated with T_3 for 2 days. The cell extracts were assayed for luciferase activity. Values are normalized to the background (luciferase activity of the cells transfected with promoterless pGL3-Basic vector and phRL-tk) and expressed as means±SEM (n=6); *$P<0.05$ vs control.

NCBI (accession numbers AY750958 and AY750959, respectively). Transcription Element Search System (TESS)[22] revealed that consensus sequences of the thyroid hormone response element were located in 585 to 600 (*TGTAC-CCCAATGAACC*) and 1009 to 1024 (*TGGAGA*CAGG*AG-GACA*) bases upstream of the putative transcription initiation sites of MGP and STC1 genes, respectively. Treatments of the cells with T_3 (15 pmol/L to 1 nmol/L) for 48 hours resulted in significant increases in transcriptional activity compared with vehicle treatment (Figure 3).

Regulation of MGP and STC1 Expression via TRs

Arterial smooth muscle cells express TRα1 and TRα2 nuclear receptor isoforms strongly and TRβ1 and TRβ2 relatively weakly.[10] To evaluate the involvement of TRs in the T_3-induced upregulation of MGP and STC1 mRNAs, RNA interference (RNAi) was performed using StealthRNAi specific for the TRα gene encoding TRα1 and TRα2. The RNAi in RAOSMCs for 2 days significantly attenuated mRNA expression of TRα1 and TRα2 by 66% and 57%, respectively, compared with the controls (Figure 4). The RNAi was also associated with upregulation of MGP mRNA by 32%

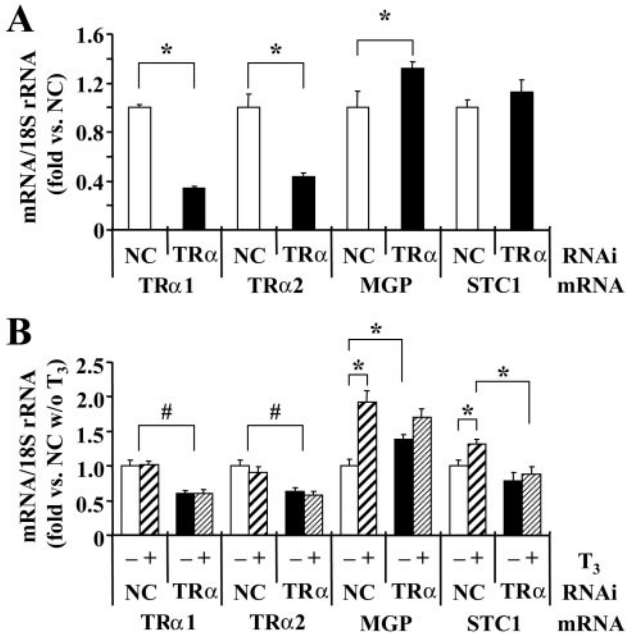

Figure 4. The RNAi against TRα gene in RAOSMCs (synthetic form). A, RNAi against TRα gene for 2 days led to significant decreases in expression of TRα1 and TRα2 to the same extent and a slight increase in the MGP expression. B, The expression of TRα1 and TRα2 remained reduced after the following incubation for 2 more days and was not altered by T_3 (15 pmol/L fT_3). The RNAi was associated with loss of responsiveness of MGP and STC1 mRNA to T_3. Values are expressed as means±SEM (n=4 to 6 [A] and 8 [B]). NC indicates negative control. *$P<0.05$ (Student *t* test [A] or Student-Newman–Keuls test [B]); #$P<0.05$ association with RNAi (two-way ANOVA).

compared with the controls, suggesting that unliganded TRα1 or TRα2, which has no affinity for thyroid hormones, had an inhibitory effect on the expression of MGP. The mRNA level of STC1 was not altered by the RNAi. Incubation with 15 pmol/L fT_3 for the following 2 days facilitated the expression of MGP and STC1 in the control group, whereas no significant effect of T_3 on MGP and STC1 gene expression was detected in the RNAi group. T_3 had no effect on the expression of the TRα isoforms.

Calcium Accumulation in Hypothyroid Rat Aorta

As examined by hematoxylin-eosin staining, the cross-sections of aorta from rats treated with MMI for 4 weeks did not show obvious calcified foci (Figure 5A). However, *o*-cresolphthalein complexone experiments indicated that the 4-week treatment with MMI significantly increased the calcium content in the rat aortic smooth muscle tissues by 33% compared with that of euthyroid animals (Figure 5B). Quantitative RT-PCRs revealed that mRNA levels of MGP, BMP4, ON, and Cbfa1 were downregulated by 68%, 87%, 69%, and 72%, respectively, by the MMI treatment. OPN, BMP2, and STC1 mRNA levels were not significantly altered (Figure 5C). MMI also attenuated protein expression of MGP by 54% (Figure 5D). Calcified foci were not observed even in aortic cross-sections from rats treated with MMI for 12 weeks (online Figure II), suggesting that the calcification was not progressive.

Calcium Content in Hyperthyroid Rat Aorta

Hyperthyroidism elicits pronounced vascular relaxation.[9] However, the effect of hyperthyroidism on vascular calcification has been unclear. Therefore, it is of interest to compare effects of hyperthyroidism on the expression profiles of calcification-associated genes with those of hypothyroidism. In contrast to the aortic smooth muscle from hypothyroid rats, daily injections of T_3 for 10 days led to a decrease in the calcium content in the rat aortic smooth muscle tissues by 11% compared with that of euthyroid animals (Figure 6A). Quantitative RT-PCRs showed that mRNA levels of MGP, OPN, and BMP2 were upregulated by 4.5-fold, 4.9-fold, and 3.4-fold, respectively, by the T_3 treatment, whereas hyperthyroidism resulted in a significant decrease in the level of STC1 mRNA (Figure 6B).

Upregulation of MGP mRNA in Cultured HCASMCs

To demonstrate that thyroid hormone regulates MGP gene expression in vascular smooth muscle cells of a different species, we determined MGP mRNA levels in HCASMCs in the presence and absence of T_3. Treatment with a physiological concentration (15 pmol/L) of T_3 for 2 days led to a significant increase in MGP mRNA by 40% (1.0±0.07 [hypothyroid 0 pmol/L T_3] versus 1.40±0.13 [euthyroid 15 pmol/L T_3] in an arbitrary unit; n=12).

Discussion

In the present study, thyroid hormone led to an upregulation of MGP in arterial smooth muscle cells in vitro regardless of culture condition, phenotype, and animal species of the cells. The transcriptional activity of the MGP gene was increased by T_3, and reduction of TRα gene expression led to a loss of responsiveness of the MGP gene to T_3, suggesting that the effect of T_3 is based on a genomic action via TRα1. Furthermore, in vivo hormone levels were positively and negatively associated with the expression of MGP and calcification in vascular smooth muscle, respectively. Because aortic smooth muscle from hypothyroid rats showed no obvious neointimal formation, the vascular calcification under the hypothyroidism is likely to be similar to that of media sclerosis. In aortic smooth muscle from hypothyroid rats, expression levels of calcification activators BMP4, ON, and Cbfa1 were decreased, whereas hyperthyroidism upregulated another calcification activator BMP2. However, calcium content in aortic smooth muscle was increased in hypothyroidism, and the opposite was observed in hyperthyroidism, suggesting that the expression or function of calcification inhibitors, such as MGP and OPN, are more dominant for the phenotypic outcome in vivo, compared with those of the calcification activators.

MGP is a mineral-binding extracellular matrix protein synthesized by vascular smooth muscle cells and chondrocytes. Luo et al have shown that ablation of MGP gene in mice causes extensive and lethal calcification and cartilaginous metaplasia of the media of all elastic arteries, indicating that MGP has an inhibitory effect on media calcification in vivo.[23] In contrast, in the same study, morphological analysis showed that heterozygous MGP knockout mice, which had a

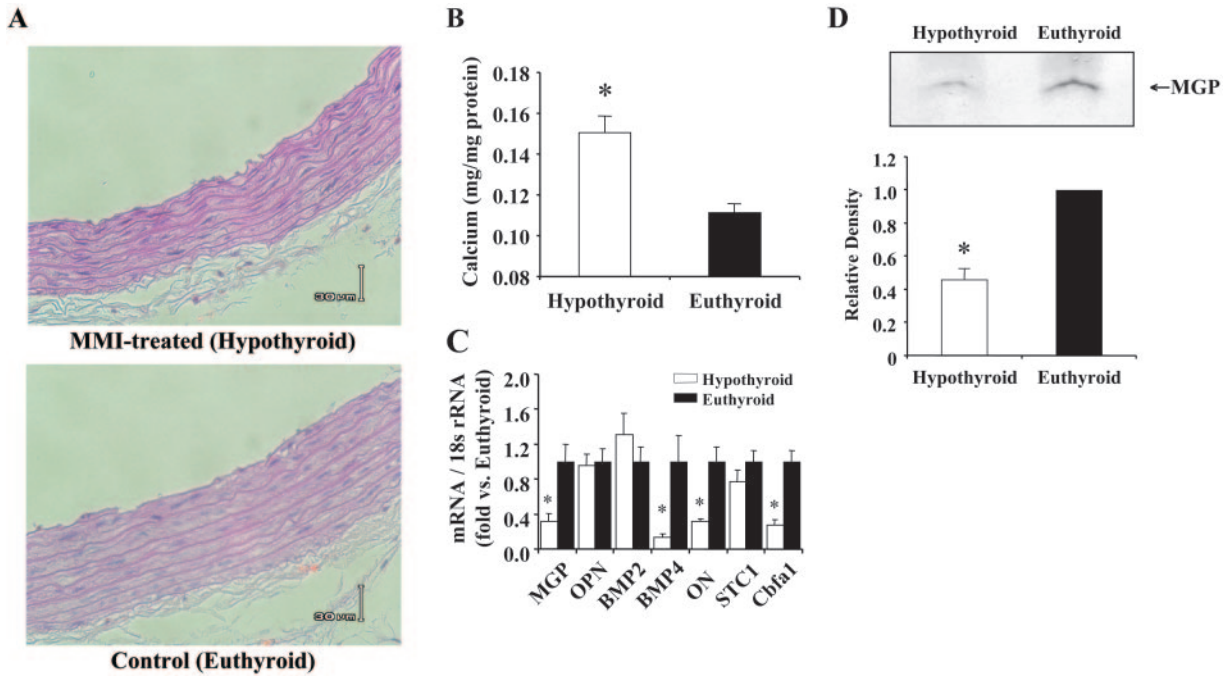

Figure 5. Hypothyroidism led to calcium accumulation in aortic smooth muscle and an altered expression profile of calcification-associated genes. Hypothyroidism was achieved by treatment of the animals with MMI (400 mg/L drinking water) for 4 weeks. A, Hematoxylin-eosin staining of cross-sections of aortae from euthyroid and hypothyroid rats. No obvious calcified foci were observed in either section. Bar=30 μm. B, Effect of the hypothyroidism on calcium deposition in rat aortic smooth muscle. C, Effect of hypothyroidism on mRNA expression of calcification-associated genes in rat aortic smooth muscle. D, Effect of hypothyroidism on MGP protein expression in rat aortic smooth muscle. Values are expressed as means±SEM (n=7 to 8); *$P<0.05$ vs euthyroid.

similar decrease in an arterial MGP level to that by the MMI treatment in the present study, had no obvious calcified foci in arterial smooth muscle. However, this does not necessarily imply that the heterozygous ablation of MGP gene does not affect calcium content in arteries because biochemical quantification for tissue calcium has not been performed. Thus, it is still possible that there is a dose-response relationship between MGP and media calcification, and that an ≈50% reduction of MGP results in some increase in calcium content, although it may not be morphologically evident.

The extracellular environment around smooth muscle cells is known to determine a functional role of MGP. Namely, calcification of vascular smooth muscle cells in the presence of a relatively high concentration of BMP2 was inhibited by MGP, whereas calcification of vascular smooth muscle cells under a low concentration of BMP2 was stimulated by MGP.[20] Moreover, extracellular matrix proteins, especially Col4, have significant influence on MGP function and vascular calcification.[21] In fact, the effect of T_3 on calcification of RAOSMCs was determined by these environmental factors. Therefore, these results suggest that T_3 regulates smooth muscle cell calcification, at least partly, by promoting MGP expression, although not only vascular cells but also migratory adventitial pericytic myofibroblasts and circulating skeletal progenitors may have some additional contributions to vascular calcification in vivo.[24]

STC1 is a mammalian homolog of stanniocalcin, the fish calcium/phosphate- regulating polypeptide that inhibits calcium flux into cells and stimulates phosphate reabsorption. In the present study, gene transcription of STC1 appeared to be regulated by T_3 via TRα in a dose-dependent manner. However, the highest mRNA expression of STC1 tended to be achieved at a euthyroid status in vitro and in vivo, and hyperthyroidism significantly attenuated the STC1 mRNA expression in vivo. Recently, STC1 was shown to accelerate osteoblast development in an autocrine/paracrine manner in cultured fetal rat calvaria cells.[25] Therefore, the downregulation of STC1 may also contribute to the low calcium content in aortic smooth muscle of hyperthyroid rats, although the mechanism that offsets the increase in the STC1 gene transcription remains to be elucidated.

OPN is known to inhibit or promote vascular smooth muscle calcification in vivo in a phosphorylation-dependent manner.[26] Therefore, the changes in its expression in the synthetic form of RAOSMCs and in smooth muscle tissue of hyperthyroid rats may be also associated with the effect of thyroid hormone on calcium accumulation. However, as shown in the in vitro and MMI experiments, a physiological concentration of thyroid hormone is unlikely to target OPN gene directly, at least in the contractile form of aortic smooth muscle cells.

BMP2 was upregulated by hyperthyroidism in vivo, whereas BMP4 was downregulated by MMI-induced hypothyroidism. BMP2 is know to antagonize the effect of MGP,[20] and BMP4 has been suggested to play a significant role as a cytokine, a growth factor or a media-calcification promoter in vascular lesions of calciphylaxis.[27] The mRNA levels of ON and Cbfa1 were also decreased in hypothyroid rat aortic smooth muscle. With the exception of BMP4, these changes in vivo did not follow on from the in vitro experiments,

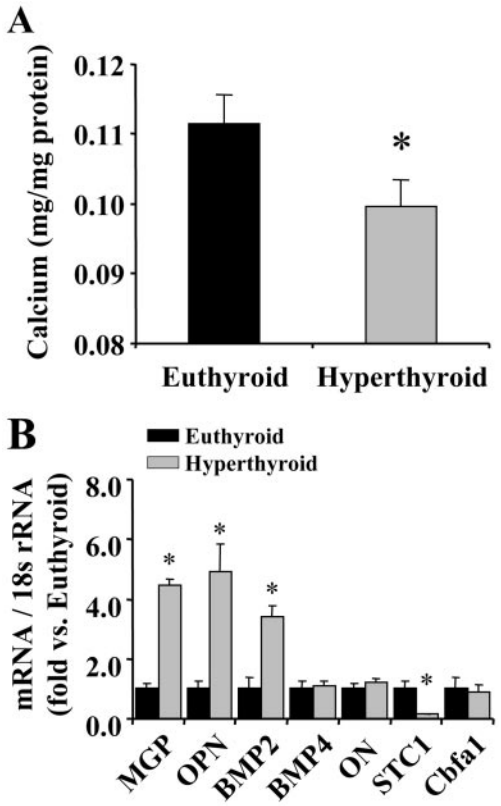

Figure 6. Hyperthyroidism decreased tissue calcium content in aortic smooth muscle and altered the expression profile of calcification-associated genes. Hyperthyroidism was achieved by daily injection of T_3 (0.2 mg/kg body weight IP) for 10 days. A, Effect of the hyperthyroidism on calcium deposition in rat aortic smooth muscle. C, Effect of hyperthyroidism on mRNA expression of calcification-associated genes in rat aortic smooth muscle. Values are expressed as means±SEM (n=7 to 8); *$P<0.05$ vs euthyroid.

suggesting that the alterations were not attributable to a direct effect of thyroid hormone on vascular smooth muscle cells. Because physiological concentrations of thyroid hormones upregulated BMP4 in the contractile form of RAOSMCs and in vivo (hypothyroid versus euthyroid), BMP4 may be another direct target of thyroid hormone in aortic smooth muscle cells. However, the expected influences of the changes in all the calcification activators above were apparently masked, aforementioned, indicating their minor roles in vascular calcification, compared with those of calcification inhibitors.

In summary, our findings, for the first time, demonstrate that a physiological concentration of thyroid hormone has significant genomic effects on vascular smooth muscle cells in vitro and in vivo, which are associated with vascular calcification. Most notably, a decrease in thyroid hormone and the concomitant increase in vascular calcification in vivo are marked by a decrease in the level of MGP expression, suggesting that a physiological concentration of thyroid hormone has a direct protective role against vascular smooth muscle calcification in vivo. Although vascular calcification has been thought to be benign, arterial calcification should alter vascular compliance. Thus, it is possible that the increased vascular stiffness underlies the high systemic vascular resistance observed in hypothyroidism. Vascular calcification can lead to some other serious problems, including vascular stenosis, calciphylaxis, and even sudden death. Recently, a polymorphism in the promoter region of MGP gene was found to have a significant association with myocardial infarction in low-risk individuals and femoral calcification in the presence of atherosclerotic plaques, suggesting involvement of this mutation in coronary artery disease.[28] In addition, a recent meta-analysis of coronary artery calcium scores suggested that the calcium score is an independent risk factor to predict coronary heart disease events.[29] Therefore, further studies on the protective role of thyroid hormone and MGP against vascular smooth muscle calcification should provide insights into novel therapeutic strategies for the high systemic vascular resistance and blood pressure of hypothyroid patients, as well as for diseases associated with cardiovascular calcification such as diabetes, chronic renal insufficiency, and hypercholesterolemia.

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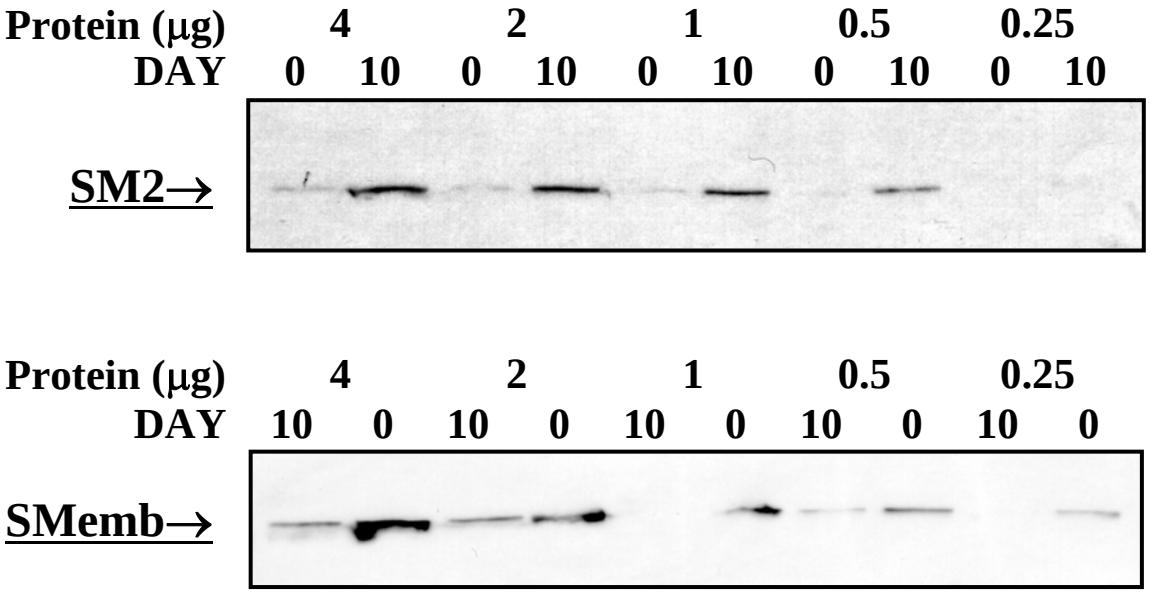
Supplement Figure 1



Representative images of immunoblots for SM2 (smooth muscle myosin heavy chain-2) and SMemb (nonmuscle myosin heavy chain) in RAOSMCs before and after treatment with the differentiation medium for 10 days. The differentiation medium altered the protein expression of SM2 and SMemb by 11.6 ± 2.8 fold (n=5) and 0.28 ± 0.04 fold (n=4), respectively.

Supplement Figure 2

MMI-treated (Hypothyroid)

Control (Euthyroid)

Hematoxylin-Eosin staining of cross sections of aortas from a hypothyroid rat treated with methimazole (MMI, 400 mg/L drinking water) for 12 weeks and a euthyroid control rat. Bar=30μm.

Suplement Table 1. Sequences of Oligonucleotides for Quantitative RT-PCRs

BMP2:	forward primer:	5’-TGAACACAGCTGGTCTCAGGTAA-3’
	reverse primer:	5’-TGTGTTTGGCTTGACGCTTT-3’
	TaqMan probe:	5’-AGTGACTTTTGGCCACGACGGTAAAGG-3’
BMP4:	forward primer:	5’-TCAGCAGCATCCCAGAGAATG-3’
	reverse primer:	5’-TTATACGGTGGAAGCCCTGTTC-3’
	TaqMan probe:	5’-TCTGCAGAGCTCCGGCTATTTCGG-3’
Cbfa1:	forward primer:	5’-GTCTTCACAAATCCTCCCCAAGT-3’
	reverse primer:	5’-GAGGCGGTCAGAGAACAAACTAG-3’
	TaqMan probe:	5’-TATTAAAGTGACAGTGGACGGTCCCCGG-3’
MGP (rat):	forward primer:	5’-CGGAGAAATGCCAACACCTT-3’
	reverse primer:	5’-CGCTCACACAGCTTGTAGTCATC-3’
	TaqMan probe:	5’-ATGGCACGCTAAAGCCCAGGAAAGA-3’
MGP (human):	forward primer:	5’-TTCATATCCCCTCAGCAGAGATG-3’
	reverse primer:	5’-GCGATTATAGGCAGCATTGTATCC-3’
	TaqMan probe:	5’-AACGCTCTAAGCCTGTCCACGAGCTCAATA-3’
ON:	forward primer:	5’-CACCTGGACTACATCGGACCAT-3’
	reverse primer:	5’-TTGTTGCCCTCATCTCTCTCGTA-3’
	TaqMan probe:	5’-CGTGACTGGCTCAAAAACGTCCTGGT-3’
OPN:	forward primer:	5’-TGACCACATGGACGATGATGA-3’
	reverse primer:	5’-GCTTGTGTGCTGGCAGTGA-3’
	TaqMan probe:	5’-ACGGAGACCATGCAGAGAGCGAGGAT-3’
STC1:	forward primer:	5’-GGATCACCTCCAAGGTCTTCCT-3’
	reverse primer:	5’-GGCAATGCTGCAAACATTGA-3’
	TaqMan probe:	5’-TTCGGAGGTGTTCTACTTTCCAGAGGATGA-3’
TRα1	forward primer:	5’-AGCTGCTGATGAAGGTGACTGA-3’
	reverse primer:	5’-GCTTAGACTTCCTGATCCTCAAAGAC-3’
	TaqMan probe:	5’- TCCACATGAAAGTCGAGTGCCCCA-3’
TRα2	forward primer:	5’-GGCAATACCTTGTCCCTTTGAG-3’
	reverse primer:	5’-CCACGTAAGCACAGACAACTATTTC-3’
	TaqMan probe:	5’-ACTCAAGTGTCACCTCCTTCCCCAGCTC-3’

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Thyroid Hormone Targets Matrix Gla Protein Gene Associated With Vascular Smooth Muscle Calcification

Yoji Sato, Ryo Nakamura, Mitsutoshi Satoh, Kayoko Fujishita, Satoko Mori, Seiichi Ishida, Teruhide Yamaguchi, Kazuhide Inoue, Taku Nagao and Yasuo Ohno

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