A Model of Type 2 Diabetes in the Guinea Pig Using Sequential Diet-Induced Glucose Intolerance and Streptozotocin Treatment

Brendan K Podell1*, David F Ackart1, Michael A Richardson1, James E DiLisio1, Bruce Pulford1, Randall J Basaraba1

1Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

Corresponding Author:
Brendan K. Podell
200 West Lake Street
1619 Campus Delivery
Fort Collins, CO 80523
Phone: (970) 491-5746
Email: brendan.podell@colostate.edu

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Summary Statement

This article demonstrates the translational manifestations of diabetes using novel methods for type 2 diabetes induction, creating a new tool for investigators interested in the guinea pig as a model species.
Abstract

Type 2 diabetes is a leading cause of morbidity and mortality among non-communicable diseases and additional animal models that more closely replicate the pathogenesis of human type 2 diabetes are needed. The goal of this study was to develop a model of type 2 diabetes in guinea pigs in which diet-induced glucose intolerance precedes β cell cytotoxicity, two processes that are critical to the development of human type 2 diabetes. Guinea pigs developed impaired glucose tolerance after eight weeks of feeding a high fat, high carbohydrate diet, as determined by oral glucose challenge. Diet-induced glucose intolerance was accompanied by β cell hyperplasia, compensatory hyperinsulinemia, and dyslipidemia with hepatocellular steatosis. Streptozotocin (STZ) treatment alone was ineffective at inducing diabetic hyperglycemia in guinea pigs, which failed to develop sustained glucose intolerance or fasting hyperglycemia and returned to euglycemia within 21 days after treatment. However, when high fat, high carbohydrate diet-fed guinea pigs were treated with STZ, glucose intolerance and fasting hyperglycemia persisted beyond 21 days post-STZ treatment. Guinea pigs with diet induced glucose intolerance then subsequently treated with STZ demonstrated an insulin secretory capacity consistent with insulin-independent diabetes. This insulin independent state was confirmed by response to oral antihyperglycemic drugs, metformin and glipizide, which resolved glucose intolerance and extended survival compared to guinea pigs with uncontrolled diabetes. In this study, we have developed a model of sequential glucose intolerance and β cell loss, through high fat, high carbohydrate diet and extensive optimization of STZ treatment in the guinea pig, which closely resembles human type 2 diabetes. This model will prove useful in the study of insulin-independent diabetes pathogenesis with or without comorbidities, where the guinea pig serves as a relevant model species.
Introduction

Among non-communicable diseases, type 2 diabetes is a major cause of morbidity and mortality worldwide. Among the different forms of diabetes, type 2 diabetes accounts for greater than 90% of the diabetic population and the prevalence is rapidly increasing on a global scale with an estimated increase from 387 million diabetic patients in 2014 to approximately 600 million by 2035 (Guariguata et al., 2014; IDF., 2015). Of particular importance is the recent rise of type 2 diabetes incidence among low- and middle-income countries, which also have the highest population of prediabetic patients worldwide (Guariguata et al., 2014; IDF., 2015). In remote global regions, approximately 50% of the diabetic population is undiagnosed and therefore is living with uncontrolled diabetes (Beagley et al., 2014; IDF., 2015). Moreover, this rise in diabetes incidence in low- and middle-income countries highlights a new convergence of non-communicable diseases with endemic communicable diseases, thereby complicating the global control of important infectious diseases as well (Remais et al., 2013). Accordingly, identification of preventive and therapeutic measures that are affordable and applicable to populations in low- and middle-income countries will be paramount to controlling the growing epidemic of type 2 diabetes. The development and testing of new prevention and treatment strategies is dependent on their initial preclinical evaluation using animal models that mimic the pathogenesis of the human disease.

Type 2 diabetes is a complex metabolic disorder resulting from chronic inflammation-associated alterations in lipid metabolism and corresponding insensitivity of tissues to the actions of insulin. Obesity, the most common risk factor for the development of type 2 diabetes, links the accumulation and inflammation of excess adipose tissue with alterations in systemic lipid metabolism and the development of impaired glucose homeostasis. This link in metabolic disturbances has been termed metabolic syndrome, and represents a combination of elevated serum triglycerides, hypertension, and evidence of insulin resistance, all of which contribute to the pathogenesis of type 2 diabetes and diabetes associated complications (Shoelson et al., 2006). Owing to the complexity of this disease, preclinical animal models used to study complications,
comorbidities, or therapeutic interventions for type 2 diabetes are essential and must accurately reflect the pathogenesis of the human disease.

A number of animal models of insulin independent type 2 diabetes and insulin dependent diabetes exist, but their application in comparative biomedical research depend on the context of disease being studied. Experimentally induced high fat diets are commonly used in inbred mouse or rat strains to model metabolic syndrome and the associated adiposity, dyslipidemia and cardiovascular disease phenotypes Srinivasan and Ramarao, 2007; Surwit et al., 1988). However, these models require lengthy feeding regimens before any decline in β cell mass is detectable. To overcome this barrier, streptozotocin has been described in the rat to experimentally deplete β cell mass after induction of diet-induced insulin resistance (Islam and Loots du, 2009; Islam and Wilson, 2012; Reed et al., 2000; Skovso, 2014). This experimental approach may best model the natural pathogenesis of overt type 2 diabetes in humans. Guinea pigs may offer additional advantages to existing rodent models of type 2 diabetes and metabolic syndrome. While guinea pigs have been previously only used as a diabetes model through induction with STZ, this serves specifically as a model of insulin dependent diabetes because it lacks the contribution of insulin resistance. Moreover, there are substantial differences published in overall susceptibility of the guinea pig species to chemical diabetes induction with STZ, which are the consequence of a wide range of experimental approaches (Aomine et al., 1990; Klein et al., 1980; Lenzen, 2007; Schlosser et al., 1984; Schlosser et al., 1987; Wehner and Majorek, 1975).

Although the guinea pig is not expected to replace more common mouse or rat rodent models of type 2 diabetes, this species does have certain advantages. In addition to inflammatory changes induced by high fat and high sugar diets (Fernandez and Volek, 2006; Ye et al., 2013), the guinea pig is widely regarded for research in specific diseases including cardiovascular disease, atherosclerosis, and arthritis, as well as a number of infectious diseases that have been linked as comorbidities with diabetes (Madsen et al., 2008; Padilla-Carlin et al., 2008; West and Fernandez, 2004). The guinea pig is critical for development of new vaccines, particularly owing
to its immunological and pathological similarities to a number of infectious diseases of humans (Hickey, 2011). Additionally, the guinea pig more so than any other rodent, shares commonalities with human lipid metabolism including cholesterol metabolism and transport, with a greater proportion carried in association with low-density lipoproteins (Ensign et al., 2002; Fernandez et al., 1999; Ye et al., 2013).

Given the significant variability and reported resistance of guinea pigs to experimental diabetogenic treatments, we investigated the impact of high fat, high carbohydrate diet on glucose tolerance and induction of insulin resistance, and the impact of preexisting glucose intolerance on induction of overt diabetes with STZ. Herein, we have developed a representative model of human insulin resistance and type 2 diabetes in the guinea pig by strategically promoting the sequential development of glucose intolerance with high fat, high sugar diet, and β cell loss through partial cytotoxicity. This experimental induction of increased insulin demand along with a reduction in functional β cell mass is designed to mimic critical manifestations in the pathogenesis of human type 2 diabetes, which can be accomplished within a condensed timeframe. (Ferrannini et al., 2005; Kahn, 2001; Weir and Bonner-Weir, 2013).

Results

HFHC-fed guinea pigs develop dyslipidemia in the absence of rapid weight gain. Weight change was monitored over time in guinea pigs fed either a normal guinea pig diet or the HFHC diet, then for three weeks following STZ treatment of HFHC-fed guinea pigs (Fig S1). Guinea pigs fed either normal diet or HFHC diet had comparable caloric intake based on body weight (Table S1) and after eight weeks of consuming the HFHC diet, control guinea pigs fed a normal diet weighed a mean 700.9 ± 48.8 g compared to 571.1 ± 45.2 g for HFHC-fed guinea pigs (P<0.05). This difference was the result of initial weight loss and subsequent static weight gain during the course of transition to HFHC diet. Following this transition period, HFHC-fed guinea pigs demonstrated a similar mean rate of weight gain (2.75 ± 0.17 g/day) compared to normal diet
controls (2.38 ± 0.15 g/day) (Fig S1). However, HFHC/STZ guinea pigs with diabetic glucose intolerance had significant weight loss (P<0.001) over the three-week period following STZ administration, with mean weight of 565.2 ± 45.2 g prior to STZ treatment and 441.6 ± 42.4 g three weeks after STZ. Both HFHC-fed and HFHC/STZ guinea pigs had elevated fasting serum triglyceride levels compared to normal diet controls; however, elevations in total serum free fatty acids were present only in the HFHC/STZ guinea pigs (Table 1). Hepatocellular triglycerides were also elevated in HFHC/STZ guinea pigs; however, in response to oral antihyperglycemic therapy with metformin and glipizide in combination, concentrations of hepatocellular triglycerides were significantly lowered compared to HFHC/STZ guinea pigs with uncontrolled diabetes (Table 1).

The majority of cholesterol in all treatment groups was contained in LDL/VLDL serum fractions, as previously reported, but HFHC feeding, both with and without STZ significantly increased LDL/VLDL cholesterol compared to normal diet controls without altering HDL cholesterol (Table 1).

**HFHC-fed guinea pigs develop impaired glucose tolerance and suppressed response to exogenous insulin.** HFHC-fed guinea pigs were evaluated for evidence of glucose intolerance based on oral glucose tolerance test. No significant difference in the glucose response was observed between normal diet controls and HFHC-fed guinea pigs after four weeks on the diet (Fig 1A). However, after 8 weeks of consuming the HFHC diet, impaired glucose tolerance was evident as increased average blood glucose at the 60-minute time point of the OGTT (197.5 ± 28.3 vs 138 ± 34.6 mg/dl) (Fig 1A). Significantly elevated fasting blood glucose was not ever observed in guinea pigs fed HFHC diet alone. To identify further evidence of insulin resistance, reduction in blood glucose was measured in HFHC-fed guinea pigs in response to exogenous insulin. Exogenous insulin treatment induced a rapid reduction in blood glucose within 30 minutes in normal diet control guinea pigs with gradual return to euglycemia over 100 minutes. Although no difference was seen at 4 weeks of HFHC feeding based on oral glucose tolerance test, the
response to exogenous insulin was suppressed after four weeks and significantly impaired after a total of eight weeks of HFHC feeding (Fig 1B).

**Optimized STZ dosing minimizes toxicity-associated mortality in the guinea pig model.** In developing a guinea pig model of STZ-induced hyperglycemia, three parameters were identified as being critical for a successful response to STZ without adverse effects, including dose, route, and preparation of the STZ-solution (Table 2, Fig S2). Since multiple doses, routes of administration, and methods for preparation have been previously reported in guinea pigs or other rodent species, an encompassing approach to optimization was taken for this guinea pig model using guinea pigs fed a normal diet. In guinea pigs receiving 300 mg/kg, regardless of SC or IP route, or single versus multiple dosing, a high level of mortality occurred within days after completing the treatment. Mortality in 300 mg/kg dosed guinea pigs ranged from 80% in those receiving a single dose by SC route (n=5), 75% in those receiving a single dose by IP route (n=4), to 100% in those receiving six consecutive daily doses of 50 mg/kg IP (n=4), using a multidose schedule shown to be effective in mice (Qi et al., 2005). Only one of 13 guinea pigs receiving a cumulative 300 mg/kg dose ever developed evidence of hyperglycemia.

Evaluation of histopathology revealed that mortality in guinea pigs was the result of acute STZ-mediated toxicity. The most severe and frequent manifestation of acute STZ toxicity was acute necrosis of the intestinal mucosal epithelium followed by acute necrosis of renal tubular epithelium (Fig S3). There were no differences in toxicity-related pathology in small intestine and kidney related to route of administration or frequency of dosing in those receiving a total of 300 mg/kg, and similar lesions in these organs were associated with mortality due to lower doses of STZ. While no significant differences in mortality were observed between IP and SC routes of administration, guinea pigs that received IP STZ developed mild peritonitis and exuberant peritoneal and serosal fibrosis on visceral organs, precluding further use of this route of administration (Fig S3).
In contrast, improved STZ tolerance and survival was achieved with 200 mg/kg as a single or multiple dose schedule, with 100% survival out to 30 days post-injection (Table 2). However, the hyperglycemia response rate was unacceptably low at this dose, with no induction at 4 doses of 50 mg/kg (n=4) and only 50% with a single dose (n=4). This rate of response was not improved upon by a slightly higher single dose of STZ at 250 mg/kg (Table 2). There was no histological evidence of β cell death within pancreatic islets in guinea pigs treated with a 300 mg/kg dose. However, acute cell death was evident within the islets of guinea pigs treated with 250 mg/kg doses of STZ (Fig 2B) and corresponded with aggregates of insulin protein presumably released from β cell necrosis (Fig 2D).

Because it is well demonstrated that the α-anomeric form of STZ is more likely to induce adverse toxicity, we evaluated the preparation of STZ as an equilibrated solution of α- and β-anomers to determine if, at an equivalent dose, the equilibrated solution of STZ is capable of producing a similar hyperglycemic response (Bell and Hye, 1983; de la Garza-Rodea et al., 2010). At a dose of 250 mg/kg, both freshly prepared and anomer-equilibrated STZ induced non-fasting hyperglycemia in 40% of treated guinea pigs, indicating a similar efficacy between both preparations (Table 2). Guinea pigs receiving anomer-equilibrated STZ demonstrated 100% survival 21 days beyond STZ treatment compared to 80% in guinea pigs receiving freshly prepared STZ, after which the experiment was terminated.

**STZ-induced hyperglycemia is enhanced with antecedent use of an α_2-adrenergic receptor antagonist.** Since the success rate of achieving hyperglycemia in guinea pigs at mid-range doses of STZ remained low regardless of dose or route of administration (Table 2), the α_2-antagonist, yohimbine, was evaluated as an adjunctive treatment, because it has been demonstrated to enhance the diabetogenic effects of STZ in mice (Nakadate et al., 1981). We evaluated the rate of hyperglycemia from anomer-equilibrated STZ treatment in guinea pigs either pre-treated with yohimbine or mock-treated with normal saline. Guinea pigs that were pre-treated with yohimbine were 100% responsive to the STZ treatment as indicated by hyperglycemia...
present for the following seven-day period (Table 2). In contrast, 20% of the mock-treated guinea pigs, developed hyperglycemia.

**STZ treatment leads to sustained diabetic glucose intolerance only in the presence of subclinical impaired glucose tolerance due to HFHC feeding.** At STZ doses of 200 and 250 mg/kg (with or without yohimbine), where hyperglycemia was manifested in 25-100% of treated guinea pigs, elevated glucose was evident within 48 hours. In the course of daily random glucose sampling, non-fasted hyperglycemia persisted in the range of 200 to 400 mg/dl, for 7 to 10 days. There was a consistent steady decline in the degree of hyperglycemia beginning at approximately day 7 and extending through day 21 post-STZ treatment. By day 14 post-STZ treatment, random glucose samples were often within the normal range of untreated, non-diabetic guinea pigs (Fig 3A). This transient response was uniform across all guinea pigs that developed hyperglycemia within 7 days of STZ treatment. Evaluation of glucose tolerance by OGTT in guinea pigs treated with STZ alone after 14 and 21 days demonstrated that glucose tolerance returned to normal over time, when compared to OGTT performed on day 7 after treatment where severe glucose intolerance was present (Fig 3B). In contrast, guinea pigs initially fed the HFHC diet for eight weeks to induce glucose intolerance then treated with yohimbine and 200 mg/kg of STZ (HFHC/STZ), developed persistent diabetic levels of glucose intolerance and fasting hyperglycemia based on human diagnostic criteria. On day 21 post-STZ treatment, HFHC/STZ guinea pigs continued to show fasting hyperglycemia, a feature that was absent from HFHC-fed guinea pigs. In response to oral glucose challenge, HFHC/STZ guinea pigs demonstrated severely impaired glucose tolerance with elevated glucose exceeding 200 mg/dl after two hours, which persisted after 21 days post-STZ treatment (Fig 3C).
HFHC-fed guinea pigs display compensatory hyperinsulinemia, which is eliminated in HFHC/STZ treated guinea pigs. Fasted serum insulin concentrations were compared along with fasting blood glucose from the same blood sample to determine if a compensatory response was present in HFHC-fed and HFHC/STZ guinea pigs (Fig 4A). Compared to normal diet controls, mean fasting insulin concentrations were 2.1-fold higher in HFHC-fed guinea pigs after consuming the diet for 8 weeks; however, fasting glucose concentrations did not differ from those of normal diet controls. In HFHC/STZ guinea pigs 21 days after STZ-treatment, serum insulin concentrations were comparable to normal diet control guinea pigs at 13.0 ± 4.7 ng/ml and 12.6 ± 4.6 ng/ml, respectively. However, fasting glucose concentrations of HFHC/STZ guinea pigs were significantly elevated over normal diet controls at 101.0 ± 30.6 mg/dl vs 58.1 ± 10.1 mg/dl, respectively (Fig 4B). To determine insulin secretory capacity in response to glucose challenge, fasting insulin concentrations were paired with stimulated samples 30 minutes after an oral glucose bolus. While fasting insulin concentrations were decreased in HFHC/STZ guinea pigs, circulating insulin increased to comparable levels after 30 minutes, indicating that when stimulated, residual insulin secretory capacity exists after STZ treatment (Fig 4C).

Islet cell hyperplasia accompanies HFHC-induced hyperinsulinemia and residual insulin-production in islets of HFHC/STZ treated guinea pigs. The pancreatic islets of HFHC-fed guinea pigs were, in general, larger in size and quantitatively more numerous, having a mean number of 1034 ± 548.4 per mm$^2$ of total evaluated pancreatic tissue after eight weeks of consuming the HFHC diet, compared to normal diet controls that had a mean number of 423.3 ± 186.8 per mm$^2$ (Fig 5A-C). In HFHC/STZ guinea pigs, 21 days post-STZ treatment, pancreatic islets were reduced in size and had indiscernible, irregular margins (Fig 5E). Immunofluorescent detection of pro-insulin in the HFHC-fed guinea pigs revealed a high frequency of insulin producing cells in all islets, including those that were significantly enlarged (Fig 5F). In contrast, immunofluorescent detection of pro-insulin in the HFHC/STZ guinea pigs 21 days post-STZ
treatment revealed persistence of insulin-producing β cells, but these were significantly reduced in frequency compared to the normal diet controls and HFHC-fed guinea pigs (Fig 5G).

**HFHC/STZ treated guinea pigs are non-insulin dependent and responsive to oral antihyperglycemic therapy.** To further confirm a state of type 2 diabetes in HFHC/STZ guinea pigs, response to oral antihyperglycemic drugs metformin and glipizide was evaluated. Prior to initiation of therapy, HFHC/STZ guinea pigs had confirmed glucose intolerance consistent with overt diabetes after 11 weeks total of HFHC feeding and 21 days post-STZ treatment. Complete reversal of diabetes-related glucose intolerance was evident in HFHC/STZ guinea pigs after 14 days of treatment with a combination of metformin and glipizide (Fig 6A). Guinea pigs treated with metformin and glipizide in combination displayed 100% survival out to 120 days while only a 25% survival rate was evident among mock-treated diabetic controls over the 120-day evaluation period (Fig 6B). Immunofluorescent detection of insulin producing β cells in the pancreas of normal diet controls, untreated HFHC/STZ guinea pigs, and HFHC/STZ guinea pigs receiving combination therapy demonstrated that compared to non-diabetic normal diet controls, there is a persistent reduction in overall β cell frequency out to 120 days post-STZ treatment in HFHC/STZ guinea pigs, regardless of whether the guinea pigs received treatment with metformin and glipizide (Fig 6C-E). However, in comparison to islet morphology and overall frequency of insulin-producing β cells in HFHC/STZ guinea pigs 21 days after STZ treatment, the frequency of β cells increases with time after STZ treatment both in the presence and absence of oral antihyperglycemic therapy.
Discussion

In this study, we developed a model of type 2 diabetes in the guinea pig through a combination of diet-induced glucose intolerance and subtotal β cell cytotoxicity. In the development of this model, we have confirmed that, similar to the rat and mouse models of diet-induced obesity, feeding of HFHC diet results in glucose intolerance in the guinea pig and compensatory responses that are consistent with insulin resistance, features which mimic a pre-diabetic state in humans. Moreover, we have identified critical factors necessary for improved survival, induction of hyperglycemia, and sustained diabetic glucose intolerance using a combination of high fat, high carbohydrate diet and STZ in the guinea pig, to yield a reproducible model of insulin independent overt type 2 diabetes.

The use of high fat diets in combination with chemical diabetes induction has gained recent traction for generation of rodent models of diabetes, particularly to be used in therapeutic testing. Although predominantly reported in the rat, this same strategy has been used in mouse models as well, but never previously evaluated in the guinea pig. There are advantages to this approach because this replicates important features of human type 2 diabetes, namely the transition from prediabetic state to overt diabetes which requires the loss of functional β cell mass. Thus, this model of diabetes induction is reflective of prototypical type 2 diabetes because it combines insulin resistance with reduced overall β cell mass, in a context where impaired systemic responsiveness to insulin is the limiting factor in the failure to control blood glucose. Thus, improving systemic insulin sensitivity is effective in controlling diabetes in this model, without requirement for insulin replacement therapy. This insulin independent phenotype is a defining feature of this guinea pig model, which has also been previously reported on multiple occasions in rats. This model is in stark contrast to chemical induction of diabetes with STZ alone, which effectively eliminates β cell populations leading to an insulin dependent phenotype and requirement for insulin therapy to maintain glucose tolerance. We show here that STZ alone is...
relatively ineffective in the guinea pig model, as this does not produce lasting hyperglycemia or
diabetic glucose intolerance even when substantial β cell mass is eliminated. This may be
explained by the ability of guinea pigs to readily compensate for lowered β cell mass, since β cell
function appears to be intact in response to glucose stimulation and β cell mass is improved with
antihyperglycemic therapy. Thus, a critical feature of this combined HFHC-diet and STZ model is
the requirement for systemic insulin resistance, that is maintained by the HFHC diet, thereby
limiting β cell compensation in the timeframe evaluated in this study.

Guinea pigs fed an HFHC diet demonstrate delayed glucose clearance in response to oral
glucose challenge, impaired glucose lowering effects of exogenous insulin treatment, as well as
high fasting serum insulin concentrations, all of which are consistent with systemic insulin
resistance. Hyperinsulinemia in the face of normal fasting glucose indicates that these guinea
pigs mount a successful β cell compensatory response to HFHC-induced glucose intolerance,
which is further supported by the development of islet hyperplasia of guinea pigs fed the HFHC
diet, a typical response of most rodent models in the face of insulin resistance, and to a lesser
extent, humans (Bruning et al., 1997; Butler et al., 2003; Jones et al., 2010; Kitamura, 2013;
Rahier et al., 2008). Additionally, functional compensation by β cells was evident in HFHC-fed
guinea pigs with increased insulin production to maintain euglycemia. We found that in this
model, a stable diabetic state and decompensated response can be achieved through STZ
treatment only after diet-induced glucose intolerance. These data suggest that the metabolic
impacts of HFHC diet are sufficient to overcome the compensatory capacity of β cells after STZ
exposure, a response that is known to be reduced in overt type 2 diabetic humans (Butler et al.,
2007) and is similar to the insulin resistant rat models, where hyperglycemia results from low
doses of STZ that would otherwise have no impact on glucose metabolism in normal wild-type
rats (Reaven and Ho, 1991). Data from this study supports a strong β cell compensatory
response in the guinea pig, making this a potentially valuable model for studying the factors that
influence β cell degeneration and function in the presence of systemic insulin resistance.
Although guinea pigs fed HFHC diet did not gain weight at a rate significantly greater than normal diet controls, the diet did alter lipid metabolism and lead to dyslipidemia when measured after a period of fasting. HFHC feeding promoted high levels of triglycerides and increased LDL/VLDL cholesterol, which were increased along with free fatty acids in the serum of HFHC/STZ guinea pigs, features that suggest altered hepatic lipid metabolism and increased lipolysis, both of which are features of insulin resistance in adipose and liver. It has been suggested that one of the greatest contributors to development of insulin resistance is the accumulation of fat within hepatocytes. Consistent with this finding in human patients, our guinea pig model also accumulated triglycerides in liver (Lim et al., 2011). Moreover, this lipid accumulation was reversible with the use of insulin sensitizing and secretagogue therapy, provided in the form of the biguanide, metformin, and sulfonylurea, glipizide, consistent with a direct link between systemic insulin resistance and the accumulation of hepatic lipid, as has been demonstrated in human patients with type 2 diabetes (Mazza et al., 2012; Taylor, 2013).

Based on the responses of this guinea pig model to HFHC diet, we show that impaired glucose tolerance from feeding HFHC diet prior to low-dose STZ treatment contributes significantly to the maintenance of stable diabetic glucose intolerance. In this model, insulin-dependent diabetes was not achieved solely through STZ-administration alone, as is seen in the rat and mouse where STZ reproducibly leads to β cell depletion and a state of insulin-deficient diabetes. In contrast, we have shown that in HFHC/STZ guinea pigs insulin production is retained in residual β cells and that fasting serum insulin circulates at concentrations comparable to that of normal diet controls. This is further supported by sustained glucose stimulated insulin secretion in HFHC/STZ guinea pigs. Although first and second phase insulin secretion cannot be distinguished without clamp techniques or isolated islets, these data suggest that β cell function remains in HFHC/STZ guinea pigs, which further supports impaired tissue sensitivity to insulin as a major contributor to sustained glucose intolerance in this model. Sustained fasting hyperglycemia was evident only after the compensatory β cell hyperplasia and hyperinsulinemia of HFHC-fed guinea pigs was abolished through single-dose STZ treatment. Therefore, the compensatory adaptations of insulin
producing β cells in guinea pigs fed a HFHC diet is a critical factor in the manifestation of overt insulin-independent diabetes following STZ treatment. These findings are similar to studies in human type 2 diabetes patients, where dietary restrictions combined with exercise can alleviate clinical diabetes, indicating that residual β cell function is sufficient to maintain blood glucose homeostasis once peripheral tissue insulin resistance is resolved (Taylor, 2013). Although this has not been confirmed through experimental glucose clamp techniques as of yet, our data indicate that HFHC/STZ guinea pigs develop overt diabetes largely due to the presence of insulin resistance, which is similar to the insulin independent state typical of human patients with type 2 diabetes. While HFHC/STZ guinea pigs have reduced β cell mass, residual function is present based on glucose stimulated insulin secretion, supporting the requirement for insulin resistance to overcome insulin compensation that occurs even in the presence of limited remaining β cell mass. In further support of an insulin independent diabetic state in this model, we have demonstrated that HFHC/STZ guinea pigs not only respond clinically to a combination of metformin and glipizide with reversal of glucose intolerance, similar to a rat model, but also that this drug combination significantly improves survival without the need for exogenous insulin therapy that is routinely required for survival in other rodent models with STZ-induced diabetes (Haughton et al., 1999; Reed et al., 2000). Moreover, guinea pigs treated with this combination therapy had improved beta cell mass, suggesting that control of tissue sensitivity affords some level of β cell protection.

In contrast to our guinea pig model, the use of STZ to establish insulin-dependent hyperglycemia in mice and rats is well established. The protocols for use of STZ in these rodents, and in comparison to what has been identified in our guinea pig model, demonstrates the wide variability in the response of multiple rodent species to STZ. The rat model requires only a single low dose of STZ for effective β cell cytotoxicity, while five consecutive doses of 50 mg/kg is generally accepted as the most effective dosing schedule for mice (Lenzen, 2007; Lenzen, 2008). Comparatively, we demonstrate here that the guinea pig generally responds more favorably to higher doses of STZ as a single bolus; however, there is a narrow toxic threshold and
hyperglycemia is not sustained. This study indicates that high mortality is associated with a cumulative STZ dose of 300 mg/kg, but doses of 100 mg/kg fail to elicit hyperglycemia in guinea pigs. Paradoxically, the lowest frequency of hyperglycemia was noted with the highest STZ dose of 300 mg/kg while midrange doses of 200 and 250 mg/kg yielded hyperglycemia at varying levels. This is further supported by a lack of histological evidence of β cell death in guinea pigs receiving the 300 mg/kg dose (data not shown). This could be explained by the level of acute toxicity seen at this dose, which precluded the establishment of diabetes due to failure to selectively target β cells for cytotoxicity (Lenzen, 2007). Additionally, the rapid clinical decline and mortality may have occurred at a rate preceding diabetogenic effects detectable by random blood glucose sampling. A lack of β cell specificity at the high 300 mg/kg dose is confirmed by pathological lesions in guinea pigs suffering early mortality post-STZ treatment that were consistent with acute STZ-induced toxicity of other major cell types expressing the GLUT2 solute transporter. GLUT2 is the cellular glucose transporter isoform previously linked to tissue specificity of STZ and cytotoxic effects on β cells, but is also expressed by intestinal epithelium, kidney tubular epithelium, and hepatocytes (Bell et al., 1990; Rees and Alcolado, 2005; Schnedl et al., 1994). In contrast, at doses capable of inducing hyperglycemia, STZ administration did lead to specific β cell death, which supports previous data that guinea pig β cells express the GLUT2 solute transporter, similar to other rodent species (Hosokawa et al., 2001; Lenzen, 2007).

Although dose appears to significantly impact STZ tolerance and the development of hyperglycemia in the guinea pig, we have also demonstrated similar efficacy of STZ solution whether administered as a freshly prepared solution or after allowing for anomer-equilibration. It is frequently suggested that the unstable α-anomer of STZ is more diabetogenic than the β-anomer and, for this reason, it is often cited that STZ must be prepared fresh and administered immediately (Ceylan-Isik et al., 2006; Gurley et al., 2006; Hegde et al., 2003; Lenzen, 2007). However, more recently it has been demonstrated that a stable equilibrium is established between α- and β-anomeric forms of STZ within two hours of dissolution, and this preparation leads to consistent hyperglycemia in mice with a significant reduction in mortality (de la Garza-
Rodea et al., 2010). These findings are recapitulated in our guinea pig model, which demonstrates that anomer-equilibrated STZ is equally effective in inducing hyperglycemia in guinea pigs compared to an equivalent dose of freshly prepared STZ. This study therefore provides additional support for the adoption of anomer equilibration in STZ chemical-induced animal models of diabetes in an attempt to improve reproducibility and reliability of studies between laboratories.

In line with previously reported resistance of guinea pigs to STZ, we experienced a rate of diabetes induction at doses of 200 and 250 mg/kg that was both variable and incomplete (Aslan et al., 2013; Kushner et al., 1969; Lenzen, 2007; Losert et al., 1971). Previously, it was reported that guinea pigs were largely insensitive to the cytotoxic effects of STZ as well as alloxan, an alternative GLUT2-dependent β cell toxin, when administered systemically; however, administration directly into the pancreatic circulation yielded uniform hyperglycemia from alloxan and isolated guinea pig β cells are susceptible to ex vivo STZ treatment (Lenzen, 2007; Lenzen, 2008). Thus, the reported level of resistance to both alloxan and STZ in the guinea pig may not be a direct reflection of the β cell itself, but rather the availability of the compound to the β cells after systemic administration. This is further supported by the fact that isolated pancreatic islets from the guinea pig are sensitive to the actions of both compounds in vitro (Griffiths, 1948; Lenzen, 2007). We were able to improve upon the response to systemic STZ administration through the use of the α2-adrenergic receptor antagonist, yohimbine, prior to administering STZ, which resulted in selective β cell death in guinea pigs analyzed within 48 hours of STZ administration. It has been previously demonstrated that STZ-induced diabetes could be improved through the use a specific α2-adrenergic receptor antagonist but not through blockade of α1 receptors (Elsner et al., 2000; Nakadate et al., 1981; Rosengren et al., 2010). The observation that increased circulating insulin coincides with enhanced STZ efficacy in mice and rats suggests that the mechanism of this synergistic effect may revolve around preventing catecholamine-adrenergic receptor signaling, which is known to inhibit insulin release from β cells (Ar’Rajab and Ahren, 1991; Jansson and Sandler, 1985; Nakadate et al., 1981; Savontaus et al.,
However, it remains to be determined if the role of insulin secretion in yohimbine sensitization to STZ is due enhanced uptake of the STZ glucose analogue or downstream intracellular actions within the β cell.

Regardless of increased STZ efficacy when combined with yohimbine, we continued to observe steadily decreasing blood glucose and improved response to oral glucose challenge over time in STZ-treated normal diet guinea pigs, indicating spontaneous recovery from diabetes induced with STZ alone. Multiple studies have utilized guinea pigs with STZ-induced diabetes, carried out for up to four weeks after STZ administration; however, the rate of fasting and non-fasting hyperglycemia, as well as response to glucose challenge over time after STZ treatment have not been well-documented (Aslan et al., 2013; Hootman et al., 1998; LePard, 2005; Saidullah et al., 2014; Tocchetti et al., 2015; Xie et al., 2013). Therefore, it is uncertain how often spontaneous recovery is observed in the guinea pigs, although this recovery is recognized in STZ-treated rats with β cell regeneration evident within 7 days of treatment (Garofano et al., 2000; Movassat et al., 1997). The improvement in glucose tolerance over time observed in the development of this model suggests that residual β cell compensation with or without regeneration may overcome the initial effects of STZ at the dose, method of preparation, and route of administration used in this study. Moreover, the data in this study support an increase in islet β cell mass over an extended period of time after STZ administration, indicating a regenerative response in STZ-treated guinea pigs. However, this is insufficient to overcome the diet-induced glucose intolerance in HFHC-fed guinea pigs.

Altogether, the guinea pig model of type 2 diabetes developed in this study closely mimics the classical pathogenesis of type 2 diabetes, where hyperinsulinemic compensation for insulin resistance is ultimately lost through diminished β cell capacity. While this guinea pig model may be applicable for certain experimental conditions including comorbidities, contributions of dyslipidemia, and compensatory or regenerative β cell responses, this model is not expected to replace other rodent models and is not without limitations. Although generation of reproducible diabetic guinea pigs using this model system requires intensive effort exceeding that required to
induce diabetes in more simple rodent models of high fat diet or STZ alone, these models do not replicate the same features of human type 2 diabetes that are seen in this guinea pig model. The important similarities between this model and human type 2 diabetes, including glucose intolerance that precedes diabetic hyperglycemia, altered lipid metabolism, reduced compensatory β cell capacity, and responsiveness to oral antihyperglycemic therapy will significantly enhance the use of the guinea pig as a model for type 2 diabetes research.

Materials and Methods

Animals and sample collection. Female, outbred Dunkin-Hartley guinea pigs, weighing between 250 and 300 g, were purchased from Charles River Laboratories or inbred Strain-13 guinea pigs were bred at the Colorado State University Laboratory Animal Resources facility and diabetogenic treatments were initiated at a weight of approximately 300 grams in mixed gender guinea pigs. For collection of serum, guinea pigs were anesthetized via isoflurane inhalation and blood collected by percutaneous venipuncture of the cranial vena cava. Blood glucose was measured using the Freestyle Lite glucometer (Abbot, Alameda, CA) from a skin prick site adjacent to the most peripheral vein on the pinna, previously validated against the glucose oxidase enzymatic assay. At the time of euthanasia, guinea pigs were administered 40 mg of ketamine and 0.5 mg of diazepam via intramuscular injection for anesthetic induction. Anesthetized guinea pigs were administered a 750 mg dose of pentobarbital via intraperitoneal route for euthanasia. All animal experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Usage Committee at Colorado State University under protocol number 10-1990A.

Preparation of streptozotocin solution for injection. All streptozotocin (STZ) preparations were prepared as 100 mg/ml solutions in sodium citrate buffer at a pH of 4.5. Sodium citrate buffer for injection was prepared initially as a 40mM citric acid solution (pH 2.9). The citric acid
solution was titrated with 40mM sodium citrate until a pH of 4.5 was reached. STZ, >75% α-anomer purity (Sigma, St. Louis, MO), was dissolved in the freshly prepared sodium citrate buffer at a concentration of 100 mg/ml. The STZ solution was passed through a 0.22 μm filter to sterilize, and administered either freshly prepared or after equilibration for two hours at 4°C and protected from light.

**Optimization of STZ treatment for induction of diabetic hyperglycemia.** Various doses of STZ and routes of administration were evaluated for diabetogenic efficacy and level of mortality in guinea pigs initially fed a normal diet and subsequently in guinea pigs with diet-induced impaired glucose tolerance. Guinea pigs were administered STZ via subcutaneous (SC) or intraperitoneal (IP) route, at doses of 300 mg/kg, 250 mg/kg, 200 mg/kg, or 100 mg/kg or as multiple daily consecutive doses of 50 mg/kg for four or six days. In a separate experiment, fresh or anomer-equilibrated STZ was administered via subcutaneous route at a dose of 200 mg/kg. Additionally, the diabetogenic efficacy of STZ was investigated for enhancement by pre-treating guinea pigs with 0.5 mg/kg of yohimbine, an α₂ adrenergic receptor antagonist, by intramuscular injection 20-30 minutes prior to administration of STZ. For the depletion of β cell mass in HFHC-fed guinea pigs, individuals with evidence of impaired glucose tolerance were administered a single 200 mg/kg dose of anomer-equilibrated STZ after pre-treatment with 0.5 mg/kg yohimbine.

**Custom Diet Formulation.** Guinea pigs were fed a custom formulated high fat and high carbohydrate (HFHC) diet (Dyets Inc., Bethlehem, PA). The custom formulated diet consisted of total calories as 30% fat, 52% carbohydrate, and 18% protein, where calories from fat were derived equally from Primex vegetable shortening and beef tallow, and carbohydrate calories were composed of 55% fructose and 45% sucrose. Fatty acid composition of the HFHC diet consisted of 41.9% saturated, 50.3% monounsaturated, and 7.8% polyunsaturated fatty acids. For comparison, control guinea pigs with normal glucose tolerance were fed a commercially
available conventional guinea pig diet containing 3% fat, 18% protein and 55% complex carbohydrates derived from grain (Harlan-Teklad #2040).

**Evaluation of impaired glucose tolerance and response to insulin.** To evaluate the glucose-lowering effects of insulin, guinea pigs fed either normal diet, HFHC diet for 4 or 8 weeks, or HFHC for 8 weeks followed by STZ treatment, were administered a subcutaneous injection of 0.5 units/kg of regular acting human recombinant insulin (Humulin-R, Eli Lilly, Indianapolis, IN). At the time of injection, glucose was measured in non-fasted guinea pigs using the Freestyle Lite glucometer then measured at 25, 50, 75, and 100 minutes after administration. To determine glucose tolerance in normal diet controls, guinea pigs fed HFHC diet (HFHC-fed) or guinea pigs rendered diabetic by HFHC feeding and STZ treatment (HFHC/STZ), a standardized oral glucose challenge consisting of a 2 g/kg bolus of D-glucose (0.5 g/ml) was administered after a 12-hour fasting period (oral glucose tolerance test, OGTT). Glucose levels were measured at times 0, 60, 90, 120 or 150 minutes post-administration with a Freestyle Lite glucometer, validated for accuracy against the glucose oxidase method for quantification of glucose in serum.

**Quantification of lipid parameters.** Total serum free fatty acid levels were measured by fluorescent detection of hydrogen peroxide generated by oxidation of acyl CoA (Cayman Chemical; Ann Arbor MI). Total serum triglycerides were quantified spectrophotometrically after sequential enzymatic conversion with lipoprotein lipase, glycerol kinase and glycerol phosphate oxidase followed by peroxidase mediated colorimetric change (Cayman Chemical, Ann Arbor, MI), as previously described (Podell et al., 2012). For measurement of total hepatocellular triglycerides, approximately 400 mg of liver tissue was homogenized in assay buffer containing protease inhibitor cocktail (Thermo Scientific, Waltham MA), centrifuged at 15,000 x g, and triglycerides measured in supernatant using the enzymatic assay. Calculated liver triglyceride concentrations were normalized to total protein in the supernatant, as measured by BCA assay (Thermo Scientific, Waltham MA). Total, HDL and LDL cholesterol was quantified in serum.
samples from normal diet, HFHC-fed, and HFHC/STZ guinea pigs from two separate experiments (total n=10 per group). Using the HDL and LDL/VLDL cholesterol assay (Cell BioLabs, San Diego, CA), LDL and VLDL particles were precipitated from serum samples and cholesterol quantified in supernatant and precipitated fractions to determine cholesterol content of HDL and LDL/VLDL particles, respectively.

Quantification of serum insulin. Due to well-documented divergence of the guinea pig insulin sequence and thereby, lack of commercially available cross-reactive immunoassays, a direct ELISA for detection of guinea pig insulin was developed based on the previously described guinea pig insulin peptide immunogen (Chan et al., 1984; Maimon et al., 1991). The C-terminal decapeptide of the guinea pig insulin β chain, amino acid sequence DDGFFYIPKD (Maimon et al., 1991), was conjugated to the c-terminus of bovine serum albumin (Bio-Synthesis Inc., Lewisville, TX). Four Balb/C mice were immunized with 100 μg of this conjugated protein initially in TiterMax adjuvant (Sigma, St. Louis, MO), then twice additionally at three-week intervals in Freund's Incomplete adjuvant (Sigma, St. Louis, MO). Serum from each mouse was evaluated for confirmation of specific antibody by direct ELISA prior to euthanasia, and specific reactivity was confirmed by specific localized binding to islet cells in guinea pig pancreas by immunofluorescent staining. For the detection of guinea pig insulin, blood was collected from fasted guinea pigs, corresponding fasting glucose levels measured, and 30 kDa filtered serum was coated on high-binding polystyrene plates overnight at 4°C at a 1:10 dilution in sodium bicarbonate buffer (pH 9.0). Guinea pig insulin was detected with pooled polyclonal mouse anti-serum (1:1000), followed by HRP-goat anti-mouse IgG antibody (1:1000) and 3,3,5,5-Tetramethylbenzidine (TMB) substrate, and concentration calculated based on a standard curve ranging from 0.1 - 7 ng/ml of insulin. Serum from unimmunized mice was used as a negative control.
Measurement of glucose stimulated insulin secretion. Guinea pigs either fed a normal diet, HFHC diet for 8 weeks, or combined HFHC diet for 8 weeks with subsequent STZ treatment, were subjected to oral glucose challenge as described for the oral glucose tolerance test. After the 12 hours fasting period, blood was collected for fasting insulin quantification then guinea pigs were fed the 2 g/kg glucose bolus. After 30 minutes, repeated blood collection was performed for quantification of insulin secretion stimulated by an oral glucose challenge. Insulin was quantified in serum by direct ELISA.

Treatment with oral antihyperglycemic therapy. Beginning at 3 weeks after STZ treatment, diabetic guinea pigs were treated with water suspensions of either metformin alone (25 mg), or with glipizide (0.25 mg) in combination, administered daily per os. Efficacy of treatment was assessed after 14 days of daily therapy based on improvement in glucose tolerance by OGTT. Normal diet controls and HFHC/STZ guinea pigs, either untreated or treated with metformin/glipizide combination therapy, were followed for survival for 120 days following initiation of oral therapy.

Histology and area morphometry of guinea pig pancreas. Sections of pancreas were sampled from either end of the pancreatic limbs and at the center of the organ at the time of necropsy and fixed in 4% buffered paraformaldehyde. The tissues were paraffin embedded and 5 μm sections were stained with hematoxylin and eosin using routine methods for histopathologic evaluation. Morphometric analysis was performed using a Nikon 80i Eclipse microscope and StereoInvestigator software, version 10.02 (MBF bioscience, Williston, VT) with tissue area estimated using the area fraction fractionator method, as previously described (31). The frequencies of islets within the quantified area were counted to determine a ratio of islets per mm² of pancreatic tissue.
Immunofluorescent detection and quantification of pro-insulin in pancreatic tissue. Paraffin embedded 5 μm sections of pancreas were deparaffinized and rehydrated in serial ethanol washes followed by antigen retrieval by boiling in target retrieval buffer (Dako, Carpinteria, CA) for 30 minutes. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ and blocking performed in 0.15 mM glycine and 10% FBS, 1%BSA in PBS. Tissue sections were incubated with anti-pro-insulin monoclonal antibody, clone K36AC10 (Abcam, Cambridge, MA), at a 1:500 dilution overnight at 4°C. After 3 washes in tween-TBS, tissue sections were incubated with HRP conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 60 minutes at room temperature, washed 3 times in tween-TBS, and the signal amplified with the tyramide signal amplification kit (Life Technologies, Grand Island, NY) employing AlexaFluor 488-labeled tyramide, as directed by the manufacturer. Purified murine IgG1 was used as an isotype control. The frequency of pro-insulin detection by immunofluorescence was quantified by image analysis using Nikon NIS Elements software. Background fluorescence was subtracted based on mouse IgG1 isotype controls and intensity thresholds set for detection of green (pro-insulin) and blue (Hoescht nuclear stain). Frequency of pro-insulin immunoreactivity was normalized to cell number based on frequency of Hoescht staining and expressed as a percent ratio.

Data Analysis. Sample size was defined by power analysis based on difference in calculated area under the curve for oral glucose tolerance test performed on non-diabetic and HFHC/STZ guinea pigs receiving the diabetogenic protocol described above with alpha set at 0.05. With a sample size of three animals per group and equivalent subjects to controls, there is an 80% probability of detecting a true mean difference of at least 5539 glucose*minutes (power of 0.8). No animals were excluded from the statistical analyses. Allocation to experimental groups was randomly determined by animal care staff at the time of animal arrival and cage assignment. All quantitative data is expressed as mean ± standard deviation (SD) for descriptive statistical representation or, where data is represented as individual points, the line represents the group mean. Statistical analyses and graphic expression of data were performed using GraphPad.
Prism, version 6 and SAS version 9.3. Comparison of data between treatment groups was performed using the unpaired t test, or two-way ANOVA followed by post-test for pair-wise comparison of means, with significance set at $P \leq 0.05$.

**Competing interests**

No competing interests declared.

**Author Contributions**

BKP, DFA, and RJB conceived and designed the experiments; BKP, DFA, MR, JED and BP performed the experiments; BKP, DFA, MR, JED and BP analyzed the data; BKP and RJB wrote and revised the manuscript; BKP and RJB provided project oversight.

**Funding**

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Table 1. Biochemical lipid parameters from guinea pigs subjected to diabetogenic treatments. (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Norma I Diet</th>
<th>HFHC-Fed</th>
<th>HFHC/STZ</th>
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<tbody>
<tr>
<td>Serum FFA (µM)</td>
<td>132.3 ± 24.9</td>
<td>146.6 ± 47.5</td>
<td>172.6 ± 47.4*</td>
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<tr>
<td>n=14</td>
<td>n=14</td>
<td>n=14</td>
<td></td>
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<tr>
<td>Serum Triglycerides</td>
<td>34.7 ± 9.4</td>
<td>93.1 ± 63.4*</td>
<td>101 ± 47**</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>n=14</td>
<td>n=14</td>
<td>n=14</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>23.5 ± 5.2</td>
<td>43.7 ± 14.8*</td>
<td>38.9 ± 13.4*</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>LDL/VLDL Cholesterol (mg/dl)</td>
<td>18.8 ± 4.9</td>
<td>37.9 ± 15.4**</td>
<td>32.2 ± 13.3**</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>4.7 ± 1.5</td>
<td>5.8 ± 0.7</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Liver Triglycerides</td>
<td>0.66 ± 0.14</td>
<td>1.41 ± 0.32**</td>
<td>3.66 ± 0.92**</td>
</tr>
<tr>
<td>(mg/g protein)</td>
<td>n=2</td>
<td>n=4</td>
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Serum parameters were collected after a total of 11-weeks of high fat, high carbohydrate diet (HFHC-fed) or in HFHC-fed guinea pigs 21 days after streptozotocin treatment (HFHC/STZ). Liver triglycerides were measured in diabetic guinea pigs after a 120 day course of treatment with metformin and glipizide (Tx) or in mock-treated guinea pigs with uncontrolled diabetes (UnTx).

*P<0.05, **P<0.01, ***P<0.001
Table 2. Survival and rate of hyperglycemia achieved with various STZ treatments.

AE = anomer-equilibrated, $\alpha_2$ = yohimbine, SQ = subcutaneous, IP = intraperitoneal

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Sample Size</th>
<th>% Survival</th>
<th>% Hyperglycemiac</th>
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<tbody>
<tr>
<td>300</td>
<td>SQ</td>
<td>5</td>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>300</td>
<td>IP</td>
<td>4</td>
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<td>0%</td>
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<td>50%</td>
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<td>200 AE</td>
<td>SQ</td>
<td>5</td>
<td>100%</td>
<td>20%</td>
</tr>
<tr>
<td>200 AE $\alpha_2$</td>
<td>SQ</td>
<td>5</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, compared to groups with 100% survival
Figure 1. Guinea pigs fed HFHC diet develop impaired glucose tolerance and suppressed response to insulin. Fasted or non-fasted guinea pigs were challenged with an oral glucose bolus or exogenous insulin, respectively, and blood glucose measured over time. (A) Response to an oral glucose challenge in guinea pigs that consumed HFHC diet for 4 or 8 weeks (n=20), compared to normal diet controls (n=20). Fasting hyperglycemia is absent but delayed glucose clearance is evident 60 minutes after glucose challenge in HFHC-fed guinea pigs. *P<0.05, two-way ANOVA (B) Response of HFHC-fed guinea pigs to an insulin tolerance test (n=5) after 4 or 8 weeks of HFHC-feeding or in HFHC/STZ guinea pigs compared to normal diet controls (n=5). Impaired tissue sensitivity to insulin is apparent in HFHC-fed and HFHC/STZ guinea pigs, based on failure to reduce blood glucose over time compared to normal diet controls. **P<0.01, based on mean area under the curve, t-test
Figure 2. Optimized streptozotocin (STZ) treatment leads to specific cytotoxicity of insulin-producing cells. Images are representative of guinea pigs developing hyperglycemia after
treatment with an optimized subcutaneous dose of anomer-equilibrated STZ at 200 mg/kg administered after an intramuscular injection of yohimbine. (A) Pancreatic islets from a non-diabetic, normal diet control guinea pig (arrow) demonstrating histologic morphology in the absence of diabetogenic treatment. (B) Morphological changes 48 hours after STZ treatment in pancreas of a guinea pig with confirmed hyperglycemia that was fed a normal diet. Cell death has occurred in the majority of islet cells (arrowheads), while a minority of the cells remain viable. (C) Immunofluorescent detection of proinsulin (green) in pancreatic islets in a normal diet control guinea pig indicates that the majority of islet cells are insulin-producing β cells. (blue, Hoechst nuclear counterstain) (D, E) Immunofluorescent detection of proinsulin in a guinea pig 48 hours after receiving an optimized dose of STZ. Disruption of cellular and nuclear morphology is uniform across all islets and is specific to insulin expressing β cells (arrows and inset). scale bar = 100 μm
Figure 3. Development of stable STZ-induced diabetes requires coexisting diet-induced impaired glucose tolerance. (A) Diagrammatic representation of optimized STZ treatment to induce hyperglycemia in guinea pigs fed either normal or HFHC diets. STZ powder is dissolved in
citrate buffer at 100 mg/ml, then incubated for two hours to allow for \( \alpha/\beta \) anomer equilibration. Twenty minutes prior to STZ treatment, guinea pigs are administered a 0.5 mg/kg dose of the \( \alpha_2 \) agonist, yohimbine, by intramuscular (IM) route. Guinea pigs are then given a single 200 mg/kg subcutaneous (SQ) injection of anomer-equilibrated STZ. Diabetic hyperglycemia is determined by oral glucose tolerance test (OGTT) at day 7, 14 and 21, based on a two-hour blood glucose \( \geq 200 \text{ mg/dl} \). (B) Blood glucose levels were measured daily at random in non-fasted, STZ-treated guinea pigs fed a normal diet (n=5). Hyperglycemia, observable 24 hours after STZ treatment, has a trend of steady decline over the course of 14 days. *P<0.05 compared to day 1 post-STZ, one-way ANOVA (C) Glucose tolerance was evaluated in STZ-treated guinea pigs (n=5) fed a normal diet by standardized oral glucose challenge on day 7, 14 and 21 after STZ treatment, and compared to normal diet controls (n=5). Fasting hyperglycemia and diabetic glucose intolerance is evident 7 days after STZ treatment, but glucose intolerance diminishes over time, returning to non-diabetic tolerance after 21 days. **P<0.01, ***P<0.001, compared to mean glucose of normal diet controls at 0, 60 and 120 minutes, or day 21 challenge of STZ guinea pigs at 150 minutes, two-way ANOVA. (D) HFHC-fed guinea pigs were treated with STZ after 8 weeks of consuming HFHC diet. Response to an oral glucose challenge is documented over 7, 14 and 21 days after STZ treatment that was preceded by 8 weeks of HFHC feeding (n=5 or 10). STZ treatment results in stable fasting hyperglycemia and diabetic glucose intolerance persisting to 21 days after treatment when guinea pigs are challenged in the presence of HFHC diet-induced impaired glucose tolerance. *P<0.05, ***P<0.001, compared to mean glucose of normal diet controls at that measurement time point, one-way ANOVA based on mean area under the curve.
Figure 4. Compensating hyperinsulinemia maintains euglycemia in HFHC-fed guinea pigs, but is lost with STZ treatment. (A) Insulin was detected by direct ELISA in serum from fasted guinea pigs either fed a normal diet (n=16), HFHC diet (n=18), or HFHC diet and treated with STZ.
Fasting hyperinsulinemia is present in guinea pigs after 8 weeks of consuming the HFHC diet. Treatment of HFHC-fed guinea pigs with STZ leads to impairment of the compensatory insulin response; however, insulin production is retained in HFHC/STZ guinea pigs at a level comparable to normal diet controls. ***P<0.001, one-way ANOVA (B) Fasting blood glucose levels were measured from fasting samples taken simultaneously for insulin quantification by direct ELISA. Fasting hyperglycemia is absent in the presence of compensating hyperinsulinemia in HFHC-fed guinea pigs. Fasting hyperglycemia is present in HFHC/STZ guinea pigs after impairment of the compensatory insulin response. ***P<0.001, one-way ANOVA (C) Glucose stimulated insulin secretion performed in guinea pigs after 8 weeks of HFHC feeding then repeated 21 days after receiving STZ. Residual function is retained in β cells after STZ treatment (n=3). *P<0.05, paired one-way ANOVA
Figure 5. Morphological alterations in pancreatic islets demonstrate a compensatory response to HFHC diet and STZ-induced β cell loss. (A) Pancreas tissue area was quantified using morphometry software and frequency of islets expressed per cm² of total pancreatic tissue (n=5 per group). HFHC-fed guinea pigs develop islet hyperplasia after 8 weeks of consuming HFHC diet, consistent with insulin resistance and a compensatory response. *P<0.05, t-test (B) Histological morphology of pancreas from HFHC-fed guinea pigs. Islets are both enlarged and more frequent compared to normal diet controls (arrows). (C) Pancreas from normal diet control guinea pigs demonstrates the typical frequency and size of pancreatic islets in the absence of diabetogenic treatment (arrows). scale bar = 100μm (D) Representative islet morphology from
guinea pigs fed HFHC diet for extended period of 6 months. Degenerative changes are present in enlarged islets, indicated by deposition of fibrous connective tissue (arrow). scale bar = 100 μm (E) Islet morphology 3 weeks after STZ treatment in HFHC/STZ guinea pigs. Islets are reduced in size after treatment with STZ (arrow), scale bar = 200 μm. (F) Immunofluorescent detection of proinsulin (green) in an enlarged islet from HFHC-fed guinea pigs after 8 weeks of consuming the diet. Enlarged islets contain a high frequency of insulin-producing β cells. Blue is Hoechst nuclear counterstain, scale bar = 100 μm (G) Immunofluorescent detection of proinsulin in an islet from HFHC/STZ guinea pigs 3 weeks after STZ treatment. There is an overall reduction in the frequency of insulin-producing β cells 3 weeks after STZ treatment. scale bar = 100 μm
Figure 6. HFHC/STZ guinea pigs develop insulin independent diabetes that is responsive to oral antihyperglycemic therapy. (A) Diabetic HFHC/STZ guinea pigs received either a combination of metformin and glipizide therapy (HFHC/STZ Tx) (n=4), initiated 21 days after STZ treatment, or remained with uncontrolled diabetes (HFHC/STZ UnTx) (n=4). Responses to an oral glucose challenge were compared with normal diet controls (n=2). Metformin and glipizide treatment in HFHC/STZ guinea pigs reverses diabetic glucose intolerance and fasting hyperglycemia. ***P<0.001, one-way ANOVA (B) Survival was tracked for 120 days in HFHC/STZ guinea pigs that were either treated with metformin and glipizide or remained with uncontrolled diabetes, compared to normal diet control guinea pigs. Antihyperglycemic therapy significantly improves survival in guinea pigs with HFHC/STZ diabetes. *P<0.05 (C) Immunofluorescent detection and quantification (inset bar graph) of pro-insulin after 120 days of treatment in HFHC/STZ guinea pigs treated with metformin and glipizide combination therapy. Reduced β cell frequency remains present after 120 days in the presence of combination therapy. (D) Immunofluorescent detection of pro-insulin in HFHC/STZ guinea pigs with uncontrolled diabetes. Insulin production appears similar to those receiving combination therapy. (E) Immunofluorescent
detection of pro-insulin in a normal diet control guinea pig from the survival group, demonstrating a greater frequency of β cells in the absence of diabetogenic treatment. (F) Quantitation of pro-insulin positive cells expressed as percent of tissue area from six representative fields per treatment group. HFHC/STZ treatment reduces total β cell mass, which increases in guinea pigs treated with metformin and glipizide. *P<0.05, ***P<0.001, compared to untreated HFHC/STZ guinea pigs, one-way ANOVA.
References


Figure S1. Graphical representation of weight change in guinea pigs over the 11-week course of HFHC or HFHC/STZ diabetogenic treatments, compared to normal diet controls.
Figure S2. Kaplan-Meier survival curve for guinea pigs receiving the range of STZ doses, as tabulated in Table 2.
Figure S3. Acute toxic effects of STZ administration in guinea pigs fed a normal diet. All images are taken from guinea pigs receiving a 300 mg/kg dose of STZ by intraperitoneal route. (A) Multiple foci of acute necrosis in the mucosal villi of the small intestine (arrows) (B) Renal tubular necrosis and cast formation in the cortex of the kidney (arrows). (C) Fibrosis and hepatocellular degeneration at the peritoneal surface of the liver (arrow).
Supplemental Table 1. Diet and water consumption over the 11 week course of diabetogenic treatment.

<table>
<thead>
<tr>
<th></th>
<th>Normal Diet</th>
<th>HFHC-Fed</th>
<th>HFHC/STZ</th>
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</thead>
<tbody>
<tr>
<td>Water Consumption (ml/day)</td>
<td>65.3 ± 8.6</td>
<td>161 ± 19.6***</td>
<td>NM</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
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</tr>
<tr>
<td>Diet Consumption (kcal/100g BW/day)</td>
<td>40 ± 3.7</td>
<td>44.1 ± 5.2</td>
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