RESEARCH

Age-dependent response to T4 overtreatment and recovery on systemic and organ level

Helena Kerp1, Kostja Renko1,2,3, Georg Sebastian Hönes1, Klaudia Brix4, Josef Köhrle2, Lars Christian Moeller1 and Dagmar Führer1

1Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, University of Duisburg-Essen, Essen, Germany
2Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institut für Experimentelle Endokrinologie, Berlin, Germany
3German Federal Institute for Risk Assessment (BfR), German Centre for the Protection of Laboratory Animals (BF3R), Berlin, Germany
4Department of Life Sciences and Chemistry, Jacobs University Bremen, Bremen, Germany

Correspondence should be addressed to D Führer: dagmar.fuehrer@uk-essen.de

Abstract
Thyroid hormone (TH) metabolism and cellular TH action are influenced by ageing. To investigate the response to thyroxine (T4) overtreatment, a kinetic study was conducted in young and aged mice with chronic hyperthyroidism and hormone withdrawal. Five and 22 months old male mice were treated with T4 or PBS over 5 weeks, followed by observation for up to 12 days. Serial analysis was performed for thyroid function parameters, transcript levels of TH target genes, deiodinase type 1 (DIO1) activity as well as serum lipids at 12, 24, 72, 144, 216, and 288 h after cessation of T4 administration. Higher FT3 concentrations and higher renal DIO1 activities were noted in aged mice 12 h after T4 withdrawal and marked thyroid-stimulating hormone elevation was found in aged mice after 12 days compared to respective controls. A biphasic expression pattern occurred for TH target genes in all organs and a hypothyroid organ state was observed at the end of the study, despite normalization of TH serum concentrations after 72 h. In line with this, mirror-image kinetics were detected for serum cholesterol and triglycerides in aged and young mice. Recovery from TH overtreatment in mice involves short- and medium-term adaption of TH metabolism on systemic and organ levels. Increased renal DIO1 activity may contribute to higher T3 concentrations and prolonged thyrotoxicosis followed by hypothyroidism in an aged-mouse organism. Translation of these findings in the clinical setting seems warranted and may lead to better management of hyperthyroidism and prevention of T4 overtreatment in aged patients.

Key Words
- thyroid hormone action
- ageing
- thyroid hormone kinetics
- gene expression

Introduction
Ageing is associated with an elevated risk for thyroid dysfunction (Hollowell et al. 2002, Pearce et al. 2016), and an excess of thyroid hormones (TH) (hyperthyroidism) is particularly harmful in elderly patients (Grossman et al. 2016). Besides endogenous causes of hyperthyroidism, for example, toxic multinodular goiter or Graves’ disease, exogenous hyperthyroidism may arise from overtreatment of hypothyroidism with thyroxine (T4). This unwanted not infrequent complication of TH substitution has been observed in several epidemiological studies (Taylor et al. 2019) and has over the past years stimulated a more vigorous decision-finding on who should receive TH treatment.
and how it should be monitored (Wiersinga et al. 2012, Jonklaas et al. 2014, Ross et al. 2016). In case of inadvertent TH overtreatment, usual practice is to stop T₄ medication immediately and to resume TH substitution at lower doses later. However, so far no studies have investigated whether T₄ withdrawal after TH overtreatment has a different impact on serum and organ status in an aged organism. In the latter context, we and others have previously shown that age modulates the biological phenotypes of chronic hyper- and hypothyroidism in a tissue-specific manner in mice, not automatically reflected by TH serum concentrations (Engels et al. 2019, Kerp et al. 2020). Furthermore, we have recently shown that both systemic and organ responses to acute T₄ challenge are highly age-dependent (Rakov et al. 2019). In the present study, we ask whether an adjustment to correction of T₄ overtreatment is different in young and aged mice and how it may affect TH metabolism and TH action in tissues. Our findings show a hitherto underappreciated age impact on systemic and organ TH response with partially incomplete recovery on organ level after T₄ excess, despite normalization of TH serum concentrations.

Material and methods

Animals

Male C57Bl/6N mice were purchased from Taconic, Denmark. Two age cohorts were investigated: 38 were 5 months old (young cohort) and 29 were 22 months old mice (aged cohort). Animals were single-housed in temperature- (23 ± 1°C) and light-controlled (inverse 12 h light:12 h darkness 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 and organ collection

Mice were randomly divided into control groups (n = 7 for 5 and 22 months) or T₄ groups sacrificed at different time points (12, 24, 72, 144, 216, and 288 h) after last T₄ treatment (n=5–6 each time point for 5 months, n=3–5 each time point for 22 months). T₄ mice were injected intraperitoneally (i.p.) for 5 weeks for every 48 h at the same time with 1 µg/g body weight T₄ as described previously (Engels et al. 2016, Rakov et al. 2016). Control mice received i.p. injections of 150 µL PBS every 48 h for 5 weeks. At indicated time points after the last injection, mice were deeply anesthetized by an i.p. injection of 200 µL ketamine/xylazine mixture (150 µL of 100 mg/mL Ketamine (Beta-pharm, Vechta, Germany) and 50 µL of 20 mg/mL xylazine (Ceva, Düsseldorf, Germany). Final blood was obtained by heart puncture. For tissue collection, mice were perfused with heparinized saline through a needle placed in the left heart ventricle. Tissues were shock-frozen in liquid nitrogen and stored at −80°C until further processing.

Serum thyroid hormone and TSH measurements

TT₄, FT₄, and fT₃ concentrations in serum of mice were measured as previously described using commercial ELISA kits according to the manufacturer’s instructions (DRG Instruments GmbH, Marburg, Germany) and serum samples with known TH concentrations as standards (provided by company). TSH serum concentration was determined using the Milliplex Map mouse pituitary magnetic bead panel (EMD Millipore Corporation, Billerica, USA; MPTMAG-49K) on the Luminex 200 system (Thermo Fisher) (Rakov et al. 2019).

Serum lipid profiles

Total cholesterol concentrations in serum were measured using an enzymatic cholesterol quantitation kit (Sigma–Aldrich (MAK043)) and total triglyceride concentrations were investigated using an enzymatic serum triglyceride determination kit (Sigma–Aldrich (TR0100)), according to the manufacturer’s instructions.

Quantitative real-time PCR

Total RNA from liver, kidney, and heart was isolated using the RNasy Kit according to the manufacturer’s instruction (Qiagen) and stored at −80°C as previously described (Rakov et al. 2019). RNA was reverse transcribed into cDNA with SuperScriptIII (Life Technologies) and hexamer primers. Quantitative real-time PCR (qRT-PCR) was performed using Roche SYBR Green I master mix (Roche). Primers were designed to be intron-spanning to exclude genomic DNA signals (sequences provided in Supplementary Table 1, see section on supplementary materials given at the end of this article). In compliance with the MIQE guidelines for RT-PCR (Bustin et al. 2009), we used a set of 2–3 reference

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genes per tissue to assure accurate normalization and calculation (liver: 18S (18S rRNA), Ppia (peptidylprolyl isomerase A, cyclophilin A); heart: Poh2a (polymerase RNA II), Gapdh (glyceraldehyde-3-phosphate dehydrogenase; kidney: Ppia, Gapdh and Actb (actin beta). Analysis and calculation of the fold-change in gene expression were done on Ct-values \( \leq 35 \) using the efficiency-corrected \( \Delta \Delta \text{Ct} \) method (Pfaffl 2001).

**Hepatic and renal deiodinase 1 activity**

Liver and kidney protein samples were prepared by mincing and sonification, adjusted to a defined protein concentration (2.5 µg/µL). In the case of lower protein concentration, the calculation of activity was adjusted by the individual protein yield of the sample. Dio1 activity was assayed as previously described (Renko et al. 2012). In brief, a 50 µL reaction mixture containing 40 µg of liver or kidney microsomal proteins, 1 mM 6-n-propyl-2-thio-uracil (PTU) for background controls, was mixed with 50 µL of freshly prepared substrate mix (20 µM rT3 (Sigma–Aldrich), 0.2 M KPO4 (pH 6.8), 2 mM ethylenediaminetetraacetic acid, and 80 mM dithiothreitol). The enzyme reaction lasted for 1 or 2 h at 37 °C, for liver and kidney samples, respectively. After centrifugation (4°C, 15,000 g, 5 min), the supernatant was used for quantification of released iodide. Dowex WS0-X2 resin columns were used for the separation of intact rT3 and the deiodinated breakdown products from the released iodide. The iodide content was determined by the Sandell–Kolthoff reaction, using cerium solution (25 mM (NH4)2Ce(SO4)3 and 0.5 M H2SO4) and arsenite solution (25 mM NaAsO2, 0.8 M NaCl, and 0.5 M H2SO4). The changes in absorption (OD at 415 nm) were determined at the reaction starting point and after 20 min. All protein samples were assayed in duplicate on two separate plates, and mean values were taken for further calculation. The tubes that contained PTU were used for background subtraction. Approximation of the enzyme activity was performed by interpolation from a separately measured iodide standard curve.

**Statistical analysis**

All data are shown as mean ± s.d. or s.e.m. as indicated. Statistical analysis was performed using GraphPad Prism 6 Software. One or two-way ANOVA followed Dunnett’s or Tukey’s post hoc analysis was applied as indicated. Values of \( *P < 0.05, **P < 0.01, ***P < 0.001, \#P < 0.0001 \) were considered statistically significant.

**Results**

Male mice of 5 and 22 months of age with exogenously induced chronic hyperthyroidism were investigated for short- and medium-term effects on systemic and organ adaption after stopping T4 treatment. Changes in circulating TH and TSH concentrations, TH target gene expression in heart, kidney, and liver, renal and hepatic DIO1 activities, and serum lipid profiles were determined in young and aged mice and were compared to age-matched controls.

**Changes in systemic thyroid hormone state after T4 withdrawal**

Age- and time-dependent changes in thyroid function were assessed by serial analysis of TT4, FT4, FT3, and TSH serum concentrations (Fig. 1A, B, C and D). In control animals, no significant difference in serum TH status was noted, although TSH concentrations tended to be higher in aged compared to young mice. As expected, T4 treatment over 5 weeks resulted in significantly increased TT4, FT4, and FT3 serum concentrations, reaching ~5-fold (TT4), ~12-fold (FT4), and ~4–5-fold (FT3) of controls. Twelve hours after the last T4 injection, FT3 concentrations were higher in aged compared to young mice (Fig. 1C). At 72 h, TH concentrations in all T4 treated mice had reached values of control animals and remained stable thereafter with no differences between aged-matched T4 treated and control mice (Fig. 1A, B and C). TSH concentrations remained below the detection limit until 72 h after T4 withdrawal, followed by a steep increase thereafter. At 12 days after cessation of T4 treatment, TSH concentrations in young mice had returned to levels of controls, while a sustained marked TSH elevation was found in aged animals (Fig. 1D).

**Impact of T4 withdrawal on TH action in target organs heart, kidney, and liver**

**Heart**

Transcript levels of deiodinases Dio2 and Dio3, that finetune cardiac T3 concentrations in the normal and diseased heart (Janssen et al. 2013), the monocarboxylate transporter 8 (Mct8), and prominent cardiac TH responsive genes myosin heavy chain 6 and 7 (Myh6 and Myh7) were quantified in mouse hearts after T4 excess and compared to expression levels of age-matched controls. Dio2 expression was constant at all time points in young mice after T4 withdrawal, while in hearts of aged mice, reduced Dio2 expression was found at 24 h (Fig. 2A and B) compared to
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Kidney

A biphasic response to T₄ withdrawal – with only minor age impact – was also noted in the kidneys. In young mice, Dio1 transcript levels increased until 72 h and then normalized to levels of controls, while Dio1 transcript levels remained suppressed in aged mice at the end of the study (Fig. 3A and B). No age differences were noted in the biphasic expression kinetics of TH-dependent 1-alpha hydroxylase (Cyp27b1). Mct8 and Mct10 expression (Fig. 3C, D, E, F, G and H) was not affected by T₄ withdrawal.

Liver

Kinetics of all four investigated TH target genes Dio1, Tbg, Trhde, and Mct8 showed a biphasic organ response to T₄ withdrawal with a thyrotoxic liver state until 72 h, followed by a hypothyroid state in both young and aged mice (Fig. 4A, B, C, D, E, F, G and H). At the end of the study, expression levels of Dio1 and Trhde mice had not completely normalized to controls suggesting an incomplete recovery of TH action in the liver. Thereby changes in Dio1 expression were more pronounced in the livers of aged mice (Fig. 4A and B), while changes in Trhde expression were more pronounced in young mice (Fig. 4E and F).

Impact of T₄ withdrawal on renal and hepatic DIO1 activity

Renal and hepatic DIO1 activities not only determine organ-specific TH concentrations but may also contribute to circulating TH concentrations under thyrotoxic conditions (van der Spek et al. 2017). In our study, renal DIO1 activity was increased 24 h after T₄ withdrawal in...
Changes in serum lipid profile after T₄ withdrawal

T₄ withdrawal after chronic T₄ treatment had a reciprocal impact on serum lipid profiles in aged and young mice (Fig. 6A). At 12 h after the last T₄ injection, serum cholesterol concentrations were significantly higher in aged compared to young mice. By 72 h, cholesterol concentrations had normalized to controls in both T₄ treatment groups followed by a steep increase in serum cholesterol concentrations reaching 180% in young and 130% in aged animals,
compared to age-matched controls at 144 h. At the end of the study, serum cholesterol concentrations were below controls in aged and above control levels in young \(T_4\)-treated mice.

For kinetics of serum triglycerides, a mirror-image pattern was observed for aged and young \(T_4\)-treated mice with significantly lower triglyceride concentrations in aged compared to young mice at the end of the study (Fig. 6B).

Expression analysis of genes involved in hepatic lipid and cholesterol metabolism showed a biphasic response for \(Scd1\), while \(Ldlr\) and \(Srebp2\) transcript levels were not altered after \(T_4\) withdrawal in the livers of young and aged mice (Supplementary Fig. 1).

### Discussion

Various clinical studies have raised the awareness for age-specific reference intervals for TH and TSH serum concentrations (Waring et al. 2012, Vadiveloo et al. 2013, Stott et al. 2017, Duntas 2018, Barbesino 2019). Even more importantly, accumulating reports of adverse effects of \(T_4\) overtreatment in older patients with subclinical or overt hypothyroidism justify the need for further studies on how correction of \(T_4\) overtreatment might affect an aged organism. To address how TH excess and its withdrawal impact on TH action systemically and organ-specifically in...
Prolonged TH excess is followed by systemic hypothyroidism in aged mice after TH overtreatment

Serial analysis of thyroid function parameters after cessation of T₄ treatment revealed two important findings with respect to ageing. First, in the early phase of recovery from TH excess, higher TH serum concentrations in particular for FT₃ were obvious in 22 months old mice and hence could portend prolonged risk of TH excess to an aged organism. Secondly, at the end of the study, markedly elevated TSH concentrations were found in aged but not young mice despite normalization of serum TH concentrations within 3 days after stopping treatment in both age cohorts, similar to observations in patients after T4 withdrawal (Carlwe et al. 2013).

Ageing has been shown to afflict the hypothalamic-pituitary-thyroid (HPT) axis on several levels (Donda et al. 1987, 1989, Reymond et al. 1992) for example, in TSH secretion, TSH bioactivity, or reduced TSH receptor...
expression in the aged thyroid gland (Persani 1998). Thyroids of old rodents have different structure and function and do not respond to TSH as sensitive as those of young mice (Studer et al. 1978, Tamura & Fujita 1981, Reymond et al. 1992). In chronically hypothyroid mice, we have previously confirmed lower TSH bioactivity in aged compared to young animals (Engels et al. 2019), using a cell-based RIA (Pohlenz et al. 1999). As TH serum concentrations were comparable for both age groups after 72 h of T₄ withdrawal, the sustained higher TSH concentrations could, in principle, result from reduced TSH bioactivity in older animals. In fact, TSH reflects local HPT state, and kinetics of readjustment of TH status results in peripheral changes of gene expression in different target organs due to the local role of deiodinases at this site of the feedback axis. However, by comparison with age-matched controls and supported by changes in organ TH action discussed below, we suggest that the observed age difference in thyroid function parameters represents systemic hypothyroidism due to prolonged recovery time of the HPT from chronic TH excess in an aged organism.

**Increased renal DIO1 activity contributes to prolonged TH excess in aged mice after TH overtreatment**

Previously we reported an age-dependent delay in hepatic DIO1 activity response upon single T₄ injection associated with lower T₃ serum and tissue concentrations (Rakov et al. 2019). In the present study, DIO1 activity in the liver was not different between young and aged mice under T₄ withdrawal, hence a compensatory effect due to chronic TH excess is likely. DIO1 activity in the liver and kidney has been described to contribute to a minor extent to circulating T₃ concentrations under euthyroid conditions in mice (Streckfuss et al. 2005, Schneider et al. 2006, van der Spek et al. 2017). However, under chronic hyperthyroidism, DIO1 may exert a counteracting function and may limit the availability of circulating T₃ as shown in a human study (Abuid & Larsen 1974) and in a fish model (Van der Geyten et al. 2005). In our study, DIO1 activities in the liver resembled hepatic Dio1 mRNA expression. Interestingly, DIO1 knockout mice showed increased fecal but reduced urinary TH clearance upon TH excess, suggesting a protective function of renal DIO1 in states of high TH availability (Streckfuss et al. 2005, Schneider et al. 2006). In the present study, renal DIO1 activity was much higher in aged compared to young mice, suggesting increased TH metabolism in kidneys of aged mice, with – at least in aged animals – a possible renal contribution to higher FT₃ serum concentrations after TH overtreatment. Thus, we hypothesize that renal DIO1 activity in young mice may be switched on/off faster, while in old mice, DIO1 response to changes in TH serum concentrations may be slower. However, one limitation of our study is the lack of data on rT₃ serum concentrations, which might have helped to explain the TH alterations, as rT₃ might be increased under T₄ treatment and rapidly disappears by DIO1 activity. In addition, the role of DIO3 during T₄ excess and recovery could not be solved and might have also influenced
organ-specific recovery similarly to its impact during acute or chronic illness (Boelen et al. 2011).

**TH action in target organs remains compromised after TH overtreatment despite normalization of TH serum concentrations**

Although TT4, FT4, and FT3 serum concentrations had normalized within 72 h after T4 withdrawal, prolonged effects on cardiac, renal, and hepatic organ TH action were observed. On the one hand, the heart was chosen because of the link between thyroid dysfunction and increased cardiac morbidity especially in aged patients (Asvold et al. 2015, Pearce et al. 2016, Jabbar et al. 2017). On the other hand, the liver and kidney play an important role in TH metabolism and fine-tuning of systemic TH serum concentrations in different conditions and ages (Streckfuss et al. 2005, Schomburg et al. 2007, Visser et al. 2016, Rakov et al. 2019, Kerp et al. 2020).

In our study and based on TH target gene expression, a biphasic response was found in the heart, kidney, and liver and was characterized by a thyrotoxic tissue state until 72 h after TH withdrawal followed by a subsequent hypothyroid state with incomplete normalization of organ TH action at the end of the study. Particularly in the heart, a prolonged hypothyroid organ state was obvious. Thereby Myh6 expression changes were more pronounced in aged, while Myh7 expression changes were more pronounced in young mice at 12 days. This is an interesting finding, as both genes are directly transcriptionally regulated by TH (Razvi et al. 2018), but particularly Myh6 expression change does not correlate to serum T3 concentrations. A possible biological relevance of this finding has been suggested in a previous study, where bradycardia and altered cardiac gene expression occurred 14 days after T4 withdrawal in T3-treated young mice (Hoefig et al. 2016). Whether this effect is due to a direct cardiac or an indirect effect of TH on the autonomous system (Herrmann et al. 2020) remains to be clarified. Functional consequences of altered Myh6 vs Myh7 expression in hearts of aged vs young mice remain to be determined and could reflect age-dependent differences in responses to, for example, myosin heavy-chain-associated lncRNA transcript (Mhrt), which has been previously described in this context (Han et al. 2014, Forini et al. 2020). However, the normalization of Myh6 expression in young mice after repression at 144 h and 216 h indicates that although serum T3 concentration is within the reference range, cardiac TH metabolism is under local control, not assessable by serum measurements.

A similar trend as in the heart was found for renal TH target gene expression, suggesting a hypothyroid kidney state 12 days after T4 withdrawal with marked Dio1 suppression in aged mice. Furthermore, in the liver, expression levels of Dio1 and Trhde still indicated a hypothyroid liver state at 12 days, that is, the end of the study. These findings are in concordance with a previous study by Ohba et al. showing incomplete hepatic recovery from chronic T3 treatment in young mice at 10 days of TH withdrawal (Ohba et al. 2016). In their study, expression of approximately 10% of investigated hepatic TH target genes had not returned to control levels and the authors suggested that these sustained changes might contribute to the persistence of metabolic and physiological abnormalities observed clinically in some patients despite normalized TH serum concentrations (Ohba et al. 2016).
Adaption to TH overtreatment involves age-specific changes in lipid profiles

TH impact mobilization, degradation of lipids, and de novo synthesis of fatty acids in the liver (Damiano et al. 2017, Sinha et al. 2019). Furthermore, TH increase bile acid flow, resulting in depletion of hepatic cholesterol and enhancement of cholesterol uptake from the circulation to the liver (Duntas & Brenta 2018). As expected, decreased cholesterol serum concentrations were found upon chronic T\textsubscript{4} treatment in young mice but this did not apply to aged mice. Interestingly, after normalization to control level, a drastic transient increase in serum cholesterol occurred in young mice, while the adaptive changes were much subtle in aged mice. Furthermore, a mirror-image pattern was found for kinetics in triglyceride serum concentrations after T\textsubscript{4} withdrawal showing normalization in young but not in aged mice, which exhibited significantly lower triglyceride concentrations than young animals at the end of the study. These observed changes in lipid profiles underpin both an age impact and a prolonged recovery of organ TH action from TH overtreatment.

In summary, recovery from TH overtreatment involves adaptation in TH metabolism and TH action with some age differences on systemic and organ level and incomplete normalization of the heart, kidney, and hepatic thyroid hormone status over 12 days in the mouse organism. Thus, although TH serum concentrations normalize fast, once the experimental source of T\textsubscript{4} excess is eliminated, TH serum status does not reflect euthyroidism. These findings may have a significant impact on hyperthyroid patients treated with antithyroid drugs, as these patients observe excessive short-term weight gain despite normal serum thyroid function tests and may also affect longer-term susceptibility to other health conditions.

Shortcomings of our study are the restriction to 12 days of observation and the lack of functional correlates of TH action in the heart and kidney, while for the liver, the identified changes in serum lipid profiles are in line with incomplete recovery from TH overtreatment in the aged liver. To our best knowledge, this is the first serial analysis of an age impact on systemic and organ response to TH overtreatment.

Our findings raise awareness that side effects of T\textsubscript{4} overtreatment extend beyond the time points of elimination of TH excess and normalization of serum TH concentrations, aspects which should be addressed in future studies.

**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/JME-21-0109.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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