NMR-based metabolomics and breath studies show lipid and protein catabolism during low dose chronic T1AM treatment.

J. A. Haviland\textsuperscript{a}, H. Reiland\textsuperscript{c}, D. E. Butz\textsuperscript{c}, M. Tonelli\textsuperscript{b}, W. P. Porter\textsuperscript{a}, R. Zucchi\textsuperscript{d}, T. S. Scanlan\textsuperscript{e}, G. Chiellini\textsuperscript{c,d,*}, F. M. Assadi-Porter\textsuperscript{b,c,*}

\textsuperscript{a}Department of Zoology, University of Wisconsin-Madison, 250 N. Mills Street, Madison, WI 53706, USA. \textsuperscript{b}National Magnetic Resonance Facility at Madison, 433 Babcock Drive, Madison, WI 53706, USA. \textsuperscript{c}Department of Biochemistry, 433 Babcock Drive, Madison, WI 53706, USA. \textsuperscript{d}Dipartimento di Scienze dell’Uomo e dell’Ambiente, Università di Pisa, Pisa 56126, Italy. \textsuperscript{e}Department of Physiology & Pharmacology and Cell & Developmental Biology, Oregon Health & Science University, Portland, OR 97239-3098, USA

*Authors of correspondence:

NMR and breath studies: Fariba Assadi-Porter, Department of Biochemistry, 433 Babcock Dr, Madison WI 53706. Phone: (608) 261-1167; Fax: (608) 262-3453; email address: fariba@nmrfam.wisc.edu

Animal design: Grazia Chiellini, Department of Biochemistry, 433 Babcock Dr, Madison WI 53706. Phone: (608)-262-3268, email address: gchiellini@biochem.wisc.edu; Dipartimento di Scienze dell’Uomo e dell’Ambiente, Università di Pisa, via Roma, 55 Pisa 56126, Italy. Phone: +39 050 2218677, email address: g.chiellini@bm.med.unipi.it

Running Title: T1AM induces weight loss and proteolysis

Word count: 3,302

Figure and Tables: 3 Figures

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/oby.20391
Abstract

Objective: 3-iodothyronamine (T1AM), an analog of thyroid hormone, is a recently discovered fast-acting endogenous metabolite. High single dose treatments of T1AM have produced rapid short-term effects, including a reduction of body temperature, bradycardia, and hyperglycemia in mice.

Design and Methods: The present study monitored the effect of daily low doses of T1AM (10mg/Kg) for eight-days on weight loss and metabolism in spontaneously overweight mice. The experiments were repeated twice (n=4). Nuclear magnetic resonance (NMR) spectroscopy of plasma and real-time analysis of exhaled 13CO2 in breath by cavity ringdown spectroscopy (CRDS) were used to detect T1M-induced lipolysis.

Results: CRDS detected increased lipolysis in breath shortly after T1AM administration that was associated with a significant weight loss but independent of food consumption. NMR spectroscopy revealed alterations in key metabolites in serum: valine, glycine, and 3-hydroxybutyrate, suggesting that the subchronic effects of T1AM include both lipolysis and protein breakdown. After discontinuation of T1AM treatment, mice regained only 1.8% of the lost weight in the following two weeks, indicating lasting effects of T1AM on weight maintenance.

Conclusions: CRDS in combination with NMR and 13C-metabolic tracing constitute a powerful method of investigation in obesity studies for identifying in vivo biochemical pathway shifts and unanticipated debilitating side effects.
Introduction

3-Iodothyronamine (T1AM) is thought to be an endogenous derivative of thyroid hormone discovered in 2004 (1). To date, the physiological effects of endogenous T1AM remain elusive, although there is increasing interest in its physiological function and pharmaceutical potential due to the role it plays in lipid and glucose metabolism (2-4). Research shows that T1AM action is mediated through non-genomic signaling (5), binding to G-protein coupled receptors such as trace amines associated receptors type 1 (TAAR-1) (6) and Alpha-2A adrenergic receptors (2). Time-course studies indicate that T1AM has a fast action and causes significant changes in body temperature, activity level and glucose metabolism (1, 4, 7). On the basis of these findings, we hypothesize that T1AM is involved in rapid regulation of lipid metabolism that is central to its metabolic functions.

Reports on measurements of endogenous T1AM concentrations remain conflicting (8-10), which is likely due to the differences in measuring unbound versus total (bound and unbound) T1AM (11). Pharmacological studies of T1AM administration (on the order of nmol/kg body weight) show that T1AM causes symptoms consistent with a hypermetabolic phase, such as increased activity and food consumption (12). However, the majority of T1AM research has used a high dose (50mg/kg body weight) that induces a severe hypometabolic state, suggesting that T1AM works in opposition to thyroid hormone. Symptoms of this hypometabolic state include increased lipid utilization resulting in a decrease in body fat, as well as decreased body temperature, chronotropy, inotropy, and physical activity (1-3, 6, 13, 14).

High doses of T1AM (i.e. 50mg/kg body weight) could have potential applications in emergency medicine because it protects against ischemic injury following stroke (15) or in space travel because it induces profound hypothermia and torpor-like symptoms (7). However, the pharmacological potential of multiple lower doses of T1AM to regulate metabolism, has not yet been explored. Previous studies have noted no observable adverse effects up to two months
following a single administration of 50 mg/kg body weight T\textsubscript{1}AM (1), indicating that long-lasting adverse side effects following the discontinuation of treatment are unlikely.

In this study, we focused on investigating the effects of chronic treatment of T\textsubscript{1}AM at a lower pharmaceutical dose on lipid metabolism in a spontaneously obese mouse model by multianalytical techniques. T\textsubscript{1}AM was administered to mice daily at a dose of 10mg/kg body weight for 8 days, while breath stable isotope ratios were monitored continuously by Cavity Ring Down spectroscopy (CRDS) and plasma samples were collected and analyzed by Nuclear Magnetic Resonance (NMR)-based metabolomics to determine the efficacy of the treatment to induce weight loss and screen for possible unexpected side effects. Untargeted NMR-based metabolomics is a powerful tool to allow general screening of potential metabolic effects of T\textsubscript{1}AM treatment while a targeted method uses \textsuperscript{13}C-metabolic labeling to specifically trace endogenous vs. exogenous metabolites contributing to the treatment. Our results show that using CRDS in combination with NMR and \textsuperscript{13}C-metabolic labeling and tracing can provide a powerful analytical tool for identifying \textit{in vivo} T\textsubscript{1}AM associated changes in energy substrate utilization.

Materials and Methods

Reagents

Purified crystalline 3-iodothyronamine was produced as previously described (1). [U\textsuperscript{13}C]-glucose was from Sigma-Aldrich Corp. (St Louis, MO, USA). 0.9% saline was from Hospira Corp. (Lake Forest, IL, USA).

Experimental procedures

All animal procedures were approved by University of Wisconsin, College of Letters and Science, Animal Care and Use Committee (Madison, WI, USA). Spontaneously obese female CD-1 mice from the F2 generation of mice bred in-house (originally obtained from Harlan, Indianapolis, IN) were used for this study. Mice were at least 1 year of age at the time of the study and weighed
between 40-67g. All animals were housed in standard polycarbonate shoebox cages except while in the metabolic chamber and had *ad libitum* access to feed (AIN-93G Harlan Teklad, Madison, WI, USA) and water except during glucose tolerance tests. Animals were maintained on a 12-hour light/dark cycle. Mice were randomly assigned to treatments of either 10mg/kg/day T$_1$AM (n=4) or sham (n=3) per each eight-day treatment time interval. Body mass and feed consumption was assessed daily at either 21:00hrs or 8:00hrs. To test reproducibility of chronic low dose T$_1$AM treatment, the experiment was repeated for a second group of animals, sham (n=4) and T$_1$AM treated animals (n=4) seven instead of eight days with 6-7 months old mice whole weights were 30-40 g mice. The breath and blood collected as described below. In the first study, animals had access to food during breath collection and blood samples were collected in non-fasting condition. In the second study animals were fasted 4 hour prior to blood collection.

**Preparation of T$_1$AM**

A stock solution of T$_1$AM (56mg in 112µL of DMSO carrier) was dissolved in 33.6mL of medical grade 0.9% saline, which was then aliquoted into volumes for single injections at final 10mg/kg body weight. A vehicle solution was made of 112µL DMSO carrier in 33.6mL 0.9% saline solution for injecting sham animals. All T$_1$AM and vehicle solutions were stored at -80°C until use.

**Preparation of glucose solution**

2g of [U$^{13}$C]-glucose in 6mL medical grade 0.9% saline and individually aliquoted and were stored at -80°C until injection time.

**Breath sample collection**

Single animals were placed in a flow through custom-made 1230cm$^3$ metabolic chamber as previously described (16, 17). The chamber contained aspen bedding and free access to food and water. Animals were acclimated to the chamber one week prior to the study to reduce stress and
associated weight loss. Furthermore, animals were placed in the chamber for two hours prior to injection to capture a steady baseline $\delta^{13}$C value prior to injection with T$_1$AM or saline. All animals were then injected at 12:00hr daily from day 0-7 intraperitoneally with either 10mg/kg T$_1$AM or vehicle (total injection volume in mL was 0.06 times body weight in grams). Immediately after injection the animals were returned to the chamber and breath delta values measured for four more hours. Then they were returned to their home cage. This process was repeated for each animal separately and the treatment was done each day at the same time for a period of one week per animal. All data were collected at the same time of day to account for diurnal variation that is known to affect $\delta^{13}$C values in mice (16). Isotopic CO$_2$ was measured from exhaust air using cavity ring-down spectrometer (CRDS) (Picarro Inc., Sunnyvale, CA) (16, 18, 19).

**Plasma sample collection**

Baseline reference plasma was collected 7 days prior to the start of injections (day -7) and then on day 0, day 7, and day 21 (followed two weeks after last day of T$_1$AM treatment). All plasma samples were collected at 16:00hr (2-hours after injection with T$_1$AM). For all blood collections animals were anesthetized with 2.5% isoflurane and blood was collected from the retro orbital venous plexus with heparinized capillary tubes. Plasma was then separated by refrigerated centrifugation at 1,000g for 10 min at 4°C and stored at -80°C until preparation for NMR data collection.

**Analysis of long-term effects of T$_1$AM on energy substrate utilization via [U-13C]-glucose.**

On day 21 of the experiment mice were fasted two hours prior to intraperitoneal injection of 20g/kg of [U-13C]-glucose. Blood was collected 2-hours later at maximum metabolic response (17). Plasma samples were prepared and stored as described above.

**Preparation of plasma samples for NMR.**
Frozen plasma samples were allowed to thaw on ice. Ice-cold methanol (2:1, v/v) precipitation was performed to remove proteins as previously described (17). The supernatant was then dried in a speed vacuum overnight. The dried supernatant was redissolved in 20mM phosphate buffer containing 1mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) and 0.1mM sodium fluoride (NaF) and the pH was adjusted to 7.4 +/- 0.05.

Collection of NMR spectra

All one-dimensional (1D) $^1$H-NMR spectra were collected at 25 °C on a 600 MHz Varian VNMRS spectrometer equipped with a cryogenic probe (17). Each 1D spectrum was accumulated for 1028 scans, with an acquisition time of ~400ms (4,096 complex points) and a 3 second repetition delay for a total collection time of ~2 hours (17). 1D data were then analyzed using Chenomx NMR suite (Chenomx Inc., Edmonton, Alberta, Canada) and Mestrela (Mestrelab Research, Santiago de Compostela, Spain) software to identify relative concentrations of metabolites of interest. In order to obtain one-dimensional (1D) spectra that contain only signals from $^{13}$C-bound protons, two different 1D spectra were collected as interleaved scans: one specific to $^{12}$C and the other isotopically non-specific ($^{12}$C+$^{13}$C) as described previously (17). Each 1D spectrum, $^{12}$C and $^{12}$C+$^{13}$C, was accumulated for 1028 scans, with an acquisition time of ~400ms (4,096 complex points) and a 3 second repetition delay for a total collection time of ~2 hours. 1D subtracted spectra were then analyzed as described above.

Statistical Analysis

Despite the small sample size (n=4), normality was confirmed by 95% of the data fitting within +/- two standard deviations of the mean. Two-tailed Student’s t-test was performed on breath delta values from 0-80 minutes after T$_1$AM injection each day for the T$_1$AM and the sham treated groups. Body weight, feed consumption and NMR metabolite data were analyzed with two-tailed Student’s t-Tests. Differences were considered significant with p<0.05 unless otherwise stated.
Results

Body Weight

Exogenous T1AM administration was associated with a body weight loss of 8.2% of initial body weight by day 9 (p=0.038) (Figure 1). On the other hand, sham mice lost only 0.1% of their initial body weight by day 9. After T1AM withdrawal, mice regained only 1.8% of initial weight in the following 2 weeks. The chronic treatment with 10 mg/kg T1AM administration was repeated for a second time, which resulted in a similar trend with a total weight loss of 8.6% of initial body weight by day 7 (data not shown).

Food intake

Weight loss was not associated with any change in food intake. No difference in food intake was observed at any time during the study between T1AM and sham treated animals. There were insignificant fluctuations in the amount of food consumed at different points in the study, which was consistent for both T1AM and sham treated animals. Pre-injection food consumption averaged 3.91g/d +/-0.17 for sham and 3.30g/d +/-0.45 for T1AM (p=0.32). Food consumption during treatment decreased slightly, (sham=3.36g/d +/-0.11; T1AM=3.12g/d +/-0.47; p=0.47). Post treatment food consumption increased slightly for both groups (sham=4.13g/d +/-0.02; T1AM=4.18g/d +/-0.14; p=0.76).

Real-time breath analysis

Upon switching to stored fat as a fuel source, the effect on breath δ¹³C values become more negative (16, 19, 20). Thus, to follow the effect of T1AM on lipid break down we measured real-time stable isotope fractionation in breath using CRDS reported as δ¹³C values (19). We determined the baseline real time breath δ¹³C values for 120 minutes prior to any treatment. The first 30 minutes allowed animals to become acclimated to the metabolic chamber. However, the breath
delta values showed a steady decline shortly after $T_1$AM injection with a minimum $\delta^{13}$C value at about 80 minutes post injection as compared to the control group that did not show a decline. Figure 2 shows the average daily difference between $T_1$AM and sham injected mice for the $\delta^{13}$C value 80 minutes post injection. The $\delta^{13}$C value was significantly lower (~3.6‰) in $T_1$AM treated mice from day 0-4 with ($p < 0.05$) (Figure 2). However, later in the treatment (day 5-7) the $T_1$AM treatment effect on lipolysis was diminished ($\delta^{13}$C value ~2‰).

*NMR plasma analysis*

Blood samples taken on day -7 (pre-$T_1$AM injection) and day 0 (the first 2 hrs post-$T_1$AM injection) revealed no differences in plasma metabolites between $T_1$AM (n=7) and sham (n=8) animals either in the fasted or the non-fasted conditions (data not shown). However, after 8 days of $T_1$AM treatment plasma 3-hydroxybutyrate, a lipid breakdown intermediate, was significantly higher in non-fasted $T_1$AM treated animals compared to sham animals (n=4) ($p=0.031$) (Figure 3A), indicating increased lipolysis in the non-fasting regime. Similarly, under fasted condition 3-hydroxybutyrate was still significantly higher in the $T_1$AM treated group as compared to the sham group (n=4) ($p=0.03$). Blood samples collected on day 21, after a uniformly labeled $^{13}$C-glucose challenge, revealed increases in unlabeled ($^{12}$C) glycine (1.35x controls, $p=0.05$) and valine (1.70x controls, $p=0.001$) in $T_1$AM treated mice (Figure 3B). Interestingly, $^{13}$C labeled acetate was 2.95 times higher in the $T_1$AM animals compared to the sham group (Figure 3C) under the non-fasting condition and still remained higher in the $T_1$AM treated mice even in the fasted condition ($p=0.286$). The increased level in $^{13}$C-acetate is consistent with the lower weight gain at day 21 seen in the $T_1$AM treated animals (1.8% weight regained by day 21).

**Discussion**

This study investigated subchronic effects of $T_1$AM treatment at a low pharmaceutical dose on lipid metabolism using three complementary technologies (CRDS, NMR-based metabolomics and $^{13}$C-
We administered 10mg/kg/day of T\textsubscript{1}AM for 8 days as a potential method for inducing weight loss. We followed the effect of T\textsubscript{1}AM for two additional weeks to evaluate long-lasting consequences of T\textsubscript{1}AM on weight gain and to assess the effectiveness of T\textsubscript{1}AM in maintaining a new reduced weight level and its side effects. Based on the combination of these analytical methods, we were able to show that chronic T\textsubscript{1}AM exposure induced a rapid increase in lipid mobilization, followed by a shift in macronutrient substrate oxidation from lipids to proteins in the last days of treatment.

T\textsubscript{1}AM treated mice showed continued reduction in body weight compared to sham animals, without reduction in food intake. Although daily reduction in body weight in T\textsubscript{1}AM treated mice did not reach statistical significance during the 8 days of treatment, weight loss was significantly greater than in sham mice at day 9 (-8.2%; p=0.038 for the first study, and -8.6%; p<0.05 in the second study). Importantly, after T\textsubscript{1}AM withdrawal, the mice that were allowed \textit{ad libitum} access to food, regained only a small part of the lost weight (1.8% of initial weight regained) in the following 2 weeks vs. sham mice.

Dhillo et al (2009) reported decreased levels of activity in mice at a high dose of T\textsubscript{1}AM, and no change in activity at a low dose of T\textsubscript{1}AM (12), consistent with our observations that the weight loss seen in our study is not due to increased activity. The results from this study suggest that T\textsubscript{1}AM, alone is capable of promoting weight loss, and may show increased efficacy for weight maintenance at a pharmaceutical level, if proteolysis is controlled. A recent study showed that changes in food consumption post T\textsubscript{1}AM administration follow a biphasic dose dependent response (21). The biphasic responses associated with T\textsubscript{1}AM may explain no changes on food consumption in our study, while other studies have shown various effects depending on the dose and route of administration (12, 21).

A single high dose injection of T\textsubscript{1}AM was shown to play a role in regulating glucose and lipid metabolism (2-4, 21). Specifically, T\textsubscript{1}AM induced a shift in energy metabolism from carbohydrates to lipids, which resulted in decreased fat mass (3). Our study provides the first evidence that T\textsubscript{1}AM
at a subchronic, lower pharmacological dose administration, plays a role in increased lipid oxidation. We measured T₁AM associated changes in energy substrate utilization by monitoring carbon stable isotope ratio ($^{13}$CO$_2$/^{12}$CO$_2$, or δ$^{13}$C value) in exhaled breath. CRDS can be used continuously for assessing lipid oxidation in real-time and non-invasively (16, 20). The results from breath analysis corroborate existing studies linking T₁AM induced weight loss to increased lipolysis.

Our results showed that two hours before treatment, the baseline δ$^{13}$C values were similar in both sham and T₁AM treatment groups. Therefore, the depression in δ$^{13}$C values following T₁AM administration indicates that the differences in observed δ$^{13}$C values between the two groups are attributed to the effect of T₁AM shifting substrate utilization to lipids and increasing rate of expired $^{12}$CO$_2$ release in breath. Because of the isotope effect of pyruvate dehydrogenase during synthesis, lipids were enriched in the lighter isotope $^{12}$C; thus during lipolysis more $^{12}$CO$_2$ is generated resulting in a more negative δ$^{13}$C value (18-20). The day 0 breath data indicated a rapid drop in δ$^{13}$C value that reached the minimum within the first 80 minutes after T₁AM injection (Figure 2). This time frame is consistent with other rapid effects of T₁AM reported in the literature (1, 3), and is indicative of its rapid lipid oxidation. Thus, breath δ$^{13}$C value can be used as a sensitive marker for early detection of lipolysis.

During the early days of T₁AM treatment (day 0-4; day 0 being the first day of treatment), there is a significant decrease (-3.6 to -4.1) in the δ$^{13}$C$_{80min}$ value in the T₁AM treated animals (Figure 2) consistent with results from microarray studies showing increased lipase gene expression in chronic T₁AM treatment in rats (M. Righi, personal communication).

Later in the treatment (day 5-7), the change in δ$^{13}$C$_{80min}$ value only decreased by 1-2 units in T₁AM treated vs. sham animals (Figure 2). These results suggest a prolonged action of T₁AM on lipolysis. The continued weight loss through day 9 in the T₁AM treated animals (Figure 1) is consistent with the breath data.
Non-targeted NMR metabolomics data confirmed the increase in lipid utilization. On the last day of treatment, \( T_1\)AM treated mice showed a significant elevation in the plasma level of the ketone body, 3-hydroxybutyrate (Figure 3A), providing further evidence that \( T_1\)AM is directly affecting lipid utilization through day 7 of treatment. Even though, Braulke et al. (3) demonstrated development of ketonuria in rodents treated with a single injection of \( T_1\)AM at 50 mg/kg, our study is the first to report that low dose chronic \( T_1\)AM treatment causes a significant increase in plasma ketone bodies.

We followed the longer-term effect \( T_1\)AM, after two weeks discontinuation, by \(^{13}\text{C}\)-isotope tracing and NMR spectroscopy. Mice were challenged with [U-\(^{13}\text{C}\)]-glucose to evaluate lipid synthesis vs. lipolysis in the post weight loss regime. Two hours after \(^{13}\text{C}\)-U-labeled glucose administration, both plasma \(^{12}\text{C}\)-glycine and \(^{12}\text{C}\)-valine were found to be elevated (Figure 3B), while the rate of \(^{13}\text{C}\)-glucose consumption was similar in \( T_1\)AM-treated vs. sham animals. The \(^{13}\text{C}\)-U-glucose injection resulted in no statistically significant labeled (\(^{13}\text{C}\)) metabolites, indicating that the elevated levels of \(^{12}\text{C}\)-valine and \(^{12}\text{C}\)-glycine found are indeed a product of endogenous protein catabolism. The increase in these amino acids further supports the breath data that showed a lesser decreased \(^{13}\delta\) values indicating a shift in macronutrient substrate oxidation from lipids to carbohydrates or proteins in day 5-7 of treatment. The rise in unlabeled amino acids thus may reflect a temporary metabolic shift resulting from the need of the body to use amino acids as precursors for energy production (pyruvate in the case of glycine and Krebs cycle intermediates, such as succinyl-CoA, in the case of valine). These longer-term changes in metabolites levels in exogenously \( T_1\)AM treated animals suggest that \( T_1\)AM may have substantially longer-term effects on energy metabolism than previously thought, with increased endogenous protein catabolism when administered subchronically. The plasma samples following the \(^{13}\text{C}\)-U-glucose injection also showed an increase in \(^{13}\text{C}\)-acetate, which was \( \sim 3\) times higher than in sham treated animals. Even though the \(^{13}\text{C}\)-acetate levels did not reach statistical significance the results are consistent with a decrease in lipid synthesis in \( T_1\)AM treated animals that still persists two weeks post termination of \( T_1\)AM treatment.
While this study begins to decode longer-term effects of T1AM on metabolism, additional studies with mildly feed restricted mice are needed to examine the effects of T1AM treatment for periods longer than 8 days. The breath $\delta^{13}C_{80min}$ value indicates that the biological response to exogenous T1AM over an 8-day period is dynamic based on the fluctuations in energy substrate utilization. Our finding that prolonged use of T1AM affects protein catabolism is consistent with prior studies showing reduced inotropism and diminished muscle contraction strength with high doses of T1AM (1). However, to date protein catabolism has not been reported following an acute administration of T1AM. The discovery that protein catabolism induction can occur after chronic application of T1AM at low concentration is important and demonstrates the power of combined analyses for anti-obesity drug evaluations to identify unexpected side effects.

ACKNOWLEDGEMENTS: We thank Dr. Louis Aronne for his insightful advice and Dermot Haughey for participation in NMR data analysis. This work was supported in part by funds from the following: the Rodale Foundation; the Farmers Advocating for Organics (FAFO) fund to W.P.P.; grants R01 DC009018 from NIH; the Wisconsin Institute of Discovery Grant (WID-135A039) to F.M.A.P.

Author Disclosures: The authors declare competing financial interests. Authors F.M.A.P., D.E.B., M.T., and W.P.P. are cofounders of Isomark LLC.
References


Figure Legends

**Figure 1** Comparison of weight loss in T1AM treated and control mice. Decrease in weight is shown as a % of body weight in T1AM treated mice (light gray bars) and sham treated mice (dark gray bars) over 21 day study period. Error bars reflect the standard error of the mean (SEM). Statistical significance (p < 0.05) compared to the vehicle treated group indicated by asterisk.

**Figure 2** Exhaled breath analysis measured by CRDS reveals lipid breakdown. Daily stable isotope breath analyses of T1AM treated animals (n=4) are reported as the average daily δ13C values. The minimum average values are at 80 minutes (δ13C80min) post injection for each treatment day. Statistical significance with p < 0.05 are indicated by asterisks.

**Figure 3** Analysis of plasma by NMR-based metabolomics reveals an increase in the lipid oxidation and protein breakdown in non-fasting condition. A) A significant increase in 3-hydroxybutyrate at day 7 of T1AM treatment. The concentration of 3-hydroxybutyrate is shown as light gray bars for T1AM treated mice (n=3) and dark gray bars for control mice (n=4) (p= 0.03). B) Increases in the plasma amino acids glycine (glucogenic) and valine (branched and ketogenic/glucogenic) in post T1AM treatment regime. Plasma samples were collected in the following two weeks after discontinuation of T1AM treatment and were two hours post [U-13C]2-glucose injection. The concentration of glycine (p=0.05) and valine (p=0.001) is shown as light gray bars for T1AM treated mice and dark gray bars for sham mice. T1AM associated increases in both of these amino acids indicate a shift in energy metabolism following glucose administration. C) Increase in the plasma acetate concentration (lipid intermediate) at day 7 of T1AM post treatment. Light gray bars are for T1AM treated mice and dark gray bars represent control mice. Error bars represent the SEM. Statistical significance (p < 0.05) compared to the sham treated group are indicated by asterisks.
Comparison of weight loss in T1AM treated and control mice. Decrease in weight is shown as a % of body weight in T1AM treated mice (light gray bars) and sham treated mice (dark gray bars) over 21 day study period. Error bars reflect the standard error of the mean (SEM). Statistical significance (p < 0.05) compared to the vehicle treated group indicated by asterisk.
Exhaled breath analysis measured by CRDS reveals lipid breakdown. Daily stable isotope breath analyses of T1AM treated animals (n=4) are reported as the average daily δ13C values. The minimum average values are at 80 minutes (δ13C80min) post injection for each treatment day. Statistical significance with p < 0.05 are indicated by asterisks.
Analysis of plasma by NMR-based metabolomics reveals an increase in the lipid oxidation and protein breakdown in non-fasting condition. A) A significant increase in 3-hydroxybutyrate at day 7 of T1AM treatment. The concentration of 3-hydroxybutyrate is shown as light gray bars for T1AM treated mice (n=3) and dark gray bars for control mice (n=4) (p= 0.03).
Analysis of plasma by NMR-based metabolomics reveals an increase in the lipid oxidation and protein breakdown in non-fasting condition. B) Increases in the plasma amino acids glycine (glucogenic) and valine (branched and ketogenic/glucogenic) in post T1AM treatment regime. Plasma samples were collected in the following two weeks after discontinuation of T1AM treatment and were two hours post [U-13C]-glucose injection. The concentration of glycine (p=0.05) and valine (p=0.001) is shown as light gray bars for T1AM treated mice and dark gray bars for sham mice. T1AM associated increases in both of these amino acids indicate a shift in energy metabolism following glucose administration.
Analysis of plasma by NMR-based metabolomics reveals an increase in the lipid oxidation and protein breakdown in non-fasting condition.

C) Increase in the plasma acetate concentration (lipid intermediate) at day 7 of T1AM post treatment. Light gray bars are for T1AM treated mice and dark gray bars represent control mice. Error bars represent the SEM. Statistical significance (p < 0.05) compared to the sham treated group are indicated by asterisks.