Targeting pyruvate carboxylase reduces gluconeogenesis and adiposity and improves insulin resistance

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Short running title: Tissue specific targeting of pyruvate carboxylase

Word Count: 4465

Number of tables and figures: 8
ABSTRACT

We measured the mRNA and protein expression of the key gluconeogenic enzymes in human liver biopsies, and found that only hepatic pyruvate carboxylase protein levels related strongly with glycemia. We assessed the role of pyruvate carboxylase in regulating glucose and lipid metabolism in rats through a loss-of-function approach using a specific antisense oligonucleotide (ASO) to decrease expression predominantly in liver and adipose tissue. Pyruvate carboxylase ASO reduced plasma glucose concentrations and the rate of endogenous glucose production in vivo. Interestingly, pyruvate carboxylase ASO also reduced adiposity, plasma lipid concentrations, and hepatic steatosis in high-fat-fed (HFF) rats and improved hepatic insulin sensitivity. Pyruvate carboxylase ASO had similar effects in ZDF rats. Pyruvate carboxylase ASO did not alter de novo fatty acid synthesis, lipolysis, or hepatocyte fatty acid oxidation. In contrast, the lipid phenotype was attributed to a decrease in hepatic and adipose glycerol synthesis, which is important for fatty acid esterification when dietary fat is in excess. Tissue specific inhibition of pyruvate carboxylase is a potential therapeutic approach for nonalcoholic fatty liver disease, hepatic insulin resistance and type 2 diabetes.
INTRODUCTION

A key step in the pathogenesis of type 2 diabetes is the development of increased hepatic gluconeogenesis and fasting hyperglycemia (1-3). Hepatic gluconeogenesis is enzymatically regulated primarily by four gluconeogenic enzymes: phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBP1), glucose-6-phosphatase (G6PC), and pyruvate carboxylase (4-7). Increased hepatic gluconeogenesis is often ascribed to transcriptional regulation of two key gluconeogenic enzymes, PEPCK and G6PC, via an intricate web of transcriptional factors and cofactors (8-12). Yet, despite the high degree of transcription regulation for these enzymes, the control they exert over gluconeogenic flux is relatively weak (13-16). We recently reported that hepatic expression of PEPCK and G6PC mRNA was not related to fasting hyperglycemia in two rodent models of type 2 diabetes and patients with type 2 diabetes (17). Thus, we hypothesized that other mechanisms must account for increased hepatic gluconeogenesis and fasting hyperglycemia in type 2 diabetes.

Pyruvate carboxylase catalyzes the first committed step for gluconeogenesis and is well poised to regulate hepatic glucose production. Pyruvate carboxylase is allosterically activated by acetyl-CoA (18). However, increased expression of pyruvate carboxylase has been reported in both rodent models of type 1 diabetes (19,20) and in obese Zucker rats (21). Here we performed a comprehensive assessment of hepatic gluconeogenic enzyme expression and discovered a strong association between pyruvate carboxylase protein expression and glycemia in humans. We then quantified the impact of pyruvate carboxylase on both glucose and lipid metabolism in vivo in multiple rodent models by using a specific antisense oligonucleotide (ASO) to decrease pyruvate carboxylase expression selectively in liver and adipose tissue. Though chemical inhibitors of pyruvate carboxylase can acutely reduce glucose production (22), these compounds lack tissue specificity. ASOs primarily decrease expression in liver and adipose, but not other key tissues, such as pancreas, muscle
or neurons (23,24). Thus, this approach permits us to chronically decrease pyruvate carboxylase expression in select tissues of adult animals, without altering expression in tissues where this enzyme supports anaplerotic flux (e.g. β-cells, astrocytes) and also avoids any potentially confounding compensatory effects that may occur in germ line gene knockout rodent studies. We assessed the effects of pyruvate carboxylase ASO in several rodent models, quantifying changes in glucose metabolism, lipid metabolism, and insulin sensitivity \textit{in vivo}.
RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats (160-180 g), ZDF rats (7 weeks age), and C57/BL6 mice (7 weeks age) were received from Charles River Laboratories (Wilmington, MA) and given at least 3 days to acclimate. Rats and mice were housed on a 12:12 hr light/dark cycle and received food and water ad libitum. Chow consisted of regular rodent chow (60 % carbohydrate, 10 % fat, 30 % protein calories) and a high-fat diet (Dyets 112245: 26 % carbohydrate, 59 % fat, 15 % protein calories, Dyets, Inc., Bethlehem, PA). ZDF rats were fed Purina Lab Diet 5008 (56.4 % carbohydrate, 16.7 % fat, 26.8 % protein calories). Body weight was monitored twice weekly. ASOs were injected intraperitoneally at a dose of 75 mg / kg per week for at least 4 weeks. For high-fat fed rats, the ASO injection was started on the same day as the high-fat diet. For fasting experiments, rats were overnight fasted (~14 hours). Rats underwent the placement of jugular venous (for blood sampling) and carotid artery (for infusion) catheters ~10 days before the terminal studies. They recovered their pre-surgical weights by 5–7 days after the operation. All procedures were approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine.

Study population

All patients who were enrolled in the Bariatric Surgery Program of the Geisinger Center for Nutrition and Weight Management between October 2004 and October 2010 were offered the opportunity to participate in this study and some others (25). Over 90 % of patients consented to participate. Patients underwent a preoperative assessment and preparation program of monthly visits, during which time a comprehensive set of clinical and laboratory measures were obtained. Although patients lost an average of ~9 % body weight over the year before surgery, they remained relatively weight stable during the preoperative period between blood
sampling and liver biopsy, with an average percent change in body weight of 0.41 %. The protocol was approved by the Institutional Review Boards of the Geisinger Clinic and Yale University, and all participants provided written informed consent.

Liver biopsies

During the bariatric surgery a wedge biopsy (250-300 mg) was obtained from the right lobe of the liver 10 cm to the left of the falciform ligament and flash frozen in liquid nitrogen for subsequent analysis.

Selection of ASOs

Rats and mice pyruvate carboxylase and control ASOs were designed and produced as previously described (26). The sequence: 5-GCCAGACTTCATGGTAGCCG-3 (ISIS-330749) was selected for both rats and mice pyruvate carboxylase and the sequence: 5-CCTTCCCTGAAGGTTCCTCC-3 (ISIS-141923) was selected as the control ASO.

RT-PCR

RT-PCR was performed as previously described (26,27). Primer sequences are described in Supplementary Table 3.

Western blotting

For gluconeogenic enzymes, liver proteins were compartmentalized into three fractions, namely a mitochondria containing, cytoplasm, and microsomal fraction as previously reported (27-29). G6PC was detected in the microsomal fraction, cytosolic PEPCK (C-PEPCK) and FBP1 were detected in the cytoplasmic fraction, and pyruvate carboxylase and mitochondrial PEPCK (M-PEPCK) were detected in the mitochondria containing fraction.
The sheep polyclonal C-PEPCK antibody was a kind gift from Daryl Granner (Vanderbilt University Medical Center). G6PC, pyruvate carboxylase, and M-PEPCK antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FBP1 was purchased from Abcam Inc. (Cambridge, MA).

For immunoprecipitation, mitochondria fraction was extracted as same as above, but 50 mM N-ethylmaleimide, 250 mM nicotinamide, and 50 mM sodium fluoride were added in the buffer. 1mg mitochondria protein was mixed with 4 µg pyruvate carboxylase antibody and protein A/G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and homogenization buffer was added up to 500 µl, and incubated overnight. After overnight incubation samples were washed with homogenization buffer containing 1 %NP-40 for three times, then 40 µl sample buffer was added and boiled for 5 minutes. Ubiquitin was detected using ubiquitin antibody (Covance, Inc., Dedham, MA) and then stripped and reprobed with pyruvate carboxylase antibody.

Whole cell lysates preparation, PKC translocation assay, and Western blotting for all the proteins were done as previously described (26,27,30).

Biochemical analysis and calculations

Plasma C-peptide was measured by radioimmunoassay kit (Millipore, Billerica, MA). Plasma lactate concentration was measured on Roche Cobas Mira Plus (Analytical Instruments, LLC, Suite 50 Golden Valley, MN) using the lactate reagent test kit (Pointe Scientific, Inc., Canton, MI). The others were measured as previously described (26,27).
Mixed meal loading test

Chronically catheterized rats treated with ASO’s were fasted overnight, and given a mixed meal (15 kcal/kg-BW Ensure Plus Ready-to-Drink Homemade Vanilla, Abbott Nutrition, Columbus, OH, 57 % carbohydrate, 28 % fat, 15 % protein calories) through a gastric catheter. Blood was taken from the venous line at the indicated time in the results.

Pyruvate tolerance test

Pyruvate tolerance test was performed as previously described (30).

Hepatic lipid metabolites assay

Hepatic triglyceride and diacylglycerol contents were determined as previously described (26,27).

Mice body composition, metabolic parameters and physical activity

Body composition was assessed by $^1$H magnetic resonance spectroscopy using a Bruker Minispec analyzer mq10 (Bruker Optics Inc., Billerica, MA). Metabolic parameters, energy expenditure, and food intake were measured using comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments, Columbus, OH).

Hyperinsulinemic-euglycemic clamp studies

Hyperinsulinemic-euglycemic clamp studies were performed as previously described (26,31). Insulin was infused at 4 mU / (kg-min) and at 12 mU / (kg-min) for high-fat fed rats and ZDF rats, respectively.
Lipid oxidation assay with primary hepatocytes

Methods were modified from the previous methods (32). Briefly, primary hepatocytes were isolated by the Yale Liver Center from high-fat fed rats treated with either control or pyruvate carboxylase ASO for 4 weeks. After recovery, cells were incubated with [1-14C]oleate (GE Healthcare Biosciences, Piscataway, NJ) or [1-14C]palmitate (PerkinElmer, Inc., San Jose, CA) in sealed flasks containing a center well supplied with a clean filter paper. After 1h, incubations were quenched with 30% perchloric acid and 2 M NaOH to collect [14CO2] which was quantified by scintillation counting. Perchloric acid-soluble 14C-radioactivity (representing ketone bodies, acyl-carnitine, and Krebs cycle intermediates) was also quantified. Counts were normalized to protein content.

\[ \text{In vivo de novo lipogenesis assay (Assessment of } ^2\text{H labeling in triglyceride-palmitate)} \]

This assay was performed as described previously (26).

\[ \text{In vivo whole body lipolysis assay} \]

This assay was performed as described previously (26).

Glyceroneogenesis assay

This assay was done as previously described (33). The ‘% total newly made triglyceride-glycerol’ was calculated using the equation (33):

\[ \% \text{ total newly made triglyceride-glycerol} = \left( \frac{^2\text{H-labeling of triglyceride-glycerol}}{^2\text{H-labeling of plasma} \times n} \right) \times 100, \]

where \(^2\text{H-labeling of triglyceride-glycerol}\) is the M1 isotopomer, the \(^2\text{H-labeling of plasma}\) is the average labeling in a given rat, and \(n\) is the number of exchangeable hydrogens. Previous studies have experimentally measured this value as 4.25 in vivo (34).
Statistical Analysis

Linear regression analysis of the data was performed using Graph-Pad Prism 5.0. Data were compared using Student’s unpaired T-test or ANOVA with the Tukey’s post-hoc test between two groups or more than two groups, respectively. For the lipid oxidation assay, the average of control ASO group was set as one every time and the assay was repeated for five times and compared using paired T-test; each replicate was a separate animal. All data are expressed as mean ± SE unless otherwise indicated. P values less than 0.05 were considered significant.
RESULTS

Pyruvate carboxylase protein was increased parallel to glycemic level in humans

We assessed both mRNA and protein expression of four gluconeogenic enzymes in human liver biopsy samples obtained from patients undergoing bariatric surgery (n = 20, Table 1) in relation to measures of glycemia (fasting plasma glucose concentration and hemoglobin A\textsubscript{1C}, or HbA\textsubscript{1C}). While none of these patients in this cohort had a prior diagnosis of type 2 diabetes, there was still a range of fasting plasma glucose concentrations and HbA\textsubscript{1C}. The protein expression of the other gluconeogenic enzymes (mitochondrial and cytosolic PEPCK, FBP1, and G6PC) did not relate to fasting plasma glucose (data not shown) or HbA\textsubscript{1C} (Supplementary Fig. 2). Expression of pyruvate carboxylase mRNA expression also did not relate with measures of glycemia (Fig. 1A and B). In humans, there are three known isoforms of pyruvate carboxylase mRNA that differ in the first exon. However, expression of these isoforms also did not correlate with glycemia (Supplementary Fig. 1). In contrast, pyruvate carboxylase protein expression closely related to plasma glucose concentrations, accounting for 52% of the variation in HbA\textsubscript{1C} (Fig. 1C and D). Thus, of all the key gluconeogenic enzymes, hepatic pyruvate carboxylase expression best relates to glycemia in humans.

Pyruvate carboxylase ASO treatment was well-tolerated and decreased plasma glucose concentrations in regular chow fed rats

To determine the extent to which pyruvate carboxylase controls endogenous glucose production \textit{in vivo}, we treated regular chow and high-fat fed (HFF) male SD rats with pyruvate carboxylase ASO. Pyruvate carboxylase ASO treatment decreased hepatic and adipose pyruvate carboxylase mRNA expressions ~80-90% in both regular chow fed and HFF rats. Hepatic and adipose pyruvate carboxylase protein expressions were decreased ~70-90% (Fig. 2). Pyruvate carboxylase mRNA expression was also slightly decreased in
gastrocnemius and kidney cortex, but this did not reduce protein expression in these tissues (Supplementary Fig. 3). Interestingly, high-fat feeding *per se* increased hepatic pyruvate carboxylase protein expression relative to regular chow fed rats, without changes in mRNA expression, reminiscent of the observation in human liver. In the cohort of rats treated with a control ASO, we found that ubiquitination of pyruvate carboxylase was decreased in livers of HFF rats relative to regular chow fed rats (Supplementary Fig. 4). This may decrease protein degradation in ubiquitin-proteasome system and allow for accumulation of pyruvate carboxylase protein out of proportion with changes in mRNA expression.

Pyruvate carboxylase ASO treatment did not have any apparent toxicity; plasma transaminase and lactate concentrations were not different from control ASO treated chow-fed or HFF rats (Supplementary Table 1). Pyruvate carboxylase ASO decreased both fasting and *ad-lib* fed plasma glucose concentrations in regular chow fed rats (Fig. 3A and C). Plasma glucose excursion after a mixed-meal tolerance test was slightly, but significantly reduced without alterations in the plasma insulin secretion (Fig. 3D and F). To assess the effect of pyruvate carboxylase ASO on glucose production from pyruvate, we performed pyruvate tolerance test in both regular chow fed and high-fat fed rats treated with either a control ASO or pyruvate carboxylase ASO. We found that glucose excursion was significantly suppressed by pyruvate carboxylase ASO in regular chow fed condition (Fig. 3G). The decrease in glucose production was even more marked in high-fat fed rats. Consistent with this observation *in vivo*, the glucose production through pyruvate from the primary hepatocytes isolated from regular chow fed SD rats was significantly reduced by pyruvate carboxylase suppression by pyruvate carboxylase ASO transfection (Supplementary Fig. 5). Taken together, pyruvate carboxylase ASO treatment reduced hepatic gluconeogenic capacity with reduction in fasting and fed glucose concentration. This was well-tolerated without evidence for hepatotoxicity, lactic acidosis or suppression of insulin secretion.
Pyruvate carboxylase ASO reduced adiposity and hepatic steatosis in HFF rats

Interestingly, pyruvate carboxylase ASO also protected HFF rats from weight gain (Fig. 4A) and adiposity (Fig. 4B). Unlike some lipoatrophic and lipodystrophic models, the reduction in adiposity was associated with a decrease in hepatic triglyceride content (Fig. 4C), which was not observed in the regular chow fed condition (Supplementary Fig. 6). There was no change in skeletal muscle triglyceride content (Fig. 3D). Of note, pyruvate carboxylase ASO also reduced plasma fatty acids and cholesterol concentrations in regular chow fed SD rats and HFF SD rats (Supplementary Table 1). To further characterize the mechanism whereby pyruvate carboxylase ASO protected animals from adiposity, we treated HFF male C57BL/6 mice with pyruvate carboxylase ASO and assessed body composition by $^1$H MRS and whole body energy expenditure and food intake in metabolic cages. As in HFF rats, pyruvate carboxylase ASO decreased body weight gain, fat mass over time. The reduction in weight gain was attributable to a decrease in fat mass; lean body mass was preserved (Supplementary Fig. 7A and B). Whole body energy balance was assessed using metabolic cages at five weeks of treatment, prior to any significant difference in body weight allowing us to assess energy balance without the confounding effects introduced with divergent body weights. Reduction in adiposity and hepatic triglyceride content occurred without any measurable increases in whole body energy expenditure or reduction in food intake in the pyruvate carboxylase ASO treated mice (Supplementary Fig. 7C and D). Though these measurements were performed when body weight was matched, we also analyzed the relationship between whole body energy expenditure and body mass, which was similar between the groups by ANCOVA analysis (i.e. the slopes were not different between the groups [P = 0.83]), suggesting that pyruvate carboxylase ASO decreased adiposity without measurable changes in whole body energy balance. In addition, there was no difference in respiratory
exchange ratio between pyruvate carboxylase and control ASO groups (0.836±0.003 and 0.831 ±0.005, respectively). Thus, in HFF rodents, decreasing pyruvate carboxylase expression in liver and adipose tissue protects against hepatic steatosis and adiposity without affecting lean body mass or measurable changes in whole body energy expenditure, and food intake.

**Pyruvate carboxylase ASO improved hepatic insulin sensitivity in HFF rats**

Hepatic steatosis has been associated with insulin resistance, at least partly via diacylglycerol (DAG) -mediated activation of PKCε and impairment of insulin signaling in both rodents and humans (27,31,35). We performed hyperinsulinemic-euglycemic clamp studies in HFF rats to assess if pyruvate carboxylase ASO altered insulin sensitivity (Fig. 5). Pyruvate carboxylase ASO reduced fasting plasma glucose concentrations and basal rates of hepatic glucose production without increasing plasma insulin concentration, as expected (Fig. 5A-C). Insulin-stimulated peripheral glucose metabolism, which largely reflects insulin-stimulated skeletal muscle glucose uptake, was unchanged (Fig. 5D-F) without any changes in muscle triglyceride content (Fig 4D). In contrast, there was an improvement in hepatic insulin sensitivity in pyruvate carboxylase ASO treated rats, reflected by a ~50% reduction in hepatic glucose production and greater suppression of endogenous glucose production compared to the control ASO treated rats during the hyperinsulinemic-euglycemic clamp (Fig. 5G and H).

To determine the mechanisms underlying the improvement in hepatic insulin sensitivity, we assessed hepatic DAG content, PKCε activation, and Akt phosphorylation. Pyruvate carboxylase ASO treatment decreased hepatic DAG content in both cytosol and membrane fraction, decreased activation of PKCε, and increased insulin-mediated hepatic Akt Ser473 phosphorylation (Fig. 6), a key node of the insulin signaling pathway (35).
Pyruvate carboxylase ASO was also effective in Zucker Diabetic Fatty rats

We also tested the efficacy of pyruvate carboxylase ASO in ZDF rats, a widely used preclinical model of type 2 diabetes. In chow-fed ZDF rats, pyruvate carboxylase ASO lowered fasting plasma glucose concentration and rates of endogenous glucose production during both basal and hyperinsulinemic periods, and suppression of endogenous glucose production by insulin was greater in pyruvate carboxylase ASO treated rats than control ASO treated rats (Supplementary Fig. 8).

Reduction in glyceroneogenesis is the primary mechanism causing reduction in adiposity and hepatic steatosis

To further assess the mechanisms underlying the reduction in adiposity and hepatic steatosis, we performed a series of studies to quantify whole body lipolysis, lipid oxidation, de novo fatty acid synthesis, and glycerol synthesis in HFF rats (Supplementary Fig 9). Pyruvate carboxylase is involved in adipogenesis (36-39). But, the adipose expressions of key genes associated with adipogenesis [e.g. Peroxisome Proliferator Activated Receptor (PPAR) γ, adiponectin, Cluster of Differentiation (CD) 36 and adipocyte Protein (aP) 2] were not altered by pyruvate carboxylase ASO (Supplementary Table 2). Pyruvate carboxylase ASO did slightly decrease adipose mRNA expression of adipocyte triglyceride lipase (ATGL) and patatin-like phospholipase domain-containing 3 (PNPLA3) (Supplementary Table 2) and also decreased plasma non-esterified fatty acid concentration (Supplementary Table 1). However, there was no difference in the rates of whole body lipolysis as assessed by glycerol turnover (Fig. 7A). There was no difference in the rates of fatty acid oxidation measured using primary hepatocytes isolated from control ASO or pyruvate carboxylase ASO treated rats (Fig. 7B and C) or in the expression of genes regulating fatty acid oxidation in liver and adipose tissue (Supplementary Table 2). We quantified hepatic de novo lipogenesis by measuring $^2$H$_2$O
incorporation into triglyceride palmitate *in vivo*. Neither the percent of *de novo* fatty acid synthesis (Fig. 7D) nor the expression of lipogenic genes in liver (Supplementary Table 2) were altered. Adipose sterol regulatory element binding transcription factor 1c (SREBP1c) mRNA expression was decreased by pyruvate carboxylase ASO treatment, but the downstream genes such as acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) were not decreased (Supplementary Table 2). However, pyruvate carboxylase ASO decreased glycerol synthesis in liver and adipose tissue, as measured by the incorporation of $^{2}$H$_{2}$O into triglyceride-glycerol (that is, the glycerol backbone of a triglyceride molecule, Fig. 7E and F). This method quantifies total new glycerol synthesis, which includes both glyceroneogenesis and formation of glycerol from glucose. However, in high-fat fed conditions, the glyceroneogenesis is though important for the production of glycerol 3-phosphate (33) for the esterification and storage of fatty acids as triglyceride (Supplementary Fig. 9). Therefore, reduced glyceroneogenesis may be the primary mechanism accounting for the reduction in adiposity and hepatic steatosis in HFF rodents (Fig. 7G).
DISCUSSION

Patients with type 2 diabetes have increased gluconeogenesis (1,3,40,41). The molecular links between islet hormones and transcription of PEPCK and G6PC supported a view that increased gluconeogenesis was a consequence of increased transcription of these enzymes (9-12). However, we previously reported that the expression of PEPCK and G6PC mRNA did not relate to fasting hyperglycemia in either rodent models of type 2 diabetes or in humans with type 2 diabetes (17). We now extend this initial observation, demonstrating that increases in pyruvate carboxylase protein expression, but not mRNA expression, better relate to glycemia than expression of the other gluconeogenic enzymes. Using an ASO approach to reduce pyruvate carboxylase protein expression, we quantified the changes in glucose and lipid metabolism in vivo. We demonstrated that liver and adipose tissue targeting pyruvate carboxylase suppression is well-tolerated and effective in decreasing basal rates of endogenous glucose production and plasma glucose concentrations. In addition, we observed a reduction of adiposity and hepatic steatosis in HFF rats with improvements in hepatic insulin sensitivity in HFF rats and ZDF rats. The changes in lipid metabolism are likely a consequence of decreased glycerol synthesis in liver and adipose tissue and highlight the importance of pyruvate carboxylase in supporting glyceroneogenesis in vivo.

We first quantified the expression of the key rate-controlling gluconeogenic enzymes in liver biopsies obtained from human subjects undergoing elective surgery and related the expression of these enzymes to plasma glucose concentration and HbA1C. Only pyruvate carboxylase protein expression correlated to glycemia in this cohort. The relationship between pyruvate carboxylase protein and HbA1C was stronger than the relationship with fasting plasma glucose concentrations, raising the possibility that hepatic pyruvate carboxylase expression impacts both fasting and postprandial glucose concentrations and thus HbA1C better relates to pyruvate carboxylase expression than fasting plasma glucose.
concentrations. However, it is also possible that a single fasting plasma glucose concentration does not accurately reflect long-term trends of fasting glycemia.

The increase in pyruvate carboxylase protein expression occurred without changes in mRNA suggesting that other mechanisms affect protein abundance (e.g. post-transcriptional modification). We observed a similar disassociation between pyruvate carboxylase protein and mRNA abundance in fat-fed rodents compared to chow-fed rodents. We used this model to explore possible mechanisms accounting for the disassociation between pyruvate carboxylase mRNA and protein expression. In HFF rat livers, pyruvate carboxylase ubiquitination is decreased relative to chow fed rat liver. This suggests that pyruvate carboxylase degradation in ubiquitin-proteasome system is decreased which may result in increased pyruvate carboxylase protein accumulation. This also may provide a possible mechanism that accounts for increased hepatic pyruvate carboxylase flux that was recently reported in humans with NAFLD (42).

To quantify the role of pyruvate carboxylase in controlling glucose and lipid metabolism, we used a loss-of-function approach. While phenylalkanoic compounds can acutely reduce hepatic glucose production, and plasma glucose concentration (22), these compounds lack tissue specificity and can potentially impair glucose-stimulated insulin secretion (43). Moreover, there are no reports of chronic inhibition of pyruvate carboxylase. ASOs have inherent tissue specificity, effectively silencing gene expression in liver and white adipose tissue but negligibly in muscle, brown adipose tissue, pancreas, brain, or stomach (23,24). This tissue specificity mirrors the two promoters that control pyruvate carboxylase expression (44). The proximal promoter element (P1) is primarily active in liver, adipose, kidney and the mammary glands. In contrast, the distal promoter element (P2) maintains pyruvate carboxylase expression in many other tissues, including skeletal muscle, β-cells and astrocytes. These discrete promoters may allow specific tissues to use pyruvate carboxylase
as a common means to different ends; for glucose and lipid metabolism in P1 predominant tissues and anaplerosis in P2 predominant tissues. Thus, this approach permits us to assess the effects of decreasing pyruvate carboxylase expression in P1 selective tissues and also serve to vet tissue-targeted inhibition of pyruvate carboxylase expression/activity as a potential treatment for type 2 diabetes.

Decreasing pyruvate carboxylase expression decreased fasting plasma glucose concentrations in regular chow fed SD rats, HFF SD rats, and ZDF rats. This was associated with a decrease in basal rates of hepatic glucose production in HFF SD rats and ZDF rats. Patients with pyruvate carboxylase deficiency can develop severe lactic acidosis at an early age (45). In contrast, the tissue specific decrease in pyruvate carboxylase expression by ASO treatment did not result in any hepatotoxicity or lactic acidosis (though there was a small increase in plasma lactate concentrations in ZDF rats). Though ASOs do not decrease β-cell gene expression, we confirmed that insulin secretion was unaffected in mixed meal tolerance tests in SD rats. Thus, tissue specific inhibition of pyruvate carboxylase by ASO treatment effectively and safely lowers hepatic glucose production in multiple rodent models in chronic treatment.

Interestingly, pyruvate carboxylase inhibition also profoundly altered lipid metabolism. Pyruvate carboxylase ASO reduced adiposity and hepatic steatosis in HFF rodents. By comparison, liver specific deletion of PEPCK and inhibition of G6PC resulted in hepatic steatosis (46,47) and inhibition of FBP1 resulted in hyperlipidemia (48). Though adipose pyruvate carboxylase expression is reported to be induced during adipogenesis and increased by PPARγ agonists, there are no data on how inhibition of pyruvate carboxylase may alter lipid metabolism (36-39). Metabolic cage studies in mice treated with pyruvate carboxylase ASO did not reveal increases in whole body energy expenditure or reduction in
food intake, though it may be possible that liver or adipose tissue specific changes are not reflected in measures of whole body energy metabolism.

To better characterize the lipid phenotype, we performed a comprehensive set of studies assessing various components of lipid metabolism. There were no differences in lipolysis, fatty acid oxidation, or de novo fatty acid synthesis. However, we demonstrated that pyruvate carboxylase ASO treatment reduced adipose and hepatic glycerol synthesis in vivo, likely due to a decrease in glyceroneogenesis. While glyceroneogenesis plays a minor role in animals fed a high-carbohydrate diets (i.e. low-fat), its contribution to total glycerol 3-phosphate synthesis increases under fat-fed conditions, accounting for ~50-90% of glycerol 3-phosphate synthesis (33,49,50). This is consistent with our observation that the reduction in adiposity is primarily apparent in fat-fed rodents. Thus, when dietary lipid is in excess, the reduction in adipose and hepatic glycerol synthesis with pyruvate carboxylase ASO may impair lipid esterification and, consequently, lipid storage. In comparison, while PEPCK is important for adipose glyceroneogenesis (51), it does not appear to be as essential for hepatic glyceroneogenesis as mice lacking PEPCK can still develop hepatic steatosis (46). By comparison, decreasing pyruvate carboxylase expression by ASO treatment protected mice and rats from both adiposity and hepatic steatosis. The subsequent improvement in hepatic insulin sensitivity could be attributed to decreased DAG content and PKCε activation and improved insulin-stimulated Akt phosphorylation (31,35,52).

In conclusion, these are the first studies to demonstrate that increased hepatic pyruvate carboxylase protein expression is specifically and closely associated with plasma glycemia in humans, suggesting that hepatic pyruvate carboxylase is a key determinant of hepatic gluconeogenesis in humans. Pyruvate carboxylase ASO decreased liver and adipose expression of this enzyme, lowered plasma glucose concentrations and hepatic glucose production in vivo without any apparent adverse toxicity. In addition, pyruvate carboxylase
ASO decreased adiposity and hepatic steatosis in fat-fed rodents by decreasing adipose and hepatic glycerol synthesis. This, in turn, improved hepatic insulin signaling and hepatic insulin responsiveness. These studies suggest that pyruvate carboxylase is a key regulator of both gluconeogenesis and glyceroneogenesis. Through the latter pyruvate carboxylase may also regulate lipid metabolism. Taken together these data demonstrate that tissue specific inhibition of pyruvate carboxylase may be a potential strategy for treating many aspects of the metabolic syndrome and type 2 diabetes.
Acknowledgements

This project was supported by grants from the United States Public Health Service: R24 DK-085638, R01 DK-40936, R01 AG-23686, R01 DK-088231, R01 DK-34989, UL1 RR-0241395, P30 DK-034989, P30 DK-45735, Manpei Suzuki Diabetes Foundation Fellowship (N.K.), a Distinguished Clinical Scientist Award (K.F.P.) and a Mentor-Based Postdoctoral Fellowship Grant (GIS) from the American Diabetes Association and a VA Merit Grant 5I01BX000901 (V.T.S.).

V.P.M. and S.B.G are employees of ISIS and may own stock in the company. The other authors have no competing interests to declare.

N.K., S.A.B., D.F.V., S.K.M., J.L.C., F.G., I.F., B.G., M.J.J., A.L.B., M.K., B.K.P., M.P., K.F.P., G.W.C., G.I.S., and V.T.S. researched data and were involved in the analysis and interpretation of data. V.P.M. and S.B. designed, screened, and generated ASOs. C.D.S. and G.S.G. obtained liver biopsies from humans. N.K., G.I.S., and V.T.S. wrote the manuscript. V.T.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Preliminary data from this study were presented at 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25-29 June 2010 and 71th Scientific Sessions of the American Diabetes Association, San Diego, California, 24-28 June 2011.

The authors thank the volunteers for participating in this study, Daryl Granner (Vanderbilt University Medical Center) for his kind gift of C-PEPCK antibody, Yanna Kosover, Jianying Dong, Kathy Harry, Dongyan Zhang, Toru Yoshimura, Shoichi Kanda, Derek M. Erion, Andreas L. Birkenfeld, Rebecca L. Pongratz, Codruta Todeasa, Maria Batsu, and Aida Groszmann (all of the Yale University School of Medicine) for their excellent technical support.
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Table 1 Characteristics of participants

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<td>Fasting plasma glucose (mg / dL)</td>
<td>99.8 ± 4.0</td>
</tr>
<tr>
<td>Fasting plasma insulin (µU / mL)</td>
<td>23.3 ± 2.0</td>
</tr>
<tr>
<td>HbA₁C (%)</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>HOMA-IR [(mg / dl)(µU / mL)]</td>
<td>5.4 ± 0.6</td>
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<tr>
<td>Alanine aminotransferase (IU / L)</td>
<td>30.8 ± 3.2</td>
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<tr>
<td>Aspartate aminotransferase (IU / L)</td>
<td>26.6 ± 2.1</td>
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<tr>
<td>LDL cholesterol (mmol / L)</td>
<td>2.84 ± 0.24</td>
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<tr>
<td>HDL cholesterol (mmol / L)</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>Triglyceride (mmol / L)</td>
<td>1.73 ± 0.28</td>
</tr>
</tbody>
</table>

HOMA-IR, Homeostatic Model Assessment of Insulin Resistance Index
Figure legends

FIG. 1. Hepatic pyruvate carboxylase (PC) protein expression levels relate to glycemic levels in humans. A and B: Hepatic PC mRNA expression in human livers in comparison to fasting plasma glucose concentration and HbA1c, respectively. C and D: Hepatic PC protein expression in human livers in comparison to fasting plasma glucose concentration and HbA1c, respectively. Representative bands are shown in panel D. PC mRNA and protein are expressed as a relative increase to the lowest expression in the data set. n = 20.

FIG. 2. Pyruvate carboxylase (PC) ASO decreased PC expression in liver and epididymal adipose tissue. A and B: PC mRNA in liver and epididymal adipose tissue, respectively. C and D: PC protein in liver and epididymal adipose tissue, respectively. ** P<0.01 and *** P<0.001 compared with control ASO group in the same diet. # P<0.05 and ### P<0.001 compared with control ASO group in regular chow fed condition. n = 3-4 per group in regular chow fed condition. n = 9-10 per group in HFF condition. Representative bands are shown in panel C and D. All rats were sacrificed and tissues were taken at four weeks treatment.

FIG. 3. Pyruvate carboxylase (PC) ASO decreased plasma glucose concentration and did not decrease insulin secretion. A and B: Fasting plasma glucose and insulin concentration, respectively, in the regular chow fed rats. n = 7-10 per group. C: Ad-lib fed plasma glucose concentration in the regular chow fed rats. n = 5 per group. D-F: Mixed-meal tolerance test in the regular chow fed rats. D: plasma glucose, E: plasma
insulin, F: plasma C peptide. n = 7-10. G: Pyruvate tolerance test in the regular chow fed and high-fat fed rats. White circles and black circles are control ASO and PC ASO in regular chow fed condition, respectively (both n = 9). White triangles and black triangles are control ASO and PC ASO in high-fat fed condition, respectively (both n = 8). * P<0.05, ** P<0.01, *** P<0.001 between control and PC ASO in regular chow fed condition, ##P<0.01 and ###P<0.001 between control and PC ASO in high-fat fed condition. Experiments were done at 4-5 weeks treatment.

FIG. 4. Pyruvate carboxylase (PC) ASO reduced adiposity and hepatic steatosis in HFF rats. A: Body weight time course in regular chow fed (n = 10-11 per group) and HFF rats (n = 12 per group). B-D: Epididymal adipose tissue weight, hepatic triglyceride content, and muscular triglyceride content at four weeks treatment in HFF rats (n = 9-10 per group), respectively. * P<0.05 and ** P<0.01 compared with control ASO group in HFF condition.

FIG. 5. Pyruvate carboxylase (PC) ASO improves hepatic insulin sensitivity in HFF rats. A and B: Fasting plasma glucose and insulin concentration, respectively (n = 9 per group). C: Basal endogenous glucose production (n = 9 per group). D and E: Plasma glucose concentration and glucose infusion rate time course during hyperinsulinemic-euglycemic [4 mU / (kg-min)] clamp, respectively (n = 7-8 per group). F-H: Insulin-stimulated peripheral glucose metabolism, endogenous glucose production, and percent suppression of endogenous glucose production, respectively during clamp (n = 7-8 per group). * P<0.05, ** P<0.01, and *** P<0.001 compared with control ASO group. Experiments were done at 4-5 weeks treatment.
**FIG. 6.** Pyruvate carboxylase (PC) ASO decreased hepatic diacylglycerol (DAG) content and protein kinase C (PKC) ε activation and increased hepatic Akt phosphorylation and in HFF rats. A: Hepatic DAG content. n = 9-10 per group. * P<0.05 compared with control ASO group. B: PKCε activation. The average of control ASO group was set as 1. n = 5 per group. $$ P<0.001 $$ compared with control ASO group. C: Akt phosphorylation (Ser473). The average expression of control ASO group in the basal condition was set as 1. n = 5 per group. # P<0.05 and ### P<0.001 compared with control ASO group in basal condition. ** P<0.01 compared with control ASO group in clamp condition. All tissues were taken at 4-5 weeks treatment.

**FIG. 7.** Pyruvate carboxylase (PC) ASO reduced hepatic and adipose glycerol synthesis. A: Whole body lipolysis as assessed by glycerol turnover in HFF rats (n = 8-9 per group). B and C: Palmitate and oleate oxidation assay with primary hepatocytes isolated from HFF and ASO treated rats, respectively (n = 5 per group). D: *in vivo* hepatic *de novo* fatty acid synthesis in HFF rats (n = 9-10 per group). E and F: Hepatic and adipose glycerol synthesis in HFF rats, respectively (n = 7-8 per group). G: Summary of this study. * P<0.05 compared with control ASO group. All experiments were done at 4-5 weeks treatment.
**FIG. 1**

**A**

R=0.19  
P=0.40

**B**

R=0.04  
P=0.87

**C**

Low  
HbA1c (%)  5.0  →  High  
HbA1c (%)  7.8

PC  
VDAC

**D**

R=0.40  
P=0.08

R=0.72  
P=0.0003

Fasting plasma glucose  (mg/dl)

PC protein  (relative)
FIG. 2

A  Liver PC mRNA

B  Adipose PC mRNA

C  Liver PC protein

D  Adipose PC protein

**  **  ***

Control ASO  PC ASO  Control ASO  PC ASO

Control ASO  PC ASO  Control ASO  PC ASO

PC  VDAC

PC  VDAC

**  ***  #

Control ASO  PC ASO  Control ASO  PC ASO

Control ASO  PC ASO  Control ASO  PC ASO

Liver PC mRNA Adipose PC mRNA

PC  VDAC

PC  VDAC

**  ***  #

Control ASO  PC ASO  Control ASO  PC ASO

Control ASO  PC ASO  Control ASO  PC ASO

Liver PC protein Adipose PC protein

PC  VDAC

PC  VDAC

**  ***  #

Control ASO  PC ASO  Control ASO  PC ASO

Control ASO  PC ASO  Control ASO  PC ASO

PC  VDAC

Liver protein Adipose protein
FIG. 3

A. Fasting plasma glucose (mg/dl)

B. Fasting plasma insulin (µU/ml)

C. Ad-lib fed glucose (mg/dl)

D. Plasma glucose (mg/dl)

E. Plasma insulin (µU/ml)

F. Plasma C peptide (µM)

G. Δ Plasma glucose (mg/dl)

Mean ± SEM

Control ASO: 109.3 ± 1.1
PC ASO: 103.2 ± 1.3

Control ASO: 6.6 ± 1.0
PC ASO: 5.7 ± 0.4

Control ASO: 144.8 ± 0.5
PC ASO: 136.0 ± 1.1

Control ASO: 109.3 ± 1.1
PC ASO: 103.2 ± 1.3

control ASO: 6.6 ± 1.0
PC ASO: 5.7 ± 0.4

control ASO: 144.8 ± 0.5
PC ASO: 136.0 ± 1.1

Mean ± SEM

Fasting plasma glucose (mg/dl)

Fasting plasma insulin (µU/ml)

Ad-lib fed glucose (mg/dl)

Plasma glucose (mg/dl)

Plasma insulin (µU/ml)

Plasma C peptide (µM)

Δ Plasma glucose (mg/dl)
FIG. 4

A

Body weight (g)

Control ASO HFF
PC ASO HFF
Control ASO RC
PC ASO RC

Time (week)

B

Epididymal adipose tissue weight [g / (kg-BW)]

Control ASO
PC ASO

C

Hepatic triglyceride content [mg / (gm-tissue)]

Control ASO
PC ASO

D

Muscle triglyceride content [mg / (gm-tissue)]

Control ASO
PC ASO
FIG. 5

A. Fasting plasma glucose (mg/dl)

B. Fasting plasma insulin (µU/ml)

C. Basal endogeneous glucose production [mg/(kg-min)]

D. Plasma glucose [mg/dl] over time (min)

E. Glucose infusion rate [mg/(kg-min)] over time (min)

F. Insulin-stimulated peripheral glucose metabolism [mg/(kg-min)]

G. Endogeneous glucose production during clamp [mg/(kg-min)]

H. % Suppression of endogeneous glucose production

Legend:
- Control ASO
- PC ASO

Statistical significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
FIG. 6

A

Cytosolic DAG [nmol / (g-liver)]

<table>
<thead>
<tr>
<th></th>
<th>Control ASO</th>
<th>PC ASO</th>
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<td><img src="image" alt="Cytosol DAG" /></td>
<td><img src="image" alt="Cytosol DAG" /></td>
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</table>
| Membrane DAG [nmol / (g-liver)]

<table>
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<th>PC ASO</th>
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<tbody>
<tr>
<td>Control</td>
<td><img src="image" alt="Membrane DAG" /></td>
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</table>

B

Membrane PKCε

Na⁺-K⁺ ATPase

Cytosol PKCε

GAPDH

PKCε Memb/Cyto

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<td>Control</td>
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C

p-Akt (Ser 473)

Akt

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<th>Clamp</th>
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<td><img src="image" alt="p-Akt Ser 473 Basal PC" /></td>
<td><img src="image" alt="p-Akt Ser 473 Clamp PC" /></td>
</tr>
</tbody>
</table>
FIG. 7

A) Whole body lipolysis
B) Palmitate oxidation
C) Oleate oxidation
D) de novo fatty acid synthesis
E) % newly synthesized glycerol in liver triglyceride
F) % newly synthesized glycerol in adipose tissue

Hepatic insulin sensitivity
Fatty liver
DAG, PKCε activity
Hepatic insulin sensitivity

PC inhibition in liver and adipose tissue
↓ gluconeogenesis
↓ glyceroneogenesis
↓ Fasting plasma glucose
↓ Adiposity
↓ Fatty liver
↓ DAG, PKCε activity
↓ Hepatic insulin sensitivity
Online Supplemental Materials

Targeting pyruvate carboxylase reduces gluconeogenesis and adiposity and improves insulin resistance

Naoki Kumashiro, Sara A. Beddow, Daniel F. Vatner, Sachin K. Majumdar,
Jennifer L. Cantley, Fitsum Guebre-Egziabher, Ioana Fat, Blas Guigni,
Michael J. Jurczak, Mario Kahn, Bryce K. Perler, Michelle Puchowicz,
Vara Prasad Manchem, Sanjay Bhanot, Christopher D. Still, Glenn S. Gerhard,
Kitt F. Petersen, Gary W. Cline, Gerald I. Shulman, and Varma T. Samuel
**SUPPLEMENTARY DATA**

**Supplementary Tables**

**Supplementary Table 1. Plasma data.**

<table>
<thead>
<tr>
<th></th>
<th>Regular chow fed SD rats</th>
<th>High fat diet fed SD rats</th>
<th>ZDF rats</th>
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<tr>
<td></td>
<td>Control ASO</td>
<td>PC ASO</td>
<td>Control ASO</td>
</tr>
<tr>
<td>AST (IU / l)</td>
<td>59.5 ± 2.6</td>
<td>59.5 ± 2.3</td>
<td>71.8 ± 3.5</td>
</tr>
<tr>
<td>ALT (IU / l)</td>
<td>15.3 ± 1.4</td>
<td>15.7 ± 0.7</td>
<td>24.8 ± 1.9</td>
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<td>Lactate (mmol / l)</td>
<td>0.19 ± 0.02</td>
<td>0.23 ± 0.05</td>
<td>0.49 ± 0.02</td>
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<td>TC (mg / dl)</td>
<td>83.3 ± 4.4</td>
<td>54.9 ± 2.0***</td>
<td>49.1 ± 2.6</td>
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<tr>
<td>LDL-C (mg / dl)</td>
<td>11.4 ± 1.0</td>
<td>6.4 ± 0.4***</td>
<td>11.4 ± 0.7</td>
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<tr>
<td>HDL-C (mg / dl)</td>
<td>13.4 ± 0.7</td>
<td>8.8 ± 0.4***</td>
<td>15.3 ± 0.8</td>
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<tr>
<td>TG (mg / dl)</td>
<td>36.5 ± 4.6</td>
<td>31.6 ± 2.9</td>
<td>58.2 ± 2.0</td>
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<tr>
<td>NEFA (mmol / l)</td>
<td>0.92 ± 0.08</td>
<td>0.72 ± 0.04*</td>
<td>0.90 ± 0.09</td>
</tr>
</tbody>
</table>

* P<0.05, ** P<0.01, *** P<0.001 compared with matched control ASO group. PC; pyruvate carboxylase, AST; Aspartate aminotransferase, ALT; Alanine Aminotransferase, TC; Total cholesterol, TG; Triglyceride, NEFA; Non-esterified fatty acid. n = 8-10 per group. Plasma samples were taken at 4-5 weeks treatment.
## SUPPLEMENTARY DATA

**Supplementary Table 2.** Lipid metabolism related gene expressions in liver and epididymal adipose tissue in HFF rats.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
<th>Liver (Control ASO)</th>
<th>Liver (PC ASO)</th>
<th>Epididymal adipose tissue (Control ASO)</th>
<th>Epididymal adipose tissue (PC ASO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipogenic pathway</td>
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<tr>
<td></td>
<td>SREBP1c</td>
<td>1.00 ± 0.21</td>
<td>0.51 ± 0.14</td>
<td>1.00 ± 0.09</td>
<td>0.72 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>ACC1</td>
<td>1.00 ± 0.13</td>
<td>0.76 ± 0.08</td>
<td>1.00 ± 0.08</td>
<td>1.20 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>FAS</td>
<td>1.00 ± 0.25</td>
<td>0.76 ± 0.21</td>
<td>1.00 ± 0.16</td>
<td>1.16 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>mGPAT</td>
<td>1.00 ± 0.09</td>
<td>0.89 ± 0.10</td>
<td>1.00 ± 0.08</td>
<td>0.88 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>AGPAT1</td>
<td>1.00 ± 0.08</td>
<td>0.83 ± 0.06</td>
<td>1.00 ± 0.10</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>AGPAT2</td>
<td>1.00 ± 0.07</td>
<td>1.18 ± 0.12</td>
<td>1.00 ± 0.07</td>
<td>1.03 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>AGPAT5</td>
<td>1.00 ± 0.06</td>
<td>1.06 ± 0.08</td>
<td>1.00 ± 0.06</td>
<td>1.22 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>DGAT2</td>
<td>1.00 ± 0.08</td>
<td>0.94 ± 0.09</td>
<td>1.00 ± 0.09</td>
<td>1.14 ± 0.15</td>
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<tr>
<td>Lipid oxidation pathway</td>
<td>PPARα</td>
<td>1.00 ± 0.10</td>
<td>0.80 ± 0.09</td>
<td>1.00 ± 0.08</td>
<td>1.07 ± 0.08</td>
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<td></td>
<td>CPT1</td>
<td>1.00 ± 0.09</td>
<td>0.93 ± 0.13</td>
<td>1.00 ± 0.06</td>
<td>1.09 ± 0.06</td>
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<td>Lipolysis pathway</td>
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<td>HSL</td>
<td>1.00 ± 0.09</td>
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<td>0.88 ± 0.08</td>
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<td>ATGL</td>
<td>1.00 ± 0.06</td>
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<td>0.79 ± 0.07*</td>
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<td>PNPLA3</td>
<td>1.00 ± 0.12</td>
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<td>0.79 ± 0.07**</td>
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<td>Adipogenesis pathway</td>
<td>PPARγ</td>
<td>1.00 ± 0.07</td>
<td>0.83 ± 0.08</td>
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<td></td>
<td>CD36</td>
<td>1.00 ± 0.07</td>
<td>1.04 ± 0.11</td>
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<td></td>
<td>Adiponectin</td>
<td>1.00 ± 0.13</td>
<td>0.83 ± 0.11</td>
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<tr>
<td></td>
<td>aP2</td>
<td>1.00 ± 0.07</td>
<td>1.11 ± 0.12</td>
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<td>Cholesterol synthesis pathway</td>
<td>HMGCoA reductase</td>
<td>1.00 ± 0.13</td>
<td>0.89 ± 0.14</td>
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<td></td>
<td>SREBP2</td>
<td>1.00 ± 0.10</td>
<td>0.80 ± 0.09</td>
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<td>Cholesterol uptake pathway</td>
<td>LDL-R</td>
<td>1.00 ± 0.14</td>
<td>0.86 ± 0.15</td>
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<td>SR-B1</td>
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<td>Cholesterol efflux pathway</td>
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<td>LXRα</td>
<td>1.00 ± 0.09</td>
<td>0.97 ± 0.07</td>
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</table>

The average expression of control ASO group in each tissue was set as 1. The average expression of control ASO group was calculated in liver and epididymal adipose tissue individually. n = 9-10 per group. * P<0.05 and ** P<0.01 compared with the control ASO group of same tissue. All tissues were taken at 4-5 weeks treatment. See Supplementary Table 3 for abbreviation.
**Supplementary Table 3. Primer sequences for RT-PCR.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
<tbody>
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<td>CTTGGGTACGGTGGAACCTTT</td>
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<tr>
<td>Tbp (R)</td>
<td>GGACTTCTGCTCCCTACCT</td>
<td>CTCAGTGCAGGGAGGGAAC</td>
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<tr>
<td>PC (H)</td>
<td>GGCCCCTGGGACATTT</td>
<td>CTGCTTTGTTGGAGCACATCC</td>
</tr>
<tr>
<td>PC1 (H)</td>
<td>GACGGGAGGAGATATCG</td>
<td>CTGTTCAAGACTCACTAC</td>
</tr>
<tr>
<td>PC2 (H)</td>
<td>GAACCTCTGACAACTGC</td>
<td>TCTTATGCTTATACCTCAG</td>
</tr>
<tr>
<td>PC3 (H)</td>
<td>GGCGTTCAAGCTCTCTAATTGATTT</td>
<td>TCGGAAACTCAGCATCCTAG</td>
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<tr>
<td>Tbp (H)</td>
<td>CAATGGATATCTCTGTTGAA</td>
<td>AAACGCTGGAGCATATATCC</td>
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<td>PC (M)</td>
<td>GGATGCACTTCAAGGAG</td>
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<td>β-actin (M)</td>
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<td>FAS (R)</td>
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<tr>
<td>ATGL (R)</td>
<td>TCTCCGCTCACCAGCTCAGC</td>
<td>GCCTGCTGCTGAGGCGA</td>
</tr>
<tr>
<td>PNPLA3 (R)</td>
<td>TCGTCCGCTCGTCGTCGTCG</td>
<td>GCCTGCTGCTGAGGCGA</td>
</tr>
<tr>
<td>LDL-R (R)</td>
<td>TCTCCGCTCACCAGCTCAGC</td>
<td>GCCTGCTGCTGAGGCGA</td>
</tr>
<tr>
<td>SR-B1 (R)</td>
<td>TCTCCGCTCACCAGCTCAGC</td>
<td>GCCTGCTGCTGAGGCGA</td>
</tr>
<tr>
<td>LXRa (R)</td>
<td>GCCGGGATGCTGAGGCGA</td>
<td>GCCTGCTGCTGAGGCGA</td>
</tr>
<tr>
<td>CYP7A1 (R)</td>
<td>ACCTGCGGATGCTGAGGCGA</td>
<td>GCCTGCTGCTGAGGCGA</td>
</tr>
</tbody>
</table>

PC: pyruvate carboxylase, Tbp; TATA box binding protein, PC1, PC2, and PC3 are transcript variants of PC, SREBP; sterol regulatory element binding transcription factor, ACC; acetyl-CoA carboxylase, FAS; fatty acid synthase, mGPAT; mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase, AGPAT; acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase, AGPAT1, AGPAT2, and AGPAT5 are transcript variants of AGPAT, DGAT; acyl-CoA:diacylglycerol acyltransferase, PPAR; peroxisome proliferator activated receptor, CD36; Cluster of Differentiation 36, Ap2; adipocyte Protein 2, CPT; carnitine palmityl transferase, HSL; hormone sensitive lipase, ATGL; adipocyte triglyceride lipase, PNPLA3; Patatin–like phospholipase domain-containing 3, SR-B1; scavenger receptor class B, member 1, LXRa; Liver X receptor α, CYP7A1; cytochrome P450, family 7, subfamily a, polypeptide 1. (R); for rats, (H); for humans, (M); for mice
Supplementary Figure 1. Hepatic pyruvate carboxylase (PC) mRNA expression was not associated with plasma glucose concentration and HbA$_1$C in insulin resistant humans including non medicated type 2 diabetes. PC mRNA expressions were assessed by transcript variants individually. The lowest expression was set as 1. n = 20.
Supplementary Figure 2. Representative western blotting bands for hepatic gluconeogenic enzymes except for pyruvate carboxylase (A) and their correlation graphs with HbA$_1$C (B-E) in insulin resistant humans including non medicated type 2 diabetes. VDAC bands are the same as for pyruvate carboxylase in Figure 1D because the detection was done with the same protein samples on the same membrane. The lowest expression was set as 1. n = 20. M-PEPCK; mitochondrial PEPCK, C-PEPCK; cytosolic PEPCK, G6PC; glucose-6-phosphatase, FBP1; fructose 1,6 bisphosphatase, VDAC; voltage-dependent anion channel.
Supplementary Figure 3. Pyruvate carboxylase (PC) ASO treatment decreased PC mRNA but not PC protein expressions in muscle and kidney. (A and B) PC mRNA in muscle and kidney, respectively. (C and D) PC protein in muscle and kidney, respectively. n = 8-10 per group for muscle and n = 3-4 per group for kidney. * P<0.05, *** P<0.001 compared with control ASO group. All rats were sacrificed and tissues were taken at four weeks treatment.
Supplementary Figure 4. Ubiquitination of pyruvate carboxylase (PC) was decreased in control ASO treated HFF rats compared to control ASO treated regular chow fed rats. n = 8-10 per group. ** P<0.01 compared to regular chow fed condition. All rats were sacrificed and tissues were taken at four weeks treatment.
Supplementary Figure 5. Pyruvate carboxylase (PC) ASO transfection into primary hepatocytes isolated from regular chow fed male SD rats suppressed glucose production through pyruvate. (A) PC mRNA expression was significantly suppressed at 24 hours after PC ASO (100nM) transfection. Experiments were repeated four times. The expression level of control ASO was set as 1 every time and the data were compared with paired T-Test. (B-D) Glucose production during three hours incubation with either non substrate, 10mM pyruvate, or 10mM glutamate, respectively, at 24 hours after transfection. Experiments were repeated four times. *P<0.05 and **P<0.01 compared to control ASO.
Supplementary Figure 6. Pyruvate carboxylase (PC) ASO did not decrease hepatic triglyceride content in the regular chow fed rats. n = 8-10 per group. Tissues were taken after 4-5 weeks treatment and overnight fasting.
Supplementary Figure 7. Pyruvate carboxylase (PC) ASO decreased fat weight without alteration of energy expenditure and food intake. (A and B) Whole body fat weight and lean body weight respectively assessed by $^1$H MRS in HFF mice after nine weeks ASO treatment. $n = 7-8$ per group. (C and D) 24 hours energy expenditure and food intake, respectively, in HFF mice after five weeks treatment, when body weight was similar. $n = 7-8$ per group. * P<0.05 compared with control ASO group.
**SUPPLEMENTARY DATA**

**Supplementary Figure 8.** Fasting plasma glucose and basal endogenous glucose production were decreased, and hepatic insulin sensitivity was increased in pruvate carboxylase (PC) ASO treated Zucker diabetic fatty rats. (A and B) Fasting plasma glucose and insulin concentration, respectively. n = 8-9 per group. (C) Basal endogenous glucose production (n = 8-9 per group). (D and E) Plasma glucose concentration and glucose infusion rate time course during euglycemic-hyperinsulinemic [12mU / (kg-min)] clamp, respectively. n = 6-7 per group. (F) Plasma insulin concentration during clamp. n = 6-7 per group. (G-I) Endogenous glucose production and % suppression of endogenous glucose production, and insulin-stimulated peripheral glucose metabolism, respectively during clamp. n = 6-7 per group. (J and K) 2-deoxy-D-glucose uptake at the end of clamp in muscle and adipose tissue, respectively. n = 6-7 per group. * P<0.05, ** P<0.01 compared with control ASO group. Experiments were done at 4-5 weeks treatment.
Supplementary Figure 9. Disposition of pyruvate carboxylase (PC) in the glucose and lipid metabolism pathways in hepatocytes. FBP1; fructose 1,6-bisphosphatase, G3P; glycerol 3-phosphate, G6PC; glucose 6-phosphatase, GA3P; Glyceraldehyde 3 phosphate, PDH; pyruvate dehydrogenase, PEP; phosphoenolpyruvate, TG; triacylglycerol.