Original Research

Sitagliptin lowers glucagon and improves glucose tolerance in prediabetic obese SHROB rats

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Abstract

The SHROB (spontaneously hypertensive rat – obese strain) is a model of prediabetes and metabolic syndrome with insulin resistance, glucose intolerance and hypertension. Inhibitors of dipeptidyl dipeptidase IV (DPP-IV) are effective hypoglycemic agents in type 2 diabetes through potentiation of incretin hormones that act in the pancreas to increase insulin and decrease glucagon release. We sought to determine whether the DPP-IV inhibitor sitagliptin might be effective in prediabetes relative to standard therapy with the sulfonylurea glyburide, by using the SHROB model. SHROB show normal fasting glucose but are insulin resistant and hyperglucagonemic. SHROB were treated for six weeks with vehicle, sitagliptin (30 mg/kg/d) or glyburide (1 mg/kg/d) and compared with untreated lean spontaneously hypertensive rats. Body weight, food intake and fasting glucose were all unchanged in all three SHROB groups, but glucagon was reduced by 33% by sitagliptin while remaining unchanged following glyburide or vehicle. In oral glucose (6 g/kg) tolerance testing, both sitagliptin and glyburide lowered plasma glucose. Both sitagliptin and glyburide shifted peak insulin secretion earlier (30 min for glyburide and 60 min for sitagliptin but 240 min for vehicle). Only sitagliptin significantly enhanced insulin secretion. Sitagliptin is effective in normalizing excess glucagon levels and delaying exaggerated insulin secretion in response to a glucose challenge in a prediabetic model.

Keywords: genetic obesity, insulin resistance, metabolic syndrome, DPP-IV inhibitors, sitagliptin, sulfonylureas, glyburide

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Introduction

A recently introduced therapy for type 2 diabetes is the inhibition of dipeptidyl dipeptidase IV (DPP-IV) in order to enhance the effectiveness of endogenous insulin-releasing gut hormones, especially glucagon-like peptide-1 (GLP-1) but also gastric inhibitory peptide (GIP).[1,2] GLP-1 has multiple actions that contribute to improved glucose homeostasis. These include promotion of insulin release in response to food intake, inhibition of glucagon release, delay of gastric emptying, and hypothalamic effects that include promotion of satiety and reduced food intake.[3] The selective and highly potent DPP-IV inhibitor sitagliptin was the first agent to be introduced clinically and has been shown effective in lowering levels of glycemia in type 2 diabetes. Sitagliptin and other DPP-IV inhibitors lower glycosylated hemoglobin HbA_{1c} by about 1%, which means that combined treatment with other agents is usually required to achieve glucose control.[4] However, little is known about the effect of these agents on the progression to type 2 diabetes, especially in its initial stages. There are reports that sitagliptin is ineffective in lowering fasting glucose in prediabetes.[5] Furthermore, little information is available on the effects of sitagliptin on metabolism apart from plasma glucose levels.

The SHROB (spontaneously hypertensive rat – obese strain) show many of the characteristics of human metabolic syndrome, a common precursor to type 2 diabetes.[6,7] These traits include insulin resistance, glucose intolerance, hyperlipidemia affecting triglycerides more than cholesterol, fatty liver and hypertension. The SHROB are spontaneously hypertensive rats (SHRs) with a naturally occurring knockout of the leptin receptor, which causes dramatic weight gain and metabolic disturbances. SHROB differ from the better-known Zucker rats in having a more complete disruption of the leptin body weight regulatory system, resulting from a naturally occurring knockout of the leptin receptor as opposed to the amino acid substitution caused by the mutation in Zucker rats.[8] Furthermore, SHROB have a hypertensive genetic background. The SHROB model shows normal fasting plasma glucose levels, but

exhibits severe insulin resistance and hyperglycemia following an oral glucose load. SHROB also show elevated glucagon levels in both fasting and postprandial states and impaired suppression of glucagon in response to an oral glucose challenge.[9] SHROB show delayed and exaggerated insulin secretion in response to an oral load. We hypothesized that increased levels of GLP-1 and GIP induced by sitagliptin treatment may reverse the abnormalities in secretion of insulin and glucagon in the SHROB. Furthermore, if these pancreatic abnormalities contribute to other aspects of the metabolic syndrome, then sitagliptin may have additional therapeutic actions in metabolic syndrome. For example, the distribution of body fat in metabolic syndrome favors accumulation of fat within the abdomen. Antidiabetic agents in the thiazolidinedione class induce remodeling of adipose tissue to favor subcutaneous depots.[10] The effects of DPP-IV inhibitors on fat distribution are not known, but glucagon may have actions on lipolysis that could redistribute fat depots.[11] DPP-IV inhibitors increase lipolysis in human subcutaneous adipocytes through unknown mechanisms.[12]

Methods

Materials

Sitagliptin was a gift from Merck (Rahway, NJ, USA). Glyburide and other chemicals were obtained from Sigma Chemical (St Louis, MO, USA) or Fisher (Pittsburgh, PA, USA) and were of analytical grade.

Animal procedure

Female SHROB and lean SHRs were obtained from Charles River laboratories at six weeks of age and maintained under standard laboratory conditions. Female animals were used because of the greater cardiovascular relative risk in women with diabetes.[13] In addition, female SHROB are acyclic and sterile and show no significant differences in glucose and insulin metabolism relative to male SHROB.[6] Animals were housed individually and were provided food (Teklad 8664, Harlan Teklad, South Easton, MA, USA) and water *ad libitum* prior to drug treatment. Animals were on a 12:12-h light–dark cycle (lights on from 7:00 to 19:00) and were maintained at a constant temperature of 21°C. These procedures were carried out with the approval of the Case Western Reserve University Animal Care and Use Committee.

Chronic drug treatment

Agents were supplied in drinking water. The vehicle drinking solution consisted of 0.7% sorbitol, 250 ppm sodium saccharine and 100 ppm sodium benzoate as a preservative. The sorbitol was necessary to solubilize the glyburide, and along with the saccharin maintained palatability and ensured adequate fluid intake. Fluid intake was allowed *ad libitum* and monitored continuously so that adjustments could be made to drug concentrations as necessary. Sitagliptin was added at 500 ppm, which provided 30 mg/kg/d at the average fluid intake of approximately 60 mL/kg body weight. Pilot studies showed that this dose was about equally effective in improving glucose tolerance as 1 mg/kg/d glyburide. The dose of 30 mg/kg/d is considerably higher than the human dose. However, sitagliptin has a half-life of two hours in rats[14] versus 13 h in humans.[2] The short half-life also necessitated continuous administration through drinking water in place of the once-a-day dosing used in humans. Glyburide was provided at 15 ppm, which provided 1 mg/kg/d, a dose previously reported to control type 2 diabetes in the rat.[15] Control untreated SHROB were provided with vehicle solution to drink. Body weight and food intake were determined twice a week, and fasting tail blood samples (0.4 mL) were obtained in heparinized capillary tubes at 9:00 every other week. Lean SHRs were provided with plain water and sacrificed at the same age as the SHROB to provide a metabolically normal comparison. They were fasted in parallel with the SHROB prior to blood collection and sacrifice.

Oral glucose tolerance test

After 60 d of treatment, an oral glucose tolerance test (OGTT) (6 g/kg glucose by oral gavage) was conducted after an 18-h fast. As previously described,[16] rats were given by oral gavage a 50% glucose solution at a dose of 6 g/kg body weight. Blood (0.2 mL) was obtained from the tail of conscious animals at baseline and 30, 60, 120, 240 and 360 min after the glucose load. Blood glucose was determined at each time point from 2.0 μL fresh whole blood from the tail using a clinical meter (One Touch Ultra, Lifescan, Milpitas, CA, USA). Area under the curve was determined for each parameter for quantification of the OGTT.

Plasma biochemical measurements

Blood samples were chilled on ice, centrifuged for 20 min at 5000*g* at 4°C, and the plasma frozen at −70°C until assayed for plasma insulin and glucagon by ELISA, using rat insulin and glucagon standards and antibodies directed against rat insulin and glucagon (ALPCO, Salem, NH, USA). Triglycerides were measured enzymatically using a standard kit (Sigma, St Louis, MO, USA). Triglycerides in 5 μL plasma were first hydrolyzed by lipoprotein lipase to liberate glycerol, which was then phosphorylated by glycerol kinase. Glycerol-1-phosphate was then oxidized by glycerol phosphate oxidase to liberate hydrogen peroxide. Peroxidase then catalyzed the coupling of O_2 with 4-aminoantipyrine and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine to produce a quinoneimine dye that shows an absorbance maximum at 540 nm proportional to the triglyceride content.

Terminal studies

At the end of the experiment, animals were anesthetized with enflurane and a 5 mL blood sample was obtained by cardiac puncture. At autopsy, we measured fat pad weights. These were classified as visceral fat: retroperitoneal, mesenteric and gonadal (epididymal/myometrial), and as subcutaneous fat: subscapular depot. Also

Table 1 Characteristics of SHROB treatment groups and untreated SHRs at study termination

Group	SHROB vehicle ($N = 14$)	SHROB sitagliptin ($N = 11$)	SHROB glyburide ($N = 9$)	SHR‡ untreated ($N = 8$)
Final body weight (g)	507 ± 27	499 ± 21	495 ± 11	244 ± 6
Total dissectible fat (g)	63 ± 6	65 ± 6	59 ± 5	9.0 ± 1.3
Visceral fat (g)	28 ± 3	25 ± 3	32 ± 3	9.0 ± 1.3
Subcutaneous fat (g)	32 ± 4	33 ± 4	29 ± 2	<1
V/S ratio	0.89 ± 0.09	0.77 ± 0.04*	1.13 ± 0.15	>10
Heart weight (g)	1.29 ± 0.04	1.29 ± 0.05	1.36 ± 0.09†	1.09 ± 0.05
Liver weight (g)	17.6 ± 1.0	17.3 ± 1.8	16.9 ± 1.2	8.3 ± 0.5
Kidney weight (g)	2.27 ± 0.12	2.48 ± 0.13	2.38 ± 0.18	1.63 ± 0.06

SHR, spontaneously hypertensive rat; SHROB, spontaneously hypertensive rat – obese strain; V/S, visceral to subcutaneous fat mass
Data are mean ± SEM
*Significantly different from glyburide-treated SHROB, $P < 0.05$ by Newman–Keuls
†Significantly different from vehicle-treated SHROB, $P < 0.05$ by Newman–Keuls
‡Each of the values for lean SHR is significantly different from all of the SHROB groups

determined were weights of the following organs: liver, heart and kidneys.

Liver glycogen determination

The method was adapted from Lo *et al*.[17] Samples of frozen liver (about 0.3 g) were homogenized in 2.5 mL of 30% KOH solution for one minute using a polytron on its highest setting (Tekmar Tissuemizer, Mason, OH, USA). The samples were then boiled at 98°C for 1–2 h, after which the samples were chilled on ice. Five milliliter of 100% ethanol was then added along with 10 μL of 4 mol/L lithium chloride as a carrier to each sample in order to precipitate glycogen. After four hours, samples were spun at 5000*g* at 4°C (Sorvall RT 600B Centrifuge, Thermo Scientific, Asheville, NC, USA) for 30 min. The supernatant was then poured off and the glycogen-enriched pellet was resuspended in 0.5 mL of 4 N HCl. This was then boiled again for one hour at 98°C to achieve hydrolysis. Samples were neutralized and re-centrifuged as before. The supernatant was analyzed for glucose by the glucose oxidase (Trinder, Sigma Chemical) method adapted for 96-well plates. Standards of 40, 20, 10, 5, 2.5, 1.25 and 0 mmol/L glucose were used in parallel with the samples (20 μL) and 200 μL of color reagent. The plates were incubated for 10 min at room temperature and read at 505 nm in a plate spectrophotometer (Tecan Rainbow, Durham, NC, USA).

Data analysis

Results are presented as mean ± standard error of the mean. Comparisons between groups were made using one- or two-way analysis of variance (ANOVA). Changes over time during the treatment interval were compared by ANOVA with repeated measures (REMANOVA). We used Prism 5.0 (Graph Pad Software, San Diego, CA, USA) with *post hoc* analyses by Neuman–Keuls test. Standard curves were analyzed by fitting a logistic equation in Prism 5.0. Glycogen was calculated as (glucose)/(liver weight × 100) = glycogen (in mg/g liver).

Results

Body weight increased in all three groups of SHROB, reaching about 500 g by the end of the experiment, more than double the body weight of lean SHR littermates (Table 1). Neither drug treatment had any effect on body weight or on food or water intake (data not shown). Consistent with the absence of any effect on body weight, total depot fat recovered at autopsy was also not changed (Table 1). Interestingly, the ratio of visceral to subcutaneous fat mass was significantly shifted by sitagliptin treatment in favor of subcutaneous relative to visceral fat. Fat distribution

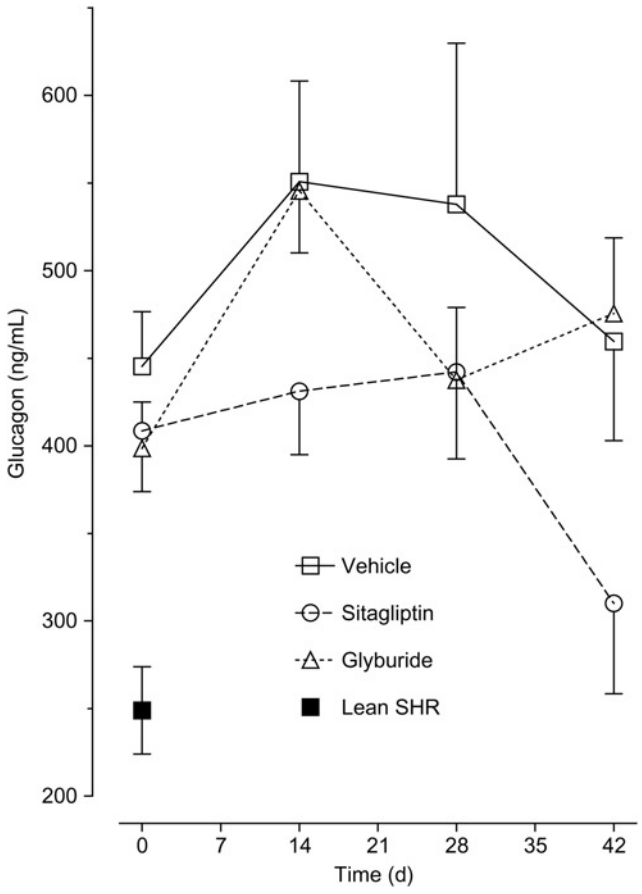

Figure 1 Progression of fasting plasma glucagon levels over time during treatment with vehicle ($N = 14$), sitagliptin ($N = 6$) and glyburide ($N = 5$). Values for an age-matched sample of lean SHR ($N = 8$) are shown at day zero for comparison. All values exceed the lean SHR group except for final values for the sitagliptin group ($P < 0.05$, Newman–Keuls). SHR, spontaneously hypertensive rat

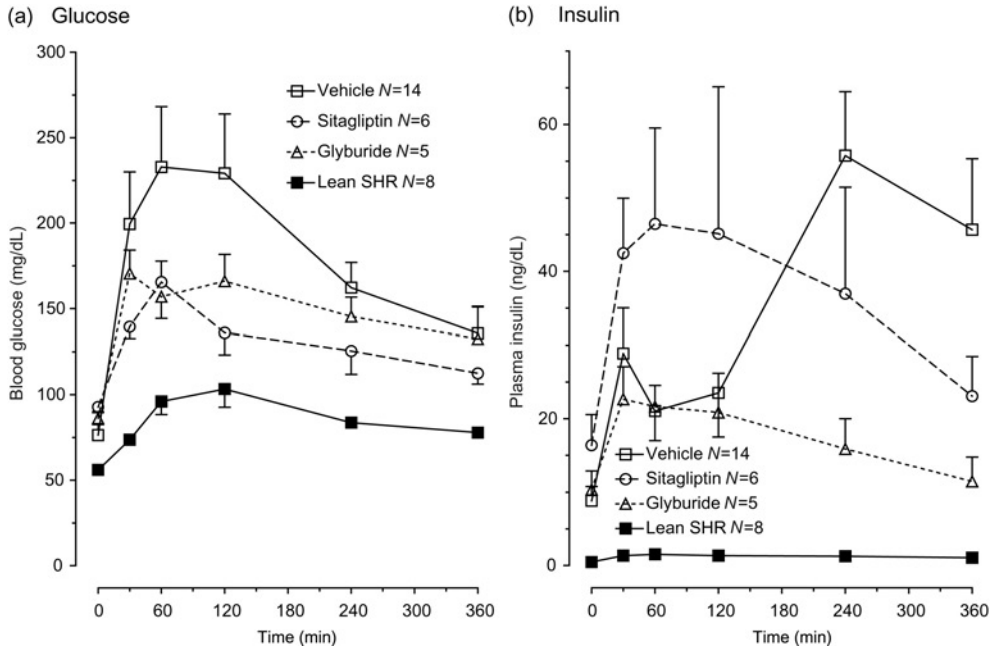

Figure 2 Oral glucose tolerance test results for glucose (a) and insulin (b). Fasting values are shown at time zero, and then the progression of glucose and insulin over time in minutes following the glucose load is shown. Where error bars are not visible, the error is smaller than the size of the symbol. Sitagliptin and glyburide are equally effective in lowering the glucose response to oral challenge. Sitagliptin but not glyburide restores the first phase of insulin secretion in the oral tolerance test. SHR, spontaneously hypertensive rat

was shifted favorably by sitagliptin relative to both the vehicle- and glyburide-treated groups.

Heart weights were increased by glyburide treatment (Table 1). This apparent cardiac hypertrophy remained significant after correction for body weight.

Fasting plasma glucose levels were within the normal range and remained relatively constant during treatment and did not differ between groups (week 4 values; vehicle 81 ± 2 mg/dL, sitagliptin 87 ± 4 mg/dL and glyburide 88 ± 6 mg/dL). Fasting insulin levels in all groups markedly elevated relative to lean SHRs.

Fasting glucagon levels were within the previously reported range, and were approximately two-fold elevated relative to lean SHRs of the same age (Figure 1). By five weeks of treatment, but not before, sitagliptin reduced glucagon levels to within the range of lean SHRs. Because glucose values determined in the same plasma samples were unchanged, changes in glucose cannot account for the difference in glucagon.

SHROB showed marked glucose intolerance relative to lean SHRs, confirming previous results. Sitagliptin and glyburide both reduced postprandial glucose levels. Glucose levels did not differ between drug-treated groups at any time point (Bonferroni tests following two-way REMANOVA). However, the overall area under the curve was reduced to a greater extent by sitagliptin than by glyburide (vehicle 35,713 mg min/dL, sitagliptin 13,655 mg min/dL and glyburide 22,788 mg min/dL).

In response to an oral glucose load, vehicle-treated SHROB showed a delayed and exaggerated rise in plasma insulin (Figure 2). Both sitagliptin and glyburide normalize the insulin response to a glucose challenge by shifting the peak of secretion earlier. Insulin peaks at 30–60 min after

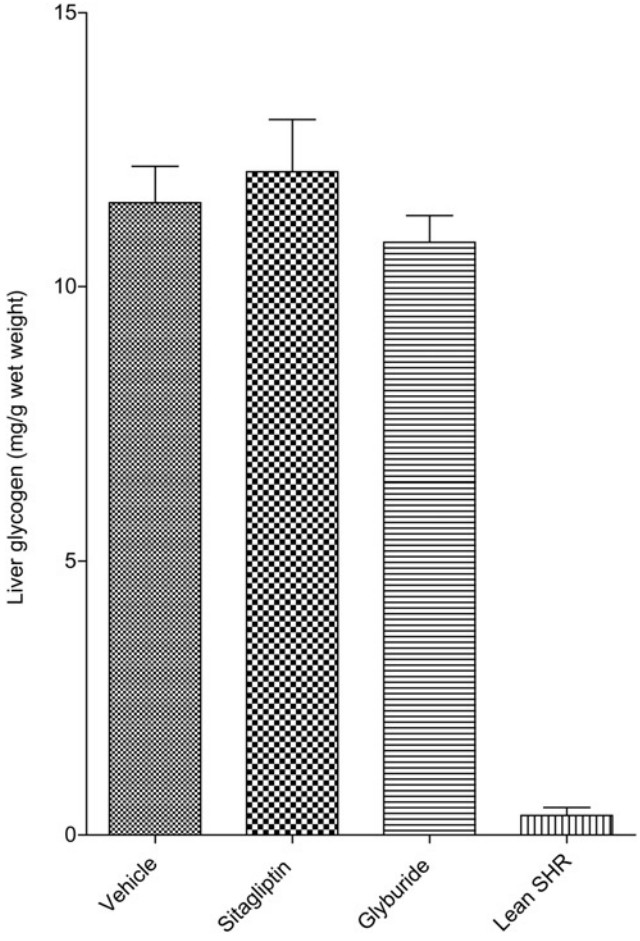

Figure 3 Liver glycogen content in SHROB treated with vehicle, sitagliptin or glyburide in comparison to untreated SHRs. SHR, spontaneously hypertensive rat; SHROB, spontaneously hypertensive rat – obese strain

glucose ingestion, whereas in controls insulin secretion continues to rise through 240 min. Only sitagliptin significantly enhanced insulin secretion at time points up to 120 min. The overall area under the curve was slightly reduced by sitagliptin relative to vehicle control (7569 versus 10,316 ng min/mL) but markedly reduced by glyburide (2557 ng min/mL).

Consistent with previous findings, plasma triglycerides were significantly higher in SHROB vehicle controls (186 ± 20 mg/dL) than in lean SHRs matched for age and sex (79 ± 21 mg/dL). Treatment with sitagliptin had no effect on triglycerides (205 ± 26 mg/dL). Glyburide likewise had no effect (217 ± 29 mg/dL).

As expected, fasted liver samples from lean SHRs showed very low levels of glycogen (0.36 ± 0.14 mg/g). Vehicle-treated SHROB showed much higher levels of glycogen (16.2 ± 1.4 mg/g), suggesting that mobilization of glycogen in the fasting state is impaired. Neither drug treatment affected liver glycogen levels (Figure 3).

Discussion

We have shown that the DPP-IV inhibitor sitagliptin has significant effects on glucose metabolism in a rat model of metabolic syndrome and prediabetes. Sitagliptin is at least as effective as the sulfonylurea compound glyburide in normalizing glucose tolerance following an oral glucose load. Sitagliptin also restored the first phase of insulin secretion in response to oral glucose more effectively than glyburide. Fasting glucagon levels, which are elevated in the SHROB model, were normalized after five weeks of treatment. The reason for the delayed fall in glucagon is not known. The target hormone GLP-1 is known to have trophic effects as well as immediate effects on the endocrine pancreas,[18] and slowly developing effects might contribute to the effects of sustained treatment. Maximum effectiveness in humans is known to require several weeks of treatment.

Previous studies of sitagliptin in rodent models have shown reduced glycemia in insulinopenic models such as streptozotocin-treated rats or mice.[19,20] In contrast, the present study used a severely hyperinsulinemic model. Sitagliptin was efficacious, despite preserved and even elevated pancreatic insulin secretion. This study is the first to demonstrate that chronic sitagliptin can lower fasting glucagon levels, an effect that is predicted based on the glucagon lowering actions of endogenous GLP-1 and GIP.[21] In human type 2 diabetes, excess hepatic production of glucose during fasting is a major component of fasting hyperglycemia, a process under the control of glucagon.[22] Reducing levels of glucagon in the postprandial period could conceivably delay the onset of diabetes in prediabetics.

Sitagliptin can acutely reduce postprandial levels of plasma triglyceride.[23] In the present study, we found no effect of chronic sitagliptin on fasting triglycerides.

In a rat model of amyloidal degeneration of the pancreas, sitagliptin induced tissue damage to the exocrine pancreas even while protecting the endocrine pancreas.[24] In the present study, careful examination of the pancreas at autopsy revealed no visible pathology in any of the SHROB groups. Moreover, all rats tolerated the drug treatments well and showed no change in rate of weight gain or food intake.

While insulinopenic diabetes prevents the activation of glycogen synthesis by insulin, insulin-resistant type 2 diabetic patients showed elevated levels of glycogen after fasting.[25] Glycogen levels are markedly increased in SHROB livers, presumably reflecting the influence of profound fasting hyperinsulinemia. The persistence of liver glycogen after a fast is all the more remarkable when considering the elevated levels of glucagon present. Neither sitagliptin nor glyburide lowered the fasting levels of insulin, so it is perhaps not surprising that they failed to normalize elevated liver glycogen in the SHROB model.

Unexpectedly, glyburide was found to be associated with increased cardiac mass. In at least one report, type 2 diabetic patients treated with glyburide show greater left ventricular mass than those treated with other agents.[26] This may reflect the influence of cardiac ATP-sensitive potassium channels.

We conclude that sitagliptin is effective in lowering fasting glucagon, improving glucose tolerance, restoring early phase insulin secretion and favorably affecting fat distribution in a prediabetic model of metabolic syndrome. If these drug actions extend to human prediabetics, then sitagliptin might delay the onset of diabetes.

Author contributions: BC prepared the first draft of the manuscript and carried out the liver glycogen analysis and assisted with the other studies. AM, LVSE, MSK and DH all worked with the animals and carried out all the assays. RJK edited the manuscript, contributed to the initial concept, and worked with the animals including all of the glucose tolerance tests. PE designed the study, worked with the animals and carried out assays, analyzed the data, prepared the graphs, and prepared the final manuscript.

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