Beneficial Endocrine but adverse Exocrine effects of Sitagliptin in the HIP rat model of Type 2 Diabetes, interactions with Metformin.

Running Title: Sitagliptin actions in pancreas

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Objective. We sought to establish the extent and by which mechanisms sitagliptin and metformin singly and in combination modify islet disease progression in human IAPP transgenic rats (HIP rats), a model for T2DM.

Research Design and Methods  HIP rats were treated with sitagliptin (SIT), metformin (MET), sitagliptin and metformin (SIT+MET) or no drug as controls for the 12 weeks. Fasting blood glucose, insulin sensitivity, beta cell mass, function and turnover were measured in each group.

Results. SIT+MET had synergistic effects to preserve beta cell mass in HIP rats. MET more than SIT inhibited beta cell apoptosis. MET enhanced hepatic insulin sensitivity, SIT enhanced extrahepatic insulin sensitivity with a synergistic effect in combination. Beta cell function was partially preserved by SIT + MET. However SIT treatment was associated with increased pancreatic ductal turnover, ductal metaplasia, and in one rat pancreatitis.

Conclusions. The combination of metformin and sitagliptin had synergistic actions to preserve beta cell mass, beta cell function and enhance insulin sensitivity in the HIP rat model of T2DM. However, adverse actions of sitagliptin treatment on exocrine pancreas raise concerns that require further evaluation.

Abbreviations: T2DM Type 2 Diabetes Mellitus; sitagliptin SIT; metformin MET; GLP-1 glucagon like peptide one, DPP-4; IAPP islet amyloid polypeptide; TUNEL terminal deoxynucleotidyl transferase biotin-dUTP nick end-labelling;
The prevalence of type 2 diabetes (T2DM) and the associated morbidity and mortality are increasing (1). There is therefore interest in strategies to slow or prevent the development of T2DM. While insulin resistance secondary to lifestyle changes likely contributes to the increased prevalence, most insulin resistant individuals increase insulin secretion and remain non diabetic (2). In contrast, in those genetically vulnerable to develop T2DM, beta cell function fails to appropriately adapt to insulin resistance leading to hyperglycemia (3; 4).

Prospective studies in humans have reported a progressive decline in beta cell function preceding development of T2DM (5; 6). Autopsy studies reveal that the islet in T2DM is characterized by an approximately 60% deficit in beta cells and islet amyloid derived from islet amyloid polypeptide (IAPP), a 37 amino acid peptide co-secreted with insulin by beta cells (7). The cause of the defect in beta cell mass in T2DM remains unresolved but is likely due, at least in part, to endoplasmic reticulum stress induced beta cell apoptosis noted both at autopsy and in isolated islets from people with T2DM (8; 9). Based on these observations, it is apparent that to favorably modify disease progression in T2DM, preservation of beta cell mass and function in the setting of insulin resistance is required.

Our primary objective in the present studies was to test the hypothesis that the combination of two potentially synergistic therapies, the DPP-4 inhibitor sitagliptin and hepatic insulin sensitizer metformin, modify progression of islet dysfunction and loss of beta cell mass in T2DM. Since it is not possible to evaluate beta cell mass or turnover in vivo in humans, we undertook these studies in the human IAPP transgenic rat (HIP) since it approximates islet and metabolic phenotype of T2DM in humans (10-12).

Metformin has previously been shown to delay onset of T2DM (13). GLP-1 has reversed loss of beta cell mass in some murine models of diabetes by both increasing new beta cell formation and decreasing beta cell apoptosis (14-16). The DPP-4 inhibitor sitagliptin increases GLP-1 concentrations (17) and modestly lowers glucose levels when used alone in T2DM (18; 19) with an additive effect in combination with metformin (20; 21).

Therefore we sought to address the following questions. First, do metformin or sitagliptin individually or in combination favorably modify disease progression (reducing beta cell loss and dysfunction) at the level of the islet in the HIP rat model of T2DM? Second, is any protection of beta cell mass accomplished by decreased beta cell apoptosis and/or increased beta cell formation? Third, what are the respective actions of these drugs on insulin sensitivity and secretion singly, and in combination, in this model of T2DM. Unexpectedly we encountered marked ductal metaplasia in 25% of HFD fed HIP rats treated with sitagliptin, and severe hemorrhagic pancreatitis in one sitagliptin treated animal. Since those findings have potentially important clinical implications we evaluated the exocrine effects of sitagliptin. These latter studies provided some insights into the reported association of GLP-1 mimetic therapy by exenatide (22) or liraglutide (23) and pancreatitis, and provide some cautions about the potential long term effects of GLP-1 mimetic therapy including by DPP-4 inhibition in diabetes.
EXPERIMENTAL DESIGN AND METHODS

Animal housing, diet and drug administration and surgical procedures. A total of 40 Sprague Dawley rats (WT; n=7) and rats expressing h-IAPP (HIP rats; n=33) were used in the current study. The generation of the h-IAPP transgenic rats has been described in detail previously (11). Rats were bred and housed individually throughout the study at the University of California Los Angeles animal housing facility and subjected to standard 12-hour light-dark cycle. The University of California Los Angeles Institutional Animal Care and Use Committee approved all surgical and experimental procedures. To establish the actions of sitagliptin and metformin singly and in combination on islet protection, 2 months of age WT and HIP rats were fed ad libitum for 12 weeks high fat diet (60% Fat, 20% protein and 20% carbohydrates, cat # D12492, Research Diets Inc, New Brunswick, NJ) and randomly assigned into 5 independent treatment groups 1) WT (no drug treatment; n=7); 2) HIP (no drug treatment; n=8); 3) HIP+SIT (200mg per kg of body weight per day sitagliptin; n=8) 4) HIP+MET (200mg per kg of body weight per day metformin; n=9); 5) HIP+SIT+MET (200mg per kg of body weight per day sitagliptin and metformin; n=8). Sitagliptin was kindly provided by the Merck Research Inc, NJ and metformin was purchased from Toronto Research Chemicals, Canada. Compounds were administered by premixing with HFD performed by Research Diets Inc, New Brunswick, NJ. Following 12 weeks of diet/drug treatment animals were anesthetized with isoflourane (2.5%) by inhalation until effect (Isoflourane Vapor 19.1, Summit Anesthesia, Portland, Ore). Indwelling catheters were then inserted into the right internal jugular vein and left carotid artery for subsequent in-vivo metabolic studies as previously described (24). All catheters were filled with 100 U/ml Heparin/Saline solution, exteriorized to the back of the neck and incased in the infusion harness (Instech Inc, PA).

Hyperglycemic Clamp and arginine bolus injection. To assess glucose and arginine-stimulated insulin secretion WT (n=6), HIP (n=8), HIP+SIT (n=8), HIP+MET (n=6) and HIP+SIT+MET (n=6) rats underwent a hyperglycemic clamp followed by an arginine bolus injection as previously described (10). In brief, following a 30 min equilibration period (-30 to 0 min) plasma samples were taken for measurements of baseline fasting glucose and insulin. Thereafter animals received an intravenous glucose bolus (375 mg/kg) followed by a variable 50% (wt/vol) glucose infusion to clamp arterial glucose at ~250 mg/dl (0-70 min). At t=60 min rats received a bolus injection of L-Arginine solution (1 mmol/kg: Sigma, St. Louis, MI). Arterial blood samples (50ul) were taken at baseline (-30 and 0 min), at 1, 5 min and every 15 minutes thereafter during the clamp for immediate determination of plasma glucose and subsequent analysis for insulin.

Hyperinsulinemic-Euglycemic Clamp + [3-3H]glucose infusion. To assess insulin sensitivity and glucose turnover WT (n=5), HIP (n=6), HIP+SIT (n=7), HIP+MET (n=6) and HIP+SIT+MET (n=6) underwent a hyperinsulinemic-euglycemic clamp with concomitant infusion of [3-3H glucose] to assess glucose turnover as previously described (10). Briefly, rats received primed (3μCi) continuous (0.05μCi/min) infusion of [3-3H glucose; Perkin Elmer, Boston, MA) for a 90 minutes basal
period increased to 0.2μCi/min for 120 minutes throughout the hyperinsulinemic-euglycemic clamp which was achieved by constant infusion of regular human insulin (Novolin, Novo Nordisk, Princeton, NJ) at 4 mU·kg⁻¹·min⁻¹, variable glucose (50% wt/vol) infusion and somatostatin infusion (Bachem, CA at 10μg/kg/min) to inhibit endogenous insulin secretion. Plasma glucose levels were determined every 10 minutes and additional blood samples (~100 μl) were collected at baseline (~30 min) and at the end of the clamp (120 and 150 min) for determination of plasma insulin. Blood samples (~150 μl) for determination of tracer specific activity at fasting were drawn from (~-40 to 0 min) and during insulin infusion from (120 to 150 min).

**Endocrine pancreas histology.** Rats were euthanised by intravenous sodium pentobarbital 120 mg/kg. The pancreas was then rapidly removed from euthanised rats and fixed in 4% paraformaldehyde overnight at 4°C. Paraffin embedded pancreatic sections were stained first for hematoxylin/eosin, and insulin (guinea-pig anti-insulin, 1:100; Zymed, Carlsbad, CA). The β-cell mass was measured by first quantifying the pancreatic cross-sectional area positive for insulin and multiplying this by the pancreatic weight. In addition, sections were co-stained by immunofluorescence for insulin (guinea-pig anti-insulin, 1:100; Zymed, Carlsbad, CA) and terminal deoxynucleotidyl transferase biotin-dUTP nick end-labelling (TUNEL method, Roche Diagnostics, Mannheim, Germany) for quantification of beta-cell apoptosis, and insulin (guinea-pig anti-insulin, 1:100; Zymed, Carlsbad, CA) and Ki-67 (mouse anti-Ki-67, 1:50; Dako, Carpinteria, CA) for determination of beta-cell replication. All beta-cells per pancreatic section (~2500 cells per section) were examined in detail and counted at ×200 magnification (×20 objective, ×10 ocular) for the total number of TUNEL and Ki-67 positive beta cells. The frequency of TUNEL and Ki-67 was presented as percentage of total beta-cells per section. Fluorescent slides were analyzed and imaged using a Leica DM600 microscope (Leica Microsystems, Wetzlar, Germany) and images acquired using OpenLab software (Improvision) microscope and analyzed using ImagePro Plus software.

**Exocrine pancreas histology.** Pancreas sections were deparaffinized in xylene and rehydrated in ethanol gradient and pancreatic sections were stained in Harris Hematoxylin Solution (Sigma, HHS16, St. Louis, MO) and Eosin Y Solution (Sigma, HT110132, St. Louis, MO). For immunofluorescence, antigen-retrieval was performed via microwave heating in citrate-buffer (Vector, H-3300, Burlingame, CA) except in the TUNEL staining which used Proteinase-K digestion (Promega, V302B, Madison, WI) at 37°C for 15 minutes. Slides were blocked in TBS (3% BSA, 0.2% TX-100, 2% Donkey Serum) for 1-hour. The following primary antibodies were used for 12 hour incubation: ductal cell marker Cytokeratin (mouse anti-pancytokeratin, 1:50; Sigma, St. Louis, MO), marker of cell fibrosis Fibrinectin (rabbit anti-Fibrinectin, 1:500; Sigma, St. Louis, MO), replication marker Ki-67 (mouse anti-Ki-67, 1:50; Dako, Carpinteria, CA), apoptosis marker deoxynucleotidyl transferase biotin-dUTP nick end-labelling (TUNEL method, Roche Diagnostics, Mannheim, Germany), a marker of T-cell (rabbit anti-CD3, Abcam, MA) and macrophage infiltration (rabbit anti-CD11C, Abcam, MA), GLP-1 receptor (rabbit anti-GLP-1 receptor, 1:100; Novus Biologicals, Denmark), PDX-1 (rabbit anti-PDX-1, 1:1000, Millipore, St. Louis, MO).
Secondary antibodies labeled with Cy3 and FITC were obtained from Jackson Laboratories (West Grove, PA, USA) and used at dilutions of 1:100 for 1-hour incubation. To determine ductal cell replication and apoptosis we quantified in each pancreatic section total number of Ki-67, TUNEL and Cytokeratin positive cells (~1000 Cytokeratin positive cells per section were counted). The frequency of ductal cell replication and apoptosis in each animal was presented as a total number of TUNEL or Ki-67 positive cells per total number of Cytokeratin positive.

**Analytical procedures.** Plasma glucose concentrations were measured by the glucose oxidase method (Beckman Glucose Analyzer 2, Fullerton, CA). Plasma insulin was measured using competitive colomeric enzyme-linked immunosorbent assay (Alpco Diagnostics, Salem NH). Plasma glucose specific activity, hepatic glucose production and glucose disposal was calculated as previously described in detail (10). Disposition index was calculated as product of first phase insulin secretion during the hyperglycemic clamp expressed as (pmol/l·min) and insulin sensitivity determined by mean glucose infusion rates (expressed in mg·kg⁻¹·min⁻¹) required to maintain euglycemia during the hyperinsulinimic-euglycemic clamp.

**Statistical analysis.** Statistical analysis was performed using ANOVA analysis with Fisher’s post-hoc were appropriate. Regression analysis was performed using Statistica, version 6 (Statsoft, Tulsa, OK). Data in graphs and tables are presented as means ± SEM. Findings were assumed statistically significant at the P<0.05.

**RESULTS**

**Blood glucose concentrations, body weight and food intake.** Prior to initiation of high fat diet blood glucose was comparable (105±4 mg/dl) in WT and HIP rats (Fig 1A). After 12 weeks of HFD plasma glucose increased to (209±12 mg/dl) in HIP rats but was unchanged (108±3 mg/dl) in WT rats. Both metformin and sitagliptin alone had a comparable effect on restraining this increase in blood glucose concentration in HIP rats (to 154±7 versus 209±12 mg/dl, P<0.05) while the combination of Sitagliptin and metformin had a synergistic effect (to 138±8 mg/dl vs. 209±12 mg/dl, P<0.01). Weight gain on the HFD was comparable in WT (from 312±5 to 628±30g, Fig 1B) and untreated HIP rats (from 291±10 to 639±14g, Fig 1B) but was ~10% less in either sitagliptin or metformin treated HIP (Fig. 1B, p<0.05) rats and ~15% less in HIP rats treated with combination therapy (Fig. 1B, p<0.05). Food intake was decreased in HIP rats treated with metformin (~10%, P<0.05 vs. HIP, Fig. 1C) and with both metformin and sitagliptin (~20%, p<0.05 vs. HIP, Fig. 1C).

**Beta cell mass, beta cell replication and beta cell apoptosis.** Beta cell mass was ~70% decreased in untreated HIP versus WT rats on a HFD (8.4±1.3 vs. 25.6±2.1 mg; P<0.05, Fig. 2), as a consequence of increased beta cell apoptosis as previously reported (12). Sitagliptin therapy alone led to preservation of beta-cell mass compared to untreated HIP rats (8.4±1.3 vs. 16.6±2.5 mg, P<0.01;Fig. 2). In HIP rats treated with metformin alone beta cell mass was not significantly different from untreated HIP rats (8.4±1.3 vs. 11.6±1.3 mg, P=0.24 for HIP vs. HIP+MET; Fig. 2), but those treated with combination therapy of sitagliptin and metformin had even better preservation of beta cell mass.
than those treated with sitagliptin alone (8.4±1.3 vs. 19.7±2.4 mg, P<0.001; for HIP vs. HIP+SIT+MET; Fig. 2).

The frequency of beta cell replication quantified by Ki-67 was increased by sitagliptin alone (0.2±0.1 vs. 0.6±0.1% for HIP vs. HIP+SIT; p<0.05, Fig. 3A,B) or with combination therapy (0.2±0.1 vs. 0.5±0.1% for HIP vs. HIP+SIT+MET; p<0.05, Fig. 3A,B). In contrast, metformin alone had no discernable effect on beta cell replication. Sitagliptin treatment alone decreased the frequency of beta cell apoptosis in HIP rats by ~55% (P<0.05), Fig. 3C,D. Metformin treatment alone was even more effective at decreasing beta cell apoptosis in HIP rats by ~75% (P<0.05), Fig. 3C,D, while sitagliptin and metformin in combination had a comparable action to suppress beta cell apoptosis to that of metformin alone (Fig. 3C,D).

**Insulin sensitivity.** The impact of metformin and/or sitagliptin on insulin sensitivity was evaluated in HIP rats by a hyperinsulinemic-euglycemic clamp and the isotope dilution technique. As expected the HFD induced insulin resistance in both WT and HIP rats (Fig. 4). Insulin sensitivity, assessed by the mean glucose infusion rates during the hyperinsulinemic period was enhanced by either metformin (14.2±1.4 vs. 5.3±1.5 mg/kg/min, P<0.001; Fig. 4, and Supplemental Table 1 which is available in the online appendix at http://diabetes.diabetesjournals.org) or sitagliptin (11.4±1.7 vs. 5.3±1.5 mg/kg/min, P<0.01) therapy alone compared to HFD HIP rats, and in combination they had a slight additive effect (15.6±1.1 vs. 5.3±1.5 mg/kg/min, P<0.001). In the fasting state, isotopically measured hepatic glucose release was ~2-fold greater in HIP versus WT rats (9.7±1.4 vs. 5.1±1.3 mg/kg/min, P<0.05; Supplemental Table 1). In contrast, metformin alone or in combination with sitagliptin led to an ~40% suppression of fasting hepatic glucose release in HIP rats, where sitagliptin alone had no measurable effect on hepatic glucose release in the fasting state in HIP rats (Supplemental Table 1). With insulin stimulation during the hyperinsulinemic euglycemic clamp, hepatic glucose release in HIP rats suppressed minimally compared to WT rats (7% vs 100% for HIP vs. WT, P<0.05; Supplemental Table 1), confirming marked hepatic insulin resistance. Metformin alone or in combination with sitagliptin partially restored hepatic insulin sensitivity in HIP rats by metformin as indicated by ~60% suppression of hepatic glucose release during the hyperinsulinemic clamp (Supplemental Table 1). Insulin stimulated glucose disposal tended to be ~30% higher in all three drug treated groups compared to non-treated HIP rats. The slightly decreased weight gain in the metformin and sitagliptin treated HIP rats may have contributed to the increased insulin sensitivity with each of these therapies.

**Beta cell function.** Glucose-mediated insulin secretion (examined by hyperglycemic clamp) was markedly attenuated in HIP rats compared to WT rats on a HFD (648±141 vs. 5423±480 pmol/l·min, P<0.05; Figure 5A). There was no significant enhancement of first (Fig. 5A) or second phase (data not shown) glucose mediated insulin secretion in HIP rats treated with sitagliptin or metformin alone when considered independently of insulin sensitivity. However, taking insulin sensitivity into account in the calculated disposition index (Fig. 5B), glucose mediated insulin secretion in HIP rats was comparably enhanced by either
metformin or sitagliptin alone, and in combination these drugs had a synergistic action to further enhance the disposition index (P<0.05; for HIP vs. HIP+SIT+MET; Figure 5B). Glucose potentiated arginine-stimulated insulin secretion was also markedly attenuated in HIP versus WT rats (3077±528 vs. 8809±1179 pmol/l, P<0.05; Figure 5C) and again there was no appreciable benefit from either sitagliptin or metformin independently or in combination on this metric (Fig.5C) that is generally considered a surrogate of beta cell mass (25). It is therefore of interest to note that the glucose-potentiated arginine elicited first phase insulin response did not reflect beta cell mass (Figure 5D) in metformin and/or sitagliptin treated HIP rats.

**Pancreatitis in a HIP rat treated with sitagliptin.** The focus on the exocrine actions of sitagliptin arose as a consequence of an unexpected observation of marked necrotizing pancreatitis in one rat out of eight treated with sitagliptin (Fig. 6 and Supplemental Figure 1 and 2). The region of pancreatitis was apparent as a mass of approximately 2 cm and histologically characterized by hemorrhagic necrosis, fibrosis, an inflammatory cell infiltrate and areas of ductal metaplasia (Supplemental Figures 1 and 2) (26-30). While pancreatitis was present in one of the 8 HIP rats treated with sitagliptin, it was not detected in any of the 17 HIP rats not treated with sitagliptin (Table 1), or any of 89 HIP rat pancreases reported previously (7; 10; 12). Given this unexpected finding we evaluated all the pancreases from this study for ductal metaplasia and increased ductal turnover, characteristics frequently present in pancreatitis in humans (29-31).

**Ductal metaplasia.** Ductal metaplasia was present in total of 3 HIP rats treated with sitagliptin (Table 1). One of the 3 sitagliptin treated HIP rats with ductal metaplasia also displayed marked pancreatitis (Table 1). These region of ductal metaplasia were located both separated from and adjacent to islets of Langerhans (Fig. 7 and Supplemental Figure 3) and consisted of angulated tubular structures, interspersed fibrosis and inflammatory cells. In some areas these were adjacent to atrophic acinar cells (Fig. 7 and Supplemental Figure 3). Ductal metaplasia was immunoreactive for cytokeratin and Ki-67 (Fig. 8A) indicating a high rate of cell turnover. Furthermore, metaplastic areas included numerous fibroblasts (by morphology and fibrinectin immunoreactivity, Fig. 8B) and were absent of PDX-1 expression (Fig.8C).

**Ductal cell turnover.** Ductal replication quantified by Ki-67 immunoreactivity was increased by four fold in untreated diabetic HIP rats versus wild type controls (0.6%±0.2 vs. 2.5±0.3%, P<0.05, Fig. 9 and 10A). Sitagliptin treatment led to an additional 3 fold increase in the frequency of ductal cell replication versus untreated HIP rats (2.5±0.3 vs. 7.3±0.7%, P<0.05, Fig. 9, 10 and Supplemental Figure 4) and a 12 fold increase compared to wild type rats. Intriguingly, metformin treatment abrogated the effects of sitagliptin on ductal cell proliferation (7.3±0.7 vs. 1.4±0.6%, p<0.05 for HIP+SIT vs. HIP+SIT+MET, Fig. 9,10A). The frequency of ductal replication was positively correlated with the fasting blood glucose concentration, with an apparent continuum between WT and HIP rats (Fig. 10C), with the sitagliptin only treated group that were displaced to a higher slope (Fig 10C). Addition of metformin to sitagliptin restored the frequency of ductal replication to the same relationship with
fasting glucose concentrations observed in rats not exposed to Sitagliptin (Fig 10C).

**GLP-1 receptor, PDX-1 and Insulin expression in exocrine ducts.** As previously reported (32) GLP-1 receptors were expressed in pancreatic ducts, but with no differences between treatment groups (Supplemental Fig. 5). PDX-1 and insulin positive ductal cells were observed following sitagliptin treatment in HIP rats (Supplemental Fig. 6A). While most beta cells are present within well-defined islets, occasional individual beta cells are present scattered in the exocrine pancreas. These scattered beta cells were ~6 fold more abundant in sitagliptin compared to untreated HIP rats (P<0.05; Supplemental Fig. 6B). Interestingly, the number of scattered beta-cells was also increased in metformin treated animals, but not in animals that received combination therapy of sitagliptin plus metformin.

**DISCUSSION**

Our primary objective was to establish if metformin or sitagliptin alone and in combination favorably modified disease progression in the HIP rat model of T2DM. While loss of beta cell beta cell mass in the HIP rat was slowed by this combination therapy, unexpected adverse actions on the exocrine pancreas were also observed.

Metformin has been shown to delay T2DM onset in humans (13). Since enhanced insulin sensitivity through lifestyle changes also delays diabetes (13), at least part of the protective effect of metformin may be mediated by metformin's actions to enhance hepatic insulin sensitivity through its actions on AMP-activated kinase (AMPK) (33). Metformin decreased beta cell apoptosis in isolated human islets from patients with T2DM (34). In the present study metformin was more effective than sitagliptin in reducing beta cell apoptosis in the HFD HIP rat. While sitagliptin alone also suppressed beta cell apoptosis, there was no added benefit of sitagliptin on metformin mediated suppression of beta cell apoptosis. Sitagliptin enhanced beta cell replication in HIP rats consistent with prior studies of GLP-1 and GLP-1 mimetic induced beta cell replication in a variety of murine models (14; 15; 32). The benefits of sitagliptin and/or metformin on beta cell mass and function reported here may have been mediated by either direct effects of the drugs on beta cells or indirectly by their actions to lower blood glucose. Hyperglycemia can contribute to both loss of beta-cell mass by increasing beta-cell apoptosis and/or loss of beta-cell function (35). The current study was designed to examine effects of sitagliptin and metformin treatment in an in-vivo model of T2DM, with the advantage of best approaching actions in humans with T2DM, but with the limitation of precluding distinction between direct and indirect effects of drugs on beta-cell mass and function.

The GLP-1 mediated increased beta cell replication has to be interpreted with caution. Juvenile rodents, in common with juvenile humans, have a period of post natal expansion of beta cell numbers mediated by beta cell replication (36; 37). Such studies (including this one) in relatively young rodents have exposed beta cells to increased GLP-1 when they remain replication competent. Recent studies have demonstrated that the capacity for new beta cell formation through beta cell replication is attenuated in adult rodents following epigenetic modifications of beta cells, and thus not surprisingly older rodents do not exhibit the same GLP-1 mediated beta cell
replication as that observed in juvenile rodents (38; 39). It is perhaps not surprising that under conditions of increased GLP-1 secretion (post gastric bypass) in humans, despite earlier predictions (40), neither beta cell replication nor the fractional area of pancreas occupied by beta cells was increased (41). Likewise, long standing exposure of non-human primates to the GLP-1 mimetic exenatide was reported to not increase beta cell mass (42). Since the incremental effect of sitagliptin on metformin to preserve beta cell mass in the present studies appeared to be mediated through its action to foster beta cell replication, it is possible that no such added benefit would be present in humans.

The unexpected finding of hemorrhagic pancreatitis in one of the sitagliptin treated rats prompted further analysis of the exocrine pancreas in this study. We report increased ductal proliferation in all sitagliptin treated rats unless also treated with metformin. We also noted ductal metaplasia in three sitagliptin treated rats, one of which was also treated with metformin. Increased ductal proliferation and ductal metaplasia are well-recognized components of pancreatitis in humans (29-31), and therefore offer a plausible mechanism for GLP-1-induced pancreatitis reported in humans treated with the GLP-1 mimetics exenatide or liraglutide (22; 23). Ductal GLP-1 receptor expression was not altered in any of the treatment groups. It cannot be assumed that the actions of sitagliptin to induce exocrine (or endocrine) pancreatic changes are mediated through GLP-1 since other regulatory peptides are also degraded by DPP-4 (43). Furthermore we cannot rule out direct actions of sitagliptin on the exocrine pancreas. However, since pancreatitis also has been reported in humans treated with GLP-1 agonists (22; 23), it seems likely that the exocrine effects of sitagliptin treatment reported here are a consequence of increased GLP-1 concentrations.

Perhaps of most concern, increased ductal cell turnover and ductal metaplasia are also well characterized risk factors for pancreatic ductal cancer (31; 44; 45), as is pancreatitis (46). As yet no increase in pancreatic cancer has been reported in patients treated with GLP-1 mimetics or DPP-4 inhibitors. However, these drugs have only been available for a relatively short period of time. Any influence that GLP-1 based therapy might have to increase the incidence of pancreatic cancer through chronically increased ductal cell turnover would be expected to take several years. The incidence of colon carcinoma associated with chronic epithelial replication and regeneration in the setting of inflammation in ulcerative colitis starts to increase 8-10 years following disease onset (47).

The present studies may also shed some light on the increased incidence of pancreatitis and pancreatic cancer in patients with diabetes. While pancreatitis or pancreatic cancer can lead to diabetes (48), epidemiological studies imply that the converse may also be the case (49-52). Exocrine pancreatic insufficiency and pancreatitis are common in both autoimmune mediated type 1 diabetes and type 2 diabetes (48; 53). In the present studies we note increased ductal turnover in the HIP rat related to plasma glucose concentrations (Fig. 10C). This implies that hyperglycemia per se may be sufficient to induce increased ductal cell turnover. While controversial, it has been proposed that there may be attempted beta cell regeneration in diabetes from
progenitor cells that are proximate to, or within, pancreatic ducts (54).

Moreover it has been proposed that GLP-1 based therapy enhances beta cell formation by increasing beta cell transdifferentiation from these putative duct related stem cells (14; 32). The action of sitagliptin treatment alone to increase ductal replication, apparently still in a glucose sensitive manner but at a higher set point, is consistent with a complimentary interaction between glucose and GLP-1 concentrations to activate ductal cell proliferation. The observed PDX-1 and insulin positive ductal cells in sitagliptin treated HIP rats support this postulate.

An intriguing finding in the present studies is the fact that addition of metformin to sitagliptin prevented the sitagliptin mediated increase in ductal replication. Since metformin therapy has been shown to increase GLP-1 levels in some studies (55), the action to counter sitagliptin mediated increased ductal replication is presumably independent of GLP-1. It is possible that the effect was mediated indirectly through metabolic actions of metformin to enhance insulin sensitivity or to decrease blood glucose concentrations (56). Alternatively, metformin might act directly on ductal cells to suppress proliferation. Anti proliferative effects of metformin have been reported in prostate cell cancer cell lines and explanted prostate cells in mice (57). Recent epidemiological studies have revealed that metformin therapy is associated with a reduced incidence of cancer, including pancreatic cancer (58). The latter might be a consequence of the metabolic actions and/or the direct antiproliferative effects of metformin.

It is unknown if sitagliptin actions on ductal turnover and/or induction of ductal metaplasia observed in the HIP rat extends to humans. It is plausible that these effects are restricted to the rat. It will be important to address this in pancreas, when available, from humans with type 2 diabetes who have been treated with GLP-1 mimetic therapy. Since the action of sitagliptin to increase ductal turnover was dependent on hyperglycemia, GLP-1 mimetic treatment on the exocrine pancreas in non-diabetic animal models as used in classical toxicology screening studies would presumably miss this effect and its potential long term adverse consequences.

In summary, sitagliptin, and metformin, had synergistic effects on preserving beta cell mass in the HIP rat model of T2DM. Metformin was most effective at suppressing beta cell apoptosis. Sitagliptin fostered increased beta cell replication, but this is likely of limited benefit in adult humans. Of concern, we noted pancreatitis in one, ductal metaplasia in three and increased ductal turnover in all sitagliptin treated HIP rats. Since the apparent adverse effects of GLP-1 mimetic therapy are at least to some extent offset by concurrent use of metformin, it is perhaps judicious to use GLP-1 mimetic therapy (including DPP-4 inhibitors) only in addition to metformin until potential long term adverse effects of GLP-1 based therapy on exocrine pancreas can be ruled out in humans with diabetes.

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Table 1: Incidence of pancreatitis, ductal metaplasia and increased ductal turnover by group.

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*Increase in ductal cell proliferation was defined as an increase in ductal proliferation 3 standard deviations above the mean of Wild Type rats.

FIGURE LEGENDS:

Figure 1. Fasting plasma glucose (A), body weight (B) mean daily food intake (C) and mean daily drug consumption following 12 week treatment with 60% high fat (HFD) chow diet wild type (n=7), HIP rats (n=8), HIP rats treated with sitagliptin (HIP+SIT, n=8), metformin (HIP+MET, n=9) and combination therapy (HIP+SIT+MET, n=8). Data are expressed as mean ± SE.

Figure 2. (A) Typical islets from wild type (WT), HIP rats, HIP rats treated with sitagliptin and sitagliptin+metformin stained for insulin (pink) and hematoxylin (blue). (B) beta-cell area and mean beta-cell mass (C) following 12 week treatment with 60% high fat (HFD) in Wild type (n=7), HIP rats (n=8), HIP rats treated with sitagliptin (HIP+SIT, n=8), metformin (HIP+MET, n=9) and combination therapy (HIP+SIT+MET, n=8). Data are expressed as mean ± SE.

Figure 3. (A) Examples of islets stained for insulin (pink) and replication marker Ki-67 (brown) and nuclear stain hematoxylin (blue) imaged at 20X. (B) Frequency of beta-cell replication in Wild type (n=7), HIP rats (n=8), HIP rats treated with Sitagliptin (HIP+MET, n=8), Metformin (HIP+MET, n=9) and combination therapy (HIP+MET+MET, n=8). (C) Examples of islets stained for insulin (green) and apoptosis marker TUNEL (red) and nuclear stain Dapi (blue) imaged at 20X. (D) Frequency of beta-cell apoptosis in Wild type (n=7), HIP rats (n=8), HIP rats treated with sitagliptin (HIP+MET, n=8), metformin (HIP+MET, n=9) and combination therapy (HIP+MET+MET, n=8). Data are expressed as mean ± SE. *P<0.05 vs. WT, HIP and HIP+MET. Arrows indicate examples of insulin positive Ki-67 and TUNEL positive cells.
Figure 4. Mean glucose infusion rates during the hyperinsulinemic-euglycemic clamp following 12 week treatment with 60% high fat (HFD) in Wild type (n=5), HIP rats (n=6), HIP rats treated with sitagliptin (HIP+SIT, n=7), metformin (HIP+MET, n=6) and combination therapy (HIP+SIT+MET, n=6). Data are expressed as mean ± SE.

Figure 5. Mean first phase insulin response during the hyperglycemic clamp (A), mean disposition index (B), mean first phase insulin response to arginine (C) and the relationship between the beta-cell mass and first phase insulin response to arginine (D) following 12 week treatment with 60% high fat (HFD) in Wild type (n=6), HIP rats (n=8), HIP rats treated with sitagliptin (HIP+SIT, n=8), metformin (HIP+MET, n=6) and combination therapy (HIP+SIT+MET, n=6). Data are expressed as mean ± SE.

Figure 6. Necrotizing Pancreatitis in a HIP rat treated with sitagliptin for 12 weeks. (A) 2X: representative image of the exocrine pancreas stained for Hematoxylin and Eosin from a HIP rat treated with sitagliptin for 12 weeks with necrotizing pancreatitis. Note partially preserved lobular configuration of the exocrine pancreas, however note the significant loss of acinar cell density and the widening of the septae (arrow) as well as a complete loss of acinar cells in some areas (circle). (B) 4X: at this higher magnification septal fibrosis and inflammation (arrows) are better appreciated as well as partial and complete loss of acinar cells (circle). (C) 20X: at this magnification, acinar cell injury and angulated tubular ductal structures within the acini are clearly seen (circle). Note the extensive septal inflammation and fibrosis (star). (D) 40X: at this higher magnification angulated tubular ductal structures and surrounding tissue fibrosis are better appreciated.

Figure 7. Extensive ductal metaplasia in HIP rats treated with sitagliptin for 12 weeks. (A and B) 10X: representative images of ductal cell metaplasia observed in a rat treated with sitagliptin for 12 weeks. Metaplastic regions consisted of angulated tubular structures, interspersed fibrosis and inflammatory cells and were located both adjacent to islets of Langerhans (stars) as well as separated from islets (circle). (C) 20X: at this higher magnification shown an apparent transition from intact acinar cells to damaged/atrophic acinar cells to angulated tubular ductal structures. (D) 40X: at this higher magnification angulated tubular ductal structures and surrounding tissue fibrosis within the metaplastic region is better appreciated.

Figure 8. Extensive ductal metaplasia in sitagliptin treated HIP rats is characterized by increased ductal cell turnover, tissue fibrosis and absence of PDX-1 expression. (A) 20X representative image of ductal cell metaplasia in a rat treated with sitagliptin for 12 weeks stained for ductal cell marker (Cytokeratin-green), replication marker (Ki-67-red) and nuclear stain (DAPI-blue). The extent of ductal cell replication within metaplasia is highlighted by co-expression of Cytokeratin and Ki-67 immunoreactivity shown in the insert. (B) The same area of ductal metaplasia was stained for ductal cell marker (Cytokeratin-green), fibroblast marker (Fibrinectin-red) and nuclear stain (DAPI-blue). (C) The same area of ductal cell metaplasia stained for ductal marker (Cytokeratin-green), PDX-1 (red) and Insulin (blue).
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Figure 10. Increased ductal cell turnover in HIP rats treated with sitagliptin. Quantification of ductal cell replication (A) and apoptosis (B) in WT, HIP rats, and HIP rats treated with either sitagliptin, metformin or combination therapy of sitagliptin and Metformin for 12 weeks. Regression analysis of the relationships between ductal cell proliferation versus fasting plasma glucose (C). *p<0.05. Note that ductal cell replication in sitagliptin treated rats was quantified only in metaplasia and pancreatitis free areas of the exocrine pancreas.
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