RESEARCH

SGLT2-i improves markers of islet endothelial cell function in *db/db* diabetic mice

Meghan F Hogan^{1,2}, Daryl J Hackney¹, Alfred C Aplin¹, Thomas O Mundinger², Megan J Larmore³, Joseph J Castillo^{1,2}, Nathalie Esser^{1,2}, Sakeneh Zraika^{1,2} and Rebecca L Hull^{1,2}

¹Division of Metabolism, Endocrinology and Nutrition, VA Puget Sound Health Care System, Seattle, Washington, USA

²Department of Medicine, University of Washington, Seattle, Washington, USA

³Department of Comparative Medicine, University of Washington, Seattle, Washington, USA

Correspondence should be addressed to R L Hull: rhull@uw.edu

Abstract

Islet endothelial cells produce paracrine factors important for islet beta-cell function and survival. Under conditions of type 2 diabetes, islet endothelial cells exhibit a dysfunctional phenotype including increased expression of genes involved in cellular adhesion and inflammation. We sought to determine whether treatment of hyperglycemia with the sodium glucose co-transporter 2 inhibitor empagliflozin, either alone or in combination with metformin, would improve markers of endothelial cell function in islets, assessed *ex vivo*, and if such an improvement is associated with improved insulin secretion in a mouse model of diabetes *in vivo*. For these studies, *db/db* diabetic mice and non-diabetic littermate controls were treated for 6 weeks with empagliflozin or metformin, either alone or in combination. For each treatment group, expression of genes indicative of islet endothelial dysfunction was quantified. Islet endothelial and beta-cell area was assessed by morphometry of immunochemically stained pancreas sections. Measurements of plasma glucose and insulin secretion during an intravenous glucose tolerance test were performed on vehicle and drug treated diabetic animals. We found that expression of endothelial dysfunction marker genes is markedly increased in diabetic mice. Treatment with either empagliflozin or metformin lowered expression of the dysfunction marker genes *ex vivo*, which correlated with improved glycemic control, and increased insulin release *in vivo*. Empagliflozin treatment was more effective than metformin alone, with a combination of the two drugs demonstrating the greatest effects. Improving islet endothelial function through strategies such as empagliflozin/metformin treatment may provide an effective approach for improving insulin release in human type 2 diabetes.

Key Words

- ▶ capillary
- ▶ *db/db* mouse
- ▶ diabetes
- ▶ empagliflozin
- ▶ IVGTT
- ▶ metformin

Journal of Endocrinology
(2021) **248**, 95–106

Introduction

In addition to their role in maintaining vascular homeostasis, endothelial cells play a crucial role in producing factors important for the function of many cell types and tissues. Within the pancreas, islets are highly vascularized with fenestrated capillaries being primarily

composed of endothelial cells. These cells are a source of paracrine signals important for both the proliferation and survival of islet beta cells and which contribute to normal insulin release (Olsson & Carlsson 2006, Richards *et al.* 2010, Peiris *et al.* 2014, Hogan & Hull 2017).

Among these factors are molecules important for maintaining vascular tone, supporting cellular growth, and production of extracellular matrix.

Hyperglycemia has been proposed to be a major factor in the development of endothelial dysfunction and microvascular complications in human diabetes (DCCT Research Group *et al.* 1993, Brownlee 2005, Maiti & Agrawal 2007). During the progression of diabetes in both animals and humans, upregulation of genes indicating endothelial dysfunction is observed throughout the vasculature (reviewed in Shi & Vanhoutte 2017). These include vasoconstrictive proteins, cell adhesion molecules and pro-inflammatory cytokines (e.g. endothelin-1, E-selectin and interleukin-6, respectively). Upregulation of these and other genes has been reported in islets isolated from diabetic GK rats (Lacruz *et al.* 2009) or *db/db* mice backcrossed on to the C57BL/6J background (B6.BKS(D)-*Lepr^{db}/J*) (Hogan *et al.* 2017). Dysregulation of *Edn1* and *Sele* was also observed in an islet endothelial cell line cultured under diabetic conditions, and secreted factors from these cells reduced glucose-stimulated insulin secretion from mouse islets (Hogan *et al.* 2017).

Loss of pancreatic islet endothelial cell function and disruption of vascular morphology are features of both human diabetes and rodent models thereof. Endothelial dysfunction as a result of hyperglycemia in diabetes has been reported in several vascular beds *in vivo*, including the retina (Stitt *et al.* 1997) and kidney glomeruli (Horie *et al.* 1997). Additionally, several studies using cultured non-islet endothelial cells have demonstrated increases in markers of vascular tone, angiogenesis, fibrinolysis, oxidative stress under high glucose conditions (Kado *et al.* 2001, Piconi *et al.* 2004). In the pancreas, morphological changes to islet microvasculature including irregular capillary shape, capillary dilation, and thickening of the microvascular walls have been described in several rodent models of diabetes (Nakamura *et al.* 1995, Shao *et al.* 2006, Lacraz *et al.* 2009). Similar abnormalities have been described in islet capillary beds from patients with type 2 diabetes (Brissova *et al.* 2015, Shah *et al.* 2016).

We hypothesized that improving islet endothelial cell function would have a positive impact on beta-cell secretory function in diabetic mice. We previously found that treatment of *db/db* diabetic mice on a C57BL/6J background for 2 weeks with the non-selective sodium-glucose co-transporter (SGLT) inhibitor phlorizin reduced plasma glucose levels, but did not reduce expression of genes indicative of endothelial dysfunction when compared to non-diabetic (*db/+;+/+*) littermate controls (Hogan *et al.* 2017). Here we investigate the effect of

normalizing plasma glucose levels over a longer time frame in the more severe diabetic *db/db* model (on the parental C57BL/KsJ background) using the SGLT2 inhibitor empagliflozin either alone or in combination with metformin. In this study, we present evidence that drug treatment results in improved glycemic control, ameliorates islet endothelial dysfunction, improves insulin release and has a minimal effect on overall islet microvascular morphology.

Materials and methods

Animals and interventions

Six-week-old male BKS.Cg-Dock7^m *+/+* *Lepr^{db}/J* mice (Stock #642, Jackson Laboratories) were group-housed under SPF conditions on a 12 h light:12 h darkness cycle. Mice homozygous for the *Lepr^{db}* mutation (*db/db*) on the C57BL/KsJ background manifest morbid obesity, chronic hyperglycemia, pancreatic beta-cell loss and deficient insulin release (Hummel *et al.* 1972). Control littermates (both *db/+;+/+*) are generally phenotypically normal, without attributes of diabetes. For this study, animals were enrolled at 6 weeks of age and treated for 6 weeks. Control ('VEH') animals of each genotype were fed PicoLab mouse chow (Diet #5058, PMI, St. Louis, MO, USA) *ad libitum* prior to sacrifice. A total of 108 mice were studied (specific *n* for each endpoint is given in each figures/figure legends). For experimental drug treatment with empagliflozin ('EMPA' group, 20 mg/kg/day target dose), animals were fed with the same diet containing EMPA (105 mg/kg; formulated by Research Diets, New Brunswick, NJ, USA). For treatment with metformin ('MET' group, 500 mg/kg/day target dose), we found administration of metformin in the diet to be ineffective (data not shown), and thus used drinking water as the route for this treatment (5 g/L). For treatment with both drugs ('EMPA+MET' groups) animals received both empagliflozin-containing food and metformin-containing water. Mice were distributed among experimental groups so that weights were matched prior to the start of each study. Animals were weighed weekly and non-fasting plasma glucose levels were measured in blood collected from lateral saphenous vein by glucose oxidase assay. Weekly food and water intake were calculated by weighing remaining food and measuring water on a per-centage basis. All procedures were approved by the VA Puget Sound Health Care System Institutional Animal Care and Use Committee, according to the USDA Animal Welfare Act and Office of Laboratory Animal Welfare guidelines.

Intravenous glucose tolerance tests

After 6 weeks of treatment, a subset of mice ($n = 3\text{--}6$ per experimental group) underwent conscious intravenous glucose tolerance tests (IVGTT). This procedure allows measurement of plasma glucose and insulin levels without the confounding effect of anesthesia (Ayala *et al.* 2010, Windelov *et al.* 2016). Briefly, an initial surgery was performed to place indwelling catheters into the carotid artery and jugular vein (based on Ayala *et al.* 2006). Surgeries were performed on a total of 44 mice. Of those, 11 died due to post-operative complications (due to peri-surgical bleeding or poor tolerance of analgesia). These deaths occurred in all groups except *db/db*-VEH mice. Anesthesia-related mortality was rectified by reducing ketoprofen from a standard dose (4 mg/kg) to 1 mg/kg (for *db/db* mice) or 2 mg/kg (for *db/+;+/+* mice), given both before and after surgery. Mice were monitored daily post-surgery for at least 3 days and were allowed to recover in their home cages; the IVGTT procedure performed in conscious, unrestrained mice 1 week after surgery. Of the 33 mice that survived surgery, 12 were removed from the study due to problems with catheter patency (this was observed in all groups except *db/db*-EMPA). Data are presented on the remaining 21 mice. Seven days post catheter placement, mice were fasted for 6 h during the middle of the light cycle and baseline blood draws taken 10 min and immediately before glucose infusion (1 g/kg) via jugular vein catheter. Blood was collected from the carotid catheter at 2, 5, 10, 20, 30, and 45 min following glucose administration. Plasma glucose was measured at each timepoint using the glucose oxidase method and insulin concentration was measured Mouse Ultrasensitive Insulin ELISA (ALPCO, Salem, NH, USA).

Islet isolation, RNA extraction, and qPCR

Islets were isolated from mouse pancreata by collagenase digestion and purified by gradient separation (Zraika *et al.* 2007). Total islet RNA ($n = 5\text{--}9$ per group) was extracted using the High Pure RNA isolation kit (Roche) cDNA was synthesized using the High-Capacity cDNA RT kit (ABI, Foster City, CA, USA). Quantitative real-time PCR (qPCR) was performed on an ABI 7500 thermocycler using validated TaqMan probes (Life Technologies). Expression levels of E-selectin (*Sele*, #Mm00441278_m1); interleukin-6 (*Il6*, #Mm00446190_m1); endothelin-1 (*Edn1*, #Mm00438656_m1); and vascular cell adhesion molecule 1 (*Vcam1*, #Mm01320970_m1) were normalized to cyclophilin (*Ppib*, #Mm00478295_m1) and fold

changes were calculated using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001).

Immunohistochemistry

Histological and immunochemical reactions were performed on formalin-fixed paraffin-embedded sections. Briefly, mouse pancreata were excised ($n = 4\text{--}12$ per treatment group), fixed overnight in 10% neutral-buffered formalin, paraffin-embedded and serially sectioned at 4 μm . Multiple sections ($n = 3\text{--}6$) from each pancreas were cut 50–100 μm in depth from one another and were processed using a BOND-MAX automatic IHC system (Leica) using heat-mediated EDTA antigen retrieval (100°C for 20 min). Sections were stained with anti-CD31 (DIA-310 Dianova, Hamburg, Germany, diluted 1:250) followed by rabbit anti-rat secondary (AI-4001 Vector Laboratories, Burlingame, CA, USA 1:300). Leica Bond Mixed Refine reagents (HRP and 3,3'-diaminobenzidine-based) were used for detection and slides were counterstained with hematoxylin. Isotype-matched IgG antibodies were included as negative controls.

For insulin and glucagon immunofluorescence, serial sections from each experimental group underwent immunohistochemistry with antibodies against either insulin (I2018 Sigma 1:2000), or glucagon (EP3070 Abcam 1:2000) followed by AlexaFluor 488 and AlexaFluor 546 secondary antibodies, respectively (Life Technologies 1:200). Nuclei were stained with Hoechst 33258 (Sigma 2 $\mu\text{g/mL}$).

CD31 image acquisition and analysis

Investigators were blinded to genotype/drug assignments of stained sections until final data analysis. Large area scans of CD31-stained sections were generated using a fully automated NiE microscope equipped with NIS Elements High Content software (Nikon Instruments). For morphometric measurements of pancreatic islet endothelial cell density, individual islets were circumscribed by hand and CD31 pixel density was computed across the section. CD31 area/islet area or pancreas area were calculated as $\sum \text{CD31 islet positive area} / \sum \text{islet area}$ or $\sum \text{pancreas area} \times 100\%$. The number of CD31+ objects/islet was computed as $\sum (\text{number of islet CD31+ objects/section})$. An average of 28 ± 2 islets for each mouse were analyzed.

Automated image acquisition and analysis of insulin- and glucagon-positive areas

Investigators were blinded to genotype/drug assignments of stained sections until final data analysis.

Pancreas sections were imaged using an automated algorithm (JOBS module of NIS Elements) on an NiE microscope (Nikon). First, large area images of each tissue section were captured at 4 $\times$. Individual islets on each section were automatically identified and, following manual verification, images of each islet were captured using four-channel fluorescence (insulin-AlexaFluor 488, glucagon-AlexaFluor 546, Hoechst 33258 and far red, the latter to detect non-specific fluorescence) at 20 $\times$ magnification. A minimum cut off of 1000 μm^2 was used for islet area.

Measurements of insulin and glucagon-positive area within islets were made using an automated analysis routine (NIS Elements, Nikon). Binary thresholding was applied on a per-section basis to measure positive fluorescence area in insulin and glucagon channels above that threshold for every islet. Binary layers for insulin and glucagon were combined through post-processing steps to calculate total islet area. Insulin-positive and glucagon-positive areas were calculated on a per-islet and a per-section basis. An average of 38 ± 2 islets for each mouse were analyzed. Pancreas tissue area was computed from large area images by binary thresholding on the Hoechst channel.

Data analysis

Data are presented as mean $\pm$ S.E.M. Time course data (plasma glucose, body weight, and IVGTTs) were analyzed using a repeated-measures general linear model. Differences among experimental groups for categorical data were compared using ANOVA. Bonferroni *post hoc* analysis was used for both statistical tests to correct for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results

Pharmacologic treatment of diabetic *db/db* mice does not affect body weight but decreases plasma glucose

In order to assess the effects of drug administration on body weight and plasma glucose levels, weekly measurements were made in diabetic C57BL/KsJ.*db/db* and non-diabetic *db/+;+/+* littermate control animals treated with either empagliflozin (EMPA group), metformin (MET group), a combination of the two drugs (EMPA+MET group), or vehicle (VEH group). We first established the route and dose of drug administration by measuring food and water consumed by animals over a 2-week period

(Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article). At 6 weeks of age, *db/db* mice were heavier compared to non-diabetic *db/+;+/+* littermates. Both diabetic and non-diabetic mice gained weight over time, however, *db/db* mice increased body mass at a higher rate (Fig. 1A). Treatment with either EMPA, MET, or the EMPA+MET combination for 6 weeks had no effect on the rate of weight gain in *db/db* mice. In contrast, after 6 weeks of treatment with the EMPA+MET, non-diabetic *db/+;+/+* mice had a slight but significant reduction in body weight vs non-treated controls.

As expected, non-diabetic *db/+;+/+* animals exhibited stable levels of plasma glucose for the duration of this study; these were not affected by any of the drug treatments (Fig. 1B). In contrast, over 6 weeks of treatment, plasma glucose levels in VEH-treated *db/db* mice progressively increased compared to *db/+;+/+* littermates and were reduced by EMPA or MET alone, while EMPA alone was more effective. When *db/db* mice were treated with the EMPA+MET combination, plasma glucose levels were reduced to near-normal levels that were indistinguishable from non-diabetic control animals (Fig. 1B). When expressed as percent change from baseline, to account for a slight (non-significant) difference in baseline glucose levels among *db/db* groups, EMPA significantly lowered plasma glucose in *db/db* mice while MET alone had no effect. Treatment with the EMPA+MET combination resulted in near-normalization of plasma glucose after 6 weeks of treatment, resulting in levels $\sim 30\%$ lower than baseline values (Fig. 1C).

Diabetic *db/db* mouse islets upregulate expression of genes indicating endothelial dysfunction

To examine alterations in expression of genes indicative of endothelial dysfunction, qPCR was used to quantify relative expression of the adhesion molecules *Sele* and *Vcam1*, the vasoconstrictive molecule *Edn1*, and the inflammatory molecule *Il6*. All four of these genes were significantly upregulated in islets isolated from diabetic *db/db* animals when compared to non-diabetic *db/+;+/+* control islets (Fig. 2).

EMPA and MET markedly reduce expression of endothelial dysfunction genes in diabetic *db/db* islets

Next, we examined the expression of endothelial dysfunctional markers in islets from diabetic *db/db* animals and non-diabetic *db/+;+/+* control mice treated for 6 weeks with EMPA or MET, either alone or in

Figure 1

Body weight (A), *ad libitum* fed plasma glucose levels (B), and the percent change in plasma glucose from baseline (C) over 6 weeks of treatment, beginning at 6 weeks of age. Treatment for both genotypes are denoted as follows: *db/+;+/+*-VEH (open circles, *n* = 9); *db/db*-VEH (black circles, *n* = 7); *db/+;+/+*-EMPA (open triangles, *n* = 5); *db/db*-EMPA (black triangles, *n* = 5); *db/+;+/+*-MET (open inverted triangles, *n* = 5); *db/db*-MET (black inverted triangles, *n* = 6); *db/+;+/+*-EMPA+MET (open squares, *n* = 5); *db/db*-EMPA+MET (black squares, *n* = 6). **P* < 0.05 *db/+;+/+*-VEH vs *db/+;+/+*-EMPA+MET, **P* < 0.05 vs *db/+;+/+*-VEH, and #*P* < 0.05 vs *db/db*-VEH.

Figure 2

Expression of endothelial genes in islets isolated from diabetic and non-diabetic animals. Relative expression of E-selectin (A, *Sele*), endothelin-1 (B, *Edn1*), interleukin-6 (C, *Il6*), and vascular cell adhesion molecule 1 (D, *Vcam1*) in *db/+;+/+* (white bars; open symbols) and *db/db* (grey bars; closed symbols) islets treated for 6 weeks with either EMPA or MET alone, or in combination (EMPA+MET). *n* = 5–9 for all conditions; **P* < 0.05 vs *db/+;+/+*-VEH, and #*P* < 0.05 vs *db/db*-VEH.

combination. In each case, *Edn1*, *Il6*, *Sele*, and *Vcam1* expression was markedly reduced by drug treatment in *db/db* islets (Fig. 2). While each treatment regimen was effective at reducing expression of these endothelial dysfunction markers, notable differences between

treatment groups were observed. Treatment with MET alone reduced islet expression of only *Il6* and *Sele* while EMPA resulted in reduced expression of all four marker genes. EMPA+MET completely normalized expression of all markers. In contrast, in islets from non-diabetic *db/+;+/+* control animals neither drug, alone nor in combination, altered gene expression. Correlation analyses showed a strong relationship between plasma glucose levels following 6 weeks of treatment and expression of each of the four genes examined, among *db/db* animals (Fig. 3).

Intra islet endothelial cell density is not affected by EMPA or MET treatment

We next determined whether EMPA and/or MET treatment resulted in alterations in pancreatic islet capillary morphology. Areas of endothelial cell CD31 positive immunoreactivity were calculated from images of stained sections taken from diabetic *db/db* and non-diabetic *db/+;+/+* mice of all treatment groups (Fig. 4A). In agreement with our previous results in *db/db* diabetic mice on a C57BL/6J background (Hogan *et al.* 2017), changes in the overall vascular area per islet were not observed in any of the treatment groups, regardless of whether diabetic *db/db* or non-diabetic *db/+;+/+* genotypes were analyzed

(Fig. 4B and C). Additionally, the total number of individual CD31+ objects per islet was equivalent across experimental groups (Fig. 4D), indicating that the overall density of islet vasculature was unchanged by drug treatment.

Plasma glucose and insulin secretion is normalized in *db/db* mice treated with empagliflozin

To determine if normalized expression of endothelial dysfunction genes is associated with improvements in insulin release, IVGTTs were performed on conscious, unrestrained diabetic *db/db* (all treatment groups) and non-diabetic *db/+;+/+* animals (VEH only). As expected, fasting plasma glucose levels and glucose levels throughout the IVGTT were significantly higher upon glucose stimulation in VEH-treated *db/db* mice when compared to *db/+;+/+* animals (Fig. 5A). When diabetic *db/db* animals were treated with EMPA alone or the EMPA+MET combination for 6 weeks, glucose responses were significantly reduced (Fig. 5A). Treatment with MET alone had little effect. VEH-treated *db/db* mice exhibited no insulin response to IV glucose (Fig. 5B). EMPA treatment alone or the EMPA+MET combination resulted in a robust increase in the insulin response throughout the IVGTT in *db/db* mice, with MET treatment having an intermediate effect.

Figure 3

Correlation of gene expression and plasma glucose levels after 6 weeks of drug treatment. All animals began treatment at 6 weeks of age. Treatments are denoted as follows: VEH, circles; EMPA, triangles; MET, inverted triangles; EMPA+MET, squares. $n = 5-9$ for each treatment group, $P < 0.0001$ for all analyses, Spearman rank correlation.

Figure 4

Relative islet capillary area in pancreatic sections from diabetic animals and non-diabetic controls. (A) Representative images of islets within pancreas sections following immunohistochemistry for CD31 (brown) with nuclear counterstaining (blue) from non-diabetic *db/+;+/+* animals (panels 1–4) and diabetic *db/db* animals (panels 5–8). Mice treated with VEH (1 and 5), EMPA (2 and 6), MET (3 and 7), and EMPA+MET (4 and 8) for 6 weeks, *db/+;+/+* (white bars; open symbols) and *db/db* (black bars; closed symbols). (B) Islet capillary area was quantified as the percentage of CD31+ islet area for each genotype and treatment group. (C) Islet CD31+ area normalized to pancreas section. (D) CD31 object count per islet. *n* = 4–13.

Figure 5

Plasma glucose (A) and insulin levels (B) measured during an IVGTT in *db/+;+/+*-VEH (open circles, *n* = 6), *db/db*-VEH (black circles, *n* = 4), *db/db*-EMPA (black triangles, *n* = 4), *db/db*-MET (black inverted triangles, *n* = 5), *db/db*-EMPA+MET (black squares, *n* = 3). For plasma glucose levels over time (panel A), *post hoc* pairwise comparisons demonstrated the following significant differences: *db/+;+/+*-VEH vs all DB groups (denoted by *); DB-VEH vs DB-EMPA, DB-MET and DB-EMPA+MET (denoted by #); DB-MET vs DB-EMPA and DB-EMPA+MET (denoted by \$). For plasma insulin levels over time (panel B), *post hoc* pairwise comparisons demonstrated the following significant differences among DB groups: DB-VEH vs DB-EMPA, DB-MET and DB-EMPA+MET (denoted by #); DB-MET vs DB-EMPA and DB-EMPA+MET (denoted by \$). For *, # and \$ *P* < 0.05 for all comparisons.

Insulin-positive area is restored in diabetic *db/db* islets by EMPA+MET combination therapy

Finally, glucagon and insulin immunoreactivity were performed in pancreas sections from diabetic *db/db* and non-diabetic *db/+;+/+* mice (all treatment groups). Representative immunofluorescent images of an islet from each experimental group are shown in Fig. 6A. For each experimental group, average pancreatic section area and islet cross-sectional area were equivalent (Fig. 6B and C). As expected, insulin-positive area expressed per islet area was reduced in VEH-treated *db/db* mice compared to non-diabetic control *db/+;+/+* animals (Fig. 6D). Drug treatment tended to increase insulin area within islets but no significant differences between groups were observed (Fig. 6D). While treatment of *db/db* animals with either EMPA or MET alone had no effect on insulin area normalized to pancreas section area, the EMPA+MET combination was effective in increasing this parameter (Fig. 6E). Glucagon positive area was not affected by any experimental treatment or genotype (Fig. 6F and G).

Discussion

Loss of pancreatic islet endothelial cell function and disruption of vascular morphology are features of human diabetes and rodent models thereof. Here we used islets isolated from diabetic C57BL/KsJ.*db/db* mice to examine the effects of SGLT2 inhibitor empagliflozin, both alone and in combination with metformin, on islet endothelial cells and on beta-cell function. We have shown that islets from *db/db* diabetic animals exhibit endothelial dysfunction which includes upregulation of *Edn1*, *Sele*, *Il6*, and *Vcam1* indicating a pro-adhesive and inflammatory phenotype, and that treatment of diabetic *db/db* mice with empagliflozin and metformin ameliorates the expression of these markers in islets.

These findings are in line with a recent study showing that empagliflozin normalizes endothelial function in the diabetic ApoE-deficient mouse model *in vivo* (Ganbaatar *et al.* 2020). In the present study, we observed a strong correlation between plasma glucose levels and the degree of expression of endothelial dysfunction marker genes, suggesting that the extent of glucose-lowering is a major determinant of the amelioration of islet endothelial dysfunction marker expression in *db/db* diabetic mice. Concomitantly, we observed a substantial increase in insulin release in *db/db* diabetic mice treated with empagliflozin alone or in combination

with metformin. These data are consistent with a model whereby improvements in islet endothelial function contribute to improvements in beta-cell function. This model is supported by our previously published *in vitro* data showing that islet endothelial cells normally release factors that enhance glucose-stimulated insulin release and that this positive effect is abolished under diabetic conditions (Hogan *et al.* 2017). Strikingly, in the present study, even though the glucose-mediated insulin release was improved ten-fold by empagliflozin treatment of *db/db* mice, beta-cell area was unchanged except for a significant increase in insulin area/pancreas area only observed in *db/db* mice treated with empagliflozin and metformin. This indicates that while these interventions may be effective in beta-cell expansion, a large capacity exists for improving beta-cell function (e.g. via factors produced by islet endothelial cells) without a requirement for coincident increases in beta-cell area.

Similarly, changes in islet capillary density or islet CD31-positive area were not observed in vehicle-treated *db/db* mice compared non-diabetic littermates. These data agree with our previous findings in C57BL/6J.*db/db* mice in which islet endothelial cell dysfunction preceded morphological abnormalities/cell loss (Hogan *et al.* 2017) and are consistent with data from human studies where no decreases in overall capillary density were observed, despite observed thickening and fragmentation of the capillaries (Brissova *et al.* 2015, Shah *et al.* 2016). Our results contrast with an electron microscopy study performed in C57BL6/Ks.*db/db* mice that reported a decrease in islet capillary number and an increase in capillary area/size from 6- to 12-month-old mice (Nakamura *et al.* 1995). However, capillary defects were not observed in younger, 4-month-old animals, consistent with results presented here. Taken together our data strongly suggests that changes in expression of functional endothelial markers can be uncoupled from changes in capillary morphology and/or density. Furthermore, profound changes in the environmental milieu surrounding islet endothelial cells do not necessarily alter vascular morphology in diabetic mice.

In humans, in addition to beneficial effects on normalizing plasma glucose levels, treatment of T2D with SGLT2 inhibitors results in multiple and diverse metabolic and cardiovascular benefits including lowering blood pressure, reducing body weight, and reducing overall cardiovascular mortality rates (Nauck *et al.* 2011, Majewski & Bakris 2015, Zinman *et al.* 2015). The precise mechanisms by which SGLT2 inhibitors mediate positive cardiovascular effects are currently a topic of intense study,

Figure 6

Quantification of insulin and glucagon area in pancreatic sections from diabetic and non-diabetic animals. Representative images (A) of islets within pancreas sections following immunohistochemistry for insulin (green), glucagon (red), and nuclei (blue) from non-diabetic *db/+;+/+* animals (panels 1–4) and diabetic *db/db* animals (panels 5–8). Mice treated with VEH (1 and 5), EMPA (2 and 6), MET (3 and 7), and EMPA+MET (4 and 8) for 6 weeks. Bar graphs show results of measurements from every islet on pancreatic sections from each experimental group. Islet area (B), pancreas section area (C), insulin/islet area, calculated as insulin positive area per islet (D) or per pancreas section area (E), glucagon positive area per islet (F) or per pancreas section area (G) from each genotype and treatment group. *db/+;+/+* (open bars; open symbols) and *db/db* (grey bars; closed symbols) animals; $n = 4$ –13; * $P < 0.05$ vs *db/+;+/+*-VEH, and # $P < 0.05$ vs *db/db*-VEH.

our results highlight the need to elucidate how these drugs affect the function of microvascular endothelial cells. In other non-islet endothelial cell types, it has been reported that SGLT inhibition (with phlorizin) can directly impact PI3K/AKT signaling and nitric oxide production (Li *et al.* 2018). However, we did not detect *Sglt2* mRNA in isolated islets from the present study (data not shown), in line with recent data showing SGLT2 protein is not detected in human beta cells, although it has been reported to be expressed on the alpha cell (Saponaro *et al.* 2020). These data suggest that the action of empagliflozin on the islet endothelial cell and beta cell is indirect. There is evidence in the literature for a direct effect of metformin on islets (Lupi *et al.* 1999, 2002, Patane *et al.* 2000), which may explain at least some of the observed effects on endothelial markers and insulin release that we observe in the present study. Finally, we observed a significant effect of empagliflozin and metformin to increase beta-cell area in *db/db* mice, suggesting that this could have contributed to the improved endothelial profile and/or beta-cell function in this group.

One limitation of this study is that it does not demonstrate a direct link between improving expression of endothelial markers and increasing beta-cell secretory function. Although our data clearly show that modulating chronic hyperglycemia with empagliflozin and metformin results in both a reduction of expression of genetic markers of endothelial dysfunction and an improvement of insulin release, it is possible that these effects occur due to decreased glucose *per se* or changes in other metabolic factors that impact islet secretory function (Lytrivi *et al.* 2020). Our results are consistent with data showing that release of paracrine factors from islet endothelial cells are required for optimal beta-cell function and survival (Johansson *et al.* 2006, 2009, Hogan *et al.* 2017). Treatment of hyperglycemia may therefore be effective in improving islet endothelial cell function, which in turn may be one mechanism contributing to improvement in beta-cell secretory function.

In summary, we have shown that long-term treatment of hyperglycemia in an aggressive mouse model of type 2 diabetes with the SGLT2 inhibitor empagliflozin in combination with metformin, decreases expression of markers of islet endothelial cell dysfunction and markedly increases insulin release. In human type 2 diabetes, combination therapy with empagliflozin and metformin has been shown to be more efficacious for glucose-lowering than either treatment alone (Hadjadj *et al.* 2016). We have demonstrated that combination therapy is also particularly effective in normalizing islet

endothelial gene expression in the diabetic *db/db* mouse model. These are the first reported data supporting the hypothesis that treatment of hyperglycemia is effective for normalizing islet endothelial function (or markers thereof). Pharmacologic targeting endothelial cells within the islet microvasculature may represent an untapped strategy for improving beta-cell function and normalizing glucose metabolism in the treatment of type 2 diabetes.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0354>.

Declaration of interest

R L Hull and M F Hogan are employees of the US Department of Veterans Affairs and/or the University of Washington and completely independent of Boehringer Ingelheim. Boehringer Ingelheim had no involvement in any data analysis and no remuneration was received for the data analysis or manuscript preparation portions of this work. The other authors have nothing to disclose.

Funding

This work was supported by the Department of Veterans Affairs, VA Puget Sound Health Care System (Seattle, WA), Seattle Institute for Biomedical and Clinical Research and National Institutes of Health grants R01 DK088082 (R L H) and P30 DK017047 (Cellular and Molecular Imaging and Cell Function and Analysis Cores of the University of Washington Diabetes Research Center). M F H was supported by F32 DK109584 and the Dick and Julia McAbee Endowed Fellowship from the University of Washington. This study was supported in part by an investigator-initiated research grant from Boehringer Ingelheim to R L Hull. The sponsor provided input regarding dosing of empagliflozin but had no other input regarding study design, data collection, analysis or interpretation, writing of the report or decision to submit the report for publication. The contents of this manuscript do not represent the views of the U.S. Department of Veteran Affairs or the United States Government.

Acknowledgements

The authors thank the staff of the University of Washington's Diabetes Research Center's Cellular and Molecular Imaging and Cell Function and Analysis Cores, M J Peters, J R Willard, B M Barrow, S J Kumar for technical support, and the Metabolism group at the VA Puget Sound Health Care System in Seattle for helpful discussions during the performance of this work.

References

- Ayala JE, Bracy DP, McGuinness OP & Wasserman DH 2006 Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes* **55** 390–397. (<https://doi.org/10.2337/diabetes.55.02.06.db05-0686>)

- Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH, Mcguinness OP & Consortium NIHMMP 2010 Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Disease Models and Mechanisms* **3** 525–534. (<https://doi.org/10.1242/dmm.006239>)
- Brissova M, Shostak A, Fligner CL, Revetta FL, Washington MK, Powers AC & Hull RL 2015 Human islets have fewer blood vessels than mouse islets and the density of islet vascular structures is increased in type 2 diabetes. *Journal of Histochemistry and Cytochemistry* **63** 637–645. (<https://doi.org/10.1369/0022155415573324>)
- Brownlee M 2005 The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* **54** 1615–1625. (<https://doi.org/10.2337/diabetes.54.6.1615>)
- DCCT Research Group, Nathan DM, Genuth S, Lachin J, Cleary P, Crofford O, Davis M, Rand L & Siebert C 1993 The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine* **329** 977–986. (<https://doi.org/10.1056/NEJM199309303291401>)
- Ganbaatar B, Fukuda D, Shinohara M, Yagi S, Kusunose K, Yamada H, Soeki T, Hirata KI & Sata M 2020 Empagliflozin ameliorates endothelial dysfunction and suppresses atherogenesis in diabetic apolipoprotein E-deficient mice. *European Journal of Pharmacology* **875** 173040. (<https://doi.org/10.1016/j.ejphar.2020.173040>)
- Hadjadj S, Rosenstock J, Meinicke T, Woerle HJ & Broedl UC 2016 Initial combination of empagliflozin and metformin in patients with type 2 diabetes. *Diabetes Care* **39** 1718–1728. (<https://doi.org/10.2337/dc16-0522>)
- Hogan MF & Hull RL 2017 The islet endothelial cell: a novel contributor to beta cell secretory dysfunction in diabetes. *Diabetologia* **60** 952–959. (<https://doi.org/10.1007/s00125-017-4272-9>)
- Hogan MF, Liu AW, Peters MJ, Willard JR, Rabbani Z, Bartholomew EC, Ottley A & Hull RL 2017 Markers of islet endothelial dysfunction occur in male B6.BKS(D)-Leprdb/J mice and may contribute to reduced insulin release. *Endocrinology* **158** 293–303. (<https://doi.org/10.1210/en.2016-1393>)
- Horie K, Miyata T, Maeda K, Miyata S, Sugiyama S, Sakai H, Van Ypersele De Strihou C, Monnier VM, Witztum JL & Kurokawa K 1997 Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. *Journal of Clinical Investigation* **100** 2995–3004. (<https://doi.org/10.1172/JCI119853>)
- Hummel KP, Coleman DL & Lane PW 1972 The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I. C57BL-KsJ and C57BL-6J strains. *Biochemical Genetics* **7** 1–13. (<https://doi.org/10.1007/BF00487005>)
- Johansson M, Mattsson G, Andersson A, Jansson L & Carlsson PO 2006 Islet endothelial cells and pancreatic beta-cell proliferation: studies in vitro and during pregnancy in adult rats. *Endocrinology* **147** 2315–2324. (<https://doi.org/10.1210/en.2005-0997>)
- Johansson A, Lau J, Sandberg M, Borg LA, Magnusson PU & Carlsson PO 2009 Endothelial cell signalling supports pancreatic beta cell function in the rat. *Diabetologia* **52** 2385–2394. (<https://doi.org/10.1007/s00125-009-1485-6>)
- Kado S, Wakatsuki T, Yamamoto M & Nagata N 2001 Expression of intercellular adhesion molecule-1 induced by high glucose concentrations in human aortic endothelial cells. *Life Sciences* **68** 727–737. ([https://doi.org/10.1016/s0024-3205\(00\)00968-1](https://doi.org/10.1016/s0024-3205(00)00968-1))
- Lacraz G, Giroix MH, Kassir N, Coulaud J, Galinier A, Noll C, Cornut M, Schmidlin F, Paul JL, Janel N, *et al.* 2009 Islet endothelial activation and oxidative stress gene expression is reduced by IL-1RA treatment in the type 2 diabetic GK rat. *PLoS ONE* **4** e6963. (<https://doi.org/10.1371/journal.pone.0006963>)
- Li CY, Wang LX, Dong SS, Hong Y, Zhou XH, Zheng WW & Zheng C 2018 Phlorizin exerts direct protective effects on palmitic acid (PA)-induced endothelial dysfunction by activating the PI3K/AKT/eNOS signaling pathway and increasing the levels of nitric oxide (NO). *Medical Science Monitor Basic Research* **24** 1–9. (<https://doi.org/10.12659/msmbr.907775>)
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* **25** 402–408. (<https://doi.org/10.1006/meth.2001.1262>)
- Lupi R, Del Guerra S, Tellini C, Giannarelli R, Coppelli A, Lorenzetti M, Carmellini M, Mosca F, Navalesi R & Marchetti P 1999 The biguanide compound metformin prevents desensitization of human pancreatic islets induced by high glucose. *European Journal of Pharmacology* **364** 205–209. ([https://doi.org/10.1016/s0014-2999\(98\)00807-3](https://doi.org/10.1016/s0014-2999(98)00807-3))
- Lupi R, Del Guerra S, Fierabracci V, Marselli L, Novelli M, Patane G, Boggi U, Mosca F, Piro S, Del Prato S, *et al.* 2002 Lipotoxicity in human pancreatic islets and the protective effect of metformin. *Diabetes* **51** (Supplement 1) S134–S137. (<https://doi.org/10.2337/diabetes.51.2007.s134>)
- Lytrivi M, Castell AL, Poitout V & Cnop M 2020 Recent insights into mechanisms of beta-cell lipo- and glucolipotoxicity in type 2 diabetes. *Journal of Molecular Biology* **432** 1514–1534. (<https://doi.org/10.1016/j.jmb.2019.09.016>)
- Maiti R & Agrawal NK 2007 Atherosclerosis in diabetes mellitus: role of inflammation. *Indian Journal of Medical Sciences* **61** 292–306. (<https://doi.org/10.4103/0019-5359.32098>)
- Majewski C & Bakris GL 2015 Blood pressure reduction: an added benefit of sodium-glucose cotransporter 2 inhibitors in patients with type 2 diabetes. *Diabetes Care* **38** 429–430. (<https://doi.org/10.2337/dc14-1596>)
- Nakamura M, Kitamura H, Konishi S, Nishimura M, Ono J, Ina K, Shimada T & Takaki R 1995 The endocrine pancreas of spontaneously diabetic db/db mice: microangiopathy as revealed by transmission electron microscopy. *Diabetes Research and Clinical Practice* **30** 89–100. ([https://doi.org/10.1016/0168-8227\(95\)01155-2](https://doi.org/10.1016/0168-8227(95)01155-2))
- Nauck MA, Del Prato S, Meier JJ, Duran-Garcia S, Rohwedder K, Elze M & Parikh SJ 2011 Dapagliflozin versus glipizide as add-on therapy in patients with type 2 diabetes who have inadequate glycemic control with metformin: a randomized, 52-week, double-blind, active-controlled noninferiority trial. *Diabetes Care* **34** 2015–2022. (<https://doi.org/10.2337/dc11-0606>)
- Olsson R & Carlsson PO 2006 The pancreatic islet endothelial cell: emerging roles in islet function and disease. *International Journal of Biochemistry and Cell Biology* **38** 492–497. (<https://doi.org/10.1016/j.biocel.2005.06.021>)
- Patane G, Piro S, Rabuazzo AM, Anello M, Vigneri R & Purrello F 2000 Metformin restores insulin secretion altered by chronic exposure to free fatty acids or high glucose: a direct metformin effect on pancreatic beta-cells. *Diabetes* **49** 735–740. (<https://doi.org/10.2337/diabetes.49.5.735>)
- Peiris H, Bonder CS, Coates PT, Keating DJ & Jessup CF 2014 The beta-cell/EC axis: how do islet cells talk to each other? *Diabetes* **63** 3–11. (<https://doi.org/10.2337/db13-0617>)
- Piconi L, Quagliari L, Da Ros R, Assaloni R, Giugliano D, Esposito K, Szabo C & Ceriello A 2004 Intermittent high glucose enhances ICAM-1, VCAM-1, E-selectin and interleukin-6 expression in human umbilical endothelial cells in culture: the role of poly(ADP-ribose) polymerase. *Journal of Thrombosis and Haemostasis* **2** 1453–1459. (<https://doi.org/10.1111/j.1538-7836.2004.00835.x>)
- Richards OC, Raines SM & Attie AD 2010 The role of blood vessels, endothelial cells, and vascular pericytes in insulin secretion and peripheral insulin action. *Endocrine Reviews* **31** 343–363. (<https://doi.org/10.1210/er.2009-0035>)
- Saponaro C, Muhlemann M, Acosta-Montalvo A, Piron A, Gmyr V, Delalleau N, Moerman E, Thevenet J, Pasquetti G, Coddeville A, *et al.* 2020 Interindividual heterogeneity of SGLT2 expression and function in human pancreatic islets. *Diabetes* **69** 902–914. (<https://doi.org/10.2337/db19-0888>)

- Shah P, Lueschen N, Ardestani A, Oberholzer J, Olerud J, Carlsson PO & Maedler K 2016 Angiotensin-2 signals do not mediate the hypervascularization of islets in type 2 diabetes. *PLoS ONE* **11** e0161834. (<https://doi.org/10.1371/journal.pone.0161834>)
- Shao J, Iwashita N, Ikeda F, Ogihara T, Uchida T, Shimizu T, Uchino H, Hirose T, Kawamori R & Watada H 2006 Beneficial effects of candesartan, an angiotensin II type 1 receptor blocker, on beta-cell function and morphology in db/db mice. *Biochemical and Biophysical Research Communications* **344** 1224–1233. (<https://doi.org/10.1016/j.bbrc.2006.04.011>)
- Shi Y & Vanhoutte PM 2017 Macro- and microvascular endothelial dysfunction in diabetes. *Journal of Diabetes* **9** 434–449. (<https://doi.org/10.1111/1753-0407.12521>)
- Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB & Vlassara H 1997 Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *American Journal of Pathology* **150** 523–531.
- Windelov JA, Pedersen J & Holst JJ 2016 Use of anesthesia dramatically alters the oral glucose tolerance and insulin secretion in C57BL/6 mice. *Physiological Reports* **4** e12824. (<https://doi.org/10.14814/phy2.12824>)
- Zinman B, Wanner C, Lachin JM, Fitchett D, Bluhmki E, Hantel S, Mattheus M, Devins T, Johansen OE, Woerle HJ, *et al.* 2015 Empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes. *New England Journal of Medicine* **373** 2117–2128. (<https://doi.org/10.1056/NEJMoa1504720>)
- Zraika S, Hull RL, Udayasankar J, Utzschneider KM, Tong J, Gerchman F & Kahn SE 2007 Glucose- and time-dependence of islet amyloid formation in vitro. *Biochemical and Biophysical Research Communications* **354** 234–239. (<https://doi.org/10.1016/j.bbrc.2006.12.187>)

Received in final form 24 November 2020

Accepted 16 December 2020

Accepted Manuscript published online 19 December 2020