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Decreased branched-chain amino acids and elevated fatty acids during antecedent hypoglycemia in type 1 diabetes

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ABSTRACT

Introduction Hypoglycemia is a major limiting factor in achieving recommended glycemic targets for people with type 1 diabetes. Exposure to recurrent hypoglycemia results in blunted hormonal counter-regulatory and symptomatic responses to hypoglycemia. Limited data on metabolic adaptation to recurrent hypoglycemia are available. This study examined the acute metabolic responses to hypoglycemia and the effect of antecedent hypoglycemia on these responses in type 1 diabetes.

Research design and methods Twenty-one outpatients with type 1 diabetes with normal or impaired awareness of hypoglycemia participated in a study assessing the response to hypoglycemia on 2 consecutive days by a hyperinsulinemic glucose clamp. Participants underwent a period of normoglycemia and a period of hypoglycemia during the hyperinsulinemic glucose clamp. Plasma samples were taken during normoglycemia and at the beginning and the end of the hypoglycemic period. Metabolomic analysis of the plasma samples was conducted using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry.

Results In total, 68 metabolites were studied. On day 1, concentrations of the branched-chain amino acids, leucine ($p=3.8\times10^{-3}$) and isoleucine ($p=2.2\times10^{-3}$), decreased during hypoglycemia. On day 2, during hypoglycemia, five amino acids (including leucine and isoleucine) significantly decreased, and two fatty acids (tetradecanoic and oleic acids) significantly increased ($p<0.05$). Although more metabolites responded to hypoglycemia on day 2, the responses of the single metabolites were not statistically significant between the 2 days.

Conclusions In individuals with type 1 diabetes, one episode of hypoglycemia decreases leucine and isoleucine concentrations. Antecedent hypoglycemia results in the decrement of five amino acids and increases the concentrations of two fatty acids, suggesting an alteration between the two hypoglycemic episodes, which could indicate a possible adaptation. However, more studies are needed to gain a comprehensive understanding of the consequences of these alterations.

Trial registration number NCT01337362.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Hypoglycemia is a major limiting factor for obtaining optimal glycemic control in individuals with insulin-treated diabetes.

⇒ Recurrent hypoglycemia attenuates the counter-regulatory hormonal response, which is responsible for the clinical symptoms and metabolic response to hypoglycemia.

WHAT THIS STUDY ADDS

⇒ This study suggests that antecedent hypoglycemia enhances the metabolic response to a subsequent hypoglycemic episode, indicating a compensatory metabolic response to the attenuation of the counter-regulatory hormonal response.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The metabolic alterations to recurrent hypoglycemia might be further explored in mechanistic studies and may potentially be a target for intervention.

INTRODUCTION

Hypoglycemia is the most common adverse event of insulin replacement therapy in type 1 diabetes (T1D).[1 2] To recover from hypoglycemia and avoid developing more severe hypoglycemia, people with diabetes depend on hormonal counter-regulatory responses that increase glycogenolysis and gluconeogenesis and the generation of warning symptoms that prompt carbohydrate ingestion. However, these responses tend to diminish with the increasing duration of diabetes.

Studies investigating the impact of hypoglycemia in individuals with T1D and healthy people have shown that antecedent hypoglycemic episodes lead to blunted counter-regulatory and symptomatic responses, indicating that short-term adaptational processes occur. In T1D, the glucagon response to hypoglycemia becomes grossly diminished in most patients after a few years.[3] Furthermore, many individuals with T1D develop counter-regulatory failure with attenuated centrally mediated autonomic responses to hypoglycemia resulting in decreased epinephrine secretion, subsequent impairment of the glucose counter-regulation

and blunting of autonomic warning symptoms, which over time leads to impaired awareness of hypoglycemia.[1] During hypoglycemia, the decrease in plasma glucose levels and elevation in counter-regulatory hormones change energy metabolism.[4] In healthy human subjects, the plasma concentrations of all amino acids are reduced[5] while the plasma concentration of free fatty acids is elevated, supporting lipolysis and stimulating gluconeogenesis.[6 7] In T1D, the metabolic response to hypoglycemia is much less understood. One study found that in T1D, isoleucine and leucine were suppressed during hypoglycemia but significantly less suppressed compared with healthy controls.[8] Another found that individuals with T1D seemed to preserve their lipid response to hypoglycemia compared with healthy controls.[9] These relatively small studies have given us insights into the metabolic responses; however, the area remains largely unexplored.

Metabolomic profiling has provided the possibility to obtain detailed metabolic insights in various conditions. In diabetes, previous studies have primarily focused on risk profiling and preconditional metabolic changes.[10–12] One study of insulin-induced hypoglycemia in type 2 diabetes used metabolomics and found a change in more than 90 metabolites during hypoglycemia that was almost absent after 24 hours.[13] The metabolic response to hypoglycemia in people with T1D using metabolomics as a platform has, to our knowledge, not previously been reported.

This study aims to assess metabolic responses to a single episode of hyperinsulinemic hypoglycemia in individuals with T1D and the short-term metabolic adaptation to a subsequent hypoglycemic episode 24 hours later. We also examine whether the state of hypoglycemia awareness is associated with these responses.

METHODS

The study is a post hoc analysis of data from an experimental mechanistic clinical study investigating electroencephalogram changes and cognitive function during antecedent hypoglycemia in participants with T1D and either hypoglycemia awareness or unawareness.[14] The protocol is registered at http://clinicaltrials.gov (NCT01337362). All participants were informed about the study procedures and provided written consent to participate.

Recruitment

Participants, both male and female over 18 years old, with T1D for over 5 years and with normal hypoglycemia awareness or unawareness were included. Participants were recruited from the outpatient clinics at Nordsjællands Hospital and Steno Diabetes Center Copenhagen, Denmark. Individuals with pregnancy, brain disorders and cardiovascular disease were excluded from the study. Other exclusion criteria included antiepileptic, β-blocker drug use, and alcohol and drug abuse. We included all the participants we acquired blood sampling from at all three time points during both experimental days (figure 1).

Hypoglycemia awareness status

Hypoglycemia awareness status was classified according to three validated methods: the Pedersen-Bjergaard method,[15] the Gold score[16] and the Clarke method.[17] Participants were classified as having normal hypoglycemia awareness if they scored within the normal awareness category on all three methods. Participants were classified as hypoglycemia unaware if they scored within the unaware category using the Pedersen-Bjergaard method, impaired using the Gold score and reduced using the Clarke method. Participants who did not meet the criteria for normal hypoglycemia awareness or hypoglycemia unawareness were excluded from the study.

Experimental design

Five days before the planned experiment, every participant was equipped with a continuous glucose monitor (CGM) (Guardian Real-Time with Enlite sensor; Medtronic, Minneapolis). Participants used a blood glucose meter (Contour Link; Bayer HealthCare, Leverkusen, Germany) to monitor and calibrate the CGM. Each participant arrived fasted at the research unit on the morning of their scheduled appointment. The experiment was postponed for 2 weeks if CGM showed glucose measurements below 3.5 mmol/L 24 hours prior to the first day. If no hypoglycemia was detected, the participant underwent a hyperinsulinemic glucose clamp in normoglycemia and hypoglycemia, acquiring plasma blood samples throughout the experiment. The following day, the same experimental procedure was performed again.

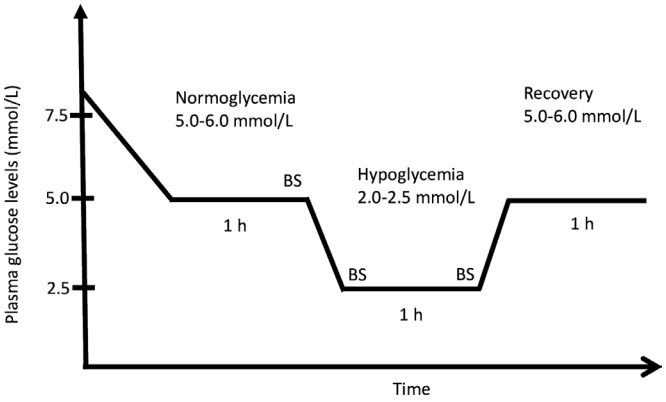

Figure 1 Timeline of the clamp set-up during day 1 and day 2. A hypoglycemic glycemic clamp was performed under normoglycemic and hypoglycemic levels. The hyperinsulinemic-normoglycemic period started when patients during the clamp reached plasma glucose between 5 and 6 mmol/L. Plasma glucose was measured every 10 min, and blood samples (BS) for counter-regulatory hormones and metabolomic analysis were drawn three times at the end of the normoglycemic period, the beginning of the hypoglycemic period and the end of the hypoglycemic period.

Hyperinsulinemic glucose clamp procedure

Insulin and glucose were infused intravenously during the entire experiment. Human insulin (Actrapid, Novo Nordisk, Ballerup, Denmark) was infused with a continuous rate of 1 mU insulin/(kg·min). Initially, a 20% glucose solution was infused at the same rate for all participants (0.015 mmol/(kg·min)) but thereafter adjusted to reach the desired blood glucose target. The blood glucose level target was 5.0–6.0 mmol/L during normoglycemia and recovery from hypoglycemia, while the target was 2.0–2.5 mmol/L during hypoglycemia. Participants were held at each target period for 1 hour (figure 1).

Blood sampling and biochemical analyses

Plasma glucose was continuously analyzed using YSI 2300 (YSI/Xylem, Yellow Springs, Ohio), and blood was sampled from the cubital vein. Furthermore, additional blood samples were acquired three times during the experiment: at the end of the normoglycemic period, the beginning of the hypoglycemic period, and the end of the hypoglycemic period. Blood samples were used to measure the counter-regulatory hormones: glucagon, epinephrine, norepinephrine, cortisol, growth hormone and substrates. Data on the hormonal counter-regulatory responses have previously been presented by Sejling *et al*.[14] Additionally, blood samples for metabolomic analysis were acquired.

Metabolomic analysis

Sample preparation and analysis were performed as described before.[18] Briefly, plasma samples (30 µL) were spiked with 400 µL methanol and 10 µL of an internal standard mixture (d4-succinic acid, d5-glutamic acid, d8-valine, and d33-heptadecanoic acid; Sigma Aldrich). Samples were vortex mixed and incubated on ice for 30 min and centrifuged (10 000 rpm, 3 min, 4°C). Finally, 180 µL of the filtered extracts was transferred to glass vials and evaporated to dryness before derivatization. The samples were derivatized using a previously described procedure.[19] Briefly, derivatization converts the reactive biological groups into trimethylsilyl derivatives, increasing the biomolecules' volatility. The polar metabolites were analyzed using a Pegasus 4D (LECO; Saint Joseph, USA) system, which combines two-dimensional chromatographic separation with time-of-flight mass spectrometric detection.

The data files obtained by the ChromaTOF software were exported to text files. In-house developed software Guineu[20] was used for aligning the data for further analyses. The original two-dimensional gas chromatography–time-of-flight mass spectrometry (GC×GC–TOFMS) data include retention times, retention indices (RI), spectral information for possible identification, spectral similarity value (S=0–999), and peak response data. The detected peaks were aligned using the Guineu software.[20] The alignment of the data was performed based on RI, second dimension retention times and spectra. Identifications were assigned using the National Institute of Standards and Technology and in-house libraries. Data were then postprocessed and analyzed in R (https://www.r-project.org/).[21]

Statistical analysis

Data were analyzed in R. Metabolite-wise mixed-effects models were used to consider the six repeated measures from each participant in the trial. Statistical tests were corrected for multiple testing and comparison with false discovery rate (FDR) using the Benjamini-Hochberg method.[22]

First, differences between the 2 days were modeled with metabolite-wise mixed-effects models, with time, day and time-day interaction as fixed effects and the participant ID as random effect. We further conducted the metabolite concentration changes found from the mentioned analysis within days. These changes were visualized in a heatmap. In the heatmap, each colored rectangle corresponds to the median concentration of the metabolite levels that were p value adjusted <0.05. Metabolites with the most substantial differences each day were visualized with curves, where the observations were grouped by time point. Finally, differences between aware and unaware groups were explored. A detailed description of the data analysis plan is available in online supplemental material 1.

RESULTS

Baseline characteristics of the participants

A total of 21 individuals with T1D, both female and male, were included in the study. The participants had a body mass index mean value of 24 kg/m^2 and a relatively long duration of disease (28 years). HbA1c mean value was 8.0% for the participants, and they used an average daily insulin dose of 41 IU. Eleven of the 21 participants were hypoglycemia unaware (table 1).

Metabolomics

Sixty-eight metabolites from the plasma samples were identified and semiquantified using untargeted GC×GC–TOFMS metabolomic analysis.

Metabolic responses on day 1

In total, two out of 68 metabolites had a change in their levels during day 1; the concentrations of two branched-chain amino acids (BCAA), isoleucine and leucine, were significantly decreased as a response to hypoglycemia compared with baseline ($p<0.05$, figure 2A); isoleucine ($\beta\pm SE$: -0.72 ± 0.16, $p=2.2\times10^{-3}$), leucine ($\beta\pm SE$: -0.78 ± 0.18, $p=3.8\times10^{-3}$). Another 10 amino acids were identified in the analysis shown in table 2. None of the other amino acids had a significant change; however, all showed a decrease in tendency. Metabolite names, 95% CI, FDR p values and slopes are given in table 2. The complete table is available in online supplemental material 2.

Table 1 Baseline characteristics

Participant characteristic	Mean±SEM (%) IQR
Males, n	12 (57%)
BMI, kg/m^2	24±0.67 18–30
Duration of T1D, years	28±2.5 8–54
HbA1c, %	8.0±0.22 6.0–9.9
mmol/mol	64±2.4 42–85
Fasting C-peptide negative, n	
<0.02 nmol/L	18 (86%)
0.02–0.04 nmol/L	3 (14%)
Daily insulin dose, IU/day	41±4.3 11–92
Hypoglycemia unaware, n	11 (52%)

Values are presented as the mean, ±SEM and IQR or number (percentage) as appropriate.
BMI, body mass index; T1D, type 1 diabetes.

Table 2 Results of the mixed-effects model

Metabolites	Slope	L95	U95	SE	FDR
Isoleucine	−0.72	−1.04	−0.41	0.16	2.2×10^{-3}
Leucine	−0.78	−1.14	−0.41	0.18	3.8×10^{-3}
Glycine	−0.31	−0.63	0.013	0.16	0.36
Methionine	−0.33	−0.67	0.019	0.17	0.36
Proline	−0.23	−0.45	−0.0094	0.11	0.36
Valine	−0.39	−0.59	−0.027	0.14	0.36
Tyrosine	−0.32	−0.65	0.0093	0.19	0.36
Phenylalanine	−0.31	−0.72	0.094	0.20	0.43
Serine	−0.30	−0.76	0.16	0.23	0.55
Threonine	−0.29	−0.77	0.19	0.24	0.61
Glutamic acid	−0.13	−0.41	0.15	0.14	0.74
Tryptophan	−0.16	−0.62	0.30	0.23	0.84
Alanine	−0.0081	−0.37	0.35	0.18	0.98
3-Hydroxybutyric acid	−0.28	−0.65	0.061	0.17	0.39

The table shows the metabolite, slope, its lower and upper CIs (L95, U95), SE, and p value of the slope (FDR) after correction for multiple testing.
FDR, false discovery rate.

Metabolic responses on day 2

During day 2, the concentration of seven metabolites (including those from day 1) was significantly changed; five amino acids (leucine, valine, isoleucine, methionine, and phenylalanine) were significantly decreased ($p<0.05$), and two fatty acids (tetradecanoic acid (myristic acid 14:0) and oleic acid) were increased (figure 2B, C). The eight other amino acids did not show significant changes as seen on day 1; however, they still had a decreasing trend. Metabolite names, 95% CI, FDR p values and slopes are given in table 3. The complete table is available in online supplemental material 3.

Adaptation and impact of hypoglycemia awareness status

Seven metabolites responded to hypoglycemia on day 2 compared with two metabolites on day 1. Metabolites responding to hypoglycemia on both days followed the same trend. However, no statistically significant differences were found when comparing the metabolic responses to hypoglycemia between the days. Additionally, no significant difference between the metabolic responses in individuals with normal or impaired awareness could be detected before and after correcting for multiple testing ($p>0.94$). Complete data sets of the comparative analysis between normal and impaired awareness are available in online supplemental materials 4 and 5.

DISCUSSION

This untargeted metabolic profiling of individuals with T1D under a hyperinsulinemic-normoglycemic and hypoglycemic clamp condition showed dynamic changes in several metabolites that increased following antecedent hypoglycemia. On day 1, we found that an event of hypoglycemia decreased the BCAAs: isoleucine and leucine. A subsequent hypoglycemic event 24 hours later resulted

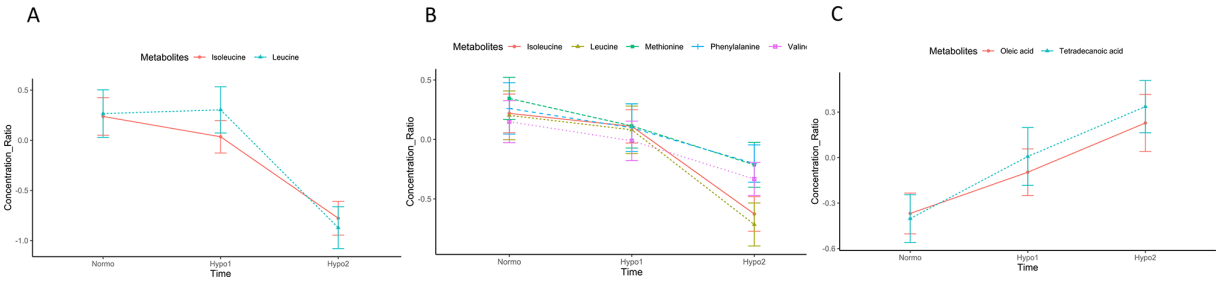

Figure 2 Metabolic responses of amino acids and free fatty acids on day 1 (A) and day 2 (B, C). SEM error bars. Samples were acquired at the end of the normoglycemic period (Normo), the beginning of the hypoglycemic period (Hypo1), and the end of the hypoglycemic period (Hypo2). Day 1: only leucine and isoleucine had significantly different levels between the end of the normoglycemic period and at the end of the hypoglycemic period ($p<0.05$). Day 2: five amino acids and two fatty acids showed significantly different levels between the end of the normoglycemic period and at the end of the hypoglycemic period ($p<0.05$).

in a decrease in additional three amino acids (phenylalanine, valine, and methionine) and an increase in two fatty acids (myristic and oleic acids). Despite this suggestion of an adaptation toward a more dynamic metabolic response to hypoglycemia on day 2, there were no statistically significant differences in the responses of the single metabolites between the 2 days. Interestingly, in this study, alanine, an important energy fuel for muscle and other tissues,[23] is unaltered during hypoglycemia on both days. However, our study cannot exclude other possibilities; one suggestion could be that BCAAs are converted into alanine, maintaining plasma alanine concentration while also being a factor in the decrement of the plasma BCAA levels.[24]

The changes in amino acids correspond to the findings in healthy human studies, which have shown a reduction in BCAA concentration during acute hyperinsulinemic hypoglycemia.[5 25] Thus, the decrease in plasma amino acids could reflect increased utilization of amino acids as substrates for ketone bodies and gluconeogenesis.[26] Battezzati *et al* have shown that the decrease in plasma concentrations of leucine and phenylalanine was significantly smaller when comparing individuals with T1D and healthy controls,[8] suggesting an impairment of this counter-regulatory response in T1D. Our study is not suited to support their findings; however, exploring this would be very interesting. Furthermore, the metabolomics platform employed in our study could only detect 12 out of the 20 standard proteinogenic amino acids, as shown in tables 2 and 3. Therefore, future studies using metabolomics should use platforms capable of identifying all amino acids. Doing so would provide a more comprehensive understanding of the alterations in amino acid levels associated with hypoglycemia.

On day 2, alongside the downregulation of five amino acids, we also observed increased levels of two fatty acids, tetradecanoic acid and oleic acid, which could suggest an increase in lipolysis. However, in our study, glycerol, another product of lipolysis, did not change in plasma levels during hypoglycemia.[27] Therefore, our study cannot determine whether the elevation in Free Fatty Acids (FFAs) results from increased lipolysis or suppression of the utilization of FFAs, which in both cases would lead to an increase in plasma FFA. However, one study examining lipolysis and hypoglycemia in T1D found an elevation in lipolysis and an increase in FFAs,[9] indicating that our findings could also result from increased lipolysis. Additionally, our data explored the fingerprint of the changes in FFA during hypoglycemia and showed a faster response in specific FFAs, oleic acid and tetradecanoic acid.

FFAs are important substrates during fasted conditions. FFAs are substrates for hepatic β oxidation providing energy in the form of ATP and forming ketone bodies

Table 3 Results of the mixed-effects model

Metabolites	Slope	L95	U95	SE	FDR
Leucine	−0.62	−0.89	−0.35	0.13	1.9×10^{-3}
Valine	−0.52	−0.75	−0.29	0.12	1.9×10^{-3}
Isoleucine	−0.59	−0.97	−0.22	0.19	2.6×10^{-2}
Methionine	−0.62	−1.01	−0.23	0.19	2.6×10^{-2}
Phenylalanine	−0.65	−1.06	−0.25	0.20	2.6×10^{-2}
Alanine	−0.22	−0.46	0.031	0.12	0.25
Proline	−0.21	−0.45	0.018	0.12	0.25
Tyrosine	−0.29	−0.60	0.019	0.15	0.25
Serine	−0.29	−0.69	0.11	0.20	0.35
Threonine	−0.31	−0.76	0.15	0.22	0.38
Tryptophan	−0.19	−0.71	0.33	0.26	0.72
Glutamic acid	−0.063	−0.30	0.18	0.12	0.82
Glycine	−0.077	−0.40	0.24	0.16	0.82
Tetradecanoic acid	0.63	0.29	0.97	0.17	1.3×10^{-2}
Oleic acid	0.50	0.22	0.79	0.14	1.6×10^{-2}
Palmitic acid	0.52	0.087	0.95	0.23	0.17
Stearic acid	0.42	0.012	0.83	0.20	0.21
Glycerol	0.064	−0.73	0.86	0.39	0.92
3-Hydroxybutyric acid	0.13	−0.15	0.40	0.14	0.64

The table shows the metabolite, slope, its lower and upper CIs (L95, U95), SE, and p value of the slope (FDR) after correction for multiple testing.
FDR, false discovery rate.

which can be transported to other extrahepatic tissues via the circulation.[28] Although β oxidation does not produce any substrates for gluconeogenesis, the ATP formed by β oxidation is used in facilitating gluconeogenesis.[28] Our study found that 3-hydroxybutyric acid (3-OHB), a ketone body, was unaltered during hypoglycemia on both days (tables 2 and 3), indicating that ketogenesis does not seem to increase during hypoglycemia in our study. Another study examining healthy participants during hypoglycemia used a bolus insulin injection to induce either hypoglycemia or normoglycemia randomly and found that while FFA increased immediately after inducing hypoglycemia, 3-OHB levels from the hypoglycemia-induced participants did not increase until plasma insulin levels were down to baseline.[29] In contrast, we used a continuous insulin infusion which probably severely suppressed ketogenesis.

The metabolic responses to hypoglycemia are primarily stimulated by the counter-regulatory hormonal response.[30] As mentioned, the hormonal responses in this study have previously been presented by Sejling *et al*.[14] Briefly, all counter-regulatory hormones (glucagon, epinephrine, cortisol and growth hormone) increased on both days. There were no differences between the 2 days for the hormonal responses except for growth hormone, which was lower during the second day. In T1D, the glucagon response to glucagon is severely diminished,[31] while growth hormone and cortisol do not seem to have an effect during the first hour of hypoglycemia.[32 33] These factors make epinephrine the pivotal counter-regulatory hormonal response during the acute phase. Previous studies investigating epinephrine infusion in healthy subjects and patients with T1D have shown a similar fall in amino acid plasma levels.[34 35] Although we did not find a statistically significant difference in amino acids between the 2 study days, we did observe more amino acids downregulated on day 2 than on day 1. This alteration in metabolic response could be a result of a change in the cellular response to epinephrine since the counter-regulatory response from epinephrine did not differ between the first and subsequent episodes of hypoglycemia.[14]

Previous studies have shown diverging results regarding the physiological responses to epinephrine. Guy *et al* found that the physiological response to epinephrine is altered in T1D, finding lower glucose levels and cardiovascular responses. However, they did see a greater increase in lipolytic response compared with healthy subjects.[36] Hypoglycemia also seems to impact the physiological response of epinephrine, reducing the cardiovascular, hepatic, and adipose tissue responses to epinephrine.[37] Another study, however, did not see the same changes.[38] All mentioned studies did not investigate the changes in different metabolites as we did; however, due to the limitation of our study, which did not specifically focus on metabolic responses to epinephrine, further studies looking at the amino acid and lipid fluxes are needed to elaborate our findings.

Although the glucagon response to hypoglycemia is deficient in T1D,[31] we did observe an increase in our study.[14] Previous studies investigating the effects of glucagon on protein metabolism in healthy subjects have shown that glucagon may lower leucine concentration but not plasma concentration of phenylalanine.[39] Another study, however, did not find a significant alteration in the plasma concentration of BCAAs in response to glucagon.[40] Thus, it is still debatable whether the changes we see in the plasma concentrations of the BCAAs are influenced by glucagon. In our study, this is further supported by the minimal glucagon responses.[14]

Another important hormone is insulin, which alters protein metabolism, lowers the plasma concentration of amino acids[41 42] and stimulates lipogenesis, thus decreasing the plasma concentration of fatty acids.[43] In our study, however, baseline normoglycemic plasma samples and plasma taken in hypoglycemia were the same during a constant hyperinsulinemic conditions, only altering the glucose infusion. Thus, the observed metabolic changes should not be a result of insulin. However, as discussed, the continuous insulin infusion in our study seems to suppress some metabolic pathways and to fully understand the metabolic consequences of the alterations, future studies aimed at investigating how these changes could facilitate energy metabolism should include experiments using bolus insulin to induce hypoglycemia, thus limiting the influence of the continuous hyperinsulinemic condition found in a clamp study.

There were no differences in the metabolic reactions of people with normal hypoglycemia awareness and unawareness on either of the days. This could be explained by the indifferent hormonal response between the two groups, reported previously by Sejling *et al*.[14] Similar findings were also observed in a previous study investigating metabolic responses to hypoglycemia in T1D.[9]

In conclusion, this study shows a significant metabolic response to hypoglycemia in people with long-standing T1D and indicates that recurrent hypoglycemia alters the metabolic response, particularly within protein and lipid metabolism. Our results could indicate that while the counter-regulatory hormonal response attenuates to recurrent hypoglycemia, the metabolism may try to adapt to these conditions. However, future studies are needed to confirm and elaborate our findings and explore the consequences. The strengths of this study include a considerable sample size and a rigorous clamp protocol. However, to further understand metabolic responses to hypoglycemia in T1D, patients with newly onset diabetes duration and healthy controls could have been included.

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Contributors RS, CL-Q and UP-B contributed to the conceptual design and data analysis of the post hoc study. A-SS contributed to the conceptual design, recruited participants and conducted the original clinical trial. IMM performed the metabolomic analysis. JP contributed to the data analysis. NHA-s and RS contributed equally to the drafting of the manuscript. All authors contributed significantly to the manuscript review and editing and approved the final manuscript. UP-B is the guarantor and is responsible for the overall content of the manuscript.

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Competing interests After conclusion of the experimental work, A-SS has been employed by Novo Nordisk. CL-q is a member of the Scientific Advisory Board of Fondation Alzheimer and Institute Pasteur, and UP-B has served on advisory panels for Novo Nordisk and Sanofi.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Regional Committee on Health Research Ethics (ID H-1-2011-024). Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available upon reasonable request.

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