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Glucose Load Following Prolonged Fasting Increases Oxidative Stress-Linked Response in Individuals With Diabetic Complications

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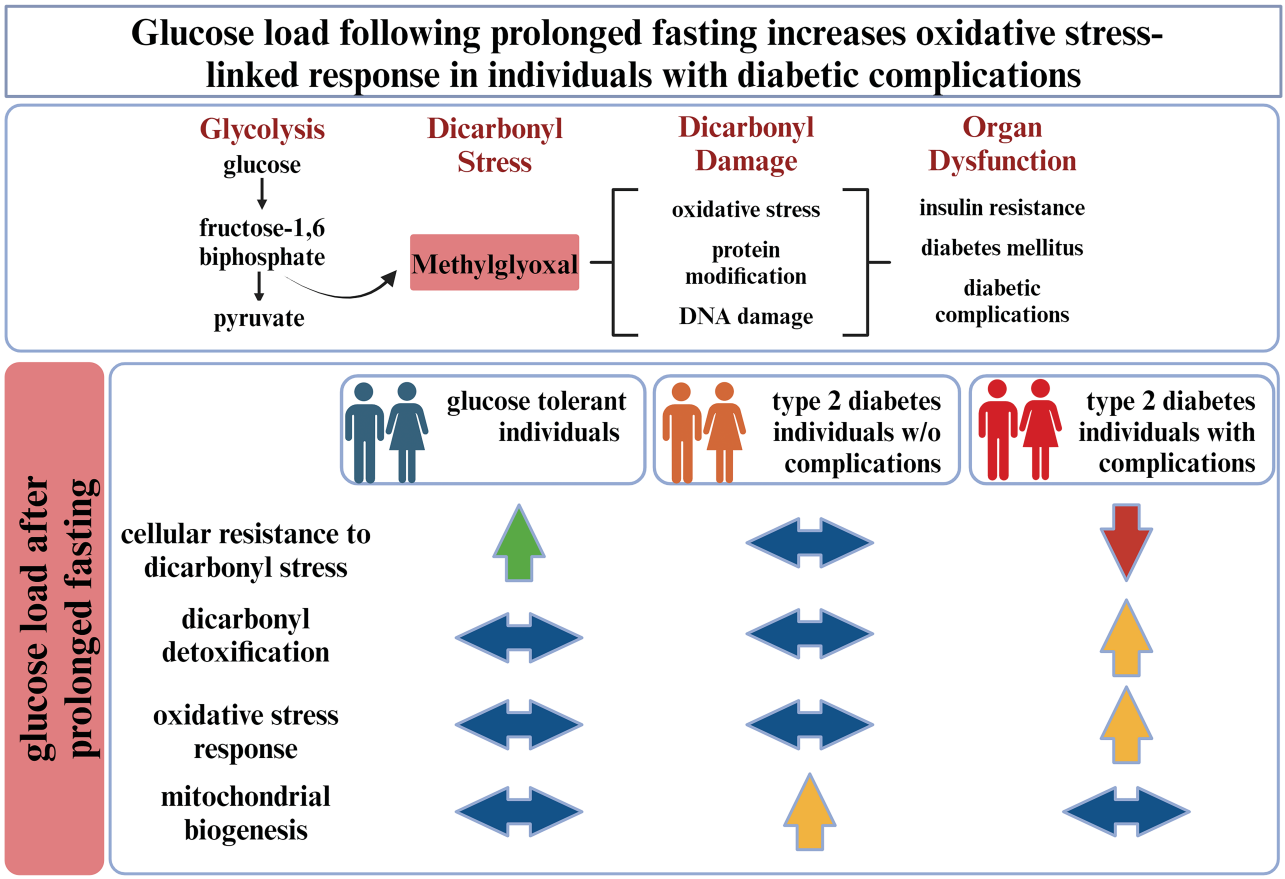

ARTICLE HIGHLIGHTS

- **Why did we undertake this study?**
  Accumulating evidence suggests that individuals with diabetes complications might not benefit from intensified lifestyle intervention. We aimed to investigate cellular resistance against dicarbonyl stress after glucose load after prolonged fasting.
- **What is the specific question(s) we wanted to answer?**
  Is cellular resistance against ex vivo exposure to dicarbonyl stress after glucose load affected by prolonged fasting in type 2 diabetes?
- **What did we find?**
  We found that cellular resistance against dicarbonyl stress is reduced after glucose load following prolonged fasting in type 2 diabetes individuals with diabetes complications, while cellular dicarbonyl detoxification and oxidative stress response are inadequately increased.
- **What are the implications of our findings?**
  Our findings indicate for increased susceptibility to glucose-induced oxidative stress after prolonged fasting in individuals with diabetes complications.

Glucose Load Following Prolonged Fasting Increases Oxidative Stress-Linked Response in Individuals With Diabetic Complications

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OBJECTIVE

Prolonged catabolic states in type 2 diabetes (T2D), exacerbated by excess substrate flux and hyperglycemia, can challenge metabolic flexibility and antioxidative capacity. We investigated cellular responses to glucose load after prolonged fasting in T2D.

RESEARCH DESIGN AND METHODS

Glucose-tolerant individuals (CON, *n* = 10), T2D individuals with (T2D+, *n* = 10) and without diabetes complications (T2D−, *n* = 10) underwent oral glucose tolerance test before and after a 5-day fasting-mimicking diet. Peripheral blood mononuclear cells' (PBMC) resistance to ex vivo dicarbonyl methylglyoxal (MG) exposure after glucose load was assessed. Markers of dicarbonyl detoxification, oxidative stress, and mitochondrial biogenesis were analyzed by quantitative PCR, with mitochondrial complex protein expression assessed by western blotting.

RESULTS

T2D+ exhibited decreased PBMC resistance against MG, while T2D− resistance remained unchanged, and CON improved postglucose load and fasting (−19.0% vs.−1.7% vs. 12.6%; all *P* = 0.017). T2D+ showed increased expression in dicarbonyl detoxification (mRNA glyoxalase-1, all *P* = 0.039), oxidative stress (mRNA glutathione-disulfide-reductase, all *P* = 0.006), and mitochondrial complex V protein (all *P* = 0.004) compared with T2D− and CON postglucose load and fasting. Citrate synthase activity remained unchanged, indicating no change in mitochondrial number. Mitochondrial biogenesis increased in T2D− compared with CON postglucose load and fasting (mRNA HspA9, *P* = 0.032). T2D−, compared with CON, exhibited increased oxidative stress postfasting, but not postglucose load, with increased mRNA expression in antioxidant defenses (mRNA forkhead box O4, *P* = 0.036, and glutathione-peroxidase-2, *P* = 0.034), and compared with T2D+ (glutathione-peroxidase-2, *P* = 0.04).

CONCLUSIONS

These findings suggest increased susceptibility to glucose-induced oxidative stress in individuals with diabetes complications after prolonged fasting and might help in diet interventions for diabetes management.

Efforts to understand the heterogeneity of type 2 diabetes (T2D) have led to the identification of distinct diabetes subtypes, enabling the possibility for precision diabetology

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(1–3). The primary findings of the Action for Health in Diabetes (Look AHEAD) trial showed that intensive lifestyle intervention does not reduce cardiovascular risk in individuals with T2D (4). However, secondary analysis has identified subgroups of study participants with increased vulnerability to intensified lifestyle interventions (5). Notably, the poor-glucose-control subgroup showed a greater cardiovascular risk following caloric restriction, despite weight loss and improved glycemic control (5). The mechanisms behind such differential responses to lifestyle interventions in individuals with T2D remain unclear. Identification of T2D individuals who are susceptible to adverse effects of lifestyle interventions will therefore help to optimize diabetes management strategies.

Recent findings show that various fasting regimens have beneficial effects in metabolic disorders and neurodegenerative diseases (6–9). Debate centers on whether prolonged fasting (≥48 h) is superior to chronic caloric restriction. Previously, we have shown that repetitive prolonged fasting in T2D individuals improved temporarily microalbuminuria, insulin resistance, and dicarbonyl stress (10), without detrimental effects on somatosensory nerve function (11). However, the improvement in dicarbonyl stress was only transient and ceased after refeeding. Studies on how fasting might challenge the metabolic flexibility and antioxidative capacity after refeeding in T2D are lacking. Such research is relevant for dietary interventions in diabetes management. Metabolic inflexibility and insulin resistance in T2D might limit the adaptation during fasting and refeeding, requiring a rapid shift from catabolism to anabolism (12–14). T2D individuals exhibit increased endogenous glucose production before and after eating, leading to fasting and postprandial hyperglycemia (15). Therefore, glucose intake after prolonged fasting may exacerbate hyperglycemic stress in T2D.

Oxidative stress is harmful to cells, yet antioxidant therapies have not proven protective in large-scale clinical studies, suggesting that the relationship between oxidative stress and disease may be more complex than previously understood (16,17). Low-level mitochondrial oxidative stress can induce changes that make cells less vulnerable to future stress, a response known as mitohormesis (18,19). Increased mitochondrial stress in yeast cells, caused by prior exposure to high glucose or to low-level dicarbonyl stress, enhances cellular survival against methylglyoxal (MG) (20). MG, a glycolysis byproduct, contributes to diabetes progression and complications (21–23). Interventions linked to enhancing mitochondrial oxidative stress have extended the lifespan in various model organisms (24–27). Hormetic pathways, involving systemic mediators of mitochondrial stress, such as GDF15 and FGF21, regulate metabolic balance (19,28,29). However, assessing mitohormesis in complex organisms remains challenging, with human studies lacking.

Based on our previous findings on hormetic response against MG, and since glucose load leads to increased glycolysis and therefore to increased production of endogenous MG (20,22), we hypothesized that glucose load after prolonged fasting increases oxidative stress-linked cellular resistance against MG in glucose-tolerant individuals. We further hypothesized that this hormetic protective response is lost in individuals with T2D and further deteriorated in those with T2D and diabetes complications.

RESEARCH DESIGN AND METHODS

Study Design and Study Population

This exploratory pilot study was performed at the Clinic of Endocrinology, Diabetology, Metabolic Diseases and Clinical Chemistry at the University Hospital of Heidelberg in Germany. The study was approved by the ethics committee of the University of Heidelberg, Heidelberg, Germany (Ethic-Nr. S–459/2018) in compliance with national guidelines and the declaration of Helsinki. This study was registered at the German Clinical Trials Register (Deutsches Register Klinischer Studien DRKS-ID: DRKS00014287). All participants gave written informed consent.

Glucose-tolerant individuals (CON, n = 10, 4/6 females/males) and individuals with T2D with (T2D+, n = 10, 4/6 females/males) and without diabetes complications (T2D−, n = 10, 4/6 females/males) were included in the study. Participants were selected from the Heidelberg Study on Diabetes and Complications (HEIST-DiC), a prospective longitudinal cohort study (ethics number S–383/2016, ClinicalTrials.gov Identifier NCT03022721). T2D groups were selected based on glucose control and thorough clinical phenotyping. They were matched for diabetes duration, glycemic control, antidiabetic medication, and medication with renin-angiotensin-aldosterone system (RAAS) inhibitors. The CON group was matched with the T2D for sex, age, and BMI. Inclusion and exclusion criteria are provided in the Supplementary Material (10). Withdrawal criteria included subject's request and lack of increase in blood and/or urine ketone bodies after the prolonged fasting phase.

Participants underwent a standardized 75-g oral glucose tolerance test (OGTT) after an overnight fast on the day before starting prolonged fasting (visit 1) and repeated the OGTT directly after the prolonged fasting (visit 2). The participants were instructed to comply with a 5-day fasting-mimicking diet (FMD) (details given below). Peripheral blood mononuclear cells (PBMC) were isolated before and 2 h after glucose intake for further analysis. The 2-h plasma glucose could not be measured in one participant of the CON group because of analytical error and therefore was excluded from the analysis of 2-h plasma glucose levels. Anthropometric measurements, clinical examination, and blood sampling were performed on both visits. Study design is shown in Fig. 1*A*.

Prolonged Fasting

Prolonged fasting was achieved by using a 5-day FMD. This diet mimics fasting-like effects on glucose and ketone bodies. Daily energy intake in day one is 4,600 kJ (11% protein, 46% fat, and 43% carbohydrates), whereas, from days 2 to 5, it is 3,000 kJ (9% protein, 44% fat, and 47% carbohydrate) (30). As previously described (10), patients on insulin therapy were instructed to discontinue short-acting insulin and to reduce the long-acting insulin by 50% when taking FMD. Oral antidiabetic therapy was also discontinued during FMD. During prolonged fasting, glycemic levels were self-monitored (fasting and 2-h postprandial levels, at least four measurements daily) with a capillary blood glucose monitoring system (Accu-Check Guide, Roche), and a 24-h telephone platform was available for participants to report hypo- or hyperglycemic episodes. All participants were instructed to avoid excessive physical activity during prolonged fasting. Safety was monitored by assessment of vital signs, physical examination, adverse events following the Common Terminology Criteria

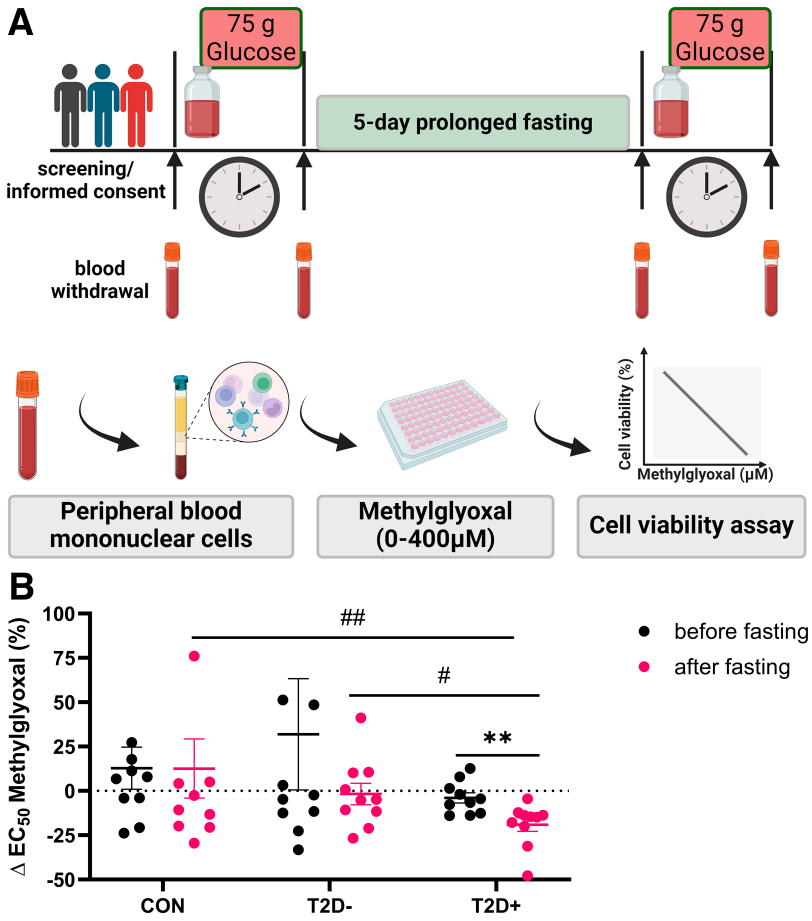

Figure 1—Study design and cellular resistance after glucose load. *A*) Study design. Created with BioRender.com. *B*) Cellular resistance to exogenous MG after glucose load. PBMC were isolated before and 2 h after glucose intake and incubated with increasing MG concentration (0–400 µmol/L) for 24 h. Cell viability was measured with a luminescent cell viability assay to determine change in EC_{50} levels for MG after glucose intake compared with those before glucose intake (ΔEC_{50}). Experiments were repeated after a 5-day prolonged fasting with an FMD. ΔEC_{50} values are shown as mean ± SEM of percentage change (n = 10; four females and six males in all study groups). $^{**}P < 0.01$ indicates significant difference of ΔEC_{50} for comparison within the study group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate significant difference of ΔEC_{50} for comparison between study groups.

for Adverse Events (v4.0), and laboratory results at each visit.

Primary and Secondary End Points

The main predictor of cellular resistance was the half-maximal effective concentration (EC_{50}) for MG measured in the PMBC with the luminescent assay CellTiter-Glo. EC_{50} value for MG represents the concentration of MG (micromolars), which reduces cell viability by 50% after 24 h of exposure to MG. EC_{50} value for each individual and condition was calculated from nonlinear regression fit analysis of normalized data (variable slope). The primary end point was within-group and between-group comparison of the change in EC_{50} levels (ΔEC_{50}) for MG of each individual after glucose load compared with before glucose load, before and after prolonged fasting. The secondary exploratory end points were within-group and between-group comparison of changes in circulating levels of dicarbonyls, in mRNA expression of markers of dicarbonyl detoxification (including glyoxalase-1 Glo1), mitochondrial oxidative stress (including glutathione-disulfide-reductase Gsr, Foxo family, glutathione peroxidases, peroxiredoxins and other peroxidases), mitochondrial biogenesis (including mitochondrial heat shock protein 70, HspA9), and protein expression of mitochondrial oxidative phosphorylation complexes after glucose load compared with before glucose load, before and after prolonged fasting.

Power Calculation

This study was an exploratory pilot study. Based on previous studies in yeast cells, exposure to low MG concentration and high glucose concentration led to a 28-fold increase in cell survival (20). Therefore, the study required a sample size of 10 patients per group to detect a mean difference among groups of 28-fold change in cell survival, assuming an SD of 15% for all groups with a two-sample t test, a two-sided significance level of α = 5%, a power of at least 90%, and a dropout rate of 20%. Sample size calculation was performed in G*Power (Version 3.1.9.7).

Blood Chemistry

Blood samples were drawn after overnight fasting and immediately processed in the Central Laboratory of the University Hospital of Heidelberg under standardized conditions. β-Hydroxybutyrate was measured at each visit in venous blood (StatStrip Glucose/Ketone Meter System, Nova Biomedical). Homeostatic model assessment indices for β-cell function (HOMA2-B), insulin resistance (HOMA2-IR), or insulin sensitivity (HOMA2-IS) were computed with fasting glucose and C-peptide levels in the participants that were not under insulin therapy.

Assessment of Cellular Viability Under MG Treatment

Directly after isolation, PBMC were seeded in a 96-well plate with RPMI medium with 10% FCS and exposed to increasing concentrations of MG (0–400 µmol/L) for 24 h at 37°C. After 24 h, a luminescent cell viability assay (CellTiter-Glo, Promega) was used, according to the manufacturer's protocol, to assess the number of viable cells.

Isolation of Plasma and PBMCs, Measurement of Dicarbonyls, RNA Isolation and Real-Time PCR, Preparation of Total Protein Extracts, Citrate Synthase Activity, Western Blotting, and ELISA

Detailed protocol is shown in the Supplementary Material.

Statistical Analysis

Descriptive data are shown as mean ± SD for normally distributed variables and median (interquartile range) for log-normally distributed variables. The EC_{50} value for each individual and condition was calculated from nonlinear regression fit analysis of normalized data (variable slope), and data are shown as mean ± SE of mean. Baseline characteristics (Table 1)

Table 1—Patient characteristics at baseline

	CON (n = 10)	T2D− (n = 10)	T2D+ (n = 10)	P
Age (years)	63.8 ± 7.3	63.8 ± 5.0	66.3 ± 6.6	0.398*
Sex, females/males	4/6	4/6	4/6	
BMI (kg/m^2)	28.8 ± 4.7	27.9 ± 3.2	29.0 ± 3.5	0.755*
WHR	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.055*
Diabetes duration (years)	—	13.4 ± 5.9	15.4 ± 4.1	0.563#
Diabetes complication, n (%)				
Nephropathy	—	—	4 (40)	
Neuropathy	—	—	6 (60)	
Retinopathy	—	—	1 (10)	
History of (%)				
Hypertension	50	90	90	
Coronary heart disease	20	—	10	
Myocardial infarction	10	20	10	
OSAS	10	—	10	
Arthrosis	30	40	20	
Thyroid nodules	20	10	30	
Thyroidectomy	—	10	—	
Diabetes therapy (%)				
Dietary measures	—	20	10	
Metformin	—	70	90	
DPP-4	—	20	—	
SGLT-2	—	30	20	
GLP-1 agonist	—	10	20	
Sulfonylureas	—	—	10	
Glinide	—	—	—	
Short-acting insulin	—	10	20	
Long-acting insulin	—	30	50	
Other medication (%)				
RAAS inhibitors	50	90	90	
β-blockers	30	20	30	
Thiazide diuretics	10	—	30	
Loop diuretics	—	—	—	
Calcium-antagonist	10	—	20	
Statin	30	70	40	
Ezetimibe	—	20	—	
Acetyl-salicylic acid	30	30	20	
Glycemic control				
HbA_{1c} (%)	5.5 ± 0.5	7.0 ± 1.30	7.4 ± 0.7	<0.0001* 0.868#
FPG (mg/dL)	93.6 ± 9.3	150.7 ± 31.0	149.4 ± 34.8	<0.0001* 0.645#
Blood pressure (mmHg)				
Systolic	145.9 ± 24.1	129.8 ± 20.2	145.9 ± 16.7	0.146*
Diastolic	88.9 ± 6.9	85.6 ± 15.2	89.4 ± 8.2	0.826*
Renal function				
Serum creatinine (mg/dL)	0.9 ± 0.2	0.8 ± 0.3	0.8 ± 0.2	0.403*
Cystatin C (mg/L)	1.1 ± 0.1	1.1 ± 0.3	1.2 ± 0.3	0.704*
eGFR CKD-EPI creatinine (mL/min/1.73 m^2)	84.0 ± 10.8	86.8 ± 17.1	89.6 ± 16.1	0.542*
eGFR from cystatin C (mL/min/1.73 m^2)	68.6 ± 10.4	71.7 ± 15.7	65.9 ± 16.8	0.708*
Albumin-to-creatinine ratio	4.0 (3.6)	5.1 (3.9)	47.8 (142.5)	0.001*
Liver transaminases (units/L)				<0.0001#
ALT	21.8 ± 7.1	27.9 ± 6.9	33.1 ± 17.8	0.120*
AST	23.2 ± 5.7	21.0 ± 5.2	26.7 ± 10.1	0.335*

Continued on p. 5

Table 1—Continued

	CON (n = 10)	T2D− (n = 10)	T2D+ (n = 10)	P
Serum lipids (mg/dL)				
Triglycerides	85.0 (31.5)	121.5 (122.3)	181.0 (159.0)	0.026*
				0.105#
Total cholesterol	193.1 ± 31.8	162.4 ± 46.3	165.8 ± 30.9	0.174*
HDL	67.0 ± 20.2	50.2 ± 11.3	41.5 ± 7.9	0.002*
				0.066#
LDL	108.1 ± 32.5	85.4 ± 41.2	89.4 ± 23.1	0.255*

Data are shown as mean ± SD for normally distributed variables, median (interquartile range) for log-normally distributed variables, or frequencies n (%) for categorical variables. When the difference among the three groups reached significance, comparison between T2D− and T2D+ was added. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; DPP-4, dipeptidyl peptidase 4; FPG, fasting plasma glucose; GLP-1, glucagon-like peptide-1; OSAS, obstructive sleep apnea syndrome; SGLT-2 sodium-glucose cotransporter 2; WHR, waist-to-hip ratio. *P values represent the difference among all three study groups. #P values represent the difference between T2D− and T2D+.

and metabolic and anthropometric parameters before and after prolonged fasting (Table 2) were compared among all three groups using Kruskal-Wallis test. When there was a significant difference among all three groups, Mann-Whitney test was used for the comparison between two groups. For primary and secondary end points, Wilcoxon matched-pair signed rank test was used for within-group comparison. Data for primary and secondary end points are presented as mean ± SE of

Table 2—Metabolic and anthropometric parameters before and after prolonged fasting

Parameter		CON (n = 10)	T2D− (n = 10)	T2D+ (n = 10)	P
Body weight (kg)	before	87.4 ± 3.8	83.7 ± 4.3	86.0 ± 3.9	0.876
	after	84.5 ± 3.6**	80.7 ± 4.1*	83.0 ± 3.8*	0.830
BMI (kg/m^2)	before	28.8 ± 1.5	27.9 ± 1.0	29.0 ± 1.1	0.803
	after	26.7 ± 1.3**	26.9 ± 1.0*	28.0 ± 1.1*	0.509
FPG (mg/dL)	before	93.7 ± 2.7	147.8 ± 9.5	145.7 ± 10.2	<0.0001
	after	86.6 ± 2.7*	118.8 ± 6.8*	125.4 ± 7.5	0.002
HbA_{1c} (%)	before	5.5 ± 0.2	7.0 ± 0.3	7.4 ± 0.2	<0.0001
	after	5.4 ± 0.2	6.8 ± 0.3	7.2 ± 0.2	<0.0001
2-h glucose (mg/dL)	before	90.8 ± 9.2	265.0 ± 25.7	285.6 ± 20.6	0.0001
	after	103.9 ± 7.1	204.8 ± 24.7	246.1 ± 15.6	<0.0001
HOMA-2IR	before	1.6 ± 0.2	2.4 ± 0.4	2.4 ± 0.4	0.188
	after	0.8 ± 0.1**	1.6 ± 0.4	1.6 ± 0.3**	0.019
HOMA-2B	before	117.1 ± 5.5	68.4 ± 13.0	66.3 ± 11.5	0.004
	after	85.2 ± 5.5**	70.7 ± 8.1	63.4 ± 5.9	0.0001
HOMA-2S	before	72.6 ± 10.3	62.8 ± 16.1	64.1 ± 18.0	0.188
	after	144.8 ± 20.2**	87.3 ± 15.7	81.8 ± 7.7**	0.666
LDL (mg/dL)	before	108.1 ± 10.3	85.4 ± 13.0	89.5 ± 7.3	0.255
	after	104.4 ± 11.8	84.1 ± 14.3	89.4 ± 8.6	0.368
HDL (mg/dL)	before	67.0 ± 6.4	50.2 ± 3.6	41.5 ± 2.5	0.002
	after	61.2 ± 5.9	44.9 ± 3.1	40.3 ± 2.2	0.009
TG (mg/dL)	before	90.2 ± 6.2	134.2 ± 27.1	195.7 ± 33.7	0.026
	after	81.6 ± 6.6	79.2 ± 7.1	112.9 ± 11.2*	0.039
Albumin-to-creatinine ratio (mg/g)	before	4.9 ± 1.1	7.1 ± 1.6	106.4 ± 49.9	0.001
	after	12.2 ± 4.3	8.9 ± 2.1	49.1 ± 32.9*	0.081
Blood ketones (mmol/L)	before	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.116
	after	0.7 ± 0.2**	0.6 ± 0.2*	0.5 ± 0.1*	0.486

Data are shown as mean ± SEM. HOMA values are computed with fasting glucose and C-peptide levels in the participants that were not under insulin therapy (CON n = 10, T2D− n = 7, and T2D+ n = 5). P values indicate significant difference of parameter among all study groups. *$P < 0.05$, **$P < 0.01$ indicates significant difference of parameters between baseline and after 5 days prolonged within the study group. FPG, fasting plasma glucose; HOMA-2IR, homeostatic model assessment indices for insulin resistance; HOMA-2B, homeostatic model assessment indices for β-cell function; HOMA-2S, homeostatic model assessment indices for insulin sensitivity; TG, triglycerides.

mean, unless otherwise stated. Because of the exploratory nature of the analyses, P value <0.05 was considered statistically significant and was not adjusted for multiplicity. All statistical data analyses and visualization were performed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA).

RESULTS

Study Participants

Thirty participants (18 male/12 female, age 64.6 [62.2, 67.0] y, BMI 28.6 [27.2, 30.0] kg/m^2) were included. HbA_{1c} was higher in T2D+ (7.4 [6.9, 7.9] %) and T2D− (7.0 [6.2, 7.7] %) as compared with CON (5.5 [5.1, 5.8] %, $P < 0.001$), while age, sex distribution, and BMI were comparable among study groups. Participants of the CON group were slightly hypertensive, with 50% of them receiving RAAS inhibitors compared with 90% of the participants in both diabetes groups. However, blood pressure values did not differ among study groups. All participants completed the study and were nonsmokers. Baseline anthropometric and metabolic measures are shown in Table 1. Study flow diagram is shown in Supplementary Fig. 1.

Metabolic and Anthropometric Changes After Prolonged Fasting

The period of prolonged fasting was well tolerated, and all participants completed the diet. The self-reported grade 1 (mild) experienced symptoms were fatigue (one participant in CON), headache (two participants in CON), flu-like body pain (one participant in T2D−), vertigo (one participants in T2D−), and nausea (one participants in CON) (Supplementary Fig. 2). One participant in CON showed, after prolonged fasting, a mild hyponatremia of 128 mmol/L (grade 2), and one participant in T2D+ showed an episode of mild hypoglycemia of 58 mg/dL (grade 1) after prolonged fasting, which did not lead to study discontinuation.

All participants showed a reduction of body weight after prolonged fasting compared with baseline (CON: −3.2 ± 0.3%, $P < 0.01$; T2D−: −3.5 ± 0.4%, $P < 0.05$; T2D+: −3.5 ± 0.31%, $P = 0.01$) and an increase in the blood ketones (CON: 711.1 ± 217.9%, $P < 0.01$; T2D−: 401.1 ± 158.8%, $P < 0.05$; T2D+: 345.0 ± 159.8%, $P < 0.05$). Change in weight loss and in increase of blood ketones after prolonged fasting did not differ among study groups (respectively, $P = 0.78$; $P = 0.12$) (Table 2).

Fasting plasma glucose decreased significantly after prolonged fasting in CON (−7.5 ± 1.7%, $P < 0.05$), in T2D− (−17.8 ± 5.2%, $P < 0.05$), and in T2D+ (−12.7 ± 5.0%, $P < 0.05$). Plasma 2-h glucose decreased in T2D− (−19.7 ± 9.4%, $P < 0.05$) and in T2D+ (−12.1 ± 4.7%, $P < 0.05$) and did not change after prolonged fasting in CON (22.3 ± 13.6%, $P = 0.426$). Insulin resistance decreased in CON and T2D+ (HOMA-2IR: CON: −46.7 ± 4.9%, $P < 0.01$; T2D+: −25.8 ± 11.4%, $P < 0.01$). β-Cell function decreased in CON (HOMA-2B: −26.6 ± 5.1%, $P < 0.01$). Insulin sensitivity increased in CON and T2D+ (HOMA-2S: CON: 104.4 ± 18.5%, $P < 0.01$; T2D+: 43.3 ± 11.2%, $P < 0.01$). Change in fasting plasma glucose, 2-h plasma glucose, homeostatic model assessment indices for insulins resistance, β-cell function, and insulin sensitivity after prolonged fasting did not differ among the three study groups (Table 2). Albumin-to-creatinine-ratio decreased in T2D+ after prolonged fasting (−36.5 ± 15.4%, $P < 0.05$). Lipid profile did not change after prolonged fasting in all study groups (Table 2).

Cellular Resistance to Exogenous MG After Glucose Load

We investigated the change of cellular resistance to exogeneous MG (ΔEC_{50}) of PBMC after glucose load before and after prolonged fasting. T2D+ showed a decreased cellular resistance against MG, while cellular resistance was unchanged in T2D− and improved in CON after glucose load and prolonged fasting (−19.0% vs.−1.7% vs. 12.6%; all $P = 0.017$) (Fig. 1*B*). Moreover, in T2D+, ΔEC_{50} of MG by glucose load was reduced after prolonged fasting as compared with before prolonged fasting (−3.9% vs.−19.0%, $P < 0.01$). In CON, ΔEC_{50} of MG by glucose load increased similarly before and after prolonged fasting (12.9% vs. 12.6%, $P = 0.922$). In T2D−, ΔEC_{50} of MG was unchanged by glucose load before and after prolonged fasting (1.8% vs.−1.7%, $P = 0.193$) (Fig. 1*B*). Full dose-toxicity curves/analysis for MG before and after glucose and before and after prolonged fasting are shown in Supplementary Fig. 3 and Supplementary Table 3.

Dicarbonyls and Markers of Dicarbonyl Detoxification, Oxidative Stress, and Mitochondrial Biogenesis

To investigate potential explanatory mechanisms for the decreased cellular resistance to exogenous MG in T2D+ after glucose load following prolonged fasting, we first investigated changes in plasma levels of dicarbonyls. Although absolute values of glyoxal and 3−Deoxyglucosone (3DG) increased after glucose load, before and after fasting in T2D− and T2D+ as compared with CON, we found no change in glyoxal, MG, 3DG, and dimethylglyoxal among study groups after glucose load before and after prolonged fasting. Absolute values of MG increased after glucose load in T2D− and T2D+ compared with CON only before fasting. Change in 3DG after glucose load increased significantly only in CON participants after prolonged fasting compared with before fasting (−4,4% vs. 21,5%, $P = 0.006$) (Supplementary Table 2).

Next, we performed qPCR analysis for several markers of dicarbonyl detoxification, oxidative defenses, and mitochondrial biogenesis in the isolated PBMC. We found no significant change among the study groups after glucose load and before prolonged fasting (Fig. 2, left panel). We found an increase by 26.9% and by 26.0% in dicarbonyl detoxification (mRNA Glo1) in T2D+ before glucose load and after prolonged fasting as compared with CON and T2D−, respectively (CON: 0.94 ± 0.04 vs. T2D+: 1.19 ± 0.14, $P = 0.024$; T2D−: 0.95 ± 0.03 vs. T2D+, $P = 0.029$) (Fig. 2, middle panel, and Supplementary Fig. 4*A*). This increase continued even after glucose load following prolonged fasting (T2D+: 1.15 ± 0.08 vs. CON: 0.92 ± 0.04, $P = 0.039$) (Fig. 2, right panel, and Supplementary Fig. 4*A*). We observed a 27.6% increase in oxidative stress (mRNA Gsr) in T2D+ after glucose load following prolonged fasting as compared with CON (1.36 ± 0.12 vs. 1.10 ± 0.05, $P = 0.006$) (Fig. 2, right panel, and Supplementary Fig. 4*B*). Before glucose load and after prolonged fasting, we found an increase in oxidative stress (mRNA Foxo4) by 42% in T2D− as compared with CON (1.70 ± 0.20 vs. 1.20 ± 0.11, $P = 0.036$) (Fig. 2, middle panel, and Supplementary Fig. 4*C*), and mRNA Gpx2 increased in T2D− (2.63 ± 0.73) compared with CON (1.18

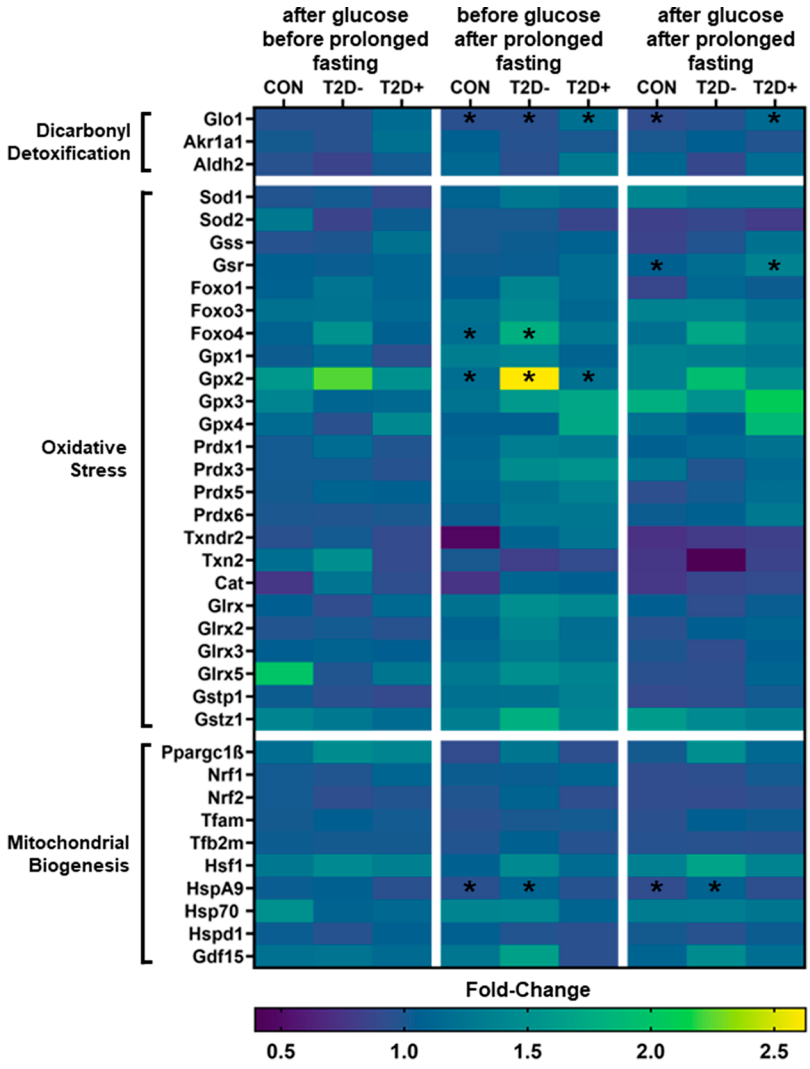

Figure 2—Changes in mRNA expression of markers for dicarbonyl detoxification, oxidative stress, and mitochondrial biogenesis in the isolated PBMC. Heat map analysis of change in mRNA expression fold change of markers for dicarbonyl detoxification, oxidative stress, and mitochondrial biogenesis in isolated PBMC 2 h after glucose intake before and after prolonged fasting compared with before glucose intake and before prolonged fasting. Baseline values of mRNA expression (before glucose intake and before prolonged fasting) are used as reference to calculate the fold change values. Data are shown as mean with SEM, $n = 9$ (three females and six males) in CON, $n = 10$ (four females and six males) in T2D−, and $n = 9$ in T2D+ (four females and five males) for Hsf1, Foxo1, Foxo3, Foxo4, Sod1, Gpx1, Gpx2, Gpx3, Gpx4, Prdx1, Prdx3, Prdx5, Prdx6, Txndr2, Txn2, Cat, Glrx, Glrx2, Glrx3, Glrx5, Gstp1, and Gstz1; $n = 10$ (four females and six males) in all study groups for the rest of the markers. *$P < 0.05$ indicates significant difference for comparison between study groups. Akr1a1, aldo-keto reductase family 1 member A1; Aldh2, aldehyde dehydrogenase; Cat, catalase; Foxo1, forkhead box O1; Foxo3, forkhead box O3; Foxo4, forkhead box O4; Gdf15, growth differentiation factor 15; Glo1, glyoxalase 1; Glrx, glutaredoxin; Glrx2, glutaredoxin 2; Glrx3, glutaredoxin 3; Glrx5, glutaredoxin 5; Gpx1, glutathione peroxidase 1; Gpx2, glutathione peroxidase 2; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Gsr, glutathione-disulfide reductase; Gss, glutathione synthetase; Gstp1, glutathione *S*-transferase π 1; Gstz1, glutathione *S*-transferase ζ; Hsp70, heat shock protein 70; HspA9, mitochondrial heat shock protein 70; Hspd1, mitochondrial heat shock protein family member D1; Nrf1, nuclear respiratory factor 1; Nrf2, nuclear respiratory factor 2; Ppargc1ß, peroxisome proliferator-activated receptors-γ coactivator-1 β; Prdx1, peroxiredoxin 1; Prdx3, peroxiredoxin 3; Prdx5, peroxiredoxin 5; Prdx6, peroxiredoxin 6; Sod1, superoxide dismutase 1; Sod2, superoxide dismutase 2; Tfam, mitochondrial transcription factor A; Tfb2m, mitochondrial transcription factor B2; Txn2, thioredoxin 2; Txndr2, thioredoxin reductase 2; 1Hsf1, heat shock transcription factor 1.

± 0.17, $P = 0.034$) and T2D+ (1.22 ± 0.22, $P = 0,04$) (Fig. 2, middle panel, and Supplementary Fig. 4*D*). Furthermore, mRNA HspA9 was increased by 18.7% in T2D− as compared with CON before glucose and after prolonged fasting (1.12 ± 0.09 vs. 0.94 ± 0.04, $P = 0.0387$) and by 19.8% after glucose load following prolonged fasting (1.09 ± 0.06 vs. 0.91 ± 0.04, $P = 0.0324$) but did not change in T2D+ (Fig. 2, middle and right panels, and Supplementary Fig. 4*E*).

Citrate synthase activity did not change among study groups after glucose load, before and after prolonged fasting (Supplementary Fig. 5). Protein expression analysis of the mitochondrial complexes showed an increase in complex V after glucose load following prolonged fasting in T2D+ (11.6 ± 5.6) compared with CON (0.60 ± 0.17, $P = 0.0030$) and with T2D− (1.3 ± 0.3, $P = 0.0029$) (Supplementary Fig. 6*A*). No change was found among study groups for complexes II, IV, and I (Supplementary Fig. 6*B–D*). Complex III was not detectable in any of the samples. Representative western blotting images are shown in Supplementary Fig. 6*E*. Samples that showed signs of overall protein degradation were excluded from the analysis: $n = 3$ (2/1 female/male) in CON, $n = 1$ (female) in T2D−, and $n = 2$ (male) in T2D (data not shown).

We analyzed serum circulating levels of the stress-responsive cytokines FGF21 and GDF15, and Hsp70 as marker for mitochondrial biogenesis. FGF21 increased in CON after prolonged fasting as compared with before prolonged fasting (7.7% vs. 115.0%, $P < 0.05$) (Supplementary Fig. 7*A*), whereas no change was observed in T2Dv and T2D+ (115.0% vs. 69.3% vs. 47.5%, $P = 0.5$). Moreover, we found no change in circulating GDF15 and Hsp70 after glucose load and after prolonged fasting in all study groups (Supplementary Fig. 7*A*). The change in FGF21 after glucose load following prolonged fasting strongly correlated with the change in β-hydroxybutyrate only in T2D+ ($r = 0.87$, $P = 0.001$) (Supplementary Fig. 7*B*). No correlation was found between change in circulating GDF15 and Hsp70 and change in β-hydroxybutyrate (data not shown).

CONCLUSIONS

This study found that 75-g glucose intake alone does not affect PBMC cellular resistance to dicarbonyl stress in either glucose-tolerant individuals or individuals with T2D. However, after prolonged fasting, T2D individuals with diabetes complications showed reduced resistance to dicarbonyl stress after glucose load, while resistance remained unchanged in those without complications and increased in

glucose-tolerant individuals. Additionally, dicarbonyl detoxification and oxidative stress response increased in T2D individuals with complications but not in those without complications or in glucose-tolerant individuals. This suggests increased susceptibility to glucose-induced oxidative stress after prolonged fasting in T2D individuals with diabetes complications.

In this study, we explored the impact of glucose load on PBMC cellular resistance against ex vivo exposure to MG. PBMC, being metabolically active and responding to insulin akin to skeletal muscle, are used as a proxy tissue for studying glucose metabolism (31,32). While glucose-tolerant individuals exhibit normal glucose disposal, those with T2D face metabolic challenges during glucose load due to increased endogenous glucose production and reduced skeletal muscle glucose utilization (15). This leads to enhanced glucose availability and altered glucose transport in other peripheral tissues, including PBMC (33,34), potentially explaining the reduced PBMC cellular resistance against dicarbonyl stress after glucose load. Moreover, increased substrate flux may enhance PBMC antioxidative capacity, as has been observed in the liver of obese individuals, with and without steatosis, which is subsequently lost with progression to steatohepatitis (35). Cell-cell interaction between PBMC and other peripheral tissues could also influence PBMC cellular resistance. Glucose load following prolonged fasting did not affect markers of insulin resistance and sensitivity markers, suggesting involvement of insulin-independent mechanisms in the glucose-induced response after prolonged fasting.

Of note, it was only after prolonged fasting that the cellular resistance against dicarbonyl stress after glucose load differed between glucose-tolerant individuals and T2D individuals. This resistance after glucose load following prolonged fasting may represent an evolutionary adaptation in healthy individuals, facilitating a safe metabolic transition between fasting and refeeding. Caloric restriction increases lifespan in various organisms and protects against age-related diseases in rhesus monkeys and humans (36). However, in T2D individuals without complications, this protective response is lost, and those with complications experience even more harmful effects, likely due to reduced dicarbonyl detoxification (37). Previous findings have demonstrated a hormetic response against dicarbonyl stress under high-glucose conditions in yeast and mammalian cells (20). While assessing mitohormesis in more complex organisms remains challenging, the results of this study associate the loss of hormetic response with diabetes complications in T2D individuals. Notably, both glucose load and prolonged fasting were necessary metabolic challenges to discern differences in hormetic response, highlighting the complexity of hormesis as a significant disease modulator in humans (19).

The increase in Glo1, and consequently MG detoxification, following glucose load and prolonged fasting in T2D individuals with complications aligns with our previous findings of prolonged fasting in T2D individuals with nephropathy, where we observed a significant decrease in MG-derived hydroimidazolone, likely due to increased detoxification (10). Enhanced MG detoxification is also supported by increased plasma MG after glucose load but decreased levels following prolonged fasting in individuals with T2D and complications. Although the MG levels in the CON group after fasting were higher overall, statistical significance was not reached. MG can form also from acetone conversion during ketogenesis (38), offering a new detoxification pathway via acetoacetate buffering (39). MG metabolism during fasting-related pathways like gluconeogenesis and ketogenesis therefore warrants further investigation. While the mild changes in Glo1 expression are comparable to changes using Glo1 inducers reported in overweight and obese subjects (40), caution is needed in interpreting these results, as they cannot impact clinical decision making.

This study primarily addressed dicarbonyl stress based on prior findings of hormetic response against MG and the link between glucose load and increased endogenous MG production. Glucose-induced changes after prolonged fasting were not observed in PBMC resistance against ex vivo exposure to H_2O_2 (Supplementary Fig. 8), suggesting specific mechanisms for MG and dicarbonyl stress.

Enhanced oxidative stress, indicated by the increase in Foxo4 and Gpx2 expression before glucose and after prolonged fasting, appears to protect T2D individuals without complications, as cellular resistance was not reduced. Moreover, increase in HspA9 expression before and after glucose following prolonged fasting in these individuals might indicate enhanced mitochondrial biogenesis. Additionally, altered Gsr in T2D individuals with complications after glucose load suggests the involvement of distinct mechanisms in glucose-induced oxidative stress following prolonged fasting. These changes cannot be explained by alterations in mitochondrial content, since citrate synthase activity was comparable among study groups.

The current study benefits from a well-controlled ex vivo experimental setting and well-matched participants. However, it has also limitations. Firstly, the study relied on previous in vitro experimental data on yeast and mammalian cells because of the absence of similar studies in mice or humans. Secondly, limited participants' material restricted the analysis of dicarbonyl detoxification and oxidative stress response through mRNA expression, lacking validation on protein expression and function. Despite analyzing mitochondria content and oxidative phosphorylation, caution is warranted, as we only analyzed isolated PBMC and lacked functional analysis on mitochondrial respiration. Complex III was not detectable in any of the samples, as this mitochondrial complex is known to be thermally unstable. A few samples showed sign of protein degradation and were excluded from the analysis. Additionally, the exploratory study's analysis was not corrected for multiple comparison. While PBMC are commonly used as a proxy tissue to study glucose metabolism, future research should explore glucose-induced responses in more metabolically relevant tissues, such as skeletal muscle. Acknowledging the small sample size, future studies should aim to validate findings in larger cohorts and using other fasting regimens to improve generalizability and practicability.

In conclusion, based on the participants' material and the methods used in this study, glucose load after prolonged fasting in individuals with T2D and diabetes complications leads to *1*) decreased cellular resistance against dicarbonyl stress, *2*) increased dicarbonyl detoxification, and *3*) increased oxidative stress response. These findings underscore the vulnerability of individuals with T2D and complications to glucose-induced cellular stress after prolonged fasting and highlight the importance

of considering such effects when designing dietary interventions for diabetes management.

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