Mechanism by which Glucose and Insulin Inhibit Net Hepatic Glycogenolysis in Humans

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Abstract

^{13}C NMR spectroscopy was used to assess flux rates of hepatic glycogen synthase and phosphorylase in overnight-fasted subjects under one of four hypoglucagonemic conditions: protocol I, hyperglycemic (~ 10 mM) –hypoinsulinemia (~ 40 pM); protocol II, euglycemic (~ 5 mM) –hyperinsulinemia (~ 400 pM); protocol III, hyperglycemic (~ 10 mM) –hyperinsulinemia (~ 400 pM); and protocol IV; euglycemic (~ 5 mM) –hypoinsulinemia (~ 40 pM). Inhibition of net hepatic glycogenolysis occurred in both protocols I and II compared to protocol IV but via a different mechanism. Inhibition of net hepatic glycogenolysis occurred in protocol I mostly due to decreased glycogen phosphorylase flux, whereas in protocol II inhibition of net hepatic glycogenolysis occurred exclusively through the activation of glycogen synthase flux. Phosphorylase flux was unaltered, resulting in extensive glycogen cycling. Relatively high rates of net hepatic glycogen synthesis were observed in protocol III due to combined stimulation of glycogen synthase flux and inhibition of glycogen phosphorylase flux. In conclusion, under hypoglucagonemic conditions: (*a*) hyperglycemia, per se, inhibits net hepatic glycogenolysis primarily through inhibition of glycogen phosphorylase flux; (*b*) hyperinsulinemia, per se, inhibits net hepatic glycogenolysis primarily through stimulation of glycogen synthase flux; (*c*) inhibition of glycogen phosphorylase and the activation of glycogen synthase are not necessarily coupled and coordinated in a reciprocal fashion; and (*d*) promotion of hepatic glycogen cycling may be the principal mechanism by which insulin inhibits net hepatic glycogenolysis and endogenous glucose production in humans under euglycemic conditions. (*J. Clin. Invest.* 1998. 101:1203–1209.) Key words: insulin • glucose • glycogenolysis • glycogenesis • NMR spectroscopy

Introduction

After a meal, inhibition of net hepatic glycogenolysis and stimulation of hepatic glycogen synthesis are key components of glucose and insulin action to inhibit hepatic glucose production, but the mechanism by which they accomplish this is unclear. Insulin and glucose could theoretically inhibit net hepatic glycogenolysis by stimulating glycogen synthase flux, inhibiting glycogen phosphorylase flux, or by some combination of the two. While there are extensive data on the roles of insulin and glucose in the regulation of hepatic glucose production (1–11) there are few studies which have examined their roles in the regulation of flux through glycogen synthase and glycogen phosphorylase because of the difficulty in measuring these fluxes in vivo. Previously, Bishop et al. found that hyperinsulinemia suppressed both glucose production and glycogenolysis in awake dogs by comparing the rate of liver glycogen breakdown obtained from serial biopsies with the rate of endogenous glucose production as determined by tracer measurements (12). In contrast, studies in rats found that euglycemic-hyperinsulinemia suppressed hepatic glucose production but did not affect hepatic glycogen content (13, 14). These results along with added observations that insulin decreased hepatocellular glucose-6-phosphate concentrations and increased fructose-2,6-biphosphate concentrations led to the hypothesis that the primary action of insulin to inhibit hepatic glucose production occurs by stimulation of hepatic glycolytic flux and not by decreased glycogenolysis (13–15). More recently Liu et al. examined this question in anesthetized rats and found that hyperinsulinemia, in the presence of euglycemia, fully suppressed hepatic glucose production and partially inhibited net hepatic glycogenolysis without affecting the phosphorylation state of glycogen phosphorylase (16). These workers concluded that insulin may modulate phosphorylase a activity via an allosteric rather than a covalent mechanism and that full suppression of hepatic glucose production in the face of significant residual glycogenolysis may also involve stimulation of glycolysis, inhibition of glucose-6-phosphatase, or both. Because of the paucity of data regarding the roles of insulin and glucose in the regulation of glycogen synthase and phosphorylase flux in vivo as well as the lack of consistent findings, we examined this question in humans. Rates of hepatic glycogen synthesis were assessed by monitoring the rate of [1-^{13}C] glucose incorporation into the C1 position of the glucosyl moieties of hepatic glycogen using ^{13}C NMR (17,18) after correcting for the relative contribution of the indirect pathway (3 carbon compounds → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → glycogen) (19). This correction was done by monitoring the relative ^{13}C enrichments in acetaminophen-glucuronide and glucose in plasma after an oral dose of acetaminophen (20, 21). Rates of hepatic glycogenoly-

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Received for publication 8 May 1997 and accepted in revised form 20 January 1998.

J. Clin. Invest.

0021-9738/98/03/1203/07 $2.00
Volume 101, Number 6, March 1998, 1203–1209
http://www.jci.org

sis were assessed by replacing the [1-^{13}C]glucose infusate with an unlabeled glucose infusate for the last 2 h of the glucose-insulin clamp (17, 18). To the extent that hepatic glycogen turnover (simultaneous synthesis and degradation) is occurring during this time, the ^{13}C labeled glycogen will be replaced by unlabeled glycogen.

Methods

Subjects. 29 healthy, sedentary, nonsmoking volunteers (20 men and 9 women), mean age 27±1 yr, mean body wt 70±2 kg, mean body mass index 23±1 kg/m^2, mean glycosylated hemoglobin 5.6±0.2% (normal range 4–8%), with body weights within 10% of ideal body weight (based upon 1983 Metropolitan Life Insurance tables) were studied in one or more of five experimental protocols. None of the subjects had a family history of diabetes mellitus, any major diseases, or was taking medication. The experimental protocols were reviewed and approved by the Yale University Human Investigation Committee and informed written consent was obtained from all subjects. An isocaloric diet consisting of 60% carbohydrate, 20% protein, and 20% fat (33 kcal/kg body wt daily) prepared by the metabolic kitchen of the Yale/New Haven Hospital General Clinical Research Center was given for 3 d before the study. During these days, no physical exercise except normal walking was performed. On the day before the study, the subjects were admitted to the Yale/New Haven Hospital General Clinical Research Center, given dinner (11 kcal/kg body wt, consisting of 60% carbohydrate, 20% fat, and 20% protein) at 6 p.m. and then fasted overnight. At 6 a.m. a Teflon® catheter was placed in an antecubital vein in the left arm for infusion of glucose and hormones and another catheter was placed in an antecubital vein in the right arm for blood withdrawal. The experiments were begun at 8:00 a.m. 11 of the subjects participated in more than one protocol, these studies were performed in random order and spaced apart by intervals of 6–12 wk. To minimize changes in ovarian hormonal effects on glucose metabolism, female subjects were studied during the follicular phase (days 0–12) of the menstrual cycle (22).

Experimental design. ^{13}C NMR spectroscopy was used to assess rates of hepatic glycogen synthesis and glycogenolysis under different plasma concentrations of glucose and insulin. Infusions of insulin, somatostatin, and glucose (1-^{13}C labeled glucose from 0–120 min followed by natural abundance ^{13}C glucose from 120–240 min) were given for 4 h to establish one of four hypoglucagonemic conditions: protocol I (n = 11); hyperglycemia (~ 10 mM) and hypoinsulinemia (~ 40 pM); protocol II (n = 5), euglycemia (~ 5 mM) –hyperinsulinemia (~ 400 pM); protocol III (n = 8), hyperglycemia (~ 10 mM) and hyperinsulinemia (~ 400 pM); and protocol IV (n = 7), euglycemia (~ 5mM) –hypoinsulinemia (~ 40 pM). An oral dose of acetaminophen (1,300 mg) was given at the start of the [1-^{13}C]glucose infusion to estimate the dilution of [1-^{13}C]glucose from the indirect pathway in the hepatic UDP-glucose pool (21). In a fifth protocol (protocol V, n = 10), hepatic glycogen measurements were performed during an overnight fast as described previously (23).

Experimental procedures. In protocol I, insulin (Humulin-R; Eli Lilly, Indianapolis, IN) was administered as a constant (24 pmol/m^2/min) infusion starting at time 0 to establish basal fasting peripheral plasma insulin concentrations (~ 40 pM) and portal vein hypoinsulinemia. Simultaneously, a primed-variable infusion of [1-^{13}C]glucose (~ 20% ^{13}C-enriched) was initiated to acutely raise and maintain the plasma glucose concentration at ~ 10 mM. To inhibit endogenous insulin and glucagon secretion, somatostatin was infused at a rate of 0.1 μg/kg body wt/min (3, 18). At 120 min, the [1-^{13}C]glucose infusate was switched to an unenriched glucose infusate which was used until the end of the experiment. A similar clamp procedure using somatostatin, to inhibit endogenous insulin and glucagon, was followed in protocols II, III, and IV. In protocol II, hyperinsulinemia (~ 400 pM) and euglycemia (~ 5 mM) were achieved by a primed-constant infusion of insulin at a rate of 240 pmol/m^2/min and glucose at a variable rate to maintain euglycemia. In protocol III, plasma insulin levels were raised to ~ 400 pM with a primed-constant infusion of insulin at a rate of 240 pmol/m^2/min and hyperglycemia (~ 10 mM) was achieved with a primed-variable infusion of [1-^{13}C]glucose (~ 20% ^{13}C-enriched). In protocol IV combined hypoinsulinemic (~ 40 pM) and euglycemia (~ 5 mM) were achieved by a constant infusion of insulin (24 pmol/m^2/min) and a variable infusion of glucose. For comparison purposes, rates of net hepatic glycogenolysis and hepatic glycogen turnover (protocol V) were also assessed during an overnight fast. For these studies, subjects were given a liquid meal (1,000 kcal; 60% carbohydrate [80% as glucose], 20% fat, and 20% protein) at 5 p.m. Hepatic glycogen concentrations were measured with ^{13}C NMR spectroscopy when hepatic glycogen content peaked (6 h after the meal) and again after 12–14 h of fasting. In a subset of experiments (protocol IV, n = 5, and protocol V, n = 6), rates of hepatic glycogenolysis as well as hepatic glycogen cycling were estimated by the relative rates of hepatic ^{13}C1 glycogenolysis versus ^{13}C2 + ^{13}C5 glycogenolysis during an infusion (0.2 mg/kg body wt min) of [1-^{13}C]glucose (99% ^{13}C-enriched).

Plasma glucose concentrations were measured every 5 min during the glucose-insulin clamps (protocols I–IV). Blood samples were taken every 15 min for determination of plasma insulin, glucagon, lactate, and FFA concentrations as well as for gas chromatography-mass spectrometry (GC-MS)[1] analysis of ^{13}C isotopic enrichment in plasma glucose and acetaminophen glucuronide. The variable infusion of glucose (either ^{13}C-enriched or natural abundance) was adjusted to maintain the desired plasma glucose concentrations. Mean glucose infusion rates during the glucose-insulin clamp studies (protocols I–IV) were calculated for 20-min intervals and corrected for change in plasma glucose concentrations and urinary glucose loss.

In vivo ^{13}C NMR spectroscopic techniques. To assess rates of hepatic glycogen synthesis and breakdown, ^{13}C NMR spectra were acquired from 60 to 155 min and from 185 to 240 min. The measurements were limited to these time periods to minimize the discomfort of the subjects from lying motionless inside the 2.1-Tesla, 1-m bore spectrometer (Biospec I; Bruker Instruments, Inc., Billerica, MA). The ^{13}C NMR signals were obtained with a 9-cm circular ^{13}C observation coil and a 12 × 14-cm coplanar butterfly ^{1}H-decoupler coil placed rigidly over the lateral aspect of the abdomen of the supine subject, and the C1 glycogen resonance (100.6 ppm) was quantitated by integration and comparison with a solution glycogen as described previously (24). Relative changes in the ^{13}C1 glycogen versus the ^{13}C2 + ^{13}C5 glycogen resonance (70 ppm) was determined by the relative change in amplitudes between the ^{13}C1 and the ^{13}C2 + ^{13}C5 glycogen resonances in the difference spectra using an amplitude conversion factor of 2.0 (determined from a glycogen solution phantom).

Analytical procedures. Plasma glucose concentrations were measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments Inc., Fullerton, CA). Plasma immunoreactive insulin and glucagon concentrations were measured using double antibody radioimmunoassay kits (insulin; Diagnostic Systems Labs., Inc., Webster, TX) (glucagon; Linco Research, Inc., St. Charles, MO). Plasma lactate concentrations were measured by using the lactate dehydrogenase method (25). Plasma FFA concentrations were determined using a microfluorimetric method (26). Plasma glucose and acetaminophen-glucuronide were derivatized for determination of ^{13}C atom percent enrichments (APE) by GC-MS. Plasma glucose was derivatized as the pentaacetate, after $Ba(OH)_2/ZnSO_4$ deproteinization and semipurification by anion/cation exchange chromatography (AG1-X8, AG50W-X8; Biorad Laboratories, Richmond, CA), as described previously (27). Plasma acetaminophen-glucuronide was derivatized by a modification of that used for amino acids (28). Plasma was deprotein-

1. *Abbreviations used in this paper:* APE, atom percent enrichments; GC-MS, gas chromatography-mass spectrometry; PI, peak integral.

ized with $Ba(OH)_2/ZnSO_4$, the supernatant was freeze-dried, and the glucuronide moiety was derivatized as the *n*-butyl ester, triacetate.

GC-MS analysis. GC-MS analysis was performed with a Hewlett-Packard 5890 gas chromatograph (HP-1 capillary column; 12 m × 0.2 mm × 0.33 μm film thickness) interfaced to a Hewlett-Packard 5971A mass selective detector operating in the positive chemical ionization mode (with methane as the reagent gas) for acetaminophen-glucuronide enrichment. Ions with m/z 197/198 were monitored for determination of isotopic enrichment in C1→C5, and m/z 299/300, 359, and 360 for enrichment in C1→C6 (28). Enrichments in C1, C2, and C6 were verified by ^{13}C NMR spectroscopy of acetaminophen-glucuronide purified from urine collections, as described previously (28). Plasma glucose enrichments were determined by GC-MS analysis using electron impact. Ions with m/z 169/170 were monitored for determination of ^{13}C isotopic enrichment in C1→C6, and m/z 200/201 for enrichment in C2→C6. Enrichment in C1 was determined from the difference in the enrichments of the two fragments. Enrichment in C6 was estimated as enrichment in the C2→C6 fragment divided by three.

Calculations and data analysis. The method for estimating rates of hepatic glycogen synthesis and simultaneous breakdown is based on measuring initial total glycogen concentration ($^{13}C + {}^{12}C$) and subsequent changes in the C1 glycogen peak integral (ΔPI) (17, 18). Briefly, the rate of hepatic accumulation of glycogen was assessed from the increase in ^{13}C-glycogen concentration during the initial [1-^{13}C]glucose infusion (0–155 min). The increase in total liver glycogen concentration for each 15-min interval is given as $[\Delta PI \cdot a \cdot 100]/[(b \cdot \textit{plasma glucose } {}^{13}C \textit{ enrichment over baseline}) + 1.1]$, where *a* is the ^{13}C glycogen concentration (1.1% times the total hepatic glycogen concentration) (mmol/liter of liver) and *b* is the dilution factor representing the fraction of UDP-glucose formed by the direct pathway. The fraction of UDP-glucose formed from the direct pathway of glycogen synthesis is determined from the ^{13}C enrichment in C1 and C6 of plasma glucose and acetaminophen-glucuronide and calculated as $b = [C1 - C6\ {}^{13}C \textit{ enrichment of plasma glucuronide}]/[C1 - C6\ {}^{13}C \textit{ enrichment of plasma glucose}]$. Since the increase in the total liver glycogen concentration was linear from 60 to 155 min during all protocols protocol I, $r = 0.934$; II, $r = 0.975$; and III, $r = 0.951$, the hepatic accumulation of glycogen in mmol glycogen · liter of liver^{-1} · min^{-1} can be assessed from the slope of the line in a plot of the total glycogen concentration versus time. A minimal rate of simultaneous glycogen breakdown, i.e., only the fraction of glycogen that escapes the hexose-1-phosphate pool, was estimated by comparing the observed change in the $^{13}C1$ glycogen concentration during the chase period (185–240 min) with the predicted change in $^{13}C1$ glycogen, assuming constant glycogen synthesis without breakdown. The observed change in the $^{13}C1$ glycogen concentration is given as $[\Delta PI \cdot a]$, whereas the predicted change in $^{13}C1$ glycogen during each 15-min time interval of the chase period is given as $\Delta T \cdot \textit{rate of glycogen synthesis} \cdot [b \cdot ({}^{13}C \textit{ plasma glucose enrichment}) + 1.1]/100$. The difference between the predicted and the observed change in $^{13}C1$ glycogen represents an estimate for the amount of $^{13}C1$ glycogen that is broken down during each 15-min time interval. The mean rate of $^{13}C1$ glycogenolysis is obtained by the slope of the line from the plot of the difference between the predicted and observed changes in $^{13}C1$ glycogen versus time. The rate of total glycogen breakdown ($[{}^{13}C + {}^{12}C]$glycogen) is obtained by dividing the slope with the maximum plasma UDP-glucose ^{13}C glucose enrichment $[(b \cdot \textit{maximum plasma } {}^{13}C \textit{ glucose enrichment}) + 1.1]$. The relative percent hepatic glycogen turnover is then calculated as 100% · (*rate of glycogenolysis*/*rate of glycogen synthesis*), and the net rate of hepatic glycogen synthesis is given by the difference between the rates of hepatic glycogen accumulation and glycogen breakdown. Glycogen cycling is estimated as the lesser rate between glycogen synthase flux and glycogen phosphorylase flux. In protocols IV and V the rate of net hepatic glycogen breakdown was calculated for each subject as the best fit line to the decrease in hepatic glycogen concentration over time. Hepatic glycogen turnover was estimated by comparing the rate of $^{13}C1$ hepatic glycogenolysis to the rate of $^{13}C2$–$C5$ hepatic glycogenolysis glycogen during the [1-^{13}C]glucose infusion. To the extent that simultaneous glycogen synthesis and degradation is occurring under these conditions, the rate of $^{13}C1$ hepatic glycogenolysis would be lower than the rate of $^{13}C2$–$C5$ hepatic glycogenolysis.

Basal endogenous glucose production rates in protocol V. Basal endogenous glucose production rates in protocol V were estimated as follows: $\textit{Basal endogenous glucose production} = f \cdot [(\textit{enrichment}^{inf}/\textit{enrichment}^{plasma}) - 1]$ where *f* is basal [1-^{13}C] glucose infusion rate (μmol/kg/min); $\textit{enrichment}^{inf}$ is [1-^{13}C] glucose infusate enrichment (APE); and $\textit{enrichment}^{plasma}$ is steady-state basal plasma[1-^{13}C] glucose enrichment (APE).

Clamped endogenous glucose production rates in protocols I–IV. Clamped endogenous glucose production rates in protocols I–IV were estimated as follows: $\textit{Clamped endogenous glucose production} = \textit{GIR} \cdot [(\textit{enrichment}^{inf}/\textit{enrichment}^{plasma}) - 1]$ where *GIR* is mean glucose infusion rate (μmol/kg/min) during the clamp procedure (90–120 minutes), $\textit{enrichment}^{inf}$ is [1-^{13}C] glucose infusate enrichment (APE), and $\textit{enrichment}^{plasma}$ is steady-state clamped plasma [1-^{13}C] glucose enrichment (APE).

All data are presented as mean±SEM. Linear regressions were calculated by the method of the least squares. Data within or between protocols were compared by ANOVA followed by the Student-Newman-Keuls post hoc test.

Results

Plasma substrate and hormone concentrations. Basal fasting plasma concentrations of glucose (protocol I, 4.8±0.2 mM; protocol II, 5±0.1 mM; protocol III, 4.9±0.2 mM; protocol IV, 5.0±0.1 mM; and protocol V, 5.0±0.02 mM), insulin (protocol I, 54±6 pM; protocol II, 42±2 pM; protocol III, 60±6 pM; protocol IV, 60±3 pM; and protocol V, 60±6 pM), and glucagon (protocol I, 52±6 ng/liter; protocol II, 44±4 ng/liter; protocol III, 47±4 ng/liter; protocol IV, 46±1 ng/liter; and protocol V, 48±6 ng/liter) were similar between the experimental protocols. Within 20 min of starting the glucose-insulin clamp studies (protocols I–IV), the desired level of glycemia was achieved and then maintained until the end of the clamp with similar steady-state concentrations between protocols I (10.3±0.1 mM) and III (10.1±0.1 mM), and between protocols II (5.6±0.3 mM) and IV (5.1±0.1 mM) (Fig. 1, *top*). In protocols II and III, plasma insulin concentrations rapidly increased and reached similar steady-state values of 438±24 pM in protocol II ($P < 0.0001$ vs. basal), and 384±24 pM in protocol III ($P < 0.0001$ vs. basal) (Fig. 1, *bottom*). Somatostatin resulted in a ∼60% decrease in plasma glucagon concentrations in all four glucose-insulin clamp protocols: protocol I, 35±4 ng/liter ($P < 0.03$ vs. basal); protocol II, 30±2 ng/liter ($P < 0.007$ vs. basal); protocol III, 25±3 ng/liter ($P < 0.002$ vs. basal); and protocol IV, 28±1 ng/liter ($P < 0.0001$ vs. basal). In protocol V plasma insulin and glucagon concentrations averaged 49±4 pmol/liter and 48±6 ng/liter, respectively, during the overnight period. Plasma lactate concentrations were similar before the experiments in all protocols (protocol I, 0.50±0.06 mM; protocol II, 0.64±0.06 mM; protocol III, 0.72±0.14 mM; protocol IV, 0.74±0.06 mM; and protocol V, 0.44±0.06 mM) and increased under the hyperinsulinemic conditions of protocols II (1.10±0.06 mM, $P = 0.005$ vs. basal) and III (1.06±0.14 mM, $P = 0.002$ vs. basal). When insulin was maintained at basal levels, plasma lactate did not change significantly from basal concentrations (protocol I, 0.72±0.06 mM, and IV, 0.66±0.06 mM). Baseline values of plasma FFAs (protocol I, 584±57 μM; protocol II, 584±38 μM; protocol III, 329±41 μM; protocol IV,

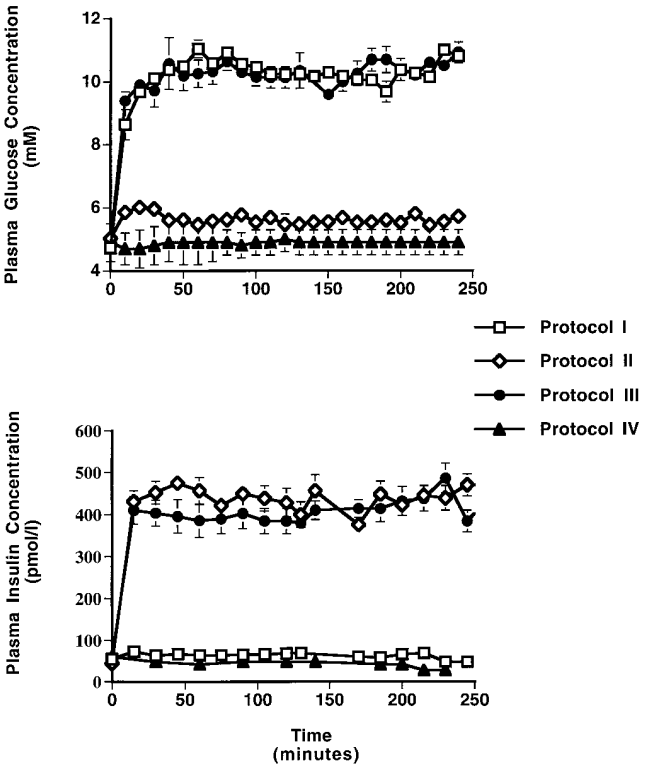

Figure 1. Mean plasma glucose concentrations (*top*) and plasma insulin concentrations (*bottom*) in protocols I–IV.

550±56 μM; and protocol V, 479±38 μM) decreased during the clamp in protocols I–III (protocol I, 235±27 μM; $P < 0.0001$ vs. basal; protocol II, 151±19 μM, $P < 0.0001$ vs. basal; and protocol III, 137±10 μM; $P = 0.018$ vs. basal), but did not change from basal in protocol IV (575±64 μM).

Glucose infusion rates and estimated endogenous glucose production rates. Mean glucose infusion rates were: 12.2±1.1 μmol/(kg/min), 44.4±3.9 μmol/(kg/min), 61.7±5.6 μmol/(kg/min) and 2.5±0.6 μmol/(kg/min) in protocols I–IV, respectively. Endogenous glucose production rates (and percent suppression) during the clamp were: 4.8±0.3 μmol/(kg/min) (66%), 2.1± 0.7 μmol/(kg/min) (85%), 0.8±0.7 μmol/(kg/min) (94%), and 10.8±1.5 μmol/(kg/min) (22%) in protocols I–IV, respectively, compared to a basal endogenous glucose production rate of 13.9±1.9 μmol/(kg/min) in protocol V.

Basal hepatic glycogen concentration, and estimated rates of glycogen synthase flux, glycogen phosphorylase flux, and net hepatic glycogen synthesis. Morning basal liver glycogen concentrations were similar before each clamp study (protocol I, 209±10 mM; protocol II, 288±47 mM; protocol III, 238±10 mM; and protocol IV, 191±13 mM). From 60 min to 155 min, total liver glycogen concentrations increased in a linear fashion during protocols I ($r = 0.934$), II ($r = 0.975$), and III ($r = 0.951$). Under similar basal concentrations of insulin and hypoglucagonemia, hyperglycemia per se caused a 75% suppression in glycogen phosphorylase flux (protocol I versus IV), but there was no net hepatic glycogen synthesis due to negligible activation of glycogen synthase flux (Table I). In contrast, under similar conditions of euglycemia and hypoglucagone-

Table I. Estimated Rates of Glycogen Synthase Flux, Glycogen Phosphorylase Flux, Net Hepatic Glycogen Synthesis, and Hepatic Glycogen Cycling in Protocols I–V Expressed as mmol · liter of liver^{-1} · min^{-1}

	Glycogen synthase flux	Glycogen phosphorylase flux	Net glycogen synthesis	Glycogen cycling rate
Protocol I				
glucose (~ 10 mM)	0.05±0.03	0.05±0.03‡	0±0.04§	0.05±0.03
insulin (~ 40 pM)				
glucagon (~ 30 ng/ml)				
Protocol II				
glucose (~ 5 mM)	0.48±0.08*	0.29±0.07	0.19±0.03‖	0.29±0.07**
insulin (~ 400 pM)				
glucagon (~ 30 ng/ml)				
Protocol III				
glucose (~ 10 mM)	0.39±0.04*	0.03±0.02‡	0.36±0.03¶	0.03±0.02
insulin (~ 400 pM)				
glucagon (~ 30 ng/ml)				
Protocol IV				
glucose (~ 5 mM)	0±0.02	0.20±0.03	−0.20±0.03	0±0.02
insulin (~ 40 pM)				
glucagon (~ 30 ng/ml)				
Protocol V				
(overnight fast)				
(without somatostatin)				
glucose (~ 5 mM)	0±0.02	0.25±0.04	−0.25±0.04	0±0.02
insulin (~ 40 pM)				
glucagon (~ 50 ng/ml)				

*$P < 0.005$ vs. protocols I, IV, and V; ‡$P < 0.05$ vs. protocols II, IV, and V; §$P < 0.0001$ vs. protocols II, III, IV, and V; ‖$P < 0.005$ vs. protocols I, III, IV, and V; ¶$P < 0.0001$ vs. protocols I, IV, and V; and **$P < 0.01$ vs. protocols I, III, IV, and V.

mia, hyperinsulinemia per se caused complete inhibition in net hepatic glycogenolysis and stimulation of net hepatic glycogen synthesis exclusively through activation of glycogen synthase flux (protocol II versus IV). Under these conditions hyperinsulinemia per se (protocol II) had no effect to inhibit phosphorylase flux (compared to protocol IV) which resulted in extensive hepatic glycogen cycling. The glycogen phosphorylase rate was ~ 60% of the glycogen synthase flux rate and rates of net hepatic glycogen synthesis were consequently relatively low. This higher rate of glycogen phosphorylase flux in protocol II occurred despite the fact that subjects in protocol II were clamped at a slightly higher plasma glucose concentration (5.6 mM) than subjects in protocol IV (5.1 mM) which, in view of the previous data, would be expected to decrease glycogen phosphorylase flux. Combined hyperglycemic-hyperinsulinemia, under similar hypoglucagonemic conditions (protocol III), resulted in relatively high rates of net hepatic glycogen synthesis through combined stimulation of glycogen synthase flux and inhibition of glycogen phosphorylase flux compared to either hyperglycemic-hypoinsulinemic (protocol I) or euglycemic-hyperinsulinemic conditions (protocol II). This combined effect resulted in a relatively large increase in the net rate of hepatic glycogen synthesis and a 90% decrease in the rate of hepatic glycogen cycling compared to euglycemic-hyperinsulinemic-hypoglucagonemic conditions (protocol II). Rates of net hepatic glycogenolysis were similar in protocols IV and V (overnight fast) and there was no detectable hepatic glycogen cycling in either protocol as reflected by similar mean rates for $^{13}C1$ and $^{13}C2 + {}^{13}C5$ hepatic glycogenolysis during the [1-^{13}C] glucose infusion: protocol IV ($^{13}C1$; 0.19±0.03 mmol · liter of liver^{-1} · min^{-1} versus $^{13}C2 + {}^{13}C5$; 0.25±0.03 mmol · liter of liver^{-1} · min^{-1}; protocol V, $^{13}C1$; 0.30±0.05 mmol · liter of liver^{-1} · min^{-1} versus $^{13}C2 + {}^{13}C5$; 0.35±0.06 mmol · liter of liver^{-1} · min^{-1}.

Pathways of hepatic glycogen synthesis. To obtain an estimate of the percent contribution of the direct versus the indirect pathways of hepatic glycogen synthesis, the ratios of ^{13}C enrichments in plasma glucose and in plasma acetaminophen-glucuronide were compared. The percent contribution of the direct pathway to hepatic glycogen synthesis was similar in all three glucose-insulin clamp protocols (protocol I, 52±4%; protocol II, 55±4%; and protocol III, 59±4%.

Discussion

In the presence of euglycemic-hypoglucagonemia, a selective increase in the plasma insulin concentration stimulated glycogen synthase flux but did not inhibit glycogen phosphorylase flux (protocol II versus IV). Despite the fact that phosphorylase flux was not inhibited, endogenous glucose production was almost completely suppressed due to extensive cycling of glucose-6-phosphate, derived from both the direct and indirect (gluconeogenic) pathways, into and out of glycogen such that glycogen phosphorylase flux was ~ 60% that of glycogen synthase flux. Due to the high rate of hepatic glycogen cycling the rate of net hepatic glycogen synthesis under euglycemic-hyperinsulinemic-hypoglucagonemic conditions (protocol II) was ~ 50% lower than that obtained under hyperglycemic-hyperinsulinemic-hypoglucagonemic conditions (protocol III) despite similar glycogen synthase fluxes. In contrast hyperglycemia, in the presence of hypoinsulinemic-hypoglucagonemia (protocol I), had a negligible effect on glycogen synthase flux but caused a ~ 75% inhibition of glycogen phosphorylase flux and a 56% suppression of endogenous glucose production when compared to these same flux rates measured under euglycemic-hypoinsulinemic-hypoglucagonemia conditions (protocol IV). These results demonstrate that hyperglycemia and hyperinsulinemia inhibit net hepatic glycogenolysis through distinct enzymatic mechanisms and not through coordinated inhibition of glycogen phosphorylase and stimulation of glycogen synthase, as thought previously (29-35). These data also demonstrate that promotion of hepatic glycogen cycling may be the principal mechanism by which hyperinsulinemia, under euglycemic conditions, inhibits net hepatic glycogenolysis and endogenous glucose production in humans. This finding may explain the relative paucity of data demonstrating a direct effect of insulin to inhibit hepatic glycogen phosphorylase activity in contrast to the abundance of data demonstrating the key role that insulin plays in stimulation of glycogen synthase activity (33, 34). While several early reports have suggested a direct action of insulin to inhibit glycogen phosphorylase a by promoting its phosphorylation to phosphorylase b (35, 36), subsequent studies have not confirmed this finding (13, 14). In contrast the effect of hyperglycemia per se (protocol I) to inhibit phosphorylase flux is consistent with its ability to promote conversion of phosphorylase a to phosphorylase b (14, 16, 37–41).

The occurrence of extensive hepatic glycogen cycling under conditions of euglycemic-hyperinsulinemia may also contribute to the underestimation of glucose turnover using isotope dilution techniques during glucose-insulin clamp studies and the resulting phenomenon of negative rates of hepatic glucose production (42). When endogenous glucose production is assessed with a tracer infusion the plasma glucose enrichment (or specific activity) will typically be higher in the basal period than in the euglycemic-hyperinsulinemia period when hepatic glucose production is suppressed and unlabeled glucose is infused to maintain euglycemia (43). Under these conditions hepatic glycogen cycling will result in some of this more highly enriched plasma glucose to become incorporated into hepatic glycogen. At a later time point hepatic glycogen cycling will cause exchange of this highly enriched glycogen with plasma glucose resulting in an increase in the plasma glucose enrichment, which in turn will result in an underestimation of the tracer-determined rate of glucose turnover. To the extent that the plasma glucose enrichment is kept constant by adding tracer to the exogenous glucose infusate (Hot Ginf), this error will be minimized (44). However, it would be predicted that rates of glucose turnover will still be underestimated with this latter approach to the extent that the plasma glucose enrichment is higher in the basal period than during the subsequent euglycemic-hyperinsulinemic period.

When hyperglycemia was combined with hyperinsulinemia (protocol III), the combined effect of hyperinsulinemia to stimulate glycogen synthase flux and hyperglycemia to inhibit glycogen phosphorylase flux resulted in a ~ 90% suppression in the rate of hepatic glycogen cycling and an approximately twofold increase in the rate of net hepatic glycogen synthesis compared to hyperinsulinemia alone (protocol II). There was no further additive effect of hyperglycemia to stimulate flux through glycogen synthase compared to hyperinsulinemia alone. The percent glycogen turnover measured in protocol III (~ 10%) was somewhat lower than we have found previously found in overnight fasted subjects studied under hyperglyce-

mic-hyperinsulinemic conditions ($\sim$ 30%) and can most likely be explained by differences in experimental design (17). In the previous study glucose alone was infused to achieve hyperglycemia which resulted in a more variable endogenous insulin and glucagon response whereas in this study somatostatin and insulin were infused to create a more reproducible suppression of endogenous insulin and glucagon release and a more consistent increase in circulating plasma insulin concentration.

Rates of net hepatic glycogenolysis were comparable during the overnight fast (protocol V) and under conditions of euglycemic-hypoinsulinemic-hypoglucagonemia (protocol IV). This suggests that, under these conditions of euglycemic-hypoinsulinemic-hypoglucagonemia (protocol IV), basal glucagon does not play a major role in promoting net hepatic glycogenolysis. Although a previous study in humans, using somatostatin to inhibit endogenous insulin and glucagon release, found that basal glucagon does plays an important role in the maintenance of basal hepatic glucose production (45), it was performed at peripheral (and portal vein) plasma insulin concentrations that were 60% higher than those in this study. Taken together, these data suggest that the lower glucagon concentration in protocol IV was counterbalanced by the lower insulin concentration, resulting in similar rates of net hepatic glycogenolysis and endogenous glucose production in the two protocols.

Hepatic glycogen cycling was also estimated in the overnight fasted state by infusing [1-^{13}C]glucose during the night while monitoring the relative changes in the ^{13}C1 and ^{13}C2 + ^{13}C5 resonances of the glucosyl units in glycogen. To the extent that hepatic glycogen turnover is occurring under these conditions, the rate of ^{13}C1 glycogenolysis would be less than that for ^{13}C2 + ^{13}C5 glycogenolysis due to the simultaneous incorporation of [1-^{13}C]glucose into C1 hepatic glycogen (under these conditions there is negligible scrambling of ^{13}C label into the C2–C6 positions of plasma glucose or hepatic UDP-glucuronide). We did not detect any differences in the rate of ^{13}C1 versus ^{13}C2 + ^{13}C5 hepatic glycogenolysis, implying that there was a negligible amount of hepatic glycogen turnover during the overnight fast. These data contrast the recent report of Hellerstein et al. who found that $\sim$ 34% of the absolute rate of hepatic glycogenolysis was returned to glycogen under similar conditions using a triple isotope-mass isotopomer method (46). The reason for this discrepancy is not clear. The signal to noise ratio of our liver glycogen ^{13}C NMR spectra typically yields a minimum detection limit of $\sim$ 15 mM change in natural abundance glycogen which translates into a rate of $\sim$ 0.02 mmol $\cdot$ liter of liver^{-1} $\cdot$ min^{-1} over the 14-h fast. Since the plasma [1-^{13}C]glucose enrichments averaged $\sim$ 11% during this period our lower limit for detectability of glycogen synthase flux can be estimated to be $\sim$ 0.004 mmol $\cdot$ liter of liver^{-1} $\cdot$ min^{-1} which translates into an upper limit for hepatic glycogen turnover of $<$ 2% under these conditions.

In summary, we found that under hypoglucagonemic conditions: (*a*) hyperinsulinemia per se inhibits net hepatic glycogenolysis exclusively through stimulation of glycogen synthase flux; (*b*) hyperglycemia per se inhibits net hepatic glycogenolysis primarily through inhibition of glycogen phosphorylase flux; (*c*) activation of glycogen synthase flux and inhibition of glycogen phosphorylase flux are not necessarily coupled and coordinated in a reciprocal fashion; and (*d*) promotion of hepatic glycogen cycling may be the principal mechanism by which insulin inhibits net hepatic glycogenolysis and endogenous glucose production in humans under euglycemic conditions. These data demonstrate that by working though separate mechanisms, both substrate and hormonal signals are simultaneously required to promote optimal rates of net hepatic glycogen synthesis. This has the protective effect of preventing hyperglycemia, in the absence of hyperinsulinemia, from promoting net hepatic glycogen synthesis and, thus, decreasing glucose availability to the brain and other vital organs in times of need.

Acknowledgments

The authors would like to thank Veronika Walton, Donna Caseria, Karynn Gerow, Parveen Vohra, and Suzanne Vogel for technical assistance and Drs. Alan Dresner, Gianluca Perseghin, Ripudaman Hundal, and Martin Krssak and the staff of the Yale/New Haven Hospital General Clinical Research Center for their help with the studies.

This work was supported by grants from the United States Public Health Service (RO1 DK-49230), the General Clinical Research Center (MO1 RR-00125), the Yale Diabetes-Endocrine Research Center (P30 DK-45735), the Juvenile Diabetes Foundation, International, (Kitt Falk Petersen), and an unrestricted grant from Bristol-Myers Squibb. Dr. Kitt Falk Petersen is the recipient of a National Institutes of Health Clinical Associate Award. Dr. Gerald I. Shulman is an investigator of the Howard Hughes Medical Institute.

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