

Mechanism of fat-induced hepatic gluconeogenesis: effect of metformin

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Song, Shaoming, Sofianos Andrikopoulos, Christine Filippis, Anne W. Thorburn, David Khan, and Joseph Proietto. Mechanism of fat-induced hepatic gluconeogenesis: effect of metformin. *Am J Physiol Endocrinol Metab* 281: E275–E282, 2001.—High-fat feeding has been shown to cause hepatic insulin resistance. The aims of this study were to investigate the biochemical steps responsible for enhanced gluconeogenesis as a result of increased dietary fat intake and the site or sites at which the antihyperglycemic agent metformin acts to inhibit this process. Male Hooded Wistar rats were fed either a standard chow diet (5% fat by weight) or a high-fat diet (60% fat by weight) for 14 days with or without metformin. Total endogenous glucose production and gluconeogenesis were determined using [6-³H]glucose and [U-¹⁴C]alanine, respectively. Gluconeogenic enzyme activity and, where appropriate, protein and mRNA levels were measured in liver tissues. The high-fat diet increased endogenous glucose production (21.9 ± 4.4 vs. 32.2 ± 4.8 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $P < 0.05$) and alanine gluconeogenesis (4.5 ± 0.9 vs. 9.6 ± 1.9 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $P < 0.05$). Metformin reduced both endogenous glucose production (32.2 ± 4.8 vs. 16.1 ± 2.1 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $P < 0.05$) and alanine gluconeogenesis (9.6 ± 1.9 vs. 4.7 ± 0.8 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $P < 0.05$) after high-fat feeding. These changes were reflected in liver fructose-1,6-bisphosphatase protein levels (4.5 ± 0.9 vs. 9.6 ± 1.9 arbitrary units, $P < 0.05$ chow vs. high-fat feeding; 9.5 ± 1.9 vs. 4.7 ± 0.8 arbitrary units, $P < 0.05$ high fat fed in the absence vs. presence of metformin) but not in changes to the activity of other gluconeogenic enzymes. There was a significant positive correlation between alanine gluconeogenesis and fructose-1,6-bisphosphatase protein levels ($r = 0.56$, $P < 0.05$). Therefore, excess supply of dietary fat stimulates alanine gluconeogenesis via an increase in fructose-1,6-bisphosphatase protein levels. Metformin predominantly inhibits alanine gluconeogenesis by preventing the fat-induced changes in fructose-1,6-bisphosphatase levels.

endogenous glucose production; fructose-1,6-bisphosphatase; fat feeding; hepatic insulin resistance

OBESITY IS A COMMON CHARACTERISTIC of type 2 diabetes and is a major contributing factor to the insulin-resistant state that is a major feature of the condition. Although there are genetic factors that contribute to insulin resistance, in many diabetic subjects insulin

resistance is induced by environmental factors, such as oversupply of dietary fat. Studies in both humans and animals have shown that an elevation in plasma free fatty acids as a result of increased dietary fat intake or Intralipid infusion results in reduced peripheral glucose uptake and impaired suppression of endogenous glucose production (EGP) in response to insulin (8, 14, 20, 42, 48).

EGP is the net result of breakdown of glucose stored as glycogen (glycogenolysis) and the synthesis of new glucose molecules from lactate, amino acids, and glycerol (gluconeogenesis). The rate of gluconeogenesis is governed by the activities of the regulatable enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G-6-Pase). Insulin can inhibit gluconeogenesis by repressing the activities of one or all of these enzymes (34).

In type 2 diabetes, gluconeogenesis has been shown to be a predominant cause of the elevated EGP, contributing ~50–65% of the released glucose (17, 46). Furthermore, gluconeogenesis from both lactate and glycerol was shown to be elevated in obese patients with type 2 diabetes and was secondary to both increased substrate availability and enhanced intrahepatic conversion (30, 36). It was hypothesized that the enhanced glycerol gluconeogenesis may be due to an increase in FBPase levels (30). We (2) have previously shown that hepatic glucose overproduction in the New Zealand Obese mouse, a model of obesity and insulin resistance, was associated with increased FBPase activity and protein levels.

Agents that inhibit gluconeogenesis can play a key role in the treatment of hyperglycemia in type 2 diabetes. The biguanide metformin is one of the most commonly used drugs for treatment of hyperglycemia in type 2 diabetes (50). The glucose-lowering effect of metformin is thought to be due to suppression of hepatic glucose production and gluconeogenesis, although its precise biochemical mechanism is not fully understood. Previous work has suggested multiple possible mechanisms, including inhibition of the respira-

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tory chain (32), impaired alanine (19) or lactate (37) uptake, increased flux through pyruvate kinase either by reducing ATP levels (6) or by enhancing fructose 1,6-bisphosphate activation of pyruvate kinase (26), antagonism of glucagon's action (1, 53), inhibition of glycogenolysis (10), and decreased flux from pyruvate to phosphoenolpyruvate (23). Many of these studies used isolated hepatocytes (1, 19, 32, 53) or perfused livers (23, 37). There have been no studies in which gluconeogenesis has been measured in vivo and correlated with the enzyme changes induced by high fat and corrected by metformin.

The aim of this study was to investigate the biochemical steps responsible for enhanced gluconeogenesis as a result of increased dietary fat intake and the site or sites at which metformin acts to inhibit this process. We show that a high-fat diet increases the levels of the gluconeogenic enzyme FBPase, resulting in increased alanine gluconeogenesis and EGP. Furthermore, metformin inhibits the changes in EGP and alanine gluconeogenesis induced by fat feeding by suppressing FBPase, thus providing another mechanism of action for this important antidiabetic drug.

MATERIALS AND METHODS

Chemicals and animals. Chemicals were purchased from Sigma Aldrich (St. Louis, MO). Enzymes and cofactors were purchased from Boehringer Mannheim (Munich, Germany). [^{14}C]alanine and [^3H]glucose tracers were purchased from Du Pont-NEN Research Products (North Ryde, NSW, Australia). Ion exchange resins were purchased from Bio-Rad Laboratories (North Ryde, NSW, Australia). Metformin (hydrochloride form) was a kind gift from Alphapharm Pty (Brisbane, QLD, Australia). Male Hooded Wistar rats (180–220 g) were purchased from Monash Animal Centre (Clayton, Victoria, Australia) and housed in the facilities within the Royal Melbourne Hospital at 1–2 animals/cage on either a standard chow or high-fat diet with or without metformin administration.

Diet composition and metformin administration. The ingredients of the high-fat diet (HFD) were adapted from Andrikopoulos and Proietto (2) and are shown in Table 1. The HFD contained 60% of fat by weight (safflower oil), 20%

carbohydrate, and 20% protein plus vitamin and mineral mixtures and was prepared every 3 days to ensure its freshness. The major fatty acid component in safflower oil is linoleic acid (18:2 $^{\Delta 9,12}$, ω -6). The standard chow diet (SCD) was commercially obtained from Barastoc (Pakenham, Victoria, Australia) and consisted of 4.5% fat by weight, 20% protein, and 75.5% carbohydrate source. Metformin was administered as a food admixture at a dose of 120 mg/kg of body weight.

Experimental design. All experimental protocols were approved by the Royal Melbourne Hospital Animal Ethics Committee. Rats were grouped according to their diet and metformin administration and kept under a 12:12-h light-dark cycle and at a room temperature of $\sim 21^\circ\text{C}$. Animals were fed ad libitum with either an HFD or SCD (with or without metformin), with water freely available at all times. Body weights were recorded at 3-day intervals. After 14 days of feeding, the rats were fasted overnight (food withdrawn at $\sim 4:00$ PM) and anesthetized at 9:00–10:00 AM on the morning of the experiment with an intraperitoneal injection of sodium pentobarbitone (60 mg/kg Nembutal; Rhone Merieux, QLD, Australia). Two catheters were inserted, one in the right jugular vein for tracer infusion and the other in the left carotid artery for blood sampling. A tracheostomy was also performed to prevent upper respiratory tract obstruction. Body temperature was maintained at 37°C with a heat lamp and monitored with a rectal probe. Animals were checked for depth of anesthesia by the toe-pressure method regularly throughout the experimental procedure, and Nembutal was administered via the carotid catheter as necessary.

A basal blood sample was obtained in sequestrene tubes for the measurement of plasma free fatty acids. Gluconeogenesis from alanine was measured as previously described (2). Briefly, a bolus of [^{14}C]alanine (3.2 μCi) and [^3H]glucose (5.8 μCi) was infused via the jugular vein for 2 min and subsequently followed by a constant infusion at 0.1 $\mu\text{Ci}/\text{min}$ for [^{14}C]alanine and 0.18 $\mu\text{Ci}/\text{min}$ for [^3H]glucose for 110 min. Blood (400 μl) was collected at 90, 100, and 110 min, and 100 μl were deproteinized immediately with 500 μl of 0.3 mol/l $\text{Ba}(\text{OH})_2$ and 500 μl of 0.3 mol/l ZnSO_4 and centrifuged, and the clear supernatant was collected and stored for further analysis. The rest of the blood was centrifuged and the plasma stored at -20°C for measurement of glucose and insulin. At the end of the experiment, a laparotomy was rapidly performed, and the liver was quickly taken into liquid nitrogen and stored at -70°C . Epididymal and infrarenal fat pads were also excised and weighed.

Analytical procedures. To determine the rate of gluconeogenesis from alanine, 400 μl of the deproteinized sample were passed down three columns. The first column contained 1.7 ml Dowex AG-50W-X8 (H^+ form, 100–200 mesh) resin, and it bound amino acids (alanine and glutamine) that were released by eluting with 4 ml of 2 mol/l NH_4OH . The second column, containing 1.7 ml AG-1-X8 (Cl^- form, 100–200 mesh) resin, bound lactate and pyruvate; they were eluted by 4 ml of 0.1 mol/l HCl and were not analyzed in this experiment. The third column contained 1.7 ml AG-1-X8 (borate form, 100–200 mesh) resin and bound glycerol and glucose. Glycerol was eluted with 5 ml of 20 mmol/l sodium tetraborate, and glucose was eluted with 4 ml of 0.5 mol/l acetic acid. All eluants were dried in a fan-forced oven at 60°C overnight, and each was resuspended in 300 μl of distilled H_2O , 200 μl of which were combined with 3.5 ml of scintillant (Ready-Value; Beckman, Palo Alto, CA). Radioactivity was determined in a Beckman LS 3081 scintillation counter (Beckman Instruments, Victoria, Australia) by a dual-label program.

Table 1. Composition of the high-fat diet

Ingredients	Amount, g/kg diet
Safflower oil	295
Casein	220
Cornstarch	230
Wheat bran	179
Methionine	4
Choline chloride	3
Vitamin mix*	18
Mineral mix†	51

*Per kilogram of vitamin mix: 3 g thiamine mononitrate, 3 g riboflavin, 3.5 g pyridoxine HCl, 15 g nicotinamide, 8 g D-calcium pantothenate, 1 g folic acid, 0.1 g D-biotin, 5 mg cyanocobalamin, 12.5 mg cholecalciferol, 25 mg acetomenaphthone, 0.6 g vitamin A acetate, 25 g D- α -tocopherol acetate, and 10 g choline chloride.

†Per kilogram of mineral mix: 30.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 65.2 g NaCl, 105.7 g KCl, 200.8 g KH_2PO_4 , 38.8 g $\text{MgCO}_3 \cdot \text{Mg}(\text{OH})_2 \cdot 3\text{H}_2\text{O}$, 40 g $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$, 515.4 g CaCO_3 , 0.8 g KI, 0.9 g NaF, 1.4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g MnSO_4 , and 0.05 g CoHN_3 .

The other 100 μ l were used to assay total glucose and alanine concentrations, as will now be described.

Determination of L-alanine and D-glucose. Both alanine and glucose were measured using standard spectrophotometric methods with a Beckman DU-50 spectrophotometer at a wavelength of 340 nm. The method to determine plasma alanine was based on Williamson (51), following the conversion of NAD⁺ to NADH. Glucose was determined by the method of Kunst et al. (21), in which NADP⁺ was converted to NADPH.

Hepatic glycogen assay. Glycogen levels were determined by measuring glucose derived from glycogen in a liver homogenate. A sample of liver (10 mg) and 200 μ l ice-cold perchloric acid (0.6 mol/l) were homogenized using a Polytron homogenizer. A sample of this homogenate (40 μ l) was added to 20 μ l KHCO₃ (1 mol/l) plus 400 μ l glycoamylase-acetate buffer (20 mg amyloglucosidase in 20 ml acetate buffer, 0.2 mol/l, pH 4.8) and incubated at 40°C for 2 h. Perchloric acid (0.6 mol/l, 200 μ l) was then added, and the sample was centrifuged at 3,000 rpm for 10 min. The remaining tissue preparation was also centrifuged. Samples of these supernatants were analyzed for glucose by a fluorometric method (33). Glycogen levels were determined by subtracting free glucose from total glucose concentrations.

Plasma assays. Plasma insulin was determined by radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden) using a double antibody technique to separate free from bound insulin. Plasma glucose was determined using a glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). Plasma free fatty acids (FFAs) were determined in samples collected in sequestrene tubes with an enzymatic colorimetric method supplied in a kit (Wako Pure Chemical Industries, Richmond, VA).

Enzyme assays and Western and Northern blotting. The activities of PEPCK, FBPase, and G-6-Pase were determined as previously described (3). FBPase protein levels were determined by Western blotting, as previously described (4). PEPCK mRNA levels were determined by Northern blotting, as previously described (3).

Calculations. Specific activity of [6-³H]glucose and [U-¹⁴C]alanine was at steady state after 90 min of infusion (Fig. 1). Rate of glucose appearance, which represents (mainly hepatic) EGP in the basal state, was calculated by dividing the infusion rate of the [6-³H]glucose tracer by its specific activity. Rate of conversion of alanine to glucose was calculated by multiplying the [¹⁴C]glucose specific activity by the rate of glucose appearance and dividing by [¹⁴C]alanine specific activity (47).

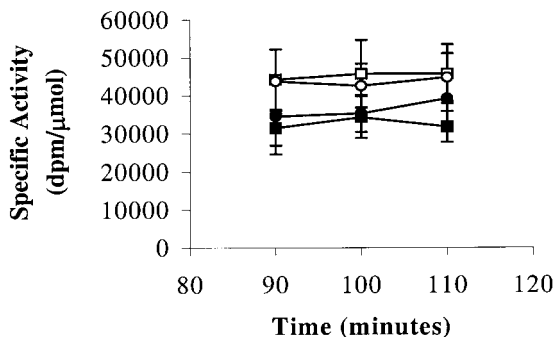


Fig. 1. Specific activity of [³H]glucose (closed symbols) and [¹⁴C]alanine (open symbols) in rats fed a standard chow (●,○) or high-fat diet (■,□).

Statistical analysis. All results are expressed as means \pm SE. Statistical significance ($P < 0.05$) was determined using the Student *t*-test.

RESULTS

Body weight and fat pad weight. Body weights and fat pad weights at the end of 2 wk of the dietary regimen are shown in Table 2. There was no difference in body weights at the start of the experiment among the four groups of animals. At the end of the experimental protocol, rats consuming an HFD gained more body weight than those consuming the SCD, although there was no difference when rats were treated with metformin. Furthermore, rats treated with an HFD showed significantly increased epididymal and infrarenal fat weight compared with SCD-fed rats. Interestingly, metformin decreased epididymal fat weight in both HFD- and SCD-fed rats, whereas it had no effect on infrarenal fat.

Plasma glucose, insulin, and FFA concentrations. Basal plasma glucose, insulin, and FFA levels are shown in Table 3. There was no difference in plasma glucose levels, and although plasma insulin concentrations tended to be high in the HFD-treated animals, there was no statistical difference among the four groups of rats. Plasma FFAs were not different between SCD- and HFD-treated rats; however, metformin decreased plasma FFA concentrations in both groups of rats.

EGP and alanine gluconeogenesis. Figure 2 shows EGP and alanine gluconeogenesis. A high-fat dietary regimen for 14 days caused a significant increase in EGP compared with rats fed the SCD (Fig. 2A). Furthermore, metformin suppressed EGP in rats fed the HFD, whereas it had no effect in rats fed the SCD.

The changes seen in EGP were mirrored in the rate of alanine gluconeogenesis. Specifically, there was an increase in alanine gluconeogenesis in rats fed an HFD compared with rats fed an SCD (Fig. 2B). This increase in the rate of alanine conversion to glucose was suppressed by metformin in rats fed an HFD, whereas it had no effect in rats fed an SCD. Thus the dietary and drug modulations in EGP are due to concomitant changes in alanine gluconeogenesis.

Hepatic glycogen concentrations. Hepatic glycogen levels in response to a diet high in fat and metformin is shown in Fig. 3. Rats fed an HFD had increased glycogen levels compared with SCD-fed rats. Furthermore, metformin decreased glycogen concentrations in HFD-fed rats, whereas it had no effect in SCD-treated rats.

Hepatic enzyme activities. To determine the biochemical changes responsible for modulation of EGP and alanine gluconeogenesis, the activities of key gluconeogenic enzymes were measured. The activities of PEPCK (27.1 ± 1.9 vs. 24.7 ± 2.7 nmol·min⁻¹·mg protein⁻¹), FBPase (39.6 ± 2.5 vs. 34.7 ± 2.8 nmol·min⁻¹·mg protein⁻¹), and G-6-Pase (23.6 ± 1.3 vs. 22.2 ± 1.1 nmol·min⁻¹·mg protein⁻¹) were comparable between rats treated with SCD and HFD. In agreement with the enzyme activity, PEPCK mRNA levels were also

Table 2. *Body weights and epididymal and infrarenal fat pad weights*

	SCD	HFD	SCD + MET	HFD + MET
Initial body weight	191.5 ± 3.4	202.8 ± 7.3	192.4 ± 6.5	189.5 ± 5.3
Final body weight	231.4 ± 8.5	249.9 ± 6.9*	230.2 ± 6.6	228.2 ± 4.6
Epididymal fat	1.53 ± 0.13	2.01 ± 0.25*	0.93 ± 0.04‡	1.38 ± 0.11‡†
Infrarenal fat	0.98 ± 0.08	1.46 ± 0.24*	0.74 ± 0.14	1.47 ± 0.13†

Values are means ± SE ($n = 8$) expressed in g. SCD, standard chow diet; HFD, high-fat diet; MET, metformin. * $P < 0.05$ vs. SCD; † $P < 0.005$ vs. SCD + MET; ‡ $P < 0.05$ vs. non-MET-treated group.

not different between SCD and HFD rats (9.8 ± 2.8 vs. 11.6 ± 1.9 arbitrary units).

FBPase protein levels. We have previously shown that a high-fat diet increases FBPase protein levels in mice (2). To determine whether FBPase levels were responsible for the changes seen in EGP and alanine gluconeogenesis, the protein levels of this enzyme were determined by immunoblotting and are shown Fig. 4. FBPase protein levels were elevated in rats fed an HFD compared with rats fed an SCD. Metformin suppressed FBPase protein levels in rats fed the HFD, whereas it had no effect in rats fed the SCD. Furthermore, there was a significant positive correlation between FBPase protein levels and alanine gluconeogenesis when all groups were considered (Fig. 5, $r = 0.56$, $P < 0.05$, $n = 19$). This association was still significant when the groups treated without ($r = 0.60$, $P < 0.05$) or with metformin ($r = 0.69$, $P < 0.05$) were analyzed separately. Thus modulation of FBPase may explain the changes seen in alanine gluconeogenesis in response to an HFD and metformin.

DISCUSSION

The aim of the present study was to evaluate the effect of a high-fat diet on EGP and alanine gluconeogenesis, ascertain the enzymatic steps responsible for these changes, and determine whether metformin can ameliorate the stimulatory effect of a high-fat diet on EGP. We show that a high-fat diet fed to rats for 14 days resulted in elevated EGP. This was contributed to by an increase in the rate of alanine gluconeogenesis. To our knowledge, this is the first study to show that oversupply of dietary fat increases the rate of alanine gluconeogenesis in vivo. Previous studies have shown that an increase in circulating lipids results in the impaired suppression of hepatic glucose production by insulin (14, 20, 42), although whether this was due to increased gluconeogenesis or glycogenolysis was not known. Recently, studies in healthy subjects in which FFAs were modulated with nicotinic acid or lipid infu-

sion resulted in reciprocal changes in gluconeogenesis (9, 39), suggesting that short-term increases in plasma lipids can stimulate the rate of gluconeogenesis. Studies in vitro using freshly isolated hepatocytes or in situ perfusion of the liver have shown that addition of fatty acids (oleate, palmitate) significantly increased the rate of gluconeogenesis from pyruvate (7, 52). The results of our study show that endogenous glucose overproduction in response to increased nutrient supply is due to an enhanced rate of alanine gluconeogenesis.

Increased EGP is a characteristic of patients with type 2 diabetes and the main contributing factor to fasting hyperglycemia. Furthermore, it has been shown that elevated gluconeogenesis accounts for

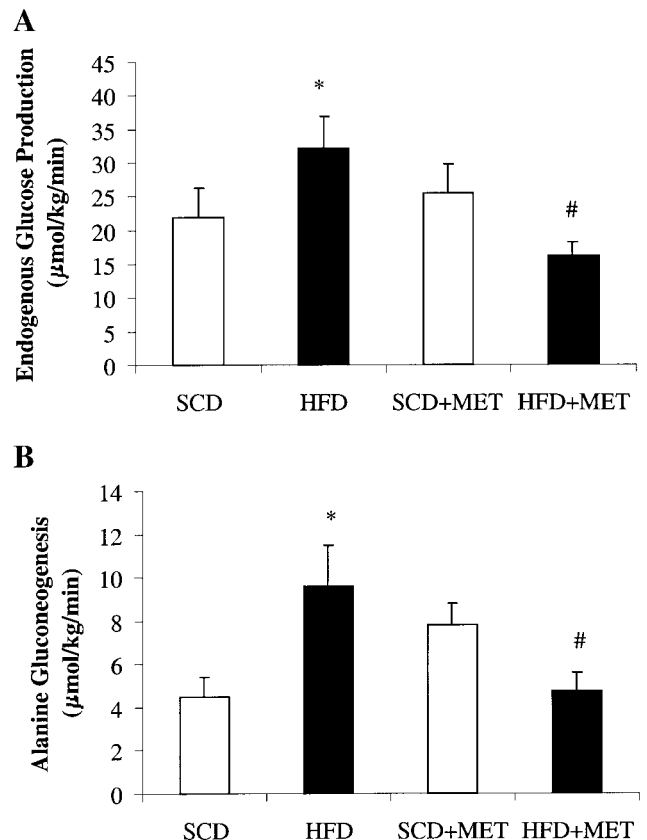


Fig. 2. Endogenous glucose production (EGP, A) and alanine gluconeogenesis (B) in rats ($n = 8$) fed a standard chow (SCD, open bars) or high-fat (HFD, closed bar) diet for 14 days in the absence or presence of metformin (MET). EGP was assessed using $[6\text{-}^3\text{H}]\text{glucose}$, and alanine gluconeogenesis was determined using $[\text{U}\text{-}^{14}\text{C}]\text{alanine}$. * $P < 0.05$ vs. SCD, # $P < 0.05$ vs. HFD.

Table 3. *Plasma glucose, insulin, and free fatty acid concentrations*

	SCD	HFD	SCD + MET	HFD + MET
Glucose, mmol/l	7.0 ± 0.4	8.0 ± 0.6	7.2 ± 0.4	7.0 ± 0.4
Insulin, ng/ml	1.36 ± 0.22	2.41 ± 0.84	1.42 ± 0.58	1.45 ± 0.13
FFA, mmol/l	0.91 ± 0.02	0.94 ± 0.02	0.79 ± 0.02*	0.74 ± 0.01*

Values are expressed as means ± SE ($n = 8$). * $P < 0.05$ vs. non-MET-treated group.

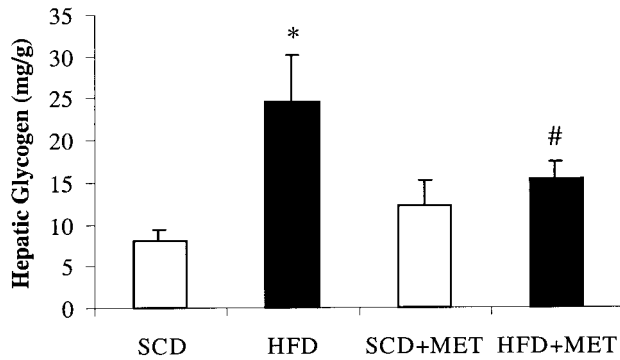


Fig. 3. Hepatic glycogen levels in rats ($n = 8$) fed an SCD (open bar) or HFD (closed bar) for 14 days in the absence or presence of MET. * $P < 0.05$ vs. SCD, # $P < 0.05$ vs. HFD.

~65% of EGP in patients with type 2 diabetes (17). However, the mechanism of increased gluconeogenesis has not been fully elucidated. A high-fat diet or Intralipid infusion in healthy humans to raise plasma FFA concentrations has been shown to cause hepatic insulin resistance. Because patients with type 2 diabetes also have elevated plasma FFA levels, it is reasonable to assume that hepatic insulin resistance is in large part due to increased lipid availability. Furthermore, calorie restriction and body weight loss have been associated with decreased EGP in patients with type 2 diabetes (16, 18, 22). Thus, use of the high-fat-fed rat model to study biochemical mechanisms of increased hepatic glucose production is most appropriate.

To provide a mechanism of increased alanine gluconeogenesis in response to a high-fat diet, we mea-

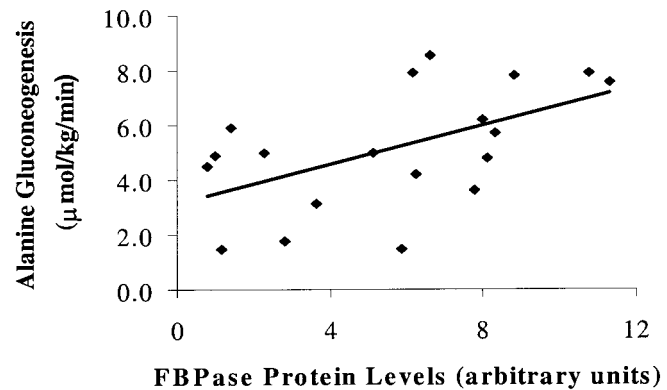


Fig. 5. Correlation between alanine gluconeogenesis and hepatic FBPase protein levels in rats fed an SCD or HFD with and without MET ($r = 0.56$, $P < 0.05$, $n = 19$).

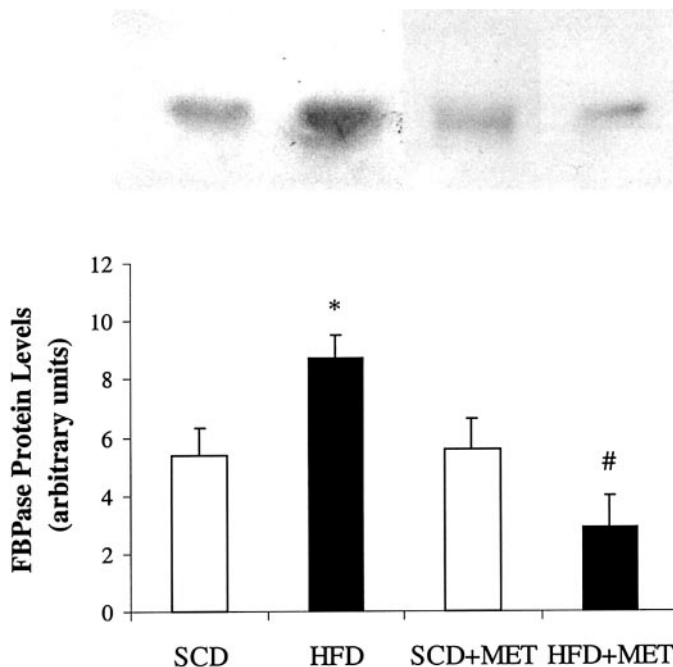


Fig. 4. Hepatic fructose-1,6-bisphosphatase (FBPase) protein levels in rats ($n = 8$) fed an SCD (open bar) or HFD (closed bar) for 14 days in the absence or presence of MET. * $P < 0.05$ vs. SCD, # $P < 0.05$ vs. HFD. A representative immunoblot is shown above the graph.

sured the gluconeogenic enzymes PEPCK, FBPase, and G-6-Pase. The activity of PEPCK was not increased in high-fat-fed rat livers. Measurement of PEPCK mRNA also failed to show any increase with high-fat feeding. PEPCK has been proposed as the rate-determining enzyme of the gluconeogenic pathway (40, 41) and might be expected to reflect the increased flux under these circumstances. However, we have previously shown no difference in PEPCK activity between mice fed a chow or a 60% fat diet for 12 days (2). Similarly, the activity of G-6-Pase was also not increased with high-fat feeding. However, lipid infusion has been shown to increase the hepatic mRNA and protein levels of G-6-Pase in rats (25). Furthermore, although a recent report in which rats were fed a 59% fat diet for 3 wk found no change in G-6-Pase activity compared with chow-fed rats, insulin infusion was not able to suppress the activity of this enzyme in the fat-fed rats (31). It is possible that, in our study, we did not see a change in G-6-Pase activity with fat feeding because overnight fasting induced its activity in all groups of rats, thus masking any additional effect of the high-fat diet.

Interestingly, the only biochemical parameter that reflected the changes in EGP and alanine gluconeogenesis was the protein levels of FBPase. This and the results of our previous study, in which we found increased FBPase activity and protein levels in control mice fed a 60% fat diet (2), suggest that lipid oversupply causes an increase in the hepatic levels of FBPase. Unlike the increase shown in our previous study in mice, this increase in FBPase protein levels was not reflected in activity measurements in the rat. This is most likely due to the fact that the rat protein contains a 27-amino acid carboxy-terminal extension that has at least two serine residues that are phosphorylated by cAMP-dependent protein kinase A (12, 27, 38). This sequence is absent in both human and mouse FBPase, such that the activity is not regulated by phosphorylation (11, 24). Phosphorylation of rat FBPase causes an increase in activity as a result of lowering the Michaelis-Menten constant, or K_m , for the substrate fructose 1,6-bisphosphate and diminishes the inhibitory effects

of AMP and fructose 2,6-bisphosphate (13, 28). Homogenization and dilution of the liver extract as it is prepared for assay may result in an altered phosphorylation state of the rat enzyme, masking any difference in activity between chow- and high-fat-fed rats despite higher protein levels. Nevertheless, the significant positive correlation between the rate of alanine conversion to glucose and FBPase protein levels emphasizes the importance of this enzyme in the regulation of alanine gluconeogenesis and hepatic glucose production.

The stimulatory effects of a high-fat diet on alanine gluconeogenesis and FBPase protein levels were counteracted by metformin, the most widely used antihyperglycemic/antidiabetic drug. The effect of metformin to inhibit gluconeogenesis has been recognized for more than 30 years (29). This drug has been shown to effectively lower plasma glucose levels in patients with type 2 diabetes by decreasing EGP as a result of inhibiting gluconeogenesis (17). However, the mechanism by which metformin inhibits gluconeogenesis has not been clear. Radziuk et al. (37) reported that the primary action of metformin is to reduce lactate uptake, although a recent study could not duplicate this finding (23). The study of Radziuk et al. was performed in perfused livers of streptozotocin-diabetic rats, and the decrease in gluconeogenesis was attributed to a decrease in the flux through PEPCK. This may not be the case in obesity and type 2 diabetes, where insulin levels are normal or elevated. Other steps in the gluconeogenic pathway (e.g., through FBPase) were not determined. A reduction in alanine gluconeogenesis in our study could be explained either by decreased flux through the pyruvate-to-phosphoenolpyruvate cycle, as proposed by Large et al. (23), a conversion catalyzed by two enzymes, pyruvate carboxylase (PC) and PEPCK, or by increased flux through pyruvate kinase, reducing fructose 1,6-bisphosphate, the substrate for FBPase. However, the failure to show modulation of PEPCK and the correlation between FBPase protein levels and alanine gluconeogenesis suggest that the main action of metformin is to inhibit FBPase protein levels. This is supported by a study in freshly isolated hepatocytes, which showed that metformin inhibited gluconeogenesis from dihydroxyacetone phosphate (6), a 3-carbon substrate that enters the pathway proximal to the step catalyzed by FBPase.

A central role for FBPase in the flux of gluconeogenesis and hepatic glucose production has been suggested by a number of other studies. First, we have shown that FBPase is the only enzyme elevated in the New Zealand Obese mouse, a model of obesity and type 2 diabetes, and it is responsible for increased gluconeogenesis from glycerol (2, 3). Second, AICA riboside, an intermediate of the de novo purine biosynthesis pathway, dose dependently inhibited gluconeogenesis by reducing the activity of FBPase (49). Furthermore, it has recently been shown that diabetic C57BL/KsJ-*db/db* mice treated with dehydroepiandrosterone and troglitazone showed a decrease in plasma glucose concentrations that was due to suppression of liver FBPase and glucose-6-phosphatase, whereas there was no

effect on PEPCK (5). Finally, an increase in the rate of glycerol gluconeogenesis in patients with type 2 diabetes was attributed to an increased intrahepatic mechanism, possibly FBPase (30). Therefore, the present and previous studies provide strong evidence for a key role of FBPase in the control of gluconeogenesis and EGP.

The enhanced rate of EGP induced by the HFD in the present study did not lead to an increase in plasma glucose concentrations. This is probably due to an increase in the rate of glucose disappearance as a result of a trend for plasma insulin levels to be elevated. No change in plasma glucose levels as a result of increased plasma insulin concentrations has also been shown in a study in which gluconeogenesis was stimulated with a lipid infusion in healthy subjects (39).

Interestingly, the levels of hepatic glycogen also reflected the changes in alanine gluconeogenesis and FBPase in response to high-fat feeding and metformin. Specifically, metformin did not affect hepatic glycogen in SCD-treated rats but significantly reduced the increase caused by the HFD. This is in contrast to a study in human subjects with type 2 diabetes in which metformin treatment was associated with decreased rates of hepatic glycogenolysis (10). Our study supports the hypothesis that a large portion of glycogen is derived from substrates generated by the gluconeogenic pathway, the so-called "indirect pathway" of glycogenesis (15). The indirect pathway is the major source of glycogen deposition in rodents; this may explain the discrepancy with the human study mentioned above. Furthermore, our results also confirm those by Radziuk et al. (37), in which glycogen formation was decreased by 60% in rat livers perfused with metformin, attributed to a decrease in gluconeogenesis. Raised levels of liver glycogen as a result of the chronic increase in gluconeogenesis would result in the excess glucose produced being released rather than stored. This may be the reason for the correlation between increased gluconeogenesis and EGP that was observed in this study. Previous work in which gluconeogenesis was acutely modulated by increasing availability of substrates (44, 45) or inhibition with alcohol (35) showed no effect on EGP, leading to the hypothesis that there is intrahepatic autoregulation. Although this may be the case with acute alterations, our study shows that chronic increases in alanine gluconeogenesis can result in increased EGP if glycogen stores are abundant.

An inhibitory effect of metformin on fat deposition despite no change in body weight was also demonstrated in this study. It is of interest that this was demonstrated in rats fed either SCD or HFD, but only in the epididymal and not infrarenal fat depot. This probably reflects the metabolic heterogeneity of different fat depots in the rat. A regulatory effect of metformin on body weight has previously been shown in obese patients with type 2 diabetes (43); this study attributed 80% of the decrease in body weight to reduced adipose tissue mass. A reduction in plasma FFA concentrations in response to metformin was also observed in both groups of rats. It is possible that the

inhibitory effect of metformin on FBPase protein levels and alanine gluconeogenesis is secondary to a suppressive effect on lipid metabolism.

In summary, we have shown that a short-term high-fat diet induced an increase in EGP in rats as a result of enhanced alanine gluconeogenesis. The antidiabetic drug metformin reduced EGP in fat-fed rats as a result of suppressing alanine gluconeogenesis. The changes in alanine gluconeogenesis were reflected in changes to FBPase protein levels, suggesting that this enzyme plays a pivotal role in endogenous glucose overproduction in type 2 diabetes.

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