Chapter 8

Thyroid hormone modulates murine coagulation through immediate and late effects on hepatic and endothelium-associated coagulation gene transcription

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Summary

Background: Thyroid dysfunction is associated with changes in coagulation.

Objective: To gain more insight in the role of thyroid hormone in coagulation control.

Methods: C57Black/6J mice received a low-iodine diet and drinking water supplemented with perchlorate to suppress endogenous triiodothyronine (T_3) and tetraiodothyronine (T_4) production. Under these conditions, the impact of exogenous T_3 on plasma coagulation, and hepatic and endothelium-associated coagulation gene transcription was studied.

Results: A single T_3 injection (0.5µg/mouse) increased hepatic transcript levels of the T_3-responsive genes deiodinase type 1 and Spot14 within 4 hours. This coincided with significantly reduced mRNA levels of fibrinogen-γ, antithrombin, protein C, protein Z and protein Z-related protease inhibitor (ZPI), and the reduction of the latter three persisted upon daily treatment with T_3 for 14 days. Prolonged T_3 treatment also induced a significant down-regulation in factor (F) II, IX and X transcript levels, while FXI and FXII levels increased. Activity levels in plasma largely paralleled these mRNA changes. Thrombomodulin transcript levels in the lung were significantly up-regulated after a single T_3 injection, which persisted upon prolonged T_3 exposure. Two-week T_3 administration also resulted in increased von Willebrand factor (VWF) and tissue factor pathway inhibitor (TFPI) mRNA levels, whereas tissue factor levels decreased.

Conclusions: Our mouse data show that T_3 has profound effects on coagulation, with fibrinogen-γ, antithrombin, protein C, protein Z, ZPI and thrombomodulin responding rapidly, making these likely direct thyroid hormone receptor targets. FII, FIX, FX, FXI, FXII, VWF, TF and TFPI are late responding genes and therefore probably indirectly regulated by T_3.

Introduction

Abnormalities of blood coagulation are common in patients with thyroid dysfunctions. In general, hyperthyroidism is associated with a hypercoagulable state and increased risk for venous thrombosis.[1] In hypothyroidism, the severity of the disorder determines whether the coagulation profile is shifted towards an anticoagulant or procoagulant state as subclinical hypothyroidism is associated with hypercoagulation whereas patients with overt hypothyroidism have a bleeding tendency.[2,3]

The mechanism of action of thyroid hormones and thyroid hormone receptors has been well established in a number of metabolic processes, including lipoprotein homeostasis, cell proliferation and mitochondrial respiration.[4-6] Experimental evidence regarding the mechanisms by which thyroid hormones modulate blood coagulation is more limited, and consists mainly of *in vitro* data showing that T_3 is able to modulate transcript levels of fibrinogen, factor (F) II, FX and antithrombin.[7-9] *In vivo* studies with thyroid hormone receptor knock-out mice identified fibrinogen-γ to be modulated by T_3, whereas T_3 administration in thyroidectomized rats was able to increase fibrinogen-α, FII and FX mRNA levels.[5,9]

Here, we used an *in vivo* approach to study the modulatory role of thyroid hormone on plasma coagulation and transcript levels of coagulation genes, by treating mice with the active thyroid hormone, i.e. triiodothyronine (T_3), under conditions of a suppressed endogenous thyroid hormone production. Our mouse data demonstrate that under these conditions, T_3 is able to modulate coagulation by controlling transcription of coagulation genes, and that these effects can be both immediate and late.

Material and Methods

Animal experiments

Eight week old C57Bl/6J mice were purchased from Charles River (Maastricht, the Netherlands) and fed a low iodine diet (ICN Biomedicals, Inc.) while drinking water was supplemented with 1% (wt/vol) potassium perchlorate (Sigma Aldrich) to suppress endogenous thyroid hormone production. 3,3′,5-Triiodo-L-thyronine sodium salt (T_3; Sigma Aldrich) stocks of 1 mg/ml were prepared in 4 mM sodium hydroxide and stored at 4°C until use. Before injections, the T_3 stock was diluted (0-10 µg T_3 /200 µl) in phosphate buffered saline (PBS) supplemented with 0.02% bovine serum albumin with a final concentration of 0.2 mM sodium hydroxide. To determine the effects of a prolonged T_3 exposure on transcription as well as plasma

levels of coagulation factors, mice received a daily intraperitoneal injection of 200 µl T_3 for 14 days. To determine which factors are rapidly modulated by T_3, a single dose of either 0.5 µg T_3/mouse or vehicle (PBS supplemented with 0.02% bovine serum albumin with a final concentration of 0.2 mM sodium hydroxide) was administered. After the last administration, mice were anesthetized by an intraperitoneal injection with a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg) after which the abdomen was opened by a midline incision and a blood sample on sodium citrate (final concentration 0.32%) was drawn from the inferior caval vein. Platelet-poor plasma was obtained and stored at -80°C until use. The liver was isolated and weighed, and part of the left liver lobule and the lungs were snap-frozen for mRNA analyses. All experimental procedures were approved by the animal welfare committee of the Leiden University.

Plasma analyses

Plasma triiodothyronine (T_3) and tetraiodothyronine (T_4) levels were measured with in-house radioimmunoassays as previously described.[10] Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were determined using routine clinical chemistry assays. Plasma FII and FX activity were analyzed by means of chromogenic substrate conversion, factor VII activity was evaluated using the commercially available Biophen FVII kit (Hyphen Biomed) and activity levels of factor VIII, IX, XI, XII were measured in APTT based assays.[11] Plasma fibrinogen and protein C antigen levels were assessed with a commercial murine ELISA kit from Affinity Biologicals and an in-house ELISA using antibodies from Haematologic Technologies Inc, respectively. Antithrombin activity was measured by means of the Coamatic Antithrombin kit (Chromogenix). For all plasma assays of individual coagulation factors, pooled normal mouse plasma was used to generate standard curves and the vehicle-treated group was subsequently set as a reference (100%).

Global coagulability of the plasma was determined by measuring the activated partial thromboplastin time (APTT) using the STA Neoplastine Plus reagent on the STart 4 analyzer (Diagnostica Stago). The prothrombin time was determined with the Simple Simon PT system (Zafena) and thrombin generation was assessed by means of the Calibrated Automated Thrombogram, using 5 pM tissue factor (Thrombinoscope) to trigger 1:6 diluted mouse plasma. Thrombin generation was measured on the Fluoroskan Ascent reader (Thermo Scientific) and the curves and

area under the curve (endogenous thrombin potential; ETP) were calculated using the Thrombinoscope software.

RNA isolation and real-time RT-PCR

Individual liver (20-30 mg) and lung samples (40-50 mg), as a substitute for the endothelium, were homogenized in RNAzol (Tel-Test) and RNA isolation and cDNA synthesis was performed as previously described.[11] Quantitative real-time PCR using SybrGreen (Applied Biosystems) and gene-specific primers (supplemental table S1) was performed on the ABI Prism 7900 HT Fast Real-Time PCR System from Applied Biosystems. Data were analyzed using the accompanying Sequence Detection System software. The comparative threshold cycle method with β-actin as internal control was used for quantification and normalization. Vehicle-treated animals were set as a reference and the ΔC_t values of the individual samples were related to the mean ΔC_t of the reference group.

Statistical analyses

Data are analyzed with the GraphPad Instat software and statistical differences between groups of the dose-finding were evaluated using a one-way analysis of variance (ANOVA) with a Dunnett post-hoc test. For studies using only one dose of thyroid hormone, a Student's t-test was used to compare the thyroid hormone- and vehicle-treated groups. A p-value of <0.05 was considered to be statistically significant.

Results

Dose-finding study

A dose-finding study was performed in which the effects of exogenous T_3 in a range from 0-10 µg/mouse/day (n=6 per group) was tested in order to determine specific T_3-induced effects on a representative panel of coagulation factors, and to establish an optimal T_3 dose to use for further evaluation.

Increasing amounts of exogenous T_3 in hypothyroid mice, i.e. having a suppressed endogenous thyroid hormone production, resulted in a dose-dependent increase in body weight up to a dose of 5 µg/day (weight gain during 14 days: 0.18±0.26 g vs. 2.26±0.36 g, $p<0.01$), whereas the liver weight dose-dependently decreased (1.51±0.07 g vs. 1.09±0.01 g, $p<0.01$; table S2). As expected, plasma T_3 levels increased dose-dependently from 0.31±0.01 nmol/L for vehicle-treated hypothyroid mice to a maximum of 51.2±5.8 nmol/L for mice treated with 10 µg T_3/day ($p<0.01$).

T_4 levels were low (range 8.8±0.6 – 14.0±1.0 nmol/L) and did not differ between treatment groups (table S2). Plasma T_3 and T_4 levels of regular C57Bl/6J mice were measured for reference values, being 1.22±0.04 nmol/L and 60.7±4.2 nmol/L respectively, indicating that the endogenous thyroid hormone production in our experimental set-up was successfully suppressed.

Circulating liver enzymes were determined as a marker of possible liver damage due to T_3 administration and showed that ALT and AST levels were not affected in T_3-treated mice as compared to vehicle treatment. However, administration of increasing amounts of T_3 (from 0.5 µg/day upwards) resulted in relatively modest increased ALP levels that differed significantly from vehicle-treated mice (61.3±1.0 U/L vs. 140.0±15.1 U/L for 0 and 0.5 µg T_3/mouse/day respectively, $p<0.01$; table S2).

Plasma levels of a limited but representative panel of coagulation factors were measured and revealed for fibrinogen antigen and factor II, X and antithrombin activity levels dose-dependent decreases (maximal effects -60%, -50%, -30% and -30% respectively for the 10 µg T_3/day dose), whereas FXII activity was increased (+30%), and as compared to the vehicle treatment these effects were significantly different from a dose of 0.5 µg upwards (table S2). FIX was not significantly affected and FVIII levels showed no unambiguous dose-dependent increase or decrease as compared to vehicle-treated mice.

As doses of 5 and 10 µg T_3/day had no additional effects on plasma coagulation factor levels, these doses were excluded from subsequent mRNA analyses. In the range from 0–1 µg T_3/mouse/day, hepatic transcript levels of deiodinase type 1 (D1), a well-established T_3 response gene,[12] was dose-dependently up-regulated as expected (table S3). Dose-dependent decreases in plasma fibrinogen and FII levels coincided with reduced transcript levels, which were significantly different in mice treated with 0.5 µg T_3/day as compared to vehicle-treated animals (-50% for fibrinogen and -35% for FII), whereas the transcript levels of FXII and antithrombin were not significantly affected (table S3). Transcript levels of the endothelium-associated coagulation factors were determined in the lung as a substitute for the endothelium since this organ is highly vascularized. Increasing T_3 doses resulted in a dose-dependent decrease of tissue factor mRNA levels (-30%) and a rise in thrombomodulin levels (+55%). Transcript levels of von Willebrand factor (VWF) and the endothelial protein C receptor (EPCR) were not affected (table S3).

Based on these data, a daily dose of 0.5 µg T_3/mouse/day was chosen for further studies, as this caused higher plasma T_3 levels as compared to regular C57Bl/6J

mice and was the lowest dose able to induce nearly maximal changes in both plasma and transcript levels of coagulation factors.

Prolonged T_3 treatment

To evaluate the effects of T_3 on coagulation in more detail, 12 mice with a suppressed thyroid hormone production per group were treated with either the vehicle (0 µg T_3/mouse/day) or a dose of 0.5 µg T_3/mouse/day for 2 weeks. This treatment regime again resulted in an increase in body weight (weight gain: 0.24±0.15 g vs. 2.12±0.20 g, $p<0.001$) and a reduction in liver weight (0.84±0.02 g vs. 0.74±0.02 g, $p<0.001$). As expected, T_3 levels were significantly higher in T_3-treated mice (5.64±0.21 nmol/L vs. 0.33±0.01 nmol/L, $p<0.001$), whereas T_4 levels were low and did not differ between treatment groups (7.2±0.2 nmol/L vs. 8.7±1.0 nmol/L).

Figure 1A shows that, in line with the dose-finding study, plasma FII and FX activity levels decreased upon T_3 treatment, while FXII levels increased and FVIII and FIX levels were not significantly affected. In addition, FVII levels did also not differ between vehicle- and T_3-treated animals, whereas FXI activity levels increased due to T_3 administration. Plasma antithrombin and protein C levels were both significantly lower in T_3-treated mice (100±1.6% vs. 92.6±1.2%, $p<0.001$ for antithrombin; 100±4.0% vs. 85.9±3.1%, $p=0.009$ for protein C).

Consistent with the lower FII and FX levels upon T_3 treatment, the prothrombin time was longer in T_3-treated animals and the increased FXI and FXII levels resulted in a shorter APTT (fig. 2). To assess the overall plasma coagulability, thrombin generation was measured, showing a lower endogenous thrombin potential in mice treated with 0.5 µg T_3/day as compared to vehicle-treated mice, which was mainly due to a lower peak height and an earlier onset of the inhibition of thrombin activity (fig. 2C).

With the exception of FIX levels, the T_3-induced effects on plasma proteins were completely paralleled by changes in hepatic transcript levels (fig. 1A and 1B). The transcript levels of additional anticoagulant genes and the fibrinolytic factors plasminogen and α_2-antiplasmin were determined and presented in figure 3A. Strikingly, T_3 administration caused only significant decreases, no increases, in mRNA levels of anticoagulant factors which included protein C, protein S, protein Z, protein Z-related protease inhibitor (PZI) and plasminogen. Antithrombin, heparin cofactor II and α_2-antiplasmin levels were not affected.

In line with the dose-finding study, tissue factor transcript levels in the lung were reduced and thrombomodulin levels were up-regulated upon two-week T_3 exposure (fig. 4A). In addition, VWF levels now showed a significant increase, as well as the mRNA levels of tissue factor pathway inhibitor (TFPI), whereas EPCR levels remained unaffected by T_3 treatment.

These data show that prolonged T_3 exposure is able to modulate the plasma coagulation profile by controlling hepatic transcript levels of coagulation genes. In addition, transcription of endothelium-associated coagulation genes, measured at the level of the lung, can also be modulated by T_3 administration.

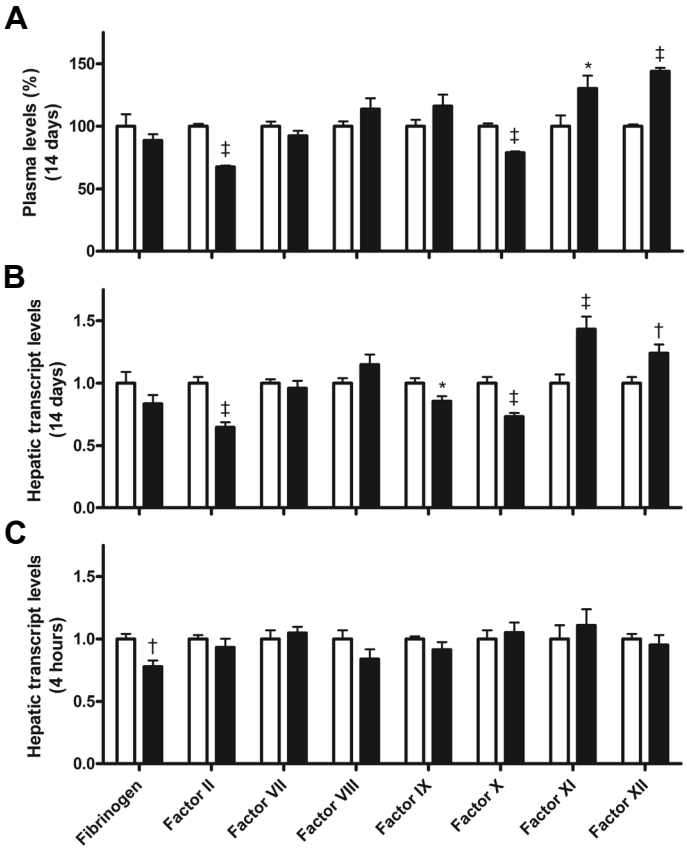

Figure 1: Plasma (A) and hepatic transcript levels (B and C) of procoagulant coagulation factors in mice treated with 0 µg T_3 (white bars) or 0.5 µg T_3 (black bars) for 14 days (panels A and B). Panel C shows the T_3-induced changes in hepatic transcript levels 4 hours after a single T_3 injection. Data are presented as mean±standard error of the mean (panel A) or mean with the error bar representing the calculated maximum expression level (panels B and C) of n=12 mice per group, with the vehicle-treated group set as a reference. Relative expression levels (B and C) were compared using the comparative threshold cycle method with ß-actin as internal control. *$p<0.05$, $^{\dagger}p<0.01$ and $^{\ddagger}p<0.001$ as compared to vehicle-treated mice.

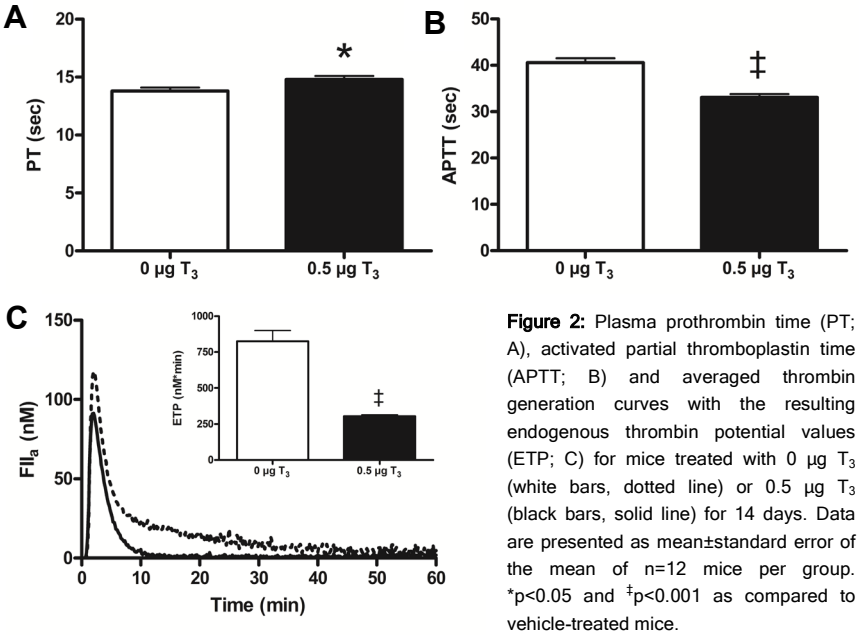

Figure 2: Plasma prothrombin time (PT; A), activated partial thromboplastin time (APTT; B) and averaged thrombin generation curves with the resulting endogenous thrombin potential values (ETP; C) for mice treated with 0 µg T_3 (white bars, dotted line) or 0.5 µg T_3 (black bars, solid line) for 14 days. Data are presented as mean±standard error of the mean of n=12 mice per group. *$p<0.05$ and ‡$p<0.001$ as compared to vehicle-treated mice.

Single T_3 dose

In order to determine whether T_3 is able to directly affect transcription of coagulation genes, i.e. via a direct interaction between the ligand-bound thyroid hormone receptor and the promoter region of coagulation genes, we examined the mRNA levels 4 hours after a single T_3 injection (0.5 µg/mouse).

Already within these 4 hours, the mRNA levels of the canonical T_3-responsive genes D1 and Spot14 were increased (1±0.18 vs. 136.4±29.3 for D1 and 1±0.36 vs. 5.80±1.26 for Spot14). Under these conditions, hepatic transcript levels of fibrinogen-γ and antithrombin were significantly reduced (fig. 1C and 3B), whereas these effects were not apparent after prolonged T_3 exposure. Although the immediate decrease of protein S mRNA levels was comparable to the reduction observed after a prolonged T_3 treatment, this did not reach statistical significance. Protein C, protein Z and PZI transcript levels were significantly affected after both the prolonged T_3 administration and a single dose of T_3. In contrast to the two-week T_3 treatment, a single T_3 dose was not able to induce significant changes in FII, FX, FXI, FXII and plasminogen transcript levels (fig. 3B). Transcript levels of most endothelium-associated clotting factors in the lung remained unaffected, however, thrombomodulin transcript levels increased markedly after a single T_3 injection (fig. 4B).

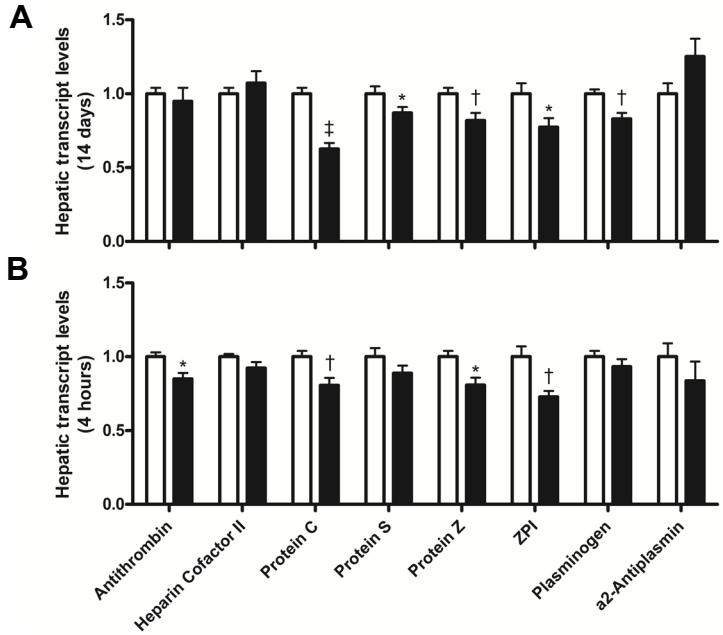

Figure 3: Hepatic transcript levels of anticoagulant and fibrinolytic factors in mice treated with 0 µg T_3 (white bars) or 0.5 µg T_3 (black bars) for 14 days (A) or given a single dose (B). Data are presented as mean with the error bar representing the calculated maximum expression level of n=12 mice per group and the vehicle-treated group set as a reference. Relative expression levels were compared using the comparative threshold cycle method with ß-actin as internal control. *p<0.05, †p<0.01 and ‡p<0.001 as compared to vehicle-treated mice.

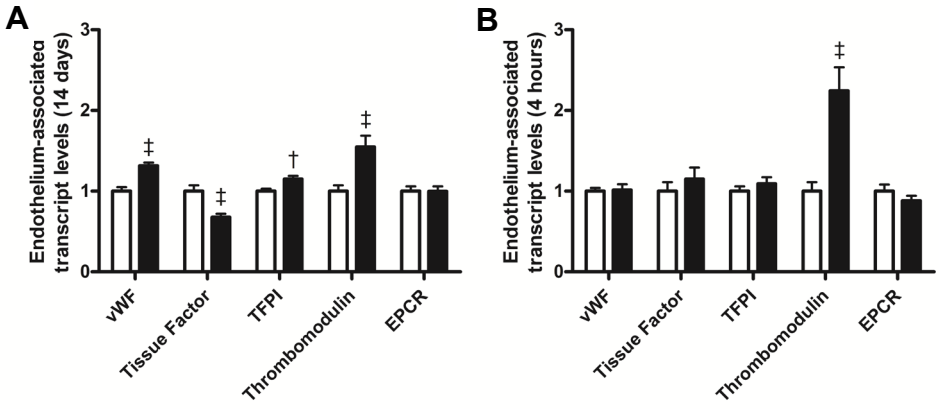

Figure 4: Transcript levels of endothelium-associated coagulation factors measured in mice treated with 0 µg T_3 (white bars) or 0.5 µg T_3 (black bars) for 14 days (A) or given a single dose (B). Data are presented as mean with the error bar representing the calculated maximum expression level of n=12 mice per group and the vehicle-treated group set as a reference. Relative expression levels were compared using the comparative threshold cycle method with ß-actin as internal control. *p<0.05, †p<0.01 and ‡p<0.001 as compared to vehicle-treated mice.

Discussion

Evidence is accumulating that overt hypothyroidism and hyperthyroidism are associated with changes in the hemostatic balance, which translates to either a bleeding tendency or an increased thrombosis risk.[1-3] However, the underlying mechanism how thyroid hormone can modulate coagulation is largely unknown. In the present study, we demonstrate that intraperitoneal administration of the thyroid hormone triiodothyronine (T_3) to mice with a suppressed endogenous thyroid hormone production is able to modulate transcription of both hepatic and endothelium-associated coagulation factors. Fibrinogen-γ, antithrombin, protein C, protein Z, protein Z-related protease inhibitor and thrombomodulin responded rapidly upon a single T_3 injection. On the other hand, factors II, IX, X, XI, XII, von Willebrand factor, tissue factor and tissue factor pathway inhibitor were only modulated after a prolonged T_3 exposure, i.e. 14 days. Although analyzed for a limited set of liver-derived coagulation factors, the changes in transcript levels were largely paralleled by changes in plasma levels. Based on these observations, we conclude that thyroid hormone can have widespread effects coagulation in mice.

Our data are in line with observations by Flores-Morales et al., who showed that T_3 can have both immediate and late effects on mouse hepatic gene transcription, and that these effects can be either up- or down-regulating.[5] The coagulation factors that responded within 4 hours after injection are likely to be directly regulated by thyroid hormone, via interaction with the thyroid hormone receptor and specific response elements in the promoter region of coagulation genes. Surprisingly, most of these genes are regulated in a negative fashion, with the exception of thrombomodulin. Although it is known that transcriptional suppression is a common action of thyroid hormones,[4-6] the mechanisms underlying this negative regulation are poorly understood and may involve binding of co-repressors like NCOR1 or post-transcriptional microRNA binding, while chromatin remodelling and DNA methylation may also play a role in transcriptional suppression.[13,14]

For the genes that require a prolonged T_3 exposure to evoke a clear transcriptional response, an indirect modulation is more likely which can involve intermediate transcription factors additional to the thyroid hormone receptor. Despite the fact that there are many intermediates possible, we hypothesized that the hepatic nuclear factor 4α (HNF4α) would be a good candidate since it is known that thyroid hormone can up-regulate HNF4 expression,[15] and the well-established HNF4α targets coagulation factor XI and XII[16] are clearly up-regulated upon prolonged T_3

exposure. However, the candidature of HNF4α was not supported by the data as we observed a 20% decrease in hepatic mRNA levels in livers of T_3 mice.

Our observations in lung samples as a substitute for the vasculature clearly indicate that also the (micro)vasculature with its endothelium-associated coagulation factors is responsive to T_3, as the levels of most factors analyzed were affected after prolonged T_3 administration. Interestingly, thrombomodulin appeared to be an immediate responder and could be induced by a T_3 dose as low as 0.05 µg/mouse/day, indicating that thrombomodulin transcription is highly sensitive to variation in T_3. Although we were not able to determine whether the changes in thrombomodulin transcript levels translated into altered protein levels, it has been reported that patients with hyperthyroidism have increased levels of circulating soluble thrombomodulin.[17]

In humans, hyperthyroidism is associated with an increased risk for thrombosis, while we showed that thyroid hormone administration in mice results in an increase in prothrombin time and a decrease in the endogenous thrombin potential, which point towards a bleeding tendency instead of a thrombotic tendency. These results can at least be partially explained by the observed decreases in plasma FII and FX levels. On the other hand, the APTT was shorter due to the increased FXI and FXII levels, suggesting a thrombosis-prone condition, which is more in line with what would be expected based on human observations. Altogether, these findings indicate that the use of mice in studying thyroid disorders in human-like coagulopathies, i.e. bleeding or thrombosis, faces limitations.

In conclusion, our mouse study demonstrates that T_3 administration to mice with suppressed endogenous thyroid hormone production has widespread effects on transcription of hepatic and endothelium-associated coagulation genes, as measured at the level of the lung. Furthermore, we identified both immediate and late responding coagulation genes, suggesting that T_3 can either directly or indirectly control transcription. In addition, the transcriptional changes resulted in altered plasma levels as analyzed for a panel of coagulation factors. We believe that this mouse study contributes to a better understanding of the relation between thyroid dysfunctions and coagulation disorders in human.

Acknowledgements

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Table 1: QPCR primer sequences

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
β-Actin	AGGTCATCACTATTGGCAACGA	CCAAGAAGGAAGGCTGGAAAA
Deiodinase type 1	TTGCCTCCACAGCCGATT	TCTTAAAAGCCCAGCCATCTG
Spot14	AAGGTGGCTGGCAACGAA	TCGGCCTCCGTTTC
Fibrinogen-γ	TGCTGCCTGCTTTTACTGTTCTC	TCTAGGATGCAACAGTTATCTCTGGTA
Factor II	GGACGCTGAGAAGGGTATCG	CCCCACACAGCAGCTCTTG
Factor V	CATGGAAACCTTACCGACAGAAA	CATGTGCCCCTTGGTATTGC
Factor VII	CGTCTGCTTCTGCCTAGA	ATTTGCACAGATCAGCTGCTCAT
Factor VIII	CTTCACCTCCAGGGAAGGACTA	TCCACTTGCAACCATTGTTTTG
Factor IX	GCAAAACCGGGTCAAATCC	ACCTCCACAGAATGCCTCAATT
Factor X	GTGGCCGGGAATGCAA	AACCCTTCATTGTCTTCGTTAATGA
Factor XI	GAAGGATACGTGCAAGGGAGATT	CAAGTGCCAGACCCCATTGT
Factor XII	GGGCTTCTCCTCCATCACCTA	GCAACTGTTGGTTTTGCTTTCC

Table 1: QPCR primer sequences (continued)

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
Antithrombin	TGGGCCTCATTGATCTCTTCA	CCTGCCTCCAGCAACGAT
Heparin Cofactor II	GAATGGCAATATGTCAGGCATCT	CACTGTGATGGTACTTTGGTGCTT
Protein C	GCGTGGAGGGCACCAA	CCCTGCGTCGCAGATCAT
Protein S	GGTGGCATCCCAGATATTTCC	CACTTCCATGCAGCCACTGT
Protein Z	GCAGCCAGAGTCAGCCTAGCT	CACGCCGGCACAGAAGTC
PZI	TGGCCCTGGAGGACTACTTG	CCATTTTCCTGGTTTTCATATTCTG
Plasminogen	TGACATTGCCCTGCTGAAAC	CAGACAAGCTGGAATGACTTTATCC
α_2-Antiplasmin	TTCTCCTCAACGCCATCCA	GGTGAGGCTCGGGTCAAAC
Von Willebrand factor	AGCTGTCAGCCAGGTTTTTCTT	GCAGAGGGCAGGCACCTT
Tissue Factor	GGAGGAGCCGCCATTTACA	CTGGCTGTCCAAGGTTTGTGT
TFPI	CCCAGGCGTCGGGATTA	AATCCACTGTCTGCTGGTTGAA
Thrombomodulin	GCGAAATGTTCTGCAATGAAAC	GGCATTCACAAACAGTAGGAGAGTT
EPCR	AACCACATCACCACGCAAAA	CCCAGGACCAGTGATGTGTAAG

PZI: protein Z-related protease inhibitor; TFPI: tissue factor pathway inhibitor; EPCR: endothelial protein C receptor

Table 2: General and plasma coagulation parameters upon increasing doses of T_3

T_3 (µg/mouse/day)	0	0.05	0.1	0.5	1	5	10	p-value
General parameters								
Δ Body weight (g)[a]	0.18±0.26	0.11±0.15	0.50±0.23	1.47±0.20†	1.92±0.15‡	2.26±0.36‡	1.77±0.33‡	<0.001
Liver weight (g)	1.51±0.07	1.30±0.06*	1.28±0.09*	1.24±0.04*	1.21±0.03†	1.09±0.01‡	1.10±0.02‡	<0.001
T_3 (nmol/L)	0.31±0.01	1.46±0.10	2.09±0.12	4.99±0.50	8.78±1.84	26.21±3.18‡	51.20±5.84‡	<0.001
T_4 (nmol/L)	12.0±1.5	9.3±0.8	8.8±0.6	9.6±1.7	11.8±0.7	14.0±1.0	10.0±0.7	0.016
ALT (U/L)	37.5±9.6	30.8±6.3	24.2±3.3	20.1±0.1	30.0±7.1	55.0±7.6	70.0±14.9	0.003
AST (U/L)	98.7±21.9	113.3±7.2	71.7±8.0	71.0±3.4	93.3±13.1	134.2±18.4	156.0±24.0	0.007
ALP (U/L)	61.3±1.0	72.5±3.4	81.7±6.0	140.0±15.1†	166.7±12.3‡	282.5±23.1‡	313.0±11.3‡	<0.001
Plasma coagulation parameters								
Fibrinogen (%)	100±5.2	97.6±3.2	91.7±15.8	59.3±7.3†	50.2±4.5‡	48.7±2.2‡	40.0±0.8‡	<0.001
Factor II (%)	100±1.6	73.3±2.8‡	74.1±2.0‡	59.4±3.5‡	50.5±3.1‡	48.1±2.8‡	50.4±2.3‡	<0.001
Factor VIII (%)	100±7.8	113.9±7.4	125.1±10.4	104.7±6.3	89.5±5.9	86.2±7.9	86.6±7.5	0.008
Factor IX (%)	100±2.9	91.6±3.8	99.2±3.2	92.2±3.5	95.5±3.7	87.7±3.8	91.8±4.2	NS
Factor X (%)	100±2.1	83.5±1.8†	88.1±2.7	79.0±3.2‡	71.3±7.2‡	65.8±2.0‡	68.4±3.4‡	<0.001
Factor XII (%)	100±3.1	117.4±3.2*	122.6±3.6‡	126.9±3.1‡	125.3±6.1‡	118.8±4.4†	128.6±2.9‡	<0.001
Antithrombin (%)	100±2.2	77.4±3.1‡	83.0±3.3‡	65.6±3.4‡	73.3±1.9‡	70.2±1.7‡	66.6±2.9‡	<0.001

[a] Difference in body weight between end and start of T3 treatment. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.
Data are presented as mean±standard error of the mean with the vehicle-treated group set as a reference in coagulation factor analyses. Overall p-values are calculated with an analysis of variance (ANOVA); *p<0.05, †p<0.01, ‡p<0.001 compared to vehicle-treated mice as calculated with a Dunnett post-hoc test.

Table 3: Transcript levels of coagulation factors upon increasing doses of T_3

T_3 (µg/mouse/day)	0	0.05	0.1	0.5	1	p-value
Hepatic transcript levels						
Deiodinase 1	1 (0.83-1.20)	24.2 (19.3-30.3)‡	76.1 (58.4-88.9)‡	181 (148-222)‡	191 (157-232)‡	<0.001
Fibrinogen-γ	1 (0.96-1.04)	1.03 (0.88-1.20)	1.07 (0.97-1.19)	0.56 (0.49-0.63)†	0.47 (0.42-0.53)‡	<0.001
Factor II	1 (0.93-1.07)	0.70 (0.60-0.82)	0.88 (0.84-0.92)	0.62 (0.56-0.68)†	0.64 (0.57-0.71)*	0.009
Factor XII	1 (0.90-1.11)	1.14 (1.04-1.25)	1.20 (1.06-1.35)	1.23 (1.14-1.32)	1.01 (0.89-1.15)	NS
Antithrombin	1 (0.97-1.04)	0.86 (0.79-0.94)	0.97 (0.95-1.00)	0.79 (0.76-0.82)	0.84 (0.75-0.95)	NS
Protein C	1 (0.95-1.05)	0.82 (0.76-0.88)	0.82 (0.78-0.86)	0.57 (0.53-0.62)‡	0.56 (0.51-0.61)‡	<0.001
Protein S	1 (0.94-1.06)	0.91 (0.85-0.98)	1.05 (0.99-1.11)	0.84 (0.81-0.87)	0.82 (0.74-0.91)	NS
Endothelium-associated transcript levels						
VWF	1 (0.95-1.05)	1.01 (0.97-1.05)	1.00 (0.94-1.05)	0.97 (0.92-1.03)	0.98 (0.93-1.03)	NS
Tissue factor	1 (0.94-1.07)	0.79 (0.75-0.83)	0.72 (0.67-0.77)*	0.66 (0.59-0.73)†	0.68 (0.61-0.76)*	0.015
Thrombomodulin	1 (0.93-1.07)	1.64 (1.54-1.73)‡	1.54 (1.43-1.66)†	1.61 (1.45-1.78)‡	1.54 (1.43-1.66)†	<0.001
EPCR	1 (0.94-1.07)	0.86 (0.76-0.97)	0.75 (0.69-0.83)	0.81 (0.71-0.91)	0.87 (0.80-0.96)	NS

VWF: Von Willebrand factor; EPCR: endothelial protein C receptor.

Data are presented as mean (range) with the vehicle-treated group set as a reference in coagulation factor analyses. Overall p-values are calculated with an analysis of variance (ANOVA); *p<0.05, †p<0.01, ‡p<0.001 compared to vehicle-treated mice as calculated with a Dunnett post-hoc test.