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Differences in Mouse Hepatic Thyroid Hormone Transporter Expression with Age and Hyperthyroidism

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Key Words
Thyroid · Hyperthyroidism · Liver · Aging

Abstract
Background: Clinical features of thyroid dysfunction vary with age, and an oligosymptomatic presentation of hyperthyroidism is frequently observed in the elderly. This suggests age modulation of thyroid hormone (TH) action, which may occur, for example, by alterations in TH production, metabolism and/or TH action in target organs. ***Objectives:*** In this paper, we address possible changes in TH transporter expression in liver tissues as a mechanism of age-dependent variation in TH action. ***Methods:*** Chronic hyperthyroidism was induced in 4- and 20-month-old C57BL6/NTac male mice (n = 8–10) by intraperitoneal injections of 1 µg/g body weight L-thyroxine (T_4) every 48 h over 7 weeks. Control animals were injected with PBS. Total RNA was isolated from liver samples for analysis of the TH transporter and TH-responsive gene expression. TH concentrations were determined in mice sera. ***Results:*** Baseline serum free T_4 (fT_4) concentrations were significantly higher in euthyroid young compared to old mice. T_4 treatment increased total T_4, fT_4 and free triiodothyronine to comparable concentrations in young and old mice. In the euthyroid state, TH transporter expression was significantly higher in old than in young mice, except for *Mct8* and *Oatp1a1* expression levels. Hyperthyroidism resulted in upregulation of *Mct10*, *Lat1* and *Lat2* in liver tissue, while *Oatp1a1*, *Oatp1b2* and *Oatp1a4* expression was downregulated. This effect was preserved in old animals. ***Conclusion:*** Here, we show age-dependent differences in TH transporter mRNA expression in the euthyroid and hyperthyroid state of mice focusing on the liver as a classical TH target organ.

Introduction

Hyperthyroidism is a pathological state characterized by excessive thyroid hormone (TH) action in the body. Classical clinical features include anxiety, nervousness, tremor, weight loss and heat intolerance, which are often obscured in elderly patients and hence diagnosis may be missed [1]. Hyperthyroidism is associated with significant morbidity and mortality, mainly from cardiovascu-

K.E. and H.R. contributed equally to this work.

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lar and cerebrovascular disease, which are conditions that are particularly relevant to an aging population [2–4].

It is widely accepted that circulating TH levels may not solely and comprehensively reflect TH organ status. In fact, TH action is complex and, in addition to different TH derivatives, involves distinct transmembrane transporters and nongenomic versus classical nuclear modes of TH action, all of which may be altered with age. However, their variations upon aging have not been thoroughly analyzed, neither in humans nor in mice.

Animal models serve as useful tools to dissect mechanisms of TH action. In this study, we aimed to investigate whether age may result in alterations of proposed TH transporters in liver as a classical TH target organ. To this aim, young (4 months) versus aged (20 months) C57BL/6NTac male mice were studied under conditions of euthyroidism and chronic hyperthyroidism. In addition to serum concentrations, total thyroxine (TT_4), free thyroxine (fT_4) and free triiodothyronine (fT_3), TH transporter mRNA levels and expression of TH-responsive genes were determined in liver tissues of euthyroid and hyperthyroid young and old mice.

Materials and Methods

Animals

Male C57BL/6NTac (Taconic Europe A/S, Denmark, and Taconic Biosciences Inc., USA) mice (n = 32) aged 4 months (young) and 20 months (old) were housed in temperature- (23 ± 1°C) and light-controlled (inverse 12:12-hour light-dark cycle) conditions. Food and water were provided ad libitum. All animal experiments were performed in accordance with the German Regulations for Laboratory Animal Science (GVSOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocols for animal studies were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV-NRW), Germany.

Treatment

For induction of a chronic hyperthyroid state, animals received freshly prepared intraperitoneal (i.p.) injections of 1 µg/g body weight (BW) T_4 [Sigma-Aldrich, USA; stock-solution: 2 mg/ml in 0.01 M NaOH, 0.1% BSA (albumin from bovine serum, Sigma-Aldrich); injection solution: stock solution diluted 1:10 with PBS] every other day. Control groups received 150 µl PBS i.p. every other day. The treatment period was 7 weeks.

Blood Sample Collection and TH Measurements

Blood samples were collected from the retrobulbar venous plexus with a heparinized micropipette at the start of experiment and after 5 weeks of T_4 treatment (48 h after the last T_4 injection) from each animal. Blood samples were stored on ice for 30 min for coagulation and serum was obtained by centrifugation at 4°C for 10 min at 10,000 *g*. Serum aliquots were stored at –80°C. TT_4, fT_3 and fT_4 concentrations in serum of mice were measured using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Germany). As standards, serum samples with known TH concentrations were used.

Collection of Mice Livers

For liver collection, mice were perfused with heparinized saline through a needle placed in the left ventricle. Livers were isolated quickly, frozen in liquid nitrogen and stored at –80°C until further processing.

Isolation of RNA

For RNA extraction, tissues were homogenized in 600 µl of RLT buffer (Qiagen, Germany) using a tissue homogenizer ULTRA-TURRAX® T25 at 4,000 rpm (Janke & Kunkel IKA, Germany). The tissue lysates were further treated with proteinase K (Qiagen) based on the manufacturers' protocol. Total RNA from liver tissue lysates was purified by using the RNeasy Mini Kit and further by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). RNA quantity and quality were determined using NanoDrop 1000 (Thermo Scientific), and the integrity of RNA was further assessed via gel electrophoresis followed by ethidium bromide staining to visualize *28S* and *18S* rRNA.

Synthesis of cDNA and Real-Time PCR

2 µg of RNA was reverse transcribed to cDNA using random hexamers and SuperScript III First-Strand Synthesis System for real-time PCR according to the instruction manuals (Life Technologies, Germany). Exon-spanning primers for amplification of TH responsive genes (online suppl. table 1; for suppl. material, see www.karger.com/doi/10.1159/000381020) were designed using PrimerBlast (NCBI) and synthesized by Eurofins (Eurofins MWG Synthesis, Germany). Quantitative real-time PCR was performed using LightCycler® DNA Master SYBR Green I and the LightCycler®480 System (Roche, Germany). The PCR program consisted of an initial denaturation step (5 min at 95°C) and 40 amplification cycles with 15 s at 95°C, 10 s at 60°C and 20 s at 72°C. Melting curve analysis was performed after each PCR, and PCR products were verified by agarose gel electrophoresis.

For normalization of gene expression, the reference genes *18S*, *Ppia* (peptidylprolyl isomerase A, cyclophilin A) and *Rpl13a* (ribosomal protein L13a) were used. The stability of the housekeeping genes was determined by calculation of the coefficient of variation on the normalized relative quantities and by calculation of the geNorm M value [5]. The geometric average of the 'best' three housekeeping genes (best keeper index) was calculated by repeated pairwise correlation analysis [5]. Fold changes were calculated by the Relative Expression Software Tool (REST©, efficiency-corrected ΔΔCt method) [6, 7].

Statistical Analysis

All data are shown as means ± SD or standard error of the mean (SEM), as indicated. Statistical analysis was performed using the unpaired Student's t test of GraphPad Prism 5 Software. For statistical analysis of real-time PCR results (in figures presented as logarithmic fold-change data) antilogarithmic data of T_4-treated mice were compared to antilogarithmic data of PBS-treated controls (variations within the control groups are not shown). Values of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and # $p < 0.0001$ were considered statistically significant.

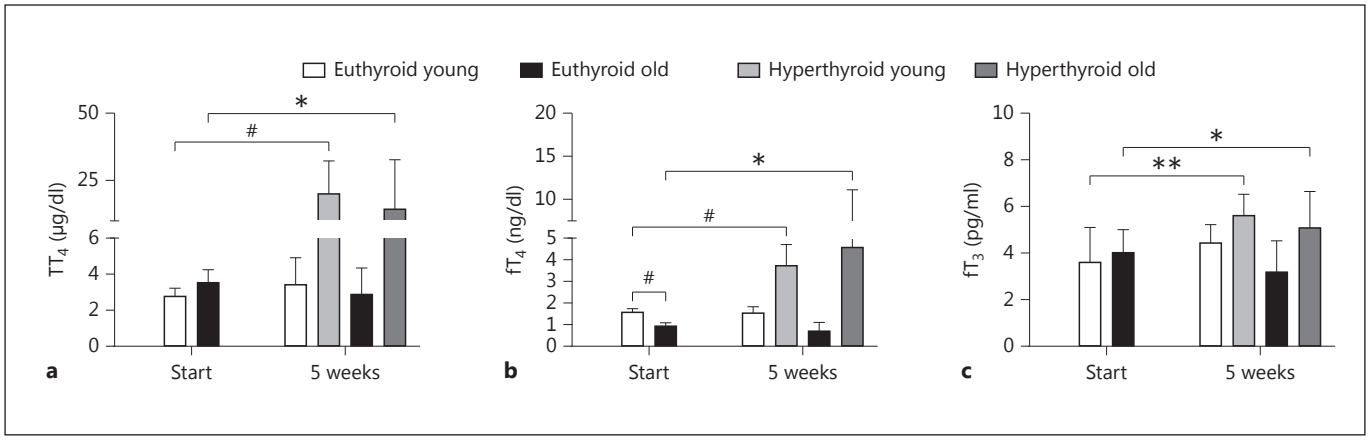

Fig. 1. Serum levels of TH in euthyroid and T_4-treated young and old male mice. TT_4 (**a**), fT_4 (**b**) and fT_3 (**c**) were determined in sera by ELISA at the start and after 5 weeks of T_4 treatment (1 µg/g BW T_4 i.p. every 48 h). A significant increase of TT_4, fT_4 and fT_3 serum parameters was found in T_4-treated young (4 months) and old (20 months) mice as compared to the euthyroid start group. The euthyroid control groups of young and old mice showed stable TH serum parameters throughout the experiments. Euthyroid old mice have significantly lower fT_4 values than euthyroid young mice. Data are presented as means ± SD; n = 16 animals for start, n = 8 animals (PBS-treated control) and n = 8 animals (hyperthyroid) for end groups; t test, * $p < 0.05$, ** $p < 0.01$, # $p < 0.0001$.

Results

Effect of T_4 Administration and Aging on TT_4, fT_4 and fT_3 Serum Concentrations in Mice

To examine TH serum changes with T_4 administration over the experimental time period, retro-orbital blood samples were collected at the beginning of and during T_4 treatment.

Under T_4 treatment, young and old male mice displayed an up to 4- to 6-fold increase in TT_4 ($p < 0.0001$ in young and $p < 0.05$ in old male mice) and a 2- to 4-fold increase in fT_4 ($p < 0.0001$ in young and $p < 0.05$ in old male mice) serum concentrations, respectively. In addition, small but significant increases of serum fT_3 concentrations ($p < 0.01$ in young and $p < 0.05$ in old male mice) were measured under T_4 treatment (fig. 1).

In the control animals, age differences in TH serum concentrations were observed for fT_4 values, which were 2-fold higher in young compared to old male mice ($p < 0.0001$; fig. 1b). In contrast, TT_4 as well as fT_3 concentrations remained unchanged with aging (fig. 1a, c).

Effect of Aging on TH Transporter Expression in Liver

To examine the impact of aging on TH transporter expression, fold changes of TH transporter expression levels in old liver were compared to young liver tissues for euthyroid (control) animals. We found an increased expression of most TH transporters with aging in livers of euthyroid mice. Thus, old control animals showed a significantly higher expression of *Mct10*, *Lat2*, *Oatp1b2* and *Ntcp* ($p < 0.05$; fig. 2) as well as of *Lat1* and *Oatp1a4* ($p < 0.01$; fig. 2) in liver tissues compared to young mice.

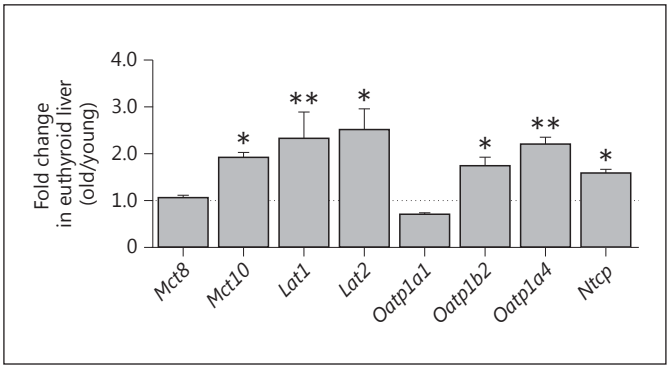

Fig. 2. Effect of aging on TH transporter mRNA expression in liver tissues of old compared to young euthyroid male mice. Gene expression levels were determined by quantitative real-time PCR in livers of old euthyroid mice (21.8 months) and were normalized to respective expression levels in livers of euthyroid young mice (5.8 months). *Mct10*, *Lat1*, *Lat2*, *Oatp1b2*, *Oatp1a4* and *Ntcp* mRNA expression levels are significantly upregulated in liver tissues of old mice compared to young animals. *18S*, *Ppia* and *Rpl13a* were used as reference genes. Data are presented as fold changes, means ± SEM; n = 7; efficiency-corrected ΔΔCt method; t test, * $p < 0.05$, ** $p < 0.01$.

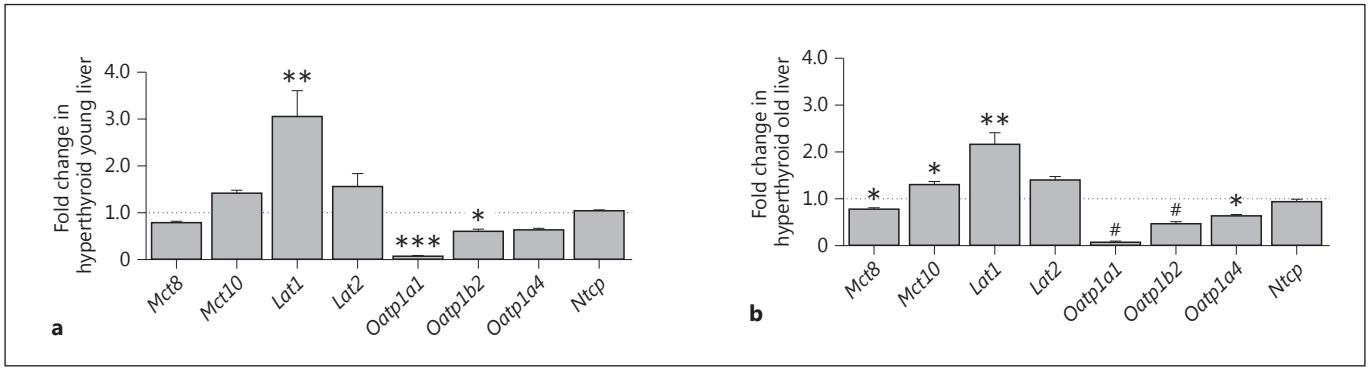

Fig. 3. Impact of hyperthyroidism on TH transporter mRNA expression in liver tissues of young and old male mice. Gene expression levels were measured by quantitative real-time PCR in liver tissues of T_4-treated (1 μg/g BW T_4 i.p. every 48 h over 7 weeks) young (5.8 months) (**a**) and old (21.8 months) (**b**) mice, and were normalized to expression levels of euthyroid (PBS-treated) age-matched controls. **a** *Lat1* mRNA expression was significantly upregulated, whereas *Oatp1a1* and *Oatp1b2* mRNA expression levels were significantly decreased in liver tissues of T_4-treated young mice compared to controls. **b** *Mct10* and *Lat1* mRNA expression levels were significantly increased, whereas *Mct8*, *Oatp1a1, Oatp1b2* and *Oatp1a4* mRNA expression levels were significantly decreased in liver tissues of T_4-treated old mice compared to controls. *18S*, *Ppia* and *Rpl13a* were used as reference genes. Data are presented as fold changes, means ± SEM; n = 7; efficiency-corrected ΔΔCt method; t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.0001$.

No changes were detected for *Mct8* mRNA levels, and *Oatp1a1* levels were marginally decreased with aging (fig. 2).

Effect of Chronic Hyperthyroidism on TH Transporter Expression in Liver of Young and Old Mice

In the hyperthyroid state, similar changes in the pattern of TH transporter mRNA expression in liver were observed for young and old mice (fig. 3). T_4 treatment led to marked downregulation of *Oatp1a1* mRNA levels (0.06-fold; $p < 0.001$ in young and $p < 0.0001$ in old mice) and significant upregulation of *Lat1* expression (3-fold in young, 2.2-fold in old mice; $p < 0.01$). Furthermore, upregulation was found for *Mct10* (1.5-fold and significant in old mice; $p < 0.05$) and downregulation was observed for *Mct8* (0.7-fold and significant in old mice; $p < 0.05$) and *Oatp1a4* mRNA (0.6-fold and significant in old mice; $p < 0.05$) in the hyperthyroid state. Downregulation of *Oatp1b2* mRNA expression was observed in both age groups after T_4 treatment [0.59-fold in young ($p < 0.05$) and 0.46-fold in old ($p < 0.0001$) mice].

Effect of Chronic Hyperthyroidism on TH Responsive Gene Expression in Liver of Young and Old Mice

To examine TH status in liver under chronic T_4 excess, mRNA transcription levels of genes proposed to respond to TH, namely *Spot14* (thyroid hormone-responsive Spot14 homolog, Thrsp), *Tbg* (TH-binding globulin) and *Dio1* (deiodinase 1), were assessed in young and old mice after T_4 treatment over 7 weeks [8, 9]. *Spot14* showed the smallest variations with regard to mRNA levels with upregulation of 1.5-fold under T_4 treatment in young ($p < 0.01$) and old ($p < 0.05$) mice. In both age groups, *Dio1* was highly expressed in the hyperthyroid state (10-fold increase; $p < 0.0001$) while *Tbg* expression was downregulated to very low levels in young and old mice ($p < 0.0001$; fig. 4).

Discussion

We examined changes of TH transporter expression during aging in a euthyroid as well as a hyperthyroid state in the TH target organ liver. T_4 treatment was chosen as the preferred TH for induction of hyperthyroidism since T_4 has been the most widely used iodothyronine in (hyperthyroid) animal experiments, allows for the assessment of T_4 to T_3 conversion in a living organism and is standard in patients requiring TH substitution.

To assess the effects of chronic T_4 exposure and aging on TH serum status, TH serum parameters were determined in euthyroid controls and T_4-treated animals. We could confirm significantly lower fT_4 concentrations in old male mice (20 months) compared to young male (4 months) mice. A decline in fT_4 values has been reported in male mice during development from 2 to 20 weeks

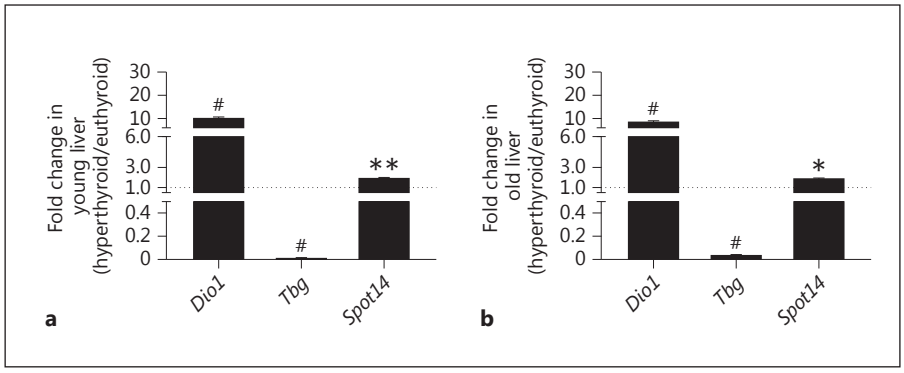

Fig. 4. Impact of T_4 treatment on expression of TH-responsive genes in liver tissues of young and old male mice. Gene expression levels of *Dio1, Spot14* and *Tbg* were measured by quantitative real-time PCR in liver tissues of T_4-treated (1 μg/g BW T_4 i.p. every 48 h over 7 weeks) young (5.8 months) (**a**) and old (21.8 months) (**b**) mice, and were normalized to expression levels of euthyroid (PBS-treated) age-matched controls. *Dio1* and *Spot14* mRNA expression levels were significantly upregulated, whereas *Tbg* mRNA expression was significantly and marked downregulated in liver tissues of T_4-treated young and old mice compared to controls. *18S*, *Ppia* and *Rpl13a* were used as reference genes. Data are presented as fold changes, means ± SEM; n = 7; efficiency-corrected ΔΔCt method; t test, * $p < 0.05$, ** $p < 0.01$, # $p < 0.0001$.

[10]. Decreasing fT_4 concentrations were also noted in aging men [11]. Under T_4 treatment, TT_4, fT_4 and fT_3 concentrations increased in young and old mice to comparable values. Investigation of gene expression in liver tissue confirmed a hyperthyroid tissue state after T_4 treatment with the expected changes in the expression of the positively TH-regulated genes *Dio1* and *Spot14* [8] and the negatively regulated gene *Tbg* [9]. Further analysis of liver tissue with regard to TH transporters showed that hyperthyroidism led to a decrease in expression of the best-studied TH transporter *Mct8* [12, 13] and an upregulation of *Mct10*. This was seen in young and old animals, and could represent a compensatory reaction. *Mct8* has been reported to have a greater impact on TH efflux than influx in liver cells, as *Mct8*$^{-/y}$ mice have higher T_3 content in liver and show upregulated *Dio1* expression and activity [14–16]. Similarly *Mct10* has been proposed to mediate TH efflux in liver tissue [16]. However, in our study the extent of regulation of both transporters remained in a significant range in livers of old mice only.

Oatp1a1 [17], *Oatp1b2* [18] and *Oatp1a4* [19] were downregulated in a comparable fashion in hyperthyroid young and old mice, with marked downregulation found for *Oatp1a1*. As secondary TH transporters, they mainly transport organic compounds, amino acids and hormones [13, 20]. Therefore, their lower expression levels might result in lower levels of amino acids available for protein synthesis in hepatocytes [21]. In contrast, *Lat1* and *Lat2* were upregulated to high levels and in a significant manner. Because *Lat1* is known to be highly expressed in hepatocytes under limited amino acid availability, these results are in line with the observed downregulation of *Oatp*s. The *Lat1* response is believed to explain how hepatocytes can still grow under limiting amino acid availability, and thus acquire a survival advantage [22, 23]. While *Lat1* prefers large neutral amino acids, *Lat2* has a broader specificity mediating transport of small neutral amino acids as well [24]. Thus an upregulation of *Lat2* might reflect the liver's need for small amino acids. *NTCp* as a multispecific organic anion transporter [13] remained unaltered during hyperthyroidism.

The most surprising finding of our observation is perhaps the upregulation of most investigated TH transporters with aging. This effect was seen in euthyroid and hyperthyroid old animals, suggesting that the liver may react similarly to TH excess in young and old age, at least at the TH transporter level. This is also in agreement with the observed age-independent effect of T_4 on TH-responsive genes *Spot14*, *Dio1* and *Tbg* in the liver of hyperthyroid old and young mice. Age-independent expression levels of *Mct8* may underline its importance in maintaining TH transport across the hepatocyte plasma membrane in TH-challenging conditions. Finally, our findings suggest a response to increased needs for amino acids and hormones in aging hepatocytes through the upregulation of, for example, *Lat1* and other secondary TH transporters like *Lat2*. Presently our findings rely on mRNA expression studies and it is unknown whether

they are representative for functional protein in the liver. However, with lack of specific antibodies for many TH transporters analyzed in this study, this issue is technically difficult to resolve in a comprehensive manner at this point.

In summary, we report changes in TH transporter mRNA expression in liver as a classical TH target organ in response to TH excess but also aging, whereby the first effect pertained in young and old age.

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Disclosure Statement

The authors state that no conflict of interest exists.

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