

Serum copper as a novel biomarker for resistance to thyroid hormone

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Thyroid hormone action is mediated by the thyroid hormone receptors TR α 1 and TR β . Defects in TR β lead to RTH (resistance to thyroid hormone) β , a syndrome characterized by high levels of thyroid hormone and non-suppressed TSH (thyroid-stimulating hormone). However, a correct diagnosis of RTH β patients is difficult as the clinical picture varies. A biochemical serum marker indicative of defects in TR β signalling is needed and could simplify the diagnosis of RTH β , in particular the differentiation to TSH-secreting pituitary adenomas, which present with clinically similar symptoms. In the present paper we show that serum copper levels are regulated by thyroid hormone, which stimulates the synthesis and the export of the hepatic copper-transport protein ceruloplasmin into the serum. This is accompanied by a concerted reduction in the mRNA levels of other copper-containing proteins such as metallothioneins 1 and 2 or superoxide dismutase 1. The

induction of serum copper is abolished in genetically hyperthyroid mice lacking TR β and human RTH β patients, demonstrating an important role of TR β for this process. Together with a previously reported TR α 1 specific regulation of serum selenium, we show that the ratio of serum copper and selenium, which is largely independent of thyroid hormone levels, volume changes or sample degradation, can constitute a valuable novel biomarker for RTH β . Moreover, it could also provide a suitable large-scale screening parameter to identify RTH α patients, which have not been identified to date.

Key words: copper, kidney, liver, resistance to thyroid hormone (RTH), thyroid hormone receptor.

INTRODUCTION

TH (thyroid hormone) is important for the development and the maintenance of almost all tissues [1,2]. This becomes most evident in congenital hypothyroidism, which, if not diagnosed and treated properly, can lead to irreversible mental retardation and a plethora of physiological defects [3]. The majority of TH action is mediated by the nuclear TRs (TH receptors) TR α and TR β , which are encoded by two separate genes located on the human chromosomes 17 and 3 respectively [4]. Defects in TR β can cause RTH (resistance to thyroid hormone) β , a syndrome characterized by elevated levels of free serum T₃ (3,3',5-tri-iodothyronine) and T₄ (thyroxine) in the presence of normal or elevated TSH (thyroid-stimulating hormone) [5]. The diagnosis of RTH β is generally difficult, as patients can be asymptomatic or clinically euthyroid, and classical symptoms such as goitre or attention-deficit-hyperactivity disorder are not consistently present [6]. If the slightly elevated or normal TSH levels are missed, RTH β can be easily misdiagnosed as thyrotoxicosis, with devastating consequences if anti-thyroid treatment is initiated as it will interfere severely with growth, particularly in children [6]. Other pathological conditions, such as a TSH-secreting pituitary adenoma [7] or abnormalities in serum hormone-binding proteins including T₄-binding globulin or albumin, may also present with altered concentrations of free T₃ and T₄ in the presence of normal or elevated TSH [5,8], thus making the conclusive diagnosis of RTH β challenging. Ultimately, genetic testing for mutations in TR β is required; however, in 15% of RTH β patients such mutations were not found [9].

Although to date more than 350 families with over 1000 affected RTH β subjects have been identified [4], surprisingly no patient

with a mutant TR α 1 has been described and the phenotype of human RTH α remains unclear. Analyses of mice heterozygous for a mutant TR α 1, however, suggest that these patients might display normal TH economy, which could hinder their identification [10]. Despite the several defects observed in the animals with a mutant TR α 1 (for a review see [11]), a reliable biochemical marker for a large-scale screening approach is missing. Although recent studies on TR α 1R384C mutant mice revealed a TR α 1-specific regulation of serum selenium (Se) [12], this parameter alone is not suitable to predict or diagnose RTH as it varies with serum TH levels.

In the present paper we report that, similar to Se, serum copper (Cu) levels are positively regulated by TH, mainly by directing the production of the Cu-transport protein ceruloplasmin. Using the TR α 1-dependent regulation of serum Se, we show that the ratio between serum Se and Cu can be used as a novel sensitive biomarker for the identification of TR-isoform-specific signalling impairments and might prove of high value for the differential diagnosis of RTH β . Moreover, the serum Se/Cu ratio might constitute a valuable screening tool in epidemiological analyses to search for RTH α patients, as it can exclude the majority of individuals with normal TR-mediated signalling, thereby narrowing down the number of potential RTH α candidates.

MATERIALS AND METHODS

Experimental animals

The mouse strain carrying the dominant-negative R384C mutation in TR α 1 has been described previously [13]. The TR α 1 + mice used for the experiments have been backcrossed to C57BL/6NCRl for eight to ten generations. In addition,

Abbreviations used: ATP7, ATPase, Cu²⁺ transporting; Mtt, metallothionein; RTH, resistance to thyroid hormone; SOD1, superoxide dismutase 1; T₃, 3,3',5-tri-iodothyronine; T₄, thyroxine; TH, thyroid hormone; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; TXRF, total reflection X-ray fluorescence.

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Table 1 Thyroid function tests in RTH β patients and first-degree relativesResults are means \pm S.E.M.

Group	<i>n</i>	Male/female	Age (years)	Free T ₄ index	Free T ₃ index	TSH (milliunits/l)	Thyroglobulin (μ g/l)
RTH patient	25	15/10	27.5 \pm 13.3	19.1 \pm 2.1**	301 \pm 48**	2.31 \pm 2.24	47 \pm 24**
First-degree relatives	20	9/11	24 \pm 10.2	8.81 \pm 1.14	157 \pm 22	2.19 \pm 1.34	18 \pm 6
Range			1.0–57	6.0–10.5	95–190	0.4–3.8	1–30

***P* < 0.01 compared with other groups.

TR α 1 + m have been crossed to TR β -deficient mice yielding TR α 1 + m TR β ^{-/-} double mutants as described in detail previously [14]. If not indicated otherwise, littermate male mutant and wild-type mice were born by wild-type females, and five animals per group were used for the experiments at the age of 4–7 months. The animals were housed at 21 °C on a 12 h light/12 h dark cycle. If required, mice were treated with T₃ via their drinking water containing 0.01 % albumin and 0.5 μ g/ml T₃ for 12 days. For the thionamide experiment, mice were kept at 30 °C for 4–6 weeks. Urine was collected by putting the animals on the surface of a mirror.

Trace element determination

Serum samples were diluted with ultrapure H₂O and a gallium standard solution was added as an internal control. Tissue samples were digested in 0.1 M nitric acid for 3 h at 150 °C and supplemented with the gallium standard. A benchtop TXRF (total reflection X-ray fluorescence) spectrometer (PicofoxTM S2, Bruker) was used to determine Cu and Se concentrations. Samples were applied to glass carriers and measured as described previously [15]. All trace element analyses were done in a blinded fashion with respect to the genotype and T₃ treatment of the mice in a remote laboratory separate from the animal facility.

Real-time PCR

RNA was isolated from snap-frozen tissues using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Subsequent cDNA synthesis of 4 μ g of RNA was carried out using oligo(dT) primers and the transcriptase first-strand cDNA synthesis Kit (Roche). Quantitative real-time PCR was performed with the 7300 Real Time PCR System (Applied Biosystems) and the FastStart Universal SYBR Green PCR Master Mix (Roche). Specificity of amplification was verified by melting-curve analyses. A standard curve was used to correct for PCR efficiency and the results were normalized using *HPRT* (hypoxanthine phosphoribosyltransferase) as the reference gene. The sequences of the primers used are available on request.

Western blotting for ceruloplasmin

For the determination of ceruloplasmin, 5 μ l of mouse serum or 10 μ l of cell culture supernatant (HepG2 cells, 40–50 % confluency, cultured for 24 h in the absence of TH or supraphysiological concentrations of 50 nM T₃) were separated on a 4–12 % Bis-Tris SDS polyacrylamide gel (NuPage) and transferred on to a nitrocellulose membrane (Whatman). Ceruloplasmin was detected using a specific polyclonal goat antibody (ab19171, 1:1000 dilution; Abcam) and a horseradish peroxidase-coupled secondary antibody with an ECL (enhanced chemiluminescence) reagent (Roche). Protein content was quantified using the NIH ImageJ software (<http://rsbweb.nih.gov/ij/>) and normalized against Ponceau S

staining of the nitrocellulose membrane for the HepG2 supernatant.

RTH β patient analysis

RTH β patients and first-degree relatives belong to the same large Azorean family of Portuguese ancestry comprising 246 members spanning five generations. All individual enrolled in the present study live on the same small island (San Miguel, Azores, Portugal), are exposed to similar environmental conditions and share a common ancestry. All affected patients harbour the same mutation of the TR β (Arg²⁴³→Gln; R243Q). None of them were taking Se supplements, were known to be exposed to heavy metals or suffer from hepatic, cardiac or renal diseases. Affected individuals have an RTH β phenotype (high serum concentrations of T₄ and tri-iodothyronine without suppressed thyrotropin), confirmed by genotyping to identify the (R243Q) mutation of the TR β gene. Free T₄ and T₃ indexes were estimated from the product of total T₄ and T₃ concentrations respectively, and the normalized resin T₄ uptake ratio. Approximately 80 % of the affected individuals presented with goitre, but no other signs or symptoms of hyperthyroidism. One patient was misdiagnosis with Grave's disease and was submitted to thyroidectomy. She presents mild hypothyroidism under levothyroxine treatment. Two affected children suffered of attention deficit hyperactivity disorder and are medicated accordingly. Unaffected first degree relatives have normal thyroid function tests.

Ethical approval

Animal care procedures were in accordance with the guidelines set by the European Community Council Directives (86/609/EEC). Required permissions were obtained from the local ethical committee (Stockholms Norra Djurförsöksetiska Nämnd, No 74/07). All clinical studies have been conducted in accordance to the Declaration of Helsinki principles. Written informed consent was received from participants prior to inclusion in the study.

Statistics

Values are presented as means \pm S.E.M. Statistical significance was calculated by an unpaired two-tailed Student's *t* test or, where indicated, by a two-way ANOVA followed by a Bonferroni post-hoc test (see Supplementary Table S1 at <http://www.BiochemJ.org/bj/443/bj4430103add.htm>) and considered significant at *P* < 0.05 (*), *P* < 0.01 (**) or *P* < 0.001 (***)

RESULTS

To identify suitable trace element patterns which are characteristic for specific defects in TR signalling, we applied TXRF spectroscopy on serum samples from wild-type mice and animals heterozygous for a mutant TR α 1R384C (TR α 1 + m) with and

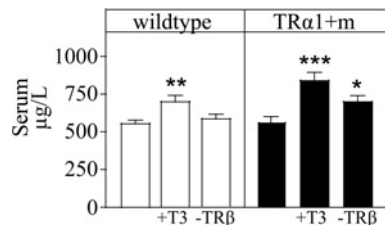


Figure 1 Serum Cu is regulated by TH

Serum Cu levels in wild-type animals and mice heterozygous for a mutant TR α 1 (TR α 1 + m) untreated ($n = 10$), T₃ treated (+ T₃; $n = 8$) or with an additional inactivation of TR β causing endogenously high levels of TH (- TR β ; $n = 5$). Results are means \pm S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared with untreated animals using two-way ANOVA).

without oral T₃ treatment [13]. The analysis revealed no difference in serum Cu between wild-type and TR α 1 + m mice, but a significant increase upon T₃ treatment in both genotypes (Figure 1 with detailed statistics in Supplementary Table S1). Other elements such as iron, calcium or potassium were not altered (results not shown). Remarkably, the increase in Cu was abrogated in both genotypes when hyperthyroidism was induced genetically by the ablation of the TR β gene [16], suggesting an important role of TR β in the regulation of serum Cu. Interestingly, independent of the cause of hyperthyroidism, serum Cu levels were always higher in hyperthyroid TR α 1 + m mutant mice as in the respective hyperthyroid controls.

To test for an effect of the metabolic rate, which is increased in TR α 1 + m mutant mice and also induced by hyperthyroidism, we reared mice for 4–6 weeks at thermoneutrality; a treatment known to reduce metabolic rate and normalize metabolism in TR α 1 + m mutant mice [17]. However, serum Cu levels did not change at thermoneutrality (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/443/bj4430103add.htm>), indicating that they are not connected to the metabolic rate of the animal. These data support our hypothesis on the usefulness of this novel trace-element-based biomarker for analysing TR-isoform-specific TH signalling effects

To elucidate the origin of the T₃-induced increase in serum Cu, we determined the Cu levels in the liver, kidney and urine of wild-type and TR α 1 + m animals with and without T₃ treatment (Figure 2 with detailed statistics in Supplementary Table S1). We observed a significant effect of the T₃ treatment on the Cu levels in the liver and kidney, indicative of increased hepatic Cu export and improved renal retention. Interestingly, the T₃ effect in the kidney was more pronounced in the TR α 1 + m mutant mice, suggesting that the stronger response in serum Cu to hyperthyroidism might originate here.

To investigate the underlying molecular mechanism, we analysed changes in gene expression induced by T₃ treatment in the liver and kidney. Real-time PCR analyses (Figure 3 with detailed statistics in Supplementary Table S1) revealed that in the liver, independent of the genotype, TH induced the expression of the two Cu-transport proteins ATP7 (ATPase, Cu²⁺ transporting) A and ATP7B as well as the major Cu-transport protein ceruloplasmin, whereas the mRNA levels of other Cu-containing enzymes such as SOD1 (superoxide dismutase 1) or Mtt (metallothionein) 1 and Mtt2 were reduced. Similarly in the kidney, the production of ceruloplasmin was induced by T₃; however, this increase was more pronounced in the TR α 1 + m mutant mice. We also observed a significant effect of the mutant TR α 1 on the expression of Mtt1 and Mtt2 mRNA levels in this tissue, which supports the hypothesis that the differences in serum Cu levels between hyperthyroid TR α 1 + m and hyperthyroid

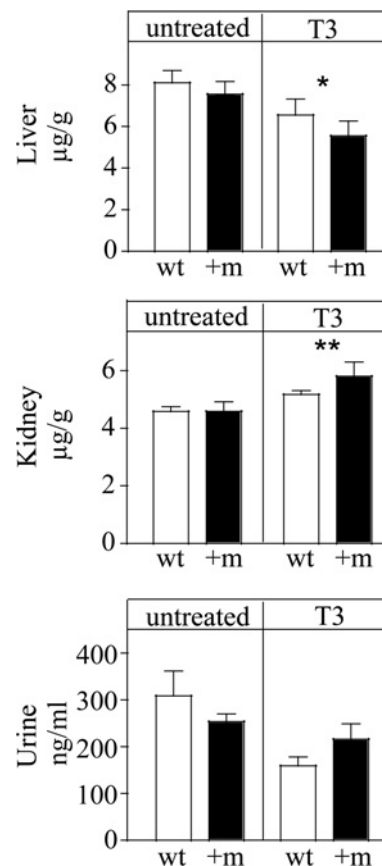


Figure 2 Changes in tissue Cu levels upon TH treatment

Levels of Cu in the liver, kidney and urine of wild-type (wt) animals and mice heterozygous for a mutant TR α 1 (+ m) untreated or T₃-treated. Results are means \pm S.E.M. * $P < 0.05$; ** $P < 0.01$ ($n = 5$, effect of T₃ treatment compared with untreated animals using two-way ANOVA).

controls originates here. However, the basal mRNA levels of all of the investigated genes did not differ between wild-type and untreated TR α 1 + m mutant mice; thus the reason for the renal oversensitivity to hyperthyroidism in TR α 1 + m mutant mice is most probably not caused by a relief of repression through the reactivation of the aporeceptor and remains somewhat enigmatic.

To test whether the increased mRNA production of ceruloplasmin also resulted in higher serum levels of this protein, we analysed ceruloplasmin protein by Western blotting in the serum of different animal models (Figure 4). As expected, we observed a 3-fold increase in ceruloplasmin in the serum of wild-type animals upon T₃ treatment, which was again slightly more pronounced in the TR α 1 + m mutants. Mice lacking TR β displayed an abrogated increase despite their severe hyperthyroidism [16], underlining the previously observed importance for TR β in this process. A similar secretion of ceruloplasmin upon T₃ treatment was also observed in the human HepG2 liver cell line (Supplementary Figure S1).

Given the interesting murine phenotype, we consequently analysed the serum Cu levels in a cohort of patients with RTH β [18]. Despite their high TH levels, serum Cu concentrations were significantly reduced in the RTH β patients when compared with control subjects from the same families ($P = 0.028$ for control compared with RTH β and no significance for male compared with female tested by two-way ANOVA; Figure 5A). As expected from our previous study [12], serum Se levels were elevated in

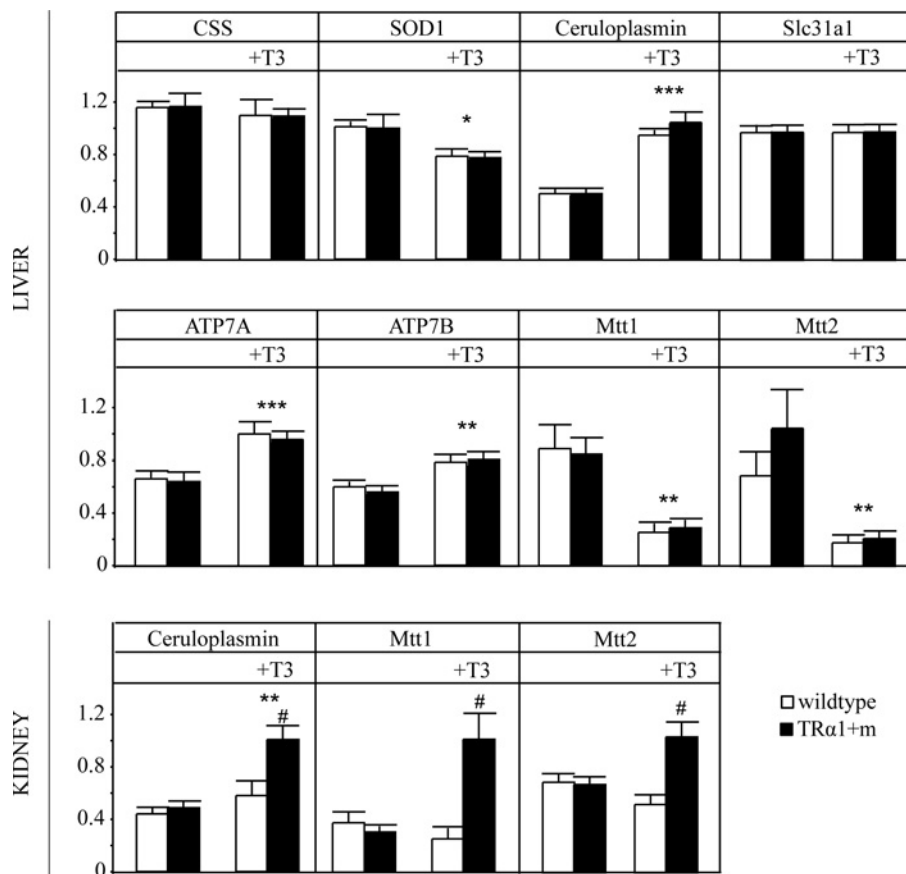


Figure 3 Changes in expression of Cu metabolic genes upon TH treatment

Quantification of mRNA expression in the liver and kidney of wild-type animals (open bars) and mice heterozygous for a mutant TR α 1 (closed bars) untreated or T₃-treated. CSS, Cu chaperone for superoxide dismutase; Slc31a1, solute carrier 31 subtype a1. Results are means \pm S.E.M. * P < 0.05; ** P < 0.01; *** P < 0.001 (n = 5, effect of T₃ treatment compared with untreated animals using two-way ANOVA).

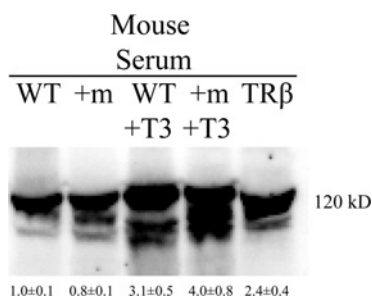


Figure 4 Serum ceruloplasmin levels

Ceruloplasmin protein levels in the serum of wild-type animals (wt) and mice heterozygous for a mutant TR α 1 (+ m) untreated, T₃-treated (T3) or mice lacking TR β .

RTH β patients (P = 0.045 for control compared with RTH β and no significance for male compared with female tested by two-way ANOVA; Figure 5A), as this regulation is mediated by the high TH levels in the patients acting on the intact TR α 1. Collectively, these findings in sera RTH β from patients correlated well with our data from the experimental animals.

We thus hypothesized that serum Cu could be used for the diagnosis of RTH; however, as it varies with changes in TH levels or blood volume, we normalized it using Se as another TH-regulated trace element. As expected, the Se/Cu ratio showed no

significant change upon T₃ treatment in the animal model (Figure 5B; the original serum Se levels in the different animal models can be found in [12]), whereas it was highly increased in the RTH β and significantly decreased in the RTH α model. Correspondingly in humans, the Se/Cu ratio was significantly increased in RTH β patients compared with the controls (P = 0.019 for control compared with RTH β and no significance for male compared with female tested by two-way ANOVA; Figure 5B).

In the light of these data, we thus conclude that the Se/Cu ratio might be helpful during the diagnosis of RTH, and we speculate that a human RTH α patient might also present with a reduced Se/Cu ratio as in the animal model (Figure 5B, right-hand column, extrapolated from the animal data in Figure 5A).

DISCUSSION

In the present study we have shown that serum Cu levels are regulated by TH, predominantly by enhancing synthesis and export of hepatic ceruloplasmin while down-regulating competing intracellular Cu-binding proteins. We generally obtained a good correlation between human and rodent data, and the serum levels were in the range expected from previous studies [19–22]; the only observed difference was a reduction in serum Cu in the human RTH β patients, whereas the TR β -deficient mice only exhibited an abrogated increase. However, this discrepancy is easily explained by the fact that the RTH β patients harbour

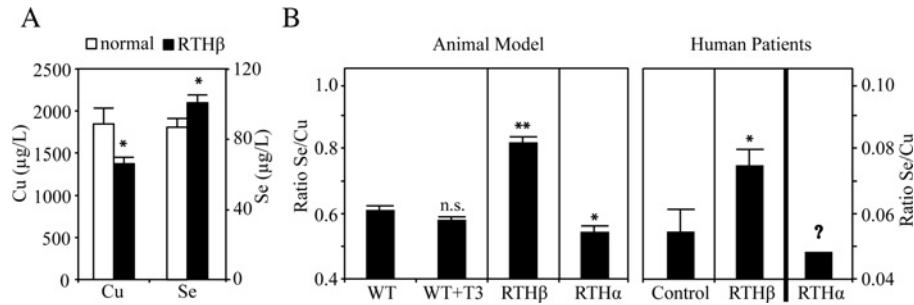


Figure 5 Serum Cu and Se as biomarker for RTHβ

(A) Serum Cu and Se levels in patients with RTHβ and controls. (B) Serum Se/Cu ratio in different animal models with defects in TRα (RTHα) or TRβ (RTHβ) and untreated (wt) or T₃-treated (wt + T₃) controls and the corresponding human situation with the predicted level for RTHα extrapolated from the animal model. Results are means ± S.E.M. **P* < 0.05; ***P* < 0.01.

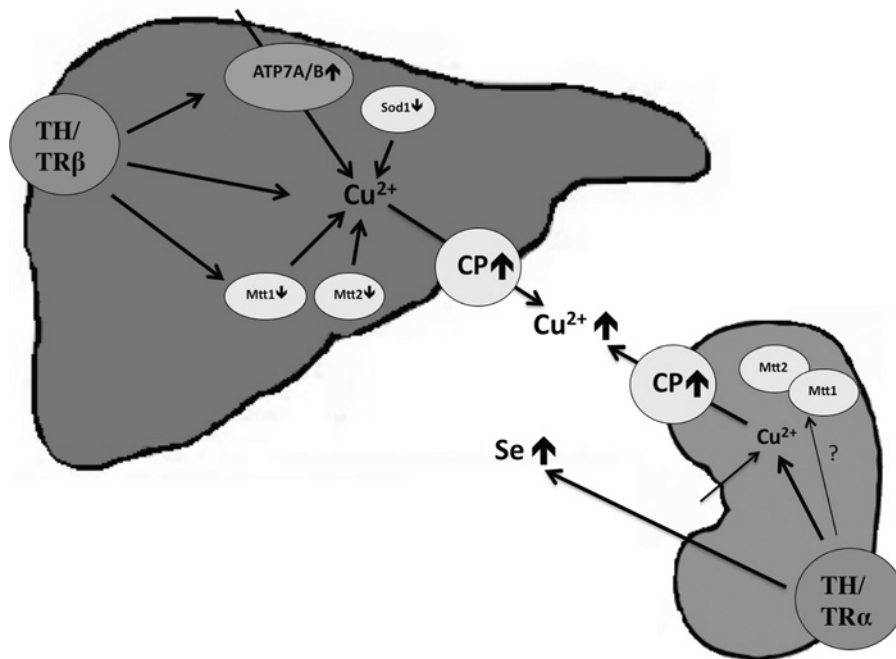


Figure 6 TH regulation of Cu

Schematic diagram indicating the concerted action of TH in the liver and kidney to increase serum Cu levels. CP, ceruloplasmin.

a mutant TRβ in a constant dominant-negative aporeceptor state, whereas the absence of the receptor as in the animal model prevents the induction by TH while still allowing basal unsuppressed expression. We therefore believe that the animal models used are valid to draw conclusions on the underlying molecular mechanism regulating serum Cu and Se, and allow an extrapolation from TRα1 + m mice to RTHα patients.

The molecular changes in liver Cu metabolism

As the liver is the key player in maintaining Cu homeostasis [23] and TRβ regulates more than 5% of all genes expressed in this tissue [4,24,25], it was not surprising that TH modulated Cu metabolism in the liver. What is remarkable, however, is the resulting change in serum Cu and ceruloplasmin, given that the synthesis of ceruloplasmin is very stable [26] and that serum Cu levels are insensitive even to minor Cu deficiency [27] or nutritional Cu supplementation [28]. Only serious genetic disorders such as Wilson's or Menke's disease, which affect

the Cu-transporting proteins ATP7B or ATP7A, are known to cause severe disturbances in Cu metabolism leading to life-threatening conditions [26]. That TH exerts such a pronounced effect on serum Cu levels seems to result from a highly concerted action of the hormone in the liver (Figure 6): an increased expression of the transporters ATP7A and ATP7B, as well as the transport protein ceruloplasmin, and a reduced production of the competing Cu-containing enzymes SOD1, Mtt1 and Mtt2. In TRα1 + m mutant mice an additional pathological mechanism seems to exist in the kidney, which upon T₃ treatment increases the production of ceruloplasmin, Mtt1 and Mtt2, thus raising renal Cu levels. This could consequently contribute to the slightly higher serum Cu levels observed in T₃-treated TRα1 + m mice or TRα1 + mTRβ^{-/-} double mutants compared with the respective controls with intact TRα1 signalling. However, it remains to be elucidated why this effect occurs specifically in TRα1 + m animals.

In general, our findings are in good agreement with the previous observation that ceruloplasmin is induced by T₄ treatment already

during neonatal development [29] and in human patients receiving T_4 after radioiodine therapy [30].

Diagnosis of RTH β

At present, the diagnosis of RTH β is based on the persistently elevated serum-free T_4 levels in the presence of unsuppressed TSH [4], often accompanied by raised free T_3 levels, increased energy expenditure and hyperphagia [31]. However, in general RTH β lacks distinct clinical manifestations [5,6], thus hampering an easy diagnosis. This is particularly problematic in otherwise asymptomatic patients, who if misdiagnosed often receive treatment with anti-thyroid drugs causing severe side-effects in adults and developmental retardation in children [6]. In particular the diagnostic distinction between a TSH-secreting pituitary adenoma and RTH β is difficult, as both present with high levels of TSH and TH. Therefore a reliable and easily detectable biochemical marker would facilitate the correct diagnosis and prevent mistreatment. The determination of the serum Se/Cu ratio could now fill this diagnostic gap: high levels of TH as in TSH-secreting adenoma would similarly increase Se and Cu in the serum with only a small net effect on the Se/Cu ratio, whereas in RTH β the induction of Cu is abolished due to the dysfunctional TR β , leading to a higher Se/Cu ratio in these patients (as seen in Figure 5B).

Identification of RTH α patients

Although several families with RTH β have been characterized [4], surprisingly no human RTH α patients have been identified to date. This is probably due to the lack of a thyroid phenotype, as inferred from animal models with a mutant TR α 1 [10]. On the basis of phenotyping of the several different TR α 1 mutant models [13,32–34], a human patient would presumably display symptoms such as a mild bradycardia, developmental delay and psychiatric deficiencies [11]. However, these parameters are difficult to translate from mouse models to humans and do not function well for a large-scale population-based screening approach. In contrast, the regulation of serum Se and Cu seems to be independent of the species and displays a characteristic fingerprint in RTH. Moreover, the Se/Cu ratio constitutes a robust biochemical marker, as serum samples do not need special pre-treatment and trace elements are stable and not subject to degradation. Even volume changes of the sample would not affect the outcome, as the ratio between two trace elements is determined.

However, both serum Se and Cu levels along with serum selenoprotein P and ceruloplasmin concentrations are acute-phase reactants, being sensitive to inflammatory cytokines and other stressors [35,36]. For this reason, it will be important to combine the results on serum trace element concentrations with additional parameters of general health and immune status in order to obtain the most meaningful results. Successfully applying the Se/Cu ratio in epidemiological screenings and for characterizing RTH individuals will critically depend on verifying a comparable baseline status of the probands and recruiting the appropriate age-, sex-, nutrition- and health-status-matched controls from the same region of residence.

Nevertheless, given the incidence of 1:50000 for RTH β and expecting a similar ratio for RTH α , the screening of large cohorts in a reasonable period of time will probably be required for the identification of RTH α patients; thus a method such as the TXRF-based analysis, which consumes only minute amounts of sample volume and relies on very robust parameters that are

virtually insensitive to the pre-analytical laboratory history of the samples (e.g. number of prior freeze–thaw cycles or non-frozen storage times), will prove helpful in narrowing down the number of possible RTH α candidates selected for DNA sequencing. In summary, we thus believe that the serum Se/Cu ratio appears advantageous for the differential diagnosis of RTH β and might even prove of key importance for the attempts to identify patients with RTH α .

AUTHOR CONTRIBUTION

Jens Mittag, Björn Vennström, Joao Anselmo and Lutz Schomburg designed the experiments; Jens Mittag, Thomas Behrends and Kristina Nordström performed the experiments; Jens Mittag, Joao Anselmo, Björn Vennström and Lutz Schomburg wrote the manuscript. All authors analysed the data, discussed the results and corrected the manuscript.

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SUPPLEMENTARY ONLINE DATA

Serum copper as a novel biomarker for resistance to thyroid hormone

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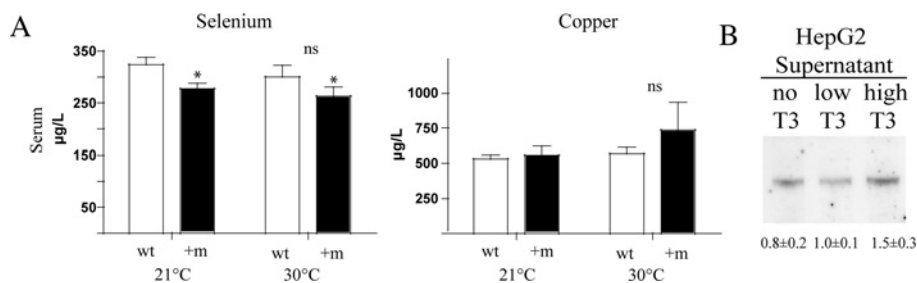


Figure S1 Serum levels of Se and Cu in mice reared at room temperature or thermoneutrality

(A) Serum levels of Se and Cu in wild-type (wt) and TR α 1 + m (mt) mice at room temperature (21°C) and thermoneutrality (30°C), showing no significant effect of environmental temperature. * $P < 0.05$ for wild-type compared with TR α 1 + m mice determined by two-way ANOVA. ns, not significant for 21°C compared with 30°C. (B) Ceruloplasmin levels in the supernatant of cultured HepG2 human liver cells in the absence of T₃ (no T₃), low TH levels (low T₃) or supraphysiological T₃ levels (high T₃). The numbers below the lanes are the quantified results of three independent experiments normalized against the overall protein content.

Table S1 Statistical analysis of the Cu levels (Figures 1 and 2) and quantitative real-time PCR results (Figure 3) using two-way ANOVA followed by a Bonferroni post-hoc test

T₃ indicates changes upon TH treatment. TR α 1 indicates a difference between the two genotypes. Interaction indicates an interaction between the two factors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. BF, Bonferroni post-hoc test; mt, mutant; wt, wild-type.

(a)						
Figure 1 (+ T ₃)	Interaction	T ₃ -induced hyperthyroidism	TR α 1	BF wt	BF mt	
Serum Cu	*	***	*	**	***	
(b)						
Figure 1 (- TR β)	Interaction	TR β -induced hyperthyroidism	TR α 1	BF wt	BF mt	
Serum Cu	-	*	-	-	*	
(c)						
Figure 2	Interaction	T ₃ -induced hyperthyroidism	TR α 1	BF wt	BF mt	
Liver Cu	-	*	-	-	-	
Kidney Cu	-	**	-	-	*	
Urine Cu	-	-	-	*	-	
(d)						
Figure 3	Interaction	T ₃ -induced hyperthyroidism	TR α 1	BF wt	BF mt	
Liver CSS	-	-	-	-	-	
Liver SOD1	-	*	-	-	-	
Liver ceruloplasmin	-	***	-	**	***	
Liver Slc31a1	-	-	-	-	-	
Liver ATP7A	-	***	-	*	*	
Liver ATP7B	-	**	-	-	*	
Liver Mit1	-	**	-	*	-	
Liver Mit2	-	**	-	-	*	
Kidney ceruloplasmin	-	**	*	-	**	
Kidney Mit1	*	-	*	-	**	
Kidney Mit2	*	-	*	-	*	

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