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NMR-based metabolomics and breath studies show lipid and protein catabolism during low dose chronic T_1AM treatment

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Abstract

Objective—3-iodothyronamine (T_1AM), an analog of thyroid hormone, is a recently discovered fast-acting endogenous metabolite. High single dose treatments of T_1AM 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 T_1AM (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 $^{13}CO_2$ in breath by cavity ringdown spectroscopy (CRDS) were used to detect T_1M-induced lipolysis.

Results—CRDS detected increased lipolysis in breath shortly after T_1AM 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 T_1AM include both lipolysis and protein breakdown. After discontinuation of T_1AM treatment, mice regained only 1.8% of the lost weight in the following two weeks, indicating lasting effects of T_1AM on weight maintenance.

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Conclusions—CRDS in combination with NMR and ^{13}C-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 (T_1AM) is thought to be an endogenous derivative of thyroid hormone discovered in 2004 (1). To date, the physiological effects of endogenous T_1AM 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 T_1AM 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 T_1AM 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 T_1AM is involved in rapid regulation of lipid metabolism that is central to its metabolic functions.

Reports on measurements of endogenous T_1AM concentrations remain conflicting (8–10), which is likely due to the differences in measuring unbound versus total (bound and unbound) T_1AM (11). Pharmacological studies of T_1AM administration (on the order of nmol/kg body weight) show that T_1AM causes symptoms consistent with a hypermetabolic phase, such as increased activity and food consumption (12). However, the majority of T_1AM research has used a high dose (50mg/kg body weight) that induces a severe hypometabolic state, suggesting that T_1AM 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 T_1AM (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 T_1AM 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_1AM (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_1AM at a lower pharmaceutical dose on lipid metabolism in a spontaneously obese mouse model by multianalytical techniques. T_1AM 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_1AM treatment while a targeted method uses ^{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 ^{13}C-metabolic labeling and tracing can provide a powerful analytical tool for identifying *in vivo* T_1AM associated changes in energy substrate utilization.

Materials and Methods

Reagents

Purified crystalline 3-iodothyronamine was produced as previously described (1). [U-^{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_1AM (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_1AM treatment, the experiment was repeated for a second group of animals, sham (n=4) and T_1AM 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_1AM

A stock solution of T_1AM (56mg in 112NL 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 112NL DMSO carrier in 33.6mL 0.9% saline solution for injecting sham animals. All T_1AM 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_1AM or saline. All animals were then injected at 12:00hr daily from day 0–7 intraperitoneally with either 10mg/kg T_1AM 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_1AM treatment). All plasma samples were collected at 16:00hr (2-hours after injection with T_1AM). 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_1AM on energy substrate utilization via [U-^{13}C]-glucose

On day 21 of the experiment mice were fasted two hours prior to intraperitoneal injection of 20g/kg of [U-^{13}C]-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 Mestranova (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_1AM injection each day for the T_1AM 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 T_1AM 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 T_1AM withdrawal, mice regained only 1.8% of initial weight in the following 2 weeks. The chronic treatment with 10 mg/kg T_1AM administration was repeated for a second time, which resulted 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 T_1AM 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 T_1AM and sham treated animals. Pre-injection food consumption averaged 3.91g/d+/−0.17 for sham and 3.30g/d +/−0.45 for T_1AM (p=0.32). Food consumption during treatment decreased slightly, (sham=3.36g/d+/−0.11; T_1AM=3.12g/d+/−0.47; p=0.47). Post treatment food consumption increased slightly for both groups (sham=4.13g/d+/−0.02; T_1AM=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 $\delta^{13}C$ 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 $\delta^{13}C$ values (19). We determined the baseline real time breath $\delta^{13}C$ 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_1AM 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_1AM 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_1AM treated mice from day 0–4 with ($p < 0.05$) (Figure 2).

However, later in the treatment (day 5–7) the T_1AM treatment effect on lipolysis was diminished ($\delta^{13}C$ value ~2‰).

NMR plasma analysis

Blood samples taken on day −7 (pre-T_1AM injection) and day 0 (the first 2 hrs post-T_1AM injection) revealed no differences in plasma metabolites between T_1AM (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_1AM treatment plasma 3-hydroxybutyrate, a lipid breakdown intermediate, was significantly higher in non-fasted T_1AM 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_1AM 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 (1.35× controls, p=0.05) and valine (1.70× controls, p=0.001) in T_1AM treated mice (Figure 3B). Interestingly, ^{13}C labeled acetate was 2.95 times higher in the T_1AM animals compared to the sham group (Figure 3C) under the non-fasting condition and still remained higher in the T_1AM 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_1AM treated animals (1.8% weight regained by day 21).

Discussion

This study investigated subchronic effects of T_1AM treatment at a low pharmaceutical dose on lipid metabolism using three complementary technologies (CRDS, NMR-based metabolomics and ^{13}C-isotope tracing). We administered 10mg/kg/day of T_1AM for 8 days as a potential method for inducing weight loss. We followed the effect of T_1AM for two additional weeks to evaluate long-lasting consequences of T_1AM on weight gain and to assess the effectiveness of T_1AM 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_1AM 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_1AM 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_1AM 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_1AM withdrawal, the mice that were allowed *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_1AM, and no change in activity at a low dose of T_1AM (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_1AM, 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_1AM administration follow a

biphasic dose dependent response (21). The biphasic responses associated with T_1AM 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_1AM was shown to play a role in regulating glucose and lipid metabolism (2–4, 21). Specifically, T_1AM 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_1AM at a subchronic, lower pharmacological dose administration, plays a role in increased lipid oxidation. We measured T_1AM associated changes in energy substrate utilization by monitoring carbon stable isotope ratio ($^{13}CO_2/^{12}CO_2$, or $\delta^{13}C$ 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 T_1AM induced weight loss to increased lipolysis.

Our results showed that two hours before treatment, the baseline $\delta^{13}C$ values were similar in both sham and T_1AM treatment groups. Therefore, the depression in $\delta^{13}C$ values following T_1AM administration indicates that the differences in observed $\delta^{13}C$ values between the two groups are attributed to the effect of T_1AM 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 $\delta^{13}C$ value (18–20). The day 0 breath data indicated a rapid drop in $\delta^{13}C$ value that reached the minimum within the first 80 minutes after T_1AM injection (Figure 2). This time frame is consistent with other rapid effects of T_1AM reported in the literature (1, 3), and is indicative of its rapid lipid oxidation. Thus, breath $\delta^{13}C$ value can be used as a sensitive marker for early detection of lipolysis.

During the early days of T_1AM treatment (day 0–4; day 0 being the first day of treatment), there is a significant decrease (−3.6 to −4.1) in the $\delta^{13}C_{80min}$ value in the T_1AM treated animals (Figure 2) consistent with results from microarray studies showing increased lipase gene expression in chronic T_1AM treatment in rats (M. Righi, personal communication).

Later in the treatment (day 5–7), the change in $\delta^{13}C_{80min}$ value only decreased by 1–2 units in T_1AM treated vs. sham animals (Figure 2). These results suggest a prolonged action of T_1AM on lipolysis. The continued weight loss through day 9 in the T_1AM 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_1AM treated mice showed a significant elevation in the plasma level of the ketone body, 3-hydroxybutyrate (Figure 3A), providing further evidence that T_1AM 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_1AM at 50 mg/kg, our study is the first to report that low dose chronic T_1AM treatment causes a significant increase in plasma ketone bodies.

We followed the longer-term effect T_1AM, after two weeks discontinuation, by ^{13}C-isotope tracing and NMR spectroscopy. Mice were challenged with [U-^{13}C]-glucose to evaluate lipid synthesis vs. lipolysis in the post weight loss regime. Two hours after ^{13}C-U-labeled

glucose administration, both plasma ^{12}C-glycine and ^{12}C-valine were found to be elevated (Figure 3B), while the rate of ^{13}C-glucose consumption was similar in T_1AM-treated vs. sham animals. The ^{13}C-U-glucose injection resulted in no statistically significant labeled (^{13}C) metabolites, indicating that the elevated levels of ^{12}C-valine and ^{12}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_1AM treated animals suggest that T_1AM 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}C-U-glucose injection also showed an increase in ^{13}C-acetate, which was ~3 times higher than in sham treated animals. Even though the ^{13}C-acetate levels did not reach statistical significance the results are consistent with a decrease in lipid synthesis in T_1AM treated animals that still persists two weeks post termination of T_1AM treatment. While this study begins to decode longer-term effects of T_1AM on metabolism, additional studies with mildly feed restricted mice are needed to examine the effects of T_1AM treatment for periods longer than 8 days. The breath $\delta^{13}C_{80min}$ value indicates that the biological response to exogenous T_1AM over an 8-day period is dynamic based on the fluctuations in energy substrate utilization. Our finding that prolonged use of T_1AM affects protein catabolism is consistent with prior studies showing reduced inotropism and diminished muscle contraction strength with high doses of T_1AM (1). However, to date protein catabolism has not been reported following an acute administration of T_1AM. The discovery that protein catabolism induction can occur after chronic application of T_1AM at low concentration is important and demonstrates the power of combined analyses for anti-obesity drug evaluations to identify unexpected side effects.

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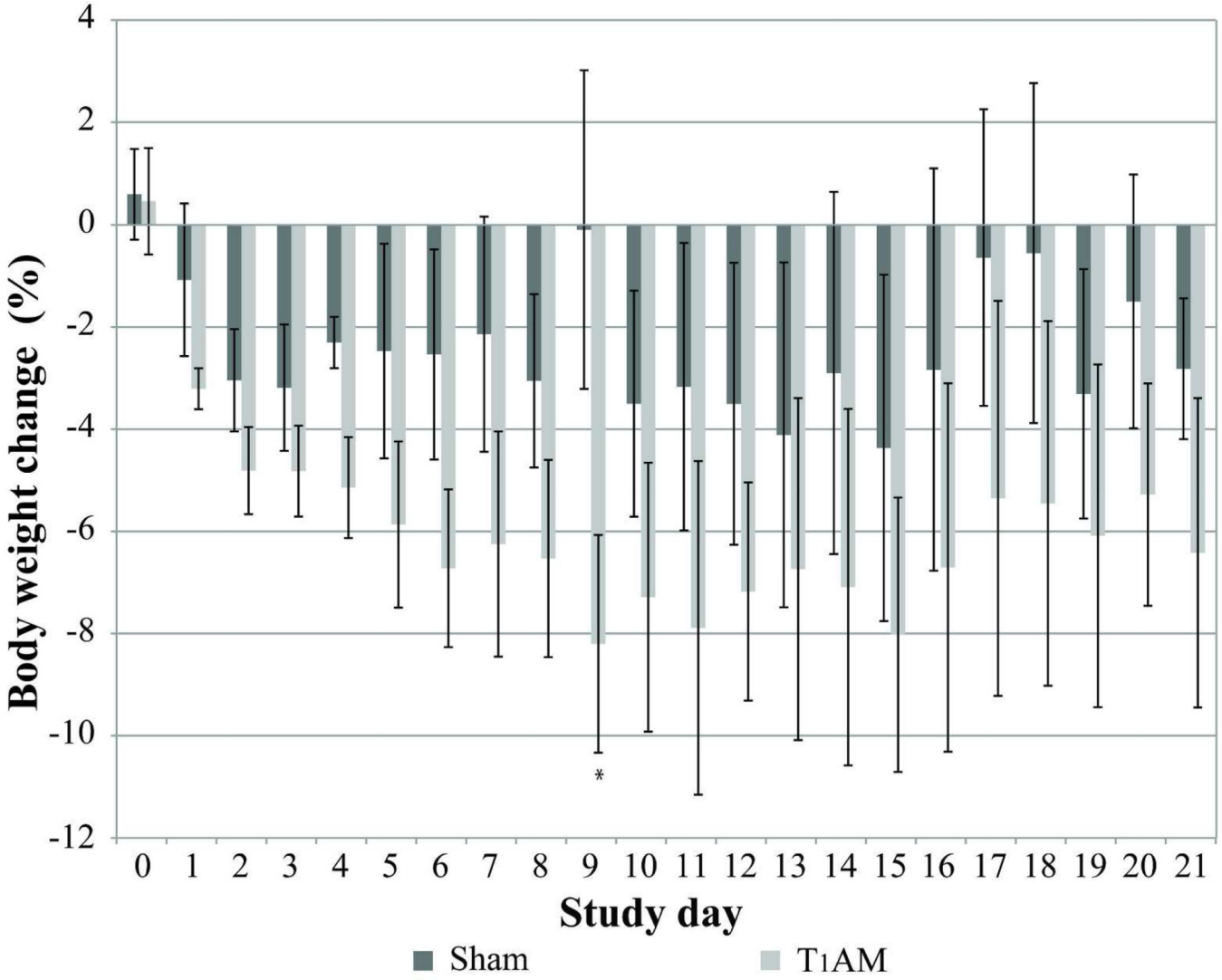


Figure 1. Comparison of weight loss in T_1AM treated and control mice

Decrease in weight is shown as a % of body weight in T_1AM 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.

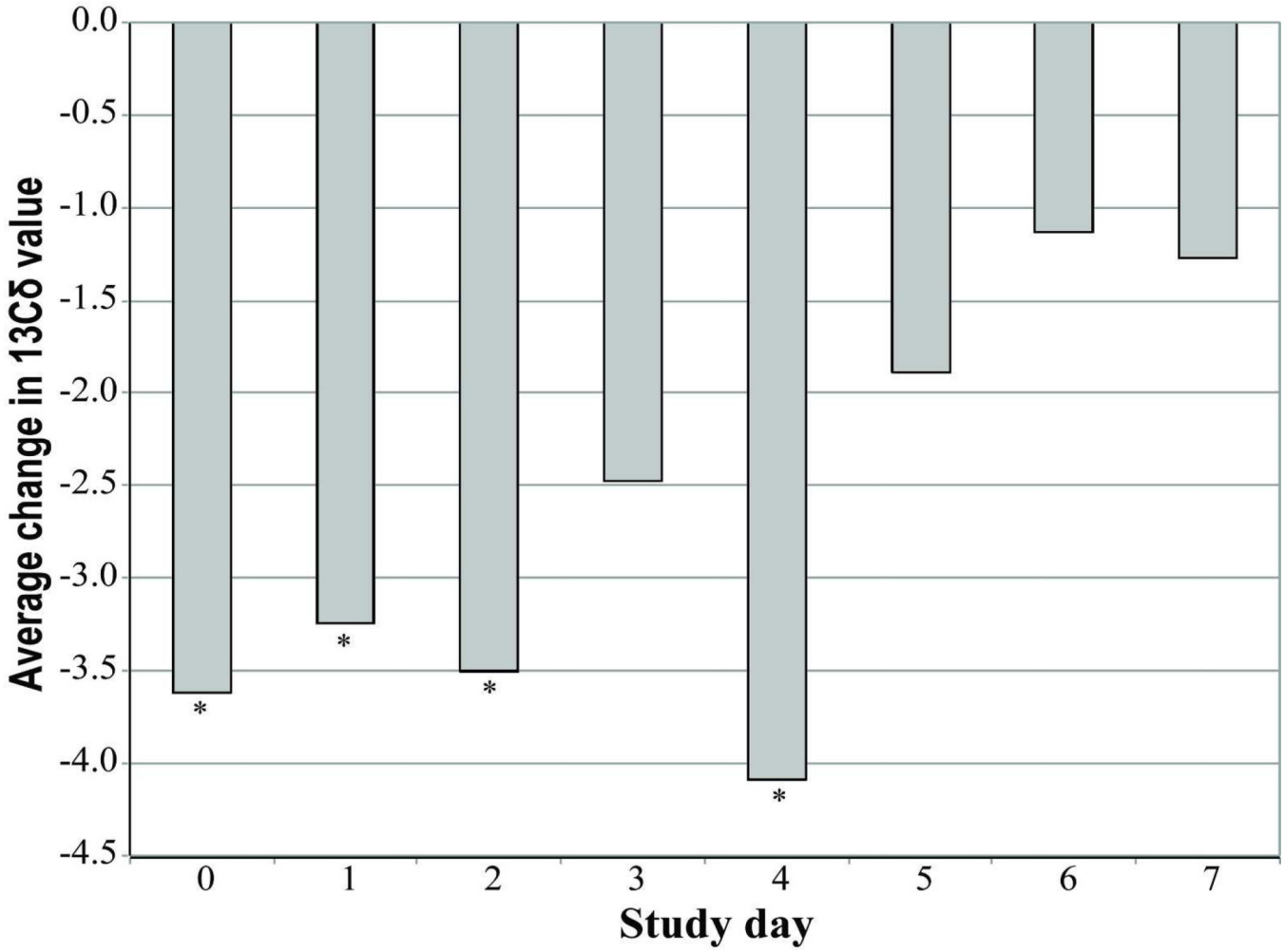
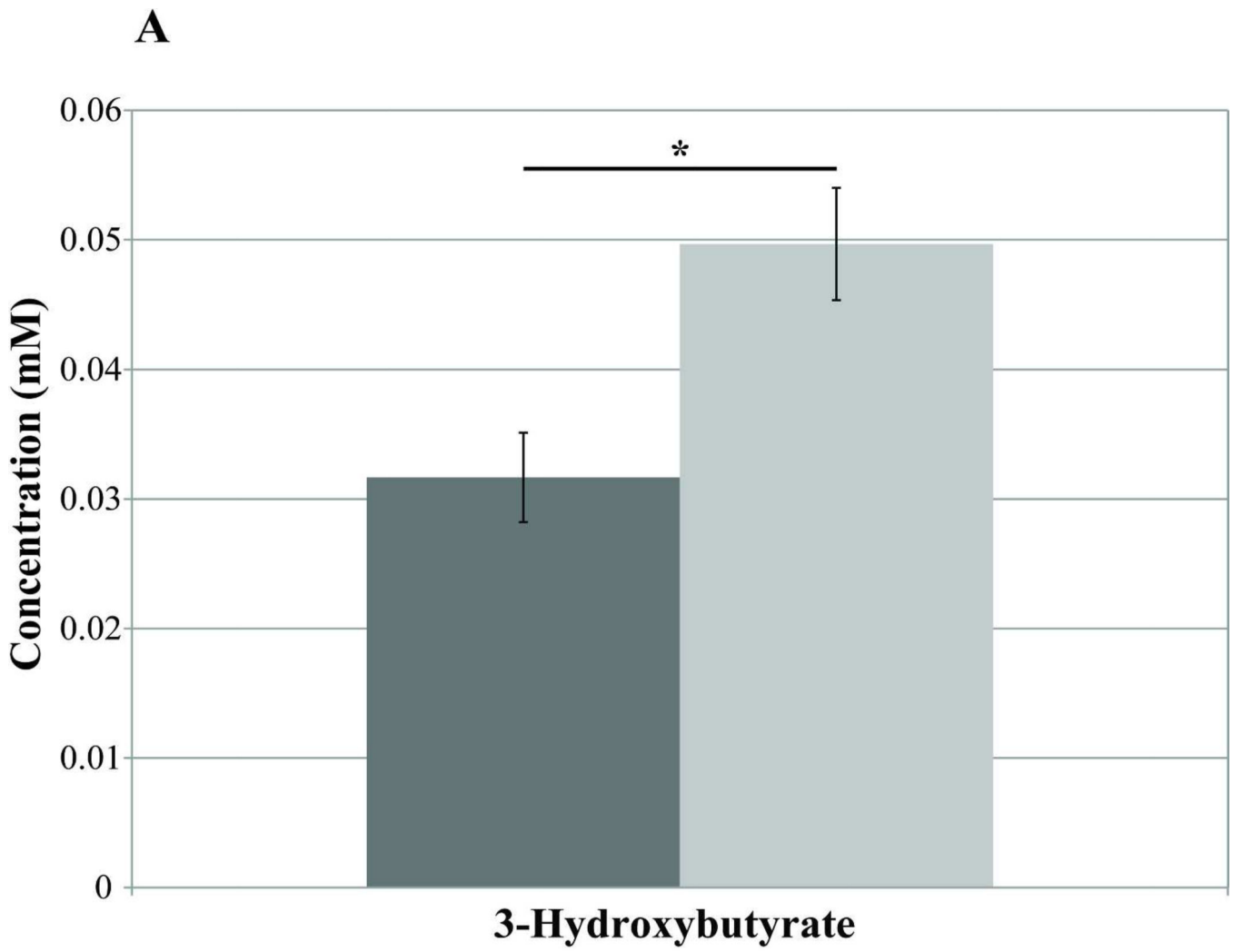
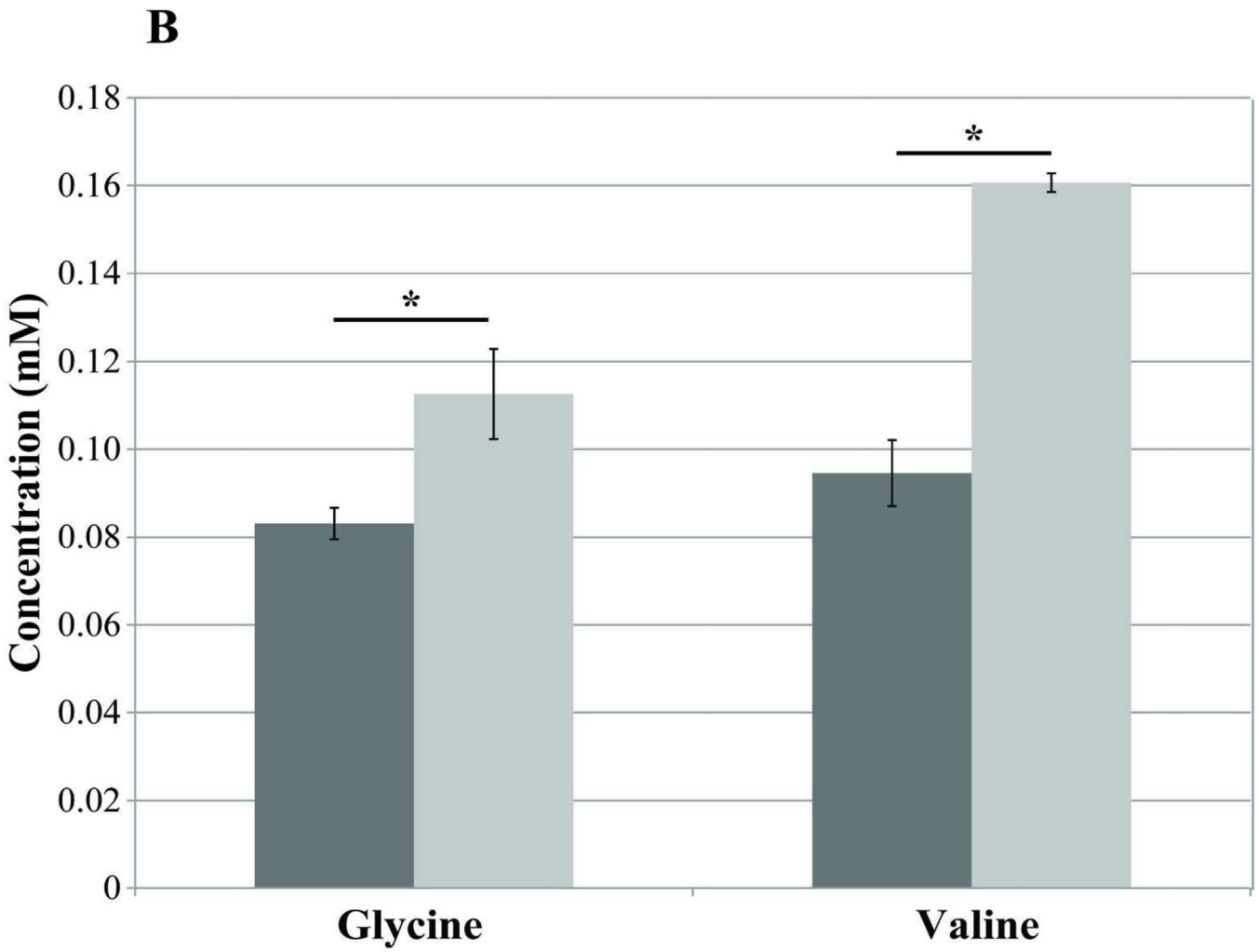


Figure 2. Exhaled breath analysis measured by CRDS reveals lipid breakdown

Daily stable isotope breath analyses of T_1AM treated animals (n=4) are reported as the average daily $\delta^{13}C$ values. The minimum average values are at 80 minutes ($\delta^{13}C_{80min}$) post injection for each treatment day. Statistical significance with $p < 0.05$ are indicated by asterisks.





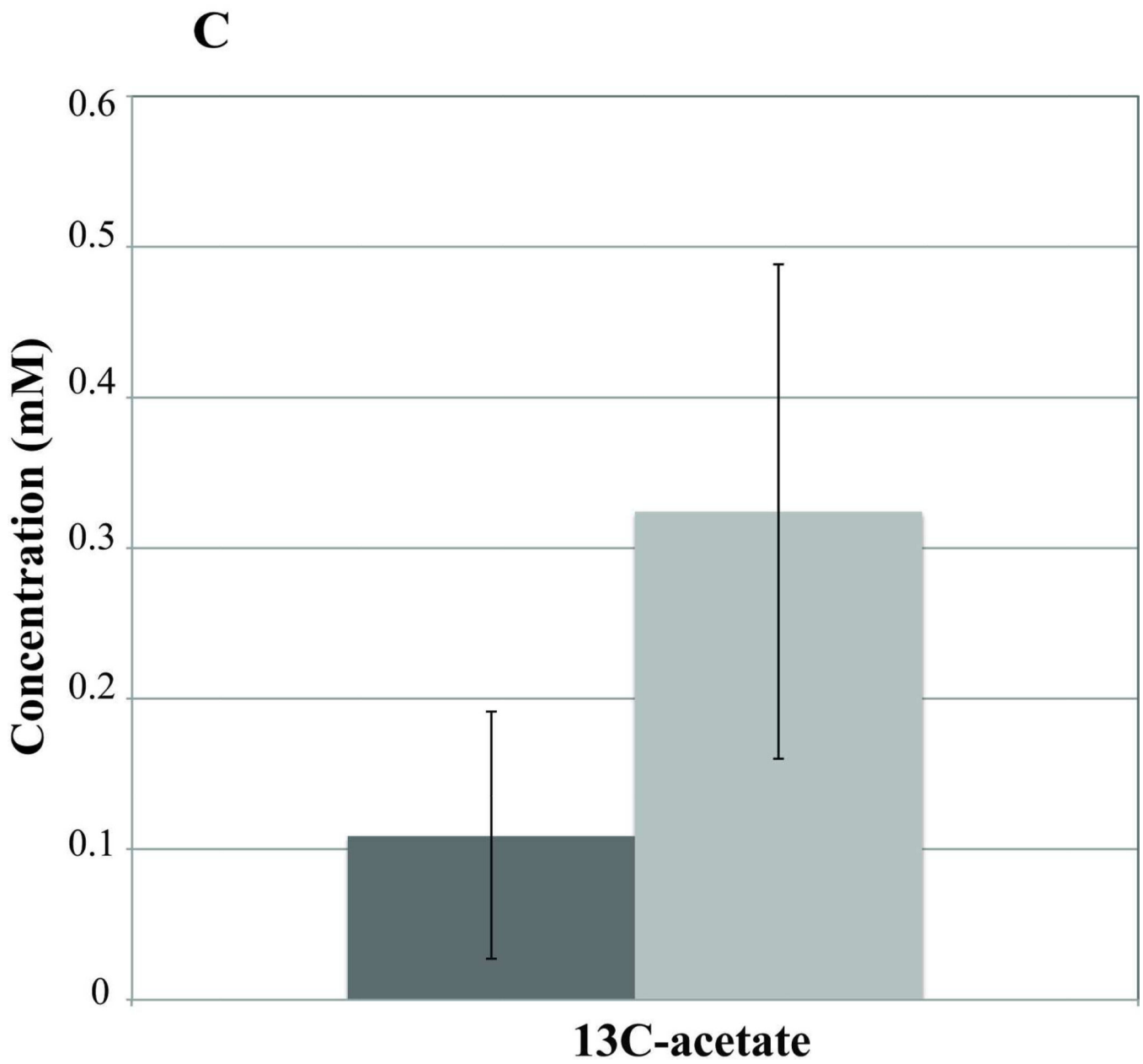


Figure 3. Analysis of plasma by NMR-based metabolomics reveals an increase in the lipid oxidation and protein break down in non-fasting condition

A) A significant increase in 3-hydroxybutyrate at day 7 of T_1AM treatment. The concentration of 3-hydroxybutyrate is shown as light gray bars for T_1AM 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 T_1AM treatment regime. Plasma samples were collected in the following two weeks after discontinuation of T_1AM treatment and were two hours post [U-^{13}C]-glucose injection. The concentration of glycine (p=0.05) and valine (p=0.001) is shown as light gray bars for T_1AM treated mice and dark gray bars for sham mice. T_1AM

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 T_1AM 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.