Targeting Pyruvate Carboxylase Reduces Gluconeogenesis and Adiposity and Improves Insulin Resistance

Naoki Kumashiro,1,2 Sara A. Beddow,3 Daniel F. Vatner,2 Sachin K. Majumdar,2 Jennifer L. Cantley,1,2 FITSUM GUEBRE-EGZIABHER,7 Ioana Fat,2 Blas Guigni,2 Michael J. Jurczak,2 Andreas L. Birkenfeld,2 Mario Kahn,2 Bryce K. Perler,2 Michelle A. Puchowicz,4 Vara Prasad Manchem,5 Sanjay Bhanot,5 Christopher D. Still,6 Glenn S. Gerhard,6 Kitt Falk Petersen,2 Gary W. Cline,2 Gerald I. Shulman,1,2,7 and Varman T. Samuel2,4

We measured the mRNA and protein expression of the key gluconeogenic enzymes in human liver biopsy specimens 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 rats and improved hepatic insulin sensitivity. Pyruvate carboxylase ASO had similar effects in Zucker Diabetic Fatty 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. Diabetes 62:2183–2194, 2013

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, through 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 in 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 rodent models of type 1 diabetes (19,20) and in obese Zucker Diabetic Fatty (ZDF) 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 effect of pyruvate carboxylase on 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. Although 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 in 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 germine 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 in vivo.

RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley (SD) rats (160–180 g), ZDF rats (7 weeks old), and C57/BL6 mice (7 weeks old) 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-h 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: 28% carbohydrate, 59% fat, 15% protein calories; Dyets, Inc., Bethlehem, PA). ZDF rats were fed Purina Laboratory Diet 5008 (56.4% carbohydrate, 16.7% fat, 26.8% protein calories). Body weight was monitored twice weekly.
Body composition was assessed by $^1$H magnetic resonance spectroscopy of mice body composition, metabolic parameters, and physical activity contents were determined as previously described (26,27).

Mixed-meal loading test. Pyruvate carboxylase protein was infused at 4 mU/kg per min for HFF rats and at 12 mU/kg per min for ZDF rats.

Liver biopsies. During the bariatric surgery, a wedge biopsy sample (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. Liver biopsies of clinical and laboratory measures were obtained. Although patients lost an average of ~9% body weight during the year before surgery, their weight remained relatively stable during the preoperative period between blood sampling and liver biopsy, with an average percentage 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.

Glyceroneogenesis assay. This assay was done as previously described (33).

RESULTS

Pyruvate carboxylase protein was increased parallel to glycemic level in humans. We assessed mRNA and protein expression of four gluconeogenic enzymes in human liver biopsy samples obtained from 20 patients undergoing bariatric surgery (Table 1) in relation to measures of glycemia assessed by fasting plasma glucose concentration and hemoglobin A1c (HbA1c). Although none of these patients had a prior diagnosis of type 2 diabetes, there was still a range of fasting plasma glucose concentrations and HbA1c. 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 HbA1c (Supplementary Fig. 2).

| TABLE 1 | Characteristics of participants |
|---------|-------------------------------|
| Participants (N) | 20 |
| Sex | Male, 14; Female, 6 |
| Age (years) | Mean, 41.5 ± 2.7 |
| BMI (kg/m²) | Mean, 48.4 ± 1.8 |
| Fasting plasma glucose (mg/dL) | Mean, 99.8 ± 4.0 |
| Fasting plasma insulin (µU/mL) | Mean, 23.3 ± 2.0 |
| HbA1c (%) | Mean, 5.8 ± 0.2 |
| HOMA-IR [(mg/dL) × (µU/mL)] | Mean, 5.4 ± 0.6 |
| Alanine aminotransferase (IU/L) | Mean, 30.8 ± 3.2 |
| Aspartate aminotransferase (IU/L) | Mean, 26.6 ± 2.1 |
| LDL cholesterol (mmol/L) | Mean, 2.84 ± 0.24 |
| HDL cholesterol (mmol/L) | Mean, 1.09 ± 0.04 |
| Triglyceride (mmol/L) | Mean, 1.73 ± 0.28 |

HOMA-IR, homeostatic model assessment of insulin resistance index.
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 HbA1c (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 in vivo, we treated regular chow-fed and HFF male SD rats with pyruvate carboxylase ASO. Pyruvate carboxylase ASO treatment decreased hepatic and adipose pyruvate carboxylase mRNA expressions ~80–90% in 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, HFF 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 the 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 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 a pyruvate

---

**FIG. 1.** Hepatic pyruvate carboxylase (PC) protein expression levels relate to glycemic levels in humans. Hepatic PC mRNA expression in human livers compared with fasting plasma glucose concentration (A) and HbA1c (B). Hepatic PC protein expression in human livers compared with fasting plasma glucose concentration (C) and HbA1c, along with representative bands (D). PC mRNA and protein are expressed as a relative increase to the lowest expression in the data set ($n = 20$). VDAC, voltage-dependent anion channel.
tolerance test in regular chow-fed and HFF rats treated with a control ASO or pyruvate carboxylase ASO. We found that glucose excursion was significantly suppressed by pyruvate carboxylase ASO in the regular chow-fed condition (Fig. 3G). The decrease in glucose production was even more marked in HFF rats. Consistent with this observation in vivo, the glucose production from pyruvate in 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 a 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 lipo dystrophic 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 in 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 1H magnetic resonance spectroscopy and also whole-body energy expenditure and food intake in metabolic cages. As in HFF rats, pyruvate carboxylase ASO decreased body weight gain and 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 5 weeks of treatment, before 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 mice treated with pyruvate carboxylase ASO (Supplementary Fig. 7C and D).

Although these measurements were preformed 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 the respiratory exchange ratio between pyruvate carboxylase and control ASO groups (0.83±0.003 and 0.83±0.005, respectively). Thus, in HFF rodents, decreasing pyruvate carboxylase expression in liver and

![FIG. 2. Pyruvate carboxylase (PC) ASO decreased PC expression in liver and epididymal adipose tissue. PC mRNA in liver (A) and epididymal adipose tissue (B). PC protein, with representative bands, is shown in liver (C) and epididymal adipose tissue (D). **P < 0.01 and ***P < 0.001 compared with control ASO group in the same diet condition. #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). All rats were killed and tissues were taken at 4 weeks of treatment. VDAC, voltage-dependent anion channel.](image-url)
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 by diacylglycerol (DAG)-mediated activation of PKC and impairment of insulin signaling in 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, pyruvate carboxylase ASO improved hepatic insulin sensitivity as reflected by a ~50% reduction in hepatic glucose production and

FIG. 3. Pyruvate carboxylase (PC) ASO decreased plasma glucose concentration and did not decrease insulin secretion. Fasting plasma glucose (A) and insulin concentration (B) 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). Results of mixed-meal tolerance test in the regular chow-fed rats for plasma glucose (D), plasma insulin (E), and plasma C-peptide (F) (n = 7–10). G: Pyruvate tolerance test in the regular chow-fed and HFF rats. ○ are control ASO and ● are PC ASO in regular chow-fed rats (both n = 9). △ are control ASO and ▲ are PC ASO in HFF rats (both n = 8). *P < 0.05, **P < 0.01, and ***P < 0.001 between control and PC ASO in regular chow-fed rats; ###P < 0.01 and ####P < 0.001 between control and PC ASO in HFF rats. Experiments were done at 4–5 weeks of treatment.
greater suppression of endogenous glucose production compared with 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 cytosol and membrane fractions, decreased activation of PKCε, and increased insulin-mediated hepatic Akt Ser367 phosphorylation (Fig. 6), a key node of the insulin-signaling pathway (35).

Pyruvate carboxylase ASO was also effective in ZDF 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 the fasting plasma glucose concentration and rates of endogenous glucose production during basal and hyperinsulinemic periods, and suppression of endogenous glucose production by insulin was greater in pyruvate carboxylase ASO–treated rats than in 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); however, the adipose expressions of key genes associated with adipogenesis, such as 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 nonesterified fatty acid concentration (Supplementary Table 1). However, no difference occurred 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 percentage of de novo fatty acid synthesis (Fig. 7D) nor the expression of lipogenic genes in liver (Supplementary Table 2) was 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 (ACCI) 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 (i.e., the glycerol backbone of a triglyceride molecule, Fig. 7E and F). This method quantifies total new glycerol synthesis, which includes glyceroneogenesis and formation of glycerol from glucose. In HFF conditions, however, glyceroneogenesis is thought 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 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 decreasing pyruvate carboxylase expression in liver and adipose tissue is well tolerated and effective in decreasing basal rates of endogenous glucose production and plasma glucose.

**FIG. 5.** Pyruvate carboxylase (PC) ASO improves hepatic insulin sensitivity in HFF rats. Fasting plasma glucose (A) and insulin concentration (B) (n = 9 per group). C: Basal endogenous glucose production (n = 9 per group). Plasma glucose concentration (D) and glucose infusion rate time course (E) during hyperinsulinemic-euglycemic (4 mU/kg per min) clamp, respectively (n = 7–8 per group). Insulin-stimulated peripheral glucose metabolism (F), endogenous glucose production (G), and percentage suppression of endogenous glucose production (H) 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 of treatment.
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 biopsy specimens 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. Thus, HbA1c

FIG. 6. Pyruvate carboxylase (PC) ASO decreased hepatic DAG content and PKCε activation and increased hepatic Akt phosphorylation 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). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. $$$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 of treatment.
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 HFF rodents compared with chow-fed rodents. We used this model to explore possible mechanisms accounting for the disassociation between pyruvate

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). Palmitate oxidation (B) and oleate oxidation (C) assay with primary hepatocytes isolated from HFF-treated and ASO-treated rats (n = 5 per group). D: In vivo hepatic de novo fatty acid synthesis in HFF rats (n = 9–10 per group). Hepatic (E) and adipose glycerol synthesis (F) in HFF rats (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 of treatment.
carboxylase mRNA and protein expression. Pyruvate carboxylase ubiquitination is decreased in HFF rat liver relative to chow-fed rat liver. This suggests that pyruvate carboxylase degradation in the ubiquitin-proteasome system is decreased, which may result in increased pyruvate carboxylase protein accumulation. This also may provide a possible mechanism that accounts for the increased hepatic pyruvate carboxylase flux that was recently reported in humans with nonalcoholic fatty liver disease (42).

To quantify the role of pyruvate carboxylase in controlling glucose and lipid metabolism, we used a loss-of-function approach. Although 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 serves to vet tissue-targeted inhibition of pyruvate carboxylase expression and 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, although there was a small increase in plasma lactate concentrations in ZDF rats. Although 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). Adipose pyruvate carboxylase expression is reported to be induced during adipogenesis and increased by PPARγ agonists, but 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 a reduction in food intake, although it may be possible that changes specific to liver or adipose tissue 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. Glyceroneogenesis plays a minor role in animals fed high-carbohydrate diets (i.e., low-fat), but 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, PEPCK is important for adipose glyceroneogenesis (51) but does not appear to be as essential for hepatic glyceroneogenesis because mice lacking PEPCK can still develop hepatic steatosis (46). By comparison, decreasing pyruvate carboxylase expression by ASO treatment protected mice and rats from adiposity and hepatic steatosis. The subsequent improvement in hepatic insulin sensitivity could be attributed to decreased DAG content and PKCe activation as well as 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 glyceremia 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 and 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.

ACKNOWLEDGMENTS

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-54089, U1L-RR-0241395, P00-DK-034989, P50-DK-45735), Manpei Suzuki Diabetes Foundation Fellowship (N.K.), a Distinguished Clinical Scientist Award (K.F.P.) and a Mentor-Based Postdoctoral Fellowship Grant (G.I.S.) from the American Diabetes Association, and a VA Merit Grant (5I01BX000901) (V.T.S.).

V.P.M. and S.B. are employees of ISIS and may own stock in the company. No other potential conflicts of interest relevant to this article were reported.

N.K., S.A.B., D.F.V., S.K.M., J.L.C., F.G.-E., I.F., B.G., M.J.J., A.L.B., M.K., B.K.P., M.A.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 biopsy specimens 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 the 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25–29 June 2010, and at the 71st 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, and Yanna Kosover, Jianying Dong, Kathy Harry, Dongyan Zhang, Toru Yoshimura, Shoichi Kanda, Derek M. Erion, Rebecca L. Pongratz, Codruta Todeasa, Maria Batsu, and Aida Groszmann (all of the Yale University School of Medicine) for their excellent technical support.

REFERENCES
1. Magnusson I, Rothman DL, Katz LD, Shulman RG, Shulman GI. Increased rate of gluconeogenesis in type II diabetes mellitus. A 13C nuclear magnetic resonance study. J Clin Invest 1990;90:1321–1327
2. Maggs DG, Buchanan TA, Burant CF, et al. Metabolic effects of trigluta-
zone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 1989;120:176–185
3. Hundal RS, Kressak M, Dufour S, et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes 2000;49:2063–2069
4. Utter MF, Keech DB. Formation of oxaloacetate from pyruvate and carbon dioxide. J Biol Chem 1960;235:PC17–PC18
5. Weber G, Cantero A. Glucose-6-phosphatase studies in fasting. Science 1954;120:851–852
6. Utter MF, Kuralashvili K. Purification of oxaloacetic carboxylase from chicken liver. J Biol Chem 1956;207:787–802
7. McGilvery RW, Mokrasch LC. Purification and properties of fructose-1, 6-diphosphatase. J Biol Chem 1949;176:909–917
8. Jurado LA, Song S, Roesler WJ, Park EA. Conserved amino acids within CCAAT enhancer-binding proteins (C/EBP[a] and beta) regulate phosphoenolpyruvate carboxylase (PEPCK) gene expression. J Biol Chem 2002;277:27066–27062
9. Koo SH, Flechner L, Qi L, et al. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 2005;437:1109–1117
10. Nakae J, Kitamura T, Silver DL, Accili D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase ex-

11. Samuel VT, Liu ZX, Wang A, et al. Inhibition of protein kinase C epsilon 6-phosphate transporter-1 ameliorates diabetes while avoiding compli-
12. Large V, Beylot M. Modifications of citric acid cycle activity and gluco-
neogenesis in streptozotocin-induced diabetes and effects of metformin. Diabetes 1999;48:1251–1257
13. Naga Y, Yonemitsu S, Erion DM, et al. The role of peroxisome proliferator-activated receptor gamma coactivator-1 beta in the pathogenesis of fruc-
tose-induced insulin resistance. Cell Metab 2009;9:252–264
14. Le Lay J, Tuteja G, White P, Dhir R, Ahima R, Kaestner KH. CRTC2 (TORC2) contributes to the transcriptional response to fasting in the liver but is not required for the maintenance of glucose homeostasis. Cell Metab 2009;10:55–62
15. Rammanan CJ, Edgerton DS, Rivero N, et al. Molecular characterization of insulin-mediated suppression of hepatic glucose production in vivo. Diabetes 2010;59:1302–1311
16. Sloop KW, Showalter AD, Cox AL, et al. Specific reduction of hepatic glu-
cose 6-phosphate transporter-1 ameliorates diabetes while avoiding complications of glycation storage disease. J Biol Chem 2007;282:19113–19121
17. Samuel VT, Beddow SA, Iwasaki T, et al. Fasting hyperglycemia is not
18. Pasi J, Skowronski E, Sze G, et al. The peroxisome proliferator-activated receptor-gamma regulates murine pyruvate carboxylase gene expression in vivo and in vitro. J Biol Chem 2005;280:27466–27476
19. Wellen KE, Uysal KT, Wiesbrock S, Yang Q, Chen H, Hotamisligil GS. In-
hibition of lipogenesis and gluconeogenesis in hepatocytes, adipose tissue, and liver. J Clin Invest 2004;113:1281–1289
44. Jitrapakdee S, Booker GW, Cassady AI, Wallace JC. The rat pyruvate carboxylase gene structure. Alternate promoters generate multiple transcripts with the 5’-end heterogeneity. J Biol Chem 1997;272:20522–20530
45. Marin-Valencia I, Roe CR, Pascual JM. Pyruvate carboxylase deficiency: mechanisms, mimics and anaplerosis. Mol Genet Metab 2010;101:9–17
46. Burgess SC, Hauser N, Merritt M, et al. Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. J Biol Chem 2004;279:48941–48949
47. Bandsma RH, Wiegman CH, Herling AW, et al. Acute inhibition of glucose-6-phosphate translocator activity leads to increased de novo lipogenesis and development of hepatic steatosis without affecting VLDL production in rats. Diabetes 2001;50:2591–2597
48. van Poelje PD, Potter SC, Chandramouli VC, Landau BR, Dang Q, Erion MD. Inhibition of fructose 1,6-bisphosphatase reduces excessive endogenous glucose production and attenuates hyperglycemia in Zucker diabetic fatty rats. Diabetes 2006;55:1747–1754
49. Chen JL, Peacock E, Samady W, et al. Physiologic and pharmacologic factors influencing glyceroneogenic contribution to triacylglyceride glycerol measured by mass isotopomer distribution analysis. J Biol Chem 2005;280:25396–25402
50. Nye CK, Hanson RW, Kalhan SC. Glyceroneogenesis is the dominant pathway for triglyceride glycerol synthesis in vivo in the rat. J Biol Chem 2008;283:27565–27574
51. Millward CA, Desantis D, Hsieh CW, et al. Phosphoenolpyruvate carboxykinase (Pck1) helps regulate the triglyceride/fatty acid cycle and development of insulin resistance in mice. J Lipid Res 2010;51:1452–1463
52. Samuel VT, Liu ZX, Qu X, et al. Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. J Biol Chem 2004;279:32345–32353