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Abbreviations

BMI	Body mass index
CV	Coefficient of variation
CVD	Cardiovascular disease
ECAR	Extracellular acidification rate
HbA_{1c}	Glycated hemoglobin
hs-CRP	High sensitive C-reactive Protein
IL	Interleukin
LPS	Lipopolysaccharide
MACS	Magnetic Activated Cell Sorting
OCR	Oxygen Consumption Rate
P3C	Pam3Cys
PBMC	Peripheral Blood Mononuclear Cells
TNF	Tumor Necrosis Factor

Abstract

Iatrogenic hypoglycaemia activates the immune system and is associated with an increased risk for atherosclerotic disease. We determined acute and long-term effects of insulin-induced hypoglycemia on inflammatory markers in humans with or without type 2 diabetes. Fifteen adults with type 2 diabetes and 16 matched controls (M/F 17/14, age 59.6±7.1 years, BMI 28.5±4.3 kg/m^2) underwent a hyperinsulinemic-euglycemic (5.31±0.32 mmol/L) hypoglycemic (2.80±0.12 mmol/L) glucose clamp. Blood was drawn during euglycemia and hypoglycemia and 1, 3 and 7 days later, to determine circulating immune cell composition, function, and inflammatory proteins. In response to hypoglycemia, absolute numbers of circulating lymphocytes and monocytes significantly increased and remained elevated for one week. The proportion of CD16$^+$-monocytes increased, and the proportion of CD14$^+$-monocytes decreased, which sustained for a week in people without diabetes. During hypoglycemia, ex vivo stimulated, monocytes released more TNF-α and IL-1β, and less IL-10, particularly in people with diabetes. Hs-CRP and 25 circulating inflammatory proteins increased, remaining significantly elevated one week after hypoglycemia. While levels at euglycemia differed, responses to hypoglycemia were broadly similar in people with or without type 2 diabetes. We conclude that hypoglycemia induces a pro-inflammatory response at the cellular and protein level that is sustained for one week in people with type 2 diabetes and controls.

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Key words: Hypoglycemia, clamp, T2DM, diabetes, inflammatory, atherosclerosis

Introduction

Type 2 diabetes is associated with the development of a wide range of microvascular complications and a 2-4 fold greater risk of cardiovascular disease (CVD), particularly in those with a more advanced stage of disease [1-3]. However, while intensive glucose lowering treatment, including insulin, has been shown to reduce the risk of microvascular complications [4], such an approach does not seem to exert similar benefits with respect to reducing macrovascular complications [5]. Some studies even reported harmful cardiovascular effects of intensive glucose lowering treament [6, 7]. It has been suggested that hypoglycemia, the most common complication of insulin treatment, explains these observations. Indeed, both International Hypoglycemia Study Group (IHSG) level 2 (glucose < 3.0 mmol/L) and level 3 (severe) hypoglycemia have been associated with increased risks of cardiovascular events in people with type 2 diabetes [8-11].

Chronic low-grade inflammation is crucial in the development of atherosclerosis, the main underlying cause of cardiovascular disease [12]. People with type 2 diabetes are characterized by a chronic pro-inflammatory state [13]. In people with type 1 diabetes and those without diabetes, we and others have shown that hypoglycemia evokes an acute immune response, illustrated by an increase in circulating monocytes and a switch in monocyte phenotype from classical ($CD14^+CD16^-$) towards non-classical ($CD14^-CD16^+$) monocytes, that are more pro-inflammatory and pro-atherogenic [14-17]. Alterations in monocyte metabolism including a switching form mitochondrial oxidative phosphorylation to glycolysis, can contribute to a more pro-inflammatory phenotype [18]. Hypoglycemia has also been shown to increase several circulating inflammatory mediators (hs-CRP) and atherogenic markers, including ICAM-1, VCAM-1, and E-selectin [16, 19].

It is currently unknown whether hypoglycemia exerts similar pro-inflammatory effects in people with type 2 diabetes who already have an activated immune system, nor how long such an effect persists after recovery from hypoglycemia. Therefore, we set out to investigate in great detail the inflammatory responses to experimental hypoglycemia for up to 7 days after the event in participants with type 2 diabetes on insulin treatment and healthy individuals without diabetes.

Research design and Methods

Study approval

This was a multi-center intervention study that was performed at the University Hospital Nordsjællands Hospital in Hillerød, Denmark and the Radboud University Medical Center in Nijmegen, The Netherlands. The study was approved by the local institutional review boards of both centers and performed according to the principles of the Declaration of Helsinki. All participants gave written informed consent prior to inclusion to the study.

Study design

Participants

We recruited people with type 2 diabetes treated with insulin for at least one year from the outpatient clinics of Internal Medicine in Nordsjællands Hospital, Hillerød in Denmark and Radboud University Medical Center in Nijmegen between August 2019 and March 2021. Healthy volunteers without diabetes, matched for age, sex and BMI with people with type 2 diabetes were also recruited. All participants were potentially eligible when they were aged between 18 and 80 years, had a BMI of 19-40 kg/m^2 and had normal blood pressure (< 140/90 mmHg) with or without treatment (ESM Methods). In addition, people with type 2 diabetes had to be on insulin treatment for at least one year and to have an HbA_{1c} below 11.3% (100 mmol/mol), while healthy controls had to have an HbA_{1c} below 42 mmol/mol (6%). Exclusion criteria were pregnancy, breastfeeding or unwillingness to undertake measures for birth control and use of immune-modifying drugs, antibiotics or statins, anti-depressive drugs, auto-inflammatory or auto-immune diseases, and infection or vaccination in the previous three months (ESM Methods). People with severe medical conditions or psychiatric disorders interfering with the perception of hypoglycemia, derived from medical records review or as judged by the treating physician, were also excluded.

Study procedure

All potentially eligible study participants were invited for a medical screening, including medical history and standard physical examination. HbA_{1c} and kidney function (serum creatinine) were determined if this had not been done in the past three months before screening.

Hyperinsulinemic euglycemic-hypoglycemic glucose clamp

On the experimental day, all subjects underwent a hyperinsulinemic euglycemic-hypoglycemic glucose clamp. Subjects were asked to attend the research facility in fasting condition at 0800h, having abstained from alcohol, caffeine-containing substances and smoking for at least 24 hours, and from strenuous exercise for 48 hours. Participants with diabetes received instructions to avoid (nocturnal) hypoglycemia the day before the clamp by reducing the basal insulin dose and to omit their usual morning insulin dose. Experiments were rescheduled in case of hypoglycemia (below 3.0 mmol/L) 24 hours before the clamp. Participants with diabetes received an intermittently scanned Continuous Glucose Monitoring (isCGM) device (Freestyle Libre 1®) for two weeks, starting 7 days prior to and remaining in place until 7 days after the experimental day. Upon arrival, an intravenous catheter was inserted into an antecubital vein of one arm, for administration of insulin (insulin aspart; Novo Nordisk, Bagsværd, Denmark) at a rate of 3.0 $mU \cdot kg^{-1} \cdot min^{-1}$ and a variable administration of glucose 20% (Baxter B.V., Deerfield, IL). In the dorsal vein of the contralateral hand, a second catheter was inserted in retrograde fashion for frequent blood sampling. The hand with the catheter for blood sampling was placed in a heated box (temperature ~55° C) to arterialize venous blood. Baseline plasma glucose levels were determined (Biosen C-Line; EKF Diagnostics, Cardiff, U.K.). Plasma glucose levels were determined at 5-min intervals to inform the amount of glucose needed to maintain glucose at predetermined levels. After 30 minutes of stable euglycemia, plasma glucose levels were allowed to drop gradually to 2.8 mmol/L and then maintained at this level for 60 minutes.

Thereafter, the insulin infusion was stopped and the glucose infusion was increased and then tapered until stable euglycemic levels were reached.

At baseline (i.e., prior to the insulin infusion) and end of hypoglycemia, blood was sampled for measurements of insulin and counterregulatory hormones (glucagon, adrenaline, noradrenaline, cortisol and growth hormone). For analyses of inflammatory parameters, blood was drawn at the end of euglycemia and hypoglycemia, and 1 day, 3 days and 1 week after hypoglycemia. For the blood drawings on days 1, 3 and 7 after the clamp, participants came to the research facility in fasting condition between 0800 and 0900h.

Measurements

Serum and plasma measurements

Serum creatinine was determined with an enzymatic assay on a Cobas 8000 c702 (Roche Diagnostics). HbA_{1c} was assessed by the TOSOH G8 and G11 HPLC-analyzer (Sysmex). Plasma adrenaline and noradrenaline were measured by HPLC in combination with fluorometric detection. Plasma insulin was analyzed with an in-house radioimmunoassay. Plasma cortisol and growth hormone were determined by a routine analysis method with an electrochemiluminescent immunoassay on a Modular Analytics E170 (Roche Diagnostics, GmbH, Mannheim, Germany). Plasma high sensitive C-reactive Protein (hs-CRP) concentrations were assessed by ELISA following manufacturer's instructions (R&D Duoset ELISA Systems). Plasma samples were used for the 92 inflammatory-related protein biomarker panel and 4 controls with Olink Proteomics (Uppsala, Sweden). Plasma samples for Olink were kept at -80°C until measurement. All samples were measured in one batch. Circulating plasma inflammatory proteins were measured using the commercially available Olink Proteomics AB Inflammation Panel (92 inflammatory proteins) (Uppsala, Sweden). Proteins are recognized by antibody pairs coupled to cDNA strands, which bind in close proximity and extend by a

polymerase reaction [20]. A threshold of 75% was used, and proteins were excluded from analysis when the threshold was not met. Quality control was performed by Olink Proteomics, which resulted in the exclusion of 3 samples. Overall, 76 of the 92 (83%) proteins were detected in at least 75% of the plasma samples and included in the analysis.

Flow cytometry

Immune cell subset numbers were calculated based on cell numbers from whole blood differences measured on a Sysmex XN-450 and Sysmex XN-9000 (Sysmex). FACS analysis was performed in one of the two participating study sites, because this method is too sensitive for confounders when performed at different sites. A total of 50µl of whole undiluted blood was incubated for a duration of 15 minutes in the dark at room temperature with the following antibodies: CD16-FITC (dilution 1:20), CD14-PC7 (1:20), CCR2-BV421 (1:20) (BD Biosciences, Vianen, the Netherlands); CD41-PC5.5 (1:20), CD11b-BV785 (1:20) (ITK Diagnostics BV, Uithoorn, the Netherlands); HLA-DR-PE (1:10), CD56-APC (1:10), CD3-APC-750 (1:10), CD45-KO (1:10), CD36-APC-700 (1:10) (Beckman Coulter, Woerden, the Netherlands). Subsequently, 1 ml of lysis buffer (BD Pharm Lyse, BD Biosciences) was added, samples were mixed, incubated for another 10 minutes and then measured on a flow cytometer (Beckman Coulter FC500). To determine the position of analysis gates, single staining and fluorescence-minus-one control stains were used. Percentages were measured with flow cytometry. To analyze the flow cytometry data, Kaluza software (Beckman, Coulter) was used.

Isolation of PBMC's and Monocytes

The peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density centrifugation over Ficoll-Paque (GE Healthcare, UK). From PBMCs, monocytes were isolated using magnetic activated cell sorting (MACS) MicroBeads (Miltenyi Biotec) for CD14 negative

selection according to the manufacturer's instructions. The purity of monocyte isolation was checked using Sysmex XN-450 and Sysmex XN-9000.

Monocyte stimulation

CD14 negative selected human monocytes (100.000 cells/well) were added to flat bottom 96-wells plates and stimulated with RPMI, 20 µg/mL of Pam3Cys (P3C), 20 ng/mL of lipopolysaccharide (LPS) from *Escherichia coli*, 2 mio of *Candida albicans*, 2 mio of *Staphylococcus aureus (S. aureus)*, 5 µg/mL of *Mycobacteria tuberculosis* (mTBC) lysate for 24 hours. The next day the supernatants were collected and stored at −20°C until cytokine measurement. The production of tumor necrosis factor-α (TNF-α) (R&D), interleukin-10 (IL-10) (R&D), interleukin 1β (IL-1β) (R&D) and Interleukin-6 (IL-6) (R&D) in supernatants determined by ELISA.

Real time mitochondrial respiration and glycolytic rate

Mitochondrial respiration and glycolytic rate were determined in 6 participants with type 2 diabetes and 6 controls. Directly after isolation, the negatively selected monocytes were seeded in quintuple in XF96 microplates (Agilent technologies, Amstelveen, The Netherlands; 200.000 cells per well) in RPMI 1640 medium and left to adhere for 30-45 min at 37°C, 5% CO_2. Next, the RPMI medium was replaced by non-buffered DMEM medium without glucose, supplemented with 1 of 2 mM L-glutamine for either the glyco-or mitostress test. The cells were kept in this medium for 45-60min in a CO_2 free incubator at 37°C. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were using a Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies). Three tests were performed to obtain a complete view of the monocyte metabolism: acute P3C stimulation, the glyco-and mitostress test. Glucose (11 mM) was the first injection, followed by P3C (10 ug/mL) during the acute P3C stimulation. During the glycostress-test, the glucose injection was followed by oligomycin

(1uM) and 2DG (22mM). Lastly, glucose was followed by oligomycin (1 uM), FCCP (1 uM) with pyruvate (1mM), and Rotenone and Antimycin A (1.25 uM and 2.5 uM) for the Mitostress test. Dotted lines in figure 4 indicate injection moments. Each condition was performed in quintuple, a well was excluded in case of a negative OCR value or a deviation of more than 2 SD's from the mean of all 5 wells, a minimum of 3 wells was required to keep the timepoint.

Statistics

All normally distributed data are shown as percentages or mean ± SD, unless otherwise indicated. All skewed distributed data were log transformed before analyzing. Independent t-tests were used for comparisons between the two participant groups. The serial data were analyzed with linear mixed models. In the mixed models analysis, the dependent variable was the result of the measured parameter of each timepoint and the independent parameter was "time". Next to "time", "participant group" was added as independent variable in the mixed models analysis to compare serial data between participants with type 2 diabetes and controls. Analysis and visualization of the Olink data were done using the R programming language and R packages "ggbiplot" and "ggplot2". A Wilcoxon matched-pairs test was performed to determine proteins that were significantly affected per timepoint compated to euglycemia. A Wilcoxon rank sum test was used to compare subgroups. Subjects with missing values were excluded from Olink analyses. Statistical analyses were performed using IBM SPSS Statistics 27 or R Studio (Version 1.4.1717). Alpha was set at 0.05 throughout, unless otherwise stated.

Results

A total of 15 participants with insulin-treated type 2 diabetes and 16 control participants without diabetes, well matched for age, sex and body-mass index (BMI), participated in the study (Table 1). All participants underwent a hyperinsulinemic 30-min euglycemic- 60-min hypoglycemic glucose clamp. The mean baseline glucose levels were higher in people with type 2 diabetes compared to the controls (9.63±4.71 vs 5.89±0.46 mmol/L, p = 0.008*).* Plasma glucose values were maintained at 5.31±0.32 mmol/L (coefficient of variation (CV), 4.73±0.78%) and 5.32±0.33 mmol/L (CV 5.69±0.49%) during the euglycemic phase of the clamp in people with type 2 diabetes and controls, respectively. During the hypoglycemic phase, glucose levels in these two groups averaged 2.85±0.15 mmol/L (CV 6.27±0.89%) and 2.75±0.06 mmol/L (CV 6.19±0.50%, p=0.029, ESM Figure 1), respectively. During the euglycemic phase, the mean GIR was numerically, but not significantly, lower in people with type 2 diabetes compared to controls (3.5±1.8 vs 5.0±2.7 $mg \cdot min^{-1} \cdot kg^{-1}$, p=0.086), whereas during the hypoglycemic phase, the mean GIR was significantly lower in people with type 2 diabetes (1.8±1.1 vs 3.8±1.2 mg/min/kg, p<0.001). Plasma adrenaline increased from 0.19±0.13 nmol/L and 0.19±0.16 nmol/L at baseline to 3.55±2.86 nmol/L and 2.91±1.74 nmol/L at the end of hypoglycemia (both p<0.001) in participants with type 2 diabetes and controls, respectively, with no significant differences between the groups.

During euglycemia, absolute levels of granulocytes and monocytes, but not lymphocytes, were higher in people with type 2 diabetes compared to controls (Figure 1). The relative increases of granulocytes, lymphocytes and monocytes induced by hypoglycemia were similar between people with or without type 2 diabetes (ESM Figure 2). The hypoglycemia-induced increase in granulocytes, lymphocytes and monocytes correlated significantly with the increase of adrenaline (ESM Table 1). In people with type 2 diabetes, granulocyte counts fell below euglycemic levels after one day, before normalizing one week after the hypoglycemic event. In

controls, granulocyte counts normalized after one day. In contrast, the levels of lymphocytes and monocytes remained elevated for up to 3 days in people with type 2 diabetes and for up to one week in controls (Figure 1, ESM Figure 2).

We subsequently performed detailed monocyte phenotyping in whole blood in 8 participants with type 2 diabetes and 6 controls. During euglycemia, monocyte phenotype did not differ between participants with or without type 2 diabetes. Hypoglycemia increased the proportion of (pro-inflammatory) $CD16^+$ monocytes and reduced the proportion of (anti-inflammatory) $CD14^+$ monocytes. Although this shift occurred in both groups to similar extent, the timing was different. The shift was only apparent during the acute phase of hypoglycemia in the participants with diabetes, while the shift persisted for up to 3 days after the hypoglycemic event in controls (Figure 2).

To further determine phenotypical changes, we investigated the effect of hypoglycemia on surface markers characterizing pro-inflammatory monocytes (CCR2 and CD11b) and monocytes participating in foam cell formation (CD36) and coagulation (CD41). Hypoglycemia decreased the proportion of monocytes characterized by CCR2, CD11b and CD36 in both groups. While these numbers returned to euglycemic levels in participants with type 2 diabetes after 24 hours, the decrease persisted for a week in control subjects, albeit that the differences between the groups failed to reach statistical significance (ESM Figure 3). The proportion of monocytes characterized by CD41 was not affected by hypoglycemia in either group.

After phenotyping the monocytes, we determined their functional properties using their cytokine production capacity upon ex vivo stimulation with a specific TLR2 agonist (Pam3Cys (P3C)) and TLR4 agonist (lipopolysaccharide (LPS). Hypoglycemia caused a significant increase in TNF-α and IL-1β production after stimulation with P3C in people with type 2

diabetes and controls. Although not statistically significant, levels appeared to remain elevated up to one week after the hypoglycemic event. Stimulation with LPS showed a similar trend (Figure 3). IL-6 production increased after P3C-stimulation (Figure 3 B) and returned to euglycemic levels over 7 days. Lastly, hypoglycemia suppressed the stimulated production of the anti-inflammatory cytokine IL-10 in both groups, which despite a gradual increase persisted for up to one week. Overall, the *ex vivo* stimulated production of TNF-α and IL-1β was higher and that of IL-10 was lower in people with type 2 diabetes compared to healthy controls (ESM Figure 4).

Since metabolism of immune cells is a key determinant of its functional output [21], we then determined the effect of hypoglycemia on mitochondrial respiration (oxidative phosphorylation) and glycolytic capacity in monocytes. No differences weer found between the groups at euglycemia. For mitochondrial respiration, basal as well as maximal oxygen consumption rate (OCR) numerically decreased one day after hypoglycemia before peaking at day 3 in both groups, yet these effects did not reach statistical significance (Figure 4 A-B). Regarding glycolytic capacity, basal extracellular acidification rate (ECAR) and maximal ECAR decreased significantly, in both groups combined, in response to hypoglycemia, followed by an increase after 3 days (Figure 4 C-F).

Finally, we determined circulating inflammatory mediators using a proteomics approach that included 93 proteins (Figure 5). During euglycemia, hs-CRP, TWEAK, TRAIL, CCL23, DNER, NT-3 and ADA were all significantly higher in people with type 2 diabetes compared to controls ($p<0.05$, ESM Figure 5). Hypoglycemia increased inflammatory protein levels to similar extent in both groups. Several proteins, including IL-6 and IL-10 ($p<0.001$), showed an immediate response with a peak during hypoglycemia and a subsequent return to baseline levels over time (Figure 5 F-J). Most proteins, including hs-CRP ($p<0.05$), FGF-21 and SLAMF-1 ($p<0.001$), reached their peak concentrations 1 day after the hypoglycemic event and remained

elevated up to one week (Figure 5 G). Other proteins, including TRANCE, FGF-23 and IFN-γ, did not reach their maximum until 3 days or a week after the hypoglycemic event ($p<0.001$) (Figure 5 H,I)**.**

Discussion

People with insulin-treated type 2 diabetes often experience episodes of hypoglycemia. Our study provides evidence for an acute *and* sustained pro-inflammatory effect of a single hypoglycemic event, as defined by changes on multiple levels. These changes included the number of immune cells, phenotypical and functional changes of monocytes, and increased levels of several pro-inflammatory mediators. While people with type 2 diabetes showed an increased inflammatory state under euglycemic conditions, the inflammatory responses to hypoglycemia, both immediate and over the longer-term, occurred largely independently of the presence of type 2 diabetes.

Previously, it was found that hypoglycemia caused an increment in the number of lymphocytes and monocytes in people with type 1 diabetes [22]. The present results extend these observations to people with type 2 diabetes and by revealing that these effects are sustained for up to one week. Given the positive association with the adrenaline response, it could be hypothesized that this counterregulatory hormone modulated the hypoglycemia-induced increase in immune cells. Indeed, adrenaline can mobilize leukocytes from the marginal pool directly during hypoglycemia [15, 23] and has the potential to stimulate the bone marrow, which may result in long-term effects through modulation of myeloid progenitor cells [24]. The shorter lifespan of granulocytes (days) compared to lymphocytes (weeks) may explain the drop below baseline in the days after hypoglycemia of the first and the persistent elevation of the latter [25] However, since the lifespan of monocytes is also relatively short, one could speculate that adrenaline stimulated the production of monocytes in the bone-marrow to explain the sustained elevation up to 1 week after the hypoglycemic event. Further studies are needed to test the potential role of adrenaline in the immune response to hypoglycemia.

Our results also revealed phenotypical changes induced by hypoglycemia. Hypoglycemia caused a shift from phagocytizing classical monocytes ($CD14^{+}CD16^{-}$) towards ROS producing

and pro-inflammatory intermediate and non-classical ($CD14^{-}CD16^{+}$) monocytes, that are more dedicated to endothelium patrolling and cytokine producing. Since classical monocytes rely heavily on glucose (and glycolysis) as their primary energy source to function properly [18], this shift may result from a lower availability of glucose during hypoglycemia. Interestingly, this shift normalized directly after hypoglycemia in the people with diabetes, yet remained visible for up to three days in the control group. To what extent this difference between people with type 2 diabetes and controls is related to prior exposure to hypoglycemia, or its absence, cannot be derived from our data.

Hypoglycemia also reduced the expression of CCR2, CD11b and CD36 receptors on the monocyte surface. These markers are relevant in the development of atherosclerosis, either by controlling adhesion of white blood cells (CD11b) and mobilization of monocytes towards the inflammatory atherosclerotic lesion (CCR2, CD11b) [26], or by controlling the uptake of OxLDL and foam cell formation in the atherosclerotic lesion (CD36) [27, 28]. Although speculative, the lower receptor expressions could be the result of monocytes binding to the endothelial wall, thus contributing to arterial wall inflammation that is known to drive the development of atherosclerosis. Alternatively, it may be that the newly derived monocytes express fewer of these receptors. Our results are in contrast with the data from Iqbal et al., who reported no effect of hypoglycemia on CD11b expression on the monocyte cell surface [29]. However, that study used a different flowcytometry panel, which could explain the different results.

In line with previous findings [15], we observed that hypoglycemia increased the release of TNF-α and IL-1β by monocytes after stimulation with LPS or P3C. This increase in pro-inflammatory cytokine production was accompanied by a decrease in anti-inflammatory IL-10 production, both of which were more profound in people with type 2 diabetes than in those without diabetes. IL-10 is essential for normal tissue homeostasis by mitigating the pro-

inflammatory immune response to pathogens [30]. The reduction in IL-10 production combined with the increase of TNF-α and IL-1β production may suggest an imbalance between pro- and anti-inflammatory cytokines. In people with type 2 diabetes, who are already characterized by a state of chronic low-grade inflammation [13], this may result in an even more pro-inflammatory environment. It has been well established that a pro-inflammatory environment contributes to increased cardiovascular risk as illustrated by recent studies showing that targeting pro-inflammatory pathways, including that of IL-1β, results in cardiovascular benefit [31].

Besides altered immune cell function, hypoglycemia reduced glycolytic capacity in human monocytes. Overall, we did not find an association between monocyte metabolism and *ex vivo* function, suggesting that alterations in metabolism are not linked to an increase in the pro-inflammatory response. A potential explanation for the reduced glycolytic capacity could be the increased proportions of non-classical monocytes directly following the hypoglycemic event, which mainly rely on oxidative phosphorylation instead of glycolysis for energy production [32]. Although the fraction of non-classical monocytes is a relatively small part of the complete monocyte population, it could still be biologically relevant because of its greater pro-inflammatory potential.

In line with a more pro-inflammatory trait of innate immune cells, we found hypoglycemia to increase circulating inflammatory proteins. Interestingly, we observed differential responses over time with some inflammatory proteins, such as IL-6 and MCP-1, responding directly to hypoglycemia, thereby contributing to the initial inflammatory response. MCP-1 is known to play a role in monocyte emigration from the bone marrow and its increase may explain the increased levels of monocytes in the circulation [33]. IL-6 is known for its driving role in hs-CRP production, which may serve to explain the increase in hs-CRP after 1 day and a week [34]. After 24h, so-called “acute and persistent proteins” peaked and remained elevated for up

to a week. Several of the inflammatory proteins demonstrating an acute and persistent response are associated with atherosclerosis developement. OPG is reported to be expressed in the atherosclerotic plaques and amplify the adverse effects of inflammation [35]. The same trend was found for hs-CRP levels, of which the increase after 1 day has been reported previously [16]. Some proteins, such as FGF-23, CXCL10 and IFN-γ, only peaked after one week, showing that a single hypoglycemic event induces a long-term pro-inflammatory response.

Type 2 diabetes has been associated with the presence of chronic low-grade inflammation [13]. Indeed, circulating immune cells, cytokine production and pro-inflammatory proteins including hs-CRP, were all higher in people with type 2 diabetes compared to subjects without diabetes. Chronic low-grade inflammation is linked to both the development of insulin resistance and the development of atherosclerosis. Several factors can contribute to this chronic low-grade inflammation in type 2 diabetes, including metabolic complications, hyperglycemia and alterations in lipid profile [13, 36, 37]. Our results suggest that hypoglycemia may contribute to or exacerbate chronic-low grade inflammation in people with type 2 diabetes, in turn contributing to the development of diabetes-related complications and a higher risk of CVD. Because antecedent hypoglycemia suppresses adrenaline responses to subsequent events, it remains to be seen to what extent such effects persist with recurrent hypoglycemia.

Strengths of our study include the well matching of the two participant groups and the extensive assessment of the entire inflammatory profile at multiple levels, including inflammatory cell counts, composition of cells, function of cells and circulating inflammatory proteins, which were measured for up to a week after the hypoglycemic event. Furthermore, only 2 participants had a level 2 hypoglycemic event (<3.0 mmol/L) in the week prior to the clamp, minimizing the effect of hypoglycemia on the results. Our study also has limitations. Hypoglycemia was induced with the glucose clamp technique, which may differ from spontaneous hypoglycemia in daily clinical practice. However, this method ensured that the hypoglycemic stimulus was

identical for all participants and is a well-established approach to examine effects of hypoglycemia. Second, the monocyte phenotyping was performed on a subgroup of the participants at one of the two participating study sites, because this method is too sensitive for confounders when performed at different sites. Third, insulin can suppress pro-inflammatory responses [19, 38]. To control for an effect of hyperinsulinemia on pro-inflammatory responses, a euglycemic control clamp could have been added to our study protoco. However, our results clearly show that the hypoglycemic event was able to overcome such a potential suppression of the pro-inflammatory response. In addition, because the experiment was already demanding for participants and involved a substantial amount of blood to be drawn, we decided against adding a euglycemic control clamp to control for hyperinsulinemia. Fourth, the study was conducted at two study sites, which may have introduced unforeseen side effects. However, this design allowed for better representativeness and we minimized this effect by doing paired analysis within each individual subject and by performing the analyses at one study site.

In conclusion, a single hypoglycemic event causes an acute inflammatory response, that persists for up to 7 days in people with or without type 2 diabetes. As such, insulin-induced hypoglycemia could interact with chronic hyperglycemia and other factors to cause a sustained pro-inflammatory state, that may exercebate vascular disease in people with type 2 diabetes treated with insulin. Whether and to what extent higher frequency of hypoglycemic events contribute to the greater risk of CVD in people with type 2 diabetes remains to be elucidated.

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Tables

Table 1: participants characteristics

	Type 2 diabetes	**Control**
Participants, *n*	15	16
Male, *n* (%)	9 (60)	8 (50.0)
Age, y	61.3 ± 7.6	57.9 ± 6.4
Duration of diabetes, y	15.0 ± 7.7	-
HbA_{1c}, %	8.0 ± 1.0*	5.4 ± 0.2*
(mmol/mol)	(63.5 ± 11.2*)	(35.6 ± 2.2*)
BMI, kg/m^2	29.0 ± 4.3	28.0 ± 4.4
Diabetes medication		
Oral	10	-
CSII	1 (6.7)	-
MDI	14 (93.3)	-

*Data are presented as number (%), mean ± SD. CSII, continuous subcutaneous insulin infusion; MDI, multiple daily injections. EQF, European quality framework. * $p < 0.05$.*

Figures and figure legends

Fig. 1 Counts ($\cdot 10^3/\mu L$) of granulocytes (B), lymphocytes (C), and monocytes (D) in participants with type 2 diabetes (black) and controls (grey), and Data are presented as mean ± SEM, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ change versus euglycemia.

Fig. 2 Distribution of classical and non-classical monocytes for participants with type 2 diabetes (A) and controls (B). Data are based on a subset of study participants (n=17) and are presented as mean ± SEM, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ change versus euglycemia.

Fig. 3 *Ex vivo* cytokine production of TNF-α (A), IL-6 (B), IL-1β (C) and IL-10 (D) upon LPS or P3C stimulation. Data presented as mean ± SEM, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ change versus euglycemia.

Fig. 4 Oxygen consumption rates (OCR) during the mitochondrial stress test, extracellular acidification rate (ECAR) during the glycolytic stress test and ECAR during acute P3C stimulation. Dotted lines indicate injection points. Basal (A) and max OCR (B) per timepoint, basal (C), after glucose (D) and max ECAR (E) per timepoint, max ECAR (F) after P3C injection per timepoint. Data based on a subset of study participants (n=12) and are presented as mean ± SEM, *$p < 0.05$ change versus euglycemia for both groups combined.

Fig. 5 Volcano plots of circulating inflammatory proteins among participants with type 2 diabetes and controls during euglycemia, where proteins in red are significantly different between the groups (A), show volcano plots from each timepoint compared to euglycemia (B-E), proteins in red are significantly different (Wilcoxon paired test, FDR<0.0001). F-J display circulating inflammatory proteins categorized based on their pattern: acute proteins (F), acute chronic proteins (G), and late proteins (H,I). Hs-CRP (J) followed mostly the pattern of acute chronic proteins.