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Research Article

The DPP4 Inhibitor Sitagliptin Increases Active GLP-1 Levels from Human Islets and May Increase Islet Cell Survival Prior to Transplantation

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Abstract

***Background*:** One of the goals of clinical islet transplantation is to achieve a single-donor transplant that is dependent on obtaining enough quality β cell mass from one donor pancreas. Human islets are routinely cultured prior to transplantation, and pro-survival factors such as GLP-1 analogues have been reported to maintain β cell mass and survival. Interestingly, human islets may secrete GLP-1 and they also express the enzyme DPP4 that proteolytically cleaves GLP-1 into an inactive form. The aim of this study is to investigate GLP-1 secretion from human islets and to test if the DPP4 inhibitor sitagliptin may increase levels of active GLP-1 to increase islet survival in culture.

***Methods*:** Active GLP-1, glucagon, and insulin were measured from non-diabetic and type 2 diabetic islets using a glucose suppression test. Human islet cultures were treated with sitagliptin, and active GLP-1 levels were taken at 48 hours. These levels were then correlated with islet isolation parameters (SI, viability, culture time, cold ischemia time, and DNA

content) and donor parameters (age, HbA1c, and BMI). A dead cell assay using Sytox Green was used to measure cell death in human islet cultures treated with sitagliptin.

***Results*:** We found that both non-diabetic and type 2 diabetic islets secrete active GLP-1. Active GLP-1 levels from human islet cultures negatively correlated with the stimulation index for insulin and this correlation was maintained when active GLP-1 levels were increased with sitagliptin. No strong correlations were found for other islet or donor parameters. The fold change in active GLP-1 in sitagliptin treated islet cultures was correlated with increased islet survival.

***Conclusions*:** Human islets have a local paracrine source of the prosurvival peptide, active GLP-1. Although low levels of active GLP-1 are associated with greater β cell function, these levels may be increased with the DPP4 inhibitor sitagliptin. The increase in active GLP-1 levels may confer protection from islet cell death and pre-treatment of islets with DPP4 inhibitors prior to transplantation has the potential to improve post-transplant islet survival.

Keywords

Human islets; survival; sitagliptin; active GLP-1; transplantation

1. Introduction

Clinical islet transplantation (CIT) is a successful treatment for T1D patients with hypoglycemic unawareness and severe hypoglycemic events who have failed to obtain adequate glycemic control with insulin therapy alone [1]. Recent studies show that CIT can restore hypoglycemia awareness, eliminate or reduce severe hypoglycemic events, and improve glycemic control [2, 3]. Furthermore, insulin independence rates at 5 years after transplantation have improved significantly since the first Edmonton Protocol and are now nearly 50% [4]. Improvements in islet isolation and culture, along with improved donor selection and immunosuppressive agents, have propelled human islet transplantation from an experimental procedure to an effective clinical treatment.

One of the goals of CIT is to obtain a single-donor transplant that is dependent on obtaining enough quality β cell mass from one pancreas [5]. Islet preparations are routinely cultured for 24-72 hours to allow for quality control and initiation of inductive immunosuppressive treatment of the recipient [1]. Although this culture period can further purify the islet preparation, it may also result in a loss of β cell mass, falling below the minimum 5000 IEQ per kg of recipient body mass required for transplant [6]. During this pre-transplant culture period there is an opportunity to retain β cell mass and precondition the islets for improved survival in the acute post-transplant setting. Indeed, the loss of β cell mass in the hepatic portal vein transplant site in the first few days post-transplant is estimated at >50% [7], suggesting that increasing β cell survival in culture may reduce overall islet requirements. Therefore, therapies already in clinical use that maintain β cell mass and improve β cell survival would further the goal of a single-donor transplant.

Glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 (DPP4) inhibitors, medications for the treatment of T2D, have shown promise in islet transplantation. Liraglutide, a long-acting GLP-1 receptor agonist, improved islet engraftment in mice and was shown to increase

survival of human islets in culture [8, 9]. DPP4 inhibitors increase circulating levels of active GLP-1 and have been shown to increase β cell mass in animals. In a clinical pilot study, the DPP4 inhibitor sitagliptin was combined with pantoprazole to successfully restore insulin independence from failing grafts, although insulin independence was lost after treatment was stopped [10]. However, in a randomized, placebo controlled study using sitagliptin in islet autotransplantation after total pancreatectomy, sitagliptin was well tolerated, although there was no metabolic benefit from treatment [11].

Human islets secrete bioactive GLP-1 from α cells and thus have a paracrine source of GLP-1 localized to β cells [12]. Furthermore, DPP4 is also expressed in the α cells of human islets allowing for the use of DPP4 inhibitors to increase active GLP-1 levels within islets. DPP4 inhibitors have been reported to increase active GLP-1 levels and insulin secretion in human islet cultures [13, 14], with one group reporting increased β cell survival [15]. However, little is known about intra-islet GLP-1 secretion and the role it may have in maintaining β cell mass, particularly in the context of DPP4 inhibition.

In this study, we characterized GLP-1 secretion in the context of human islet isolation and donor parameters. We also investigated the potential to improve islet survival by increasing active GLP-1 levels with the DPP4 inhibitor sitagliptin. Here we show that both nondiabetic and type 2 diabetic islets secrete active GLP-1. We also observed that GLP-1 levels negatively correlate with the stimulation index for insulin secretion, suggesting that islets with a low stimulation index tend to show enhanced GLP-1 secretion, perhaps as a result of damage during isolation and culture. Sitagliptin treatment of human islets increased active GLP-1 levels ~7 fold. Increased active GLP-1 levels with sitagliptin also negatively correlated with the SI for insulin secretion. However, when human islets were treated with sitagliptin, the increased active GLP-1 levels correlated with islet cell survival, suggesting a protective effect against cell death.

2. Materials and Methods

2.1 Human Islets

De-identified human primary islets isolated from deceased donors were obtained from the Alberta Diabetes Institute IsletCore and the Clinical Islet Laboratory, University of Alberta, in accordance with institutional human ethics guidelines. If the islet preparation was <90% pure, islets were handpicked to obtain >90% purity. Islets were cultured in DMEM (5.5 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin for static incubations and islet culture experiments. Diagnosis of type 2 diabetes was determined from medical records and a HbA1c > 6.5%.

2.2 Compounds

Sitagliptin phosphate monohydrate was purchased from Biovision, reconstituted in water, and used at 200 nM concentration for islet culture experiments and dead cell assay. IL-1β (Genscript, Z02922-10) was reconstituted in PBS and used at 50 ng/ml for islet culture experiments.

2.3 Static Incubations

Static incubations were performed using 500 µL KRBH buffer containing (mM) 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 0.1% BSA, (pH 7.4) supplemented with the appropriate concentration of glucose. 50 islets were pre-incubated at 37°C with 2.8 mM glucose buffer for 2 hours, transferred to 2.8 mM glucose buffer for 1 hour, followed by 11.1 mM glucose buffer for 1 hour. Supernatant was taken and stored at −20°C for insulin, glucagon, or active GLP-1 analysis.

2.4 Islet Culture Experiments

Using a 96 well plate with optical bottoms, 40 human islets were cultured in 200uL of Ham's F10 (6.1 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin for 48 hours with or without 200 nM sitagliptin at 37°C. A parallel group was coincubated with 50 ng/ml IL-1β. Media samples were taken after 48 hours of culture, spiked with 100 uM sitagliptin to prevent further degradation by DPP4, and frozen at −20°C for active GLP-1 analysis. The islets were imaged with an AMG EVOS fluorescent microscope, 10X objective, using the brightfield channel. Images were analyzed using ImageJ and the total area of the islets was measured and calculated using the polygon tool. For the serum-free 8 hour experiment, 50 islets were cultured in 500uL of Ham's F10 supplemented with 10 mM glucose, 10 mM nicotinamide, 2 mM L-glutamine, 1.6 mM calcium chloride, 0.5% BSA, 50 uM IBMX, and 0.5% penicillin/streptomycin.

2.5 Hormone Secretion Assays

Active GLP-1 levels from islet culture media, the dead cell assay, and supernatants from static islet incubations were quantified using the electrochemiluminescent assay Active GLP-1 (v2) Kit (K150JWC-1), MesoScale Discovery. Insulin levels in supernatants from static incubations were quantified with Stellux Chemiluminescent Human Insulin ELISA, Alpco. Glucagon levels in supernatants from static incubations were quantified with HTRF Glucagon Assay, Cisbio.

2.6 Dead Cell Assay

Using a 96 well plate with optical bottoms, 40 islets were cultured at 37°C in 200uL of Ham's F10 (6.1 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin. The remaining wells of the 96 well plate were filled with PBS to reduce evaporation. The islets were treated with or without 200 nM sitagliptin for 48 hours. The time in culture was used to challenge the islets and promote cell death. After 48 hours, media samples were taken and Sytox Green (Molecular Probes, Eugene, OR) was added to each well (final concentration 100 nM). The islets were imaged with an AMG EVOS fluorescent microscope, 10X objective, using the brightfield and green channels. Images were analyzed using ImageJ where the dead cells (green spots) were counted using a threshold technique and the area of the islets was quantified. Dead cell density was calculated by dividing the dead cells by the area of the islets.

2.7 Immunoblotting

Islets were washed three times with PBS and then lysed with a lysis buffer containing (mM) 50 Tris-HCL, 1 EDTA, 1 EGTA, 1 sodium orthovanadate, 50 sodium fluoride, 5 sodium pyrophosphate,

and 0.27M sucrose. This lysis buffer was supplemented with a protease cocktail (BS387, Bio Basic Inc., 1:100 dilution), 0.1% Triton X 100, and 1 mM DTT. Islet lysates were subjected to SDS-PAGE on 8% gels transferred to nitrocellulose membranes and probed with the primary antibody, hDPPIV polyclonal Goat IgG, AF1180, R & D Systems (1:5000). Detection was with peroxidase-conjugated secondary donkey anti-goat, sc-2020, Santa Cruz (1:1000), and visualization by chemiluminescence with ECL-Plus (GE Healthcare). Images were acquired using a Kodak In-Vivo Multispectral Imaging System (Carestream MI software) and analyzed using ImageJ.

2.8 Islet Isolation Parameters

For assessment of in vitro function of islets, static glucose stimulation was performed sequentially on the same sample. After overnight culture, triplicate islet samples were incubated with 2.8 mM glucose and then with 28.0 mM glucose in Connaught Medical Research Laboratory 1066 medium. Stimulation index was calculated as the ratio of stimulated to basal insulin release during 60 minute incubation intervals. Islet preparations were assessed for cell viability using membrane exclusion dyes. A sample of islet preparations was stained using SYTO Green (SYTO-13; Molecular Probes, Eugene, OR) and ethidium bromide. Following one minute incubation at room temperature, preparations were analyzed under fluorescent microscopy for proportion of live and dead cells, which then was expressed as a percentage. Culture time is the time islets were in the CO_2 incubator prior to release. Cold ischemia time was defined as time from cross-clamp of aorta to initiation of islet isolation. DNA content was measured using the Quant-iT DNA quantification assay.

2.9 Donor Parameters

Age is obtained from the donor chart. HbA1c is assayed from a donor whole blood sample. BMI is calculated from the height and weight recorded in the donor chart.

Approval for the use of isolated human islets for research has been obtained each year for the duration of the study, and most recently approved on Nov. 22, 2018 from the Health Research Ethics Board (HREB) - Biomedical Panel under the Study ID: Pro00014814. The HREB - Biomedical Panel carries out its functions in a manner consistent with Good Clinical Practices and the Canadian General Standards Board (CAN/CGSB-101.1-2013). De-identified human primary islets isolated from deceased donors were obtained from the Alberta Diabetes Institute IsletCore and the Clinical Islet Laboratory, University of Alberta, in accordance with institutional human ethics guidelines.

3. Results

3.1 Nondiabetic and Type 2 Diabetic Islets Secrete Active GLP-1

As α cell-derived peptides are important for normal insulin secretion [16] and contribute to glucose homeostasis, we first measured active GLP-1 and glucagon from nondiabetic islets (Figure 1A). We performed a glucose suppression test where we observed robust amounts of active GLP-1 secreted at low glucose that surprisingly were not suppressed at high glucose (Figure 1B). To test if the lack of suppression was unique to GLP-1 we also measured glucagon at low and high glucose

and found no suppression at high glucose as well (Figure 1C). This indicates that in terms of suppression of secretion at high glucose, GLP-1 and glucagon secretion from whole islets is similar in our experiments. However, this contrasts with the expected decrease in glucagon secretion from islets at high glucose.

It has been proposed that failing transplanted islets resemble the dysfunction seen in type 2 diabetic islets [17]. To understand the effect of prolonged islet dysfunction on secretion of α cell-derived peptides, we repeated the glucose suppression test on type 2 diabetic islets. Although glucose-stimulated insulin secretion was reduced compared to nondiabetic islets (Figure 1D), we observed robust amounts of GLP-1 secreted at low glucose that were not suppressed at high glucose, indicating that islet dysfunction does not reduce GLP-1 secretion (Figure 1E). Moreover, glucagon secretion was comparable with nondiabetic islets (Figure 1F).

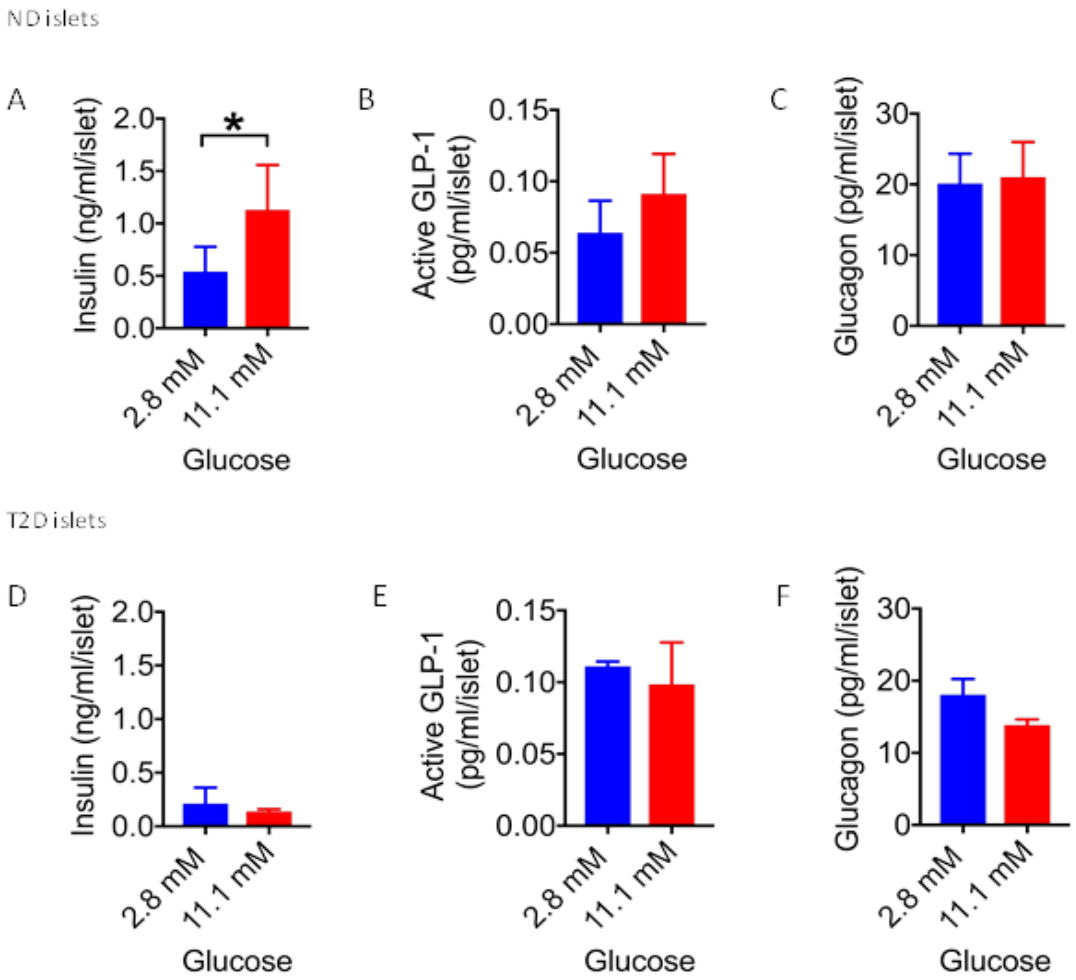

Figure 1 Nondiabetic and type 2 diabetic (T2D) islets secrete active GLP-1. A: Glucose-stimulated insulin secretion confirming secretory phenotype of nondiabetic islets. B, C: Glucose suppression test of active GLP-1 and glucagon secretion from nondiabetic islets. D: Glucose-stimulated insulin secretion confirming secretory phenotype of T2D islets. E, F: Glucose suppression test of active GLP-1 and glucagon secretion from T2D islets. Nondiabetic islets, N=5 donors. T2D islets, N=2 donors. Statistical significance for the data was determined using a paired Student's t test. *, P<0.05. Error bars indicate SEM.

3.2 Low Active GLP-1 Levels Associate with Greater β Cell Function in Human Islets

The insulin response to glucose varies among isolated islets [18], so we compared GLP-1 secretion from insulin responders and insulin non-responders. As shown in Figure 1, insulin responders secrete GLP-1 that is not suppressed at high glucose (Figure 2A, Figure 2B). In contrast, insulin non-responders show increased GLP-1 secretion that is suppressed at high glucose (Figure 2C, Figure 2D). Surprisingly, insulin non-responders show the expected α cell secretion pattern with GLP-1, while insulin responders do not.

We then examined the relationship between the stimulation index (SI) for insulin at isolation and active GLP-1 levels measured after 48 hours of culture for five donors. Consistent with the GLP-1 secretion measured in the glucose suppression tests, we observed a strong negative correlation between SI and active GLP-1 levels (Figure 2E). In other words, low GLP-1 levels associate with normal β cell function, while high GLP-1 levels associate with compromised β cell function. We them examined the relationship between GLP-1 levels and the islet isolation parameters viability, culture time, cold ischemia time, and DNA content (Figure 2F-I). However, GLP-1 levels did not strongly correlate with these parameters. Also, the donor parameters of age, A1c, and BMI did not strongly correlate with active GLP-1 levels (Figure 2J-L).

3.3 Active GLP-1 Levels are Increased with Sitagliptin and Negatively Correlate with β Cell Function

To test if sitagliptin can increase active GLP-1 levels in human islets we first probed for DPP4 protein in human islet lysates. As others have shown [13], we confirmed with western blot that human islets express DPP4. DPP4 is variably glycosylated and as expected we observe multiple bands (Figure 3A). We then treated human islets with sitagliptin and measured active GLP-1 levels after 48 hours. Active GLP-1 levels increased ~7 fold with sitagliptin treatment and there was no significant increase in the presence of the pro-inflammatory cytokine IL-1β (Figure 3B). To establish that sitagliptin is inhibiting islet DPP4 and not DPP4 present in serum, we treated islets in serum-free culture with sitagliptin where we also observe an increase in active GLP-1 levels (Figure 3C).

With islets from the same five donors as before, we examined the relationship between the increased levels of active GLP-1 with sitagliptin and the initial SI for insulin at the time of isolation. Active GLP-1 levels with sitagliptin treatment should approximate total GLP-1 levels and give an indication of GLP-1 secretion without the modifying effect of DPP4 enzymatic activity. Here too we observe a strong negative correlation between increased active GLP-1 levels and the SI for insulin (Figure 3D), suggesting that the variation in active GLP-1 levels from donor islets is due to secretion rather than DPP4 enzymatic activity. Consistent with active GLP-1 levels at baseline, increased active GLP-1 levels did not strongly correlate with islet isolation or donor parameters (Figure 3E-K).

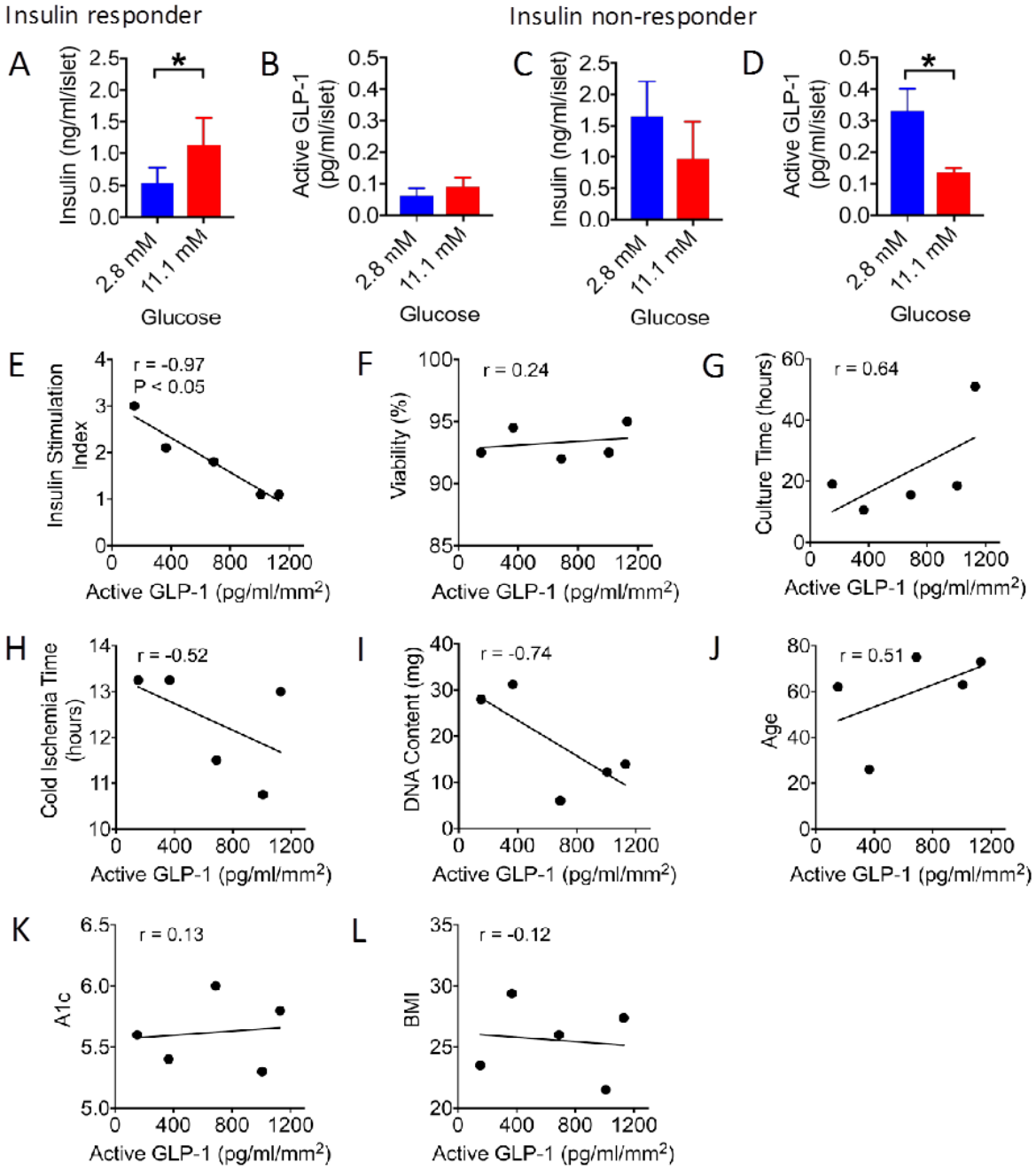

Figure 2 Low active GLP-1 levels associate with greater β cell function in human islets. A: Glucose-stimulated insulin secretion of insulin responder. B: Active GLP-1 secretion is not suppressed at high (11 mM) glucose of insulin responder. N=5 donors. C: Glucose-stimulated insulin secretion of insulin non-responder. D: Active GLP-1 secretion is suppressed at high (11 mM) glucose of insulin non-responder. N=3 donors. E-I: Active GLP-1 levels from human islets after 48 hours of culture plotted against islet isolation parameters. E: Insulin stimulation index F: Viability. G: Culture time before islet preparation release. H: Cold ischemia time. I: DNA content of islet preparation. J-L: Active GLP-1 levels are plotted against donor parameters. J: Age (years). K: HgA1c (%). L: BMI (kg/m^2). N=5 donors. Statistical significance for the data was determined using a paired Student's t test or Pearson's r. *, P<0.05. Error bars indicate SEM.

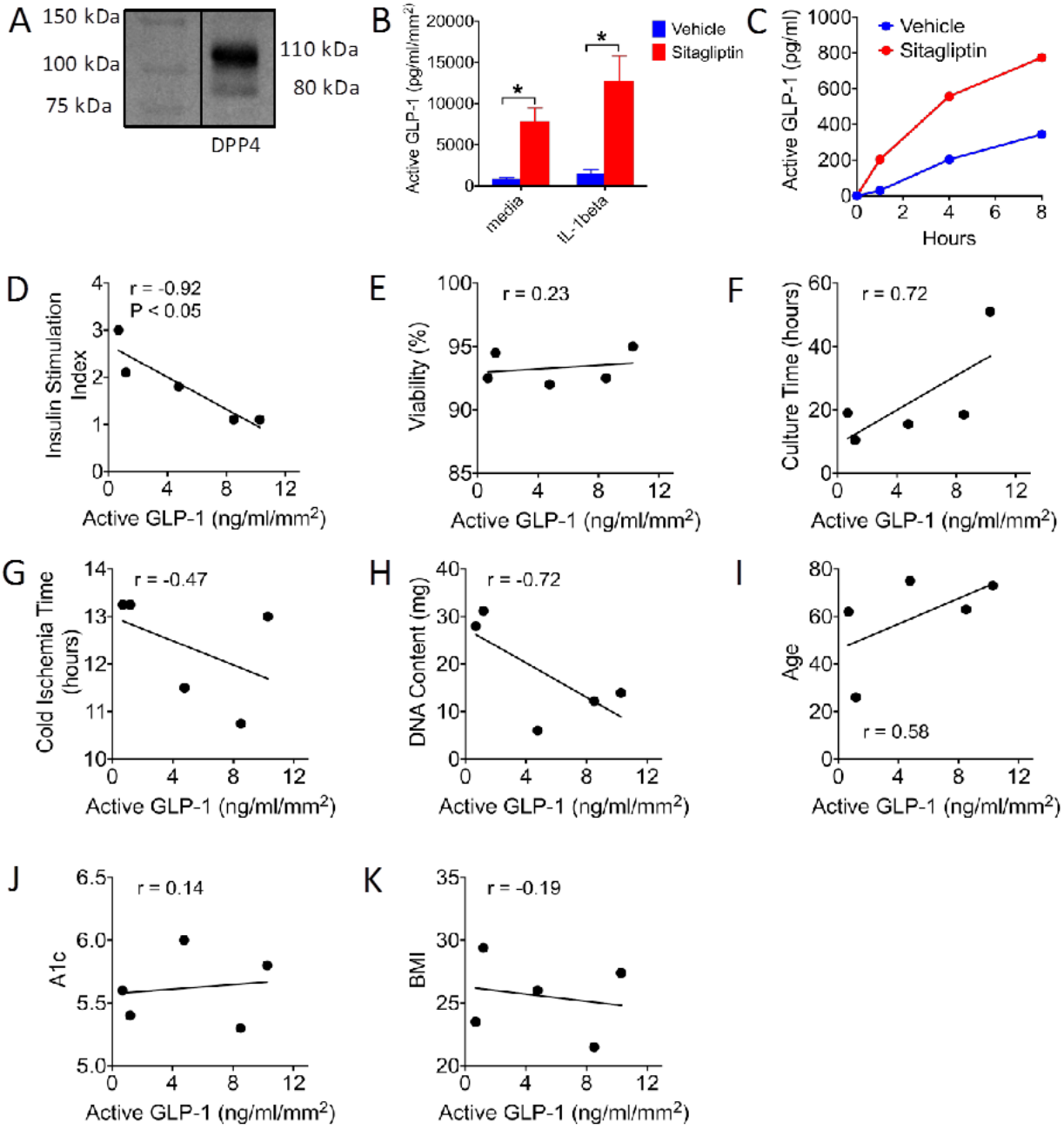

Figure 3 Active GLP-1 levels are increased with sitagliptin and negatively correlate with β cell function. A: Western blot of DPP4 in human islet lysates. The two main bands correspond with different levels of glycosylation. B: Human islets treated with sitagliptin alone or in the presence of IL-1β. N=3 donors. C: Time course of active GLP-1 secretion from human islets treated with sitagliptin in serum-free culture. N=1 donor. D-H: Active GLP-1 levels after 48 hours from human islets treated with sitagliptin plotted against islet isolation parameters. D: Insulin stimulation index. E: Viability. F: Culture time before islet preparation release. G: Cold ischemia time. H: DNA content of islet preparation. I-K: Active GLP-1 levels are plotted against donor parameters. I: Age (years). J: HgA1c (%). K: BMI (kg/m^2). N=5 donors. Statistical significance for the data was determined using a paired Student's t test or Pearson's r. *, P<0.05. Error bars indicate SEM.

3.4 Increase in Active GLP-1 with Sitagliptin Correlates with Islet Cell Survival

As GLP-1 has been reported to decrease apoptosis and increase cell survival in mouse islets [19], we investigated the effect of increasing active GLP-1 levels with sitagliptin on cell survival in human islets. Although active GLP-1 levels did not correlate with initial viability testing at isolation (Figure 2C), we hypothesised that a sustained increase in active GLP-1 levels may increase islet cell survival. Using Sytox Green to identify dead cells, we developed a dead cell assay to measure cell death in whole islets after 48 hours (Figure 4A, Figure 4B). Active GLP-1 levels were increased except for one unhealthy donor preparation, but the effect on cell survival was variable (Figure 4C, Figure 4D). However, when the relationship between the increase in active GLP-1 levels and the dead cell count is examined we observe a strong negative correlation (Figure 4E). This suggests that the increase in active GLP-1 levels with sitagliptin treatment confers a protective effect from cell death.

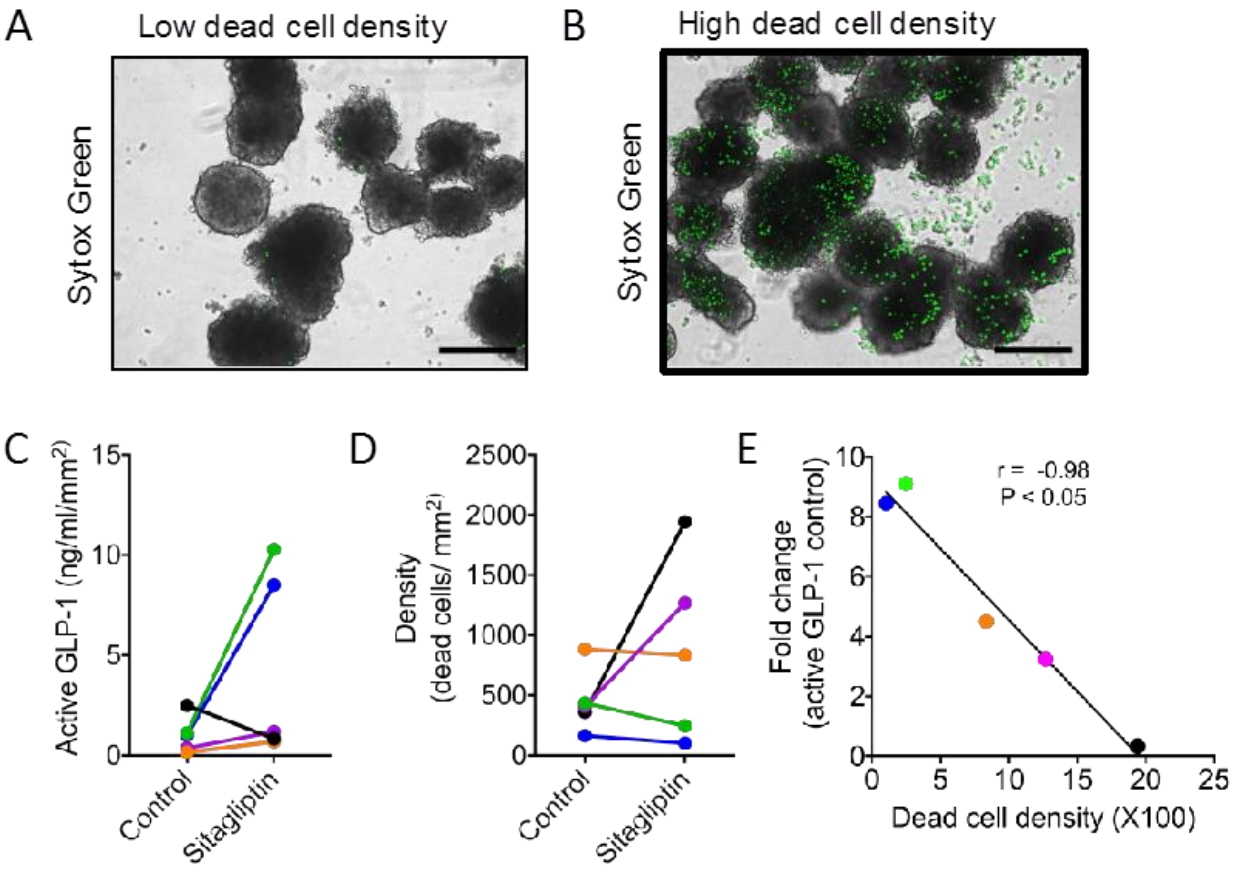

Figure 4 Increase in active GLP-1 with sitagliptin correlates with islet cell survival. A, B: Representative images of low and high dead cell density using the cell impermeable DNA binding dye, Sytox Green. Scale bar: 200 µm. C: Difference in active GLP-1 levels after 48 hours with or with sitagliptin treatment. D: Dead cell density after 48 hours with or without sitagliptin. Dead cell densities correspond with active GLP-1 levels in C. E: Fold change in active GLP-1 levels with sitagliptin plotted again dead cell densities. N=5 donors. Statistical significance for the data was determined using Pearson's r. *, P<0.05.

4. Discussion

The observation that GLP-1 levels negatively correlate with β cell function suggests that GLP-1 may play a role in an adaptive paracrine response to maintain insulin secretion. Although GLP-1 potentiates insulin secretion and a positive correlation may be expected, an islet not responsive to glucose may increase GLP-1 secretion in a failed attempt to maintain insulin secretion. This is suggested by the high GLP-1 secretion at low glucose with insulin non-responder islets. β cell injury has been shown to increase GLP-1 secretion [20] and isolated islets are inflamed due to cold ischemia of the pancreas and the islet isolation process [21]. However, we did not observe a strong correlation between GLP-1 levels and cold ischemia time. Furthermore, we did not observe a correlation between the donor parameters age, sex, BMI, and GLP-1 levels, suggesting that the SI for insulin secretion is a more important determinant of GLP-1 secretion from human islets. Since insulin secretion from β cells is known to suppress glucagon secretion [22, 23], a diminished paracrine signal from human islets with a low SI for insulin may be responsible for the increased levels of active GLP-1.

We have shown that sitagliptin treatment of islets increases active GLP-1 levels and that the increase (fold change) in active GLP-1 correlates with islet cell survival in whole islets. This suggests that increased activation of the GLP-1 receptor on islet cells increases survival under post-isolation culture conditions. Linagliptin, another DPP4 inhibitor, has been show to increase GLP-1 levels and reduce apoptosis in human islet cultures [15]. Our correlation results are also consistent with the effect of the long-acting GLP-1 receptor agonist liraglutide on human islet survival. In human islet cultures, liraglutide has been shown to reduce apoptosis and preserve islet mass [9]. A strict observation of the association between high active GLP-1 levels and a lower SI for insulin secretion may suggest that a GLP-1 receptor antagonist, such as exendin-9, would benefit islet function in pre-transplant human islet cultures, particularly in islet cultures with a poor SI for insulin. However, the increased survival of islet cells and increased active GLP-1 levels observed with sitagliptin treatment in our studies, along with the observed benefits of GLP-1 receptor agonism on β cells documented in the literature, suggests the use of GLP-1 receptor antagonists would be detrimental to preserving β cell mass and function during pre-transplant culture of human islets.

It is possible that other DPP4 substrates, such as the chemokine SDF-1, may be responsible for the correlation between sitagliptin treatment and increased cell survival in our data. SDF-1 signaling has been show to protect and preserve function β cell mass [24]. When β cells are injured, a regenerative process is induced where SDF-1 is expressed and secreted from β cells. Indeed, the process of islet isolation may be enough to induce SDF-1 expression. The model proposed by Liu et al. for islet survival describes SDF-1 acting at β cell CXCR4 receptors to increase survival and at α cell CXCR4 receptors to induce GLP-1 secretion by PC1/3 expression [20]. GLP-1 would then activate the GLP-1 receptor on β cells to increase survival. Interestingly, both GLP-1 and SDF-1 are physiological substrates of DPP4 and inhibiting DPP4 may increase levels of both hormones for a greater impact on islet cell survival.

Sitagliptin may not only benefit islet culture, but could improve engraftment and islet cell survival in the hepatic portal vein. DPP4 has recently been identified as a hepatokine indicating that DPP4 is expressed and active in the liver [25, 26]. This raises the possibility that islet-derived and circulating GLP-1 may be degraded within the hepatic portal vein, thus reducing levels local to

the islet graft. SDF-1 levels may also be lowered, thus curtailing an adaptive mechanism for β cell survival. Therefore, inhibition of hepatic and islet DPP4 with sitagliptin may promote survival of the islet graft.

In summary, we show that human islets secrete GLP-1 and thus have a local paracrine source of this prosurvival hormone. Although a reduction in glucose-stimulated insulin secretion may be associated with increased GLP-1 secretion, the prosurvival properties of GLP-1 may be harnessed to increase islet survival during pre-transplant culture. We show that sitagliptin treatment of human islets inhibits islet DPP4 and increases active levels of GLP-1. The increase in active GLP-1 may confer protection from cell death and has the potential to improve islet viability prior to islet transplantation, leading to improved islet survival post-transplant.

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Author Contributions

S.A.C designed and performed experiments, analyzed data, and wrote the manuscript. M.H., J.J, and N.S. performed experiments. P.E.L contributed to the design of the study and reviewed the manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

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