β-Hydroxybutyrate promotes basal insulin secretion while decreasing glucagon secretion in mouse and human islets

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ABSTRACT

Dietary carbohydrates raise blood glucose and limiting carbohydrate intake improves glycemia in patients with T2D. Low carbohydrate intake (< 25 g) allows the body to utilize fat as its primary fuel. As a consequence of increased fatty-acid oxidation, the liver produces ketones to serve as an alternative energy source. β-Hydroxybutyrate (βHB) is the most abundant ketone. While βHB has a wide range of functions outside of the pancreas, its effect on islet cell function remains understudied. In this study, we examined islet response to racemic βHB treatment, and observed an increase in insulin secretion in human islets treated with βHB at basal glucose concentrations (3 mM glucose). Because βHB is a chiral molecule, existing as both R and S forms, we further examined insulin and glucagon secretion following acute treatment with individual βHB enantiomers in human and C57BL6/J mouse islets. We found that acute treatment with R-βHB increases insulin secretion and decreases glucagon secretion at physiological glucose concentrations in human and mouse islets. Proteomic analysis of human islets treated with R-βHB over 72 h showed altered abundance of proteins that may promote islet cell health and survival. However, live cell imaging revealed that neither enantiomer of βHB protected dispersed mouse islet cells from cell death. Collectively, our data show that physiological concentrations of βHB influence hormone secretion and signaling within pancreatic islets.

INTRODUCTION

The secretion of insulin and glucagon from the endocrine pancreas is essential for maintaining glucose homeostasis and regulating metabolism throughout the body (1). Although glucose is the primary regulator of islet hormone secretion, other nutrients and metabolites are also implicated (1). Numerous studies have investigated the effects of non-glucose substrates on islet cell function (2,3), but some metabolites, such as ketone bodies, remain understudied.

Ketone bodies are produced as a byproduct of protein and fatty acid metabolism in the liver and are used as an energy source in the absence of glucose (4). Of the 3 ketone bodies (beta-hydroxybutyrate, acetoacetate, and acetyl coA) found in mammals, beta-hydroxybutyrate (βHB) is the most abundant (5). βHB exists in two enantiomeric forms – R-βHB and S-βHB (5). R-βHB is the physiological enantiomer, while S-βHB, is isomerized in the liver into the physiological R enantiomer before being metabolized (5–7). The serum concentration of βHB in humans is <1 mM (6,8,9), but this level increases to > 2 mM during exercise, prolonged fasting and/or strict carbohydrate restriction, as seen with ketogenic diets (6, 10,11). This physiologic state of ketosis is different from diabetic ketoacidosis (DKA), a serious medical condition where ketone concentrations in the blood can reach as high as 20 mM (12,13).

Investigations into the role of βHB in various cell types have shown that its metabolism in cardiomyocytes is upregulated for use as an energy source in the failing heart (14), and that βHB protects hepatocytes from ER-stress-induced cell death (15). Studies highlighting the benefits of the ketogenic diet have even led to an increase in the popularity of exogenous ketone supplements in an effort to further promote weight loss and slow the onset of T2D in prediabetic patients (16). Recently, clinical studies have shown that while exogenous ketone supplements lower blood glucose levels in healthy individuals (17), this effect does not extend to adults with T2D, where exogenous ketone supplements did not improve glycemic control or lower blood glucose following acute or chronic treatment (18).

However, the direct effect of βHB on pancreatic islet function has not been studied in detail. Early studies (19–22) suggest that βHB can potentiate insulin release in rat islets, and newer studies show that ketone esters can stimulate insulin release in obese, but not lean, C57BL/6J mice (23). However, there is currently little to no evidence outlining the effect of βHB on hormone secretion from human islets or on their survival following exposure to stress-induced stimuli.

In this study, we investigated the effect of acute and chronic βHB treatment on insulin and glucagon secretion in both human and mouse pancreatic islets (Figure 1A,B). We examined hormone secretion in response to racemic βHB, as well as individual enantiomers to delineate any possible enantiomer-specific effects. In addition, we used mass spectrometry (MS)-based proteomics to identify proteins involved in islet response to prolonged ketone exposure, and live cell imaging to determine whether this prolonged exposure affects beta cell health and survival under conditions of ER stress. We found that R-βHB increases insulin secretion and decreases glucagon secretion under physiological glucose concentrations in both human and mouse islets. Proteomic analysis revealed differential abundance of proteins involved in cell proliferation, amino acid biosynthesis and metabolism upon R-βHB treatment. Interestingly, neither enantiomer of βHB had a significant effect on beta cell survival under conditions of ER stress. By utilizing a systematic *in-vitro* approach, the results of our study now implicate a role for βHB in altering hormone release in human and mouse islets. Considering the growing popularity of exogenous ketone supplements and the ketogenic diet, our study illustrates the need for additional investigations into the effects of ketone bodies on islet function and glucose homeostasis.

MATERIALS AND METHODS

Human islet culture

Human islets for research were provided by the Alberta Diabetes Institute IsletCore at the University of Alberta in Edmonton (www.bcell.org/adi-isletcore), used with approval of the Human Research Ethics Board at the University of Alberta (Pro00013094; Pro00001754) and the University of British Columbia (UBC) Clinical Research Ethics Board (H13-01865). All donors' families gave informed consent for the use of pancreatic tissue in research. Information about individual donors can be found in Supplementary Table 1. Details regarding the islet isolation procedures can be found in the in the protocols.io repositor (24).

Each islet preparation was received in a 50 ml conical centrifuge tube containing CMRL culture media (Corning, 15-110-CV) (24). Upon arrival, islets were further purified by hand-picking under a stereomicroscope. Islets were then suspended in RPMI 1640 medium (Thermo Fisher Scientific, Cat#, 11879-020) supplemented with 5.5 mM glucose, 10% fetal bovine serum (FBS), and 100 units/mL penicillin/streptomycin, and cultured in 10 cm non-tissue culture coated (NTCC) petri dishes (ThermoFisher Scientific, Cat # FB0875713) overnight at 37ºC, 5% CO_2.

For static secretion experiments with racemic βHB, 260 islets were picked into NTCC dishes with 10 mL culture media. For static secretion experiments with individual enantiomers of βHB, 3 islets in 100 µL of culture media were picked into a 96 V-well plate (Corning: #CLS3894) per replicate. Islets were given ~48 hours to adhere to the V-well prior to start of experiment.

Mouse islet isolation and culture

C57BL/6J mice were purchased from the Jackson Laboratory and housed in the (temperature-controlled) UBC Modified Barrier Facility using UBC Animal Care Committee-approved protocols and following international guidelines. The mice were on a 12 h light/dark cycle and fed a chow diet (LabDiet #5053) with water *ad libitum*. Mouse islets were isolated by ductal collagenase (Type

XI, Sigma, Cat. # C7657) injection as described previously (25). Islets were then hand-picked to purity. Mouse islets were cultured overnight in RPMI media with 11.1 mM D-glucose supplemented with 10% vol/vol fetal bovine serum (Thermo Fisher Scientific, Cat. #12483020) and 1% vol/vol Penicillin-Streptomycin (Gibco, Cat #15140-148) at 37°C with 5% CO_2. For static secretion experiments, 3 islets in 100 µL of culture media were picked into a 96 V-well plate (Corning: #CLS3894) per replicate.

Glucose stimulated insulin secretion of human islets treated with racemic βHB

200 mL of Krebs-Ringer HEPES (KRBH) Buffer (pH 7.2, 2580 mM NaCl, 600 mM KCl, 24 mM $MgSO_4$, 24 mM KH_2PO_4, 50 mM $CaCl_2$, 100 mM $NaHCO_3$, 1 M HEPES, 1 g BSA, and ddH_2O to volume) was prepared at glucose concentrations of 3 mM and 15 mM. Islets were then transferred into a new NTCC dish containing 10 mL 3 mM glucose KRBH and incubated at 37ºC, 5% CO_2 for 1 h. Following this, 12 samples of 20 islets were set up in 1.5 mL microcentrifuge tubes with 500 µL of either 3 mM glucose KRBH, 15 mM glucose KRBH, or the corresponding vehicle controls (each in triplicates), and incubated at 37ºC, 5% CO_2 for 30 min. The tubes were centrifuged at 1000 RPM for 1 min and the supernatant was collected and stored at -20ºC; 500 µL of acid-ethanol extraction buffer (75 % of 95 % ethanol, 23.5 % concentrated acetic acid, 1.5 % concentrated HCl) was added to the cell pellet and stored at -20ºC prior to being assayed for insulin using human insulin specific RIA (Millipore Cat# HI-14K).

Glucose stimulated insulin secretion (GSIS) and glucagon secretion in human and mouse islets treated with individual enantiomers of βHB

200 mL of Krebs-Ringer HEPES (KRBH) Buffer (pH 7.2, 2580 mM NaCl, 600 mM KCl, 24 mM $MgSO_4$, 24 mM KH_2PO_4, 50 mM $CaCl_2$, 100 mM $NaHCO_3$, 1 M HEPES, 1 g BSA, and ddH_2O to volume) was prepared at glucose concentrations of 3 mM, 6 mM, 10 mM, and 15 mM. 3mM R-

βHB, S-βHB, and control (ddH_2O) treatments were prepared in 3 mM, 6 mM, 10 mM, and 15 mM glucose KRBH solutions.

To measure insulin secretion, 3 islets/well in 96 V-well plates (Corning: #CLS3894) were pre-incubated in 3 mM glucose for 1 h. Following this, the islets were treated with either enantiomer of βHB or vehicle in different glucose concentrations for 45 min. The supernatant was collected, and islets were stored in 100 µL of extraction buffer (75 % of 95 % ethanol, 23.5 % concentrated acetic acid, 1.5 % concentrated HCl) at −20 °C. Human and mouse insulin secretion was quantified using human insulin radioimmunoassay and rat insulin radioimmunoassay kits, respectively (Millipore Cat# HI-14K, HI-13K). Samples treated with 3 mM glucose KRBH were measured undiluted, all other samples were diluted 1:10. Samples assayed for islet insulin content were diluted 1:500 in assay buffer.

To measure glucagon secretion, 3 islets/well in 96 V-well plates (Corning: #CLS3894) were pre-incubated in 10 mM glucose for 1 h. (The plate layout used is outlined in (**S-Figure 1.)**) Following this, the islets were treated with either enantiomer of βHB or vehicle in different glucose concentrations for 45 min. The supernatant was collected, and islets were stored in 100 µL of extraction buffer and 5 µg/mL of aprotinin (Sigma-Aldrich: A6279) at -20ºC prior to quantification of glucagon secretion in both human and mouse islets using radioimmunoassay (Millipore-Sigma, GL-32K).

Proteomics

3 mM solutions of either R-βHB or vehicle (sterile ddH_2O) were prepared in human islet culture media and added to 6 cm petri-dishes (Falcon, Cat. # 351007). 200 islets were added to each plate, and incubated for 72 h at 37ºC, 5% CO_2. The islets were then picked into 1.5 mL microcentrifuge tubes and washed with 200 µL of 1X PBS. The islet pellets from each treatment were flash-frozen and stored at -70ºC.

Protein was extracted by treating frozen pellets with 60 µL of SDS lysis buffer (4 % SDS, 100 mM Tris, pH= 8), vortexing, and then heating for 10 min at 100ºC. The lysates were then centrifuged at 4ºC and 10000 rpm for 10 min. Protein concentration was measured from the supernatant using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Cat # 23225).

Islet lysate samples were run on a 10% SDS-PAGE gel, visualized using Coomassie Blue, and the bands were excised from the gel. The resulting proteins were reduced, alkylated, and digested by incubating with trypsin. The purified peptides were analyzed using a Bruker TIMS-ToF Pro II mass spectrometer coupled to a Bruker Nanoelute UPLC. The TIMS-ToF Pro II was set to Data-Independent Acquisition Parallel Accumulation-Serial Fragmentation (diaPASEF) scan mode for DIA acquisition scanning 100 – 1700 m/z. The capillary voltage was set to 1800V, drying gas to 3L/min, and drying temperature to 180°C. Acquired diaPASEF data were then searched using FragPipe computational platform (v. 17.1) with MSFragger (v. 3.4) (26), Philosopher (v. 3.8)(27), EasyPQP (v. 0.1.27) and DIA-NN (v. 1.8) to obtain DIA quantification, with use of the spectral library generated from a highly fractionated sample. Quantification mode was set to “Any LC (high precision)”. All other settings were left default.

Cell death screening

Mouse islets were isolated and dispersed into 384 well plates in culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS) at a density of 5000 cells/well as previously described[16]. Islet viability was measured with the TC20 Automated Cell Counter (Bio-Rad: 1450102) with Trypan Blue (Bio-Rad: 1450021). Cells were stained with 50 ng/mL Hoechst 33342 (Invitrogen) (to stain all cells), and 0.5 µg/mL propidium iodide (Sigma-Aldrich) in RPMI 1640 medium (Invitrogen) (to stain for dead cells) with 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen), 10% vol/vol FBS (Invitrogen). After one hour of staining, the cells were treated with either vehicle, 3 mM R-βHB, or 3 mM S-βHB. The 384-well plate was placed in the environmentally controlled (37ºC, 5% CO_2) ImageExpress^{MICRO} high content imaging system. To

measure cell death, the cells were imaged every 2-6 hrs for 96 h. Dispersed islet cells from C57BL6/J mice treated with 10 μM Thapsigargin in DMSO along with R-βHB, S-βHB, or vehicle (DMSO) were imaged every 2 h for 72 h. Cell death was quantified as the number of propidium iodide positive cells/number of Hoescht 33342 positive cells.

Statistics and data analysis

Insulin secretion levels were normalized to the total islet insulin content. GraphPad Prism 9 was used to conduct statistical analyses on the obtained data. Grubb's test (a=0.05) was used to identify and eliminate statistical outliers. Insulin and glucagon secretion data were analyzed using a simple linear regression model on GraphPad Prism. Significance was assessed by Kruskal-Wallis test and Dunn's multiple comparison test at 95% confidence, using $p<0.05$ to define statistical significance.

Differences between treatment groups were compared using 2-tailed t-test of $\log_2$(abundance). Only proteins detected in >80% of donors were considered for analysis. A p-value cutoff was applied to identify significance in differential abundance of proteins between treatments ($p<0.05$, fold change ≥1.2 for upregulated proteins, and fold change ≤0.8 for downregulated proteins). A list of significant protein IDs was uploaded to WebGestalt (28), a KEGG-based gene ontology enrichment software, to identify biological processes involving these proteins.

RESULTS

βHB increases basal insulin secretion in human islets

To determine whether βHB affects islet hormone secretion, we first examined insulin secretion in islets obtained from cadaveric human donors (n=22, ADI IsletCore, UAlberta) in response to acute racemic βHB treatment at both basal (3 mM) and stimulatory (15 mM) glucose concentrations. We observed a 44% decrease in fold-insulin secretion (n=22, $p<0.01$, **Figure 1C-inset**), however this was strictly driven by an increase in insulin secretion at low glucose, since insulin secretion at 15 mM glucose was unchanged (**Figure 1C**). Total insulin content remained unchanged upon βHB treatment (**Figure 1D**).

Considering that R-βHB is the physiological enantiomer in humans (5), we next sought to investigate whether βHB affected hormone secretion from human islets in an enantiomer-specific manner. To also study whether the effects of βHB enantiomers are glucose concentration-dependent, islets from cadaveric human donors (n=10, ADI IsletCore, UAlberta) were treated acutely with either 3 mM R-βHB or S-βHB at multiple glucose concentrations (3, 6, 10, and 15 mM). To account for heterogeneity in individual islet size and composition, we normalized insulin secretion to the total islet insulin content, which was statistically unchanged overall following our acute treatments (**Supplementary Figure 2**). We observed a 1.7-fold increase in insulin secretion in islets treated with 3 mM R-βHB under basal glucose conditions (3 mM) (n=10, $p<0.01$, **Figure 2A**). There was no change in insulin secretion upon treatment with either enantiomer of βHB at glucose concentrations at or above 6 mM glucose concentrations (**Figure 2A**). However, we did observe considerable inter-islet differences in response to βHB, consistent with our recent study showing considerable heterogeneity in insulin secretion in response to macronutrient stimuli (2). To explore this further, we stratified insulin secretion in response to either R-βHB or S-βHB treatment by donor BMI. We observed a 5-fold decrease in BHB-potentiated insulin secretion (fold change compared to vehicle) in islets from donors with BMI >25 (overweight or obese) at a

suprathreshold glucose concentration of 6 mM and R-βHB treatment ($p<0.001$, **Figure 2B**). In S-βHB treated islets, there was a ≥2-fold decrease in insulin secretion in islets from donors with BMI > 25 compared to those from donors with BMI < 25 at both 3- and 6-mM glucose (**Figure 2C, D**), suggesting an enantiomer-specific BMI-dependency in insulin secretion in human islets. We then repeated this experiment in islets from donors with T2D. While we observed trends similar to those observed in islets from donors without diabetes, the limited availability of donors with T2D, restricts these observations to islets from a single donor (**Supplementary Figure 3A**).

Overall, our results indicate that while racemic βHB leads to an increase in insulin secretion in human islets, this increase is driven by R-βHB at physiological glucose concentrations and might be influenced by individual donor characteristics such as BMI.

βHB increases glucose-stimulated insulin secretion in mouse islets

Given that previous studies have identified a role for βHB in potentiating insulin release in rodent islets(29), we next sought to investigate any enantiomer-specific effects of βHB on islets from C57BL/6J mice. Islets were treated acutely with either 3 mM R-βHB or S-βHB at glucose concentrations of 3 mM, 10 mM, or 15 mM. At 10 mM glucose, we observed a 2.6-fold increase in insulin released upon R-βHB treatment ($p<0.01$, $n=13$) (**Figure 2E**). Interestingly, there was no change in insulin release upon treatment with S-βHB at 10 mM glucose, or with either enantiomer at 3- or 15-mM glucose (**Figure 2E**), suggesting species-specific differences in ketone body-potentiated insulin signaling.

βHB decreases glucagon secretion in both mouse and human islets

Glucagon secretion from islets is dependent on local glucose concentrations. In a hyperglycemic environment, insulin is shown to suppress glucagon secretion via the PI3K/Akt signaling pathway (30). We thus next assessed glucagon secretion in human islets treated with 3 mM R-βHB, or S-βHB at glucose concentrations of 3 mM, 6 mM, 10 mM, and 15 mM. Interestingly,

we observed a ~46% decrease in glucagon secretion in islets treated with R-βHB, but not with S-βHB. ($p<0.05$, n=11, **Figure 3A**), implicating a direct role of ketone bodies on glucagon secretion. There was no change in glucagon secretion in islets treated with either enantiomer in the presence of higher glucose concentrations when we examined all donors. However, when we again stratified βHB-mediated changes in glucagon secretion by donor BMI, we observed a 1.5-fold increase in glucagon secretion at 10 mM glucose (**Figure 3B**).

In islets treated with S-βHB, we also observed higher βHB-potentiated glucagon secretion (fold change compared to vehicle) at 10 mM glucose in donors with BMI > 25 compared to those with BMI > 25 ($p<0.05$, **Figure 3C**). Interestingly, in islets treated with S-βHB at 15 mM glucose, donors with BMI > 25 displayed lower βHB-potentiated glucagon secretion compared to those with BMI< 25 ($p<0.05$, **Figure 3D**). We repeated these experiments in islets from a single diabetic human donor (**Supplementary figure 3B**)– while we again see trends in glucagon secretion similar to those seen in donors without diabetes, our observations are limited to n=1.

When we examined islets isolated from C57BL6/J mice, we observed a 23% decrease in glucagon secretion upon treatment with R-βHB at 10 mM compared to vehicle control (n=13, $p<0.01$, **Figure 3E**). There was no change in glucagon release in islets treated with R-βHB at 3- or 15 mM glucose, or S-βHB at any glucose concentration (**Figure 3E**).

Overall, our results indicate that R-βHB treatment leads to a decrease in glucagon secretion at physiological glucose concentrations in both human and mouse islets. Similar to our observations in the context of insulin secretion in human islets, this change might be influenced by donor characteristics such as BMI at glucose concentrations higher than 3 mM.

βHB impacts cellular proliferation and nutrient-response signaling pathways

We next aimed to understand whether prolonged exposure to βHB (for example, to mimic the effects of exogenous ketone supplements) would impact molecular signaling pathways within human islets. We performed LC-MS-based proteomics in human islets treated with R-βHB for 72

h– we focused on R-βHB specifically because under chronic conditions we would expect S-BHB to be converted to R-βHB within the body (5). Our proteomic analysis identified >7000 unique proteins, of which 110 displayed significant differences in abundance upon R-βHB treatment ($p<0.05$) (**Figure 4A**). Upon stratifying by fold change, 68 gene products were either upregulated (fold change > 1.2), or downregulated (fold change <0.8) following βHB treatment (**Figure 4B**). Web Gestalt Over-Representation Analysis (ORA) revealed that proteins involved in response to ketones (CDK4, NCOA2, FOXO1, FDX1, YAP1, TXNIP), and hormone receptor binding (SOCS2, NCOA2) are enriched in islets treated with R-βHB. We also observed an enrichment in biological processes associated with cellular response to drugs (BNIP3, CDK4, DNMT3, FDX1, FOXO1, PDE8A, SSH1, TRIM41) and the Wnt signaling pathway (CDH2, FOXO1, GID8, HMGXB4, MCC, YAP1) (**Supplementary figure 4**). Additionally, DNAL1 and CEP131, primarily associated with beta-cell ciliary movement in response to glucose (31), had lower abundances upon R- βHB treatment. BNIP3, a protein implicated in apoptotic, necrotic, and autophagic cell death (32,33), as well as CCDC92, a protein known to promote NLRP3 inflammasome activity (34), were less abundant following R-βHB treatment. Interestingly, the proteins YAP1, FOXO1, and PDCD2 were increased in abundance in R-βHB -treated islets: these proteins are all involved in islet response to free fatty acids such as palmitate. In mice, FOXO1 is involved in maintaining metabolic homeostasis in the islet and regulates insulin signaling in response to oxidative stress, contributing to beta-cell compensation (35).

Collectively, these data identify numerous differentially abundant proteins upon chronic R-βHB treatment and implicate several cellular signaling pathways including regulation of cell death and inflammatory signaling, autophagy, vesicle transport, and fatty acid-induced signaling.

Chronic βHB treatment does not protect islet cells from ER-stress induced cell death

The results of our proteomics analysis revealed a decreased abundance of proteins involved in cell death and inflammatory signaling. βHB has additionally been shown to promote cell survival

and proliferation in hepatocytes (15). Thus, to determine whether either enantiomer of βHB has a similar protective effect on islets, we used kinetic cell death screening to measure cell death in response to prolonged R-βHB and S-βHB exposure. We observed no significant effect of treatment with either enantiomer of βHB compared to vehicle controls in islets from both male and female mice (**Figure 5A,B**, respectively). Islet cells from male mice had high % PI-positive cells after 96 h of treatment with either enantiomer of βHB; however, female mice did not exceed 40% cell death under the same conditions (**Supplementary Figure 5A,B**). Next, we repeated this experiment using an ER stressor, Thapsigargin (TG), in combination with βHB treatments to determine whether βHB can protect islet cells from ER stress-induced cell death. Dispersed islets from C57BL6/J mice (n=3, 2 male, 1 female mouse) were treated with either enantiomer of βHB in the presence and absence of 10μM TG. We observed no significant change in cell death in islet cells treated with either R or S-βHB compared to vehicle both in the presence and absence of TG overall (**Figure 5C, D**), and between individual male and female mice (**Supplementary Figure 5C, D)**. Treatment with TG+βHB did not have a significant effect on islets from female mice, in contrast with islets from male mice, which showed a modest but significant reduction in cell death upon treatment with TG + R-βHB compared to TG + S-βHB**,** suggesting a sex-specific and enantiomer-specific effect of βHB on islet cell survival upon ER stress that requires further investigation (**Supplementary figure 5E, F**). This agrees with previous studies, which have shown that female β cells are more resilient to factors inducing cell-death, such as ER stress (25). Overall, our observations indicate that there may be slight differences in the effect of individual enantiomers of βHB on islet survival compared to each other, but that neither enantiomer plays a significant role in the health and survival of mouse islet cells, both in the presence and absence of the ER stressor thapsigargin.

DISCUSSION

The aim our study was to systematically investigate the effects of βHB in both human and mouse pancreatic islets. In human islets, we found that racemic βHB treatment led to an increase in insulin secretion at 3 mM glucose concentration – this effect was driven by the physiological enantiomer of βHB, R-βHB. R-βHB treatment also led to a corresponding decrease in glucagon secretion in human islets at 3 mM glucose. In mouse islets as well, R-βHB led to an increase in insulin secretion and a decrease in glucagon secretion at 10 mM glucose. Heterogeneity in donor characteristics influenced the effect of βHB on human islets; islets from donors with BMI greater than 25 (overweight/obese) had a smaller increase in insulin secretion compared to islets from donors with BMI less than 25 at a glucose concentration of 6 mM. Our data indicate that the effect of βHB on islets is dependent on several factors, including individual donor characteristics.

We studied the effects of βHB on both major hormones of the endocrine pancreas. The inverse relationship between insulin and glucagon secretion is supported by studies detailing their negative feedback loop (30,36). With increasing glucose concentrations, there is an expected increase in insulin release, which should then lead to the suppression of glucagon secretion. Thus, whether the observed decrease in glucagon seen upon βHB treatment at insulin-stimulatory glucose concentrations is the result of the combination of an increase in insulin secretion and βHB treatment requires further investigation.

To discern molecular mechanisms underlying islet response to chronic βHB treatment, we performed proteomic analysis on human islets treated with R-βHB over a period of 72 h. Several proteins involved in cell death signaling and nutrient-response pathways were observed to have differential abundance in βHB-treated islets. Specifically, we observed an upregulation of YAP1– overexpression of YAP1 in isolated human islets induced β-cell proliferation and protected INS1 cells from apoptotic cell death under conditions of high glucose and inflammatory cytokine treatment (37). As well, the downregulation of BNIP3, an inducer of apoptosis in MIN6 mouse pancreatic cells (33), suggest that βHB treatment might have a protective effect on islets in the

context of cell death. The downregulation of cyclin-dependent kinases (CDK4, CDK18) that are involved in cell cycle regulation and beta cell proliferation (38), on the other hand, point to the possibility that βHB might not protect islets from cell death; CDK4 overexpression contributes to beta cell proliferation in mouse islets (38), so the decreased abundance of this protein in βHB-treated islets might suggest that CDK4 does not promote islet cell health and survival. In addition, we observed an upregulation in SOCS2; interestingly transgenic mice overexpressing SOCS2 show impaired insulin secretion and eventual beta cell failure (39).

Overall, these contrasting trends led us to investigate whether either enantiomer of βHB would protect dispersed islet cells from cell death. Given the observed effect of βHB on pathways involved in autophagy, necroptosis, and apoptosis in islets, we expected to see this effect translated into βHB having a protective effect on islets in the context of cell death. Surprisingly, our results showed that neither enantiomer of βHB appeared to protect islet cells from cell death – possibly suggesting that there could be downstream effectors that compensate for the observed downregulation of the abundance of these proteins. However, we did observe that female islets displayed a much lower cell death compared to their male counterparts, in support of previous studies on ER-stress mediated islet cell death (25). Our proteomics data indicate an increase in SOCS2 abundance, a suppressor of cytokine signalling, and a downregulation of CDK4, which is involved in cell cycle regulation at the G1/S checkpoint. Therefore, future experiments should investigate whether βHB treatment protects islet cells from cytokine-induced cell death.

To our knowledge, ours is the first study to characterize the effects of individual enantiomers of βHB on hormone secretion and cell death in human and mouse islets. Overall, our results show that the effects of acute R-βHB on both human and mouse islets is significant and resembles the effect of racemic βHB. Chronic βHB treatment, indicative of either a prolonged ketogenic diet or chronic consumption of exogenous ketone supplements, alters the abundance of proteins involved in nutrient response and cell death in islets, however, neither enantiomer of βHB significantly improves islet cell survival, in the presence or absence of ER stress. Considering the

growing popularity of the ketogenic diet, our findings contribute to the mechanistic understanding of how increasing concentrations of ketone bodies affect pancreatic islet function and survival.

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Figure 1

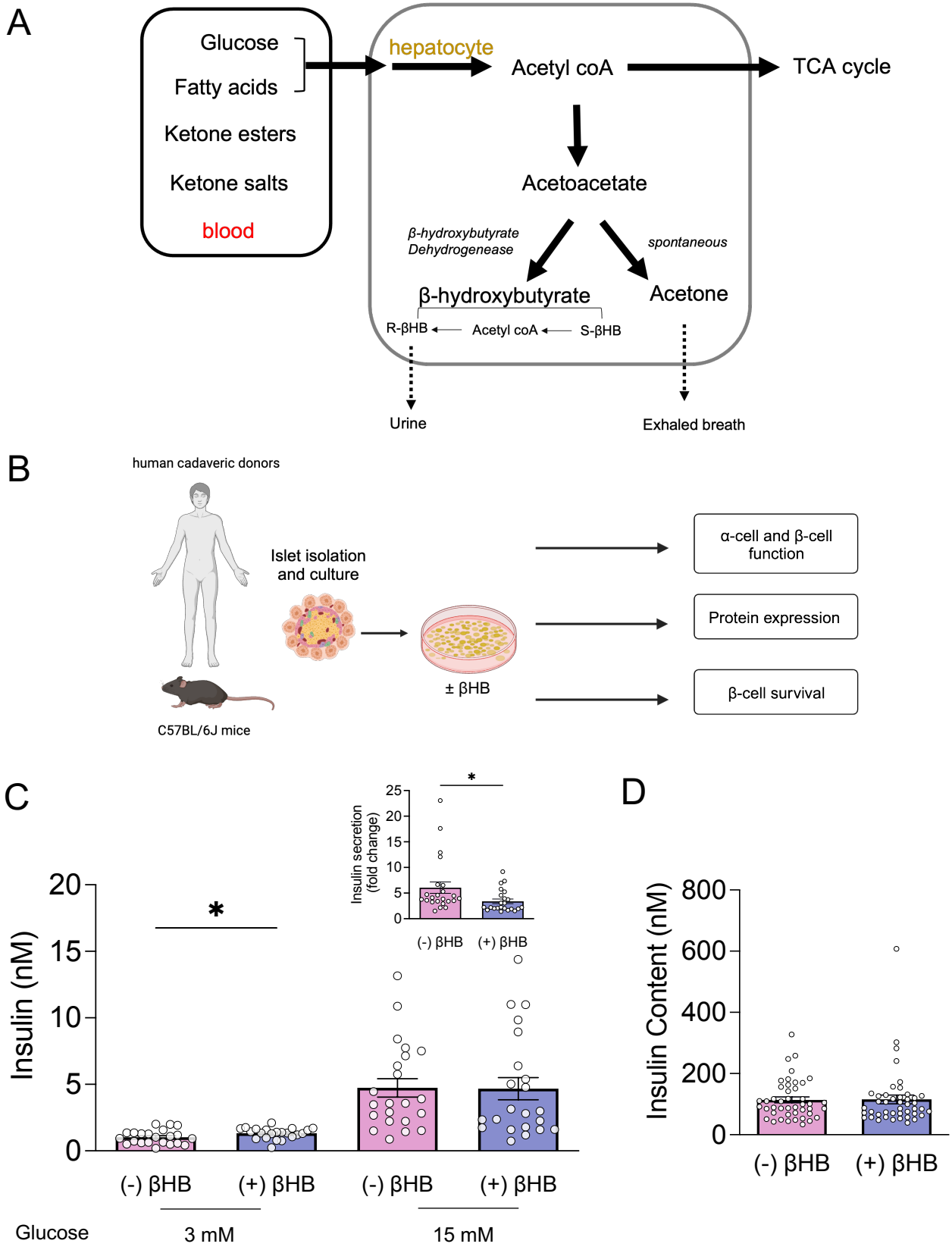

A
Glucose
Fatty acids
Ketone esters
Ketone salts
blood
hepatocyte
Acetyl coA
TCA cycle
Acetoacetate
β-hydroxybutyrate Dehydrogenease
spontaneous
β-hydroxybutyrate
Acetone
R-βHB
Acetyl coA
S-βHB
Urine
Exhaled breath
B
human cadaveric donors
Islet isolation and culture
± βHB
C57BL/6J mice
α-cell and β-cell function
Protein expression
β-cell survival
C
Insulin (nM)
20
15
10
5
0
*
(-) βHB
(+) βHB
(-) βHB
(+) βHB
Glucose
3 mM
15 mM
Insulin secretion (fold change)
25
20
15
10
5
0
*
(-) βHB
(+) βHB
D
Insulin Content (nM)
800
600
400
200
0
(-) βHB
(+) βHB

Figure 1. Experimental overview and effect of βHB on insulin secretion in human islets. (A) Modes of ketone body production and metabolism in the human body. **(B)** Experimental design for study of βHB on high-quality primary human islets isolated from cadaveric donors and from C57BL6/J mice. **(C)** Insulin secretion in islets from cadaveric human donors (n=22) treated acutely with 3 mM racemic βHB at low (3 mM) or high (15 mM) glucose is shown*. (Inset)* Overall fold-change in insulin secretion (15 mM / 3 mM glucose) with or without βHB is calculated. **(D)** Total insulin content from islets used in (A) is shown. Bars represent mean ± SEM. Each circle represents the mean of three technical replicates. Significance levels are indicated as asterisks over the corresponding bars. * indicates $p<0.05$.

Figure 2

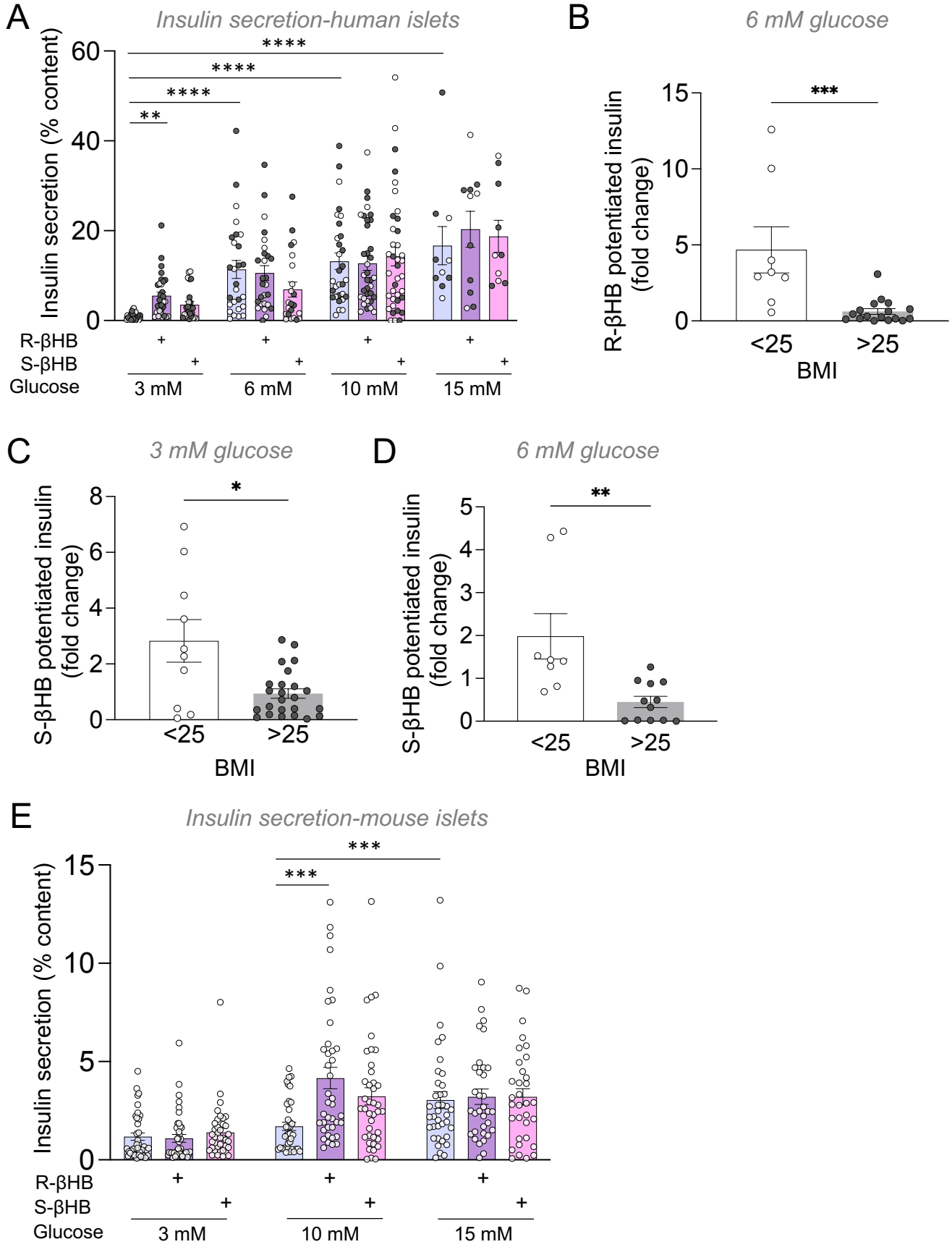

A
Insulin secretion-human islets
Insulin secretion (% content)
60
40
20
0
****
****
****
**
R-βHB
S-βHB
Glucose
3 mM
6 mM
10 mM
15 mM
B
6 mM glucose
R-βHB potentiated insulin (fold change)
15
10
5
0
***
<25
>25
BMI
C
3 mM glucose
S-βHB potentiated insulin (fold change)
8
6
4
2
0
*
<25
>25
BMI
D
6 mM glucose
S-βHB potentiated insulin (fold change)
5
4
3
2
1
0
**
<25
>25
BMI
E
Insulin secretion-mouse islets
Insulin secretion (% content)
15
10
5
0
***
***
R-βHB
S-βHB
Glucose
3 mM
10 mM
15 mM

Figure 2. The effect of individual enantiomers of βHB on insulin secretion in human and mouse islets. **(A)** Insulin secretion as % of total islet content in islets from cadaveric human donors (n=10) treated acutely with 3 mM R- or S- βHB at 3 mM, 6 mM, 10 mM or 15 mM glucose is shown. Each circle represents a technical replicate. Black circles correspond to secretion values from donors with BMI > 25 and white circles correspond to secretion values from donors with BMI <25. **(B)** Fold-change in insulin secretion values in islets treated with 6 mM glucose and 3 mM R-βHB is shown for donors with BMI > 25 or <25 from (A). **(C)** Fold-change in insulin secretion values in islets treated with 3 mM glucose and 3 mM S-βHB is shown for donors with BMI > 25 or <25 from (A). **(D)** Fold-change in insulin secretion values in islets treated with 6 mM glucose and 3 mM R-βHB is shown for donors with BMI > 25 or <25 from (A). **(E)** Insulin secretion as % of total islet content in islets from C57BL6/J mice (n=13) treated acutely with 3 mM R- or S- βHB at 3 mM, 10 mM or 15 mM glucose is shown. Each circle represents a technical replicate. Significance levels are indicated as asterisks over the corresponding bars. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, and **** indicates $p<0.0001$.

Figure 3

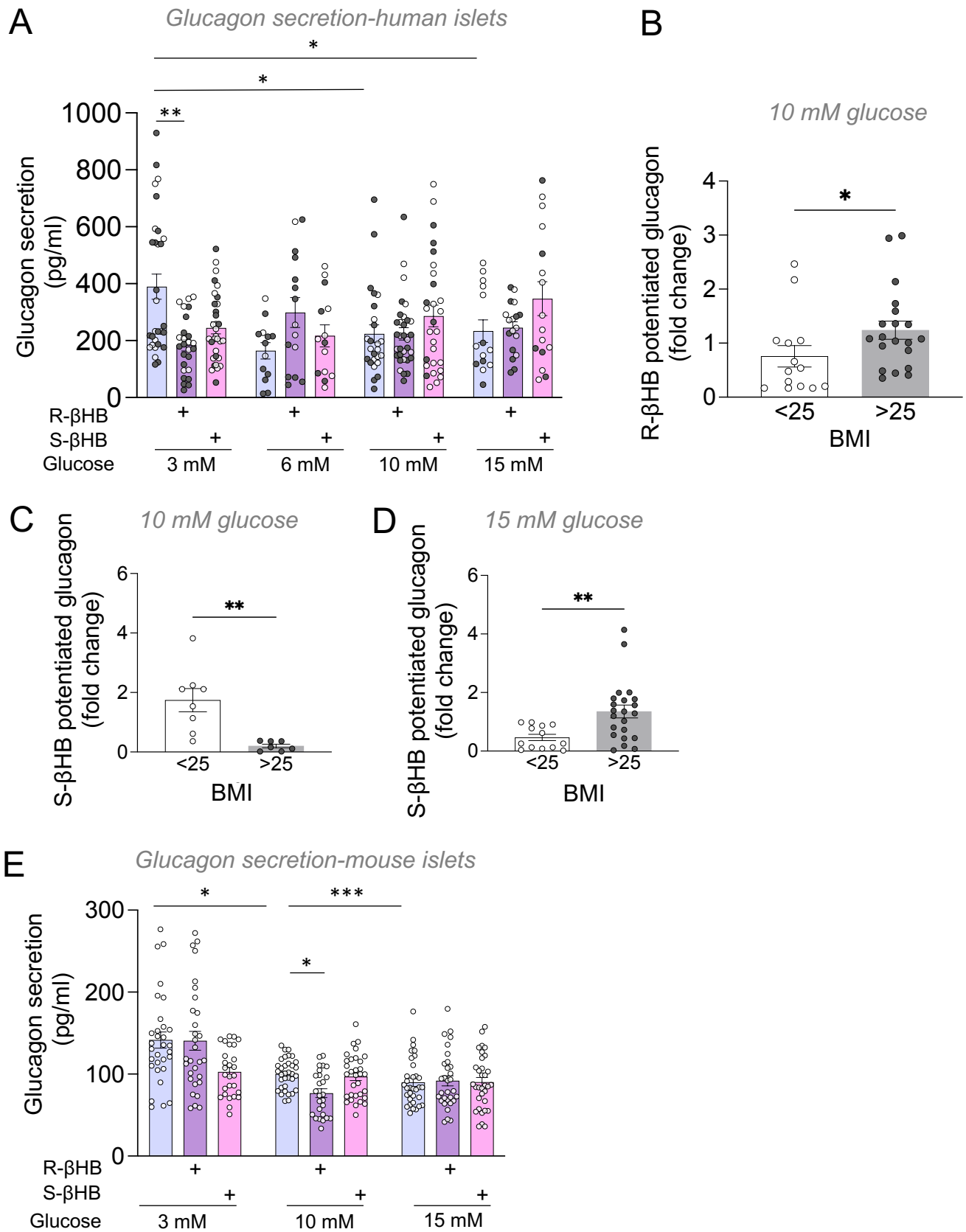
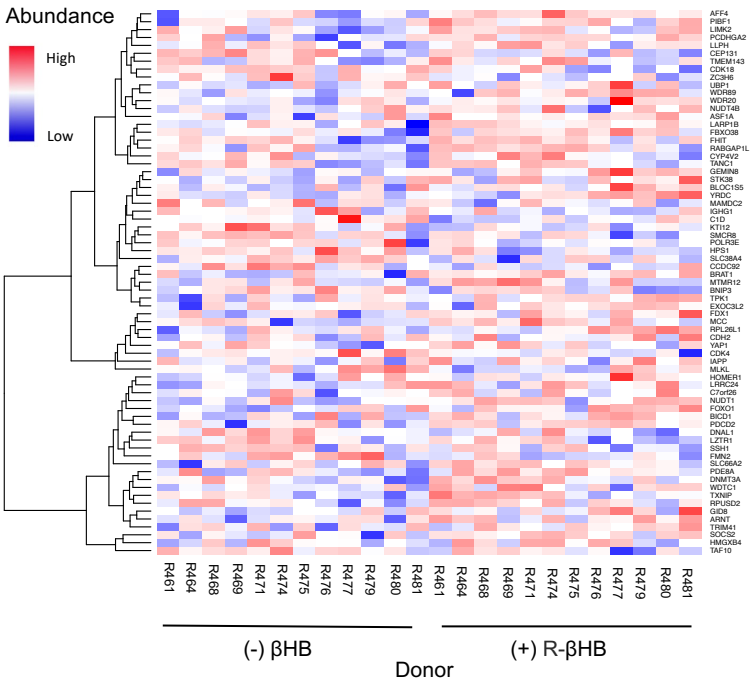

A
Glucagon secretion-human islets
Glucagon secretion (pg/ml)
1000
800
600
400
200
0
*
*
**
R-βHB
S-βHB
Glucose
3 mM
6 mM
10 mM
15 mM
B
10 mM glucose
R-βHB potentiated glucagon (fold change)
4
3
2
1
0
*
<25
>25
BMI
C
10 mM glucose
S-βHB potentiated glucagon (fold change)
6
4
2
0
**
<25
>25
BMI
D
15 mM glucose
S-βHB potentiated glucagon (fold change)
6
4
2
0
**
<25
>25
BMI
E
Glucagon secretion-mouse islets
Glucagon secretion (pg/ml)
300
200
100
0
*
***
*
R-βHB
S-βHB
Glucose
3 mM
10 mM
15 mM

Figure 3. The effect of individual enantiomers of βHB on glucagon secretion in human and mouse islets. **(A)** Glucagon secretion in islets from cadaveric human donors (n=10) treated acutely with 3 mM R- or S- βHB at 3 mM, 6 mM, 10 mM or 15 mM glucose is shown. Each circle represents a technical replicate. Black circles correspond to secretion values from donors with BMI > 25 and white circles correspond to secretion values from donors with BMI <25. **(B)** Fold-change in glucagon secretion values in islets treated with 10 mM glucose and 3 mM R-βHB is shown for donors with BMI > 25 or <25 from (A). **(C)** Fold-change in glucagon secretion values in islets treated with 10 mM glucose and 3 mM S-βHB is shown for donors with BMI > 25 or <25 from (A). **(D)** Fold-change in glucagon secretion values in islets treated with 15 mM glucose and 3 mM S-βHB is shown for donors with BMI > 25 or <25 from (A). **(E)** Glucagon secretion in islets from C57BL6/J mice (n=9) treated acutely with 3 mM R- or S- βHB at 3 mM, 10 mM or 15 mM glucose is shown. Each circle represents a technical replicate. Significance levels are indicated as asterisks over the corresponding bars. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, and **** indicates $p<0.0001$.

Figure 4

A


Abundance
High
Low
R461
R464
R468
R469
R471
R474
R475
R476
R477
R479
R480
R481
(-) βHB
(+) R-βHB
Donor

B

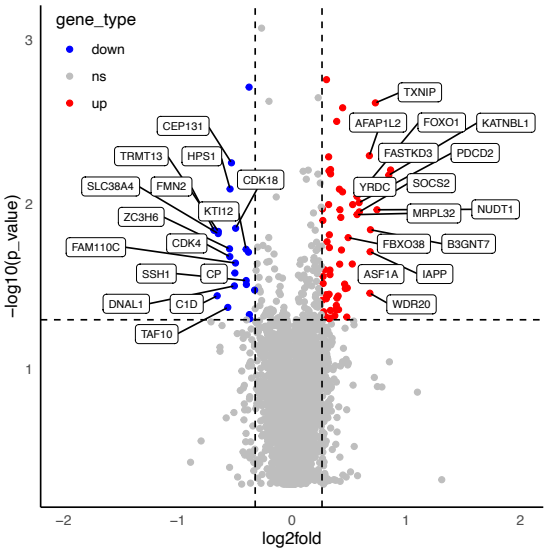

gene_type
down
ns
up
TXNIP
AFAP1L2
FOXO1
KATNBL1
CEP131
FASTKD3
PDCD2
TRMT13
HPS1
CDK18
YRDC
SOCS2
SLC38A4
FMN2
MRPL32
NUDT1
KTI12
ZC3H6
FBXO38
B3GNT7
FAM110C
CDK4
ASF1A
IAPP
SSH1
CP
WDR20
DNAL1
C1D
TAF10
−log10(p_value)
log2fold

Figure 4. Proteomic analysis of human islets treated with R-βHB. (A) Heatmap showing fold change (vs. vehicle) of proteins displaying differential abundance ($p<0.05$) upon prolonged R-βHB treatment (72 h) in human islets (n=12 cadaveric human donors). **(B)** Volcano plot showing proteins displaying differential abundance upon chronic R-βHB treatment in human islets. 15 proteins displaying highest and lowest fold change are labelled– upregulated proteins are shown in red and downregulated proteins are shown in blue. Grey dots indicate proteins that do not show significant differential expression between treatment conditions (ns).

Figure 5

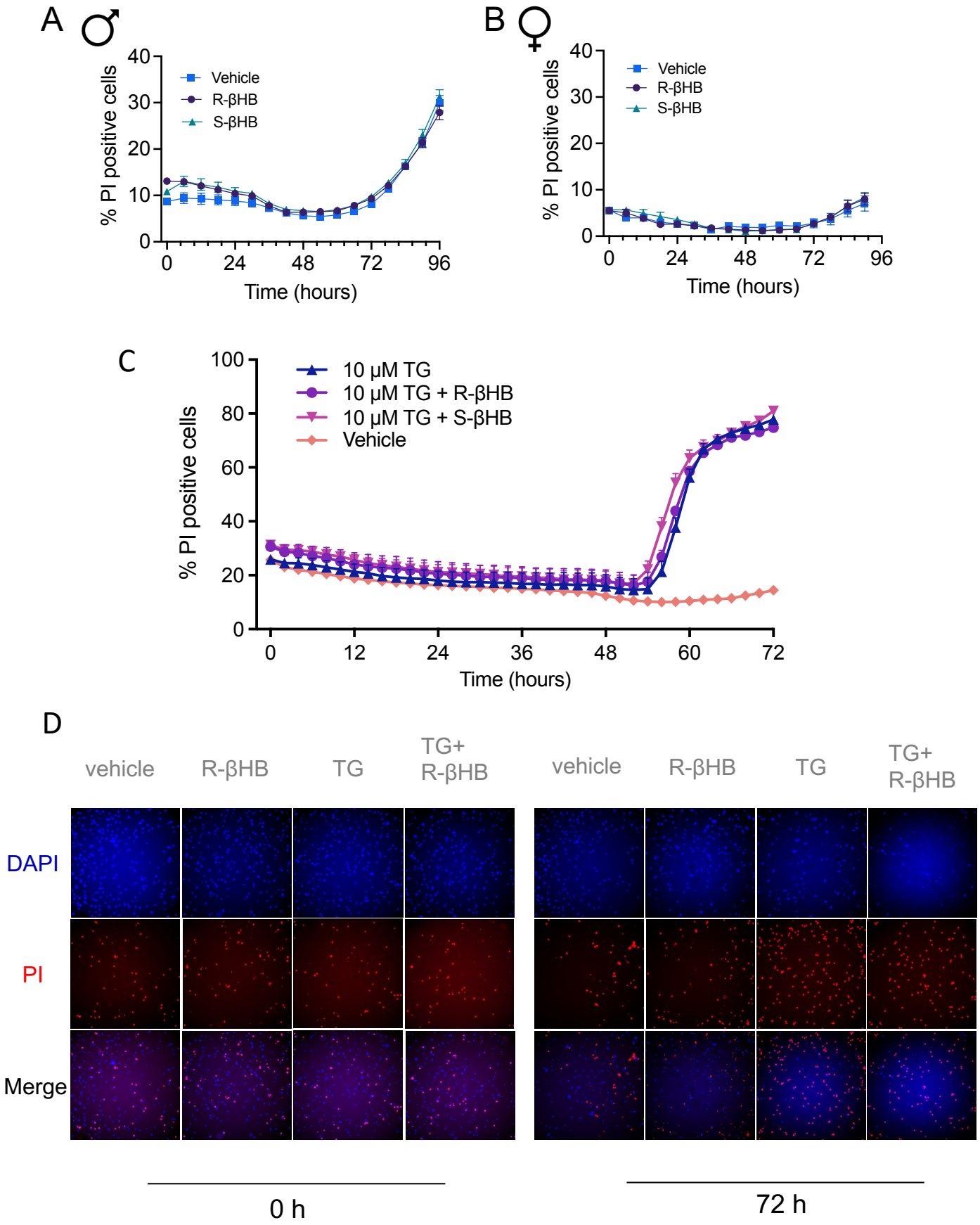

A
♂
% PI positive cells
Vehicle
R-βHB
S-βHB
Time (hours)
B
♀
% PI positive cells
Vehicle
R-βHB
S-βHB
Time (hours)
C
% PI positive cells
10 μM TG
10 μM TG + R-βHB
10 μM TG + S-βHB
Vehicle
Time (hours)
D
vehicle
R-βHB
TG
TG+ R-βHB
vehicle
R-βHB
TG
TG+ R-βHB
DAPI
PI
Merge
0 h
72 h

Figure 5. Human islet cell survival on chronic βHB treatment. (A, B) Cell death as % PI positive cells in dispersed islet cells treated with either R- or S-βHB in male (**A**) or female **(B)** mice. **(C)** Cell death as % PI positive cells in dispersed islet cells treated with the ER stressor thapsigargin ± R-βHB or S-βHB is shown. **(D)** Representative images of dispersed islet cells at 0 h and 72 h. Results shown are quantified from n=6 male and n=2 female mice over 2 separate runs.