Effect of SGLT2 inhibitor on NLRP3 inflammasome activity and ketone bodies metabolism in type 2 diabetes with cardiovascular disease

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Directed by Professor Yong-ho Lee

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ABSTRACT

Effect of SGLT2 inhibitor on NLRP3 inflammasome activity and ketone bodies metabolism in type 2 diabetes with cardiovascular disease

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In large clinical trials, sodium-glucose cotransporter 2 (SGLT2) inhibitors reduce cardiovascular events in humans with type 2 diabetes (T2D); SGLT2 inhibitors correct metabolic and hemodynamic abnormalities that are risk factors for cardiovascular disease, by decreasing serum glucose, body weight, and blood pressure and by increasing diuresis. However, other underlying mechanisms to explain the cardioprotective effects of SGLT2 inhibitors are as yet unclear. Activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome and subsequent interleukin (IL)-1β release induces

atherosclerosis and heart failure. It was revealed that ketone bodies, i.e. β-hydroxybutyrate (BHB) suppresses activation of NLRP3 inflammasome in macrophages. As SGLT2 inhibitors cause increases in serum BHB by pharmacologic profile, we show the effect of SGLT2 inhibitor empagliflozin on NLRP3 inflammasome activity, which could explain its cardioprotective effects.

In a randomized, active-controlled study, a total of 61 patients with T2D and high cardiovascular risk (mean age and glycated hemoglobin are 64.4 yrs and 7.32 %, respectively) receive SGLT2 inhibitor (n = 29) or sulfonylurea (n = 32) for 30 days. To exclude glucose-lowering effects on inhibition of NLRP3 inflammasome activation, we use an active comparator, namely sulfonylurea instead of placebo. NLRP3 inflammasome activation is analyzed in macrophages and the serum metabolic parameters including glucose, BHB, and insulin from baseline to the end of treatment are tested. In addition, using a 3-day isocaloric ketogenic diet trial in healthy subjects (n = 15) which significantly increases circulating BHB levels in humans, we evaluate whether increased circulating levels of BHB block NLRP3 inflammasome activation.

While SGLT2 inhibitor's glucose-lowering capacity is similar to sulfonylurea, it shows a greater reduction in IL-1β secretion compared to sulfonylurea accompanied by increased serum BHB and decreased serum insulin, while sulfonylurea has no significant effects on these measurements. Ex vivo experiments with macrophages for assessing the direct effects of metabolites on NLRP3 inflammasome activation and a human study with isocaloric ketogenic diet verify the inhibitory effects of high BHB and low insulin levels on NLRP3 inflammasome activation.

In conclusion, SGLT2 inhibitor attenuates NLRP3 inflammasome activation

and subsequent secretion of IL-1β in human macrophages, via increased serum BHB levels and decreased serum levels of insulin, among patients with T2D and cardiovascular disease, regardless of glycemic control. Given that previous studies show a beneficial role of IL-1β blockade in the pathological processes of cardiovascular disease, the present data suggest that these mechanisms might help to explain the cardioprotective effects of SGLT2 inhibitor in humans.

Key words : SGLT2 inhibitor, type 2 diabetes, NLRP3, inflammasome, IL-1β, cardiovascular disease, beta-hydroxybutyrate, ketogenic diet

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I. INTRODUCTION

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in patients with type 2 diabetes (T2D), with a two- to four-fold increase in incidence compared with patients without diabetes.[1] Diabetes produces an abnormal metabolic state, including chronic hyperglycemia, dyslipidemia, insulin resistance, and inflammation, which can lead to the development of atherosclerosis, a pathologic process implicated in symptomatic CVD events. A large, multiprotein complex oligomerized in the cytoplasm of innate immune cells, known as the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome, is stimulated to secrete pathogenic inflammatory cytokines, specifically interleukin-1β (IL-1β).[2] This is involved in the molecular etiology of numerous chronic inflammatory diseases, including diabetes, non-alcoholic steatohepatitis, gout, atherosclerosis, and heart failure.[2-7]

Sodium-glucose cotransporter 2 (SGLT2) inhibitors lower serum glucose by increasing urinary glucose excretion.[8] The recently published EMPA-REG

OUTCOME study demonstrated that, in patients with T2D and high CVD risk, empagliflozin reduced adverse cardiac events by 14%, which resulted in a 38% reduction in cardiovascular (CV) mortality.[9] It is the first antidiabetic agent that reduced CV events beyond glycemic control. Subsequently, the CANVAS Program also achieved comparable effects with another SGLT2 inhibitor.[10] The unique action of SGLT2 inhibitors corrects several metabolic and hemodynamic abnormalities that are risk factors for CVD, by decreasing serum glucose, body weight, and blood pressure and by increasing diuresis.[8] However, other underlying mechanisms to explain the cardioprotective effects of SGLT2 inhibitors are as yet unclear.

Based on their pharmacologic profile, SGLT2 inhibitors cause mild increases in serum ketone bodies, i.e., β-hydroxybutyrate (BHB).[8] BHB is a convenient carrier of energy from adipocytes to peripheral tissues, particularly in brain, heart, and skeletal muscles during prolonged fasting or exercise.[11] Beyond acting as a metabolite, BHB has important cellular signaling roles. Youm et al. revealed that BHB suppresses activation of the NLRP3 inflammasome and reduces IL-1β production in macrophages and mice.[12] However, there is no report of whether increased serum BHB concentration inhibits NLRP3 inflammasome activation in humans. A recent study demonstrated that fasting and refeeding can differentially regulate NLRP3 inflammasome activation in human peripheral blood mononuclear cells (PBMCs).[13] This indicates that the dynamic regulation of NLRP3 inflammasome activation can be assessed using human PBMCs.

On the basis of the pathogenic effect of the NLRP3 inflammasome on CVD and the therapeutic role of BHB on its inhibition, the present study demonstrates that the SGLT2 inhibitor blocks NLRP3 inflammasome activation by raising

circulating levels of BHB in patients with T2D at high CV risk, which could explain their cardioprotective effects. To exclude glucose-lowering effects on inhibition of NLRP3 inflammasome activation, we used an active comparator, namely sulfonylurea instead of placebo. In addition, using a ketogenic diet (KD) trial which significantly increases circulating BHB levels in humans,[14] we verify that increased circulating levels of BHB block NLRP3 inflammasome activation.

II. MATERIALS AND METHODS

1. Study design

The present prospective, randomized, open, active-controlled, 2-arm parallel interventional study was carried out at Severance Hospital between November 2016 and July 2017. This randomized controlled trial (RCT) complied with the Declaration of Helsinki, and is registered at clinicaltrials.gov. (NCT02964572). The protocol was approved by the Institutional Review Board at Severance Hospital (4-2016-0795). All participants provided their written informed consent.

2. Patients

Eligible participants were aged 20-79 yrs with a diagnosis of T2D and high CV risk, having inadequate blood glucose control with metformin-based oral hypoglycemic agents (OHAs). High CV risk was defined as the presence of ≥1 of the following based on previous literatures:[9,15] 1) history of acute coronary syndrome (myocardial infarction (MI) or unstable angina); 2) evidence of multi-vessel coronary artery disease documented by coronary angiography; 3) evidence of occlusive peripheral artery disease

documented by angiography or ankle brachial index; 4) evidence of carotid atherosclerosis defined as presence of carotid artery plaques or ≥0.9 mm of peak carotid intima-media thickness documented by common carotid arterial ultrasound examinations;[16] 5) body mass index (BMI) ≥25 kg/m^2, with at least 2 of the following: history of hypertension, current smoking, or steatohepatitis. Inadequate blood glucose control was defined as: 1) glycated hemoglobin (HbA_{1C}) ≥6.5% checked within the last 3 months; 2) fasting serum glucose >120 mg/dL; or 3) random serum glucose >180 mg/dL. Key exclusion criteria included: 1) type 1 diabetes; 2) pregnant women; 3) estimated glomerular filtration rate ≤45mL/min per 1.73 m^2; 4) active cancer; 5) any uncontrolled endocrine disorder; or 6) active infection. The inclusion criteria were amended from HbA_1c ≥7% or fasting serum glucose ≥130 mg/dL to HbA_1c ≥6.5% or fasting serum glucose >120 mg/dL early in the course of the study.

3. Randomization

Participants who met the eligibility criteria were randomly assigned in a 1:1 ratio to receive empagliflozin or glimepiride once daily, stratifying for baseline HbA_{1C} (<8% or ≥8%) and BMI (<25 kg/m^2 or ≥25 kg/m^2) using a computer-generated permuted block randomization (a block size of four). Each participant received 10 mg or 25 mg once-daily dose of empagliflozin, or glimepiride at an individualized dose according to risk of hypoglycemia and status of glycemic control (average dose was 2 mg). In participants who were previously treated with metformin-based OHAs, non-metformin OHAs were substituted with a study drug (either empagliflozin or glimepiride), or a study drug was added to OHAs. In drug naïve participants, monotherapy with a study drug was initiated. OHA

administration was maintained throughout the 30-day study period. A schematic illustrating the temporal organization of the study is shown in Fig. 1.

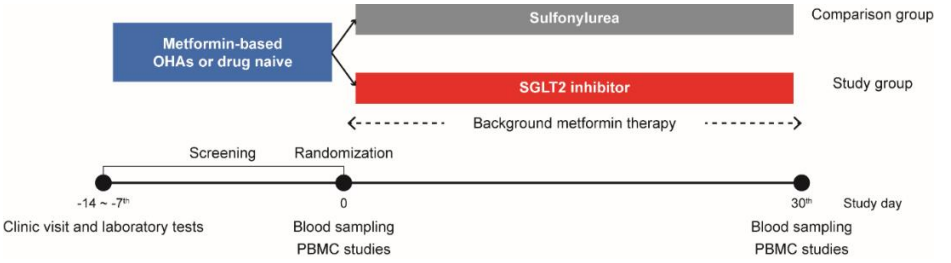

Fig. 1. Study design. OHAs, oral hypoglycemic agents; PBMC, peripheral blood mononuclear cell; SGLT2, sodium-glucose cotransporter 2.

4. Outcome measures

The primary endpoint was the group difference in the levels of IL-1β secreted from macrophages, before and after the administration of SGLT2 inhibitor or sulfonylurea. The secondary endpoints were the group differences in the levels of tumor necrosis factor-α (TNF-α) secreted from macrophages, serum levels of BHB, insulin secretory/resistant indices, and other biochemical profiles (glycated albumin, glucose, uric acid, liver enzymes, lipid profiles, creatinine, and free fatty acid (FFA)), urinary glucose excretion, and body weight, before and after the administration of SGLT2 inhibitor or sulfonylurea. Quantitative polymerase chain reaction (qPCR) and immunoblot analyses for NLRP3, IL-1β, and TNF-α were performed and compared between two treatment groups. For post-hoc analyses, ribonucleic acid (RNA) sequencing and gene ontology (GO) enrichment analyses for differentially expressed genes between two groups

were performed using macrophages before and after treatment. In addition, changes in serum IL-1β and IL-18 levels from baseline to end of treatment were analyzed.

5. Clinical and laboratory measurements

A complete physical examination was conducted and current medications were recorded for all participants. For laboratory parameters, after overnight fasting, serum BHB was determined by an enzymatic assay using a commercial reagent from Nittobo Medical Co., LTD (Tokyo, Japan) and the Hitachi 7600 analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Serum glucose, glycated albumin, uric acid, FFA, lipid profiles, and creatinine were measured using a Hitachi 7600 automated chemistry analyzer. Fasting serum insulin was measured by electrochemiluminescence immunoassay using a Cobas e601 analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). In addition, spot urine glucose and creatinine were measured on an AU680 chemistry system (Beckman Coulter, Inc., Brea, CA, USA) immediately prior to study initiation and at end of treatment. Baseline HbA_{1C} was measured by immunoassay using an Integra 800 CTS (Roche Diagnostics). Pancreatic beta cell function and insulin sensitivity were assessed using the following indices:[17] homeostatic model assessment of pancreatic β-cell function (HOMA-β) = [(fasting serum insulin [μU/mL] × 20)/(fasting serum glucose [mmol/L] – 3.5)]; homeostatic model assessment of insulin resistance (HOMA-IR) = [(fasting serum insulin [μU/mL] × fasting serum glucose [mmol/L])/22.5]; and quantitative insulin sensitivity check index (QUICKI) = [1/(logarithm(base 10) (log)(fasting serum glucose [mg/dL]) + log(fasting serum insulin [μU/mL]))].[18] The estimated glomerular filtration rate was

derived from the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI).[19] Serum IL-1β and IL-18 were measured with enzyme-linked immunosorbent assay (ELISA) using human Quantikine HS ELISA kits (R&D Systems, Minneapolis, MN, USA). The assay sensitivities (the minimum detectable levels) for IL-1β and IL-18 were 0.033 pg/mL and 1.25 pg/mL, respectively, determined by zero standard +2 SD.

6. Isolation and culture of peripheral blood mononuclear cells

Samples of whole blood were collected into acid citrate dextrose tubes. PBMCs were isolated from blood by density centrifugation (20 min at 1,600 × *g* (without brakes) at 18-20 °C) using Ficoll Medium (Ficoll-Paque PLUS, GE Healthcare Life Science, Uppsala, Sweden).[13] After removing the top layer of clear plasma, the PBMC-containing layer was aspirated and the cells were washed with Dulbecco's phosphate-buffered saline. Then the cells were re-suspended in RPMI-1640 supplemented with 1% penicillin, 1% streptomycin, and 10% fetal bovine serum. To generate human macrophages, cells were incubated at 1×10^6 cells per milliliter in 24-well plates in RPMI medium plus 10% fetal bovine serum for 2 hrs and then incubated with 20 ng/mL macrophage colony-stimulating factor (M-CSF) for 3 days. After 3 days, the cells were then incubated with fresh RPMI medium plus 10% fetal bovine serum containing 10 ng/mL M-CSF and the medium was freshly replaced every 2 days (total 7 days) as previously described.[13]

7. Cell stimulation and cytokine assays

Human macrophages were incubated at 1×10^6 cells per milliliter in 24-

well plates in RPMI medium plus 10% fetal bovine serum with 0.1 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA, L6529) for 4 hrs.[13] To stimulate the release of IL-1β, 2 mM adenosine triphosphate (ATP) (Sigma-Aldrich) or 0.2 mM palmitate (Sigma-Aldrich, P9767) was added for the last 1 or 12 hrs of incubation, respectively. In addition, to evaluate the direct inhibitory or stimulating effects on NLRP3 inflammasome activation, different concentrations of BHB (1, 10, 20, and 30 mM), glucose (11.5 and 25 mM) and 2-deoxyglucose (2-DG) as a calorie restriction mimetic, or insulin (10 nM) were pre-treated for 5 hrs before adding LPS and ATP. Supernatants were collected, centrifuged to remove cells and debris, and stored at -80 °C for later analysis. IL-1β and TNF-α was measured using ELISA (eBioscience, Waltham, MA, USA, human 88-7261-88 and human 88-7346-88, respectively). Results were normalized to cell number, as determined by the CyQuant cell proliferation assay (Invitrogen, Waltham, MA, USA). Experiments on the participants were repeated up to three times per sample. The examiner conducting these experiments remained blinded to the subject's clinical status and treatment group assignment throughout the study.

8. Immunoblot analysis

Total cell lysates with or without stimulation by LPS and ATP were prepared by lysis of human macrophages with RIPA buffer (cell signaling, Danvers, MA, USA, 9806) (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate) and the protein contents were measured using the Bradford assay (Bio-Rad, Hercules, CA, USA, 500-0006). Equivalent amounts of each protein extract were heat-denatured in 5 × sample buffer (2% sodium dodecyl sulfate, 62.5 mM Tris

(pH 6.8), 0.01% bromophenol blue, 1.43 mM mercaptoethanol, and 0.1% glycerol), separated on 10% polyacrylamide gels, and electrophoretically transferred onto a polyvinylidenefluoride membrane (Bio-Rad, 1620175). After blocking, membranes were treated with the following antibodies: anti-p-nuclear factor kappa-light-chain-enhancer of activated B cells (pNF-κB) (Ser536; Cell Signaling Technology, 3033), anti-NF-κB (Cell Signaling Technology, 8242), anti-NLRP3 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-66846), and anti-IL-1β (Santa Cruz Biotechnology, sc-7884). Immunostaining was performed using chemiluminescent reagents (SuperSignal West Pico Luminol/Enhancer solution; Thermo Scientific, 34080) and Agfa medical X-ray film (Mortsel, CURIX 60). Actin protein levels were used as a loading control.

9. Quantitative PCR analysis

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, 15596–018) following the manufacturer's instructions, and then 2 μg total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA, 4368814). The cDNA was then amplified in the ABI 7500 sequence detection system (Applied Biosystems, 4350584) using Power SYBR® Green PCR Master Mix (Applied Biosystems, 4367659) with the following cycling conditions: 40 cycles of 95 °C for 5s, 58 °C for 10s, and 72 °C for 20s. Target gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) or *Actin*, and quantitative analyses were conducted using the ΔΔcycle threshold method and StepOne Software version 2.2.2. The primer sets used for qPCR were as follows: *IL1B*, forward 5'-GGA CAA GCT GAG

GAA GAT GC-3' and reverse 5'-TCG TTA TCC CAT GTG TCG AA-3'; *TNFA*, forward 5'–GTG ACA AGC CTG TAG CCC AT-3' and reverse 5'–TAT CTC TCA GCT CCA CGC CA-3'; *NLRP3*, forward 5'–TCT CAC GCA CCT TTA CCT GC-3' and reverse 5'–GAT CCC AGC AGC AGT GTG AC-3'; *β–actin*, forward 5'-GGA CTT CGA GCA AGA GAT GG-3' and reverse 5'-AGC ACT GTG TTG GCG TAC AG-3'.

10. RNA extraction and sequencing

Total RNA was isolated from macrophage lysates obtained from PBMCs of 7 individuals (3 from the sulfonylurea arm, 4 from the SGLT2 inhibitor arm) before and after treatment using a Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA). Among 7 individuals, 3 of 3 in sulfonylurea group and 3 of 4 in SGLT2 inhibitor group were treated with metformin before and throughout the study period. Total RNA quality and quantity was verified on a NanoCrop1000 spectrometer (Thermo Scientific, Wilmington, DE, USA) and Bioanalyzer 2100 (Agilent technologies, Palo Alto, CA, USA). RNA sequencing was performed by Macrogen (Seoul, Korea). Libraries were constructed with the TruSeq RNA Access Library Prep kit (Illumina, San Diego, CA, USA) and the enriched library was sequenced on an Illumina HiSeq 4000 system. In brief, we started with 1 μg of total RNA, messenger RNA (mRNA) was first purified using polyA selection, then chemically fragmented and converted into single-stranded cDNA using random hexamer priming. Next, the second strand was generated to create double-stranded (ds)-cDNA that was ready for the TruSeq library construction. The short ds-cDNA fragments were then connected with sequencing adapters, and suitable fragments were separated by agarose gel electrophoresis. Finally, TruSeq strand-specific mRNA libraries were built

by PCR amplification and quantified using qPCR according to the qPCR Quantification Protocol Guide and qualified using Bioanalyzer 2100 (Agilent technologies).

11. Bioinformatics of RNA-seq data

Sequence reads were mapped against the human reference genome (NCBI hg19) and analyzed using CLC Genomics Workbench v.9.0.1 software (CLC Bio, Cambridge, MA, USA). Among a total of 57,773 genes annotated for expression values based on transcripts, differentially expressed genes among two groups with statistical significance (*P* value <0.05) using the Baggerley's test were selected for further analysis. Afterwards, fold change calculated as mean Reads Per Kilobase per Million mapped reads (RPKM) values of drug group divided by mean RPKM values of control group (pre-treatment samples) was utilized for further filtering step with cut-off value of 1.3 for upregulation and 0.77 for downregulation.

12. GO enrichment analysis

GO enrichment analyses were carried out using database for annotation, visualization and integrated discovery (DAVID) (https://david.ncifcrf.gov/). ENSEMBL gene IDs were used instead of official gene symbols. The background species was selected as Homo Sapiens for genes with upregulation and downregulation values. Annotation of genes into GO terms with functional categories, general annotations, and protein interactions was executed, and biological process was used for GO category. Afterwards, GO terms with statistical significance (Benjamini corrected *P*

value <0.05) were further visualized using reduce visualize gene ontology (REViGO) (http://revigo.irb.hr/) using a cut-off of 0.7 for SimRel similarity scores. Finally, we determined major representative GO terms and grouped all GO terms into clusters according to treemap view. Additionally, individual GO terms with statistical significance were ranked by degree of statistical significance according to values of –log(Benjamini corrected *P* value).

13. Isocaloric ketogenic diet in healthy subjects

Eligible participants of observational KD study were healthy adults (19-44 yrs of age) with a BMI of at least 18 kg/m^2. Exclusion criteria included any disease including diabetes, hypertension, and dyslipidemia; any current medication; or pregnant women. We recruited 15 healthy volunteers aged 24-38 yrs (7 men and 8 women) and placed them on a diet restricting intake of carbohydrates (75% fat, 20% protein, 5% carbohydrate) but not energy, i.e., isocaloric KD, for 3 days (Fig. 2).

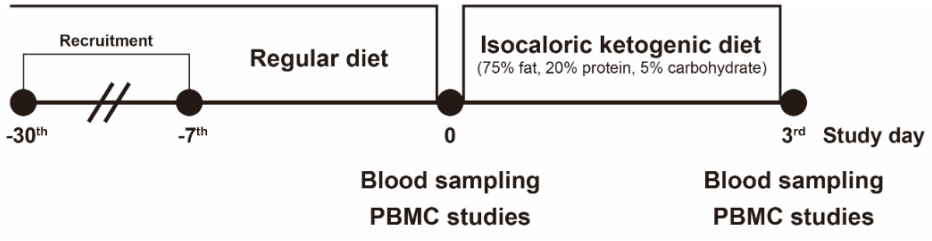

Fig. 2. Schematic of the isocaloric ketogenic diet protocol in healthy subjects. PBMC, peripheral blood mononuclear cell.

Total calories per day were determined by 'Dietary Reference Intakes for Koreans':[20] men, 2,600 kcal/day for age under 30 and 2,400 kcal/day for

age 30 or older; women, 2,100 kcal/day for age under 30 and 1,900 kcal/day for age 30 or older. However, in participants with a BMI ≥23 kg/m^2, total calories per day were restricted to 20 kcal/kg/day. Mean age and BMI were 28.9 yrs and 23.8 kg/m^2, respectively. Participants' body composition was analyzed by bioelectrical impedance (InBody 720, BioSpace Co., Seoul, Korea) before and after KD at the same time of day (early morning). Changes in secretion levels of IL-1β (designated as the primary endpoint) and TNF-α from supernatants of macrophages, the serum levels of glucose, BHB, insulin, FFA, and other biochemical profiles, and clinical variables (designated as secondary endpoints) from baseline to the end of treatment were tested as described above. All outcomes in this KD study were pre-specified in the study protocol. This KD study was approved by the Institutional Review Board at Severance Hospital (the same as previously described), and complied with the Declaration of Helsinki. All participants provided their written informed consent.

14. Statistical analysis

All statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corp., Armonk, NY, USA). A normality test was performed for all continuous variables. The characteristics of the study participants were analyzed according to groups using a Mann-Whitney U or two-sample Student's t-test for continuous variables and a Pearson χ^2 test for categorical variables. The effects of empagliflozin or glimepiride treatment and KD on secretion of IL-1β and TNF-α from macrophages and metabolic parameters were assessed by two-sided paired t-test or Wilcoxon signed rank test. Statistical significance for the time × group interaction was evaluated by using repeat-measures analysis of variance (ANOVA). Non-normally

distributed variables were logarithm transformed for analysis and back transformed for presentation. The Pearson's correlation coefficient was used for correlation. One-way ANOVA using Tukey's test or a two-tailed Student's t-test with the Bonferroni method for adjusting *P* values for the number of comparisons being made were used to examine differences between treatments in ex vivo experiments. All *P*-values <0.05 were considered statistically significant.

III. RESULTS

1. Baseline characteristics of study participants

88 participants who were diagnosed with T2D and treated with metformin-based OHAs or drug naïve had undergone screening for eligibility. Among them, 71 met the inclusion criteria and were randomly assigned to treatment with empagliflozin (SGLT2 inhibitor) or glimepiride (sulfonylurea). 10 participants withdrew prematurely from the trial, which led to 61 participants completing the analysis for effects of each treatment on NLRP3 inflammasome activation and various metabolic parameters (empagliflozin, 29; and glimepiride, 32) (Fig. 3). Baseline characteristics of the study participants are summarized in Table 1. Participants were matched for demographic and biochemical variables. As metformin is first-line therapy, most participants in both groups were taking it and continued on the same dosage during the study period. Adverse events related to study drugs were uncommon, although discontinuation of study drug occurred once in each group (Table 2).

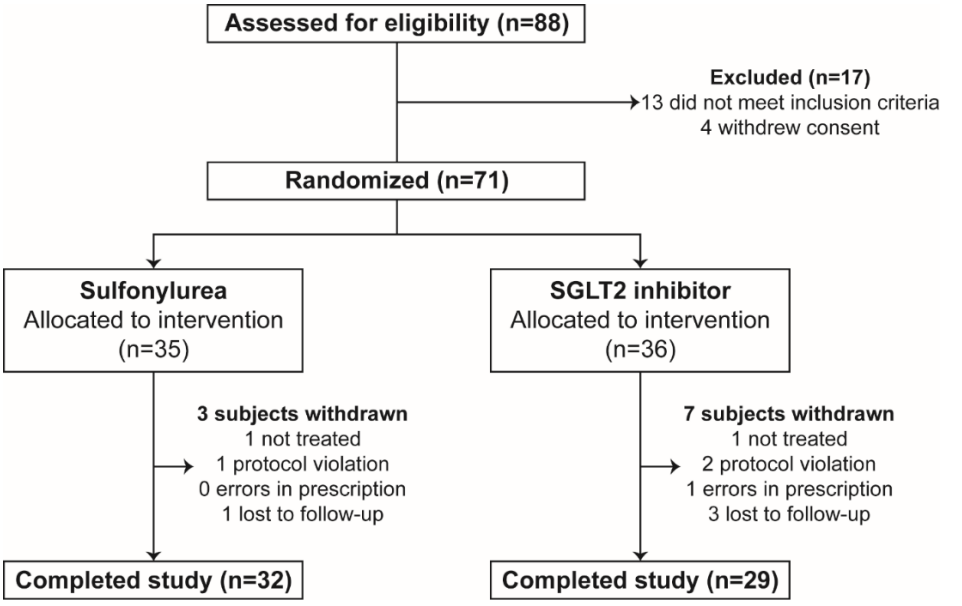

Fig. 3. Participant flow diagram. Numbers of participants who were initially screened, underwent random allocation, withdrew, and were included in the final analysis. SGLT2, sodium-glucose cotransporter 2.

Table 1. Baseline characteristics of study participants

Baseline characteristics	Sulfonylurea (*n* = 32)	SGLT2 inhibitor (*n* = 29)	*P* values
Demographics			
Age (yrs)	64.9±8.60	63.9±9.18	0.64
Male Sex [*n* (%)]	22 (68.8)	24 (82.8)	0.20
Body weight (kg)	69.5±11.0	73.0±14.8	0.30
BMI (kg/m^2)	26.0 (24.3-27.9)	26.3 (24.3-28.0)	0.98
Currently smoking [*n* (%)]	3 (9.38)	7 (24.1)	0.17
Systolic blood pressure (mmHg)	129.3±14.0	127.8±15.4	0.71
Diastolic blood pressure (mmHg)	73.6±10.1	76.3±11.3	0.33
Duration of diabetes (yrs)	7.58 (3.39-14.1)	7.58 (4.00-12.6)	0.83
Cardiovascular disease			
History of ACS[1] [*n* (%)]	13 (40.6)	16 (55.2)	0.26
History of AMI [*n* (%)]	9 (28.1)	8 (27.6)	0.96
Multi-vessel CAD [*n* (%)]	22 (68.8)	23 (79.3)	0.35

Table 1. Continued

Baseline characteristics	Sulfonylurea (*n* = 32)	SGLT2 inhibitor (*n* = 29)	*P* values
PTCA [*n* (%)]	23 (71.9)	18 (62.1)	0.42
Coronary artery bypass graft [*n* (%)]	3 (9.38)	3 (10.3)	>0.99
Gluco-metabolic indices			
HbA_{1C} (%)	7.25 (6.75-8.00)	6.90 (6.45-7.80)	0.25
Glycated albumin (%)	17.4 (15.7-21.0)	17.2 (15.3-19.9)	0.50
Fasting serum glucose (mg/dL)	139.0 (127.8-171.5)	128.0 (123.5-147.0)	0.05
Fasting serum BHB (mM)	0.07±0.07	0.06±0.04	0.21
Uric acid (mg/dL)	4.96±1.43	4.75±1.19	0.54
AST (IU/L)	24.0 (19.3-33.0)	22.0 (19.5-29.0)	0.41
ALT (IU/L)	24.0 (19.0-34.0)	27.0 (18.5-35.0)	0.60
Total cholesterol (mg/dL)	139.0 (118.5-158.0)	133.0 (117.5-147.0)	0.30
Triglyceride (mg/dL)	126.5 (101.3-183.5)	143.0 (104.0-188.0)	0.76
HDL cholesterol (mg/dL)	43.6±10.2	42.0±9.77	0.54

Table 1. Continued

Baseline characteristics	Sulfonylurea (n = 32)	SGLT2 inhibitor (n = 29)	P values
LDL cholesterol (mg/dL)	65.2 (43.2-75.2)	63.4 (47.4-71.2)	0.40
Creatinine (mg/dL)	0.85 (0.73-1.00)	0.84 (0.77-0.92)	0.61
eGFR CKD-EPI (mL/min per 1.73 m^2)	86.0 (74.8-94.5)	92.0 (83.0-98.5)	0.08
Insulin secretory/resistant indices			
Fasting serum insulin (μU/mL)	9.60 (7.36-16.8)	8.09 (5.31-11.9)	0.08
Fasting serum FFA (μEq/L)	426.5 (351.5-527.0)	412.0 (284.0-517.0)	0.41
HOMA-IR	3.89 (2.32-7.42)	2.72 (1.74-4.40)	0.05
QUICKI	0.32±0.03	0.33±0.03	0.05
HOMA-β (%)	46.2 (29.8-92.4)	48.7 (27.6-55.5)	0.42
Drug use			
Antiplatelet/anticoagulant agents [n (%)]	30 (93.8)	27 (93.1)	>0.99
Statin [n (%)]	29 (90.6)	27 (93.1)	>0.99
Fibrate [n (%)]	3 (9.38)	4 (13.8)	0.70

Table 1. Continued

Baseline characteristics	Sulfonylurea (*n* = 32)	SGLT2 inhibitor (*n* = 29)	*P* values
ACE inhibitor/ARB [*n* (%)]	20 (62.5)	18 (62.1)	0.97
Diuretics [*n* (%)]	4 (12.5)	2 (6.90)	0.67
Calcium channel blockers [*n* (%)]	6 (18.8)	4 (13.8)	0.74
Beta blockers [*n* (%)]	22 (68.8)	15 (51.7)	0.17
Metformin [*n* (%)]	31 (96.9)	26 (89.7)	0.34

[1]history of AMI or unstable angina. Mann-Whitney U or two-sample Student's t-test for continuous variables and a Pearson χ^2 test for categorical variables; continuous variables are described as mean ± SD for parametric variables and as median (interquartile range) for nonparametric variables. ACE, angiotensin-converting enzyme; ACS, acute coronary syndrome; ALT, alanine aminotransferase; AMI, acute myocardial infarction; ARB, angiotensin II receptor blocker; AST, aspartate aminotransferase; BHB, β-hydroxybutyrate; BMI, body mass index; CAD, coronary artery disease; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate; FFA, free fatty acid; HbA_{1C}, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-β, homeostatic model assessment of pancreatic β-cell function; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; n, number of patients; PTCA,

percutaneous transluminal coronary angioplasty; QUICKI, quantitative insulin sensitivity check index; SGLT2, sodium-glucose cotransporter 2.

Table 2. Adverse events for sulfonylurea and SGLT2 inhibitor groups

Adverse events	Sulfonylurea (*n* = 32)	SGLT2 inhibitor (*n* = 29)
Any adverse event [*n* (%)]	3 (9.38)	2 (6.90)
Adverse event leading to discontinuation of a study drug [*n* (%)]	1[1] (3.13)	1[2] (3.45)
Hypoglycemia [*n* (%)]	1 (3.13)	0
Urinary tract infection [*n* (%)]	0	0
Genital infection [*n* (%)]	0	1 (3.45)
AST and/or ALT ≥2 times upper normal limit [*n* (%)]	0	0
eGFR decline ≥30% baseline [*n* (%)]	0	1[3] (3.45)
Gastrointestinal disorders [*n* (%)]	2 (6.25)	0

[1]Nausea; [2]Genital infection; [3]decline of eGFR 31% baseline. ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; SGLT2, sodium-glucose cotransporter 2.

2. Effects of SGLT2 inhibitor on metabolic parameters

Despite a similar glucose lowering effect in the two groups (Table 3 and Fig. 4a), distinct patterns of change in metabolic parameters were observed in the SGLT2 inhibitor group. SGLT2 inhibitor caused a significant increase in fasting serum BHB, approximately 3.9-fold from baseline (Fig. 4b) and a significant decrease in serum uric acid and fasting serum insulin (Fig. 4c and d, respectively) accompanied by an increase in fasting serum FFA (Fig. 4e), while sulfonylurea had no significant effects on these measurements. SGLT2 inhibitor induced significant improvement in insulin sensitivity (Fig. 4f, g), while sulfonylurea led to increased insulin secretion (Fig. 4h). SGLT2 inhibitor significantly decreased body weight, with a mean change of -2.5% (Fig. 4i).

Table 3. Effects of sulfonylurea and SGLT2 inhibitor on metabolic parameters

Metabolic parameters	Sulfonylurea (n = 32)			SGLT2 inhibitor (n = 29)			
	Baseline	Day 30	P	Baseline	Day 30	P	$P_{time \times group}$
Body weight (kg)	69.5±11.0	69.1±10.3	0.47	**73.0±14.8**	**71.2±14.1**	**<0.001**	**<0.001**
Glycated albumin (%)	**17.4 (15.7-21.0)**	**16.9 (14.8-18.9)**	**<0.001**	**17.2 (15.3-19.9)**	**16.3 (14.4-18.3)**	**0.01**	0.32†
Fasting serum glucose (mg/dL)	139.0 (127.8-171.5)	131.0 (112.0-165.8)	0.14	128.0 (123.5-147.0)	124.0 (112.5-138.0)	0.13	0.64†
Fasting serum BHB (mM)	0.07±0.07	0.07±0.07	0.60	**0.06±0.04**	**0.20±0.19**	**<0.001**	**<0.001**
Fasting serum BHB (fold increase)	1	1.29		1	3.91		
Uric acid (mg/dL)	4.96±1.43	5.00±1.34	0.94	**4.75±1.19**	**4.39±1.08**	**0.01**	0.08
AST (IU/L)	24.0 (19.3-33.0)	25.0 (20.5-31.5)	0.95	22.0 (19.5-29.0)	23.0 (19.0-27.0)	0.54	0.45†
ALT (IU/L)	24.0 (19.0-34.0)	27.5 (20.3-36.0)	0.94	27.0 (18.5-35.0)	24.0 (17.0-28.5)	0.07	0.06†
Total cholesterol (mg/dL)	139.0 (118.5-158.0)	137.5 (128.0-147.8)	0.48	133.0 (117.5-147.0)	129.0 (115.0-146.0)	0.86	0.61†

Table 3. Continued

Metabolic parameters	Sulfonylurea (*n* = 32)			SGLT2 inhibitor (*n* = 29)			
	Baseline	Day 30	*P*	Baseline	Day 30	*P*	$P_{time \times group}$
Triglyceride (mg/dL)	126.5 (101.3-183.5)	142.5 (99.3-182.3)	0.96	143.0 (104.0-188.0)	113.0 (87.5-180.5)	0.15	0.18†
HDL cholesterol (mg/dL)	43.6±10.2	42.6±8.46	0.36	42.0±9.77	43.7±10.7	0.27	0.15
LDL cholesterol (mg/dL)	65.2 (43.2-75.2)	67.2 (53.5-82.0)	0.11	63.4 (47.4-71.2)	57.2 (47.6-73.3)	0.96	0.76†
Creatinine (mg/dL)	0.85 (0.73-1.00)	0.86 (0.73-1.00)	0.41	0.84 (0.77-0.92)	0.84 (0.77-0.95)	0.14	0.54†
eGFR CKD-EPI (mL/min per 1.73 m^2)	86.0 (74.8-94.5)	85.5 (74.3-93.5)	0.39	92.0 (83.0-98.5)	91.0 (78.5-98.0)	0.16	0.59†
Fasting serum insulin (μU/mL)	9.60 (7.36-16.8)	10.7 (6.15-19.6)	0.34	**8.09 (5.31-11.9)**	**6.09 (4.50-8.94)**	**<0.001**	**0.002†**
Fasting serum FFA (μEq/L)	426.5 (351.5-527.0)	416.0 (333.5-536.8)	0.60	**412.0 (284.0-517.0)**	**523.0 (417.5-637.5)**	**<0.001**	**0.01†**
HOMA-IR	3.89 (2.32-7.42)	3.46 (2.06-7.05)	0.98	**2.72 (1.74-4.40)**	**1.92 (1.32-2.91)**	**<0.001**	**0.01†**

Table 3. Continued

Metabolic parameters	Sulfonylurea (n = 32)			SGLT2 inhibitor (n = 29)			
	Baseline	Day 30	P	Baseline	Day 30	P	$P_{time \times group}$
QUICKI	0.32±0.03	0.32±0.03	0.85	**0.33±0.03**	**0.35±0.03**	**<0.001**	**0.01**
HOMA-β (%)	**46.2 (29.8-92.4)**	**70.7 (25.8-112.3)**	**0.02**	48.7 (27.6-55.5)	38.6 (26.5-52.4)	0.09	**0.004**†
Spot urine UGCR (mg/mg)[1]	0.07 (0.05-0.26)	0.07 (0.05-0.16)	0.22	**0.11 (0.06-0.38)**	**43.3 (33.1-55.1)**	**<0.001**	**<0.001**†

Bold values indicate statistical significance. [1]Urinary glucose levels are expressed as UGCR to minimize the influence of variations of kidney function using the following formula: spot urine UGCR (mg/mg) = [spot urine glucose (mg/dL) / spot urine creatinine (mg/dL)]. Statistical significance for the time × group interaction (i.e. the group difference in the levels of metabolic parameters, before and after the administration of sulfonylurea or SGLT2 inhibitor) was evaluated by using repeat-measures analysis of variance (ANOVA) (†Non-normally distributed variables were logarithm transformed for analysis and back transformed for presentation). Two-sided paired t-test or Wilcoxon signed rank test; continuous variables are described as mean ± SD for parametric variables and as median (interquartile range) for nonparametric variables. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHB, β-hydroxybutyrate; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate; FFA, free fatty acid; HDL, high-density lipoprotein; HOMA-β, homeostatic model assessment of pancreatic β-cell function; HOMA-IR, homeostatic model assessment of insulin

resistance; LDL, low-density lipoprotein; QUICKI, quantitative insulin sensitivity check index; SGLT2, sodium-glucose cotransporter 2; UGCR, urinary glucose-to-creatinine ratio.

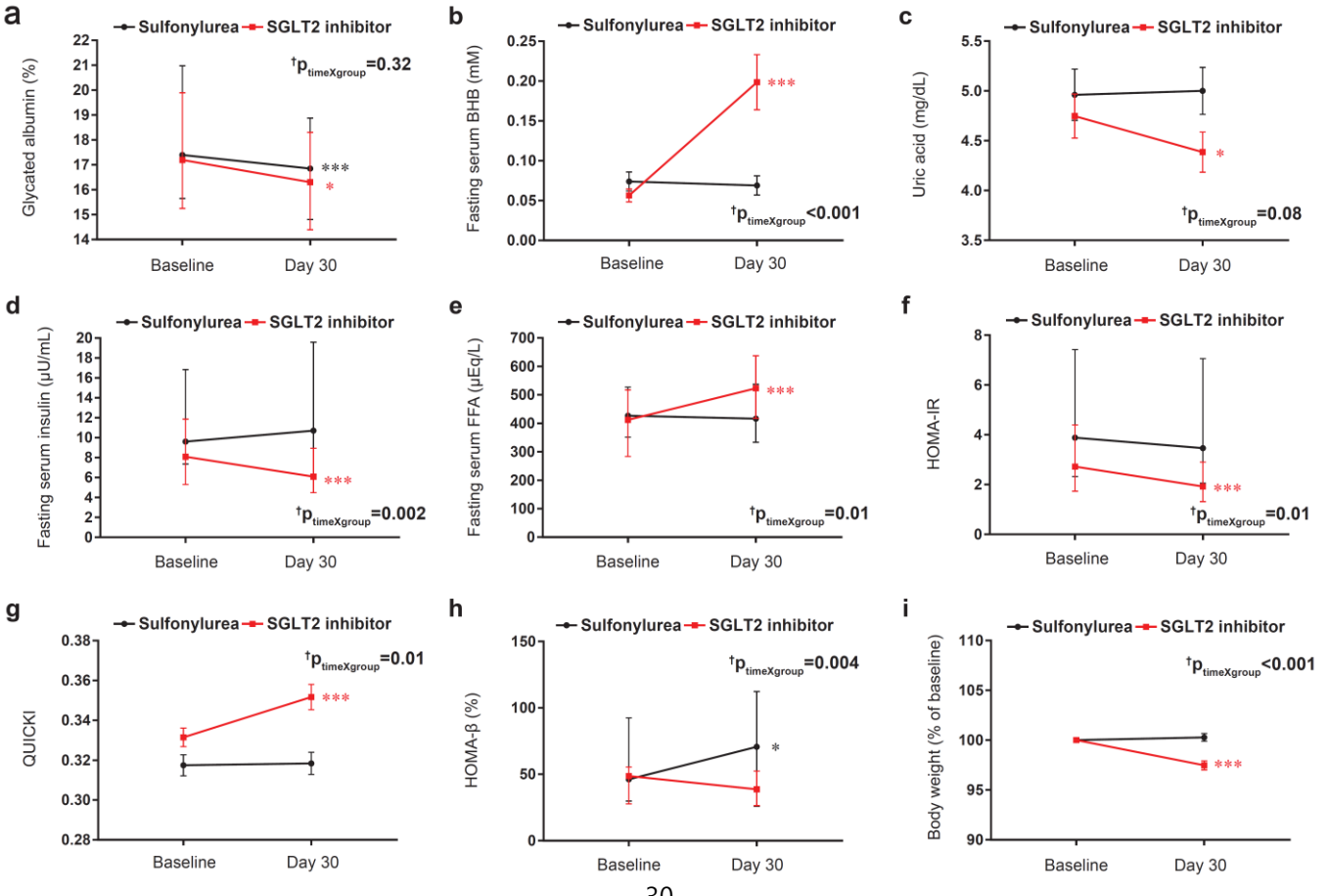

Fig. 4. Effects of sulfonylurea and SGLT2 inhibitor on metabolic parameters. a-i Changes in metabolic parameters from baseline to end of treatment (sulfonylurea group: $n = 32$, SGLT2 inhibitor group: $n = 29$). [†]Statistical significance for the time × group interaction (i.e. the group difference in the levels of metabolic parameters, before and after the administration of sulfonylurea or SGLT2 inhibitor) evaluated by repeat-measures analysis of variance (ANOVA) (Non-normally distributed variables were logarithm transformed for analysis and back transformed for presentation). Data are represented as mean ± SEM or median (interquartile range). Two-sided paired t-test or Wilcoxon signed rank test; *P <0.05 and ***P <0.001 versus baseline. BHB, β-hydroxybutyrate; FFA, free fatty acid; HOMA-β, homeostatic model assessment of pancreatic β-cell function; HOMA-IR, homeostatic model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index; SGLT2, sodium-glucose cotransporter 2.

3. SGLT2 inhibitor suppresses NLRP3 inflammasome activation

Regarding the primary endpoint of the current study, IL-1β secretion levels were measured from baseline to end of treatment in isolated macrophages after exposure to 2 mM ATP or 0.2 mM palmitate as an NLRP3 trigger, with 0.1 μg/mL LPS priming. In response to ATP stimulation, IL-1β secretion was significantly reduced after both SGLT2 inhibitor and sulfonylurea treatment (3,733 ± 360 to 2,549 ± 320 pg/mL, P <0.001; and 3,777 ± 485 to 3,121 ± 345 pg/mL, P = 0.01, respectively) (Fig. 5a). However, SGLT2 inhibitor showed a greater reduction in IL-1β secretion compared to sulfonylurea (time × group interaction P = 0.002), which remained significant after adjustment for body weight change (time × group interaction P = 0.02). This tendency was potentiated under the condition of inflammasome stimulation by palmitate (time × group interaction P <0.001, Fig. 5b).

In parallel with the IL-1β effect, TNF-α secretion was significantly reduced after SGLT2 inhibitor treatment in response to ATP and palmitate stimulation (262 ± 61 to 145 ± 22 pg/mL, P <0.001; and 271 ± 54 to 148 ± 26 pg/mL, P <0.001, respectively) (Fig. 5c, d). Alternatively, sulfonylurea treatment had no effect on TNF-α secretion in response to ATP and palmitate stimulation (210 ± 32 to 204 ± 30 pg/mL, P = 0.41; and 206 ± 35 to 215 ± 31 pg/mL, P = 0.64, respectively). A previous study found that TNF-α may cause atherosclerosis through pro-inflammatory actions on leukocytes, endothelial cells, and adipocytes.[21] A number of studies have described the protective effects of TNF antagonists on vascular diseases.[22]

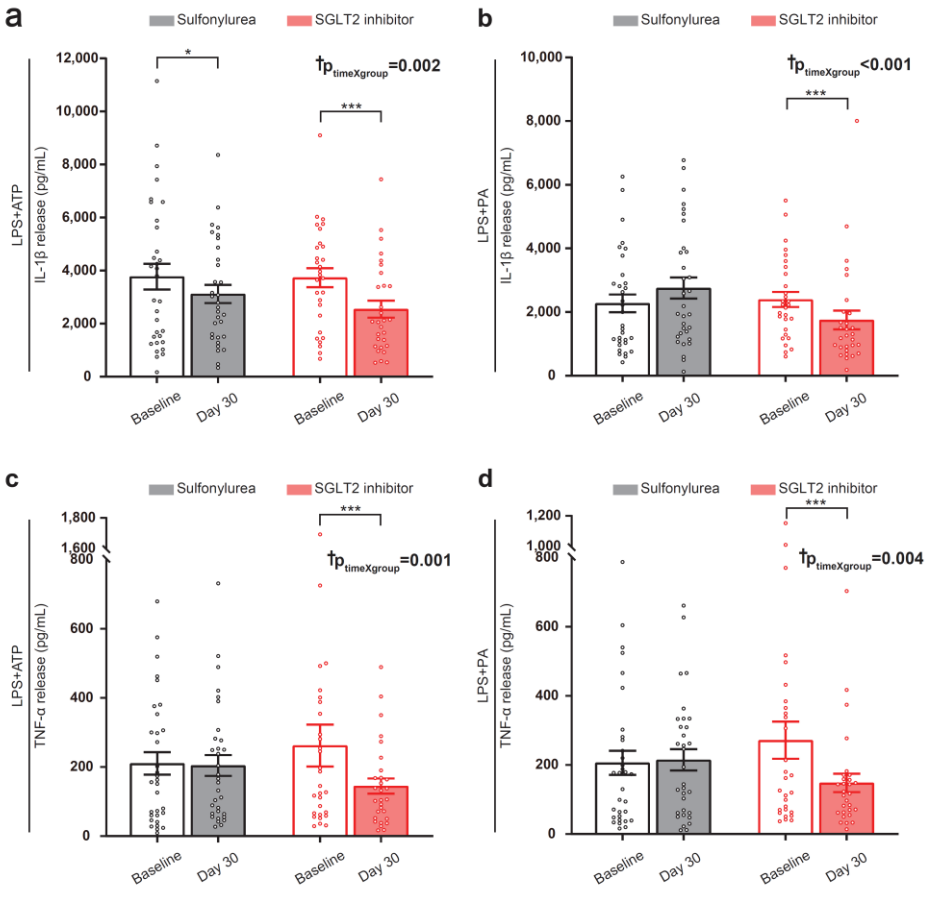

Fig. 5. Effects of sulfonylurea and SGLT2 inhibitor on secretion of IL-1β and TNF-α from macrophages. a, b ELISA assay measurement of IL-1β secretion from macrophages exposed to 2 mM ATP (a) or 0.2 mM palmitate (b) with 0.1 μg/mL LPS priming (sulfonylurea group: n = 32, SGLT2 inhibitor group: n = 29). c, d TNF-α secretion from macrophages exposed to ATP (c) or palmitate (d). Experiments were repeated twice or three times per sample; bar graphs are drawn using mean values of those results per sample, whereas the statistical significances are derived from raw data. Data are represented as mean ± SEM. Two-sided paired t-test; *P <0.05 and ***P <0.001 versus baseline.

†Statistical significance for the time × group interaction (i.e. the group difference in the levels of secretion of IL-1β and TNF-α from macrophages, before and after the administration of sulfonylurea or SGLT2 inhibitor) evaluated by repeat-measures analysis of variance (ANOVA) (Non-normally distributed variables were logarithm transformed for analysis and back transformed for presentation). ATP, adenosine triphosphate; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; LPS, lipopolysaccharide; PA, palmitate; SGLT2, sodium-glucose cotransporter 2; TNF-α, tumor necrosis factor-α.

To further evaluate metabolic factors associated with changes in inflammasome activity, correlation analyses were conducted. Changes in fasting serum insulin or BHB levels were significantly correlated with changes in NLRP3 inflammasome activity (Fig. 6). There was no significant correlation between changes in body weight and changes in IL-1β or TNF-α release (Fig. 7).

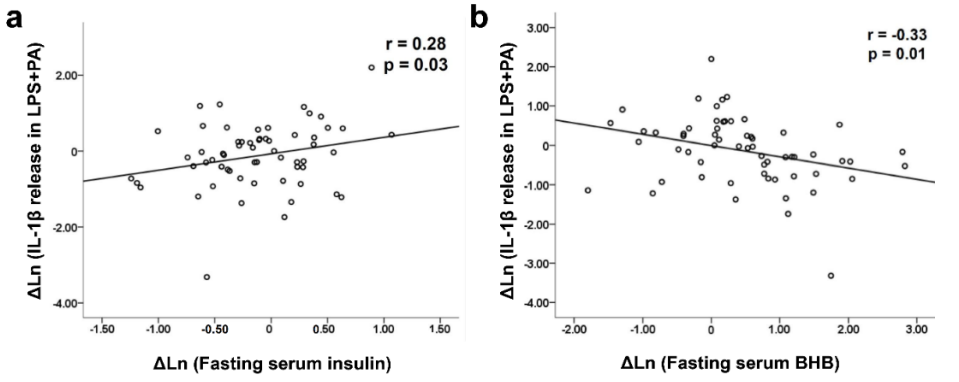

Fig. 6. Correlation between changes in fasting serum insulin (a) or BHB (b) levels and changes in IL-1β release (*n* = 61). Open circles are data points from individual subjects. Pearson's correlation coefficient. Variables were logarithm transformed for analysis. 0.1 μg/mL LPS; 0.2 mM PA. ΔLn (Fasting serum insulin) = [Ln (Fasting serum insulin at end of treatment (μU/mL)) – Ln (Fasting serum insulin at baseline (μU/mL))]; ΔLn (Fasting serum BHB) = [Ln (Fasting serum BHB at end of treatment (mM)) – Ln (Fasting serum BHB at baseline (mM))]; ΔLn (IL-1β release) = [Ln (IL-1β release at end of treatment (pg/mL)) – Ln (IL-1β release at baseline (pg/mL))]; IL-1β levels are the mean values of measurements repeated twice or three times per sample. BHB, β-hydroxybutyrate; IL-1β, interleukin-1β; Ln, logarithm(natural); LPS, lipopolysaccharide; PA, palmitate.

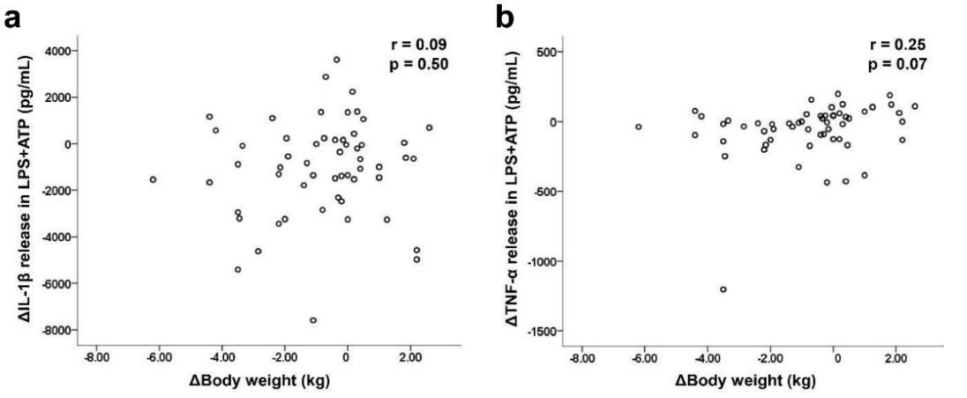

Fig. 7. Correlation between changes in body weight and changes in IL-1β (a) and TNF-α (b) release (*n* = 61). Open circles are data points from individual subjects. Pearson's correlation coefficient. 0.1 μg/mL LPS; 2 mM ATP. ΔBody weight (kg) = [Body weight at end of treatment (kg) – Body weight at baseline (kg)]; ΔIL-1β release (pg/mL) = [IL-1β release at end of treatment (pg/mL) – IL-1β release at baseline (pg/mL)]; ΔTNF-α release (pg/mL) = [TNF-α release at end of treatment (pg/mL) – TNF-α release at baseline (pg/mL)]; IL-1β and TNF-α levels are the mean values of measurements repeated twice or three times per sample. ATP, adenosine triphosphate; IL-1β, interleukin-1β; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

Next, we measured the mRNA levels of *IL1B*, *TNFA*, and *NLRP3* in unstimulated macrophages following 30-day treatment with sulfonylurea or SGLT2 inhibitor. The transcripts encoding IL-1β were significantly decreased following SGLT2 inhibitor treatment (Fig. 8a). Transcript levels of *TNFA* and *NLRP3* tended to decrease after SGLT2 inhibitor treatment, but not statistically significant (Fig. 8b, c). SGLT2 inhibitor significantly decreased the LPS- and ATP-induced processing of the biologically active form of IL-1β in cell lysates (Fig. 8d). RNA sequencing with GO enrichment analysis identified 2 clusters of upregulated and downregulated genes in SGLT2 inhibitor group compared to sulfonylurea group. The downregulated genes were highly enriched for immune-related receptor signaling pathway, activation of immune response, and regulation of immune system process, whereas the upregulated cluster was enriched for genes involved in cellular components such as vesicles and extracellular region (Fig. 9).

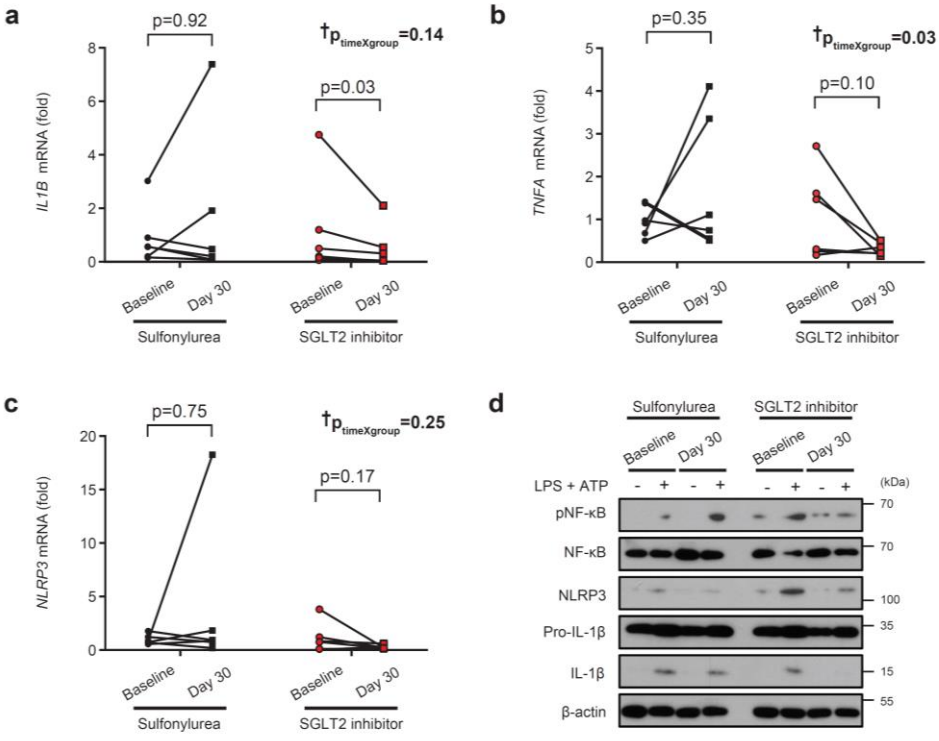

Fig. 8. mRNA and protein levels of molecules regarding NLRP3 inflammasome activation in macrophages following treatment with sulfonylurea or SGLT2 inhibitor. a-c mRNA levels encoding *IL1B* (a), *TNFA* (b), and *NLRP3* (c) (n = 6 per group). Two-sided paired t-test or Wilcoxon signed rank test. †Statistical significance for the time × group interaction (i.e. the group difference in the mRNA levels, before and after the administration of sulfonylurea or SGLT2 inhibitor) evaluated by repeat-measures analysis of variance (ANOVA) (Non-normally distributed variables were logarithm transformed for analysis and back transformed for presentation). d Representative protein levels of molecules regarding NLRP3 inflammasome activation with or without LPS and ATP stimulation. ATP, adenosine triphosphate; IL-1β, interleukin-1β; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor kappa-light-chain-enhancer

of activated B cells; NLRP3, NLR family, pyrin domain-containing 3; SGLT2, sodium-glucose cotransporter 2; TNF-α, tumor necrosis factor-α.

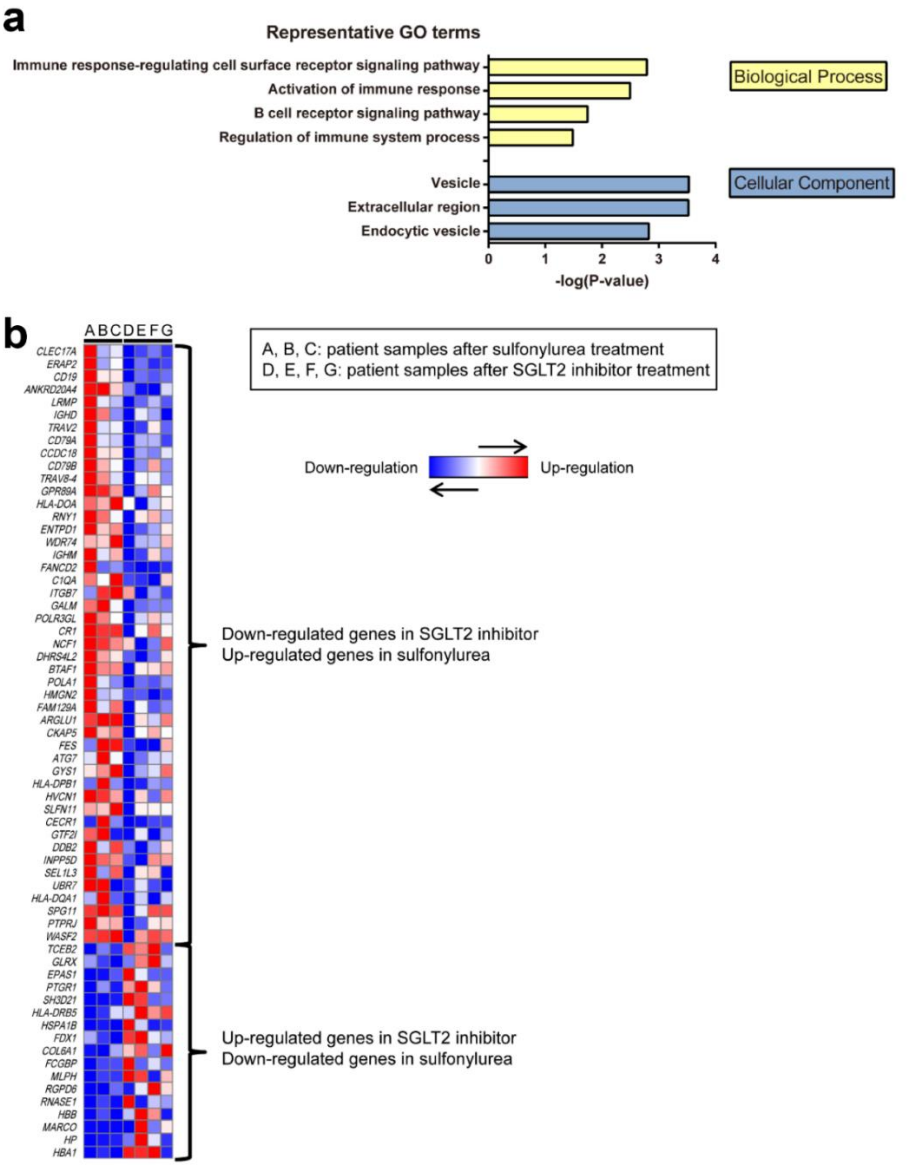

Fig. 9. GO analysis for differentially expressed genes between two groups (sulfonylurea group (*n* = 3) vs. SGLT2 inhibitor group (*n* = 4)). a Enrichment of biological processes in clusters of downregulated (yellow bars) and upregulated (blue bars) macrophage genes in SGLT2 inhibitor group

compared to sulfonylurea group. b Heatmap representation of macrophage genes of these 2 clusters. GO, gene ontology; log, logarithm(base 10); SGLT2, sodium-glucose cotransporter 2.

Regarding serum IL-1β and IL-18 levels, despite a tendency to decrease after SGLT2 inhibitor treatment, there were no significant changes in those concentrations (Table 4). This may be attributable to the following factors: First, serum IL-1β levels are often undetectable even in diseases with clear evidence of increased IL-1 activity.[5,23-25] IL-1β exerts its effect in an autocrine/paracrine fashion and, consequently, the detected levels of IL-1β in the serum may be very low;[26] in our study, 37 out of 61 participants, had serum IL-1β levels below the detection limits of the assay used. Second, extensive use of aspirin or statin in both sulfonylurea and SGLT2 inhibitor groups might attenuate changes in serum proinflammatory cytokines.[27] Finally, as activation of the NLRP3 inflammasome requires two independent steps: priming and triggering,[28] IL-1β signaling in the immune cells of vessel tissues or of atherosclerotic lesions enriched for various endogenous danger signals, rather than circulating IL-1β levels, might be a major determinant of atheroma formation or heart failure.[5,29,30] In the present study, we identified that treatment with an SGLT2 inhibitor can reduce the activation of NLRP3 inflammasome in human macrophages after stimulation with LPS and ATP/palmitate.

Table 4. Effects of sulfonylurea and SGLT2 inhibitor on serum IL-1β and IL-18 levels

	Sulfonylurea (*n* = 32)			SGLT2 inhibitor (*n* = 29)		
	Baseline	Day 30	*P*	Baseline	Day 30	*P*
Serum IL-1β (pg/mL)[1]	0.17 (0.08-0.53)	0.13 (0.05-0.84)	0.83	0.11 (0.07-0.35)	0.08 (0.06-0.23)	0.29
Serum IL-18 (pg/mL)	227.7 (168.8-283.0)	243.4 (181.2-266.4)	0.54	218.2 (174.4-262.8)	216.6 (164.7-286.9)	0.57

Statistical significance was evaluated by Wilcoxon signed rank test; values are described as median (interquartile range). [1]Not detectable (<0.033 pg/mL) in 37 (18 in sulfonylurea group and 19 in SGLT2 inhibitor group) out of 61 participants; excluded from the analyses. IL-1β, interleukin-1β; IL-18, interleukin-18; SGLT2, sodium-glucose cotransporter 2.

4. Effects of BHB, glucose, and insulin on NLRP3 inflammasome

Although it has been reported that BHB blocks activation of the NLRP3 inflammasome-IL-1β process by preventing K^+ efflux and reducing apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization and speck formation,[12] we found that its effective concentration was rather high compared to the modest increase seen after SGLT2 inhibitor treatment. Therefore, other mediators besides BHB may play important roles in modulating NLRP3 inflammasome activity in humans. As SGLT2 inhibitor significantly reduced fasting serum levels of insulin as well as glucose, we performed ex vivo experiments using human macrophages to investigate whether these metabolites could affect NLRP3 inflammasome activity.

We confirmed that BHB dose-dependently inhibited IL-1β secretion from human macrophages stimulated by LPS and ATP (Fig. 10a). Co-treatment with 2-DG, a non-metabolizable glucose analogue that blocks glycolysis and has been used to mimic a condition of glucose starvation,[31] significantly decreased IL-1β secretion in a dose-dependent manner (Fig. 10b). Inversely, a high glucose condition (25 mM) markedly increased IL-1β production, which was blocked by administration of 2-DG (Fig. 10c). Regarding the role of insulin on NLRP3 inflammasome, 10 nM insulin significantly elevated IL-1β secretion (Fig. 11a). Furthermore, co-treatment with insulin attenuated the inhibitory effect of BHB on NLRP3 inflammasome activation (Fig. 11b).

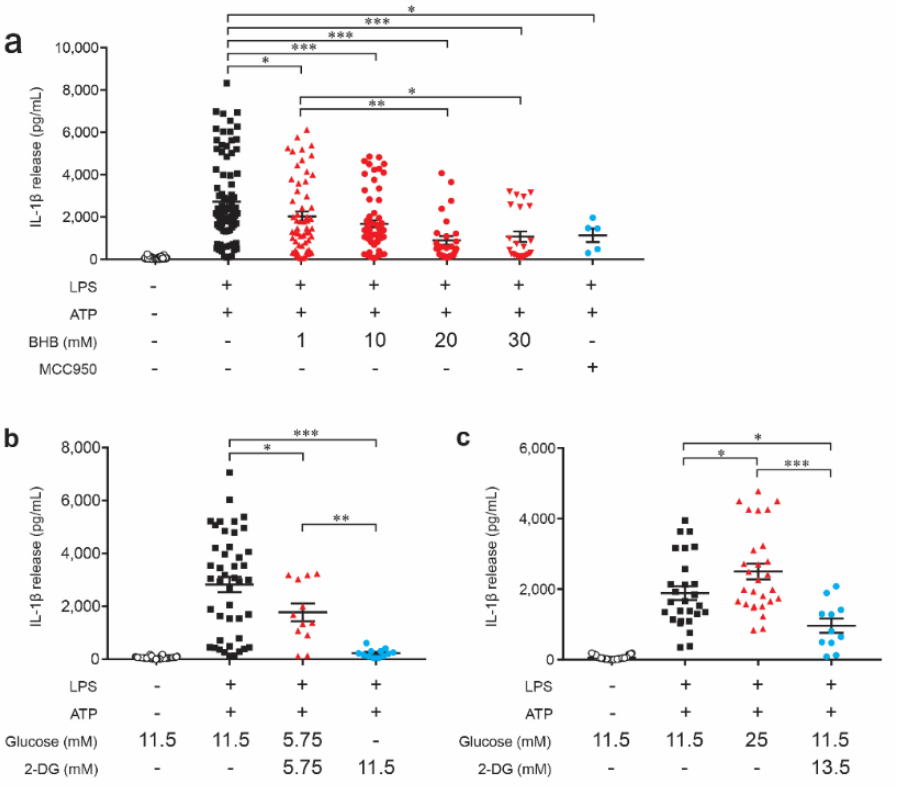

Fig. 10. Effects of BHB and glucose on NLRP3 inflammasome activation in human macrophages by ELISA assay. IL-1β secretion when exposed to vehicle or 2 mM ATP with 0.1 μg/mL LPS priming and increased BHB (1, 10, 20, and 30 mM) or 100 nM MCC950, a small-molecule inhibitor of NLRP3 inflammasome (administered for the last 5 hrs as a positive control) (a); increased 2-DG (b); increased glucose (11.5 mM vs. 25 mM) (c). 5 to 64 biologically independent samples per treatment; experiments on each treatment were repeated up to three times per sample. Symbols are data points from independent experiments: *n* = 137, 90, 58, 64, 28, 24, 5 (**a**, left to right), 66, 44, 12, 12 (**b,** left to right), 39, 27, 28, 11 (**c**, left to right). Data are represented as mean ± SEM. One-way analysis of variance (ANOVA) using Tukey's test or a two-tailed Student's t-test with the Bonferroni method for adjusting *P* values;

*P <0.05, **P <0.01, and ***P <0.001. ATP, adenosine triphosphate; BHB, β-hydroxybutyrate; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NLRP3, NLR family, pyrin domain-containing 3; 2-DG, 2-deoxyglucose.

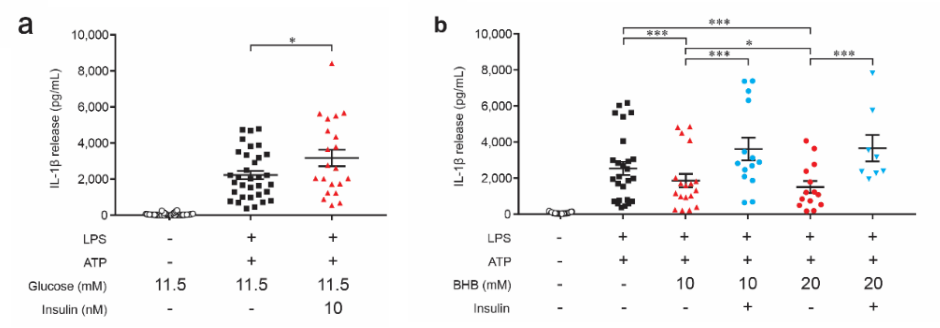

Fig. 11. Effects of insulin on NLRP3 inflammasome activation in human macrophages by ELISA assay. IL-1β secretion when exposed to vehicle or 2 mM ATP with 0.1 μg/mL LPS priming and 11.5 mM glucose with/without 10 nM insulin (a); increased BHB (10 mM vs. 20 mM) with/without 10 nM insulin (b). 5 to 64 biologically independent samples per treatment; experiments on each treatment were repeated up to three times per sample. Symbols are data points from independent experiments: n = 48, 33, 21 (**a**, left to right), 39, 26, 18, 14, 14, 8 (**b**, left to right). Data are represented as mean ± SEM. One-way analysis of variance (ANOVA) using Tukey's test or a two-tailed Student's t-test with the Bonferroni method for adjusting P values; *P <0.05 and ***P <0.001. ATP, adenosine triphosphate; BHB, β-hydroxybutyrate; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NLRP3, NLR family, pyrin domain-containing 3.

5. Short-term isocaloric ketogenic diet suppresses NLRP3 inflammasome activation in humans

Similar to the metabolic condition after SGLT2 inhibitor treatment, previous reports demonstrate that KD can induce elevated serum BHB levels, while decreasing fasting glucose and insulin levels.[14,32] To validate the in vivo effect of high BHB levels and low insulin levels on NLRP3 inflammasome activation in our study, 15 healthy subjects underwent isocaloric KD for 3 days.

Fasting serum glucose levels were not significantly changed after KD in our study (Fig. 12a). KD caused a significant increase in fasting serum BHB concentration (median [interquartile range], 0.03 [0.02 - 0.06] to 0.49 [0.36 - 1.25] mM, P <0.001) (Fig. 12b) and a decrease in fasting serum insulin levels (mean ± SD, 7.00 ± 3.87 to 4.61 ± 3.54 μU/mL, P = 0.01) (Fig. 12c). Total cholesterol and fasting serum FFA were increased after KD (185.6 ± 31.8 to 208.4 ± 27.9 mg/dL, P <0.001; and 504.6 ± 288.3 to 998.4 ± 411.1 μEq/L, P = 0.002, respectively) (Fig. 12d, e). KD significantly improved insulin resistance indices (Fig. 12f, g) and reduced body weight (67.8 ± 15.6 to 65.9 ± 15.2 kg, P <0.001; mean change of -2.7% (-1.90 kg), Fig. 12h). Loss of total body water was significant, whereas body fat mass was not changed (Table 5).

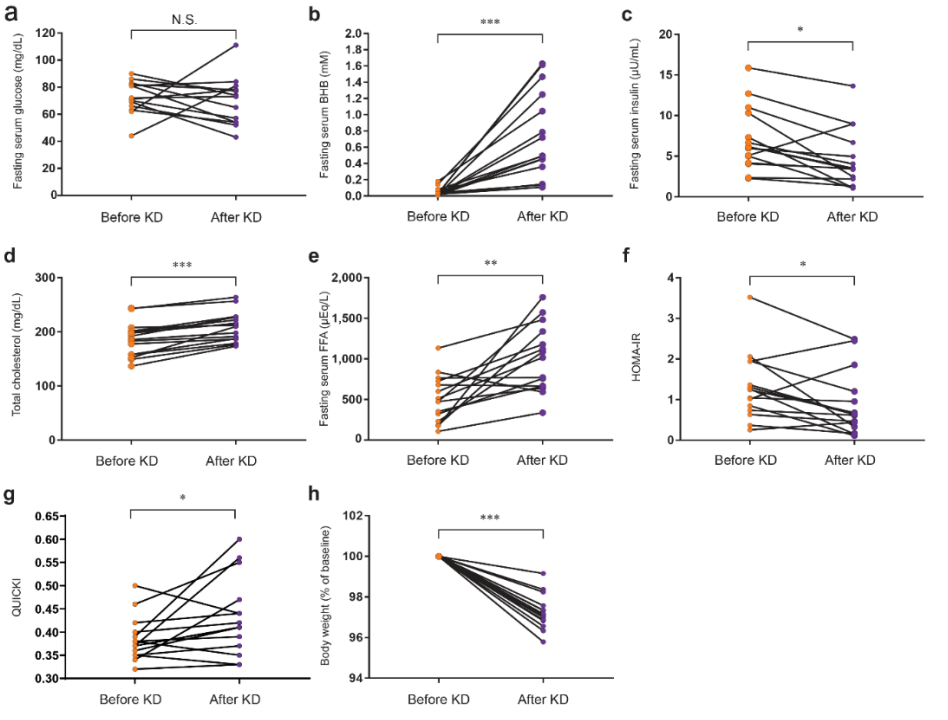

Fig. 12. Effects of isocaloric ketogenic diet on metabolic parameters. a-h Changes in metabolic parameters from baseline to day 3 of isocaloric KD (n = 15). Two-sided paired t-test or Wilcoxon signed rank test; *P <0.05, **P <0.01, and ***P <0.001 versus baseline. BHB, β-hydroxybutyrate; FFA, free fatty acid; HOMA-IR, homeostatic model assessment of insulin resistance; KD, ketogenic diet; N.S., non-significant; QUICKI, quantitative insulin sensitivity check index.

Table 5. Body composition changes after isocaloric ketogenic diet in healthy subjects (*n* = 15)

Body composition	Before KD	After KD	Mean Change[1]	*P*
Fat-free mass (kg)	50.0 (40.4-59.7)	47.9 (39.2-57.8)	-1.55	0.001
Total body water (kg)	36.7 (29.5-44.1)	35.2 (28.6-42.6)	-1.19	0.001
Fat mass (kg)	13.4 (11.7-18.6)	12.8 (11.9-18.5)	-0.32	0.06
Percentage of body fat (%)	23.2 (18.0-25.3)	23.1 (17.7-26.2)	0.20	0.46

Statistical significance was evaluated by Wilcoxon signed rank test; values are described as median (interquartile range).

[1]Mean of values of [at Day 3 – at baseline]. KD, ketogenic diet.

IL-1β secretion in response to both ATP and palmitate stimulation in macrophages decreased significantly after KD (2,532 [1,882 - 5,645] to 1,719 [840 - 3,883] pg/mL, P <0.001; and 1,637 [1,047 - 2,659] to 1,045 [470 - 1,441] pg/mL, P <0.001, respectively) (Fig. 13a, b). The secretion of TNF-α in response to both ATP and palmitate stimulation also decreased significantly after KD (76 [57 - 212] to 64 [43 - 160] pg/mL, P = 0.02; and 65 [38 - 139] to 58 [24 - 93] pg/mL, P = 0.004, respectively) (Fig. 13c, d).

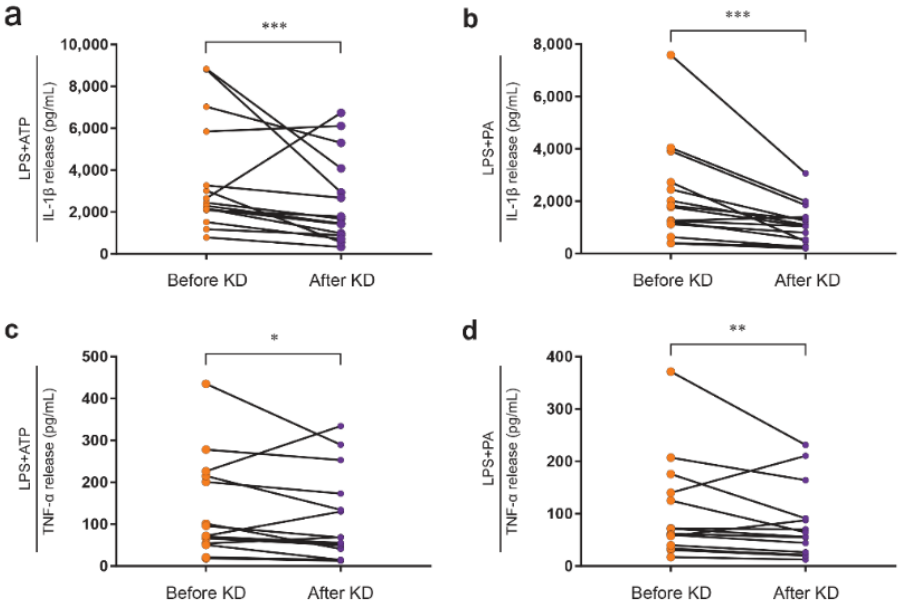

Fig. 13. Effects of isocaloric ketogenic diet on secretion of IL-1β and TNF-α from healthy subjects (*n* = 15)' macrophages. a-d Changes in secretion of IL-1β and TNF-α from macrophages from baseline to day 3 of isocaloric KD: ELISA assay measurement of IL-1β secretion from macrophages exposed to 2 mM ATP (a) or 0.2 mM palmitate (b) with 0.1 μg/mL LPS priming; TNF-α secretion from macrophages exposed to ATP (c) or palmitate (d). Experiments were repeated twice or three times per sample; graphs are drawn using mean values of those results per sample, whereas the statistical significances are derived from raw data. Two-sided paired t-test or Wilcoxon signed rank test; **P* <0.05, ***P* <0.01, and ****P* <0.001 versus baseline. ATP, adenosine triphosphate; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; KD, ketogenic diet; LPS, lipopolysaccharide; PA, palmitate; TNF-α, tumor necrosis factor-α.

IV. DISCUSSION

We demonstrate that an SGLT2 inhibitor significantly suppresses NLRP3 inflammasome activation and subsequent secretion of IL-1β in human macrophages, via increased serum BHB levels and decreased serum levels of insulin, among patients with T2D and CVD, regardless of glycemic control. Furthermore, ex vivo experiments with human macrophages and KD study in healthy subjects verify the mediatory roles of BHB, insulin, and glucose on the modulating activity of the NLRP3 inflammasome.

NLRP3 inflammasome is involved in the molecular etiology of atherosclerosis and heart failure.[2,4,5,7] Mice with constitutively increased IL-1 signaling because of IL-1 receptor antagonist deficiency develop a transmural arterial inflammation leading to lethal aneurysms.[33] In human atherosclerotic plaques, the expression of IL-1β appears to correlate with the progression of atherosclerotic plaques.[34] In an in vitro study, IL-1β and TNF-α decreased collagen synthesis and activated collagen degradation in rat cardiac fibroblasts, suggesting that IL-1β and TNF-α may contribute to ventricular dilation and heart failure.[3]

Although there is a paucity of large clinical trial data, a number of observational, pilot, and preclinical studies suggest a beneficial role of IL-1 blockade in the pathological processes of CVD. Experimental studies in atherosclerosis-prone animals have consistently shown that genetic deletion or pharmacological inhibition of IL-1 signaling reduces the formation and progression of atherosclerotic plaques.[5,7] For example, transplantation of bone marrow cells from *NLRP3*- or *IL1*-deficient mice into mice models of atherosclerosis resulted in significant amelioration of aortic atherogenesis.[4] In

terms of heart failure, when exposed to ischemia-reperfusion injury, mice that lack the *NLRP3* gene have a smaller infarct size and better cardiac function than wild-type mice.[35] In the recent CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study) trial, canakinumab, a monoclonal antibody against IL-1β, significantly prevented recurrent CV events among patients with a prior MI and high levels of proinflammatory biomarkers.[36] This RCT implicates that reducing low-grade inflammation in CVD patients without affecting blood cholesterol levels can decrease the risk of developing CV events. In addition, it confirms the clinical importance of the proatherogenic characteristics of IL-1β in humans.

Previously, subgroup analyses from EMPA-REG OUTCOME or CANVAS Program did not show a significant reduction in MI or stroke by SGLT2 inhibitors;[9,10] however, these secondary endpoints could not be proven with relatively small numbers of trial participants. In a recent meta-analysis of 71 RCTs[37] and the large multinational CVD-REAL 2 study,[38] SGLT2 inhibitors significantly decreased MI and stroke events, as well as all-cause death and hospitalization for heart failure, suggesting their anti-atherothrombotic effects. Our study suggests that SGLT2 inhibitors offer not only improvements in metabolic profiles but also inhibition of IL-1β secretion and other pro-inflammatory cytokines, potentially reducing the development of CVD in high risk patients with diabetes.

Glycosuria resulting from SGLT2 inhibition subtracts glucose from blood, and relative hypoinsulinemia reduces tissue glucose uptake, producing a compensatory increase in lipid oxidation and a concomitant rise in serum BHB levels.[8] Along with reproducible outcomes of the inhibitory effect of BHB on NLRP3 inflammasome in our ex vivo study, we assumed that decreases in

serum insulin level induced by SGLT2 inhibitor might also play a role in NLRP3 inflammasome inhibition. A recent study reported that insulin reinforced a pro-inflammatory pattern of macrophages via increased glucose uptake followed by subsequent glucose catabolism and production of reactive oxygen species, which eventually secreted IL-1β mediated by NLRP3 inflammasome activation.[39] Indeed, sulfonylurea increases insulin secretion, in turn reducing blood glucose levels. In contrast, SGLT2 inhibitor decreases blood glucose by renal glucose excretion, in turn decreasing serum insulin levels. These opposing changes in insulin levels might differentially regulate NLRP3 inflammasome in the two groups. In addition, similar to our findings, SGLT2 inhibitors have been shown to reduce serum uric acid levels through increasing renal clearance of uric acid,[40] a potent activator of NLRP3 inflammasome.[12] Taken together, not only increased serum BHB, but also decreased serum levels of insulin, glucose, and uric acid, account for the overall inhibitory effect of SGLT2 inhibitor on the NLRP3 inflammasome.

Using a short-term isocaloric KD protocol, which alters body composition primarily by water rather than metabolically active tissues, we demonstrate that a reduction in NLRP3 inflammasome activity results from increased serum BHB concentrations and decreased insulin secretion rather than fat loss per se. Similar to our data, the activity of NLRP3 inflammasome was suppressed in healthy individuals after 24-h fasting,[13] which can significantly increase BHB levels, although this study did not measure serum levels of BHB in the fasted states.

A limitation of this study is that we did not assess whether the changes in inflammasome activity by SGLT2 inhibitor could be linked to the improvement in CV outcomes due to a relatively short-term trial design. However, both

experimental evidence using *NLRP3/IL1* knockout mice and the recent large-scale CANTOS trial indicate that therapeutic interference with IL-1β production or function can improve long-term CV outcomes.[4,7,36] The present study is a proof-of-concept RCT aimed to elucidate the glucose-independent mechanism of SGLT2 inhibitors regarding CV protection. Preventive benefits were observed after 3 months of treatment in the EMPA-REG OUTCOME study; the present study demonstrates that only 1 month of SGLT2 inhibitor treatment or 3 days of KD can suppress inflammasome activation in individuals with T2D at high risk of CVD or healthy people, respectively. Besides our findings, it is important to bear in mind that there may be multiplicity of other pathways or mechanisms which can be directly or indirectly involved in the protection of CVD by SGLT2 inhibitors. Nevertheless, the present study provides distinct evidence that SGLT2 inhibitor suppresses NLRP3 inflammasome activation in patients with T2D at high risk of CVD.

V. CONCLUSION

The present study shows that SGLT2 inhibitor treatment in patients with T2D at high risk of CVD attenuates NLRP3 inflammasome activation and secretion of IL-1β, which has a pathogenic effect on both T2D and CVD, in part via increased serum BHB and decreased serum insulin, glucose, and uric acid (Fig. 14). Moreover, the effects of BHB and insulin on NLRP3 inflammasome activation have also been verified ex vivo and in a human study of KD. The present data suggest that these mechanisms might help to explain the cardioprotective effects of SGLT2 inhibitor in humans.

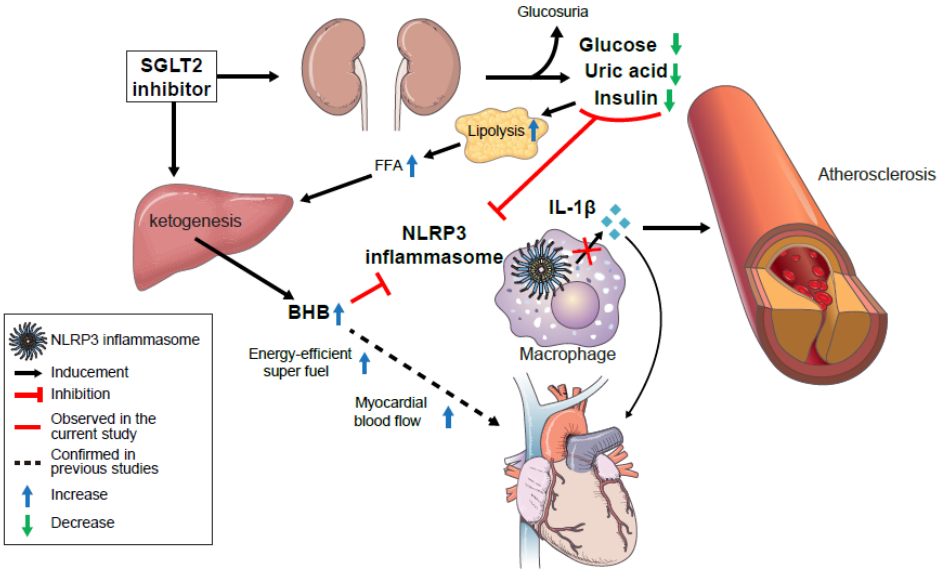

Fig. 14. Scheme representing the proposed effects of SGLT2 inhibitor on NLRP3 inflammasome activation. BHB, β-hydroxybutyrate; FFA, free fatty acid; IL-1β, interleukin-1β; NLRP3, NLR family, pyrin domain-containing 3; SGLT2, sodium-glucose cotransporter 2.

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ABSTRACT(IN KOREAN)

SGLT2 억제제가 심혈관계 질환이 있는 제2형 당뇨병 환자에서 NLRP3 인플라마솜 활성도와 케톤체 대사에 미치는 영향

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김소라

SGLT2 (Sodium-glucose cotransporter 2) 억제제는 이전의 여러 대규모 임상연구에서, 제2형 당뇨병 환자에서 심혈관계 질환의 발생을 감소시켰다. SGLT2 억제제는 혈당, 체중, 혈압을 감소시키고, 이뇨작용을 유발하는 등 심혈관계 질환 발생의 위험인자가 되는 여러 대사적, 혈역학적 이상을 교정한다. 그러나 이외에도 심혈관 보호 효과에 관한 다른 기전들이 있을 것으로 생각되지만, 아직 충분히 밝혀지지 않았다. NLRP3 (NLR family, pyrin domain-containing 3) 인플라마솜의 활성화와 이로 인한 IL (interleukin)-1β의 분비는 동맥경화와 심부전의 병인에 관여한다. 한편, 케톤체, 특히 β-hydroxybutyrate는 대식세포내의 NLRP3 인플라마솜의 활성화를 억제한다는 것이 알려져 있다. SGLT2 억제제는 그 약리학적 특성에 의해 혈청 β-hydroxybutyrate 농도를 증가시킨다. 따라서 본 연구에서는 SGLT2 억제제가 NLRP3 인플라마솜 활성에 미치는 영향을 확인하여, SGLT2 억제제의 심혈관 보호 효과에 관한 기전을 제시하고자 하였다.

본 연구는 무작위 활성대조군 연구로서, 심혈관계 질환의 고위험 인자를 가진 제2형 당뇨병 환자 총 61명(평균 나이 64.4세, 평균 당화혈색소 7.32%)에게 SGLT2 억제제(29명) 또는 설폰요소제(32명)를 무작위 배정하여 30일간 투여하였다. NLRP3 인플라마솜 활성 억제에 있어 혈당 강하의 영향을 배제하기 위해, 위약 대신 설폰요소제를 활성대조군으로 이용하였다. 약물 투여 전, 후에 각각 전혈로부터 분리한 대식세포를 이용하여 NLRP3 인플라마솜의 활성도를 분석하고, 혈청 포도당, β-hydroxybutyrate, 인슐린 농도 등을 포함한 여러 혈청 대사 지표들을 측정하여 비교하였다. 또한, 체내 혈청 β-hydroxybutyrate 농도를 유의하게 증가시킨다고 알려진 키톤유발식이를 건강인 15명을 대상으로 3일간 시행하여, 혈청 β-hydroxybutyrate 농도의 증가가 NLRP3 인플라마솜의 활성을 억제하는지 확인하였다.

그 결과, SGLT2 억제제와 설폰요소제의 혈당 강하 효과는 비슷하였지만, SGLT2 억제제 투여군에서 설폰요소제 투여군에 비해 대식세포로부터의 IL-1β 분비량이 더 크게 감소하였다. SGLT2 억제제 투여군에서는 혈청 β-hydroxybutyrate 농도의 증가와 혈청 인슐린 농도의 감소를 보였으나, 설폰요소제 투여군에서는 이들 농도에 변화가 없었다. 또한, β-hydroxybutyrate, 인슐린 등이 NLRP3 인플라마솜 활성에 대해 직접적인 효과를 갖는지 확인하기 위해 시행한 대식세포를 이용한 ex vivo 실험과, 건강인을 대상으로 한 키톤유발식이 연구를 통해 고농도 β-hydroxybutyrate 와 저농도 인슐린이 갖는 NLRP3 인플라마솜 활성 억제 효과를 검증하였다.

결론적으로, 본 연구에서는 SGLT2 억제제 투여가 심혈관계 질환의 고위험 인자를 가진 제2형 당뇨병 환자의 체내에서 NLRP3 인플라마솜의 활성을 억제하여 IL-1β 분비를 감소시킨다는 것을 확인하였다. 이는 혈당 조절과는 독립적으로, 혈청 β-hydroxybutyrate 농도의 증가와 혈청 인슐린 농도의 감소에 의한 것임을 확인하였다. 이전의 여러 연구들은 IL-1β의 작용을 억제하는 것이 심혈관계 질환의 병태생리학적 과정을 차단하는데에 효과가 있음을 보여주었다. 따라서, 본 연구의 결과는 SGLT2 억제제의 심혈관 보호 효과의 기전을 설명하는 데에 중요한 이론적 근거를 제시할 것이다.

핵심되는 말 : SGLT2 억제제, 제2형 당뇨병, NLRP3, 인플라마솜, IL-1β, 심혈관계 질환, beta-hydroxybutyrate, 키톤유발식이

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