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N- and O-Acetylated 3-Iodothyronamines Have No Metabolic or Thermogenic Effects in Male Mice

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Keywords
Thyronamines · Thyroid hormone · Metabolism · Thermoregulation · Body temperature · Brown adipose tissue

Abstract
Introduction: Injection of 3-iodothyronamine into experimental animals profoundly affects their metabolism and body temperature. As 3-iodothyronamine is rapidly acetylated in vivo after injection, it was hypothesized that the metabolites N- or O-acetyl-3-iodothyronamines could constitute the active hormones. ***Methods:*** Adult male mice were injected once daily with one of the metabolites (5 mg/kg body weight intraperitoneally dissolved in 60% DMSO in PBS) or solvent. Metabolism was monitored by indirect calorimetry, body temperature by infrared thermography, and body composition by nuclear magnetic resonance analysis. Signaling activities in brown fat or liver were assessed by studying target gene transcription by qPCR including uncoupling protein 1 or deiodinase type 1 or 2, and Western blot. ***Results:*** The markers of metabolism, body composition, or temperature tested were similar in the mice injected with solvent and those injected with one of the acetylated 3-iodothyronamines. ***Conclusions:*** In our experimental setup, N- and O-acetyl-3-iodothyronamine do not constitute compounds contributing to the metabolic or temperature effects described for 3-iodothyronamine. The acetylation of 3-iodothyronamine observed in vivo may thus rather serve degradation and elimination purposes.

Introduction

Thyronamines are decarboxylated and deiodinated metabolites of thyroid hormone [1, 2]. The most prominent member of this group is 3-iodothyronamine

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(3-T1AM), which, upon intraperitoneal (i.p.) injection at 50 mg/kg into mice induces a strong anapyrexia, with a reduction of several degrees in body temperature [3] caused by massive vasodilation and heat loss over the tail surface [4]. This metabolite has been detected in vivo in several organs and in the blood [3, 5–7]. In vivo, it is rapidly converted within minutes after injection [8], i.e., by deamination to 3-iodothyroacetic acid (3-T1A), deiodination to the iodine-free thyronamine (T0AM), or acetylation to N- or O-acetyl-3-iodothyronamine (NAcT1AM or OAcT1AM). 3-T1A and T0AM have been previously studied with regard to their metabolic and thermoregulatory properties [9, 10], but neither compound caused detectable changes in body temperature, food intake, or interscapular brown adipose tissue (iBAT) thermogenesis when given for 7 days (5 mg/kg) to mice. However, the biological activity of the acetylated 3-iodothyronamines has so far not been tested in vivo. As acetylation constitutes a common modification for several biogenic amines or proteins [11, 12], we hypothesized that NAcT1AM and OAcT1AM may also possess some unique biological activities that could, for instance, contribute to the prominent effects of 3-T1AM.

Here, we demonstrate that the administration of NAcT1AM or OAcT1AM at 5 mg/kg i.p. over 10 days in mice had no detectable effect on metabolic parameters or body temperature regulation, suggesting that the acetylation of 3-T1AM constitutes an inactivation mechanism to terminate its powerful anapyrexic effect.

Materials and Methods

Experimental Animals and Drug Administration

C57Bl6 male mice were obtained from Charles River (Germany) at the age of 8–12 weeks and housed in single cages at an ambient temperature of 22 °C with a 12 h dark/light cycle at the GTH University of Lübeck, Lübeck, Germany, or the KMW Animal Facility of the Karolinska Institute, Stockholm, Sweden. The animals were injected (i.p.) once per day with either NAcT1AM or OAcT1AM at a dose of 5 mg/kg body weight dissolved in 60% DMSO in PBS, comparable to a previous study with 3-T1AM [4]. The animals were monitored for 7 consecutive days in metabolic cages. Their body composition was then assessed on day 8 and infrared thermography data were obtained on days 9 and 10 before sacrificing the animals. OAcT1AM was obtained from ASM Research Chemicals (Art. 9004008, Lot 16165, purity min. 95%, Burgwedel, Germany). NAcT1AM was obtained from the Department of Physiology and Pharmacology, Oregon Health and Science University (purity min. 95%, Portland, OR, USA).

Liquid Chromatography/Mass Spectrometry

Liquid chromatography/mass spectrometry (LC-MS) analyses were performed by Metasafe AB (Södertälje, Sweden) using a LTQ Orbitrap XL system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Rheos Allegro HPLC (Flux Instruments, Switzerland) and a CTC HTC PAL autosampler (CTC PAL Analytics, Switzerland). Chromatographic separation of thyronamines was achieved using the Acquity UPLC BEH C18 analytical column (1.7 μm, 2.1 × 50 mm; Waters Corp., Milford, MA, USA) with a gradient elution program (98% A 0–1 min, from 98 to 10% A in 10 min, 10% A from 10 to 11 min, and 98% A from 11 to 12 min) at a flow rate of 300 μL/min. For HPLC separation, 0.1% formic acid in MilliQ water was used as the aqueous mobile phase, and 100% acetonitrile was used as the organic mobile phase. MS was operated with an electrospray interface in the positive ion mode. The heated capillary temperature and spray voltages were set to 275 °C and 4.0 kV, respectively. The instrument was operated in SIM mode at a resolution of 7,500 with 2 scan segments: segment 1 scan 355–361, 0–5.7 min, and segment 2 scan 395–401, 5.7–1 min. The software Xcalibur 2.07 SR2 (Thermo Fisher Scientific) was used for data acquisition and processing, and control of the mass spectrometer. The accuracy and precision at the lower limit of quantitation (LLQ) were estimated by analyzing a spiked serum sample at a nominal concentration of 0.1 nmol/L. The following parameters were used for the compounds: 3-T1AM retention time 5.12, m/z = 356.00–356.02; and NAcT1AM retention time 6.37, m/z = 398.01–398.03. The recovery rate from spiked mouse serum was 60% for 3-T1AM and 90% for NAcT1AM.

Preanalytical Extraction of Serum and Liver Samples

Mouse serum samples were pH adjusted with an equal volume of 0.1 M NH_4HCO_3 (pH 7.8), and 100 μL internal standard (D4-T1AM) was added. Serum was extracted with 2 mL diethyl ether for 10 min. The organic phase was separated, dried under N_2 and reconstituted in 150 μL 20% methanol in water with 0.1% formic acid. White adipose tissue (WAT) and liver acetylation samples were diluted with 20% methanol in water with 0.1% formic acid. Liver samples (0.15–0.2 g) were homogenized in 0.3 mL sodium acetate buffer (20 mM, pH 4.8). 100 μL of the liver homogenate was mixed with 500 μL 0.1 M NH_4HCO_3 buffer at pH 7.8 and 20 μL of 1 μmol/L internal standard, homogenized twice for 20 s and extracted with 4 mL ether. The ether phase was evaporated and reconstituted in 200 μL 40% MeOH and 0.5% formic acid in water. The extraction yield was approximately 70%. 30 μL of all samples were injected on LC-MS.

Acetylation Activity Assay

Tissue samples from C57BL/6J male mice (liver, WAT) were homogenized in 5 mL ice-cold PBS and diluted 1:40 in 10 mM sodium phosphate buffer (pH 7.4). The diluted homogenates were preincubated with acetyl-coenzyme A (final concentration 0.53 mg/mL) at 37 °C for 5 min. Subsequently, assay buffer with or without the substrate 3-T1AM (1 mM in 25 mM Tris-HCl, pH 7.5) was added, and the samples incubated for 4 h at 37 °C. The reaction was stopped with 1:4 volume of stopping buffer containing 55 mM guanidinium hydrochloride, 83 mM Tris-HCl pH 7.5, and 1.7 mM 5,5'-dithio-bis-2-nitrobenzoic acid, vortexed, and incubated for 10 min before MS analysis.

Nuclear Magnetic Resonance and Indirect Calorimetry

Body composition was measured using nuclear magnetic resonance (Minispec LF110) and Minispec Plus software v6.0 (Bruker Corp., Billerica, MA, USA). Measurement of daily energy expen-

diture was performed in single-housed animals via indirect calorimetry using an open respirometry system (sample interval of 20 min) and a temperature-controlled climate chamber (22 °C ± 1 °C, Phenomaster, TSE Systems, Germany). Oxygen consumption (VO_2) as readout for energy expenditure and respiratory exchange ratio (RER) were calculated with Phenomaster software (TSE Systems, Germany).

Infrared Thermography

Infrared pictures of the inner ear, iBAT, and tail were taken on conscious and freely moving animals with a compact infrared camera (T335, FLIR, Sweden) [13], and the maximum value of several pictures per animals was obtained with the software provided by the manufacturer, and then averaged per group.

RNA Isolation and qPCR

For gene expression analysis, RNA was isolated using Qiagen RNeasy Kits (Qiagen, Germany), transcribed into cDNA (Molecular Biology RevertAid Strand cDNA Kit, Thermo Fisher Scientific) following the manufacturer's instructions. The RNA isolation procedure includes a DNAse digestion step to remove contamination by genomic DNA. qPCR analysis was performed using SYBR Green PCR Master Mix (Roche, Germany) and QuantStudio Applied Biosystems (Thermo Fisher Scientific). Efficiency of the PCR was calculated using standard curves and levels of gene expression were normalized to a stable housekeeping gene (hypoxanthine phosphoribosyltransferase) using the ΔΔCT method. A control sample without reverse transcriptase is routinely used to confirm the absence of genomic DNA. Primer sequences have been published previously [14, 15].

Western Blot Analysis

Western blot analysis of iBAT proteins was performed as described previously [14]. HSP90 was used as a loading control.

Statistical Analysis

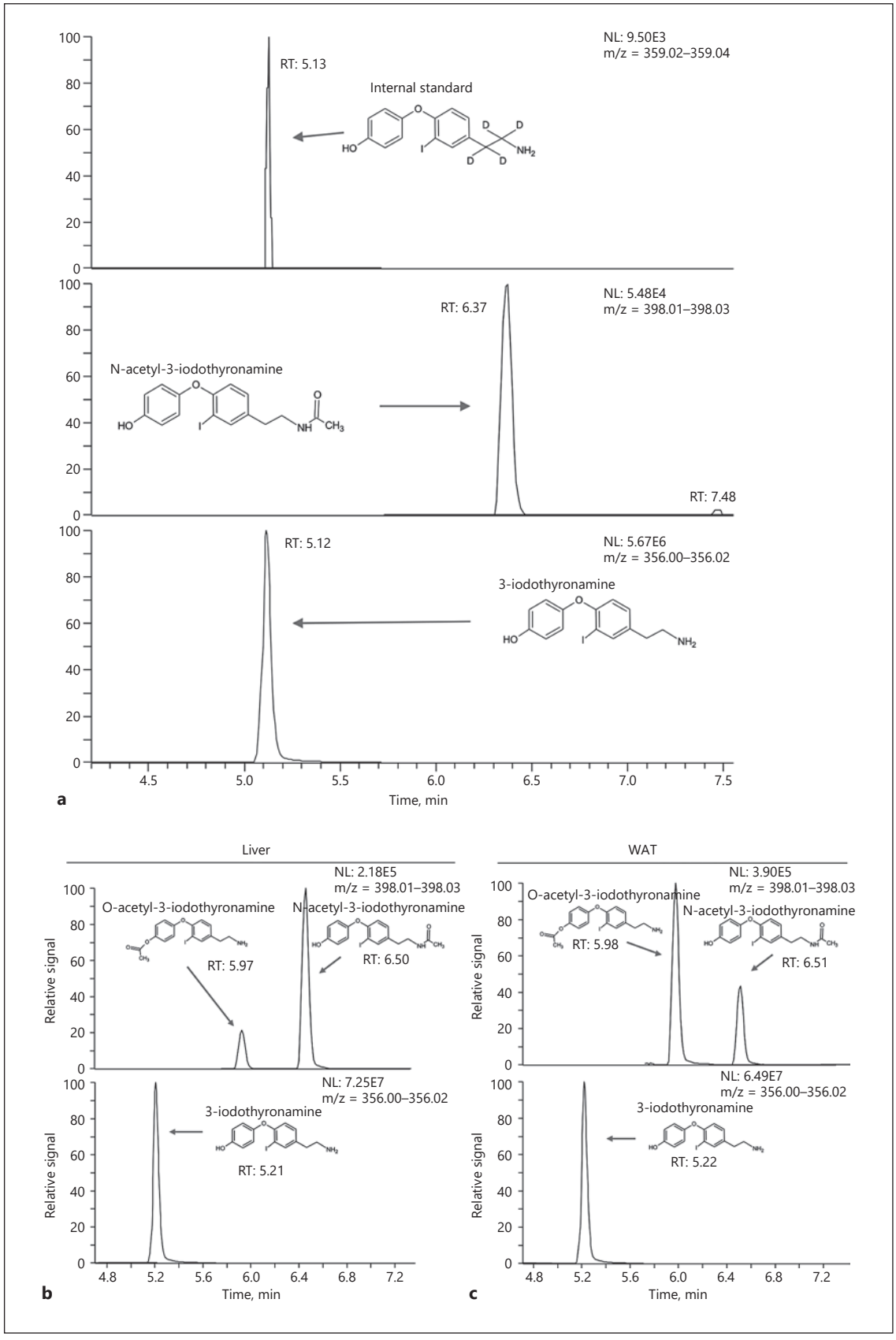
The results were analyzed using GraphPad Prism (La Jolla, CA, USA). For simple comparisons between 2 groups, two-tailed nonpaired Students' *t* test was used. For TSE data, a repeated measures ANOVA was used. $p > 0.05$ were considered nonsignificant.

Results

Concurring with previous reports [8], the formation of NAcT1AM can be readily observed in mouse serum 20 min after an injection of 25 mg/kg 3-T1AM as analyzed by MS (Fig. 1a, normalization level [NL] ratio 3-T1AM to NAcT1AM approx. 100:1). To identify tissues that could mediate this acetylation of 3-T1AM, we incubated mouse WAT and liver homogenates with 1 mM 3-T1AM for 4 h at 37 °C, and then analyzed the products by MS. The analysis showed that WAT and liver samples contained significant amounts of NAcT1AM after the incubation (Fig. 1b, c: upper panel, right peak) in addition to nonmetabolized 3-T1AM substrate (Fig. 1b, c: lower panel). Furthermore, a second product with the same molecular weight was detected, most likely OAcT1AM (Fig. 1b, c: upper panel, left peak).

Subsequently, we applied the validated MS method to test for endogenous NAcT1AM in untreated mice. While NAcT1AM was not detected in the serum of juvenile mice ($n = 3$, age 2 months, not shown), we obtained a signal for NAcT1AM in that of older mice ($n = 3$, age 1 year, not shown), although the concentrations were below the limit of quantification at 0.1 nmol/L. In contrast, endogenous NAcT1AM as well as 3-T1AM could also be quantified in mouse liver samples (3-T1AM 171.8 ± 51.4 and NAcT1AM 42.7 ± 9.4 pmol/g; $n = 4$, age 6 months). OAcT1AM was not measured in any of the mouse samples. Taken together, these data show that NAcT1AM and OAcT1AM can be formed in vivo by conversion of 3-T1AM; however, their endogenous levels are presumably even several-fold lower than those of 3-T1AM, which was reported to be >0.3 nmol/L, although the precise levels are still somewhat debatable [2].

To identify potential metabolic effects of acetylated 3-T1AMs, mice were injected once daily for 10 days with 5 mg/kg i.p. of either NAcT1AM or OAcT1AM or solvent. No detectable effects of NAcT1AM (Fig. 2a–c) or OAcT1AM (Fig. 2d–f) were recorded on body weight development, food intake, or body composition when compared to animals receiving a daily injection of solvent. The animals were then also put into metabolic cages that record daily energy expenditure in a high resolution. Under these conditions, again, no detectable effects of either compound on VO_2 or respiratory quotient were recorded (Fig. 3a–d). It should be noted that the animals were injected daily at 10:30 a.m., and that there were no acute effects observed in the minutes after the injection. When we studied body temperature regulation in greater detail using our established infrared camera system (Fig. 4a) [13, 14], again, no detectable effects of NAcT1AM on inner ear temperature, skin temperature above the iBAT, or tail temperature were recorded, demonstrating that the compound did not affect heat loss or facultative thermogenesis (Fig. 4b–d). A similar observation was made for OAcT1AM (Fig. 4e–g). On the molecular level, a number of potential target genes known to respond to changes in thermoregulation were studied, including cell death-inducing DFFA-like effector A (*Cidea*), hormone-sensitive lipase (*Hsl*), uncoupling protein 1 (*Ucp1*) or deiodinase type 2 (*Dio2*) in iBAT. Neither compound elicited any modulating effect on the expression of these potential target genes after 10 days (Fig. 5a–b), with the exception of a significant decrease in *Cidea* and a trend toward reduc-



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tion in *Ucp1* mRNA upon NAcT1AM treatment. Mice treated with T4 were used as a positive control (Fig. 5c) and showed the expected reduction in *Ucp1* [16]. Likewise, in the liver, no significant differences were observed in the mRNA levels of deiodinase type I (*Dio1*), *Spot14*, selenoprotein p (*Selenop*), or glutathione peroxidase 1 (*Gpx1*); the only notable exception was a minor reduction in superoxide dismutase 1 (*Sod1*) mRNA concentrations after NAcT1AM administration (Fig. 5d–e). Mice treated with T4 were used as a positive control (Fig. 5f) and showed the expected induction in hepatic *Dio1* [16]. To test whether the changes in iBAT *Ucp1* and *Cidea* mRNA would be translated into detectable changes in protein levels, we performed Western blot analysis. The results revealed similar tendencies; however, the differences in CIDEA and UCP1 failed to reach significance after quantification (Fig. 5g).

Discussion

This study tested the potential activities of NAcT1AM and OAcT1AM as immediate downstream metabolites of 3-T1AM in vivo, assuming that part of the pronounced effects of 3-T1AM on metabolism or temperature regulation are mediated by these 3-T1AM derivatives. The results of our study did not support this hypothesis, and rather suggest that NAcT1AM and OAcT1AM may constitute iodine-poor degradation products potentially destined for excretion and catabolism.

Comparison to 3-T1AM

Although discovered over 50 years ago, the interest in iodothyronamines and their potential endocrine activity was not sparked until recently when it was shown that a single injection of 3-T1AM into mice and Djungarian

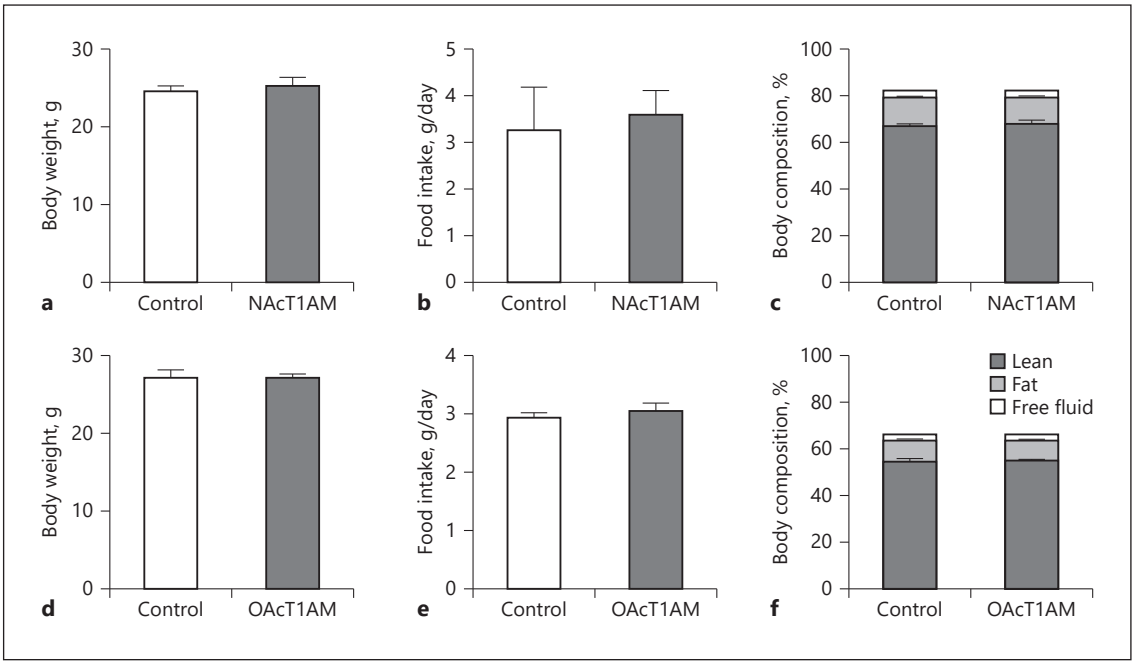

Fig. 2. a Body weight of animals injected daily with 5 mg/kg NAcT1AM or controls after 10 days. **b** Food intake of these animals as an average of measurements on days 7–10. **c** Body composition of these animals after 8 days of injection. **d** Body weight of animals injected daily with 5 mg/kg OAcT1AM or controls after 10 days. **e** Food intake of these animals averaged on the last 3 days of measurement. **f** Body composition of these animals after 8 days of injection. All values are means ± SEM of 5–6 animals per group.

Fig. 1. a In vivo production of NAc-3-T1AM after 3-T1AM injection. Representative mass spectrometry chromatograms for the internal standard D4-3-T1AM (top panel), NAc-3-T1AM (middle panel), and 3-T1AM (bottom panel) from mouse serum 20 min after 3-T1AM (25 mg/kg) injection. **b**, **c** Biosynthesis of NAc-3-T1AM in liver (**b**) and white adipose tissue (WAT) (**c**) samples incubated with 3-T1AM. The substrate 3-T1AM is still present in the sample (lower panel) and acetylated products, namely OAc-3-T1AM (left peak) and NAc-3-T1AM (right peak), have been generated (upper panel). m/z: mass-to-charge ratio. RT, retention time; NL, normalization level (base peak intensity).

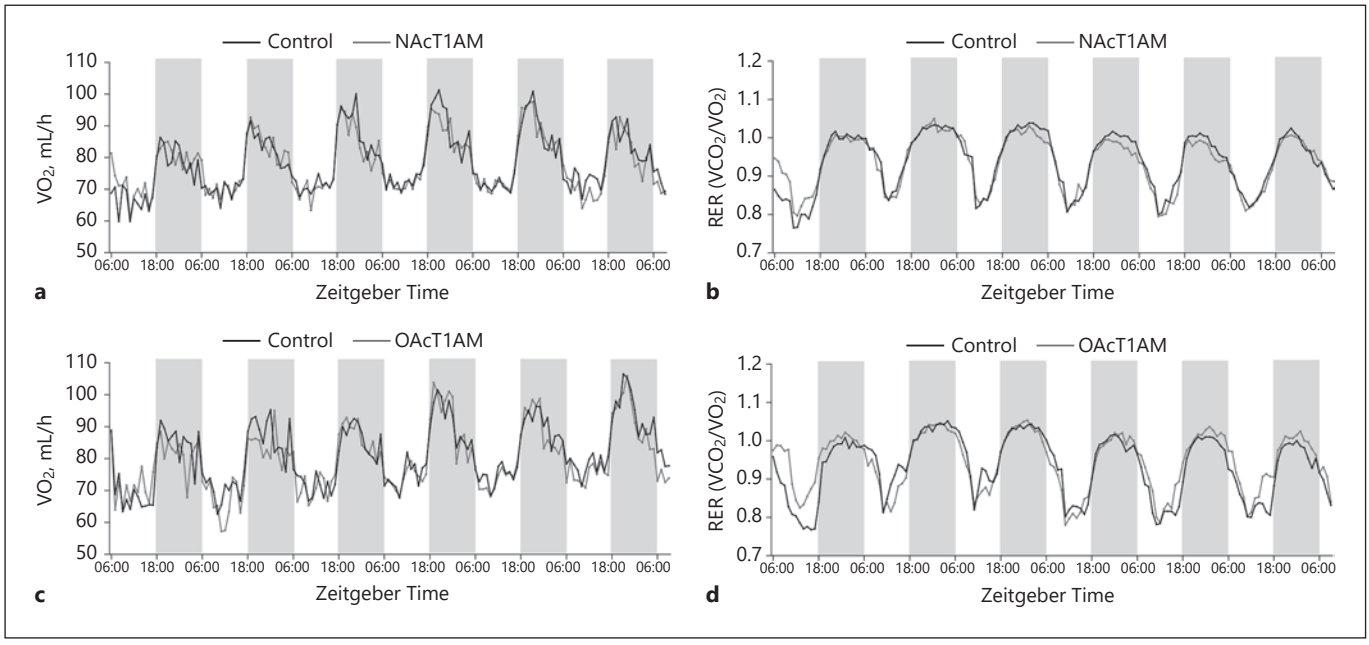

Fig. 3. a Oxygen consumption (VO_2) over the course of 6 days in animals injected daily with 5 mg/kg NAcT1AM or controls. **b** Respiratory exchange ratio (RER) over the course of 6 days in animals injected daily with 5 mg/kg NAcT1AM or controls. **c** VO_2 over the course of 6 days in animals injected daily with 5 mg/kg OAcT1AM or controls. **d** Respiratory quotient over the course of 6 days in animals injected daily with 5 mg/kg OAcT1AM or controls. All values are means ± SEM of 5–6 animals per group.

hamsters rapidly caused major decreases in body temperature [3, 17]. Despite these profound effects, the biosynthesis of 3-T1AM and its target receptor(s), as well as the molecular mechanisms behind the hypothermia remained unclear [4, 6, 18]. Recently, 3-T1AM generation by the intestine was shown, involving decarboxylation of thyroxine through ornithine decarboxylase and further deiodination [19, 20]. The initial hypothesis that the trace amine-associated receptor 1 (TAAR1) would be the physiological mediator of the 3-T1AM-induced hypothermia was questioned when studies revealed that the hypothermic response to peripheral 3-T1AM administration was maintained in TAAR1-deficient mice [21]. To date, there is some evidence that 3-T1AM might act on thermosensitive TRP channels in the hypothalamus [2, 22], as the injection of the compound is rapidly followed by a response comparable to a centrally mounted heat stress, including tail vasodilation and a lack of iBAT activation, despite a strong decline in body temperature [4]. Interestingly, we confirmed that, upon systemic administration, 3-T1AM is rapidly converted to several metabolites as shown previously [8], raising the question whether downstream products might contribute to the pharmacological effects observed after 3-T1AM administration. Indeed, there is evidence suggesting that 3-T1A, the oxidatively deaminated metabolite of 3-T1AM, mediates some of the central effects of T1AM other than thermal regulation [23]. However, the data generated in this study demonstrate that the acetylated metabolites NAcT1AM and OAcT1AM do not mediate any change in peripheral metabolism or body temperature regulation. Since a cationic basic amine is generally critical for the biological actions of biogenic amines, it is conceivable that acetylation at the nitrogen of 3-T1AM is a protective mechanism that blocks most, if not all, of the T1AM actions. A similar deactivation of 3-T1AM's heart and peripheral metabolic effects by conversion to 3-T1A has been shown [10]. Likewise, the deiodination to T0AM seems to reduce the thermogenic and metabolic action of 3-T1AM [9]. However, both the oxidative deamination of 3-T1AM to 3-T1A and the deiodination to T0AM are chemically irreversible whereas the acetylation of T1AM is, at least in principle, reversible via the action of a hydrolase. As such, the acetylation/deacetylation of T1AM may constitute a switch to regulate the activity of T1AM in vivo.

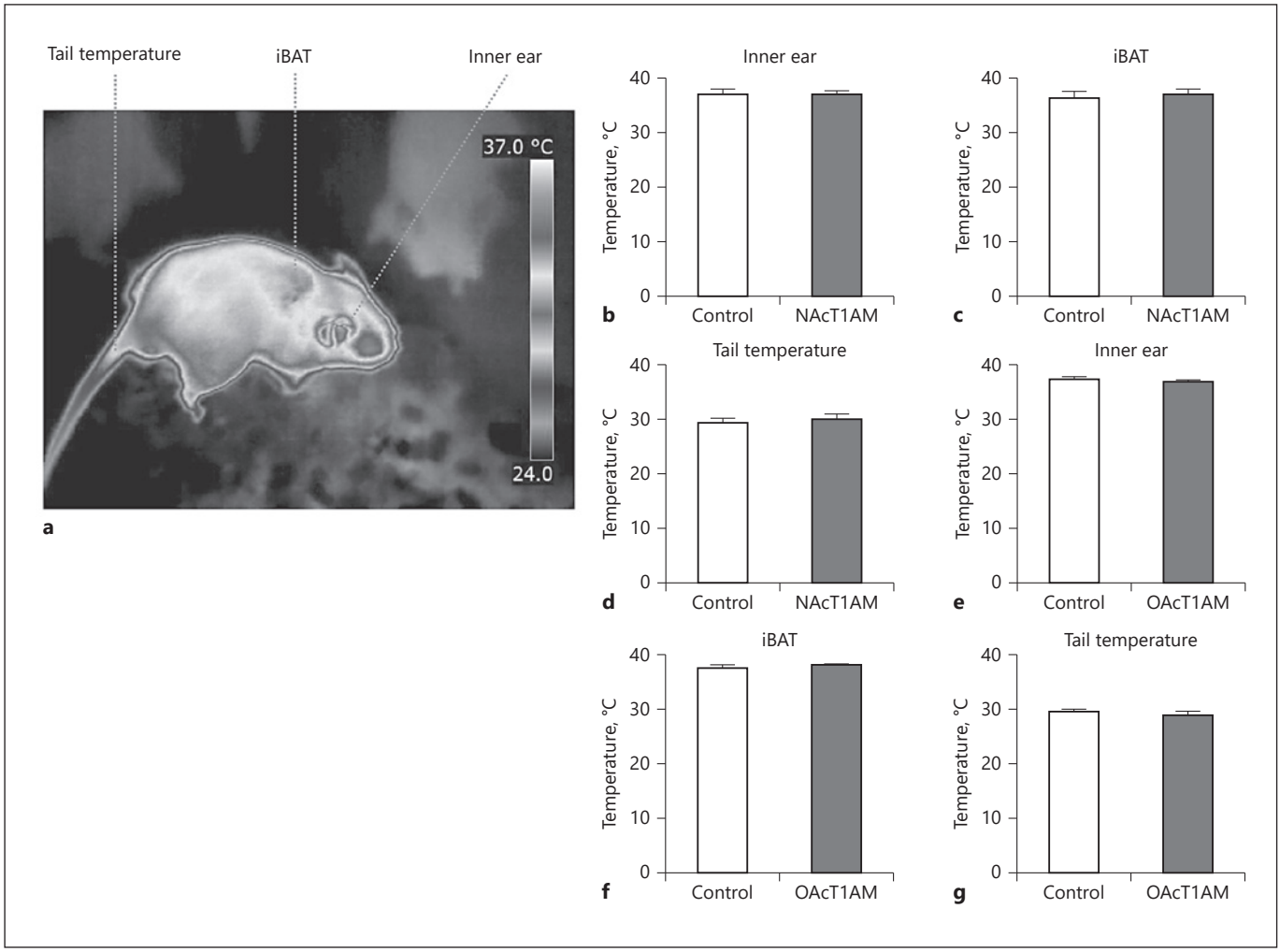

Fig. 4. a Representative thermography image of a mouse depicting the location of the tail, interscapular brown adipose tissue (iBAT), and inner ear for temperature recordings. Temperature of the inner ear (**b**), skin surface over the iBAT (**c**), or tail base (**d**) of animals injected daily with 5 mg/kg NAcT1AM or controls after 9 days. Temperature of the inner ear (**e**), skin surface over the iBAT (**f**), or tail base (**g**) of animals injected daily with 5 mg/kg OAcT1AM or controls after 9 days. All values are means ± SEM of 5–6 animals per group.

Possible Interference with Thyroid Function

Recent studies suggested that 3-T1AM may also affect thyroid function [24]. However, as already shown for 3-T1A and T0AM [9, 10], this seems unlikely for NAcT1AM or OAcT1AM, as we did not observe any changes in the highly thyroid hormone-sensitive genes *Dio1* and *Spot14* in the liver or *Dio2* in iBAT. Likewise, hepatic *Gpx1* expression, which was induced by thyroid hormone in a previous study [15], was unaltered by either compound. The only significant alteration in gene expression observed was a minor reduction in iBAT *Cidea* mRNA, which was not translated to the protein level, and hepatic *Sod1* mRNA, which codes for superoxide dismutase 1, a key enzyme in the regulation of antioxidative defenses [25]. This could suggest that NAcT1AM might have an effect on the oxidative stress in the liver; however, this is not supported by the unaltered levels of *Gpx1*, which is another key enzyme protecting cells from oxidative damage [26].

Study Limitations

A major limitation of our study is the fact that the half-life of NAcT1AM and OAcT1AM is not known, and that the endogenous concentrations are difficult to quantify. However, our dose of 5 mg/kg was clearly in the upper pharmacological range, comparable to what has been

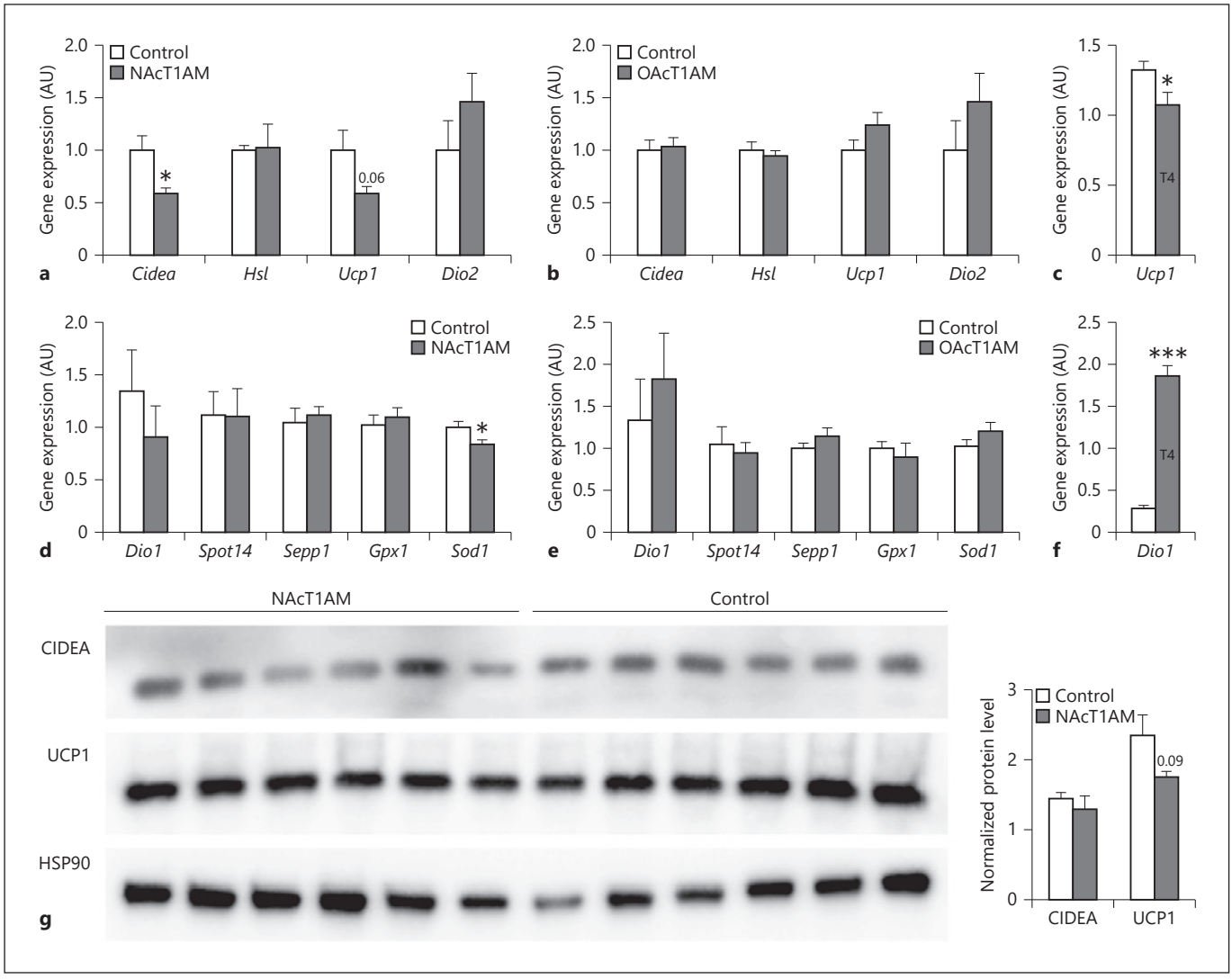

Fig. 5. Expression of iBAT genes in animals injected daily with 5 mg/kg NAcT1AM (**a**) or OAcT1AM (**b**) for 10 days or controls. Mice treated with T4 were used as positive control to show repression of iBAT *Ucp1* (**c**). Expression of hepatic genes in animals injected daily with 5 mg/kg NAcT1AM (**d**) or OAcT1AM (**e**) for 10 days or controls. **f** Mice treated with T4 were used as positive controls to show induction of *Dio1*. **g** Western blot analysis of iBAT CIDEA and UCP1 protein content with HSP90 as loading control. Cidea, cell death-inducing DFFA-like effector A; Hsl, hormone-sensitive lipase; Ucp1, uncoupling protein 1; Dio2, deiodinase type 2; Dio1, deiodinase type I; Sepp1, selenoprotein p; Gpx1, glutathione peroxidase 1; Sod1, superoxide dismutase 1. * $p < 0.05$. All values are means ± SEM of 5–6 animals per group.

used previously for 3-T1A [10] or T0AM [9], given that the serum concentrations of NAcT1AM and OAcT1AM seem to be even several-fold lower than 3-T1AM. More importantly, our data recorded in the metabolic cages had a high temporal resolution and any transient short-lived metabolic effects that would only occur directly within a few hours after the injection would have been detected by altered energy expenditure. However, it cannot be fully excluded that other doses of NAc- or OAcT1AM could induce noticeable effects, given that, for instance, 3-T1AM causes biphasic effects on feeding or locomotion [27].

Open Questions

As our data demonstrate that the N- or O-acetylation of 3-T1AM terminates the powerful effects of the precursor, it would be interesting to identify the enzyme respon-

sible for this conversion. There are several candidate acetylases that could accept 3-T1AM as substrate, e.g., NAT1 [28], and our findings of conversion in liver and white adipose tissue could very well serve as a starting point to identify these enzymes. It is therefore tempting to speculate that an inhibition of these acetylating enzymes may lead to an accumulation of endogenous 3-T1AM, which helps to reaching the pharmacological concentrations necessary for exerting anapyrexic effects [4].

Acknowledgements

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Statement of Ethics

All animal experiments were approved by the respective local authorities Djurförsöksetiska Nämnd (Stockholm Norra, Sweden), or Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung (Schleswig-Holstein, Germany).

Disclosure Statement

The authors have no conflicts of interest to disclose.

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Author Contributions

S.G., R.O., B.H., Q.S., and C.S.H. performed the in vivo studies, the qPCRs and Western blots, analyzed the data, and prepared the figures. T.S.S. provided compounds and discussed and analyzed the data. H.B., L.S., J.M. supervised the study and analyzed and discussed the data. J.M. wrote the manuscript. All authors discussed and edited the manuscript.

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