

# Mechanism by Which Metformin Reduces Glucose Production in Type 2 Diabetes

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To examine the mechanism by which metformin lowers endogenous glucose production in type 2 diabetic patients, we studied seven type 2 diabetic subjects, with fasting hyperglycemia ( $15.5 \pm 1.3$  mmol/l), before and after 3 months of metformin treatment. Seven healthy subjects, matched for sex, age, and BMI, served as control subjects. Rates of net hepatic glycogenolysis, estimated by  $^{13}\text{C}$  nuclear magnetic resonance spectroscopy, were combined with estimates of contributions to glucose production of gluconeogenesis and glycogenolysis, measured by labeling of blood glucose by  $^2\text{H}$  from ingested  $^2\text{H}_2\text{O}$ . Glucose production was measured using  $[6,6\text{-}^2\text{H}_2]\text{glucose}$ . The rate of glucose production was twice as high in the diabetic subjects as in control subjects ( $0.70 \pm 0.05$  vs.  $0.36 \pm 0.03$  mmol  $\cdot$  m $^{-2}$   $\cdot$  min $^{-1}$ ,  $P < 0.0001$ ). Metformin reduced that rate by 24% (to  $0.53 \pm 0.03$  mmol  $\cdot$  m $^{-2}$   $\cdot$  min $^{-1}$ ,  $P = 0.0009$ ) and fasting plasma glucose concentration by 30% (to  $10.8 \pm 0.9$  mmol/l,  $P = 0.0002$ ). The rate of gluconeogenesis was three times higher in the diabetic subjects than in the control subjects ( $0.59 \pm 0.03$  vs.  $0.18 \pm 0.03$  mmol  $\cdot$  m $^{-2}$   $\cdot$  min $^{-1}$ ) and metformin reduced that rate by 36% (to  $0.38 \pm 0.03$  mmol  $\cdot$  m $^{-2}$   $\cdot$  min $^{-1}$ ,  $P = 0.01$ ). By the  $^2\text{H}_2\text{O}$  method, there was a twofold increase in rates of gluconeogenesis in diabetic subjects ( $0.42 \pm 0.04$  mmol  $\cdot$  m $^{-2}$   $\cdot$  min $^{-1}$ ), which decreased by 33% after metformin treatment ( $0.28 \pm 0.03$  mmol  $\cdot$  m $^{-2}$   $\cdot$  min $^{-1}$ ,  $P = 0.0002$ ). There was no glycogen cycling in the control subjects, but in the diabetic subjects, glycogen cycling contributed to 25% of glucose production and explains the differences between the two methods used. In conclusion, patients with poorly controlled type 2 diabetes have increased rates of endogenous glucose production, which can be attributed to increased rates of gluconeogenesis. Metformin lowered the rate of glucose production in these patients through a reduction in gluconeogenesis. *Diabetes* 49:2063–2069, 2000

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GCMS, gas chromatograph–mass spectrometer; GCRC, General Clinical Research Center; HMT, hexamethylenetetramine; NMR, nuclear magnetic resonance.

**A**lthough it is generally agreed that metformin reduces fasting plasma glucose concentrations by reducing rates of hepatic glucose production (1,2), its effect on the relative contributions of hepatic glycogenolysis and gluconeogenesis remains controversial. Some studies conclude that metformin works mostly by reducing rates of gluconeogenesis (3); others, that it works by reducing rates of hepatic glycogenolysis (4,5).

Because of limitations of the methods used in the previous studies to assess gluconeogenesis and glycogenolysis, we used two independent and complementary methods to assess these processes in patients with poorly controlled type 2 diabetes before and after 3 months of metformin therapy.  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy was used to directly measure rates of net hepatic glycogenolysis, in combination with  $[6,6\text{-}^2\text{H}_2]\text{glucose}$  administration, to calculate the rates of endogenous glucose production (6). Rates of gluconeogenesis were estimated by subtracting the rates of net hepatic glycogenolysis from the rates of endogenous glucose production. In addition, fractional contributions of gluconeogenesis and glycogenolysis were estimated from the ratios of the  $^2\text{H}$  enrichments of the hydrogens bound to C5 and C2 of blood glucose after oral intake of  $^2\text{H}_2\text{O}$  (7,8). Rates of gluconeogenesis were calculated by multiplying those fractional contributions by the rates of glucose production. In addition, because the NMR method measures rates of net hepatic glycogenolysis whereas the  $^2\text{H}_2\text{O}$  method measures rates of total hepatic glycogenolysis (net hepatic glycogenolysis + glycogen cycling), estimates of glycogen cycling were made by subtracting rates of net hepatic glycogenolysis (NMR measured) from the rates of total hepatic glycogenolysis ( $^2\text{H}_2\text{O}$  measured).

## RESEARCH DESIGN AND METHODS

**Subjects.** Nine subjects with history of type 2 diabetes (seven men and two women, aged  $55 \pm 4$  years, weight  $90 \pm 5$  kg, body surface area  $2.1 \pm 0.1$  m $^2$ ) were studied before and after 3 months of treatment with metformin. At baseline, these subjects were in poor glycemic control, as reflected by fasting plasma glucose concentration of  $15.3 \pm 1.1$  mmol/l and glycosylated hemoglobin of  $12.9 \pm 1.2\%$ . All of the patients were initially screened to rule out any other systemic disease or any biochemical evidence of abnormal renal or hepatic functions. Patients with a history of alcohol abuse, symptomatic coronary heart disease, current use of insulin for glycemic control, significant hepatic enzyme elevation ( $>2$  times the upper limit of normal), serum creatinine  $>1.5$  mg/dl, or a metallic device/fragment in their body were excluded from the study. Because the purpose of the study was to assess the mechanism by which metformin lowers glucose production, two diabetic subjects whose glucose production failed to decrease after metformin treatment were excluded

from the final data analysis. The control group consisted of seven healthy volunteers matched for age and body weight (five men and two women, aged  $46 \pm 2$  years, weight  $86 \pm 8$  kg, body surface area  $2.0 \pm 0.1$  m<sup>2</sup>) who were studied once at baseline. The diabetic subjects were asked to discontinue their antidiabetic medications during the washout period (i.e., 2–3 weeks before the first study). Two subjects were taking sulfonylureas and one subject was taking both metformin and a sulfonylurea. The other four subjects were newly diagnosed with type 2 diabetes and were not taking any oral hypoglycemic medications. All diabetic subjects monitored their blood glucose levels during the washout period. All study subjects were given a diet containing 40–45 kcal/kg body wt, consisting of 60% carbohydrate, 20% fat, and 20% protein (prepared by the Yale–New Haven Hospital General Clinical Research Center [GCRC] metabolic kitchen) and asked to abstain from alcohol, caffeine, and exercise, except for normal daily activities, for 3 days before the inpatient study. The protocol was reviewed and approved by the Yale University Human Investigation Committee. Informed written consent was obtained from each subject.

**Study protocol.** The subjects were admitted to the Yale–New Haven Hospital GCRC between 3:00 and 4:00 P.M. on the day of study. At 5:00 P.M., a liquid meal consisting of 15 kcal/kg body wt (60% carbohydrate, 20% fat, and 20% protein) was ingested over a 15-min period. At 10:00 P.M., a catheter was placed in the antecubital vein for blood collection.

The subjects were brought by wheelchair to the Yale Magnetic Resonance Center at 10:45 P.M. They were placed in a 2.1 T NMR spectrometer (Bruker Biospec Spectrometer; Billerica, MA), where liver glycogen concentrations were measured periodically from 11:00 P.M. to 7:00 A.M. the next morning with <sup>13</sup>C NMR spectroscopy. The subjects also drank 5 ml/kg body water of <sup>2</sup>H<sub>2</sub>O (99.9% <sup>2</sup>H; Isotec, Miamisburg, OH), divided into four equal portions and given 45 min apart. The first dose was given at midnight and the last dose at 2:15 A.M. to minimize any side effects (8). From 2:30 until 8:30 A.M., blood was drawn every 90 min for the determination of <sup>2</sup>H enrichment of the hydrogen bound to C2 and C5 of blood glucose. Water ingested ad libitum during the fast was enriched by 0.5% with <sup>2</sup>H<sub>2</sub>O to maintain isotopic steady state.

Subjects were returned by wheelchair to the GCRC at 7:00 A.M. A second catheter was then placed retrogradely in a hand vein for blood collection. This hand was kept in a hot box at 70°C to arterialize the blood samples, and basal samples were drawn for determining plasma glucose, insulin, C-peptide, free fatty acid, glucagon, lactate, and cortisol concentrations. At 7:30 A.M., the catheter placed earlier in the antecubital vein was used for 4-h infusion of [6,6-<sup>2</sup>H<sub>2</sub>]glucose (Cambridge Isotopes Laboratories, Andover, MA), which was administered as a prime adjusted for fasting plasma glucose concentration (range 260–990 μmol/m<sup>2</sup>) and then continuous infusion (2.28–6.68 μmol · m<sup>-2</sup> · min<sup>-1</sup>). During the final 40 min of the 4-h infusion period, blood samples were drawn at 10-min intervals for the determination of enrichment of plasma [6,6-<sup>2</sup>H<sub>2</sub>]glucose, plasma glucose, and other hormone concentrations (as determined during the basal period, before the start of the infusion). Continuous indirect calorimetry was performed to determine whole-body glucose oxidation as previously described (9). At the end of infusion, urine was collected for the measurement of nitrogen excretion.

Liver volume was measured on admission by clinical imaging in a 1.5 T magnet (Sigma II; General Electric, Milwaukee, WI) using multiecho axial scanning (TE 20/80, TR 2000). The data were transferred to an independent workstation (ISG, Toronto, Ontario, Canada) and processed with the CAMRA S200 program (ISG) that provided a three-dimensional image reconstruction and volume determinations. Accuracy of the measurement was assessed with water-filled phantoms of known volume and was determined to be  $\pm 5\%$  with a coefficient of variation of  $\pm 1\%$ . In one diabetic subject and three control subjects, the liver volumes were not obtained, so the average of liver volumes of all other subjects (1.7 L) was used for the calculation of net hepatic glycogenolysis in these subjects.

The diabetic subjects were started on metformin 850 mg once a day after the baseline study. After 1 week, the dose was increased to twice a day, and in the third week, it was increased to 850 mg three times a day. This stepwise increase was done to minimize any gastrointestinal side effects of metformin. Subjects returned to the GCRC monthly, where their weight, plasma fasting glucose, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, creatinine, and glycosylated hemoglobin levels were obtained. They were instructed to maintain their weight stable during the course of study and to continue their routine activities as before, without performing any strenuous exercise. After 3 months of metformin therapy, they were again admitted to the GCRC to undergo the same protocol as before.

**<sup>13</sup>C NMR spectroscopy.** Subjects were placed supine in the NMR spectrometer from 11:00 P.M. until 7:00 A.M. and were given breaks as needed to minimize discomfort. <sup>13</sup>C NMR spectra were obtained with a 9-cm circular <sup>13</sup>C observation coil, and a 12 × 14-cm coplanar butterfly <sup>1</sup>H decoupler coil placed rigidly over the lateral aspect of the abdomen, as previously described (10). In brief, initial coil placement was determined by percussion of the borders

of the liver, and the position was confirmed by imaging the liver from the surface coil with a multislice gradient echo image. Before taking the subjects out for breaks, the position of the coil was marked on the abdomen and an image was obtained to confirm the coil position, when the measurements were to be restarted. The magnet was shimmed with the water signal obtained from the decoupling coil. Localized <sup>13</sup>C NMR liver spectra were obtained with a modified one-dimensional inversion-based sequence for surface suppression. On the basis of <sup>1</sup>H density profiles of the sequence, <15% of the glycogen signal could arise from tissues within 2.5 cm of the surface of the subject, assuming equal glycogen concentration throughout the volume of observation. In each subject, all skin and muscles were within 2.5 cm of the surface as assessed by the image. Spectra were processed with a 30-Hz Lorentzian-to-Gaussian filter and a 500-Hz convolution difference. After processing, the glycogen line width was 70–90 Hz. Resonance intensity was measured by integrating over a band width of  $\pm 120$  Hz. The hepatic glycogen concentration was determined by comparing the signal intensity obtained with the localized spectroscopy sequence in vivo with the signal intensity obtained from a volume-selected rectangular phantom containing a solution of KCl (50 mmol/l) and glycogen (150 mmol/l) at the same coordinates relative to the coil. The phantom was raised 2.5 cm above the surface of the coil to simulate liver depth. Spectrometer sensitivity was normalized between phantom and in vivo measurements with the signal from the formate sphere as a calibration standard. Resonances from the C-1 glucosyl unit of liver glycogen have been shown to be  $\sim 100\%$  visible in vivo by this method. Reproducibility of the glycogen concentration measurement has been assessed in an earlier study, with a coefficient of variation between the pairs of measurements of  $\sim 7\%$ . The standard deviation in the <sup>13</sup>C NMR glycogen concentration measurement due to spectral noise was  $\pm 5\%$  (6).

**Analyses.** Plasma glucose concentrations were measured by a glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Palo Alto, CA). Plasma lactate concentrations were measured using a lactate dehydrogenase method (11). Plasma insulin, glucagon, C-peptide, and cortisol concentrations were measured using commercial double-antibody radioimmunoassay kits (insulin: Diagnostic Systems Laboratories, Webster, TX; glucagon: Linco Research, St. Charles, MO; cortisol: Diagnostic Product, Los Angeles, CA). Plasma free fatty acid concentrations were determined using a microfluorimetric method (12).

**<sup>2</sup>H<sub>2</sub>O method.** Body water was calculated as 50% of body weight in women and 60% in men (13). Enrichments of the hydrogen at C2 and C5 of blood glucose were determined by procedures previously described (7,8). ZnSO<sub>4</sub> (0.3N) and Ba(OH)<sub>2</sub> (0.3N) were added to the blood to precipitate protein and the mixture was centrifuged. The supernatant was deionized by passage through a column of AG 1–8 X in the formate form and AG 50W–8 X in the hydrogen form (Bio-Rad Laboratories, Hercules, CA). Glucose in the effluent was isolated by high-performance liquid chromatography using a Bio-Rad HPX–87P column with water as solvent at 80°C and a flow rate of 0.5 ml/min. An aliquot of the glucose was converted to xylose. The xylose was oxidized with periodate to yield C5 of glucose with its hydrogen in formaldehyde. Hexamethylenetetramine (HMT) was prepared from the formaldehyde and assayed for mass m+1 using a gas chromatograph–mass spectrometer (GCMS) (7,8,14). HMTs from [1-<sup>2</sup>H]sorbitol of known enrichments 0.125 to 1.0% provided standards for the assays. Another aliquot of the glucose was converted to ribulose-5-phosphate, which was reduced to a mixture of ribitol-5-phosphate and arabitol-5-phosphate. These were also oxidized with periodate, yielding formaldehyde containing C2 with its hydrogen. Again the formaldehyde was condensed to form an HMT, which was assayed by GCMS for mass m+1. Enrichments of plasma water were determined by Metabolic Solutions, (Nashua, NH), using an isotope ratio mass spectrometer. Plasma water weight was assumed to be 94% of plasma volume (7,15).

For determining glucose production, plasma glucose collected at 10-min intervals in the last hour of infusion of [6,6-<sup>2</sup>H<sub>2</sub>]glucose infusion was derivatized as the penta-acetate, following Ba(OH)<sub>2</sub>/ZnSO<sub>4</sub> deproteinization and semipurification by anion/cation exchange chromatography (AG 1–8X, AG 50W–8X; Bio-Rad Laboratories, Richmond, CA), as previously described (16). GCMS analysis was performed with a Hewlett-Packard 5890 gas chromatograph (HP-1 capillary column, 12 mm × 0.2 mm × 0.33 μm film thickness) interfaced to a Hewlett-Packard 5971A mass-selective detector operating in the electron impact ionization mode. For glucose–penta-acetate, GCMS analysis was isothermal at 200°C. Selected ion monitoring was used to determine enrichment in various molecular ion fragments. [6,6-<sup>2</sup>H<sub>2</sub>]Glucose m+2 enrichment was determined from the ratio of m/z 202 to 200 of fragment ion consisting of C2→C6 (17).

#### Calculations

**Endogenous glucose production rates.** Rate of endogenous glucose production (mmol · m<sup>-2</sup> · min<sup>-1</sup>) equals the infusion rate × [(enrichment<sup>inf</sup>/enrichment<sup>plasma</sup>) – 1]. The infusion rate is the rate that [6,6-<sup>2</sup>H<sub>2</sub>]glucose is infused

TABLE 1

Enrichments in plasma glucose after 200–230 min of [6,6-<sup>2</sup>H<sub>2</sub>]glucose infusion as a percentage of enrichment at 240 min (11:30 A.M.)\*

| Subjects                           | n | Time (min)  |            |             |             |
|------------------------------------|---|-------------|------------|-------------|-------------|
|                                    |   | 200         | 210        | 220         | 230         |
| Control subjects                   | 7 | 98.9 ± 4.3  | 104 ± 5.5  | 99.3 ± 3.3  | 97.4 ± 3.8  |
| Diabetic subjects before metformin | 7 | 104.3 ± 4.5 | 99 ± 6.0   | 102.8 ± 3.4 | 106.8 ± 5.6 |
| Diabetic subjects after metformin  | 7 | 94 ± 1.0    | 92.8 ± 3.0 | 95 ± 3.2    | 98.8 ± 2.7  |

Data are means ± SE. \*Mean percent excess enrichments at 240 min in control subjects was 1.14%; in diabetic subjects premetformin, 0.82%; and in diabetic subjects postmetformin 1.02%. Natural abundance was less than one-seventh of these enrichments.

(mmol · m<sup>-2</sup> · min<sup>-1</sup>), enrichment<sup>inf</sup> is the enrichment of the hydrogen bound to carbon 6 of the [6,6-<sup>2</sup>H<sub>2</sub>] glucose (i.e., 99%) and enrichment<sup>plasma</sup> is the percent enrichment in the hydrogen bound to carbon 6 of plasma glucose. For enrichment<sup>plasma</sup>, the mean of the enrichments in the plasma glucose collections at 10-min intervals, for 40 min ending at 11:30 A.M., 240 min into the infusion, was used. In Table 1, enrichments in glucose collected at 200–230 min are recorded as percents of the enrichments at 240 min.

**<sup>13</sup>C NMR-determined rates of net hepatic glycogenolysis and gluconeogenesis.** The slope of the decrease in the liver glycogen concentration (mmol/l) from the maximum liver glycogen concentration ~5–6 h after ingestion of the meal to the concentration measured at 7:00 A.M. after ~14 h of fasting, was multiplied by the liver volume (liters) and divided by body surface area (meters squared) to calculate the rate of net hepatic glycogenolysis, in mmol · m<sup>-2</sup> · min<sup>-1</sup>. The NMR-determined rate of gluconeogenesis (as defined by the flux of three carbon units into glucose) was calculated as the difference between the rate of glucose production and net hepatic glycogenolysis.

**<sup>2</sup>H<sub>2</sub>O-determined rates of total hepatic glycogenolysis and gluconeogenesis.** Fractional contributions of gluconeogenesis and total hepatic glycogenolysis were estimated from the ratios of the <sup>2</sup>H enrichments of the hydrogens bound to C5 and C2 of blood glucose after oral intake of <sup>2</sup>H<sub>2</sub>O (7,8). Rates of gluconeogenesis were calculated by multiplying those fractional contributions by the rates of glucose production. The rate of total hepatic glycogenolysis was calculated by subtracting the rates of gluconeogenesis from the rate of glucose production.

**Rates of hepatic glycogen cycling.** Because the NMR method directly measures rates of net hepatic glycogenolysis, and because the <sup>2</sup>H<sub>2</sub>O method measures rates of total hepatic glycogenolysis (net hepatic glycogenolysis + hepatic glycogen cycling), estimates of hepatic glycogen cycling were made by subtracting rates of net hepatic glycogenolysis (NMR measured) from the rates of total hepatic glycogenolysis (<sup>2</sup>H<sub>2</sub>O measured).

**Glucose clearance rate.** Glucose clearance rate (ml · m<sup>-2</sup> · min<sup>-1</sup>) was calculated as the rate of glucose disappearance divided by the plasma glucose concentration.

**Statistics.** Data are expressed as means ± SE. Differences between groups at baseline were assessed by the Student's *t* test and paired *t* test for differences in the diabetic subjects before and after treatment.

## RESULTS

**Subject characteristics and chemistries.** Baseline characteristics of control and diabetic subjects are shown in Table 2. Weight, age, and body surface area were not significantly different between the two groups. There was also no significant difference in insulin concentrations, but the diabetic subjects were relatively insulinopenic in the presence of a threefold higher fasting plasma glucose concentration (Table 3). Plasma triglyceride levels were significantly higher in the diabetic subjects. Plasma concentrations of glucagon, lactate, cortisol, free fatty acids, total cholesterol, HDL, and LDL were similar.

**Endogenous glucose production and clearance rate.** The rate of endogenous glucose production in the diabetic subjects was twice that of the control subjects. (Table 4). The glucose clearance rate was significantly decreased in diabetic subjects compared with that in the control subjects

(46.9 ± 4.5 ml · m<sup>-2</sup> · min<sup>-1</sup> in diabetic subjects vs. 64.3 ± 5.1 ml · m<sup>-2</sup> · min<sup>-1</sup> in control subjects, *P* = 0.02).

**NMR results.** Two of the seven diabetic subjects could not complete both sets of the NMR study, so only their NMR data were excluded from the final analysis. Hepatic glycogen content was lower in the diabetic subjects than in the control subjects (at 12:00 A.M.: 200 ± 13 vs. 288 ± 12 mmol/l in control subjects, *P* = 0.0005. At 7:00 A.M.: 153 ± 16 vs. 207 ± 13 mmol/l in control subjects, *P* = 0.02 [Fig. 1]). Rates of net hepatic glycogenolysis during the overnight fast were linear in both groups. Diabetic subjects had a 46% lower rate of net hepatic glycogenolysis than the control subjects (0.120 mmol/l of liver volume per min vs. 0.221 mmol/l of liver volume per min in control subjects, *P* = 0.02). On the basis of total liver volume and body surface area, the diabetic subjects had an ~40% reduction in the rate of net hepatic glycogenolysis compared with control subjects (*P* = 0.08) (Table 4). The NMR-determined rate of gluconeogenesis was threefold higher in the diabetic subjects than in the control subjects.

**<sup>2</sup>H<sub>2</sub>O results.** The fractional contributions of gluconeogenesis to glucose production, expressed as the C5-to-C2 ratio (%), gradually increased during the overnight fast (Table 3). However, there was no more than a small difference in the percentages between the diabetic and control subjects. The rate of gluconeogenesis using the C5-to-C2 ratio at 8:30 A.M. was approximately twofold higher in the diabetic subjects than in the control subjects (Table 4). Because the C5-to-C2 ratio was similar in control and diabetic subjects, the rate of total hepatic glycogenolysis was also approximately twofold higher in the diabetic subjects. Enrichment of the hydrogen

TABLE 2

Study subjects' characteristics

| Characteristic                      | Control subjects | Diabetic subjects | Diabetic subjects |
|-------------------------------------|------------------|-------------------|-------------------|
|                                     |                  | premetformin      | postmetformin     |
| n                                   | 7                | 7                 | 7                 |
| Age (years)                         | 46 ± 2           | 52 ± 5            | 52 ± 5            |
| Weight (kg)                         | 86 ± 8           | 90 ± 6            | 91 ± 6            |
| BMI (kg/m <sup>2</sup> )            | 27 ± 1           | 29 ± 1            | 29 ± 1            |
| Body surface area (m <sup>2</sup> ) | 2.0 ± 0.1        | 2.1 ± 0.1         | 2.1 ± 0.1         |
| Fasting plasma glucose (mmol/l)     | 5.6 ± 0.2        | 15.5 ± 1.3*       | 10.8 ± 0.9†       |
| Ghb (%)                             | 5.9 ± 0.3        | 14.1 ± 1.2*       | 9.7 ± 0.5‡        |

Data are means ± SE. \**P* < 0.0001 vs. control subjects; †*P* = 0.0002 vs. premetformin; ‡*P* = 0.02 vs. premetformin.

TABLE 3  
Plasma metabolite concentrations

|                           | Control subjects | Diabetic subjects premetformin | Diabetic subjects postmetformin |
|---------------------------|------------------|--------------------------------|---------------------------------|
| Insulin (pmol/l)          | 63 ± 8           | 77 ± 13                        | 88 ± 13                         |
| C-peptide (nmol/l)        | 0.67 ± 0.11      | 0.70 ± 0.10                    | 0.75 ± 0.10                     |
| Glucagon (ng/l)           | 48 ± 6           | 64 ± 7                         | 60 ± 5                          |
| Lactate (mmol/l)          | 0.65 ± 0.09      | 0.93 ± 0.14                    | 1.13 ± 0.25                     |
| Free fatty acids (μmol/l) | 639 ± 65         | 894 ± 132                      | 627 ± 89*                       |
| Cortisol (nmol/l)         | 475 ± 57         | 570 ± 103                      | 472 ± 79                        |
| Cholesterol (mmol/l)      | 5.5 ± 0.3        | 5.8 ± 0.5                      | 4.4 ± 0.2†                      |
| Triglycerides (mmol/l)    | 1.1 ± 0.3        | 2.6 ± 0.6‡                     | 2.1 ± 0.5                       |
| HDL (mmol/l)              | 1.4 ± 0.3        | 1.1 ± 0.04                     | 1.0 ± 0.06                      |
| LDL (mmol/l)              | 3.5 ± 0.4        | 3.2 ± 0.3                      | 2.4 ± 0.2                       |

Data are means ± SE. \**P* = 0.01 vs. premetformin; †*P* = 0.04 vs. premetformin; ‡*P* = 0.04 vs. control subjects.

bound to C2 of blood glucose at 8:30 A.M. was 93 ± 2.5% of the enrichment in plasma water in the control subjects, 95.3 ± 1.7% in the diabetic subjects before treatment, and 94.6 ± 2.3% after treatment. This is in accord with essentially complete equilibration of the hydrogen bound to carbon 2 of glucose with that in body water (7,8). Measurements of the C5-to-C2 ratios were made during the period that glycogen contents were measured (i.e., from 7 to 15 h of fasting). However, technical limitations prevented measurement of glucose production during that time. Instead, production was measured during the 18th h of fasting. Those differences in time could affect the quantitation to some extent, but not the conclusions from those quantitations. It is known that glucose production decreases with duration of fasting. In normal subjects, it decreased ~20% from 14 to 22 h of fasting (7). In type 2 diabetic subjects with fasting plasma glucose concentrations ~10 mmol/l, production declined 25% from 17 to 22 h of fasting (A. Wajngot, V.C., W.C.S., K. Ekberg, P.K. Jones, S. Efendic, B.R.L., unpublished data). Therefore, at 8:30 A.M. production might have been 10–15% more than that at 11:30 A.M. However, m+2 enrichments used to estimate production were not different over a 40-min interval ending at 11:30 A.M. (Table 1). The C5-to-C2 ratio also increased with time (Table 5), by perhaps 1–2% per hour, and the ratio in glucose

TABLE 4  
Hepatic glucose metabolism

|                                                                                                                                          | Control subjects | Diabetic subjects premetformin | Diabetic subjects postmetformin |
|------------------------------------------------------------------------------------------------------------------------------------------|------------------|--------------------------------|---------------------------------|
| Endogenous glucose production (mmol · m <sup>-2</sup> · min <sup>-1</sup> )                                                              | 0.36 ± 0.03      | 0.70 ± 0.05*                   | 0.53 ± 0.03†                    |
| Net hepatic glycogenolysis (by <sup>13</sup> C NMR) (mmol · m <sup>-2</sup> · min <sup>-1</sup> )                                        | 0.18 ± 0.03      | 0.11 ± 0.02                    | 0.15 ± 0.02                     |
| Total hepatic glycogenolysis (by <sup>2</sup> H <sub>2</sub> O method) (mmol · m <sup>-2</sup> · min <sup>-1</sup> )                     | 0.16 ± 0.02      | 0.28 ± 0.02‡                   | 0.25 ± 0.02                     |
| Glycogen cycling (mmol · m <sup>-2</sup> · min <sup>-1</sup> )                                                                           | -0.02 ± 0.03     | 0.17 ± 0.03¶                   | 0.10 ± 0.04                     |
| Gluconeogenesis (by <sup>13</sup> C NMR) (mmol · m <sup>-2</sup> · min <sup>-1</sup> )                                                   | 0.18 ± 0.03      | 0.59 ± 0.07§                   | 0.37 ± 0.03                     |
| Gluconeogenesis (minus hepatic glycogen cycling) (by <sup>2</sup> H <sub>2</sub> O method) (mmol · m <sup>-2</sup> · min <sup>-1</sup> ) | 0.20 ± 0.02      | 0.42 ± 0.04¶                   | 0.28 ± 0.03#                    |

Data are means ± SE. \**P* < 0.0001 vs. control subjects; †*P* = 0.0009 vs. premetformin; ‡*P* = 0.0002 vs. control subjects; §*P* = 0.0001 vs. control subjects; ||*P* = 0.01 vs. premetformin; ¶*P* = 0.0005 vs. control subjects; #*P* = 0.0002 vs. premetformin.

collected at 8:30 A.M. was then a composite of the ratios in glucose entering the blood at that time with decreasing amounts from the earlier times. Thus, the ratio at 8:30 A.M., used for calculation, is also an underestimate by a small amount. Justification for assuming a constant rate of net hepatic glycogenolysis, from midnight to 7 A.M., is evidenced in the figure.

**Hepatic glycogen cycling.** There was no detectable hepatic glycogen cycling in the control subjects (Table 4). In contrast, there was a significant amount of hepatic glycogen cycling in the poorly controlled type 2 diabetic subjects, accounting for ~25% of endogenous glucose production (Table 4).

**Indirect calorimetry.** There were no significant differences between the two groups in the basal rates of carbohydrate, lipid, or protein oxidation (data not shown).

**Metformin treatment.** After 3 months of metformin treatment, there was a 30% decrease in fasting plasma glucose concentration and glycosylated hemoglobin levels compared with the pretreatment values (Table 2). There was no significant change in body weight or plasma insulin, C-peptide, glucagon, lactate, and cortisol levels. Metformin treatment resulted in a 24% reduction in cholesterol and a 30% reduction in free fatty acid concentrations in plasma (Table 3).

Three months of metformin treatment also lowered post-treatment glucose production by 25% in comparison with pretreatment (Table 4). There was no significant increase in the glucose clearance rate (46.9 ± 4.5 ml · m<sup>-2</sup> · min<sup>-1</sup> before metformin vs. 50.9 ± 3.6 ml · m<sup>-2</sup> · min<sup>-1</sup> after metformin). Both hepatic glycogen content and the rate of net hepatic glycogenolysis tended to increase with metformin treatment, as determined by <sup>13</sup>C NMR. There was no significant change in the rate of total hepatic glycogenolysis as determined indirectly by the <sup>2</sup>H<sub>2</sub>O method. The rate of gluconeogenesis as determined by <sup>13</sup>C NMR was 37% less after treatment. Similarly, there was a 33% reduction in the rate of gluconeogenesis measured with the <sup>2</sup>H<sub>2</sub>O method. There was also a trend for metformin to cause a 40% reduction in rates of hepatic glycogen cycling (Table 4). Metformin treatment did not significantly change the rates of carbohydrate, lipid, or protein oxidation (data not shown).

## DISCUSSION

Increased rates of glucose production are strongly correlated with increased fasting plasma glucose concentrations in patients with type 2 diabetes (18–21). We found that the rate of glucose production was increased twofold in the diabetic subjects as compared with that in the control subjects, which

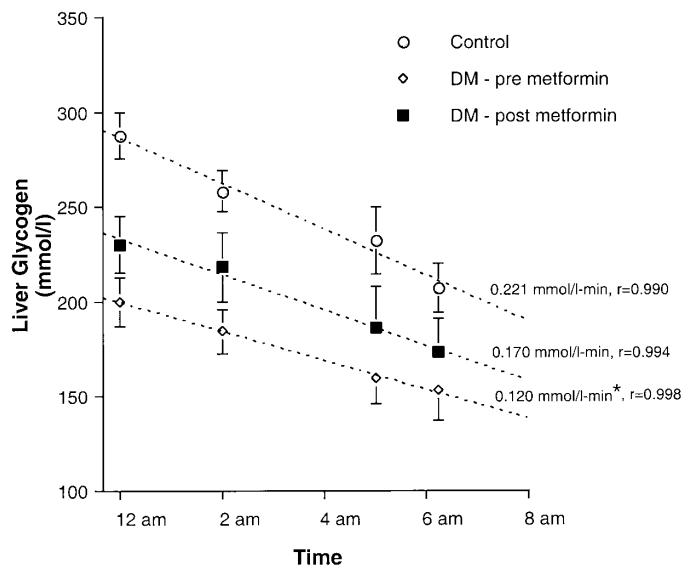


FIG. 1. Serial  $^{13}\text{C}$  NMR measurements of hepatic glycogen concentration during the overnight fast in control and diabetic subjects before and after metformin treatment (net hepatic glycogenolysis in  $\text{mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$ , \* $P = 0.02$ ).

could be entirely attributed to an approximately threefold increase in the rate of gluconeogenesis as assessed by the  $^{13}\text{C}$  NMR method and mostly to a roughly twofold increase in the rate of gluconeogenesis as assessed by the  $^2\text{H}_2\text{O}$  method. These results are consistent with our previous study examining the rates of gluconeogenesis in normal and poorly controlled type 2 diabetic subjects, using  $^{13}\text{C}$  NMR (23). Metformin treatment resulted in a 25–30% reduction in fasting plasma glucose concentrations and glucose production, which is consistent with the results of previous studies (2,22,24,25). This decrease in glucose production could be entirely accounted for by a reduction in the rates of gluconeogenesis as determined by both methods.

Because the NMR method measures rates of net hepatic glycogenolysis, whereas the  $^2\text{H}_2\text{O}$  method measures rates of total hepatic glycogenolysis (net hepatic glycogenolysis + hepatic glycogen cycling), estimates of hepatic glycogen cycling were made by subtracting rates of net hepatic glycogenolysis (NMR measured) from the rates of total hepatic glycogenolysis ( $^2\text{H}_2\text{O}$  measured). There was no detectable hepatic glycogen cycling in the control subjects, which is consistent with an earlier study using these two methods (26). In contrast, there was considerable glycogen cycling in the poorly controlled diabetic subjects, which accounted for ~25% of endogenous glucose production (Table 4). Hyperglucagonemia and hyperinsulinemia, which are typically present in patients with type 2 diabetes, have both been shown to promote glycogen cycling (27–29). Although the plasma concentrations of these hormones tended to be higher in the diabetic subjects than in the control subjects, the differences were not significant; however, it is likely that differences in portal vein concentrations of these hormones were much greater.

Three different studies have previously examined the effect of metformin on the rates of net hepatic glycogenolysis and gluconeogenesis in patients with type 2 diabetes with conflicting results. Stumvoll et al. (3) studied 10 obese diabetic

TABLE 5  
Serial C5/C2 measurements (%)

| Time      | Control subjects | Diabetic subjects premetformin | Diabetic subjects postmetformin |
|-----------|------------------|--------------------------------|---------------------------------|
| 2:30 A.M. | 46.4 ± 3.4       | 46.2 ± 2.4                     | 49.0 ± 6.1                      |
| 4:00 A.M. | 51.5 ± 1.9       | 46.0 ± 2.7                     | 49.4 ± 5.2                      |
| 5:30 A.M. | 49.8 ± 2.7       | 55.6 ± 1.1                     | 50.0 ± 5.0                      |
| 7:00 A.M. | 49.6 ± 1.8       | 55.7 ± 2.7                     | 53.3 ± 4.8                      |
| 8:30 A.M. | 55.5 ± 2.7       | 59.1 ± 2.5                     | 52.3 ± 4.7                      |

Data are means ± SE.

individuals before and after 16 weeks of treatment with metformin (2,550 mg/day), using  $[3-^{14}\text{C}]$ lactate to estimate the rates of gluconeogenesis. Using this approach, these investigators found that metformin decreased endogenous glucose production through a 37% reduction in rates of gluconeogenesis. However, because of unknown dilution of the  $^{14}\text{C}$  label in the tricarboxylic acid cycle, this approach does not accurately quantify rates of gluconeogenesis. In addition, the diabetic subjects lost on average ~3 kg of body wt during this study, which may have obscured the independent role of metformin in lowering the rates of glucose production.

Cusi et al. (4) used the same  $[3-^{14}\text{C}]$ lactate approach to assess rates of gluconeogenesis in 20 type 2 diabetic subjects before and after 15 weeks of metformin treatment (2,500 mg/day) in a randomized double-blind placebo-controlled trial. Fifteen of the subjects were taking sulfonylureas at the time of enrollment. This medication was continued during the trial, and metformin was added. These investigators reported that metformin treatment significantly decreased glucose production, but they found no change in the contribution of gluconeogenesis from lactate. They therefore concluded that metformin decreased glucose production by inhibiting hepatic glycogenolysis. It is unclear why these investigators arrived at a result opposite to that of Stumvoll et al. (3) despite using the identical approach.

More recently, Christiansen et al. (5) examined the mechanism of metformin's action in diabetic subjects using mass isotopomer distribution analysis to estimate the rates of gluconeogenesis using  $[2-^{13}\text{C}]$ glycerol. Five obese subjects (BMI  $38 \pm 3$ ) with type 2 diabetes were studied before and after 4 weeks of treatment with metformin. In this study, gluconeogenesis contributed only ~24% of overall glucose production in the diabetic subjects. These investigators therefore concluded that an increased rate of glycogenolysis was entirely responsible for the increased rate of glucose production, which is contrary to the current and previous  $^{13}\text{C}$  NMR studies (23). These investigators also found that metformin treatment led to a 17% reduction in the rate of glucose production, with no change in the absolute rate of gluconeogenesis and concluded that this occurred through a 21% reduction in hepatic glycogenolysis. It is unclear why Christiansen et al. obtained the opposite results from those obtained in the present study. However, recent studies have demonstrated limitations in their approach, resulting in underestimation of gluconeogenic rates (30–33).

The data from the present study are consistent with in vitro studies demonstrating an inhibitory effect of metformin

on gluconeogenesis. Radziuk et al. (34) reported that metformin inhibited gluconeogenesis in perfused liver, primarily through inhibition of hepatic lactate uptake. Argaud et al. (35) found that metformin decreased ATP concentration in isolated rat hepatocytes. Because ATP is an allosteric inhibitor of pyruvate kinase, these investigators hypothesized that the metformin-induced reduction in glucose production was the result of increased pyruvate kinase flux. However, Large and Beylot (36) observed no decrease in ATP concentration or in the uptake of gluconeogenic precursors by perfused livers obtained from fasted streptozocin-injected diabetic rats after metformin treatment. They hypothesized that metformin decreased gluconeogenic flux through inhibition of pyruvate carboxylase-phosphoenolpyruvate carboxykinase activity and possibly through increased conversion of pyruvate to alanine. However, it should be noted that all of these in vitro studies used very high doses of metformin (250–350 mg/kg), which are 8- to 12-fold higher than the doses used in the treatment of diabetic patients.

Metformin treatment also lowered the plasma free fatty acid concentrations by 30% in the diabetic subjects, which is consistent with the results of some (37–39) but not all previous studies (3,4,40). Plasma free fatty acids have been shown to play an important role in the regulation of hepatic glucose production (41–44). It is possible that reduction in plasma free fatty acid concentration after metformin treatment also contributed to the reduced rates of gluconeogenesis.

In summary, we examined the effect of metformin treatment on rates of total and net hepatic glycogenolysis, gluconeogenesis, and hepatic glycogen cycling in poorly controlled type 2 diabetic patients who were matched to healthy control subjects using  $^{13}\text{C}$  NMR and  $^2\text{H}_2\text{O}$  techniques. We found the following: 1) Poorly controlled diabetic subjects had a twofold increase in rates of glucose production and extensive hepatic glycogen cycling accounting for ~25% of their glucose production; 2) the increased rate of glucose production in the diabetic subjects could be attributed to an increased rate of gluconeogenesis; and 3) metformin treatment in the diabetic subjects decreased rates of glucose production through a reduction in the rate of gluconeogenesis.

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