

Impact of a Glycogen Phosphorylase Inhibitor and Metformin on Basal and Glucagon-Stimulated Hepatic Glucose Flux in Conscious Dogs

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ABSTRACT

The effects of a glycogen phosphorylase inhibitor (GPI) and metformin (MT) on hepatic glucose fluxes ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) in the presence of basal and 4-fold basal levels of plasma glucagon were investigated in 18-h fasted conscious dogs. Compared with the vehicle treatment, GPI infusion suppressed net hepatic glucose output (NHGO) completely (-3.8 ± 1.3 versus 9.9 ± 2.8) despite increased glucose 6-phosphate (G-6-P) neogenesis from gluconeogenic precursors (8.1 ± 1.1 versus 5.5 ± 1.1). MT infusion did not alter those parameters. In response to a 4-fold rise in plasma glucagon levels, in the vehicle group, plasma glucose levels were increased 2-fold, and NHGO was increased (43.9 ± 5.7 at 10 min and 22.7 ± 3.4 at steady state) without altering G-6-P neogenesis (3.7 ± 1.5 and 5.5 ± 0.5 , respectively). In the GPI group, there was no

increase in NHGO due to decreased glucose-6-phosphatase flux associated with reduced G-6-P concentration. A lower G-6-P concentration was the result of increased net glycogenesis without altering G-6-P neogenesis. In the MT group, the increment in NHGO (22.2 ± 4.4 at 10 min and 12.1 ± 3.6 at steady state) was approximately half of that of the vehicle group. The lesser NHGO was associated with reduced glucose-6-phosphatase flux but a rise in G-6-P concentration and only a small incorporation of plasma glucose into glycogen. In conclusion, the inhibition of glycogen phosphorylase activity decreases basal and glucagon-induced NHGO via redirecting glucose 6-phosphate flux from glucose toward glycogen, and MT decreases glucagon-induced NHGO by inhibiting glucose-6-phosphatase flux and thereby reducing glycogen breakdown.

Introduction

The liver produces glucose via glycogen breakdown and/or gluconeogenesis, and the relative contribution of each to total glucose production changes with altered nutritional and metabolic states. Several studies in dogs and humans have shown that increased delivery of gluconeogenic precursors, such as alanine (Diamond et al., 1988; Wolfe et al., 1988), glycerol (Jahoor et al., 1990), or lactate (Jenssen et al., 1990; Connolly et al., 1993), to the liver has no acute effect on the amount of glucose produced by that organ. Gluconeogenic

precursors can alter hepatic glycogen metabolism by exerting regulatory effects on glycogen phosphorylase and synthase in addition to serving as substrates for glycogen synthesis (Youn and Bergman, 1990), of which glucose 6-phosphate (G-6-P), an intermediate at a central cross point between the metabolic pathways of glycogen metabolism and gluconeogenesis, has been shown in studies using isolated hepatocytes to regulate glycogen synthase (Ciudad et al., 1986) and phosphorylase activity within a physiological range (Aiston et al., 2003, 2004). The above data suggest the existence of autoregulatory control of glycogenolysis by gluconeogenesis within the liver, such that the desired rate of hepatic glucose output can be maintained regardless of the gluconeogenic precursor supply. In contrast, Staehr et al. (2007) reported that a galactose-induced increase in hepatic glycogenolysis resulted in a concomitant decrease in hepatic gluconeogenesis in 44-h fasted healthy humans. In keeping with this, we

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ABBREVIATIONS: G-6-P, glucose 6-phosphate; GPI, glycogen phosphorylase inhibitor; MT, metformin; NHGO, net hepatic glucose output; PEG, polyethylene glycol; NHGU, net hepatic glucose uptake; M-HGR, minimal estimate of unidirectional hepatic glucose release; CP-316819, (3S,2R)-3-(5-chloroindole-2-carbonyl)amino-2-hydroxy-4-phenylbutyric acid *N*-methyl-*N*-methoxyamide; SA, specific activity; M-HGU, minimal estimate of unidirectional hepatic glucose uptake; GK, glucokinase; G-6-Pase, glucose-6-phosphatase; BAY R3401, isopropyl 4-(2-chlorophenyl)-1-ethyl-2-methyl-5-oxo-1,4,5,7-tetrahydro-furo[3,4-*b*]pyridine-3-carboxylate.

showed that a concomitant increase in hepatic gluconeogenesis resulted from an inhibition of hepatic glycogenolysis (Shiota et al., 1997), although this was not confirmed by others (Fosgerau et al., 2001). These findings suggest the existence of an autoregulatory mechanism between net glycogenolysis and gluconeogenesis within the liver to maintain the desired rate of hepatic glucose output. Furthermore, the flux from glucose to glycogen has two highly regulated steps, glucose phosphorylation by glucokinase and the formation of a glycoside bond between C1 of the activated glucose, UDP-glucose, and C4 of a terminal glucose residue of glycogen by glycogen synthase. It has been reported that increasing both glucokinase and glycogen synthase activity synergistically increases glycogen synthesis from glucose in cultured hepatocytes isolated from normal rats (Gomis et al., 2000; Hampson and Agius, 2005). Therefore, net glycogen flux may be tightly linked to fluxes in other pathways, including gluconeogenesis, glucose phosphorylation, and glucose 6-phosphate dephosphorylation. It is possible that alteration of net hepatic glucose output resulting from a modification in glycogenolytic flux involves a secondary change in other metabolic pathway(s).

In patients and animals with type 2 diabetes, the diabetic hyperglycemia is associated with inappropriately increased endogenous glucose production, a lesser suppression of endogenous glucose production and a blunted glucose disposal in response to increased plasma glucose and insulin (Firth et al., 1986; Consoli, 1992; Iozzo et al., 2003). The blunted response of hepatic glucose flux to raised insulin and glucose is associated with blunted response of net hepatic glycogen flux (Krssak et al., 2004). The normalization or reduction of net hepatic glycogenolysis has attracted attention as a potential therapeutic strategy. During the past decade, a specific inhibitor of glycogen phosphorylase that catalyzes glycogen breakdown to glucose 1-phosphate, a rate-limiting step of glycogenolysis, was generated to directly decrease glycogen breakdown. Treatment with the inhibitor has been shown to reduce hyperglycemia acutely in a model of type 2 diabetes (Treadway et al., 2001; Ogawa et al., 2003). Metformin (*N,N*-dimethylbiguanide; MT) has been used for decades to improve glycemic control in diabetic patients and is thought to decrease blood glucose levels by reducing hepatic glucose output (Bailey and Turner, 1996; Kirpichnikov et al., 2002). The most widely accepted mechanism of MT action is the inhibition of transcription of key gluconeogenic genes in the liver (Shaw et al., 2005; Viollet et al., 2009). On the other hand, it has been reported that MT reduces net hepatic glucose production acutely by decreasing glycogenolysis in the normal dog (Chu et al., 2000).

To verify the mechanism by which glycogen phosphorylase inhibition decreases net hepatic glucose output (NHGO), we examined whether any alteration of glucose phosphorylation, gluconeogenesis or glycogen synthesis, is associated with the inhibition of glycogen phosphorylase. Furthermore, to assess the mechanism by which MT decreases net glycogenolysis, we compared the alterations in liver glucose flux caused by MT with those caused by a glycogen phosphorylase inhibitor.

Materials and Methods

Animals and Surgical Procedures. Experiments were performed on 15 overnight-fasted mongrel dogs (17.4–29.0 kg, mean

22.4 ± 1.1 kg) of either sex, which had been fed once daily a standard meat and chow diet (34% protein, 46% carbohydrate, 14% fat, and 6% fiber based on dry weight; Kal Kan, Vernon, CA; and Purina Lab Canine Diet 5006; Purina Mills, St. Louis, MO). The dogs were housed in a facility that met American Association for the Accreditation of Laboratory Animal Care guidelines, and the protocols were approved by the Vanderbilt University Medical Center Animal Care Committee. At least 16 days before an experiment, a laparotomy was performed under general endotracheal anesthesia (15 mg/kg b.wt. pentothal sodium presurgery and 1.0% isoflurane as an inhalation anesthetic during surgery), and catheters for blood sampling were placed into a femoral artery and the portal, a hepatic, a jejunal, and a splenic vein as described previously (Diamond et al., 1988; Conolly et al., 1993). A catheter for drug infusion was placed into the stomach as described previously (Moore et al., 1994). On the day of the experiment, the catheters were exteriorized under local anesthesia (2% lidocaine; Abbott Laboratories, Chicago, IL), their contents were aspirated, and they were flushed with saline. Angiocaths (20 gauge; Abbott Laboratories) were inserted into both cephalic veins for infusion of indocyanine green, radioactive tracers, and glucose and a saphenous vein for the infusion of somatostatin.

On the day before an experiment the leukocyte count and hematocrit were determined. Dogs were used for an experiment only if they had 1) a leukocyte count <18,000/mm³, 2) a hematocrit >38%, 3) a good appetite, and 4) normal stools.

Experimental Design. Each experiment consisted of a 100-min tracer and dye equilibration period (–140 to –40 min), a 40-min control period (–40 to 0 min), and two test periods: Test Period I (0–90 min) and Test Period II (90–210 min). A priming dose of [³-H]glucose (41.7 μCi) was given at –140 min. Continuous infusions of [³-H]glucose (0.34 μCi/min) and indocyanine green (0.1 mg/m² · min^{–1}) were also started at –140 min and were continued throughout the experiment. At –140 min, a peripheral infusion of somatostatin (0.8 μg · kg^{–1} · min^{–1}) was started to inhibit endogenous insulin and glucagon secretion. Intraportal replacement infusions of insulin (0.25 mU · kg^{–1} · min^{–1}) and glucagon (0.6 ng · kg^{–1} · min^{–1}) were started simultaneously with initiation of the somatostatin infusion. Plasma glucose was then monitored every 5 min, and the rate of insulin infusion was adjusted until the level of plasma glucose was stabilized at a euglycemic value. Once stabilization was achieved, the insulin infusion rate was left unchanged. Three experimental protocols were used: 1) vehicle (placebo) group, an intragastric bolus (0.5 ml/kg) of polyethylene glycol (PEG)-500 (10%) was given at 0 min, and saline was infused into the portal vein at 7 μl · kg^{–1} · min^{–1} during the test periods. 2) GPI group, an intragastric bolus (10 mg/kg) of GPI, (3*S*,2*R*)-3-(5-chloroindole-2-carbonyl)amino-2-hydroxy-4-phenylbutyric acid *N*-methyl-*N*-methoxyamide (CP-316819; Pfizer, Inc., Groton, CT) (Treadway et al., 2001), with PEG-500 (0.5 ml/kg) was given at 0 min because of its low solubility in vehicle, and then saline was infused into the portal vein at 7 μl · kg^{–1} · min^{–1} during the test periods. 3) MT group, an intragastric bolus of PEG-500 (0.5 ml/kg) was given at 0 min, and MT was infused into the portal vein at 0.15 mg · kg^{–1} · min^{–1} with 7 μl of saline · kg^{–1} · min^{–1} during the test periods because MT causes diarrhea if given orally. Based on hepatic blood flow rate (approximately 30 ml · kg^{–1} · min^{–1}), hematocrit (approximately 40%), and the infusion rate of MT (150 μg · kg^{–1} · min^{–1}), hepatic sinusoidal plasma concentrations of MT were expected to be >8 μg/ml, which are 10 times higher than plasma concentrations of MT (in range of 0.5 to 2.0 μg/ml) seen when MT was given at 1 g per day in patients with type 2 diabetes (Scheen, 1996). After a 90-min test period, the infusion rate of glucagon was increased 4-fold in all groups for another 120 min.

Analytical Procedures. Plasma glucose concentrations and plasma glucose radioactivity were determined as described previously (Fujimoto et al., 2006; Torres et al., 2009). Blood concentrations of gluconeogenic precursors (lactate, alanine, and glycerol) and plasma concentration of nonesterified fatty acids were determined according to the methods reported previously (Fujimoto et al., 2006;

Torres et al., 2009). Individual blood amino acid (alanine, serine, threonine, and glycine) levels were assessed by high-performance liquid chromatography methods with an interassay coefficient of variation of 4% (Venkatakrisnan et al., 1996). Plasma arterial and hepatic vein indocyanine green concentrations were determined spectrophotometrically at 805 nm (Leevy et al., 1962). Immunoreactive plasma insulin, glucagon, and cortisol as well as plasma epinephrine and norepinephrine were determined as described previously (Wada et al., 1995).

Liver samples were obtained at the end of each experiment by euthanizing the dog with pentobarbital sodium, exposing the liver by laparotomy, and freeze-clamping approximately 5 g of liver sections from each lobe. The time elapsed from euthanization to freeze-clamping was less than 4 min. The entire liver was then removed from the dog and weighed. The frozen liver samples were stored at approximately -70°C for subsequent analysis. Glycogen and ^3H in glycogen were determined as described previously (Fujimoto et al., 2006; Torres et al., 2009). Liver content of UDP-glucose and UDP-galactose were obtained through two sequential chromatographic separations, and the amount of ^3H radioactivity in each fraction was measured as described previously (Fujimoto et al., 2006). Glycogen synthase and phosphorylase activities were measured as reported previously (Fujimoto et al., 2006; Torres et al., 2009). It has been reported that barbiturate derivatives do not have a direct effect on activity of liver phosphorylase *in vitro* (Brunner and Haugaard, 1965). However, Mikines et al. (1986) showed a time-dependent decrease in glycogen phosphorylase *a* activity in liver after anesthesia of rats with pentobarbital. Thus, there is a possibility that the activity of liver glycogen phosphorylase was altered to some extent during the time between euthanasia and freeze-clamping the tissue.

Materials. [^3H] glucose (PerkinElmer Life and Analytical Sciences, Waltham, MA) was used as the glucose tracer (500 $\mu\text{Ci}/0.005$ mg). Indocyanine green (Hynson, Westcott, and Dunning, Baltimore, MD) was prepared in sterile water. Insulin (Squibb-Novo, Princeton, NJ), glucagon (Eli Lilly, Indianapolis, IN), and cyclic somatostatin (Bachem, Torrance, CA) were prepared with normal saline and contained 3% (v/v) of the dog's own plasma. Cortisol radioimmunoassay kits were obtained from Micromedex Systems (Horsham, PA). GPI (CP-316819; see Hoover et al., 2001), was synthesized at Pfizer Global Research and Development (Groton, CT).

Calculations. Hepatic blood flow was assessed by measuring hepatic extraction of indocyanine green (Leevy et al., 1962). Based on data from Greenway and Stark (1971), the proportions of the hepatic blood supply provided by the hepatic artery and portal vein were assumed to be 28 and 72%, respectively, which conforms to data we obtained with Doppler flow probes during pancreatic clamps (Myers et al., 1991). These proportions were assumed to remain constant throughout all experiments because treatment did not significantly affect hepatic blood flow. Net hepatic substrate balance was calculated using the formula $[\text{H} - (0.28\text{A} + 0.72\text{P})] \times \text{HF}$, where A, P, and H are the arterial, portal vein, and hepatic vein substrate concentrations, respectively, and HF is the hepatic blood or plasma flow. When substrate levels in blood were measured, blood flow was used in the calculation, whereas plasma flow was used when plasma substrate levels were measured.

For calculation of minimal estimates of unidirectional hepatic glucose uptake (M-HGU), which does not include glucose taken up by liver and then returned (glucose cycling), the net hepatic [^3H]glucose uptake was divided by average sinusoidal [^3H]glucose-specific activity (SA). The sinusoidal [^3H]glucose SA was calculated using the formula $[(0.28 \times [\text{HSA in artery}] + (0.72 \times [\text{HSA in portal vein}])]$. Minimal estimates of unidirectional hepatic glucose release (M-HGR), which does not contain glucose released via glucose cycling, was determined by adding M-HGU to net hepatic glucose balance, which is NHGO when the balance is positive and net hepatic glucose uptake (NHGU) when the balance is negative. Assuming that there is 100% conversion of gluconeogenic precursors taken up by the liver into G-6-P (Goldstein et al., 2002), the gluconeogenic

flux to G-6-P (G-6-P neogenesis) was determined by summation of net hepatic uptake rates of the gluconeogenic precursors (alanine, glycine, serine, threonine, glycerol, and lactate), converting the sum to glucose equivalents and dividing by two to account for the incorporation of three carbon precursors into the six-carbon molecules. When lactate was released, net lactate uptake was calculated as zero. The fractional contribution of plasma glucose via the direct pathway to UDP-glucose flux was calculated as the ratio of [^3H]SA in hepatic UDP-glucose to the liver sinusoidal [^3H]glucose SA. The remaining G-6-P was formed via other pathways, mainly glycogenolysis and G-6-P neogenesis. Glucose-6-phosphatase (G-6-Pase) flux was calculated using the formula $[\text{M-HGR}/(1 - [\text{HSA in hepatic UDP-glucose}/[\text{HSA in plasma glucose}]])]$. Glucose cycling was calculated as the difference between G-6-Pase flux rate and M-HGR.

Hepatic glycogen content was determined by multiplying the glycogen concentration (milligram per gram of liver) by liver weight. The amount of glycogen synthesized from glucose (the direct pathway) was calculated by dividing ^3H radioactivity incorporated into liver glycogen by the average [^3H]SA in arterial plasma glucose between 0 and 180 min. Net glycogenolysis was calculated as the difference between M-HGR and G-6-P neogenesis, with the assumption that all of the G-6-P derived from G-6-P neogenesis was converted to glucose.

Statistical Analysis. Data are expressed as means \pm S.E. Statistical comparisons were made using two-way analysis of variance with repeated-measures design. Post hoc analysis was performed using either paired *t* test or unpaired *t* test.

Results

Hematocrit, Hepatic Blood Flow, and Hormone Levels. Arterial hematocrit (Fig. 1A) and hepatic arterial and portal vein blood flow (Fig. 1B) were not significantly different over time among the three groups. Portal and arterial insulin levels remained at basal levels throughout the study among all three groups (Fig. 1C). Arterial and portal glucagon levels were basal and unchanged in all protocols during the first two time periods, and then in the third period (Test Period II), they rose approximately 2- and 3-fold, respectively, when the glucagon infusion rate was increased (Fig. 1D). Arterial cortisol, norepinephrine, and epinephrine levels were not significantly changed with either GPI or MT treatment (Table 1).

Hepatic Glucose Balance. Plasma glucose levels were maintained at basal during the control period and Test Period I (Fig. 2A). During the control period, there were no significant differences in NHGO and M-HGU among the groups (Fig. 2, C and D). During Test Period I, in the vehicle group, glucose infusion ($5.6 \pm 1.6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was necessary to maintain euglycemia (Fig. 2B). Net hepatic balance of glucose and hepatic glucose uptake changed minimally (Fig. 2, C and D). In the GPI group, glucose needed to be infused at $13.3 \pm 2.6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ to maintain euglycemia in Test Period I. NHGO was completely suppressed by 60 min ($-1.5 \pm 2.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and, thereafter, switched to NHGU ($3.8 \pm 1.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) by 90 min (Fig. 2C). Hepatic glucose uptake more than doubled in response to the GPI. In the MT group, small amount of glucose ($2.3 \pm 0.6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) needed to be infused to maintain euglycemia. NHGO tended to decrease from basal ($8.9 \pm 0.9 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) to $7.6 \pm 1.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and was not significantly different from that of the vehicle group (Fig. 2C). Hepatic glucose uptake was not altered by MT treatment.

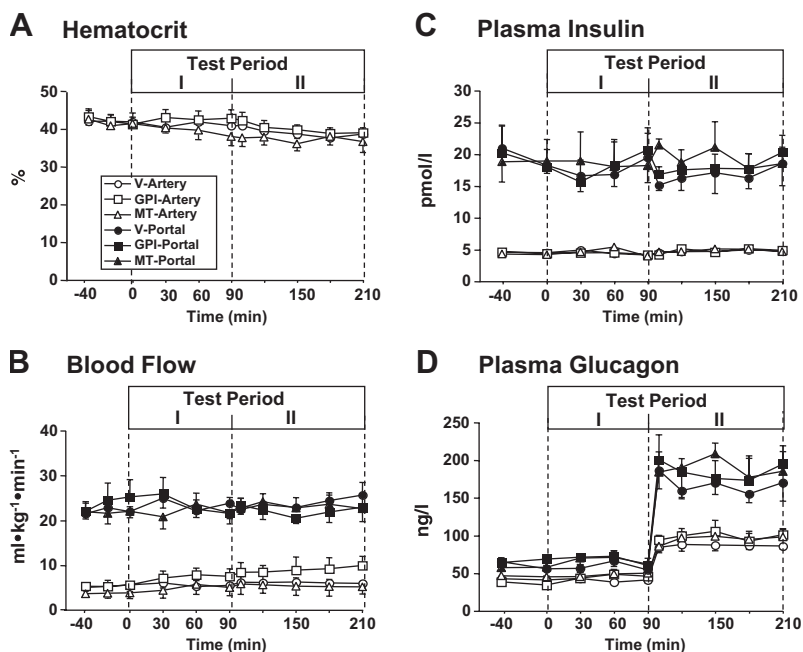


Fig. 1. Arterial blood hematocrit (A), hepatic arterial and portal blood flows (B), arterial and portal plasma levels of insulin (C), and glucagon (D) before (control) and after drug treatment (Test Period I) and subsequent increase of plasma glucagon (Test Period II) in 18-h fasted conscious dogs. The animals received intragastric bolus injection of vehicle or GPI (10 mg/kg) at 0 min, continuous infusion of metformin, or vehicle into the portal vein ($0.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) from 0 min, and a 4-fold increase in intraportal glucagon brought about from 90 min. Data are means \pm S.E. for five experiments.

TABLE 1

Arterial plasma levels of norepinephrine, epinephrine and cortisol before (control) and after drug treatment (Test Period I) and subsequent increase of plasma glucagon (Test Period II) in 18-h fasted conscious dogs
Values are means \pm S.E. for five experiments. V, vehicle.

Hormones	Groups	Control (0 min)	Study Periods			
			Test Period I		Test Period II	
			60 min	90 min	150 min	210 min
Norepinephrine (pM)	V	673 \pm 59	682 \pm 118	532 \pm 127	751 \pm 136	790 \pm 129
	GPI	523 \pm 138	489 \pm 142	488 \pm 105	519 \pm 175	689 \pm 189
	MT	761 \pm 227	592 \pm 184	697 \pm 245	767 \pm 250	871 \pm 291
Epinephrine (pM)	V	345 \pm 144	383 \pm 160	237 \pm 110	315 \pm 101	535 \pm 230
	GPI	332 \pm 152	658 \pm 373	509 \pm 157	350 \pm 157	624 \pm 317
	MT	824 \pm 277	527 \pm 215	713 \pm 248	589 \pm 204	520 \pm 245
Cortisol (nM)	V	62 \pm 12	41 \pm 5	54 \pm 18	58 \pm 8	46 \pm 4
	GPI	79 \pm 16	101 \pm 31	83 \pm 19	96 \pm 22	95 \pm 24
	MT	69 \pm 6	87 \pm 19	84 \pm 12	94 \pm 22	97 \pm 10

In response to the rise in plasma glucagon levels, in the vehicle group, the plasma glucose level rose from 5.8 ± 0.2 to 11.1 ± 1.0 mM by the end of the study (Fig. 1A). NHGO increased by $42 \pm 6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in 10 min and then fell but remained elevated relative to the previous period (Fig. 2C), and there was no change in hepatic glucose uptake (Fig. 2D).

In the GPI group, the plasma glucose levels were completely matched to the levels seen in the vehicle group by infusing glucose at $35 \pm 5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (Fig. 2, A and B). NHGO was not observed in response to the rise in plasma glucagon (Fig. 2C). Net and absolute hepatic uptake of glucose was increased in parallel (Fig. 2, C and D). In the MT group, NHGO was increased by $21 \pm 5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ within 10 min, after which it returned close to the rate evident in the previous period (Fig. 2C), although the increment of NHGO was significantly smaller than that in the vehicle group (Fig. 2C). Hepatic glucose balance uptake did not change significantly in response to MT treatment.

Net Hepatic Balances of Gluconeogenic Precursors.

As shown in Tables 2 and 3, the arterial level and net hepatic balance of all of measured gluconeogenic precursors were not

significantly changed from control values by treatment with vehicle or by the rise in glucagon in the presence of vehicle (Test Period I). On the other hand, treatment with GPI during Test Period I caused markedly decreased net hepatic lactate release and brought about a switch to hepatic lactate uptake (Table 3). The sum of net hepatic gluconeogenic precursors uptake increased significantly (Fig. 3). The rise in glucagon in the presence of GPI treatment brought about an immediate decrease in the sum of net hepatic gluconeogenic precursor uptake, which was due to a sudden decrease in net hepatic lactate uptake. Treatment with metformin tended to decrease net hepatic gluconeogenic precursor uptake (Fig. 3; Table 3). In response to the rise in plasma glucagon and glucose, in all of the three groups, arterial amino acids levels tended to decrease (Table 2) with a tendency toward an increase in net hepatic uptake and fractional extraction (data not shown) of these precursors (Table 3; Fig. 3).

Arterial Nonesterified Fatty Acids and Net Hepatic Balance. Compared with the control period, arterial levels and net hepatic uptake of Nonesterified Fatty Acids did not change significantly during Test Period I with either GPI or MT treatment. Likewise, the rise in plasma glucagon levels

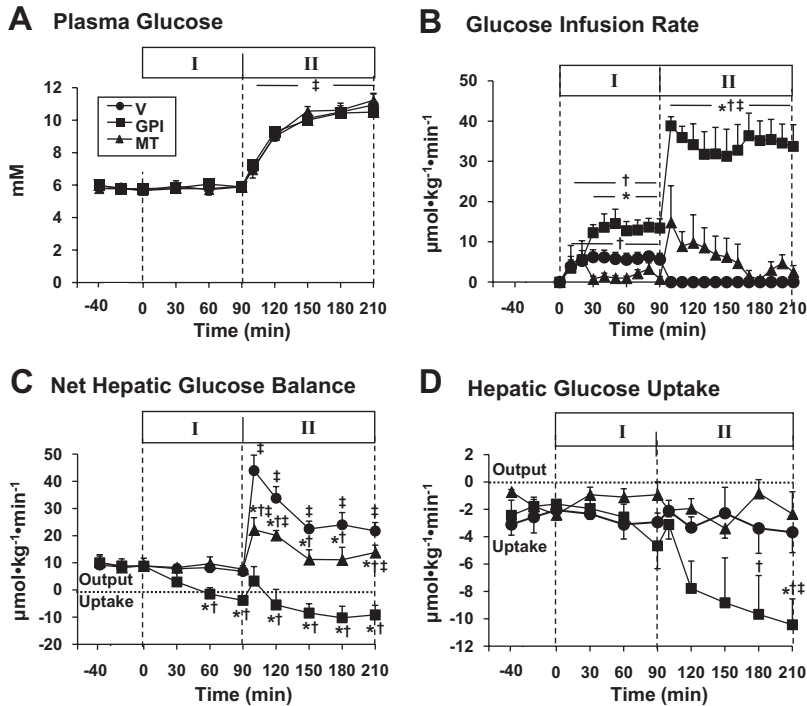


Fig. 2. Arterial plasma glucose levels (A), glucose infusion rates (B), and changes in net hepatic glucose (C), and [^3H]glucose balance (D) before (control) and after drug treatment (Test Period I) and subsequent increase of plasma glucagon (Test Period II) in 18-h fasted conscious dogs. The animals received intragastric bolus injection of vehicle or GPI (10 mg/kg) at 0 min, continuous infusion of metformin, or vehicle into the portal vein ($0.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) from 0 min, and a 4-fold increase in intraportal glucagon brought about from 90 min. Values are means \pm S.E. for five experiments. *, significantly different from the corresponding value in vehicle group ($P < 0.05$). †, significantly different from the control period with the same group ($P < 0.05$). ‡, significantly different from the corresponding value at 90 min in the Test Period I with the same group ($P < 0.05$).

TABLE 2

Arterial levels of lactate, alanine, serine, threonine, glycine, glycerol, and free fatty acids (FFAs) before (control) and after drug treatment (Test Period I) and subsequent increase of plasma glucagon (Test Period II) in 18-h fasted conscious dogs

Blood (BL) and plasma concentration (PL) of metabolites are described in micromolars. Values are means \pm S.E. for five experiments. V, vehicle.

Metabolites	Group		Study Period								
			Control	Test Period I				Test Period II			
				30	60	90	100	120	150	180	210
Lactate	V	BL	495 \pm 30	504 \pm 64	448 \pm 26	465 \pm 61	632 \pm 49	577 \pm 53	506 \pm 51	511 \pm 39	609 \pm 87
	GPI	BL	810 \pm 150	608 \pm 93	578 \pm 112	471 \pm 129	567 \pm 111	662 \pm 174	587 \pm 156	523 \pm 100	508 \pm 117
	MT	BL	830 \pm 111	822 \pm 125	849 \pm 115	903 \pm 121	981 \pm 159	1042 \pm 159	952 \pm 125	1030 \pm 224	986 \pm 187
Alanine	V	BL	507 \pm 47	553 \pm 28	503 \pm 39	521 \pm 58	556 \pm 32	543 \pm 39	487 \pm 32	426 \pm 61	394 \pm 43
	GPI	BL	509 \pm 74	457 \pm 73	398 \pm 71	388 \pm 65	408 \pm 92	428 \pm 123	369 \pm 104	308 \pm 84	311 \pm 87
	MT	BL	556 \pm 26	577 \pm 36	540 \pm 23	544 \pm 38	582 \pm 83	611 \pm 53	567 \pm 64	563 \pm 42	501 \pm 66
Serine	V	BL	198 \pm 19	187 \pm 10	194 \pm 19	180 \pm 15	165 \pm 12	154 \pm 14	152 \pm 12	142 \pm 8	127 \pm 8
	GPI	BL	174 \pm 14	174 \pm 22	184 \pm 29	177 \pm 14	148 \pm 20	133 \pm 13	144 \pm 20	128 \pm 14	132 \pm 14
	MT	BL	188 \pm 26	174 \pm 26	176 \pm 27	181 \pm 25	135 \pm 35	165 \pm 17	155 \pm 19	153 \pm 18	136 \pm 21
Threonine	V	BL	276 \pm 32	264 \pm 36	287 \pm 39	269 \pm 31	242 \pm 22	228 \pm 15	234 \pm 23	212 \pm 24	190 \pm 14
	GPI	BL	277 \pm 36	270 \pm 51	288 \pm 58	262 \pm 31	248 \pm 45	216 \pm 32	224 \pm 42	203 \pm 32	213 \pm 42
	MT	BL	303 \pm 47	287 \pm 36	296 \pm 29	299 \pm 38	197 \pm 53	298 \pm 33	255 \pm 14	269 \pm 23	242 \pm 34
Glycine	V	BL	267 \pm 27	253 \pm 21	263 \pm 26	250 \pm 19	233 \pm 19	214 \pm 19	207 \pm 20	193 \pm 16	171 \pm 15
	GPI	BL	253 \pm 32	257 \pm 39	264 \pm 48	269 \pm 39	221 \pm 33	189 \pm 22	210 \pm 42	176 \pm 26	183 \pm 27
	MT	BL	285 \pm 30	278 \pm 30	281 \pm 24	278 \pm 24	212 \pm 56	262 \pm 12	229 \pm 15	230 \pm 18	195 \pm 22
Glycerol	V	PL	124 \pm 14	112 \pm 12	104 \pm 10	110 \pm 15	127 \pm 15	98 \pm 5	73 \pm 10	93 \pm 15	125 \pm 26
	GPI	PL	164 \pm 14	195 \pm 24	155 \pm 13	180 \pm 10	180 \pm 12	144 \pm 23	158 \pm 19	149 \pm 15	157 \pm 23
	MT	PL	171 \pm 29	152 \pm 19	156 \pm 25	149 \pm 18	158 \pm 18	156 \pm 17	131 \pm 16	139 \pm 15	132 \pm 14
FFAs	V	PL	285 \pm 75	244 \pm 62	297 \pm 51	225 \pm 51	222 \pm 56	221 \pm 61	164 \pm 47	158 \pm 33	271 \pm 93
	GPI	PL	315 \pm 82	304 \pm 73	311 \pm 61	383 \pm 72	355 \pm 58	283 \pm 69	229 \pm 48	211 \pm 76	204 \pm 87
	MT	PL	331 \pm 85	254 \pm 53	258 \pm 68	239 \pm 35	224 \pm 59	203 \pm 58	144 \pm 17	153 \pm 29	211 \pm 47

that occurred during Test Period II (Tables 2 and 3) had no effect. Likewise, there were no changes in lipolysis as indicated by the stability in plasma glycerol levels.

Intermediary Metabolism in the Liver. At the end of Test Period II, compared with the vehicle group, the MT group had a 2-fold higher G-6-P content but 50% lower UDP-glucose content (Fig. 4, A and B). The ratio of ^3H -specific activity of UDP-glucose to that of plasma glucose, reflecting the percentage contribution of plasma glucose to UDP-glucose flux, was similar (Fig. 4C). Hepatic glycogen content was similar, and the amounts of ^3H in hepatic glycogen were

similarly very low but slightly elevated with metformin (Fig. 4, D and E). In the GPI group compared with the vehicle group, both G-6-P and UDP-glucose contents were markedly lower. The percentage contribution of plasma glucose to UDP-glucose flux was increased by 40% ($55 \pm 6\%$ in GPI versus $42 \pm 2\%$ in vehicle). The hepatic glycogen content was higher by 30%, and the incorporated amount of [^3H]glucose into hepatic glycogen was 100-times greater (Fig. 4, D and E).

Glycogen Phosphorylase and Synthase Activity. Compared with the vehicle group, as shown in Fig. 5, in the GPI group, glycogen phosphorylase *a* activity was markedly

TABLE 3

Net hepatic balance of lactate, alanine, serine, threonine, glycine, glycerol and free fatty acids (FFAs) before (control) and after drug treatment (Test Period I) and subsequent increase of plasma glucagon (Test Period II) in 18-h fasted conscious dogs

Net hepatic balance of metabolites are described as $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Values are means \pm S.E. for five experiments. Negative values indicate net hepatic uptake. V, vehicle.

Metabolites	Group	Control	Study Period									
			Test Period I					Test Period II				
			30 min	60 min	90 min	100 min	120 min	150 min	180 min	210 min		
Lactate	V	7.64 \pm 2.81	8.19 \pm 2.75	6.88 \pm 2.93	5.26 \pm 2.05	8.22 \pm 1.28	8.09 \pm 1.28	6.62 \pm 2.02	5.48 \pm 1.74	6.48 \pm 2.40		
	GPI	9.39 \pm 3.99	3.49 \pm 3.64	-5.02 \pm 1.77*	-4.87 \pm 2.58*	-0.21 \pm 3.73*	2.82 \pm 4.23	1.31 \pm 3.78	-0.40 \pm 3.76	0.71 \pm 3.99		
Alanine	MT	7.00 \pm 2.31	5.35 \pm 1.25	3.62 \pm 1.44	4.20 \pm 1.04	8.87 \pm 2.99	6.23 \pm 2.23	7.84 \pm 2.20	4.02 \pm 1.44	3.15 \pm 2.53		
	V	-2.87 \pm 0.71	-6.04 \pm 1.25	-3.81 \pm 1.71	-4.56 \pm 1.20	-3.19 \pm 0.64	-5.08 \pm 1.60	-5.84 \pm 1.46	-5.13 \pm 0.78	-7.02 \pm 0.95		
Serine	GPI	-2.59 \pm 0.77	-3.04 \pm 0.52	-2.69 \pm 0.79	-4.40 \pm 1.09	-4.19 \pm 0.89	-6.40 \pm 2.41	-4.93 \pm 2.04	-5.86 \pm 1.61	-6.58 \pm 2.19		
	MT	-3.11 \pm 0.48	-2.76 \pm 0.83	-1.84 \pm 0.6	-3.26 \pm 0.74	-2.35 \pm 0.5	-2.31 \pm 0.5	-3.39 \pm 0.83	-4.9 \pm 0.51	-3.41 \pm 1.13		
Threonine	V	-1.48 \pm 0.27	-1.09 \pm 0.23	-1.40 \pm 0.36	-1.43 \pm 0.33	-1.16 \pm 0.15	-1.17 \pm 0.14	-0.96 \pm 0.3	-1.53 \pm 0.19	-1.24 \pm 0.13		
	GPI	-0.79 \pm 0.35	-0.97 \pm 0.23	-0.92 \pm 0.25	-1.11 \pm 0.23	-1.09 \pm 0.31	-1.25 \pm 0.62	-1.40 \pm 0.3	-1.57 \pm 0.33	-0.97 \pm 0.24		
Glycine	MT	-0.85 \pm 0.24	-0.77 \pm 0.22	-0.85 \pm 0.28	-0.51 \pm 0.21	-0.71 \pm 0.11	-0.79 \pm 0.46	-0.85 \pm 0.24	-0.80 \pm 0.24	-0.82 \pm 0.23		
	V	-1.13 \pm 0.4	-0.21 \pm 0.35	-0.79 \pm 0.48	-1.15 \pm 0.35	-0.60 \pm 0.28	-1.14 \pm 0.41	-1.15 \pm 0.39	-1.29 \pm 0.48	-0.91 \pm 0.15		
Glycerol	GPI	-0.72 \pm 0.33	-0.93 \pm 0.32	-0.99 \pm 0.25	-1.21 \pm 0.50	-0.69 \pm 0.21	-0.81 \pm 0.14	-0.69 \pm 0.49	-0.79 \pm 0.36	-0.42 \pm 0.15		
	MT	-0.63 \pm 0.26	-0.48 \pm 0.09	-0.72 \pm 0.26	-0.34 \pm 0.17	-0.22 \pm 0.23	-0.72 \pm 0.32	-0.64 \pm 0.15	-0.47 \pm 0.36	-0.36 \pm 0.19		
FFAs	V	-1.69 \pm 0.39	-1.10 \pm 0.48	-1.60 \pm 0.33	-2.26 \pm 0.58	-1.36 \pm 0.27	-1.47 \pm 0.17	-0.90 \pm 0.35	-1.80 \pm 0.37	-1.45 \pm 0.16		
	GPI	-0.88 \pm 0.41	-1.14 \pm 0.39	-1.13 \pm 0.45	-1.53 \pm 0.42	-0.95 \pm 0.33	-0.70 \pm 0.29	-1.36 \pm 0.46	-1.20 \pm 0.38	-1.10 \pm 0.31		
FFAs	MT	-1.21 \pm 0.50	-0.75 \pm 0.19	-1.13 \pm 0.50	-0.57 \pm 0.29	-0.77 \pm 0.29	-1.29 \pm 0.69	-1.32 \pm 0.21	-1.14 \pm 0.25	-1.09 \pm 0.18		
	V	-0.83 \pm 0.49	-1.52 \pm 0.81	-1.09 \pm 0.21	-1.54 \pm 0.25	-1.64 \pm 0.66	-0.83 \pm 0.36	-1.00 \pm 0.27	-1.27 \pm 0.31	-0.91 \pm 0.19		
FFAs	GPI	-1.80 \pm 0.38	-2.20 \pm 0.51	-1.77 \pm 0.61	-2.55 \pm 0.71	-1.76 \pm 0.48	-1.11 \pm 0.41	-1.09 \pm 0.47	-1.37 \pm 0.42	-1.55 \pm 0.29		
	MT	-1.59 \pm 0.49	-1.25 \pm 0.43	-1.91 \pm 0.66	-1.29 \pm 0.18	-1.28 \pm 0.33	-1.57 \pm 0.31	-1.18 \pm 0.34	-1.44 \pm 0.35	-1.01 \pm 0.31		
FFAs	V	-3.13 \pm 0.77	-1.96 \pm 0.42	-2.60 \pm 0.83	-2.44 \pm 0.69	-2.48 \pm 0.64	-2.11 \pm 0.61	-1.73 \pm 0.33	-1.82 \pm 0.33	-2.28 \pm 0.36		
	GPI	-3.08 \pm 1.23	-3.05 \pm 0.95	-2.69 \pm 0.63	-3.44 \pm 1.01	-3.80 \pm 0.95	-1.88 \pm 0.62	-1.62 \pm 0.30	-2.60 \pm 0.81	-2.71 \pm 1.05		
FFAs	MT	-2.76 \pm 1.18	-2.58 \pm 0.43	-2.97 \pm 0.74	-2.11 \pm 0.39	-2.20 \pm 0.40	-1.96 \pm 0.95	-1.60 \pm 0.36	-1.68 \pm 0.26	-2.76 \pm 1.09		

* Significantly different from the corresponding value in vehicle group ($P < 0.05$).

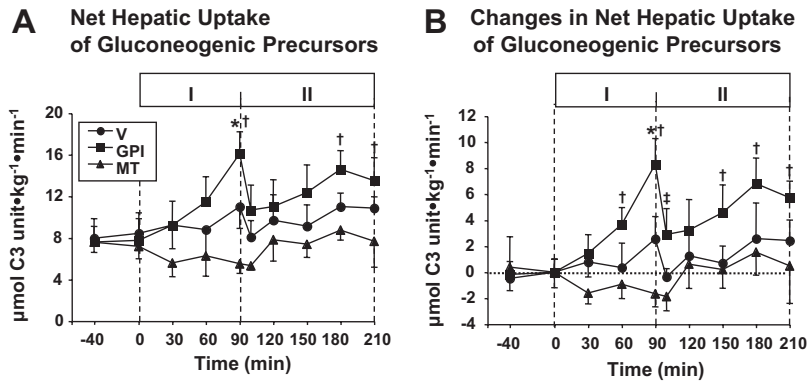


Fig. 3. Net hepatic gluconeogenesis precursor uptake (A) before (control) and after drug treatment (Test Period I) and subsequent increase of plasma glucagon (Test Period II) and changes in net hepatic gluconeogenesis precursor uptake from that in the control period (B) in 18-h fasted conscious dogs. The animals received intragastric bolus injection of vehicle or GPI (10 mg/kg) at 0 min, continuous infusion of metformin, or vehicle into the portal vein ($0.15 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) from 0 min, and a 4-fold increase in intraportal glucagon brought about from 90 min. The rate was obtained by summation of net hepatic uptake rates of lactate, glycerol, pyruvate, and all gluconeogenic amino acids. Lactate uptake was considered as zero when lactate was released by the liver. Values are means \pm S.E. for five experiments. *, significantly different from the corresponding value in the vehicle group ($P < 0.05$). †, significantly different from the control period ($P < 0.05$). ‡, significantly different from the corresponding value at 90 min in the Test Period I with the same group ($P < 0.05$).

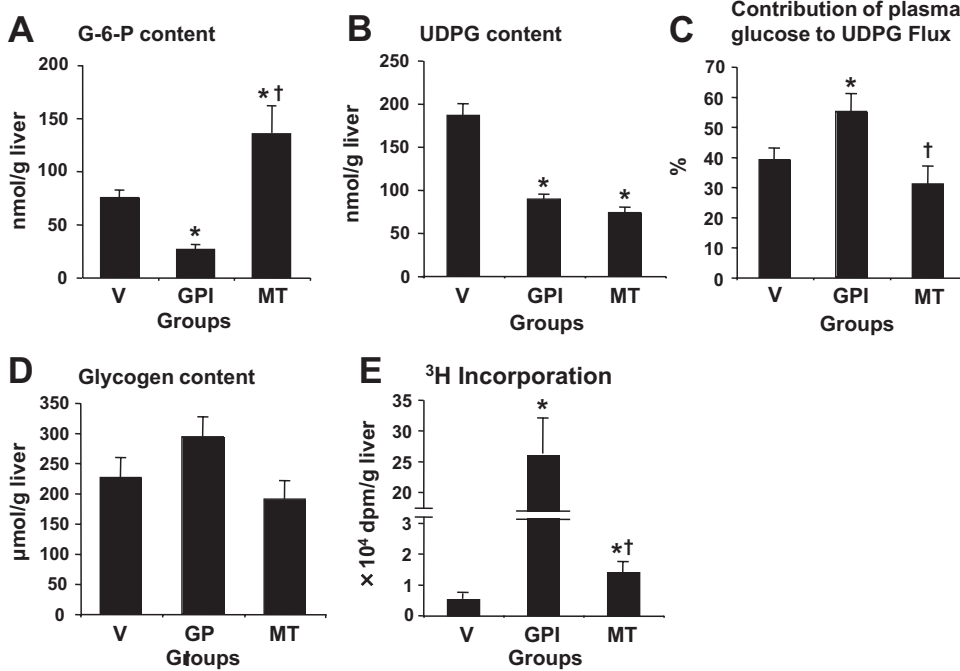


Fig. 4. Hepatic content of G-6-P (A), UDP-glucose (B), and glycogen content (D), percentage contribution of plasma glucose to UDP-glucose flux (C), and amount of [^3H]glucose incorporated into glycogen (E) at the end of the Test Period II. Data are means \pm S.E. for five experiments. *, significantly different from the corresponding value in the vehicle group ($P < 0.05$). †, significantly different from the control period ($P < 0.05$).

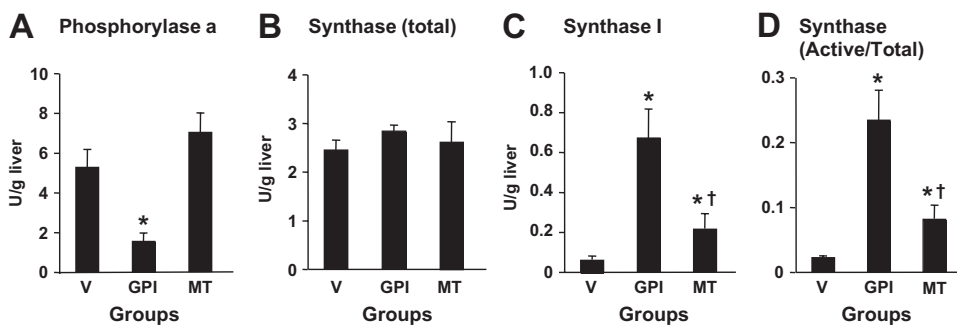


Fig. 5. Phosphorylase activity (A), total (B), and active form (C) activities, and the ratio of active form to total activity in the liver at the end of the Test Period II. Data are means \pm S.E. for five experiments. *, significantly different from the corresponding value in the vehicle group ($P < 0.05$). †, significantly different from the control period ($P < 0.05$).

lower, and in contrast, glycogen synthase I activity and the ratio of active form (I) to total activity were higher. In the MT group, glycogen phosphorylase *a* activity was similar to that in the vehicle group. However, the active form of glycogen synthase and the ratio of active to total activity were slightly but significantly higher.

Estimated Hepatic Glucose Fluxes. During the control period, there were no significant differences in NHGO, M-HGU, M-HGR, G-6-P neogenesis, net glycogenolytic rate, and percentage contribution of gluconeogenesis and glycogenolysis to M-HGR among the groups (Table 4). During Test Pe-

riod I, in the vehicle group, NHGO and M-HGR tended to reduce with significant decrease in the rate and the contribution of glycogenolysis to M-HGR from that in the control period. On the other hand, G-6-P neogenesis was not changed, and the percentage contribution of gluconeogenesis to M-HGR rose relative to that in the control period. In the GPI group, GPI treatment switched NHGB from NHGO to NHGU, with a marked decrease in M-HGR and net glycogenolytic rate and an increase in the gluconeogenic rate. In the MT group, NHGO, M-HGR, net glycogenolysis, and G-6-P neogenesis tended to decrease from that in the control period.

gree of phosphorylation of phosphorylase in hepatocytes, thus leading to inhibition of glycogen synthase (Latsis et al., 2002). However, 1,4-dideroxy-1,4-imino-D-arabinitol had no effect on glycogen synthesis in hepatocytes (Andersen et al., 1999; Latsis et al., 2002). On the other hand, a 1,4-dihydroxy-pyridine-2,3-dicarboxylate derivative (BAY R3401) and an indole 2-carboxamide (CP-316819), which were used in the previous (Shiota et al., 1997) and present studies, respectively, cause conversion of phosphorylase *a* into phosphorylase *b* and activation of glycogen synthase and, as a result, promote net glycogenesis (Shiota et al., 1997; Bergans et al., 2000); thereby, BAY R3401 and CP-316819 might pull gluconeogenic flux by increasing G-6-P flux toward glycogen via stimulation of net glycogenesis. Therefore, the observed differences in the effects of GPI on gluconeogenesis between Fosgerau et al. (2001) and us (Shiota et al., 1997; Bergans et al., 2000) may be explained as a difference in the mechanism of action among the compounds used.

MT tended to decrease NHGO and M-HGR that was associated with a decrease in both net glycogenolysis and gluconeogenesis. The percentage contribution of gluconeogenesis and glycogenolysis to M-HGR was not changed by MT. Therefore, it is likely that decreased NHGO by MT result from an inhibition of common site in the metabolic pathway where glucose is produced via glycogenolysis and gluconeogenesis.

Effect of Glucagon on Hepatic Glucose Flux in the Presence of GPI. The increment of NHGO brought about by glucagon was associated exclusively with increased net glycogenolysis in the placebo group. Glucagon failed to increase NHGO in the presence of GPI. Net hepatic glucose flux is a balance between glucose phosphorylation rate mediated by glucokinase and G-6-P dephosphorylation rate mediated by G-6-Pase. A failure of glucagon to increase NHGO in the presence of GPI was associated with a failure to increase in G-6-Pase flux, because glucokinase flux was not altered. Hepatic content of G-6-P, a substrate for G-6-Pase, was less than half of that in the vehicle group, implying that the failure of glucagon to increase G-6-Pase flux arose from the failure of the hormone to maintain or increase the G-6-P pool. The rate of glucose phosphorylation (GK flux) or G-6-P neogenesis was not significantly altered; therefore, the decreased G-6-P content resulted from greater conversion of G-6-P into glycogen.

Glucagon increases net glycogenolysis via activation of glycogen phosphorylase and by inhibition of glycogen synthesis via inactivation of glycogen synthase. Compared with the vehicle group, glycogen phosphorylase *a* activity was significantly lower, and glycogen synthase activity was higher with GPI treatment. Activation of glycogen phosphorylase via protein kinase A and phosphorylase kinase results in enhanced phosphorylation of the inactive form (glycogen phosphorylase *b*), which promotes the formation of an active enzyme complex (glycogen phosphorylase *a*) (Bollen et al., 1998). The GPI, CP-316819, has been reported to bind the indole inhibitor site of glycogen phosphorylase by enhancing the dephosphorylation of the glycogen phosphorylase *a* complex, converting it to an inactive form (glycogen phosphorylase *b*) (Treadway et al., 2001). This is thought to occur as a result of a binding-induced conformational change that makes glycogen phosphorylase *a* better substrate for the regulatory phosphatase (protein phosphatase 1) (Kasvinsky et al., 1981) and/or interference with the binding of phosphorylase *a* to the C-terminal domain of the glycogen-targeting subunit of

protein phosphatase 1 (Kellsall et al., 2007; Pautsch et al., 2008). The indole-2-carboxamide (CP-91149; Pfizer, Inc.) was shown to counteract the phosphorylation caused by glucagon (Latsis et al., 2002). Therefore, the treatment with GPI may interfere with the action of glucagon by activation of protein phosphatase 1.

The inactivation of glycogen synthase is mediated by enhancing the phosphorylation of the active form (glycogen synthase I) and converting it to an inactive form (glycogen synthase D) via activation of protein kinase A and mediated by protein phosphatase 1 (Bollen et al., 1998). Glycogen phosphorylase *a* may inhibit protein phosphatase 1 allosterically through the binding of the enzyme to the C-terminal domain of the glycogen-targeting subunit of protein phosphatase 1 (Armstrong et al., 1998). The failure of glucagon to inactivate glycogen synthase in the presence of GPI probably results from a defective activation of glycogen phosphorylase by this hormone.

Insulin inactivates glycogen phosphorylase *a* by activation of protein phosphatase 1 via activation of protein kinase B/Akt (Aiston et al., 2006). It has been reported that inhibition of phosphorylase activity is critical to activate glycogen synthase (glycogen synthesis) by elevation of glucose and insulin in cultured hepatocytes isolated from normal rats (Aiston et al., 2003b). Therefore, it is likely that acute alteration of net glycogen flux in the liver by glucagon and insulin is primarily mediated by regulating glycogen phosphorylase activity.

Effect of Glucagon on Hepatic Glucose Flux in the Presence of MT. Partial reduction of the glucagon-induced increase in NHGO in the presence of MT was also associated with decreased G-6-Pase flux without an alteration of GK flux. In contrast to the decreased G-6-Pase flux in the GPI group with a marked reduction in metabolites in the GPI group, the decreased G-6-Pase flux with MT treatment was accompanied by a markedly greater content of G-6-P compared with the vehicle group. This increase in the G-6-P pool occurred without an increase in glucose phosphorylation (GK flux), G-6-P neogenesis, or glycogenolysis. Furthermore, the lower G-6-Pase flux was associated with equally lower rates of gluconeogenesis, glucose cycling, and glycogenolysis without changes in the percentage contribution of glycogenolysis, gluconeogenesis, and glucose cycling to G-6-Pase flux. These results suggest that the reduced glucagon-induced NHGO brought about by treatment with MT resulted primarily from an inhibition of G-6-Pase activity by MT.

Decreased glucagon-induced glycogenolysis in the presence of MT was brought about without decreased phosphorylase *a* activity. On the other hand, glycogen synthase activity and incorporation of glucose into glycogen were slightly but significantly higher in the MT group compared with that in the vehicle group. The greater glycogen synthase activity in the MT group might have arisen due to the increased G-6-P content, because the latter is a strong activator of glycogen synthase. Our results are consistent with the report by Mithieux et al. (2002) that the suppression of hepatic glucose production by chronic treatment with MT in insulin-resistant high-fat diet fed rats is accompanied by increased content of G-6-P in liver. Therefore, it is likely that inhibition of G-6-Pase activity by MT decreased net glycogenolysis by promoting glycogen sparing through increased G-6-P content.

The glucose-lowering effects of MT are mainly a consequence of reduced hepatic glucose output through inhibition of both gluconeogenesis and glycogenolysis in patients with type 2 diabetes (Hundal et al., 2000; Kirpichnikov et al., 2002). The in vitro studies using isolated hepatocytes (Argaud et al., 1993) or perfused liver (Radziuk et al., 1997) demonstrated the acute effect of MT in inhibiting gluconeogenesis. Although its primary action has been proposed to be mitochondrial function, the exact mechanism through which MT reduces hepatic gluconeogenesis remains unclear (Kirpichnikov et al., 2002). The present study demonstrates that the primary action of MT to reduce net glycogenolysis may not be through glycogen phosphorylase inhibition. Glucose production via glycogenolysis and gluconeogenesis shares one common step in their metabolic pathway, dephosphorylation of G-6-P by G-6-Pase. Therefore, the present data suggest that G-6-Pase is one of the primary sites of MT's action.

Authorship Contributions

Participated in research design: Shiota and Treadway.

Conducted experiments: Torres, Sasaki, Lacy, Donahue, and Shiota.

Contributed new reagents or analytic tools: Treadway.

Performed data analysis: Shiota and Cherrington.

Wrote or contributed to the writing of the manuscript: Shiota, Printz, and Cherrington.

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