Abstract

The endogenous thyroid hormone (TH) metabolite 3,5-diiodo-L-thyronine (3,5-T₂) acts as a metabolically active substance affecting whole-body energy metabolism and hepatic lipid handling in a desirable manner. Considering possible adverse effects regarding thyromimetic action of 3,5-T₂ treatment in rodents, the current literature remains largely controversial. To obtain further insights into molecular mechanisms and to identify novel target genes of 3,5-T₂ in liver, we performed a microarray-based liver tissue transcriptome analysis of male lean and diet-induced obese euthyroid mice treated for 4 weeks with a dose of 2.5 µg/g bw 3,5-T₂. Our results revealed that 3,5-T₂ modulates the expression of genes encoding Phase I and Phase II enzymes as well as Phase III transporters, which play central roles in metabolism and detoxification of xenobiotics. Additionally, 3,5-T₂ changes the expression of TH responsive genes, suggesting a thyromimetic action of 3,5-T₂ in mouse liver. Interestingly, 3,5-T₂ in obese but not in lean mice influences the expression of genes relevant for cholesterol and steroid biosynthesis, suggesting a novel role of 3,5-T₂ in steroid metabolism of obese mice. We concluded that treatment with 3,5-T₂ in lean and diet-induced obese male mice alters the expression of genes encoding hepatic xenobiotic-metabolizing enzymes that play a substantial role in catabolism and inactivation of xenobiotics and TH and are also involved in hepatic steroid and lipid metabolism. The administration of this high dose of 3,5-T₂ might exert adverse hepatic effects. Accordingly, the conceivable use of 3,5-T₂ as pharmacological hypolipidemic agent should be considered with caution.

Introduction

Thyroid hormones (TH) regulate multiple metabolic processes and play an essential role in normal growth, development, and energy homeostasis. Moreover, they are powerful agents in counteracting hyperlipidemia and reducing body weight. However, unwanted effects on heart, bone, or muscle restrict their use as therapeutically active compounds for obesity-related diseases (Angelin & Rudling 2010).

In the last few decades, several TH derivatives have been designed as treatment options to counteract obesity and
related diseases, without inducing adverse thyromimetic effects even in a dose-escalating pilot study in two volunteers (Antonelli et al. 2011, Coppola et al. 2014). One of these is the endogenous TH metabolite 3,5-diiodo-l-thyronine (3,5-T2) (Lehmphul et al. 2014), which has attracted scientific attention because of its marked effects on metabolism. In vivo and in vitro studies revealed that 3,5-T2 stimulates hepatic fatty acid oxidation and increases cellular and mitochondrial respiration. 3,5-T2 administered mainly to rats fed with a high-fat diet (HFD) prevented hepatic lipid accumulation and steatosis without inducing thyromimetic effects known for the classical TH 3,5,3′-triiodo-l-thyronine (T3) and l-thyroxine (T4) (Lanni et al. 2005, Mollica et al. 2009, de Lange et al. 2011, Moreno et al. 2011). Recently, central effects of 3,5-T2 on adrenergic and sympathetic signaling have been demonstrated with peripheral consequences such as innervation and vascularization of brown adipose tissue (Lombardi et al. 2015).

However, apart from these unambiguous effects on energy metabolism, 3,5-T2 might also exert thyromimetic side effects at other targets. In a recently published study, chronic treatment with 3,5-T2 (2.5 µg/g body weight (bw)) in diet-induced obese euthyroid mice resulted in reduction of body fat mass and improvement of hepatic lipid status. But in contrast to these beneficial effects, an undesired negative feedback inhibition of the hypothalamus-pituitary–thyroid (HPT) axis accompanied by increased heart weight was observed at the dose effectively improving hepatic lipid metabolism and systemic lipid status (Jonas et al. 2014). Such observations on dose-related thyromimetic actions, among others also on the pituitary and the heart, are in agreement with published results on mice and rats treated with 3,5-T2 (Horst et al. 1995, Goldberg et al. 2012, Padron et al. 2014).

The results in diet-induced obese mice by Jonas et al. (2014) strongly suggest that 3,5-T2 dose-dependently influences the expression of TH responsive genes possibly through binding and activating the TH receptor (TR) as shown for teleosts (Garcia et al. 2007, Mendoza et al. 2013, Navarrete-Ramirez et al. 2014) and rats (Ball et al. 1997).

Most of the beneficial effects of 3,5-T2 reported on mitochondrial function, lipid mobilization, and fatty acid oxidation were observed in experimental models of obesity or chronic hypothyroidism in rodents, mainly rats (de Lange et al. 2011, Grasselli et al. 2012, Lombardi et al. 2012, Cavallo et al. 2013, Silvestri et al. 2010), while only scarce data exist regarding the effects of 3,5-T2 on metabolism in euthyroid mammals on standard or on HFD in a mouse model (Goldberg et al. 2012).

To identify novel target genes of 3,5-T2 previously unknown to be TH responsive, we compared liver tissue transcriptomes of diet-induced obese mice that were treated with either 0.25 or 2.5 µg/g bw 3,5-T2. Furthermore, we performed a comparative transcriptome analysis of liver tissue from lean mice treated with the higher dose of 2.5 µg/g bw 3,5-T2 to compare the effects of 3,5-T2 between euthyroid lean and obese mice.

The results obtained suggest a specific signature of 3,5-T2-dependent gene expression. Furthermore, it became obvious that 3,5-T2 treatment induces the expression of genes encoding xenobiotic-metabolizing enzymes in lean as well as in obese mice, an effect of 3,5-T2 also demonstrated for the constitutive androstane receptor (CAR) target gene Sult1a1 in murine primary hepatocytes. Interestingly, only in diet-induced obese mice 3,5-T2 additionally alters the expression of genes encoding proteins involved in the modulation of endogenous cholesterol and bile acid synthesis.

**Materials and methods**

**Animals and treatment**

C57BL/6j breeding pairs were obtained from Jackson Laboratories and were further bred at the animal care unit of the German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany. Mice were kept under standard conditions (22°C, 12 h light:12 h darkness cycle). Free access to water and a standard diet for mice (ssniff, Soest, Germany) was assured. After weaning, mice were fed either a normal diet (ND: 10kJ% fat, 55% soybean oil, 44% lard, D12450B, Research Diets, New Brunswick, NJ, USA) or a high-fat diet (HFD: 60kJ% fat; 9% soybean oil, 90% lard, D12492, Research Diets) ad libitum.

Twenty weeks after weaning, male mice on ND or HFD were randomly divided into groups and single-housed before starting the experiment. In each group ($n$ = 6–8), bw was normally distributed (ND: 29.9 ± 2.3 g, HFD: 50.2 ± 3.8 g). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by ethics committee of the State Agency of Environment, Health and Consumer Protection (State of Brandenburg, Germany).

We carried out an initial experiment in which HFD male mice were injected for 14 days daily i.p. either with 0.25 or 2.5 µg/g bw 3,5-T2 (purity >99.9%) dissolved in physiological saline solution containing 0.15% NaOH (Sigma-Aldrich) or with the solvent control (Fig. 1A).
Primary culture of mouse hepatocytes

Three-month-old C57BL/6N male mice weighing 20–30g were anesthetized and primary hepatocytes were isolated by digestion of the liver with perfusion of collagenase type II, as described previously (Rathmann et al. 2015).

Cell viability was assessed by trypan blue staining and 4×10⁵ cells/well were seeded on collagen-coated 6-well plates and cultured in DMEM (Biochrom GmbH, Berlin, Germany) supplemented with 4.5g/L glucose, 1% glutamine, 10% fetal calf serum (FCS), 100IU/mL penicillin G, 100IU/mL streptomycin, and 100nM Na₂SeO₃ at 37°C and 5% CO₂. After 3h, the medium was renewed. For ex vivo stimulation, primary hepatocytes were starved in FCS-free medium for 24h and afterward incubated in DMEM containing 4.5g/L glucose, 1% glutamine, 0.5% DMSO, 100IU/mL penicillin G, 100IU/mL streptomycin, and 100nM Na₂SeO₃ with or without 3,5-T₂ (10nM to 10µM 3,5-T₂ in DMSO/30mM HCl) for 24h.

Microarray analysis

Total RNA was extracted from frozen liver tissues using a modified phenol extraction with TRIzol reagent combined with homogenization in a bead mill dismembrator. After total RNA purification and quality control using an Agilent 2100 Bioanalyzer (Agilent Technologies), individual RNA samples (n=3) were subjected to transcriptome analysis using GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix). Target preparation and array hybridization were performed according to the manufacturer's instructions using the WT Expression Kit and GeneChip WT Terminal Labeling Kit (GeneAtlas WT Expression Kit User Manual, Affymetrix). Quality assessment of hybridizations was monitored using Expression Console software (Affymetrix). Microarray data analysis was performed using Rosetta Resolver software system (Rosetta Biosoftware, Seattle, WA, USA). For a detailed description of microarray analysis refer to Supplementary Document 1.

Genes exhibiting significantly different expression on the mRNA level were identified using the following cut-off criteria: one-way ANOVA with Benjamini and Hochberg false discovery rate (P≤0.05), signal correction statistics (Ratio Builder, P≤0.05), and fold-change ≥1.5-fold.

In silico pathway and functional analysis of differentially expressed genes was carried out using the commercial systems biology oriented package, Ingenuity Pathway Analysis (Ingenuity Systems, Inc. Redwood City, CA, USA).

Total RNA preparation, cDNA synthesis, and quantitative real-time PCR

Total RNA from liver was isolated by TRIzol reagent (Invitrogen) following the manufacturer’s protocol and stored at −80°C until use. Total RNA from primary hepatocytes was isolated using the Aurum Kit (Bio-Rad) following the manufacturer’s instructions. RNA purity and concentration as well as performance of cDNA synthesis and RT-qPCR were monitored as described in a previous publication, with respect to their HPT feedback axis and lipid metabolism (Jonas et al. 2014).

After treatment, mice were restricted from food for 6h before being killed under anesthesia with isoflurane. Livers were immediately excised, weighed, and subsequently snap-frozen in liquid nitrogen and stored at −80°C until use.

Figure 1
Experimental design. (A) Groups of mice fed high-fat diet (HFD) for either 2 or 4 weeks. (B) Groups of mice fed normal diet (ND) for 4 weeks.

The low dose of 3,5-T₂ (0.25 µg/g bw), corresponding to that used in previous rat experiments, did not significantly change hepatic gene expression compared with saline application. Therefore, in a second and third experiment, HFD as well as ND mice were treated with a higher dose of 3,5-T₂ (2.5 µg/g bw) or with the solvent control for 28 days to study long-term treatment and to compare the effects of 3,5-T₂ in mice receiving different dietary fat supply (Fig. 1A and B). These treatment groups were partially identical to those already described in a previous publication, with respect to their HPT feedback axis and lipid metabolism (Jonas et al. 2014).

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Statistical analyses

Results generated by RT-qPCR are expressed as mean ± S.E.M. Statistical differences between more than two treatment groups and control groups were determined by Kruskal–Wallis test (Dunn’s post-test) otherwise Mann–Whitney U test was used to compare between two groups. All statistical analyses and the identification of outliers were done by GraphPad Prism and QuickCalcs software (version 5, GraphPad Software). Statistical significance between treatment group and control group was defined as *P* ≤ 0.05.

Results

Impact of 3,5-T₂ on the liver transcriptome of lean and obese mice

Based on the results of the liver transcriptome analysis, treatment of HFD mice for 14 days with the 3,5-T₂ dose that was described to be effective after the same treatment time in the liver of HFD rats (de Lange et al. 2011), that is, 0.25 μg/g bw 3,5-T₂, did not significantly change the murine hepatic transcriptome, while a 10-fold higher dose caused marked effects as illustrated by Venn diagram (Fig. 2).

As expected, treatment of HFD mice with 3,5-T₂ (2.5 μg/g bw) for 4 weeks resulted in a higher number of differentially expressed genes compared with the 2-week treatment. Approximately, half of those transcripts already affected after 2 weeks were also changed after 4 weeks (Fig. 2). Interestingly, while in ND animals, 221 genes exhibited different hepatic transcript levels after the 4-week treatment, this was only the case for 106 genes in HFD mice, with a partial overlap of 20 genes with increased and 30 with decreased expression (Fig. 2).

3,5-T₂ exerts effects on TH responsive genes

Treatment of HFD as well as ND mice with 3,5-T₂ significantly increased the transcript levels of TH responsive genes such as Dio1 encoding deiodinase 1, and decreased those of Serpina7 encoding serpin peptidase inhibitor, clade A, member 7, also known as thyroxine-binding globulin. These expression profiles were validated by RT-qPCR analyses (Fig. 3).

3,5-T₂ regulates gene expression of drug-metabolizing enzymes and transporters

The significantly associated canonical pathways identified using ingenuity pathway analysis (IPA) mainly comprised genes related to xenobiotic metabolism and detoxification, including the IPA pathways ‘glutathione-mediated detoxification’ and ‘nicotine degradation’. Interestingly, the analysis of genes differentially expressed after 4 weeks of treatment in HFD mice specifically identified gene ontology-classified pathways in the context of farnesyl, cholesterol, and zymosterol biosynthesis (Supplementary Table 2).

The greatest impact on hepatic genes expressing drug-metabolizing enzymes (DME) was observed for ND mice treated for 4 weeks. Most DME-related genes with significant expression changes in HFD mice exhibited the same profile in ND mice. However, 12 genes (Cyp1a2, Cyp39a1, Cypt46a1, Cyp51, Cyp2d9, Ces1(f,g) and 2a, Sult1b1, Slc13a3, Slc39a4, Gpx6) were exclusively differentially expressed in HFD mice (Fig. 4).

With regard to Phase I enzymes, the transcriptome data indicated that 3,5-T₂ affects the expression of several genes coding for cytochrome P450 enzymes (P450), especially for those of the Cyp1, −2, −4, −17, −39, −46, and −51 families (Fig. 4), which are known to fulfill several physiological functions (Table 1). Moreover, 3,5-T₂ also modulated the transcript levels of Ces1 (Ces1g), 2, and 4 encoding carboxylesterases that comprise a family of proteins catalyzing neutral lipid hydrolysis and biotransformation of structurally diverse drugs (Staudinger et al. 2010) (Fig. 4). A more detailed look at Phase II enzymes revealed that treatment of ND and HFD mice significantly increased the mRNA levels of Sult5a1 and Ugt2b37, also known as Ugtb25, encoding the conjugation enzymes sulfotransferase family 5A, member 1 and UDP-glucuronosyltransferase 2 family, polypeptide B37. Interestingly, 3,5-T₂ treatment of ND mice also increased the expression of Papss2 encoding 3′-phosphoadenosine 5′-phosphosulfate (PAPS) synthetase, which catalyzes the synthesis of PAPS, the activated sulfate source in sulfotransferase reactions. The transcriptome
3,5-T2 alters murine genes

Data also demonstrated that 3,5-T2 affects glutathione metabolism, as treatment of ND and HFD mice reduced the expression of several glutathione S-transferase encoding genes (e.g. Gsta2 and Gsta4) (Fig. 4).

Phase III (efflux) transporter encoding genes were differentially affected: Slc22a7 which codes for the organic anion transporter 2 (OAT2) was strongly induced in ND and HFD mice. On the other hand, the strongest decrease of gene expression after 3,5-T2 treatment in both ND and HFD mice was observed for Abcc3 encoding the multidrug resistance-associated protein MRP3 (Fig. 4).

To confirm and validate the transcriptome data, we analyzed representative genes encoding Phase I and Phase II enzymes or Phase III transporters via RT-qPCR. The results obtained correlated well with the microarray-based expression profiles obtained for HFD mice treated for 2 weeks and ND mice treated for 4 weeks (Fig. 5A, B, and C).

CAR as a potential candidate for an intermediate factor of 3,5-T2 action on drug-metabolizing enzymes

Literature to date suggests a major role for the CAR encoded by Nrl13 as a transcriptional regulator of DME and ATP-binding cassette/solute carrier family transporters (Chen et al. 2012, Xu et al. 2005). The transcriptome data indicated that several of those genes differentially expressed after 3,5-T2 treatment are potential targets of CAR (Table 2).

The results obtained from the in vitro model of primary hepatocytes demonstrated that 3,5-T2 is also able to regulate the transcription of genes involved in xenobiotic metabolism in murine hepatocytes exposed for 24 h. The transcript concentration of Sult1a1, a potential CAR target, was concentration-dependently increased by 3,5-T2 in a range from 10 nM to 10 µM (Fig. 6).

3,5-T2 enhances the expression of genes involved in farnesyl, cholesterol, and bile acid synthesis

Treatment of HFD mice with 3,5-T2 for 4 weeks increased the mRNA levels of several genes encoding enzymes known to be involved in farnesyl and zymosterol synthesis in rats, namely of Idi1, Sgle, Cyp51, Sc4mol (Msno1), Nsdhl, and Hsd17b7 (Supplementary Table 2) (Nemoto et al. 2013). Furthermore, enhanced Cyp39a1
3,5-T₂ alters murine genes

expression and reduced Cyp46a1 expression was exclusively observed in HFD but not in ND mice, suggesting a potential modulation of bile acid synthesis by 3,5-T₂ under HFD conditions (Table 1). Figure 7 shows the validation of microarray data via RT-qPCR for Sgle and Hsd17b7 encoding squalene epoxidase and hydroxysteroid (17-) dehydrogenase 7, respectively, two key enzymes of steroid synthesis (Fig. 7A and B).

<table>
<thead>
<tr>
<th>Phase I enzyme</th>
<th>HFD ± 3,5-T₂ 2 weeks fold change</th>
<th>HFD ± 3,5-T₂ 4 weeks fold change</th>
<th>ND ± 3,5-T₂ 2 weeks fold change</th>
<th>HFD + control 2 weeks</th>
<th>HFD + control 4 weeks</th>
<th>ND + control 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp2b9</td>
<td>3.5</td>
<td>2.8</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp17a1</td>
<td>2.9</td>
<td>2.8</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp39a1</td>
<td>2.1</td>
<td>2.8</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp51</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp2b13</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp2c39</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ces4a</td>
<td>2.5</td>
<td>2.4</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp2c38</td>
<td></td>
<td>1.7</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ces1f</td>
<td></td>
<td>-1.6</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ces1g</td>
<td>-1.9</td>
<td>-2.0</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp46a1</td>
<td>-2.3</td>
<td>-2.3</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp2c37</td>
<td>-2.0</td>
<td>-2.0</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp1a2</td>
<td>-2.1</td>
<td>-2.1</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ces2a</td>
<td>-2.2</td>
<td>-2.2</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp2u1</td>
<td>-2.2</td>
<td>-2.2</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp2d9</td>
<td>-2.4</td>
<td>-2.4</td>
<td></td>
<td>*</td>
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<td>*</td>
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<td>Cyp2c44</td>
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<td></td>
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<td>*</td>
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<tr>
<td>Cyp4a12a</td>
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<td>-5.3</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cyp4a12b</td>
<td>-10.3</td>
<td>-6.5</td>
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<td>*</td>
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</table>

**Figure 4**
The influence of 3,5-T₂ treatment on the expression of genes encoding drug-metabolizing enzymes (DME). Genes differentially expressed after 3,5-T₂ treatment for 2 and 4 weeks in mice fed high-fat diet (HFD) or for 4 weeks in mice fed normal diet (ND) are shown. Gene products were classified in drug-metabolizing enzymes (DME) including Phase I and Phase II enzymes as well as Phase III transporters. The heat map on the right-hand side shows significantly differentially expressed genes (*P < 0.05) compared with the corresponding control groups (ND or HFD control) after 3,5-T₂ treatment (n = 3 per group). Each cell in the heat map indicates the expression level of the corresponding gene for one animal. The highest expression levels are given in black, the lowest in white.
Table 1  Putative functions of proteins encoded by P450 genes differentially expressed after 3,5-T2 treatment in mice fed normal or high-fat diet.

<table>
<thead>
<tr>
<th>P450 family</th>
<th>Isoforms modulated by 3,5-T2 treatment</th>
<th>Putative hepatic function</th>
<th>Reference</th>
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<tr>
<td>Cyp1a</td>
<td>2</td>
<td>Oxidation of xenobiots</td>
<td>(Toison &amp; Wang 2010)</td>
</tr>
<tr>
<td>Cyp2b</td>
<td>9, 13</td>
<td>Oxidation of xenobiots</td>
<td>(Wang &amp; Negishi 2003)</td>
</tr>
<tr>
<td>Cyp2c</td>
<td>29, 37, 38, 39, 44</td>
<td>Metabolism of steroids and acute-phase response</td>
<td>(Corton et al. 1998)</td>
</tr>
<tr>
<td>Cyp2d</td>
<td>9</td>
<td>Steroid biosynthesis</td>
<td>(Jarukamjorn et al. 2006)</td>
</tr>
<tr>
<td>Cyp2u</td>
<td>1</td>
<td>ω-hydroxylation of fatty acids</td>
<td>(Chuang et al. 2004)</td>
</tr>
<tr>
<td>Cyp4a</td>
<td>12a, b</td>
<td>ω-hydroxylation of fatty acids and related compounds</td>
<td>(Sue Masters &amp; Marohnic 2006)</td>
</tr>
<tr>
<td>Cyp17a</td>
<td>1</td>
<td>Steroid biosynthesis</td>
<td>(Liu et al. 2005)</td>
</tr>
<tr>
<td>Cyp29a</td>
<td>1</td>
<td>Bile acid synthesis</td>
<td>(Li-Hawkins et al. 2000)</td>
</tr>
<tr>
<td>Cyp46a</td>
<td>1</td>
<td>Bile acid synthesis</td>
<td>(Lund et al. 2003, Lorbek et al. 2012)</td>
</tr>
<tr>
<td>Cyp51</td>
<td></td>
<td>Cholesterol and steroid biosynthesis</td>
<td>(Debeljak et al. 2003)</td>
</tr>
</tbody>
</table>

Discussion

This study demonstrates that treatment with 3,5-T2 (2.5 µg/g bw) in ND and HFD mice strongly affects the hepatic gene expression. The results of the comparative analysis of 3,5-T2-treated vs saline-treated mice suggest an important role for 3,5-T2 in modulating the expression of genes encoding enzymes involved in thyroid and steroid hormone as well as drug metabolism. The liver is the primary organ expressing genes coding for DME including Phase I and Phase II enzymes as well as Phase III (efflux) transporters, thereby representing a defense system that metabolizes and eliminates xenobiots und endobiots to protect the organism (Xu et al. 2005).

Compared with ND mice, the hepatic transcriptome of 3,5-T2-treated HFD mice demonstrated a specific and broad expression pattern of DME genes, but fewer differentially expressed genes. Recent studies have revealed different hepatic expression profiles of genes involved in xenobiots processing in animal models on HFD or caloric restriction (Ghose et al. 2011, Pu & Klaassen 2014). Such differences might be explained by an already modified expression of genes encoding nuclear receptors and DME in fatty liver due to pronounced oxidative stress and/or hepatic inflammation and crosstalk between proinflammatory factors and biotransformation enzymes (Pascussi et al. 2003, Ghose et al. 2011), which might also affect the elimination of exogenously added 3,5-T2. Alternatively, expression profiles of most genes modulated by 3,5-T2 might overlap with those altered by HFD and administration of 3,5-T2 to mice will not act additively or synergistically with HFD.

3,5-T2 altered the transcript concentration of genes encoding cytochrome P450 enzymes (P450), which comprise heme-containing microsomal mono-oxygenases and exert different important physiological functions including steroid, lipid, and xenobiots metabolism (Table 1). Previous studies have shown that classical TH regulate numerous enzymes associated with the mixed function oxidation system (Ram & Waxman 1991, 1992, O’Leary et al. 1997, Ortiz de Montellano 2005), while no such effects were reported for 3,5-T2.

In addition, the transcriptome analyses revealed increased expression of certain genes specifying Phase II enzymes including Sult5a1 and Ugt2b37, which encode sulfotransferase and UDP-glucuronosyltransferase. Previous studies have shown that Sult and Ugt isoforms are of physiological importance in iodothyronine conjugation and enterohepatic recycling as well as local prereceptor control of T3 availability for TR binding and activation (Visser 1996, Wu et al. 2005, Runge-Morris et al. 2013). Silvestri et al. observed no change in the protein levels of SULT1A1 in the liver of HFD rats treated over 30 days with 0.25 µg/g bw 3,5-T2 compared with sham, though differences between ND and HFD rats were detected (Silvestri et al. 2010). We demonstrated in primary hepatocytes that 3,5-T2 concentration-dependently increased the expression of Sult1a1 (Fig. 6), potentially resulting in a subsequent inactivation of 3,5-T2 as it is known for T3 (Anderson et al. 1995).

Treatment of HFD mice with 3,5-T2 reduced the expression of Gpx6 encoding glutathione peroxidase 6 that is involved in the detoxification of hydrogen peroxide. Moreover, various Gst-encoded isoenzymes catalyzing the conjugation of a wide variety of xenobiots with glutathione (McCarver & Hines 2002) were downregulated in ND as well as HFD mice (Fig. 4). Previous studies on hyperthyroid rats showed a decline in hepatic glutathione content and reduced enzyme activities of GST and GPX (Beckett et al. 1988; Chattopadhyay et al. 2007). Our findings suggest a potential role of 3,5-T2 in altering...
hepatic cellular redox status and influencing synthesis and turnover of glutathione possibly by mimicking a hyperthyroid status of the liver.

Efflux transporters are localized to basolateral and canalicular membranes transferring bile acids, drugs, and metabolites from hepatocytes to blood or bile. We detected the strongest alterations in transporter gene expression after 3,5-T₂ treatment for Abcc3 and Slc22a7 encoding the multidrug resistance-associated protein MRP3 and the organic anion transporter OAT2, respectively. MRP3 is responsible for the basolateral export of bile acids, xenobiotics, and glucuronide metabolites into sinusoidal blood, while OAT2 is involved in the transport of organic anions (Maher et al. 2005, Zamek-Gliszczynski et al. 2006, Burchardt 2012). Hepatic expression of Abcc3 and Slc22a7 is altered by age, sex, and diet composition (Cheng et al. 2008, Ghose et al. 2011, Fu et al. 2012, Fu & Klaassen 2014). Beside this, Dong et al. (2007) demonstrated in liver of hypothyroid animals a reversed expression pattern of Slc22a7 and Abcc3, where Slc22a7 transcript levels were significantly decreased, while Abcc3 expression was increased. We speculate that 3,5-T₂ modulates the expression of efflux transporter genes resulting in a higher clearance of the corresponding metabolites. Interestingly, 3,5-T₂ treatment of ND as well as HFD mice enhanced the transcript levels of Slc25a30 and Slc25a45, both encoding...
Table 2  Known CAR target genes identified to be differentially expressed after 3,5-T₂ treatment by transcriptome analysis in this study.

<table>
<thead>
<tr>
<th>Annotated genes</th>
<th>Modulation by 3,5-T₂</th>
<th>Drug-metabolizing enzymes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp1a2</td>
<td>down</td>
<td>Phase I enzyme</td>
<td>(Tolson &amp; Wang 2010)</td>
</tr>
<tr>
<td>Cyp2b9</td>
<td>up</td>
<td>Phase I enzyme</td>
<td>(Rivera-Rivera et al. 2003)</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>down</td>
<td>Phase I enzyme</td>
<td>(Jackson et al. 2004)</td>
</tr>
<tr>
<td>Cyp39a1</td>
<td>up</td>
<td>Phase I enzyme</td>
<td>(Beilke et al. 2009)</td>
</tr>
<tr>
<td>Ces2a</td>
<td>down</td>
<td>Phase I enzyme</td>
<td>(Zhang et al. 2012, Jones et al. 2013)</td>
</tr>
<tr>
<td>Sult5a1</td>
<td>up</td>
<td>Phase II enzyme</td>
<td>(Aleksunes &amp; Klaassen 2012)</td>
</tr>
<tr>
<td>Ugt2b37</td>
<td>up</td>
<td>Phase II enzyme</td>
<td>(Richardson et al. 2008)</td>
</tr>
<tr>
<td>Gsta2</td>
<td>down</td>
<td>Phase II enzyme</td>
<td>(Roques et al. 2013)</td>
</tr>
<tr>
<td>Slik22a7</td>
<td>up</td>
<td>Phase III transporter</td>
<td>(Jigorel et al. 2006)</td>
</tr>
<tr>
<td>Abcc3</td>
<td>down</td>
<td>Phase III transporter</td>
<td>(Staudinger et al. 2013)</td>
</tr>
</tbody>
</table>

mitochondrial carrier proteins (Haitina et al. 2006), which is in line with pronounced effects of 3,5-T₂ on the mitochondrial compartment (Arnold et al. 1998; Lombardi et al. 1998). Additionally, Paquette et al. reported a TH responsive element in the promoter region of Slc25a45, though the physiological function of the encoded transporter in liver is still unknown (Paquette et al. 2011).

Many of the DME genes affected by 3,5-T₂ are known to be regulated by CAR. CAR is a nuclear receptor and xenobiotic sensor that modulates gene expression by forming heterodimers with retinoid X receptor and binding to xenobiotic responsive elements (PBREM) in the promoter regions of genes encoding DME (Timsit & Negishi 2007). Beyond this, studies elucidated that CAR is also linked to hepatic lipid and glucose metabolism (Wada et al. 2009; Xiao et al. 2013). In vitro data from the hepatic Huh7 cell line and cryopreserved small hepatocytes have shown that TH may regulate the expression of specific CAR target genes possibly by binding of the activated TH receptor to the PBREM region (Ooe et al. 2009, Bing et al. 2014). In addition, it was shown that CAR itself can regulate the TH responsive gene Thsps through binding to TRE sequences of the promoter region (Breuker et al. 2010). Since xenobiotics are known to exert strong influence on TH metabolism, it is important to consider that activation of CAR by 3,5-T₂ might alter hepatic TH homeostasis via secondary mechanisms through induced liver DME synthesis and enhanced TH clearance (Maglich et al. 2004, Qatanani et al. 2005, Radovic et al. 2010, Roques et al. 2013, Schraplau et al. 2014). Previous studies demonstrated a strong overlap and cross talk between hepatic transcription factors regulating different DME encoding genes such as Sult5a1 or Mrp3 (Xu et al. 2005, Aleksunes & Klaassen 2012). Therefore, genes encoding DME and modulated by 3,5-T₂ may be additionally controlled via an indirect or independent mechanism possibly involving further transcription factors (Ahr, Pxr, and Nrf2).

Aside from DME, the transcriptome analysis demonstrated that 3,5-T₂ is also modulating the expression of genes (Serpina7, Dio1) known to be TH responsive (Hayashi et al. 1993, Zhang et al. 1998). These findings might be explained by potential binding of 3,5-T₂ to the hepatic TH receptor β1 mediating the transcription of target genes. This would be in agreement with conclusions from recent studies on 3,5-T₂ action in rat hepatic nuclei and in tilapia (Koerner et al. 1975, Mendoza et al. 2013, Navarrete-Ramirez et al. 2014).

Our transcriptome analysis reveals that several genes involved in farnesyl, cholesterol, and bile acid synthesis are modulated by 3,5-T₂ treatment for 4 weeks in HFD mice, but not in ND mice. Induced gene expression was observed for enzymes involved in the biosynthesis of zymosterol, a precursor of cholesterol (Nemoto et al. 2013). In addition, 3,5-T₂ modulated the transcript levels of genes (Cyps46a1, Cyp39a1) involved in bile acid synthesis (Beilke et al. 2009). Regarding the potential effects of 3,5-T₂ on CAR activation, it is interesting to note that HFD mice treated with the CAR activator TCPBOP exhibited a comparable induction of hepatic genes involved in cholesterol and bile acid syntheses as well as reduced total serum and liver cholesterol levels (Rezen et al. 2009). 3,5-T₂ might stimulate hepatic cholesterol synthesis accompanied by increased secretion of biliary cholesterol, the main route for cholesterol elimination from the body. This would be in line with published data showing the antisteatotic ability of 3,5-T₂ application in HFD rats (Mollica et al. 2009, Grasselli et al. 2012).

To our knowledge, this is the first transcriptome analysis of liver tissue from euthyroid lean and obese mice treated with 3,5-T₂. Proteomic approaches in HFD rats that were treated with 3,5-T₂ (0.25 µg/g bw) or TRC150094, a novel 3,5-T₂ analogue, showed strong effects on hepatic mitochondrial and fatty acid metabolism; however, only minor similarities with regard to the expression profiles
reported in this study were observed (Silvestri et al. 2010; Silvestri et al. 2012).

Treatment of mice with 2.5 µg/g bw 3,5-T₂ exerted marked transcriptional effects in liver. A tenfold lower dose (0.25 µg/g bw) did not change the hepatic gene expression pattern. However, the same dose already affected the HPT axis by lowering pituitary transcript of thyroid-stimulating hormone beta as well as serum T₃ concentration (Jonas et al. 2014). These findings are surprising since studies in rats using the same dose exhibited alterations in hepatic gene expression as well as changes in metabolic parameters without interfering with the HPT axis (Goglia 2005, 2014). This might be due to species differences in hepatic and pituitary 3,5-T₂ uptake, metabolism, action, and elimination.

The differences between published 3,5-T₂ effects might be explained in part by short- vs long-term 3,5-T₂ treatment in vivo or by using biomaterials originating from 3,5-T₂ treatment of hypothyroid vs euthyroid animal models to study the effects of 3,5-T₂ in vitro. 3,5-T₂ treatment might indirectly affect the hepatic gene expression resulting from a 3,5-T₂-induced altered balance of further locally active hepatic TH metabolites. Hepatocytes exhibit pronounced deiodination capacity (Rathmann et al. 2015). It might be that TH metabolism in liver generates decarboxylated and/or deiodinated further TH metabolites that may affect gene expression even via the classical T₃ receptor.

Therefore, it should be taken into account that murine effects of 3,5-T₂ on energy and TH metabolism are possibly accompanied by toxic hepatic side effects, such as the observed induction of drug-metabolizing enzymes. We cannot exclude that the effects of 3,5-T₂ are mediated by various different receptors which have not been characterized in detail yet. Such action might include modulation of classical T₃ receptors by high local 3,5-T₂ concentrations.

Overall, administration of 3,5-T₂ might exert adverse hepatic effects demonstrated by altered expression of TH responsive genes and activation of xenobiotic metabolism in liver. Therefore, the potential use of 3,5-T₂ as an antiobesity drug, as it is suggested in several publications or commentaries of the internet body-building and wellness scene, should be viewed with caution. Anyhow, recently described clinical associations between human 3,5-T₂ serum concentration and serum metabolic parameters such as TSH, fasting glucose, and leptin (Pietzner et al. 2015) might find prospective molecular explanations with the identification of these and further hepatic 3,5-T₂ target genes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0159.

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.

Funding
This work was supported by DFG grants KFO 218/2 TP3; the DFG priority program ThyroidTransAct KO 922/17-1 to JK, WA1328/5-1 to GH and UV, and the German Ministry of Education and Research (BMBF, DZD, grant 01GI0922).
Acknowledgments
The authors gratefully acknowledge Anja Fischbach and Antje Kretschmer for their excellent technical assistance. They are thankful to Eva K Wirth for her encouraging advice and support and to Maria Hartmann for the generation of the heat map.

References


Angelini B & Rudling M 2010 Lipid lowering with thyroid hormone and thyminometrics. Current Opinion in Lipidology 21 499–506. (doi:10.1097/GIM.0b013e328340326c)


Fu ZD, Csanaky IL & Klaassen CD 2012 Effects of aging on mRNA profiles for drug-metabolizing enzymes and transporters in livers of male and female mice. Drug Metabolism and Disposition 40 1216–1225. (doi:10.1124/dmd.111.044461)


Goglia F 2005 Biological effects of 3,5-diiodothyronine (T2). Biochemical Pharmacology 70 164–172. (doi:10.1016/j.bcp.2004.05.007)


Jarukamjorn K, Sakuma T, Jaruchotikamol A, Oguro M & Nemoto N 2005 3,5-diiodothyronine (3,5-T2) alters murine genes and others.


O'Leary KA, Li HC, Ram PA, McQuiddy P, Waxman DJ & Kasper CB 1997 Thyroid regulation of NADPH: cytochrome P450 oxidoreductase: identification of a thyroid-responsive element in the 5'-flank of the oxidoreductase gene. Molecular Pharmacology 52 46–53.

Ohe O, Kon J, Oshima H & Mitaka T 2009 Thyroid hormone is necessary for expression of constitutive androstane receptor in rat hepatocytes. Drug Metabolism and Disposition 37 1963–1969. (doi:10.1124/dmd.108.029905)


Ram PA & Waxman DJ 1991 Hepatic P450 expression in hypothyroid rats: differential responsiveness of male-specific P450 forms 2a (IIA2), 2c (IIC1), and RLM2 (IIA2) to thyroid hormone. Molecular Endocrinology 5 13–20. (doi:10.1210/mend-5-1-13)

Ram PA & Waxman DJ 1992 Thyroid hormone stimulation of NADPH P450 reductase expression in liver and extrahepatic tissues. Regulation by multiple mechanisms. Journal of Biological Chemistry 267 3294–3301. (doi:10.1074/jbc.M004755200)


Received in final form 13 February 2016
Accepted 22 February 2016
Accepted Preprint published online 22 February 2016