Biochem. J. (2012) **443**, 103–109 (Printed in Great Britain) doi:10.1042/BJ20111817

online data

Serum copper as a novel biomarker for resistance to thyroid hormone

Jens MITTAG*[1], Thomas BEHRENDS†, Kristina NORDSTRÖM*, Joao ANSELMO‡, Björn VENNSTRÖM* and Lutz SCHOMBURG†

*Department of Cell and Molecular Biology, Karolinska Institutet, 17177 Stockholm, Sweden, †Institut fuer Experimentelle Endokrinologie, Charité Universitätsmedizin, 13353 Berlin, Germany, and ‡Unit of Endocrinology, Hospital Divino Espiríto Santo, Ponta Delgada 9500, Portugal

Thyroid hormone action is mediated by the thyroid hormone receptors TRα1 and TRβ. Defects in TRβ 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 RTHβ patients is difficult as the clinical picture varies. A biochemical serum marker indicative of defects in TRβ signalling is needed and could simplify the diagnosis of RTHβ, 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 TRβ and human RTHβ patients, demonstrating an important role of TRβ for this process. Together with a previously reported TRα1 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 RTHβ. Moreover, it could also provide a suitable large-scale screening parameter to identify RTHα 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) TRα and TRβ, which are encoded by two separate genes located on the human chromosomes 17 and 3 respectively [4]. Defects in TRβ can cause RTH (resistance to thyroid hormone) β, a syndrome characterized by elevated levels of free serum T_3 (3,3′,5-tri-iodothyronine) and T_4 (thyroxine) in the presence of normal or elevated TSH (thyroid-stimulating hormone) [5]. The diagnosis of RTHβ 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, RTHβ 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 T_4-binding globulin or albumin, may also present with altered concentrations of free T_3 and T_4 in the presence of normal or elevated TSH [5,8], thus making the conclusive diagnosis of RTHβ challenging. Ultimately, genetic testing for mutations in TRβ is required; however, in 15 % of RTHβ patients such mutations were not found [9].

Although to date more than 350 families with over 1000 affected RTHβ subjects have been identified [4], surprisingly no patient with a mutant TRα1 has been described and the phenotype of human RTHα remains unclear. Analyses of mice heterozygous for a mutant TRα1, 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 TRα1 (for a review see [11]), a reliable biochemical marker for a large-scale screening approach is missing. Although recent studies on TRα1R384C mutant mice revealed a TRα1-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 TRα1-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 RTHβ. Moreover, the serum Se/Cu ratio might constitute a valuable screening tool in epidemiological analyses to search for RTHα patients, as it can exclude the majority of individuals with normal TR-mediated signalling, thereby narrowing down the number of potential RTHα candidates.

MATERIALS AND METHODS

Experimental animals

The mouse strain carrying the dominant-negative R384C mutation in TRα1 has been described previously [13]. The TRα1 + m mice used for the experiments have been backcrossed to C57BL6/6NCrl for eight to ten generations. In addition,

Abbreviations used: ATP7, ATPase, Cu^{2+} transporting; Mtt, metallothionein; RTH, resistance to thyroid hormone; SOD1, superoxide dismutase 1; T_3, 3,3′,5-tri-iodothyronine; T_4, thyroxine; TH, thyroid hormone; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; TXRF, total reflection X-ray fluorescence.

[1] To whom correspondence should be addressed (email jens.mittag@ki.se).

Table 1 Thyroid function tests in RTHβ patients and first-degree relatives

Results are means ± S.E.M.

Group	*n*	Male/female	Age (years)	Free T_4 index	Free T_3 index	TSH (milliunits/l)	Thyroglobulin (μg/l)
RTH patient	25	15/10	27.5 ± 13.3	19.1 ± 2.1**	301 ± 48**	2.31 ± 2.24	47 ± 24**
First-degree relatives	20	9/11	24 ± 10.2	8.81 ± 1.14	157 ± 22	2.19 ± 1.34	18 ± 6
Range			1.0–57	6.0–10.5	95–190	0.4–3.8	1–30

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

TRα1 + m have been crossed to TRβ-deficient mice yielding TRα1 + m TR$\beta^{-/-}$ 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 T_3 via their drinking water containing 0.01 % albumin and 0.5 μg/ml T_3 for 12 days. For the thermoneutrality 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 H_2O 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 (Picofox™ 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 T_3 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 μg of RNA was carried out using oligo(dT) primers and the transcriptor 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 μl of mouse serum or 10 μl of cell culture supernatant (HepG2 cells, 40–50 % confluency, cultured for 24 h in the absence of TH or supraphysiological concentrations of 50 nM T_3) 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.

RTHβ patient analysis

RTHβ 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 TRβ ($Arg^{243} \rightarrow Gln$; 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 RTHβ phenotype (high serum concentrations of T_4 and tri-iodothyronine without suppressed thyrotropin), confirmed by genotyping to identify the (R243Q) mutation of the TRβ gene. Free T_4 and T_3 indexes were estimated from the product of total T_4 and T_3 concentrations respectively, and the normalized resin T_4 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 ± 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 TRα1R384C (TRα1 + m) with and

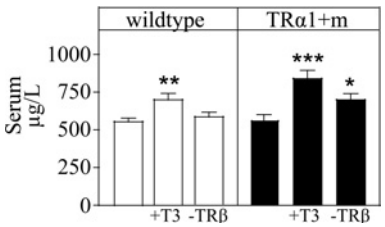

Figure 1 Serum Cu is regulated by TH

Serum Cu levels in wild-type animals and mice heterozygous for a mutant TRα1 (TRα1 + m) untreated ($n = 10$), T_3 treated (+ T3; $n = 8$) or with an additional inactivation of TRβ causing endogenously high levels of TH (− TRβ; $n = 5$). Results are means ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (compared with untreated animals using two-way ANOVA).

without oral T_3 treatment [13]. The analysis revealed no difference in serum Cu between wild-type and TRα1 + m mice, but a significant increase upon T_3 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 TRβ gene [16], suggesting an important role of TRβ in the regulation of serum Cu. Interestingly, independent of the cause of hyperthyroidism, serum Cu levels were always higher in hyperthyroid TRα1 + m mutant mice as in the respective hyperthyroid controls.

To test for an effect of the metabolic rate, which is increased in TRα1 + 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 TRα1 + 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 T_3-induced increase in serum Cu, we determined the Cu levels in the liver, kidney and urine of wild-type and TRα1 + m animals with and without T_3 treatment (Figure 2 with detailed statistics in Supplementary Table S1). We observed a significant effect of the T_3 treatment on the Cu levels in the liver and kidney, indicative of increased hepatic Cu export and improved renal retention. Interestingly, the T_3 effect in the kidney was more pronounced in the TRα1 + 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 T_3 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, Cu^{2+} 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 T_3; however, this increase was more pronounced in the TRα1 + m mutant mice. We also observed a significant effect of the mutant TRα1 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 TRα1 + m and hyperthyroid

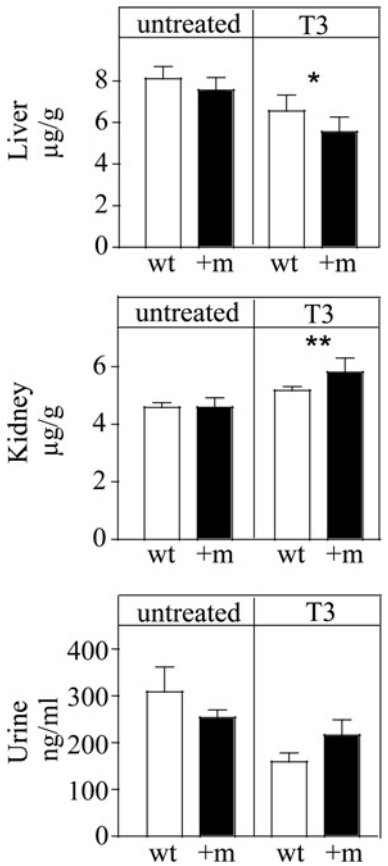

Figure 2 Changes in tissue Cu levels upon TH treatment

Levels of Cu in the liver, kidney and urine of wild-type (wt) animals and mice heterozygous for a mutant TRα1 (+ m) untreated or T_3-treated. Results are means ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$ ($n = 5$, effect of T_3 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 TRα1 + m mutant mice; thus the reason for the renal oversensitivity to hyperthyroidism in TRα1 + 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 T_3 treatment, which was again slightly more pronounced in the TRα1 + m mutants. Mice lacking TRβ displayed an abrogated increase despite their severe hyperthyroidism [16], underlining the previously observed importance for TRβ in this process. A similar secretion of ceruloplasmin upon T_3 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 RTHβ [18]. Despite their high TH levels, serum Cu concentrations were significantly reduced in the RTHβ patients when compared with control subjects from the same families ($P = 0.028$ for control compared with RTHβ 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

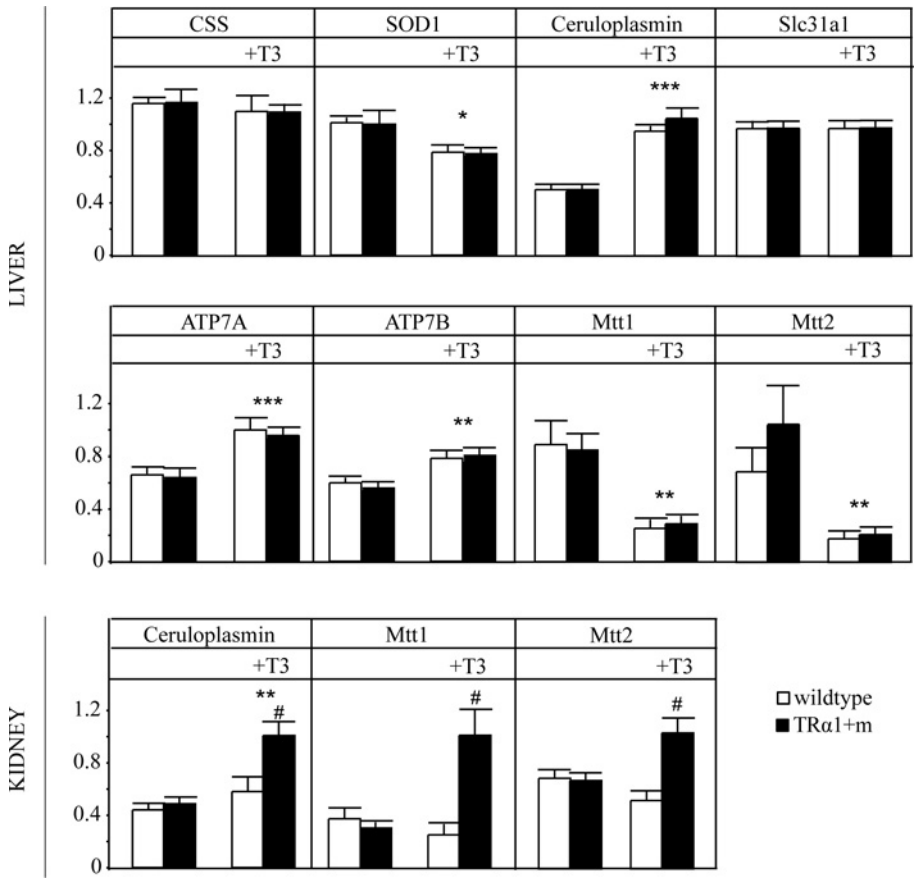

Figure 3 Changes in expression of Cu metabolic genes upon TH treatment

Quantification of mRNA expression in the liver and kidney of wild-type animals (open bars) and mice heterozygous for a mutant TRα1 (closed bars) untreated or T_3-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 T_3 treatment compared with untreated animals using two-way ANOVA).

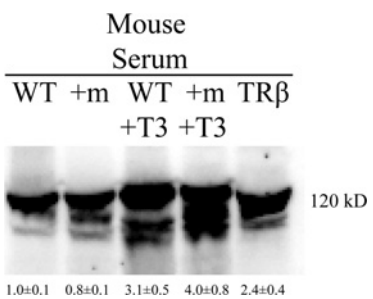

Figure 4 Serum ceruloplasmin levels

Ceruloplasmin protein levels in the serum of wild-type animals (wt) and mice heterozygous for a mutant TRα1 ($+$ m) untreated, T_3-treated (T3) or mice lacking TRβ.

RTHβ patients ($P = 0.045$ for control compared with RTHβ 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 TRα1. Collectively, these findings in sera RTHβ 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 T_3 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 RTHβ and significantly decreased in the RTHα model. Correspondingly in humans, the Se/Cu ratio was significantly increased in RTHβ patients compared with the controls ($P = 0.019$ for control compared with RTHβ 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 RTHα 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 RTHβ patients, whereas the TRβ-deficient mice only exhibited an abrogated increase. However, this discrepancy is easily explained by the fact that the RTHβ patients harbour

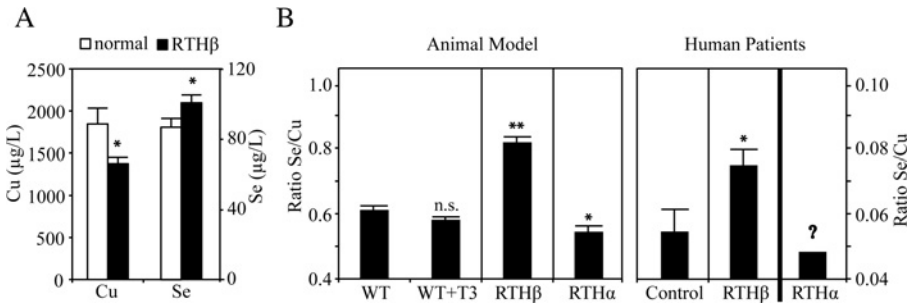

Figure 5 Serum Cu and Se as biomarker for RTHβ

(**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 T_3-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. $^*P < 0.05$; $^{**}P < 0.01$.

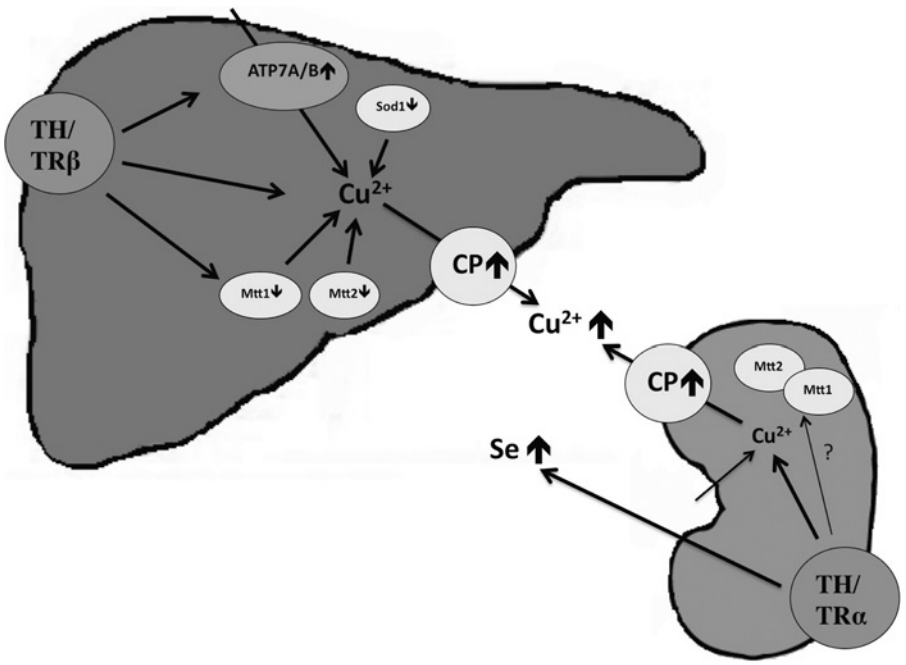

Figure 6 TH regulation of Cu

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 homoeostasis [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 ATP7A, 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 T_3 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 T_3-treated TRα1 + m mice or TRα1 + mTR$\beta^{-/-}$ 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 T_4 treatment already

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

Diagnosis of RTHβ

At present, the diagnosis of RTHβ 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 RTHβ 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 RTHβ 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 RTHβ the induction of Cu is abolished due to the dysfunctional TRβ, leading to a higher Se/Cu ratio in these patients (as seen in Figure 5B).

Identification of RTHα patients

Although several families with RTHβ have been characterized [4], surprisingly no human RTHα 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 TRα1 [10]. On the basis of phenotyping of the several different TRα1 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 1:50 000 for RTHβ and expecting a similar ratio for RTHα, the screening of large cohorts in a reasonable period of time will probably be required for the identification of RTHα 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 RTHα candidates selected for DNA sequencing. In summary, we thus believe that the serum Se/Cu ratio appears advantageous for the differential diagnosis of RTHβ and might even prove of key importance for the attempts to identify patients with RTHα.

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.

ACKNOWLEDGEMENTS

We thank our colleagues at our animal facilities for their excellent handling of the animals used in the present study.

FUNDING

This work was supported by Vetenskapsrådet (to J.M. and B.V.); the Karolinska Institutet Foundation (to J.M. and B.V.); Söderbergstiftelse (to B.V.); the Deutsche Krebshilfe [grant number 10-1792 Scho2 to (L.S.)] and the Deutsche Forschungsgemeinschaft [grant numbers GraKo 1208/2 and Scho 849/2-2 to (L.S.)].

REFERENCES

1 Roberts, C. G. and Ladenson, P. W. (2004) Hypothyroidism. Lancet **363**, 793–803
2 Yen, P. M. (2001) Physiological and molecular basis of thyroid hormone action. Physiol. Rev. **81**, 1097–1142
3 De Felice, M. and Di Lauro, R. (2004) Thyroid development and its disorders: genetics and molecular mechanisms. Endocr. Rev. **25**, 722–746
4 Cheng, S. Y., Leonard, J. L. and Davis, P. J. (2010) Molecular aspects of thyroid hormone actions. Endocr. Rev. **31**, 139–170
5 Weiss, R. E. and Refetoff, S. (2000) Resistance to thyroid hormone. Rev. Endocr. Metab. Disord. **1**, 97–108
6 Weiss, R. E., Dumitrescu, A. and Refetoff, S. (2010) Approach to the patient with resistance to thyroid hormone and pregnancy. J. Clin. Endocr. Metab. **95**, 3094–3102
7 Beck-Peccoz, P., Persani, L., Mantovani, S., Cortelazzi, D. and Asteria, C. (1996) Thyrotropin-secreting pituitary adenomas. Metab. Clin. Exp. **45**, 75–79
8 Kvistad, P. H. and Myking, O. L. (2001) Hereditary thyroxin-binding globulin deficiency-changed thyroid function tests. Tidsskr Nor Laegeforen. **121**, 1336–1338
9 Reutrakul, S., Sadow, P. M., Pannain, S., Pohlenz, J., Carvalho, G. A., Macchia, P. E., Weiss, R. E. and Refetoff, S. (2000) Search for abnormalities of nuclear corepressors, coactivators, and a coregulator in families with resistance to thyroid hormone without mutations in thyroid hormone receptor β or α genes. J. Clin. Endocr. Metab. **85**, 3609–3617
10 Vennstrom, B., Mittag, J. and Wallis, K. (2008) Severe psychomotor and metabolic damages caused by a mutant thyroid hormone receptor α1 in mice: can patients with a similar mutation be found and treated? Acta Paediatr. **97**, 1605–1610
11 Mittag, J., Wallis, K. and Vennstrom, B. (2010) Physiological consequences of the TRα1 aporeceptor state. Heart Failure Rev. **15**, 111–115
12 Mittag, J., Behrends, T., Hoefig, C. S., Vennstrom, B. and Schomburg, L. (2010) Thyroid hormones regulate selenoprotein expression and selenium status in mice. PLos ONE. **5**, e12931
13 Tinnikov, A., Nordstrom, K., Thoren, P., Kindblom, J. M., Malin, S., Rozell, B., Adams, M., Rajanayagam, O., Pettersson, S., Ohlsson, C. et al. (2002) Retardation of post-natal development caused by a negatively acting thyroid hormone receptor α1. EMBO J. **21**, 5079–5087
14 Wallis, K., Sjogren, M., van Hogerlinden, M., Silberberg, G., Fisahn, A., Nordstrom, K., Larsson, L., Westerblad, H., Morreale de Escobar, G., Shupliakov, O. and Vennstrom, B. (2008) Locomotor deficiencies and aberrant development of subtype-specific GABAergic interneurons caused by an unliganded thyroid hormone receptor α1. J. Neurosci. **28**, 1904–1915
15 Stosnach, H. (2005) Environmental trace-element analysis using a benchtop total reflection X-ray fluorescence spectrometer. Anal. Sci. **21**, 873–876

16 Forrest, D., Erway, L. C., Ng, L., Altschuler, R. and Curran, T. (1996) Thyroid hormone receptor beta is essential for development of auditory function. Nat. Gen. **13**, 354–357
17 Sjogren, M., Alkemade, A., Mittag, J., Nordstrom, K., Katz, A., Rozell, B., Westerblad, H., Arner, A. and Vennstrom, B. (2007) Hypermetabolism in mice caused by the central action of an unliganded thyroid hormone receptor α1. EMBO J. **26**, 4535–4545
18 Anselmo, J., Cao, D., Karrison, T., Weiss, R. E. and Refetoff, S. (2004) Fetal loss associated with excess thyroid hormone exposure. JAMA, J. Am. Med. Assoc. **292**, 691–695
19 Kim, H., Son, H. Y., Bailey, S. M. and Lee, J. (2009) Deletion of hepatic Ctr1 reveals its function in copper acquisition and compensatory mechanisms for copper homeostasis. Am. J. Physiol. Gastrointest. Liver Physiol. **296**, G356–G364
20 Massie, H. R., Ofosu-Appiah, W. and Aiello, V. R. (1993) Elevated serum copper is associated with reduced immune response in aging mice. Gerontology **39**, 136–145
21 Merle, U., Tuma, S., Herrmann, T., Muntean, V., Volkmann, M., Gehrke, S. G. and Stremmel, W. (2010) Evidence for a critical role of ceruloplasmin oxidase activity in iron metabolism of Wilson disease gene knockout mice. J. Gastro. Hepatol. **25**, 1144–1150
22 Tanaka, A., Kaneto, H., Miyatsuka, T., Yamamoto, K., Yoshiuchi, K., Yamasaki, Y., Shimomura, I., Matsuoka, T. A. and Matsuhisa, M. (2009) Role of copper ion in the pathogenesis of type 2 diabetes. Endocr. J. **56**, 699–706
23 Kaplan, J. H. and Lutsenko, S. (2009) Copper transport in mammalian cells: special care for a metal with special needs. J. Biol. Chem. **284**, 25461–25465
24 Feng, X., Jiang, Y., Meltzer, P. and Yen, P. M. (2000) Thyroid hormone regulation of hepatic genes *in vivo* detected by complementary DNA microarray. Mol. Endocrinol. **14**, 947–955
25 Flores-Morales, A., Gullberg, H., Fernandez, L., Stahlberg, N., Lee, N. H., Vennstrom, B. and Norstedt, G. (2002) Patterns of liver gene expression governed by TRβ. Mol. Endocrinol. **16**, 1257–1268
26 Lalioti, V., Muruais, G., Tsuchiya, Y., Pulido, D. and Sandoval, I. V. (2009) Molecular mechanisms of copper homeostasis. Front. Biosci. **14**, 4878–4903
27 Turnlund, J. R., Keen, C. L. and Smith, R. G. (1990) Copper status and urinary and salivary copper in young men at three levels of dietary copper. Am. J. Clin. Nutr. **51**, 658–664
28 Kehoe, C. A., Turley, E., Bonham, M. P., O'Connor, J. M., McKeown, A., Faughnan, M. S., Coulter, J. S., Gilmore, W. S., Howard, A. N. and Strain, J. J. (2000) Response of putative indices of copper status to copper supplementation in human subjects. Br. J. Nutr. **84**, 151–156
29 Fitch, C. A., Song, Y. and Levenson, C. W. (1999) Developmental regulation of hepatic ceruloplasmin mRNA and serum activity by exogenous thyroxine and dexamethasone. Proc. Soc. Exp. Biol. Med. **221**, 27–31
30 Zhang, F., Liu, N., Wang, X., Zhu, L. and Chai, Z. (2004) Study of trace elements in blood of thyroid disorder subjects before and after ^{131}I therapy. Biol. Trace Elem. Res. **97**, 125–134
31 Mitchell, C. S., Savage, D. B., Dufour, S., Schoenmakers, N., Murgatroyd, P., Befroy, D., Halsall, D., Northcott, S., Raymond-Barker, P., Curran, S. et al. (2010) Resistance to thyroid hormone is associated with raised energy expenditure, muscle mitochondrial uncoupling, and hyperphagia. J. Clin. Invest. **120**, 1345–1354
32 Itoh, Y., Esaki, T., Kaneshige, M., Suzuki, H., Cook, M., Sokoloff, L., Cheng, S. Y. and Nunez, J. (2001) Brain glucose utilization in mice with a targeted mutation in the thyroid hormone α or β receptor gene. Proc. Natl. Acad. Sci. U.S.A. **98**, 9913–9918
33 Liu, Y. Y., Schultz, J. J. and Brent, G. A. (2003) A thyroid hormone receptor α gene mutation (P398H) is associated with visceral adiposity and impaired catecholamine-stimulated lipolysis in mice. J. Biol. Chem. **278**, 38913–38920
34 Quignodon, L., Vincent, S., Winter, H., Samarut, J. and Flamant, F. (2007) A point mutation in the activation function 2 domain of thyroid hormone receptor α1 expressed after CRE-mediated recombination partially recapitulates hypothyroidism. Mol. Endocrinol. **21**, 2350–2360
35 Conley, L., Geurs, T. L. and Levin, L. A. (2005) Transcriptional regulation of ceruloplasmin by an IL-6 response element pathway. Brain Res. **139**, 235–241
36 Renko, K., Hofmann, P. J., Stoedter, M., Hollenbach, B., Behrends, T., Kohrle, J., Schweizer, U. and Schomburg, L. (2009) Down-regulation of the hepatic selenoprotein biosynthesis machinery impairs selenium metabolism during the acute phase response in mice. FASEB J. **23**, 1758–1765

Received 11 October 2011/16 December 2011; accepted 6 January 2012
Published as BJ Immediate Publication 6 January 2012, doi:10.1042/BJ20111817

Biochem. J. (2012) **443**, 103–109 (Printed in Great Britain) doi:10.1042/BJ20111817

SUPPLEMENTARY ONLINE DATA

Serum copper as a novel biomarker for resistance to thyroid hormone

Jens MITTAG*[1], Thomas BEHRENDS†, Kristina NORDSTRÖM*, Joao ANSELMO‡, Björn VENNSTRÖM* and Lutz SCHOMBURG†

*Department of Cell and Molecular Biology, Karolinska Institutet, 17177 Stockholm, Sweden, †Institut fuer Experimentelle Endokrinologie, Charité Universitätsmedizin, 13353 Berlin, Germany, and ‡Unit of Endocrinology, Hospital Divino Espiríto Santo, Ponta Delgada 9500, Portugal

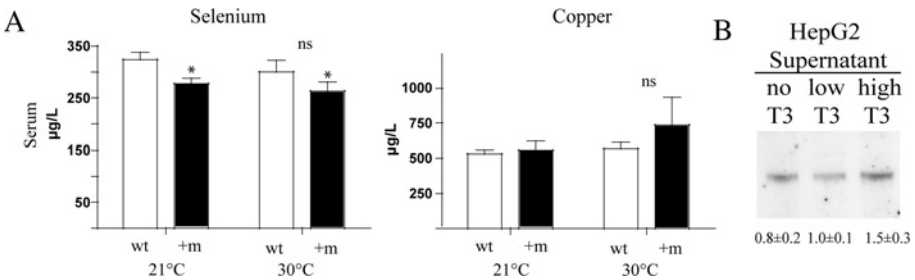

Figure S1 Serum levels of Se and Cu in mice reared at room temperature or thermoneutrality

(**A**) Serum levels of Se and Cu in wild-type (wt) and TRα1 + 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 TRα1 + 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 T_3 (no T3), low TH levels (low T3) or supraphysiological T_3 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

T_3 indicates changes upon TH treatment. TRα1 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	TRα1	BF wt	BF mt
Serum Cu	*	***	*	**	***

(b)

Figure 1 (− TRβ)	Interaction	TRβ-induced hyperthyroidism	TRα1	BF wt	BF mt
Serum Cu	-	*	-	-	*

(c)

Figure 2	Interaction	T_3-induced hyperthyroidism	TRα1	BF wt	BF mt
Liver Cu	-	*	-	-	-
Kidney Cu	-	**	-	-	*
Urine Cu	-	-	-	*	-

(d)

Figure 3	Interaction	T_3-induced hyperthyroidism	TRα1	BF wt	BF mt
Liver CSS	-	-	-	-	-
Liver SOD1	-	*	-	-	-
Liver ceruloplasmin	-	***	-	**	***
Liver Slc31a1	-	-	-	-	-
Liver ATP7A	-	***	-	*	*
Liver ATP7B	-	**	-	-	*
Liver Mtt1	-	**	-	*	-
Liver Mtt2	-	**	-	-	*
Kidney ceruloplasmin	-	**	*	-	**
Kidney Mtt1	*	-	*	-	**
Kidney Mtt2	*	-	*	-	*

Received 11 October 2011/16 December 2011; accepted 6 January 2012
Published as BJ Immediate Publication 6 January 2012, doi:10.1042/BJ20111817

[1] To whom correspondence should be addressed (email jens.mittag@ki.se).