Expression of thyroid hormone transporters during critical illness

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Abstract

Objective: Prolonged critically ill patients have low circulating thyroid hormone (TH) levels without a rise in TSH, a condition labeled ‘the low tri-iodothyronine (T₃) syndrome’. Currently, it is not clear whether this represents an adaptive response. We examined the role of TH transporters monocarboxylate transporter 8 (MCT8, also known as SLC16A2) and MCT10 in the pathogenesis of the low T₃ syndrome in prolonged critical illness.

Methods: A clinical observational study in critically ill patients and an intervention study in an in vivo animal model of critical illness. Gene expression levels of MCT8 and MCT10 were measured by real-time PCR.

Results: In prolonged critically ill patients, we measured increased MCT8 but not MCT10 gene expression levels in liver and skeletal muscle as compared with patients undergoing acute surgical stress. In a rabbit model of prolonged critical illness, gene expression levels of MCT8 in liver and of MCT10 in skeletal muscle were increased as compared with healthy controls. Treatment of prolonged critically ill rabbits with TH (thyroxine + T₃) resulted in a downregulation of gene expression levels of MCT8 in liver and of MCT10 in muscle. Transporter expression levels correlated inversely with circulating TH parameters.

Conclusions: These data suggest that alterations in the expression of TH transporters do not play a major role in the pathogenesis of the ‘low T₃ syndrome’ but rather reflect a compensatory effort in response to hypothyroidism.

Introduction

Critically ill patients have low circulating tri-iodothyronine (T₃) levels while rT₃ levels are increased (1, 2). TSH levels, measured in a single sample, are normal to low (3). These changes are the result of complex alterations that occur in the hypothalamic–pituitary–thyroid (HPT) axis and are commonly referred to as the ‘low T₃ syndrome’ or ‘non-thyroidal illness’ or ‘euthyroid sick syndrome’. Importantly, there are distinct differences between the acute phase and the chronic phase of critical illness (4). In the acute phase, the alterations predominantly occur in peripheral tissues, resulting in inactivation of thyroid hormone (TH). Subtle changes in the central part of the HPT axis, however, can already be observed in the acute phase of critical illness with a rise in hypothalamic D₂ (5, 6) and a transient rise in serum levels of thyrotropin (7). These disturbances are uniformly present in different types of acute illnesses and could be interpreted as an attempt of the body to reduce energy expenditure. In the prolonged phase of critical illness, there is an additional neuroendocrine component characterized by suppressed hypothalamic TRH expression levels (8, 9). It is unclear whether in this unnatural state, low circulating TH levels remain beneficial. However, there is no clear clinical evidence advocating TH treatment in such patients.

The peripheral conversion of TH is regulated by the intracellular enzymes iodothyronine deiodinases (10). It has recently been shown that disturbances of the peripheral metabolism of TH may play an important role in the pathogenesis of the ‘low T₃ syndrome’ (11, 12). However, before TH can be converted, it needs to be transported over the plasma membrane in order to be available for subsequent metabolism (13). Monocarboxylate transporter 8 (MCT8, also known as SLC16A2) has recently been shown to be a very active and specific TH transporter (14, 15). It was originally named X-linked PEST-containing transporter (16) and has affinity for thyroxine (T₄), T₃, and rT₃ (14). Human MCT8 is shown to be expressed in various tissues such as heart, brain, liver, skeletal muscle, and kidney (16). Recently, analysis of two MCT8 knockout models
showed marked alterations in circulating TH concentrations, with increased T\(_3\) and TSH and decreased T\(_4\) levels, as seen in affected humans (19, 20). In these knockout mice, cell-specific changes in intracellular TH content coincided with differences in intracellular deiodinase activity levels (17, 18). Previous studies suggested the involvement of a T-type amino acid transporter in the uptake of iodothyronines in different cell types (21–23). MCT10 was identified as a T-type amino-acid transporter (24, 25) and was recently shown to be very active for TH transport (26). It is also a member of the MCT family and shows a particular high homology with MCT8. Human MCT10 (TAT1, SLC16A10) is expressed in heart, skeletal muscle, kidney, placenta, and intestine (25).

The precise role of TH transporters in the pathogenesis of the low T\(_3\) syndrome in prolonged critical illness remains to be unknown. We analyzed MCT8 and MCT10 expression levels in human skeletal muscle and liver biopsies from prolonged critically ill patients in comparison with those from patients undergoing acute surgical stress. In addition, we used an in vivo animal model of prolonged critical illness to examine the gene expression levels of MCT8 and MCT10 in liver, muscle, and kidney. We also studied the effect of intravenous T\(_4\) and T\(_3\) infusion, resulting in an increase of the circulating TH levels, on transporter expression levels in order to assess the ability of the thyroid status to regulate TH transporters in this condition of critical illness.

Materials and methods

Human model

We studied prolonged critically ill patients who participated in a large randomized controlled study on the effects of intensive insulin treatment in intensive care unit (ICU) patients (\(n=1548\)), of whom the major clinical outcomes have been published in detail previously (27). From 98 patients who had died in the ICU, postmortem biopsy samples of skeletal muscle (right musculus rectus abdominis) and liver were taken minutes after death. Of these, 21 patients had been treated with TH at some time during the course of critical illness and were excluded from this study. Ultimately, good quality RNA from postmortem liver and skeletal muscle biopsies was available from 44 patients. Tissue samples were snap-frozen in liquid nitrogen and stored at \(–80^\circ\text{C}\) until further analysis. Daily blood samples were collected and stored at \(–80^\circ\text{C}\). Analysis was done on blood samples from the last morning before death.

For comparison, we studied skeletal muscle (right musculus rectus abdominis) and liver biopsy samples harvested from 22 age, gender, and body mass index matched patients (Table 1) during elective abdominal surgery.

| Table 1 Demographic characteristics of the studied prolonged critically ill patients and patients undergoing acute surgical stress. |
|---|---|---|---|
|          | Acute surgical stress | Prolonged critically ill |          |
| Gender (\(n, \text{male}\)) | 14 (64%) | 31 (70%) | 0.6 |
| Age (years; mean±s.d.) | 69±12 | 69±14 | 0.8 |
| BMI (mean±s.d.) | 25.1±2.6 | 25.7±3.7 | 0.5 |
| Days in ICU before death (median – IQR) | – | 11 (6–28) | – |

P values for age and BMI represent Student t-tests. P value for gender represents \(\chi^2\) test.

All protocols were approved by the Institutional Review Board of the Leuven University. Written informed consent was obtained from all healthy volunteers and from the patients or the closest family member, when the patient was unable to give consent.

In vivo animal experiment

All animals were treated according to the Principles of Laboratory Animal Care formulated by the US National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health. The study protocol was approved by the Leuven University ethical review board for animal research (P03052).

The model has been described in detail previously (11, 28). In brief, male New Zealand white rabbits were anesthetized and a full thickness burn injury equaling 15–20% of the total body surface area was imposed. Blood glucose levels were kept below 180 mg/dl by frequent blood glucose monitoring and titration of insulin infusion (100 IU/ml; Actrapid Novolet, Novo Nordisk, Bagsvaerd, Denmark; via an SE200B infusion pump, Vial Medical, Brezins, France) when necessary. This model has been validated as representative of the human critically ill condition (11, 28). After 4 days, surviving rabbits were randomized by sealed envelopes to receive a 4 day continuous infusion of 0.9% NaCl (saline group, \(n=7\)) or 5 \(\mu\)g/kg per day T\(_3\) plus 9 \(\mu\)g/kg per day T\(_4\) (T\(_4\) group, \(n=8\)) (Fig. 1). We first used T\(_4\) and T\(_3\) substitution doses but these did not result in significantly elevated serum TH levels, therefore we increased the doses to ensure elevated levels of TT\(_4\) and TT\(_3\) in the circulation. On day 8, animals were killed and samples were taken from upper limb skeletal muscle, liver and kidney and snap-frozen in liquid nitrogen, after which they were stored at \(–80^\circ\text{C}\) until further analysis. For comparison, four healthy rabbits were killed at the day of arrival and tissue samples were collected as described above.
Determination of T₄, T₃, and rT₃ concentrations in serum

**Human samples** Commercial RIAs were used to determine total serum T₄, T₃, TSH (Immunotech SAS, Marseille, France), and rT₃ (Biocode Hycel, Liège, Belgium) concentrations. The detection limits were 13 nmol/l, 0.1 nmol/l, 0.025 mIU/l, and 0.008 nmol/l for T₄, T₃, TSH, and rT₃ respectively. The intra-assay coefficient of variation (CV) for each was 5.1, 3.3, 3.7 and 3% respectively. All samples were assayed in duplicate. Results were compared with previously reported reference values from 25 matched healthy controls (29).

**Rabbit samples** Plasma concentrations of TSH were measured by a specific RIA (reagents provided by Dr A Parlow, National Pituitary Agency). The detection limit was 1.2 mIU, and the intra-assay CV was 5.3%. For samples below detection limit, a value representing half the detection limit was entered. Total concentrations of plasma T4, T3, and rT3 were determined by an in-house RIA (30). The detection limits were 0.005, 0.002, and 0.005 pmol/l respectively. The intra-assay CV were 9.2, 6.4, and 9.7% respectively. No free hormone determinations were done because blood sampling was performed with heparinized catheters which substantially interferes with the assay used to quantify free concentrations of TH (31).

**Determination of T₄, T₃, and rT₃ concentrations in human skeletal muscle**

T₄, T₃, and rT₁ were determined by highly sensitive and specific RIAs after extraction and purification of the iodothyronines from skeletal muscle, as described previously (32–34). [¹³¹I]T₄ and [¹²⁵I]T₃ of two thousand counts per minute were added to each sample as internal tracers for recovery calculations. Average recovery was 55.4% for [¹³¹I]T₄ and 72.3% for [¹²⁵I]T₃. Owing to the limited amount of available tissue, [¹²⁵I]T₄ was also used as a recovery tracer for the determination of rT₃. No corrections for the amounts of iodothyronines contributed by the blood trapped in the tissue aliquot were performed.

**Cloning of rabbit genes**

Total RNA was isolated from rabbit liver tissue using Qiazol lysis reagent (Qiagen) and subsequently purified using the RNeasy mini RNA isolation kit (Qiagen). cDNA was obtained by reverse transcription of 2 μg total RNA using random hexamer primers. Oligonucleotides homologous to sequences surrounding the start or stop codons of human, mouse, and rat Mct8 and Mct10 were designed and used for PCR. The amplified fragments were cloned into the pGEM-T vector followed by sequence analysis. These sequences showed high amino acid identity with the corresponding genes from other mammalian species and data have been submitted to the GenBank database under accession numbers.

<table>
<thead>
<tr>
<th>Gene name (Genbank accession number)</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>ocMCT8 (EF420874) Forward: 5'-CCATGTGGCCTTCTACTTTGC-3'</td>
<td></td>
</tr>
<tr>
<td>Probe: 5'-CCCCCCCATCATTGGAGCTGTATC-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5'-TGACCTCACAGGGACAAAGAAA-3'</td>
<td></td>
</tr>
<tr>
<td>ocMCT10 (EF489851) Forward: 5'-TCCAGAAGAAATCCATGCG-3'</td>
<td></td>
</tr>
<tr>
<td>Probe: 5'-TGGGAGCACATCCCTGGGACC-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5'-GGCTCCCATGCTGGGACAG-3'</td>
<td></td>
</tr>
<tr>
<td>ocHPRT (AF020294) Forward: 5'-TGAGATTATATTCCAGAAGCAGATTGCTTTG-3'</td>
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<tr>
<td>Probe: 5'-TCCAGATTAGGTTGAGAGACGATCTCACCACGAT-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5'-AAGGAAGGATGCTTGGAT-3'</td>
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<tr>
<td>hsMCT8 (NM_006517) Forward: 5'-CCTGTTGCCGCTTACCC-3'</td>
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<tr>
<td>Probe: 5'-CTTGGGACATCCCTGGGACC-3'</td>
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<td>Reverse: 5'-GGCTCCATGCTGGGACAG-3'</td>
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<tr>
<td>hsMCT10 (NM_018593) Forward: 5'-GGCTCCTAATGATGGCAGATTG-3'</td>
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<tr>
<td>Probe: 5'-CTGCGGTGAAGCTCCCTCCCGATTG-3'</td>
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<tr>
<td>Reverse: 5'-TCCACCGGGATAAAAAAAGCA-3'</td>
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<tr>
<td>hsHPRT (NM_000194.1) Forward: 5'-TGAGATATG GCCCTTGGGACAGATTG-3'</td>
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<tr>
<td>Reverse: 5'-AATAGGACCTGAGATTTGCAGA-3'</td>
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EF420874 (MCT8) and EF489851 (MCT10). Based on these sequences, we designed specific primers and probes for real-time PCR analysis using Primer Express software v 2.0 (Applied Biosystems, Foster City, CA, USA).

RNA isolation and real-time PCR

Total RNA from skeletal muscle, liver, and kidney was isolated using Qiazol lysis reagent (Qiagen) and subsequently purified using the RNeasy mini RNA isolation kit (Qiagen) and quantified by Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE, USA). Samples were treated with DNase to remove all contaminating genomic DNA. In total 1 μg RNA was reverse-transcribed with Super-script III Reverse Transcriptase (Invitrogen) using random primers (Invitrogen). All samples were reverse transcribed simultaneously. Reactions lacking reverse transcriptase were also run as a control for genomic DNA contamination.

Human and rabbit MCT8 and MCT10 mRNA levels were quantified in real time with the ABI PRISM 7700 sequence detector (Applied Biosystems) which uses TaqMan chemistry for highly accurate quantification of mRNA levels. Sequences of the primers and probes are given in Table 2. The 10 μl real time reaction mixture contained 5 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.5 μl forward primer, 0.5 μl reverse primer, 0.5 μl TaqMan probe ([5′]6-FAM [3′]BHQ-1 labeled), 0.5 μl water, and 3 μl cDNA (7.5 ng). Unknown samples were run in duplicate and individual samples with a C_t value standard deviation >0.3 were reanalyzed. Data were analyzed using the comparative C_t method. Gene expression of hypoxanthine guanine phosphoribosyl transferase (HPRT) remained stable in all experimental groups of our human and rabbit study population and was therefore used as an internal control.

Statistical analysis

All statistical analyses were done using StatView software (SAS Institute Inc., Cary, NC, USA). Data were analyzed using one-way ANOVA tests with a post hoc Fisher’s least significant difference test for multiple comparisons and χ² tests, when appropriate. Data are presented as means ± S.D. or medians and interquartile ranges when appropriate. Statistical significance was assumed for a two-sided P value <0.05.

Results

Human model

Prolonged critically ill patients (n=44) showed lower circulating total T₃ (TT₃), TT₄, and TSH levels and higher rT₃ levels as compared with patients undergoing acute surgical stress (n=22; Fig. 2A) which is characteristic for the low T₃ syndrome. Eighteen prolonged critically ill patients (41%) had been treated with dopamine at some time during their ICU stay, but this did not result in significantly different circulating TT₃ (P=0.5), TT₄ (P=0.8), TSH (P=0.2) or rT₃ (P=0.5) levels on the moment of the assay as compared with the patients who had not received dopamine.

We analyzed gene expression of TH transporter MCT8 and observed a marked increase in both liver and skeletal muscle of prolonged ill patients as compared with patients undergoing acute surgical stress (Fig. 2B). Expression of the TH transporter MCT10 in liver and skeletal muscle was not significantly different between acute and chronically ill patients (Fig. 2B).
MCT8 and MCT10 protein expression analysis in liver and skeletal muscle samples was tested but did not yield satisfactory results.

Since we observed the most dramatic change in gene expression for MCT8 in skeletal muscle, we examined in this tissue whether the change in MCT8 gene expression was associated with changes in circulating TH concentrations and/or with tissue TH concentrations. In the entire study population (acute surgical stress controls and prolonged critically ill patients), we found a significant inverse correlation between skeletal muscle MCT8 mRNA and serum TT3 (P<0.001, R = −0.6), TT4 (P<0.001, R = −0.5), and T3/rT3 (P<0.001, R = −0.6). Serum iodothyronine levels are positively correlated with tissue levels, as described previously (34). Within the prolonged critically ill patients, selectively, we found a significant positive correlation between skeletal muscle MCT8 mRNA and tissue T3 (P = 0.05, R = 0.4) and T4 (P = 0.02, R = 0.4).

In vivo animal experiment

Our animal model of prolonged critical illness is characterized by low circulating T3 (P<0.001 versus healthy controls) concentrations and low-normal T4 levels (Fig. 3A), as previously described (11).

We first analyzed MCT8 and MCT10 gene expression in liver, skeletal muscle, and kidney. In liver of prolonged critically ill rabbits, we found an increase in MCT8 but not MCT10 gene expression levels as compared with healthy control animals (Fig. 3B). In skeletal muscle of prolonged critically ill rabbits, MCT10 but not MCT8 mRNA was strongly increased as compared with healthy controls (Fig. 3B). In kidney, there was no significant change in MCT8 or MCT10 gene expression levels between prolonged ill and control rabbits.

We used our rabbit model of prolonged critical illness to examine, in a controlled setting, whether MCT8 or MCT10 gene expression can be affected by alterations in circulating TH levels during prolonged critical illness. Treatment of prolonged critically ill rabbits with a 4-day infusion of T3 and T4 significantly increased circulating T3 and T4 levels (Fig. 3A). The exogenous administration of T3 and T4 reduced MCT8 mRNA in liver and MCT10 mRNA levels in skeletal muscle as compared with saline-treated ill rabbits (Fig. 3B). In the liver, we found a significant negative correlation between MCT8 mRNA levels and plasma T3 (P = 0.03, R = −0.5). MCT10 mRNA in skeletal muscle showed a significant negative correlation with plasma T3 (P<0.001, R = −0.7), T4 (P = 0.002, R = −0.6), and TSH (P = 0.008, R = −0.6). In kidney, no effect of TH treatment was observed on gene expression levels of the studied transporters.

Discussion

We set out to study the role of TH transporters in the pathogenesis of the low T3 syndrome, and its regulation by thyroid status during prolonged critical illness.

In the human study, prolonged critically ill patients showed increased MCT8 gene expression in liver as well as skeletal muscle. MCT10 gene expression was not altered in human liver or in human skeletal muscle. Interestingly, we found a significant inverse correlation between circulating TH parameters and MCT8 gene expression in skeletal muscle. This means that patients with the lowest serum TT3 and TT4 levels show the highest upregulation of MCT8 mRNA. This is also reflected by an inverse correlation between the ratio of active over inactive TH, T3/rT3 ratio, and MCT8 gene expression, meaning that the sickest patients, with the lowest T3/rT3 ratio, have a higher induction of MCT8 gene expression. We were not successful in measuring MCT8 protein expression levels, and hence we do not know whether increased MCT8 gene expression was
effectively associated with increased transporter activity. However, within the prolonged critically ill patient group, MCT8 mRNA showed a significant positive correlation with skeletal muscle T₃ and T₄. At the first sight, this may seem contradictory, as in the entire study population (acutely stressed controls and prolonged critically ill patient studied together), there was an inverse correlation. The positive correlation in the patients between MCT8 mRNA and the low tissue T₃ levels, could suggest that the upregulated MCT8 may have evoked a small increase in tissue T₃ levels, although these remained low when compared to controls. Hence, this seemingly adaptive response, if present, was clearly insufficient to normalize tissue TH levels, possibly in part related to a reduced TH availability in the circulation.

In the rabbit model of prolonged critical illness, in the presence of low circulating T₃, MCT8 expression was increased in liver and MCT10 in skeletal muscle as compared with healthy controls. No changes were observed in kidney. Despite these dissimilarities between the human and animal observations, the net result was similar, being an increase in TH transporter gene expression levels in skeletal muscle and liver during prolonged critical illness. The difference between human and rabbit TH transporter expression can be due to species differences as has been described for the expression of TH transporters in the brain of mice, rats, and humans (35).

We were interested to test the hypothesis that circulating TH levels can affect TH transporter gene expression during critical illness. This cannot be done in a clinical setting as only the sickest patients receive TH treatment. We used our rabbit model of prolonged critical illness to examine in a controlled setting, independent of other illness associated factors, whether MCT8 or MCT10 gene expression is regulated by circulating TH levels during prolonged critical illness. Treatment of prolonged critically ill rabbits with a combination of T₃ and T₄, thereby increasing circulating levels of T₃ and T₄ to supranormal levels, reduced transporter expression levels in all tissues studied except kidney. Also, circulating TH parameters correlated inversely with the different transporter gene expression levels. Previous studies by our group showed that tissue iodothyronine levels in prolonged critically ill rabbits correlate well with circulating iodothyronine levels (36). Together, these data suggest that TH transporter expression levels are regulated by the TH status during prolonged critical illness resulting in increased MCT8 or MCT10 expression levels when circulating and tissue iodothyronine levels are low and a decrease in MCT8 and MCT10 expression when circulating and tissue iodothyronine levels are high. We acknowledge that infusion of T₃ + T₄ resulted in higher plasma T₃ and T₄ concentrations than healthy control rabbits have. However, this relative overdosing is not disadvantageous in the context of our study. Although liver and skeletal muscle of prolonged ill animals showed increased MCT8 or MCT10 expression levels, there was no upregulation in kidney. This does not exclude that other TH transporting molecules may be upregulated in this tissue (37).

Together, the data suggest that TH transporters are not involved in the pathogenesis of the ‘low T₃ syndrome’ during prolonged critical illness. Early studies have reported decreased T₄ transport into tissues in patients with low T₃ syndrome (38–40). These findings could be reconciled with our observations if other TH transporters would be decreased. Alternatively, since patients with low T₃ syndrome are often in a negative energy balance, and may suffer from mitochondrial dysfunction (41), hepatic ATP depletion may impair energy dependent TH transport (42, 43). Furthermore, serum of critically ill patients can inhibit the uptake of T₄ into cultured hepatocytes (44–46). This observation has led to the identification of several inhibitors, such as indoxyl sulfate, non-esterified fatty acids, and bilirubin which circulate in increased concentrations during critical illness (44–47).

Some limitations of the two experiments should be highlighted. First, biopsies from prolonged ill patients were obtained within minutes after death in contrast to those obtained during anesthesia from patients undergoing acute surgical stress. However, there was no correlation between TH transporter expression levels and postmortem time delay. Second, our animal model of burn injury-induced critical illness may mirror only part of the complex entity of human critical illness. This may explain the observed discrepancy between MCT10 expression levels in human and rabbit skeletal muscle during prolonged illness.

Our conclusions are that gene expression level of the TH transporter MCT8 and/or MCT10 is upregulated during prolonged critical illness and this upregulation is positively associated with the severity of the low T₃ syndrome. When circulating TH levels are increased by exogenous infusion during prolonged critical illness, TH transporter gene expression levels are lowered. These data show that some tissues may try to adapt to the low circulating T₃ levels by increasing expression of TH transporters. This could increase the tissue availability of TH, without resulting in a normalization of the tissue TH concentrations. This may suggest that the ‘low T₃ syndrome’ in prolonged critical illness may not necessarily represent an adaptive ‘euthyroid state’ for all organ systems.

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

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