

β -Hydroxybutyrate improves β -cell mitochondrial function and survival



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Pharmacological interventions aimed at improving outcomes in type 2 diabetes and achieving normoglycaemia, including insulin therapy, are increasingly common, despite the potential for substantial side effects. Carbohydrate-restricted diets that result in increased ketogenesis have effectively been used to improve insulin resistance, a fundamental feature of type 2 diabetes. In addition, limited evidence suggests that states of ketogenesis may also improve β -cell function in type 2 diabetics. Considering how little is known regarding the effects of ketones on β -cell function, we sought to determine the specific effects of β -Hydroxybutyrate (β HB) on pancreatic β -cell physiology and mitochondrial function. β HB treatment increased β -cell survival and proliferation, while also increasing mitochondrial mass, respiration and adenosine triphosphate (ATP) production. Despite these improvements, we were unable to detect an increase in β -cell or islet insulin production and secretion. Collectively, these findings have two implications. Firstly, they indicate that β -cells have improved survival and proliferation in the midst of β HB, the circulating form of ketones. Secondly, insulin secretion does not appear to be directly related to apparent improvements in mitochondrial function and cellular proliferation.

Introduction

Because of conventional clinical perspectives, type 1 (T1D) and type 2 diabetes (T2D) are both considered diseases of hyperglycaemia (based on glucose-induced polyuria), despite each representing an opposite end of a spectrum with regard to insulin: too little, in the case of T1D, or often initially too much, in the case of T2D.¹ Hyperinsulinemia is inseparably connected with reduced insulin responsiveness, at least initially, which is the fundamental feature of T2D.² In the absence of intervention, most cases of insulin resistance will progress to the point that pancreatic β -cell insulin secretion, despite being elevated, is no longer sufficient to control blood glucose. Ultimately, this damaging environment will result in the dedifferentiation and loss of the pancreatic β -cell mass (1).

Given the complications associated with chronic hyperglycaemia, including neuropathy³ and vascular damage,⁴ interventions to control blood glucose are rightly a priority in improving health of insulin-resistant and T2D patients. Human trials have found that carbohydrate restriction is an effective intervention for improving every clinical outcome related to diabetes, including glucose control and insulin levels.⁵ Indeed, these results are sufficiently effective that many scientists and physicians promote such a dietary change as a 'first approach' to managing diabetes.⁵ Such a perspective is based on evidence indicating reduced or complete cessation of insulin therapy,⁶ as well as improved β -cell function,^{7,8} in response to dietary intervention.

A common theme among successful interventions to improve β -cell function is a reduction in refined carbohydrates and caloric restriction.⁹ Because of the lower glucose load, carbohydrate-restricted diets result in reduced circulating insulin levels, which decrease the inhibitory effect that insulin has on hepatic ketogenesis,¹⁰ thereby increasing blood ketones. Interestingly, both of these interventions, that is, carbohydrate and caloric restriction, individually or combined, induce hepatic ketogenesis, which introduces the possibility that ketones are independently relevant in the benefits of the intervention. Ketones have long been known to improve survival of nervous cells and tissue^{11,12} and, interestingly, despite arising from different germ cell lines, β -cells share significant common genetic and physiological characteristics with neurons.¹³ Many of ketones' benefits appear to be mitochondrial specific, including enhanced biogenesis¹⁴ and improving redox states.¹⁵

Despite the evidence of ketogenic states improving mitochondrial function and survival in diverse cells, the degree to which ketones affect pancreatic β -cells is unclear. Considering the improvements in β -cell function with diets that lower the need for insulin production, thereby promoting ketogenesis,^{16,17,18,19,20} the purpose of this study was to determine the effect of ketones on pancreatic β -cell physiology and survival.

Materials and methods

Animal husbandry and islet isolation

Wistar rat breeding pairs were purchased from Harlan and maintained on standard chow diet (Teklad 7001; Harlan). Pups were weaned at 21 days, at which point female rats were euthanised. Male rats were fed *ad libitum* and maintained on a 12-h light dark cycle and were age-matched for all islet experiments. Pancreatic islets were isolated as previously described.^{21,22,23} All animal studies were approved and performed in accordance with Brigham Young University's animal research committee's guidelines.

Cell culture

The INS-1 derived 832/13 rat insulinoma cell line was acquired from Dr Christopher Newgard, Duke University. The 832/13 cells were maintained in complete RPMI 1640 medium with L-glutamine and 11.2 mM glucose supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES buffer, 10% foetal bovine serum (FBS) and an INS-1 supplement with a final concentration of 2 mM L-glutamine, 1 mM sodium-pyruvate and 0.05 mM 2-mercaptoethanol, as described previously.²⁴

Proliferation assays

The 832/13 INS-1 β-cells were cultured as previously described.²⁵ Cells were plated at a concentration of 2×10^5 cells/mL in 24-well plates (at 1 ml/well) or in 96-well plates (at 100 µL/well), then cultured for 48 h total with vehicle (water; CON) or 10 mM β-Hydroxybutyrate (βHB). Cellular proliferation was determined by cell counts,²⁶ MTT assays (Sigma-Aldrich)²⁴ and Alamar Blue assays (Sigma-Aldrich).²⁴ Absorbance for the MTT and Alamar Blue assays were determined on a BioTek Synergy 2 plate reader.

Cell viability assay

The 832/13 INS-1 β-cells were plated at a concentration of 2×10^5 cells/mL in 24-well plates (at 1 ml/well), then cultured for 48 h with CON or 10 mM βHB. At 24 h of culture with CON or βHB, 1 mM palmitate (superphysiological) or water was added to the culture media for the final 24 h of culture. Following 48 h total of culture, the cells were trypsinised, resuspended in PBS, and counted using a haemocytometer. Percent cell viability was determined by dividing the number of cells counted for the CON or βHB treatment after palmitate treatment by the number of cells counted with water treatment after the respective CON or βHB treatment (i.e. Cell count_{βHB+Palmitate}/Cell count_{βHB-Palmitate}).

MitoTracker assay

The 832/13 INS-1 β-cells were plated at a concentration of 2×10^5 cells/mL in 96-well plates (at 100 µL/well), then cultured for 48 h total with CON or 10 mM βHB. Cells were loaded with 25 nM MitoTracker Red CMXRos-FM for 15 min, following the manufacturer's suggested protocol (Life Technologies). Relative mitochondrial

content was measured by fluorescence in a BioTek Synergy 2 plate reader.²⁴

Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) was performed as described previously, using INS-1 832/13 cells of primary rat islets.²⁴ Cells or islets were cultured for 48 h total with CON or 10 mM βHB. For 832/13 INS-1 cells, GSIS was performed once the cells reached ~80% confluency and preincubated in secretion assay buffer (SAB) containing 2.5 mM glucose for 2 h (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, and 0.2% BSA, pH 7.2). GSIS was performed by incubating 12 replicate wells of cells in SAB containing 2.5 mM glucose for 1 h, followed by 1 h in SAB with 16.7 mM glucose, each followed by collection of the respective buffers, as described previously. For total insulin content, cells were lysed in RIPA buffer with protease inhibitors (Life Technologies). Secreted insulin and total insulin were measured in SAB using a rat insulin RIA kit (MP Biomedicals; Santa Ana, CA, USA), as described previously.²⁵

Mitochondrial respiration

Cells were prepared for mitochondrial respiration as described previously^{27,28} before being transferred to respirometer chambers using the Oroboros O2K oxygraph. Electron flow through complex I was supported by glutamate + malate (10 mM and 2 mM, respectively) to determine leak oxygen consumption (GM_L). Following stabilisation, adenosine diphosphate (ADP) (2.5 mM) was added to determine oxidative phosphorylation capacity (GM_D). Succinate was added (GMS_D) for complex I + II electron flow into the Q-junction. To determine full electron transport system capacity in cells over oxidative phosphorylation, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 µM, followed by 0.025 µM steps until maximal O₂ flux was reached). Mitochondrial membrane integrity was tested in all experiments by adding cytochrome c (not shown; 10 µM). Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 µM) to block complex III action, effectively stopping any electron flow, which provides a baseline rate of respiration. Following respiration protocol, samples were removed from the chambers and used for further analysis, including protein quantification.

ATP production and amount

ATP production was determined using the Oroboros O2K oxygraph, as described previously.²⁹ Briefly, ATP production was determined after the addition of ADP (2.5 mM) by measuring changes in free extramitochondrial (Mg⁺) using Magnesium Green (MgG; Life Technologies). Fluorescence was measured with 503 nm and 530 nm excitation and emission, respectively. Additionally, total ATP (Life Technologies) was determined in identical conditions used to measure insulin secretion, described previously.²⁴ Briefly,

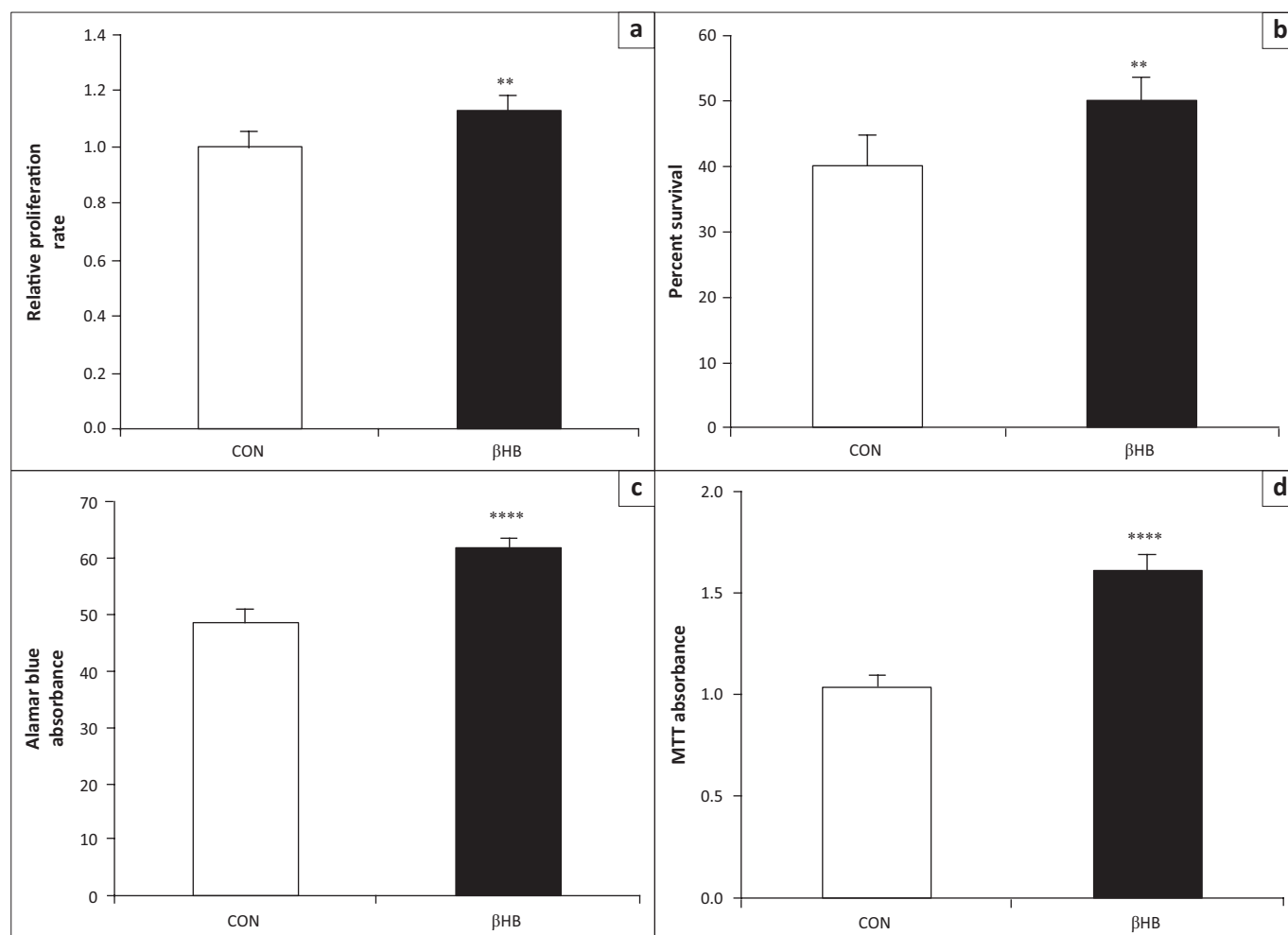


FIGURE 1: β -Hydroxybutyrate increases INS-1 832/13 β -cell replication and maintains cell survival rate. (a; $n = 6$) INS-1 832/13 β -cells proliferation rate was calculated by counting cells following 48 h of culture with vehicle (water; CON) or 10 mM β -Hydroxybutyrate (β HB), measured for viability after treatment with 1 mM Palmitate for the final 24 h of culture (b; $n = 6$), stained with Alamar Blue (c; $n = 6$), or with MTT (d; $n = 6$) to determine metabolic activity. ** $P < 0.01$ for β HB versus CON. **** $P < 0.0001$ for β HB versus CON.

following the culture period, cells were transferred to 2.5 mM glucose in SAB buffer for 2 h, followed by transfer to either 2.5 mM glucose SAB buffer or 16.7 mM glucose SAB buffer for 1 h. Cells were washed with PBS, harvested by trypsinisation and pelleted by centrifugation. The cells were lysed in 150 μ L 1M perchloric acid on ice to precipitate cellular proteins. Lysate was centrifuged at 20 000 \times g for 10 min, after which 150 μ L supernatant was transferred to a new tube with 150 μ L 1M KOH.

Statistical methods

Data are presented as means \pm SEM. Data were compared with Student's t -test (Graphpad Prism; Microsoft Excel). Significance was set at $P < 0.05$.

Results

β -Hydroxybutyrate increases INS-1 832/13 β -cell replication while maintaining cell survival. Given the potentially beneficial effects of ketogenic diets for individuals with T2D, we determined the effect of culturing the INS-1 derived 832/13 β -cell line in the presence of 10 mM β HB. 832/13 cells were cultured in the presence of β HB for 48 h, after which β -cell

replication was measured. Interestingly, 832/13 cells treated with β HB showed an 11% increase in proliferation relative to controls (Figure 1a).

The ability of β HB to protect 832/13 β -cells from palmitate-induced apoptosis was also measured. Elevated free fatty acids is a hallmark of T2D disease progression, and elevated levels of palmitate have been shown to induce apoptosis in β -cells. Therefore, 832/13 cells were cultured in the presence or absence of β HB for 48 h, and with palmitate being added to a final concentration of 1 mM for the last 24 h of that culture period. Measurements of β -cell survival revealed that β HB treated β -cells had a 50% survival rate, compared to 40% in controls (Figure 1b; $p < 0.01$). These data demonstrate that culturing 832/13 β -cells with β HB results in enhanced proliferation and increased cellular survival and suggests a beneficial effect of ketones at the pancreatic β -cell.

Given the proliferative and cell survival effects seen with 832/13 β -cells cultured in the presence of β HB, we measured the effect of β HB culture on cell viability and metabolic activity. 832/13 β -cells cultured in the presence of β HB demonstrated a 29% change in cell viability as determined by Alamar Blue assay (Figure 1c). We subsequently determined

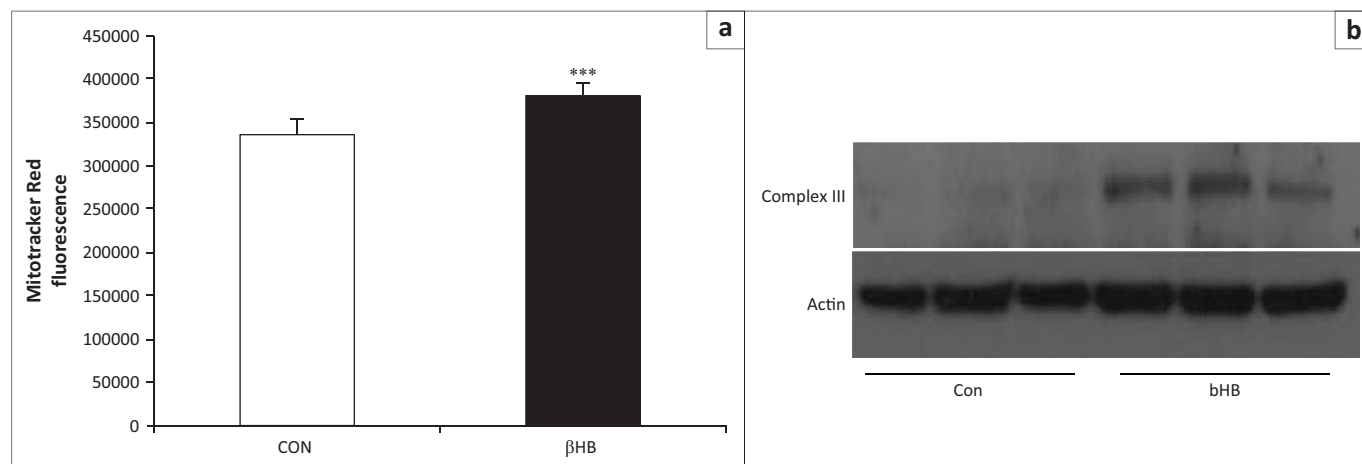


FIGURE 2: β -Hydroxybutyrate increases mitochondrial content and function. INS-1 832/13 β -cells were treated with vehicle (water; CON) or with β -Hydroxybutyrate (β HB; 10 mM) for 24 h then incubated with MitoTracker and fluorescence was measured by plate reader assay (a; $n = 6$). In parallel, cells were used to detect mitochondrial complex III expression via Western blot (b; $n = 3$). *** $P < 0.001$ for β HB versus CON.

the effect of β HB treatment on 832/13 β -cell MTT reduction. These studies demonstrated that culture with β HB resulted in a 56% change in MTT absorption (Figure 1d). As both assays measure cellular NADPH and NADH levels, these data demonstrate increased viability through either increased proliferation or mitochondrial function.

β -Hydroxybutyrate increases β -cell mitochondria and favourably alters mitochondrial function. MitoTracker staining indicated a significant increase in mitochondrial content after culture with β HB (Figure 2a), though only complex III, among mitochondrial complexes, was increased when detected via Western blot (Figure 2b). Moreover, β HB resulted in a significant increase in mitochondrial respiration (Figure 3a), though no pronounced difference in respiratory control ratio (Figure 3b), a general indicator of mitochondrial 'fitness'. Moreover, β HB resulted in a stunted uncoupling control ratio, an indicator of sensitivity to the chemical uncoupler FCCP (Figure 3c). This is likely an artefact since β HB-treated cells are already closer to maximal rate of respiration without the use of chemical uncoupling. In addition to respiration, differences were noted in rate of ATP production, namely β HB elicited an increase in ATP production (Figure 3d) and an elevated P:O ratio (Figure 3e), suggesting enhanced ATP production per unit oxygen consumed. These data substantiate the hypothesis that culture of 832/13 β -cells with β HB enhances mitochondrial function. Additionally, specific post hoc analysis of the difference in respiration rates with the addition of succinate (i.e. GMS_D vs. GM_D) revealed a significant increase in complex II-mediated respiration with β HB treatment (Figure 3f).

β -Hydroxybutyrate maintains insulin content and glucose-stimulated insulin secretion in primary rat islets and INS-1 832/13 β -cells. Our data demonstrating that culture of 832/13 β -cells with β HB enhances mitochondrial function suggest that ketone treatment may increase insulin secretion. To determine the effect of β HB on β -cell function, we measured GSIS in 832/13 β -cells and primary rat islets cultured in the presence of β HB. The increased respiration and cellular ATP production suggest that culture with β HB may increase GSIS.

As previously described, 832/13 β -cells or primary rat islets were cultured for 48 h in the presence or absence of β HB, after which the GSIS was measured. We observed no increase in total insulin content of islets (Figure 4a) or 832/13 β -cells (Figure 4c), demonstrating that culture with β HB does not enhance insulin production. Furthermore, static incubation of islets (Figure 4b) and 832/13 β -cells (Figure 4d) with unstimulatory (2.5 mM) or stimulatory (16.7 mM) levels of glucose did not result in increased insulin secretion. Moreover, similar glucose exposures resulted in no significant difference in total ATP amount (Figure 4e), and the ATP:insulin ratio was similar between CON and β HB treatment conditions (Figure 4f).

Discussion

β -cell status in insulin resistance and frank type 2 diabetes mellitus (T2DM) remains poorly understood. In conditions where reduced β -cell survival and insulin production are noted, previous reports have found that lifestyle interventions may be sufficient in some cases of T2DM to improve β -cell function. Considering the pathogenicity of insulin therapy with T2DM,³⁰ interventions aimed at restoring inherent β -cell function should be encouraged to help avoid exogenous insulin therapy and its consequences.

Pharmacological interventions aimed at improving β -cell insulin release are successful in achieving normoglycaemia, but carry substantial side effects as a result of the artificially induced hyperinsulinaemia. Insulin therapy itself, for example, increases the risk of weight gain,³¹ cardiovascular events³⁰ and cancer.³² Moreover, sulfonylureas, which increase β -cell insulin release, also increase these pathologies.^{33,34} For these reasons, increasing attention needs to focus on alternative strategies to both improve diabetes progression and mitigate risk of related diseases.

The evidence is clear that with β -cell health in T2DM, diet matters. Lim et al.³⁵ reported that T2DM patients placed on a calorie-restricted diet consisting of roughly 40% carbohydrates experienced significant improvements in insulin sensitivity

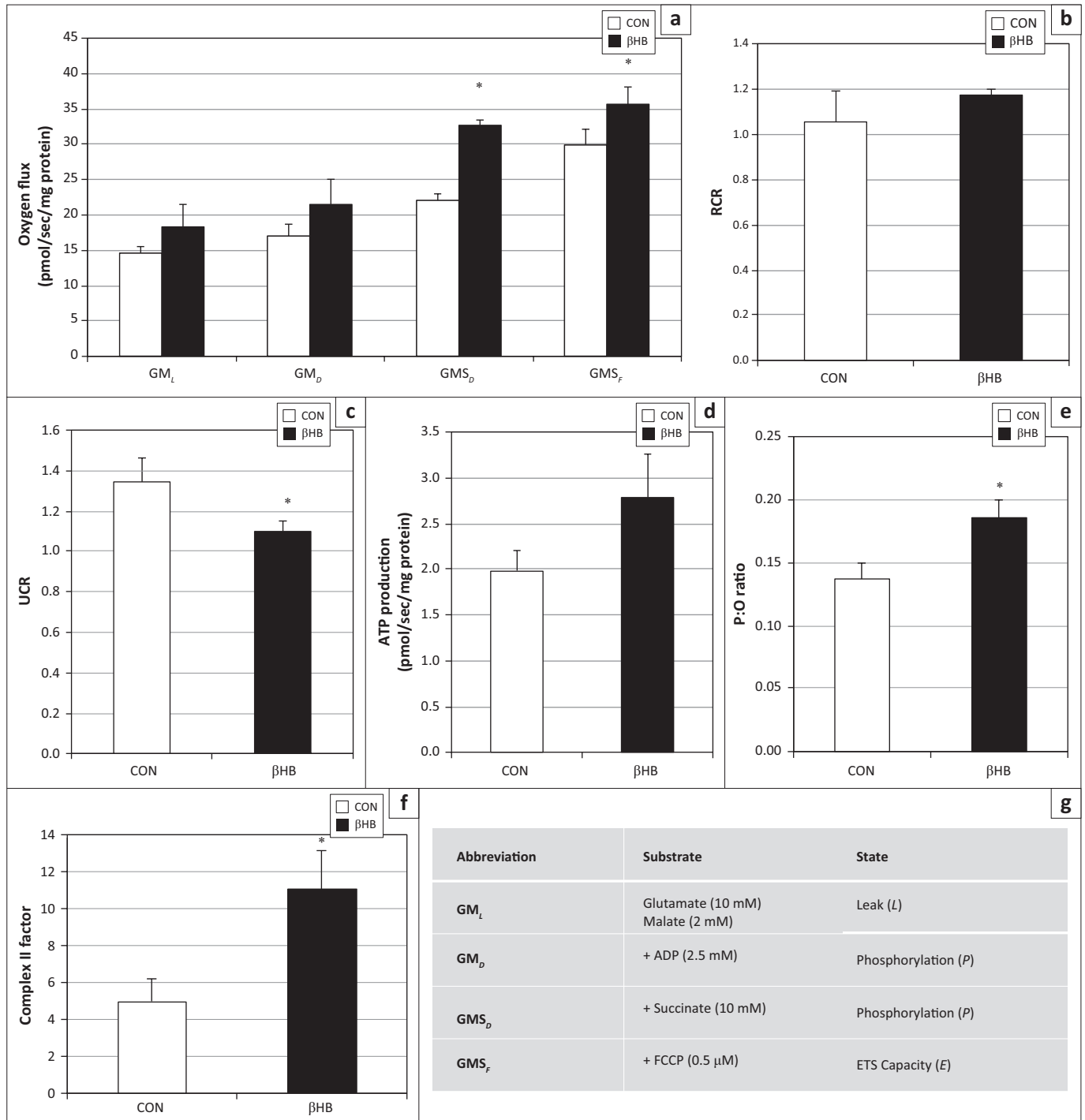
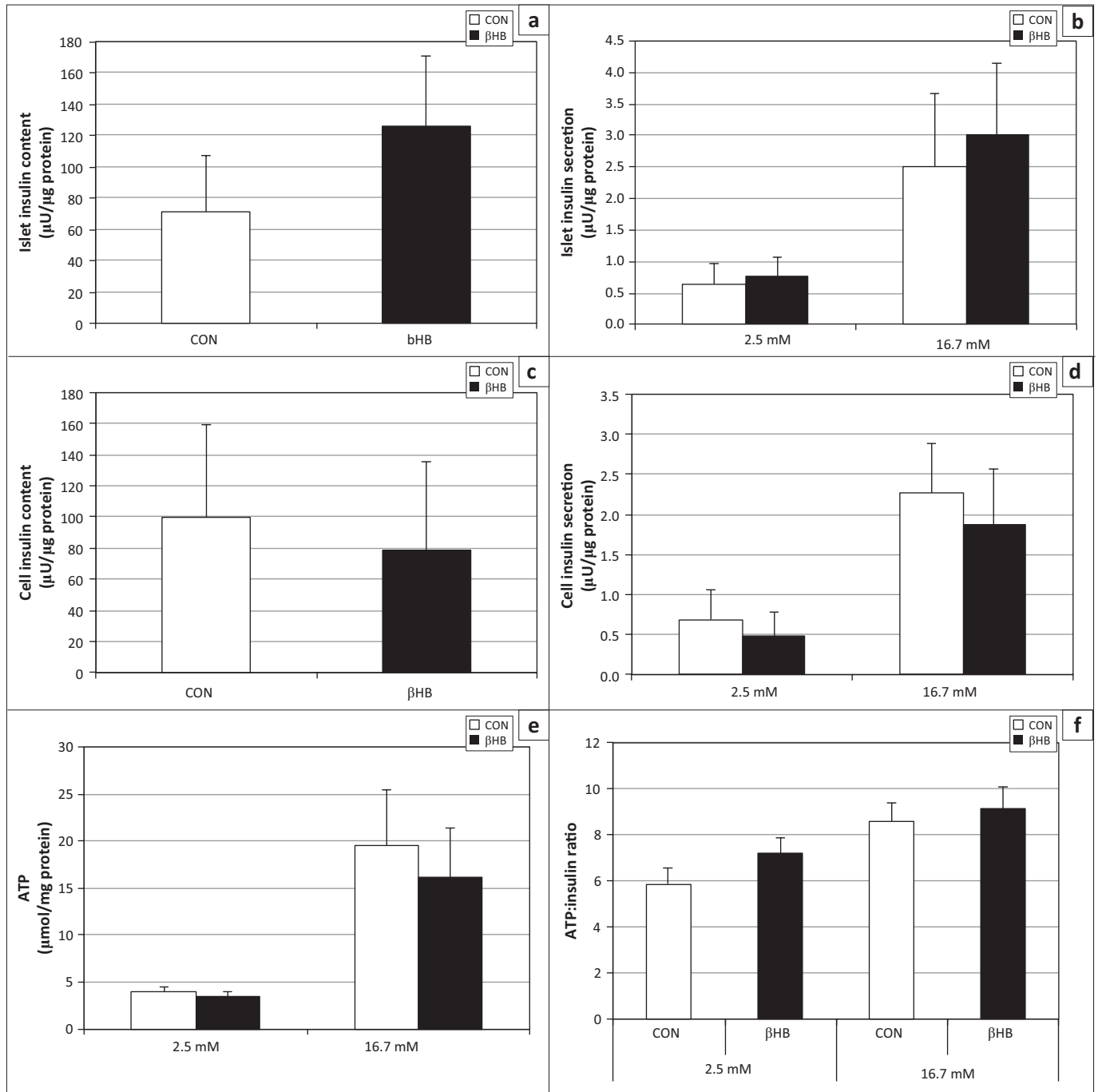


FIGURE 3: β -Hydroxybutyrate favourably alters mitochondrial function. INS-1 832/13 β -cells were treated with vehicle (water; CON) or with β -Hydroxybutyrate (β HB; 10 mM) for 24 h. To measure mitochondrial respiration (a; $n = 6$), cells were treated with GML: glutamate (10 mM) + malate (2 mM); GMD: + ADP (2.5 mM); GMSD: + succinate (10 mM); GMSF: + FCCP (0.05 μ M) [details in (g)]. Respiratory control ratio (b; RCR; GM_D/GM_L), uncoupling control ratio (c; UCR; GMS_D/GMS_D) and complex II factor (f; GMS_D-GM_D) were determined from the respiration assay. ATP production (d) and P:O ratio (e; peak ATP production/O₂ use at that same time) were determined by measuring fluorescence (503 mM/530 mM) with MgG. * $P < 0.05$; $n = 6$.

and β -cell insulin production. Concomitant with these improvements, hepatic and pancreatic fat significantly diminished. However, the ability of lifestyle changes to promote β -cell 'recovery' is not universal. By introducing lifestyle changes, including alterations in nutrient content to reduce carbohydrate consumption among patients, McFarlane et al.⁷ found that roughly half achieved a complete remission of T2D, including a normalisation of β -cell function. Despite a lack of complete functional restoration of β -cells, the

remaining patients experienced improvements in insulin sensitivity with reduced reliance on pharmacological therapies.

In addition to the clear and justified focus on diet, exercise is also commonly used as a tool to improve insulin-glucose health in insulin resistance and T2DM, including beneficial changes to β -cell health.³⁶ Like dietary changes, exercise is similarly an intervention known to be ketogenic.^{37,38,39} Future research efforts will serve to establish the role



Source: Accili D, Talchai SC, Kim-Muller JY, et al. When beta-cells fail: Lessons from dedifferentiation. *Diabetes Obes Metab.* 2016;18(Suppl 1):117–22.

FIGURE 4: β -Hydroxybutyrate maintains insulin content and glucose-stimulated insulin secretion in primary rat islets and INS-1 832/13 β -cells. Primary rat islets and INS-1 832/13 β -cells were cultured for 48 h with vehicle (CON) or 10 mM β -Hydroxybutyrate (bHB) after which (a and c; $n = 3$) total insulin content and (b and d; $n = 3$) glucose-stimulated insulin secretion was measured for islets (a and b) or INS-1 832/13 β -cells (c and d). Total ATP was measured in identical conditions (e; $n = 3$), and the ATP:insulin ratio was determined (f; $n = 3$).

of ketones, if any, in mediating improved β -cell function with exercise.

Considering evidence of improved clinical outcomes in T2DM in states of elevated ketones (e.g. ketogenic diet),⁵ it's not surprising that several studies have previously explored the relationship between ketones, particularly β HB and β -cell function, albeit with conflicting results. While Zhou et al.⁴⁰ found that rat β -cell function, particularly insulin secretion, was diminished when exposed to β HB, Madison et al.⁴¹ observed β HB stimulated β -cell action and

insulin release, a finding corroborated by others.^{42,43} In addition to experimental model (species, *in vivo*, etc.), other notable differences abound between these studies that may explain disparate results, including the addition of elevated fatty acids, which are known to alter β -cell insulin production.⁴⁴ Importantly, plasma triglycerides and free fatty acids are generally reduced in type 2 diabetics on a carbohydrate-restricted diet.^{8,45} Thus, a condition of elevated ketones *and* elevated FFA is not generally reflective of a type 2 diabetic on a carbohydrate-restricted diet. Nevertheless, we observed an interesting trend in insulin

secretion with β HB treatment: whereas primary islets tended towards enhanced insulin secretion, INS-1 832/13 β -cells tended towards reduced insulin secretion. These disparate responses may be a result of the primary versus immortalised cells and their inherent responsiveness to β HB and may shed light on a physiological improvement in insulin secretion from β -cells with increased β HB.

Importantly, in addition to the similar focus on insulin secretion between our work and previous studies, we are unaware of any research that has explored changes in β -cell mitochondrial bioenergetics with β HB exposure. Our results suggest that β -cells, like neurons, thrive with exposure to β HB, the predominant circulating form of ketones). Indeed, β -cell replication and survival were significantly increased when treated with β HB (Figure 1). Moreover, β HB exposure increased β -cell viability, mitochondrial biogenesis and ATP production (Figures 2 and 3). Importantly, the increased ATP production occurred despite a lack of significant change in actual oxygen use with the addition of ADP to induce oxidative phosphorylation through complex I alone (i.e. GM_L and GM_D). That respiration rates between CON and β HB-treated β -cells departed significantly only with the addition of succinate (Figure 2a and f) corroborates previous findings in neurons – namely, that β HB increases succinate dehydrogenase activity.⁴⁶ Nevertheless, an important caveat with this research is that the levels of β HB used in culture (10 mM) are unlikely to be reached in healthy individuals, where insulin is always sufficiently present to inhibit such a degree of ketogenesis. Future efforts will determine the efficacy of ketones to improve β -cell function at more achievable levels of ketones in humans (i.e. 1 mM–3 mM).

A provocative paradigm arises from a general overview of these results: that β -cells appear to respond so favourably to ketones may be a physiological attempt to adapt to a pathological condition. In particular, the untreated type 1 diabetic has dangerously high ketones (i.e. ketoacidosis) as a result of too little or no insulin because of β -cell loss. Perhaps in response to and in parallel with the immune-mediated loss of pancreatic β -cells, hepatic ketogenesis is a mechanism that, in addition to other ends, attempts to maintain and even restore β -cell survival.

Conclusion

In conclusion, efforts to explore the benefits of lifestyle or pharmacological interventions in improving native β -cell function are necessary and should be timely. A very recent meta-analysis involving almost 19 000 patients revealed no long-term benefit to exogenous insulin therapy in type 2 diabetics, but rather found only potential harm,⁴⁷ highlighting the need to focus on mechanisms that improve endogenous glucose control. Altogether, our findings suggest that β HB may be involved in improved β -cell outcomes in states of increased ketogenesis and provide a focus for future therapies to enhance β -cell maintenance in type 2 diabetes.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

B.T.B. conceived and managed the project. M.S., D.R.L., B.W.D., C.D., J.D.R., K.B.K., B.A.P., J.L.G., and J.S.G. performed and analysed all experiments. J.S.T. made conceptual contributions and data analysis. M.S., D.R.L., J.S.T., and B.T.B. prepared the manuscript.

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