Plasma C5 Glucose–to–^{2}H$_2$O Ratio Does Not Provide an Accurate Assessment of Gluconeogenesis During Hyperinsulinemic-Euglycemic Clamps in Either Nondiabetic or Diabetic Humans

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OBJECTIVE—Measurement of plasma C2 glucose enrichment is cumbersome. Therefore, the plasma C5 glucose–to–^{2}H$_2$O rather than the plasma C5-to-C2 glucose ratio commonly has been used to measure gluconeogenesis and glycogenolysis during hyperinsulinemic-euglycemic clamps. The validity of this approach is unknown.

RESEARCH DESIGN AND METHODS—Ten nondiabetic and 10 diabetic subjects ingested ^{2}H$_2$O the evening before study. The following morning, insulin was infused at a rate of 0.6 mU · kg^{-1} · min^{-1} and glucose was clamped at ~5.3 mmol/l for 5 h. Plasma C5 glucose, C2 glucose, and ^{2}H$_2$O enrichments were measured hourly from 2 h onward.

RESULTS—Plasma C2 glucose and plasma ^{2}H$_2$O enrichment were equal in both groups before the clamp, resulting in equivalent estimates of gluconeogenesis and glycogenolysis. In contrast, plasma C2 glucose and plasma C5 glucose enrichments fell throughout the clamp, whereas plasma ^{2}H$_2$O enrichment remained unchanged. Since the C5 glucose concentration and, hence, the C5 glucose–to–^{2}H$_2$O ratio is influenced by both gluconeogenesis and glucose clearance, whereas the C5-to-C2 glucose ratio is only influenced by gluconeogenesis, the C5 glucose–to–^{2}H$_2$O ratio overestimated ($P < 0.01$) gluconeogenesis during the clamp. This resulted in biologically implausible negative (i.e., calculated rates of gluconeogenesis exceeding total endogenous glucose production) rates of glycogenolysis in both the nondiabetic and diabetic subjects.

CONCLUSIONS—Plasma C5 glucose–to–^{2}H$_2$O ratio does not provide an accurate assessment of gluconeogenesis in nondiabetic or diabetic subjects during a traditional (i.e., 2–3 h) hyperinsulinemic-euglycemic clamp. The conclusions of studies that have used this approach need to be reevaluated. *Diabetes* **57:1800–1804, 2008**

Measurement of gluconeogenesis in humans is difficult. The deuterated water method is widely used for this purpose (1–17). This method relies on the fact that the fifth carbon of glucose is labeled during gluconeogenesis, whereas the second carbon of glucose is labeled with deuterium during equilibration of glucose-6-phosphate and fructose-6-phoshate (1,2). Therefore, at steady state, the ratio of plasma glucose with deuterium on the fifth carbon (C5 glucose) to plasma glucose labeled on the second carbon (C2 glucose) equals the percentage of plasma glucose derived from gluconeogenesis (1,2). Measurement of C2 glucose enrichment is cumbersome. Since ^{2}H$_2$O and C2 glucose enrichments are equal at steady state, many investigators have used the plasma C5 glucose–to–^{2}H$_2$O ratio to calculate gluconeogenesis after an overnight fast (4–7,9–12,18). The C5 glucose–to–^{2}H$_2$O ratio has also been used to measure gluconeogenesis during glucose clamps (4,6,7,11,12,18). However, the validity of this approach is uncertain. We have reported that the rate of gluconeogenesis measured during the final hour of a 3-h hyperinsulinemic-euglycemic clamp in lean nondiabetic subjects using the C5-to-C2 glucose ratio correlated with that measured in the same subjects using the C5 glucose–to–^{2}H$_2$O ratio (7). However, in those as well as other glucose clamp experiments (4,6,7,11,12,18), gluconeogenesis calculated using the C5 glucose–to–^{2}H$_2$O ratio commonly exceeded total endogenous glucose production. Since endogenous glucose production equals the sum of glucose derived via gluconeogenesis and glycogenolysis, this result was biologically implausible.

Plasma C5 glucose concentrations are determined both by the rate of appearance of C5 glucose into and the rate of disappearance of C5 glucose from the plasma pool. Therefore, the use of the C5 glucose–to–^{2}H$_2$O ratio during a clamp is only accurate when C5 glucose has achieved a new steady state. Since hyperinsulinemic clamps typically are relatively short (e.g., 2–3 h) and the glucose pool large, we became concerned that plasma C5 glucose concentration was artificially elevated because the clearance was not sufficiently rapid for C5 glucose concentration to have reachieved a steady state at a lower concentration. If so, this would be particularly problematic when the C5 glucose–to–plasma ^{2}H$_2$O ratio was used to assess gluconeogenesis in groups in whom insulin action, and therefore glucose clearance, differed. The present experiments addressed this question by measuring both plasma C5-to-C2 glucose and the C5 glucose–to–^{2}H$_2$O ratios in diabetic and non-
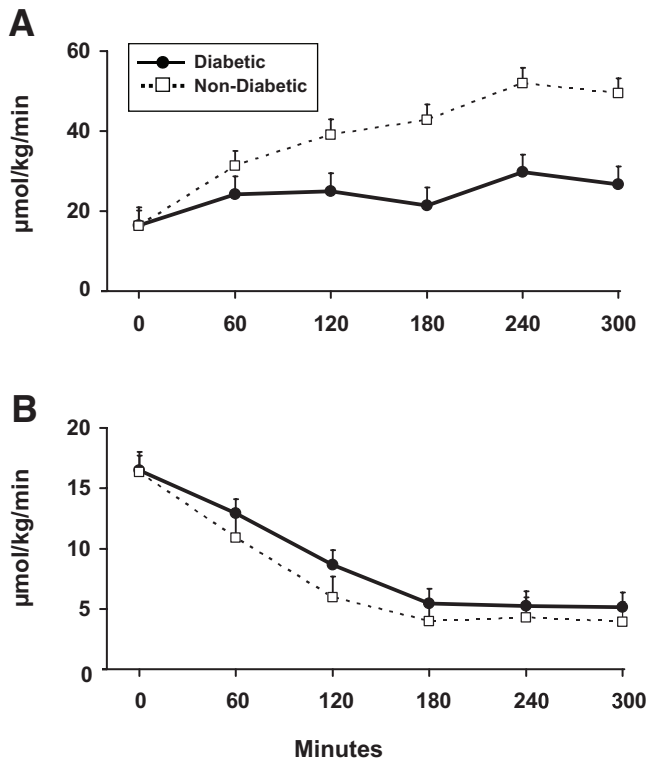
A

B

FIG. 1. Glucose disappearance (*A*) and endogenous glucose production (*B*). An insulin infusion was started at time zero.

diabetic subjects before and every hour from 2 h onward during a 5-h hyperinsulinemic-euglycemic clamp.

RESEARCH DESIGN AND METHODS

Results for this report are derived from 10 nondiabetic subjects and 10 subjects with type 2 diabetes in whom sufficient plasma was available to permit measurement of C5 glucose, C2 glucose, and ^2H_2O enrichment at hourly intervals during a prolonged hyperinsulinemic-euglycemic clamp. Subject characteristics are given in supplemental Table 1 (available in an online appendix at http://dx.doi.org/10.2337/db08-0195).

Details of the experimental design have been described in detail elsewhere (19). Subjects were admitted to the Mayo Clinical Research Unit on the evening before study and given a standard meal at 1700 h and 1.67 g ^2H_2O/kg body water in divided doses at 1800, 2000, and 2200 h. Insulin was infused in the diabetic subjects during the night to maintain glucose at ~5.5 mmol/l. A primed (fasting glucose divided by 5.5 mmol/l times 12 μCi), continuous (0.12 μCi/min) infusion of [3-^3H] glucose was started at 0700 h; infusions of insulin (0.6 mU · kg^{-1} · min^{-1}), somatostatin (60 ng · kg^{-1} · min^{-1}), growth hormone (3 ng · kg^{-1} · min^{-1}), and glucagon (0.65 ng · kg^{-1} · min^{-1}) were started at 1000 h (time 0 min). Glucose containing [3-^3H] glucose was infused in amounts sufficient to maintain euglycemia, as previously described (20). Plasma glucose, insulin, [3-^3H] glucose specific activity, and enrichment of deuterium on the 2nd and 5th carbons of plasma glucose were measured as previously described (1,2,21).

Calculations. Rates are expressed in the figures and text as micromoles per kilogram lean body mass per min. Rates of glucose appearance and disappearance and endogenous glucose production were calculated using the steady-state equations of Steele et al. (22) as previously described (19). Rates of gluconeogenesis were calculated either by multiplying the plasma C5-to-C2 glucose ratio by endogenous glucose production or plasma C5 glucose–to–^2H_2O ratio by the total rate of glucose appearance (2). Glycogenolysis was calculated by subtracting the rate of gluconeogenesis from endogenous glucose production. The results from the diabetic subjects have previously been published, in part, elsewhere (19).

Statistical analysis. Data in the text and figures are expressed as means ± SEM. Student's paired *t* test was used to determine whether rates calculated using the plasma C5-to-C2 glucose ratio differed from those calculated using the plasma C5 glucose/^2H_2O ratio. A *P* value <0.05 was considered statistically significant.

RESULTS

Plasma glucose and insulin concentrations. Plasma glucose concentrations (supplemental Fig. 1 in the nondiabetic and diabetic subjects averaged 5.31 ± 0.16 and 5.41 ± 0.10 mmol/l, respectively, before the clamp and did not change during the clamp. Plasma insulin concentrations in the nondiabetic and diabetic subjects averaged 31 ± 4 and 178 ± 41 pmol/l, respectively, before the clamp and increased to 177 ± 11 and 203 ± 14 pmol/l during the clamp.

Glucose infusion rate required to maintain euglycemia and [3-^3H] glucose specific activity. The glucose infusion rate required to maintain euglycemia increased in both groups during the first 4 h of the clamp and plateaued thereafter. The glucose infusion rate required to maintain euglycemia during the final hour of study was higher (*P* < 0.01) in the nondiabetic than in the diabetic subjects (45.9 ± 3.6 vs. 21.9 ± 5.6 μmol · kg^{-1} · min^{-1}, respectively). Plasma [3-^3H] glucose specific activity remained constant in both groups during the clamp, enabling accurate measurement of glucose turnover (supplemental Fig. 2).

Glucose disappearance and endogenous glucose production. Glucose disappearance increased in both groups during the first 4 h of the clamp and remained unchanged thereafter. In contrast, endogenous glucose production was maximally suppressed in both groups within 3 hours. Glucose disappearance during the final hour of study was higher (*P* < 0.01) in the nondiabetic than diabetic subjects, whereas endogenous glucose production did not differ between groups (Fig. 1).
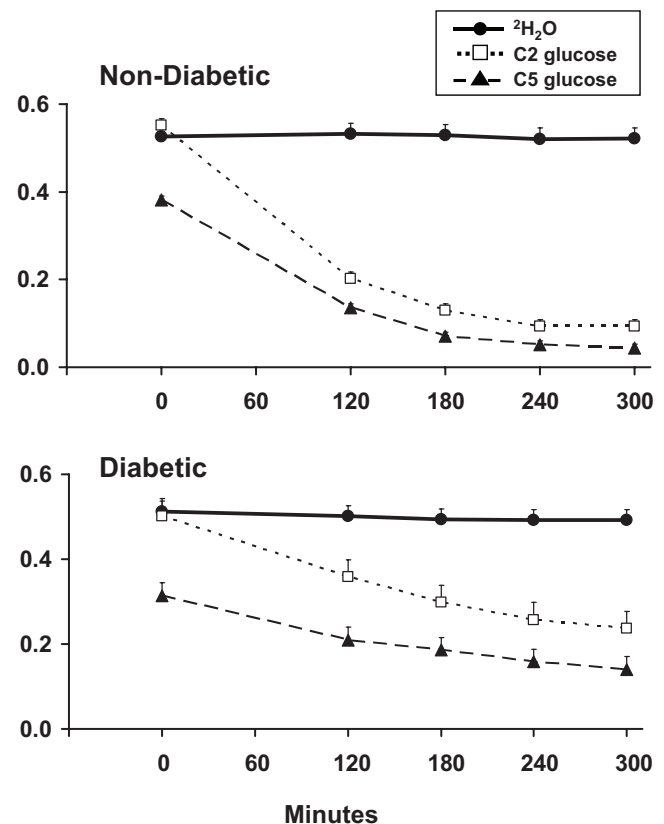

FIG. 2. Plasma enrichment of ^2H_2O, C2 glucose, and C5 glucose observed in the nondiabetic and diabetic subjects. An insulin infusion was started at time zero.
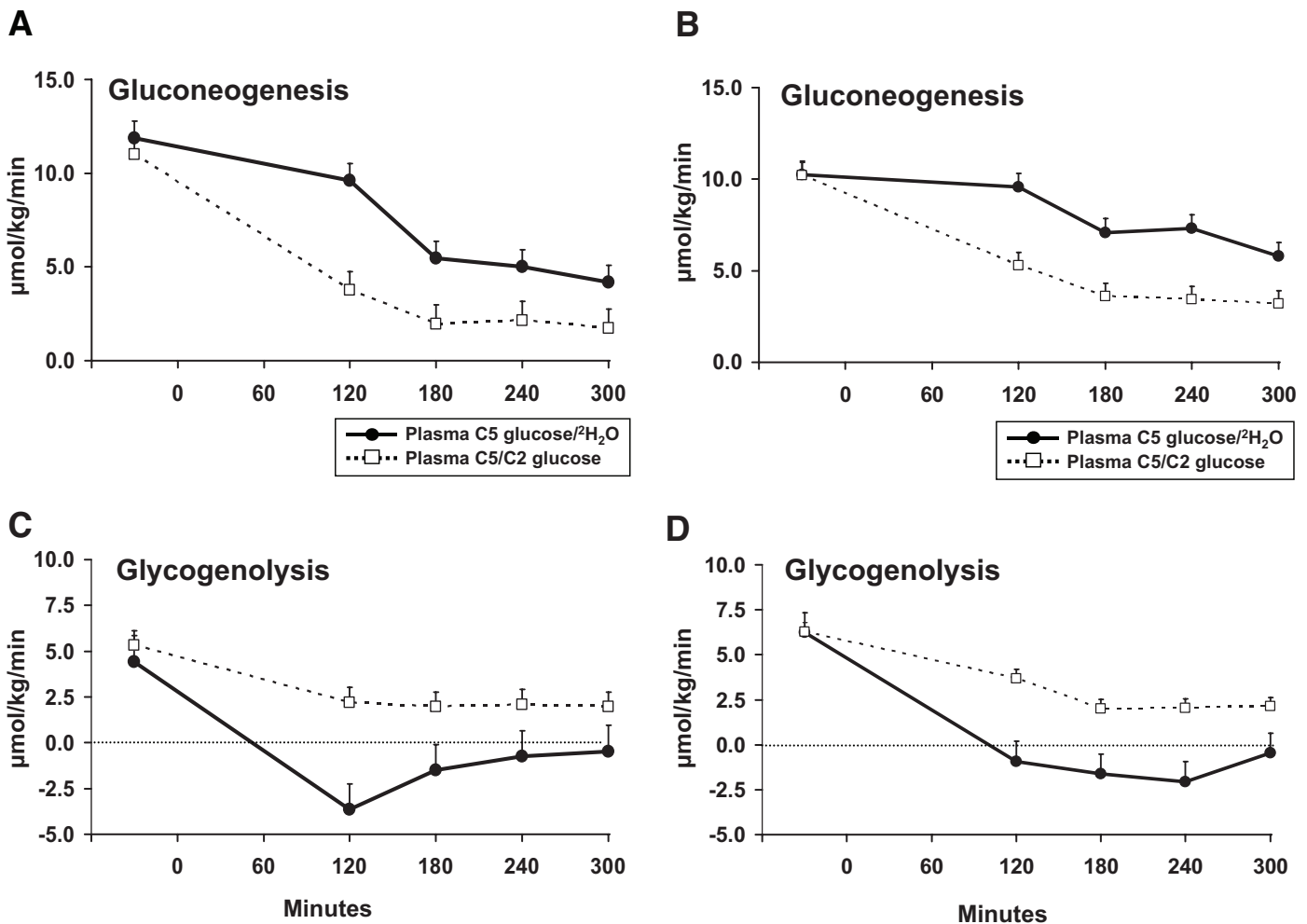
FIG. 3. Rates of gluconeogenesis and glycogenolysis measured in the nondiabetic (*A* and *C*) and diabetic (*B* and *D*) subjects using the plasma C5-to-C2 glucose or plasma C5 glucose–to–^{2}H$_2$O ratios. An insulin infusion was started at time zero.

Plasma ^{2}H$_2$O, C2 glucose, and C5 glucose enrichment. Plasma ^{2}H$_2$O and C2 glucose enrichment did not differ in either the nondiabetic or diabetic subjects before the clamp. Plasma ^{2}H$_2$O enrichment remained unchanged in both groups during the clamp. In contrast, plasma C2 glucose and C5 glucose enrichment decreased ($P < 0.01$) in both groups during the clamp (Fig. 2).

Rates of gluconeogenesis and glycogenolysis calculated using the plasma C5 glucose–to–^{2}H$_2$O and plasma C5-to-C2 glucose ratios. Rates of gluconeogenesis and glycogenolysis measured before the clamp using the plasma C5 glucose–to–^{2}H$_2$O ratio did not differ from those measured using the plasma C5-to-C2 glucose ratio in either group. In contrast, rates of gluconeogenesis measured during the clamp using the plasma C5 glucose–to–^{2}H$_2$O ratio were consistently greater ($P < 0.01$) and glycogenolysis consistently lower ($P < 0.01$) than those measured using the plasma C5-to-C2 glucose ratio in both the nondiabetic and diabetic subjects, with the difference most evident during the first 2 h of the clamp (Fig. 3).

Of note, rates of glycogenolysis calculated using the plasma C5 glucose–to–^{2}H$_2$O ratio were lower ($P < 0.05$) than zero at 120 min in the nondiabetic subjects, indicating that calculated rates of gluconeogenesis exceeded endogenous glucose production. Rates of glycogenolysis calculated using the plasma C5 glucose–to–^{2}H$_2$O ratio converged toward zero thereafter. A similar pattern was observed in the diabetic subjects, with rates of glycogenolysis calculated using the C5 glucose–to–^{2}H$_2$O ratio decreasing to less than zero at 120, 180, and 240 min and converging toward zero thereafter.

Correlations. Rates of gluconeogenesis measured in the nondiabetic subjects using the C5 glucose–to–^{2}H$_2$O ratio correlated ($P < 0.05$) with rates measured using the C5-to-C2 glucose ratio at all time points (Fig. 4). However, the slopes did not equal one, and the y intercepts did not equal zero. Rates of gluconeogenesis measured in the diabetic subjects using the C5 glucose–to–^{2}H$_2$O ratio were not significantly correlated with those measured using the C5-to-C2 glucose ratio until 240 min, implying that the contribution of gluconeogenesis relative to the contribution of glucose clearance to C5 glucose concentration increased with time.

DISCUSSION

The present studies confirm that the C5 glucose–to–^{2}H$_2$O and the C5-to-C2 glucose ratios provide equivalent estimates of gluconeogenesis and glycogenolysis following an overnight fast in both nondiabetic and diabetic subjects. In contrast, use of the C5 glucose–to–^{2}H$_2$O ratio during an euglycemic-hyperinsulinemic clamp systematically overestimates gluconeogenesis and underestimates glycogenolysis.
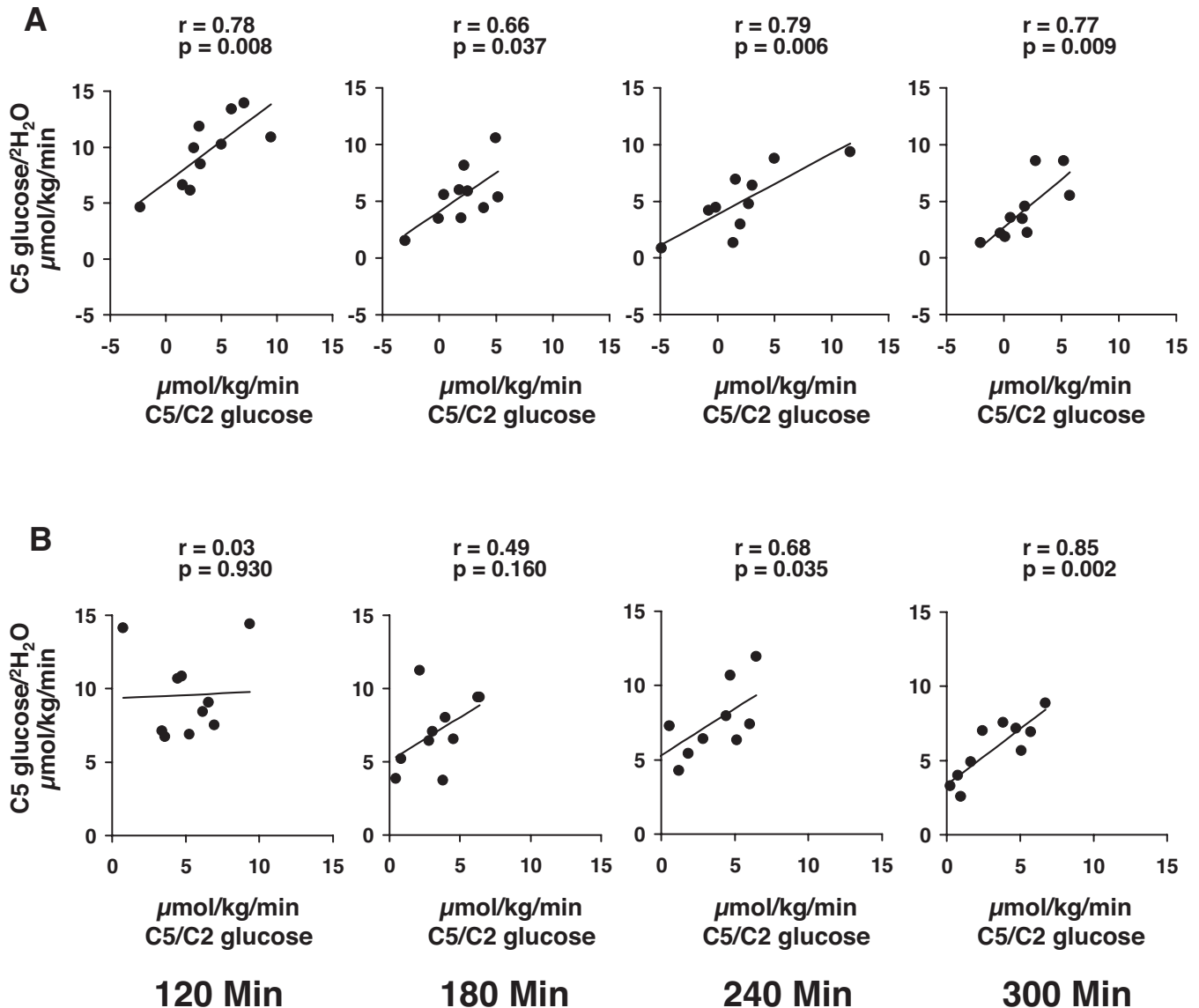
FIG. 4. Correlation between the rates of gluconeogenesis in nondiabetic (*A*) and diabetic (*B*) subjects, measured using the C5 glucose–to–^2H_2O ratio and the C5-to-C2 glucose ratio.

In the presence of ^2H_2O, the fifth carbon of glucose is labeled with deuterium during gluconeogenesis (1,2). The second carbon of glucose is labeled with deuterium during equilibration of glucose-6-phosphate and fructose-6 phosphate (1,2). At equilibrium, the enrichment of deuterium on the second carbon of glucose equals that of plasma water. Since plasma glucose derived from either gluconeogenesis or glycogenolysis passes though the glucose-6-phosphate pool, at steady state either the C5 glucose–to–^2H_2O or the C5-to-C2 glucose ratio equals the proportion of plasma glucose derived from gluconeogenesis (1,2). The situation changes during a hyperinsulinemic-euglycemic clamp, when the rate of release of C5 glucose decreases as a result of suppression of glucose production and the rate of clearance of C5 glucose increases as a result of stimulation of glucose uptake. As is evident from Fig. 2, C5 glucose enrichment fell throughout the 5 h of the study in the diabetic subjects, indicating that equilibrium was never reached. While C5 glucose enrichment also fell during the clamp in the nondiabetic subjects, it appeared to approach steady state during the final hour of

the study. Therefore, plasma C5 glucose concentrations during the first portion of the clamp were higher than those that would be present when a new steady state was eventually achieved. In contrast, since C5 glucose and C2 glucose are cleared in parallel, the C5-to-C2 glucose ratio is only influenced by the rates of release of C5 glucose and C2 glucose, enabling assessment of gluconeogenesis.

Since plasma C5 glucose enrichment is diluted by both endogenously produced and exogenously infused glucose, gluconeogenesis is calculated by multiplying the C5 glucose–to–^2H_2O ratio by total rate of glucose appearance. Use of this approach resulted in rates of gluconeogenesis that were not only greater than those calculated using the C5-toC2 glucose ratio but that were also greater than endogenous glucose production. This yielded biologically implausible (i.e., negative) rates of glycogenolysis. A similar pattern has been observed in previous studies that have used the C5 glucose–to–^2H_2O ratio to calculate gluconeogenesis during a hyperinsulinemic clamp (4,6,7,11,12,18).

The present study suffers from several limitations. Uptake and re-release of glucose (i.e., plasma glucose to hepatic glucose-6-phosphate to plasma glucose) will cause labeling of second carbon of glucose (1,2). This will reduce the C5-to-C2 glucose ratio and will cause an underestimation of gluconeogenesis. Although limited hepatic glucose uptake occurs in the presence of euglycemia (23,24), the impact of such cycling on the C5-to-C2 glucose ratio during a hyperinsulinemic-euglycemic clamp is not known. There is no gold standard with which in vivo measurements of gluconeogenesis can be compared. The present data merely indicate that since both gluconeogenesis and glucose clearance rates influence the C5 glucose concentration, clamps must be conducted for a sufficient duration for plasma C5 glucose enrichment to reachieve a steady state; otherwise, use of the C5 glucose–to–^{2}H$_2$O ratio yields erroneous estimates of both gluconeogenesis and glycogenolysis.

In summary, the present studies indicate that while use of the C5 glucose–to–^{2}H$_2$O and C5-to-C2 glucose ratios provide a comparable estimate of gluconeogenesis after an overnight fast, they do not do so during a traditional 2-h hyperinsulinemic-euglycemic clamp. Use of the C5–to–glucose/^{2}H$_2$O ratio during a glucose clamp overestimates gluconeogenesis and underestimates glycogenolysis. In contrast, since the C5-to-C2 glucose ratio only is influenced by gluconeogenesis, estimates obtained using this ratio will not be altered by changes in glucose clearance. We therefore recommend using the C5-to-C2 glucose ratio to assess gluconeogenesis during a hyperinsulinemic clamp. We also recommend performing the clamp for a sufficient duration (e.g., at least 3 h) so that the effects of insulin on gluconeogenesis have time to become readily evident.

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