Increased Potency and Efficacy of Combined Phosphorylase Inactivation and Glucokinase Activation in Control of Hepatocyte Glycogen Metabolism

Laura J. Hampson and Loranne Agius

Glucokinase and phosphorylase both have a high control strength over hepatocyte glycogen metabolism and are potential therapeutic targets for type 2 diabetes. We tested whether combined phosphorylase inactivation and glucokinase activation is a more effective strategy for controlling hepatic glycogen metabolism than single-site targeting. Activation of glucokinase by enzyme overexpression combined with selective dephosphorylation of phosphorylase-α by an indole carboxamide that favors the T conformation of phosphorylase caused a greater stimulation of glycogen synthesis than the sum of either treatment alone. This result is explained by the complementary roles of elevated glucose-6-phosphate (G6P; a positive modulator) and depleted phosphorylase-α (a negative modulator) in activating glycogen synthase and also by synergistic inactivation of phosphorylase-α by glucokinase activation and the indole carboxamide. Inactivation of phosphorylase-α by the indole carboxamide was counteracted by 5-aminoimidazole-4-carboxamide 1-β-d-ribofuranoside, which is metabolized to an AMP analog; this effect was reversed by G6P. Our findings provide further evidence for the inverse roles of G6P and AMP in regulating the activation state of hepatic phosphorylase. It is proposed that dual targeting of glucokinase and phosphorylase-α enables improved potency and efficacy in controlling hepatic glucose metabolism. Diabetes 54:617–623, 2005

In type 2 diabetes, the liver’s failure to maintain blood glucose homeostasis is a significant contributing factor to hyperglycemia during both the absorptive and the fasting state (1,2). Accordingly, targeting hepatic glucose utilization or production is a potential therapeutic strategy for type 2 diabetes. The optimal targets for drug intervention are proteins with a high control strength over glucose metabolism (3), such as glucokinase (4) and phosphorylase (5).

Activators of glucokinase lower blood glucose in animal models of type 2 diabetes by potentiating glucose-induced insulin secretion (6) and stimulating hepatic glucose metabolism (7). Their efficacy in lowering blood glucose concentrations is consistent with the effect of activating mutations of the glucokinase gene that cause hyperinsulinemia and hypoglycemia in humans (8). The high control strength of glucokinase on liver metabolism is explained by the reciprocal control of glucokinase activity by its regulatory protein (9) and the role of glucose-6-phosphate (G6P), the product of the glucokinase-catalyzed reaction, as an activator of glycogen synthase (10,11) and inactivator of phosphorylase (12,13).

Inhibitors of liver phosphorylase also lower blood glucose in animal models of type 2 diabetes (14–16). Phosphorylase catalyzes the flux-generating step in glycogen degradation (17) and is a key regulatory component of the mechanism by which insulin activates glycogen synthase in liver (18,19). Various classes of phosphorylase inhibitors have been identified that inhibit hepatic glycogenolysis by interacting with diverse binding sites (20). They include glucose analogs (21), dihydropyridine derivatives that bind to the adenine nucleotide activation site (22,23), flavopiridol derivatives that bind to the purine nucleoside inhibitor site (24), and indole carboxamides that bind to a novel site (14,25). Many, but not all, of these compounds promote dephosphorylation of phosphorylase-α (conversion of phosphorylase-b) by favoring the T conformation, which is a better substrate for phosphorylase phosphatase (26). Because phosphorylase-α is a potent allosteric inhibitor of glycogen synthase phosphatase associated with the glycogen-targeting protein G_{T}, phosphorylase inhibitors that promote dephosphorylation of phosphorylase-α also cause stimulation of glycogen synthesis (5,17,26).

Targeting of a single site in a metabolic pathway is predicted to have a limited effect on metabolic flux (27). In this study, we tested the hypothesis that simultaneous targeting of two enzymes with a high control strength over glycogen metabolism is more effective at increasing pathway flux than targeting a single site. We determined the combined effects of glucokinase overexpression and inactivation of phosphorylase with an indole carboxamide (14) on hepatocyte glycogen synthesis. Our results demonstrate the increased potency of the phosphorylase inhibitors as well as the increased efficacy achieved by combined targeting of the two enzymes.
RESEARCH DESIGN AND METHODS

CP-91149 was a kind gift from Pfizer Global Research and Development (Groton/New London Laboratories, Groton, CT); the adenovirus for glucokinase expression (28) was a kind gift from Dr. C. Newgard (Duke University, Durham, NC).

Hepatocytes were isolated by collagenase perfusion of the liver from male Wistar rats (180–250 g body wt; B&K, Hull, U.K.) fed ad libitum. The hepatocytes were cultured in monolayer in minimum essential medium (MEM) containing 6% vol/vol newborn calf serum for 2 h to allow cell attachment (5). This was followed by a 2-h incubation in serum-free medium with varying titers of adenovirus encoding rat liver glucokinase (28). The medium was then replaced by serum-free MEM containing 10 nmol/l dexamethasone, and the hepatocytes were cultured for 16 h.

Glycogen synthesis was determined during a 3-h incubation in MEM containing 10 mmol/l glucose and [U-14C]glucose (5). Incubations without radiolabel were performed to determine enzyme activity and G6P levels (12). Glucokinase activity was measured spectrometrically after hepatocytes were permeabilized with digitonin (4). To determine phosphorylase-a and glycogen synthase, hepatocyte monolayers were snap frozen in liquid N2. Phosphorylase-a levels were measured spectrometrically (29). The concentration of CP-91149 necessary to cause the half-maximal effect (EC50) was determined using the Fig.P program (Biosoft, Cambridge, U.K.). Glycogen synthase activity was determined in homogenate extracts from the incorporation of [3H]glucose from uridine diphosphate [3H]glucose into glycogen in the absence or presence of G6P, representing active and total enzyme, respectively (30). Translocation of glycogen synthase was determined from the distribution of total glycogen synthase (assayed with G6P) in the 13,000-g supernatant and pellet fractions of homogenates and is expressed as total glycogen synthase in the pellet percent (supernatant + pellet) fractions (13).

Enzyme activities are expressed as milliunits per milligram of cell protein, where one milliunit is the amount converting 1 nmol of substrate/min.

Data are expressed as means ± SE for the number of hepatocyte preparations indicated. Statistical analysis was by the paired t test.

RESULTS

Combined effects of glucokinase overexpression and CP-91149 on glycogen synthesis. Previous studies have shown that graded overexpression of glucokinase (4) or inactivation of phosphorylase with CP-91149, an indole carboxamide (5), stimulates glycogen synthesis. We determined the combined effects of graded glucokinase overexpression and CP-91149 on glycogen synthesis (Fig. 1). Overexpression of glucokinase by up to fivefold relative to endogenous activity caused a progressive increase in glycogen synthesis, and CP-91149 (2.5 μmol/l) further increased glycogen synthesis (Fig. 1 A and B). The increment in glycogen synthesis caused by CP-91149 was significantly greater in cells overexpressing glucokinase (Fig. 1C); similarly, the increment caused by glucokinase overexpression was greater in incubations with CP-91149 than in controls without the inhibitor (Fig. 1D), indicating that the combined effects of glucokinase overexpression and CP-91149 were more than additive.

FIG. 1. Combined effects of glucokinase overexpression and CP-91149 on glycogen synthesis. Hepatocytes were either untreated (1.0-fold) or treated with four titers of adenovirus for expression of glucokinase by 1.3-, 1.8-, 2.9-, and 5.2-fold relative to endogenous activity. After a 16-h culture, they were incubated for 3 h in MEM containing 10 mmol/l glucose and [U-14C]glucose without or with 2.5 μmol/l CP-91149 for determination of glycogen synthesis. A: Rates of glycogen synthesis. B: Double log plot of glycogen synthesis versus glucokinase activity. C: Increment in glycogen synthesis caused by CP-91149. D: Increment in glycogen synthesis caused by glucokinase overexpression. Data are means ± SE, n = 4. *P < 0.05 vs. untreated hepatocytes (1.0-fold); **P < 0.05 vs. no inhibitor.
Role of phosphorylase inactivation and glycogen synthase activation. Because both CP-91149 and glucokinase overexpression cause inactivation of phosphorylase (5,12,13) and activation of glycogen synthase (5,31,32), we tested whether glycolysis synthesis correlates with the activation state of phosphorylase or glycogen synthase. The combined effects of glucokinase overexpression and CP-91149 on inactivation of phosphorylase (Fig. 2A) and activation of glycogen synthase (Fig. 3A) were greater than the effects of either treatment alone.

A plot of glycogen synthesis against phosphorylase-α activity (Fig. 2B) shows that CP-91149 caused greater inactivation of phosphorylase than glucokinase expression for corresponding rates of glycogen synthesis. This finding is consistent with the selective action of CP-91149 on phosphorylase inactivation (14,26) and with the activation of glycogen synthase by G6P (10,11). In contrast, when glycogen synthesis was plotted against glycogen synthase activity, the data for incubations without and with CP-91149 were superimposed (Fig. 3B). Similar to activation of glycogen synthase, both glucokinase overexpression and CP-91149 caused movement of glycogen synthase from a soluble to a pellet fraction (Fig. 3C), and this translocation correlated with glycogen synthesis (Fig. 3D).

This observation is consistent with the role of activation and translocation of glycogen synthase in the mechanism(s) by which phosphorylase inactivation and glucokinase activity regulate glycogen synthesis (13).

The cellular G6P content was increased by glucokinase overexpression by 4- to 12-fold in the absence of CP-91149, a result in agreement with previous findings (31,32). Although CP-91149 alone did not affect the G6P content in control conditions, it did suppress the increase by glucokinase overexpression to two- to sixfold (Fig. 4).

Gluco kinase expression increases the affinity for CP-91149 on glycogen synthesis. To determine the basis for the synergistic stimulation of glycogen synthesis by increased glucokinase activity and phosphorylase inactivation, we tested whether glucokinase overexpression increases the affinity for CP-91149 on glycogen synthesis. The concentration of CP-91149 that caused the EC<sub>50</sub> was decreased (2.9 ± 0.3 to 1.8 ± 0.3 μmol/l; P < 0.002) (Fig. 5A) in cells with twofold glucokinase overexpression, and the increase in glycogen synthesis in cells overexpressing glucokinase and incubated with CP-91149 (Fig. 5B) was greater than the arithmetic sum of the stimulation by CP-91149 and glucokinase expression alone (Fig. 5B).

Converse effects of glucokinase overexpression and 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside on phosphorylase inactivation by CP-91149. Because glucokinase overexpression is associated with an increase in G6P (32) and the effects of G6P on phosphorylase are counteracted by 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), which is metabolized to an AMP analog (13), we tested the separate and combined effects of glucokinase overexpression and AICAR on the inactivation of phosphorylase-α by CP-91149. AICAR activated phosphorylase (Fig. 6A) and increased the EC<sub>50</sub> for CP-91149 (Fig. 6B), whereas glucokinase overexpression partially reversed the effect of AICAR.

Because inactivation of phosphorylase by a high glucose concentration is in part due to the increase in G6P (12,13), we tested whether the affinity for CP-91149 is affected by a high glucose concentration and whether this involves a role for G6P. 5-Thioglucose (5TG), a glucokinase inhibitor (13), suppressed the increase in G6P caused by 25 mmol/l glucose (5 mmol/l glucose, 0.27 ± 0.07; 25 mmol/l glucose, 1.54 ± 0.18; 25 mmol/l glucose + 3 mmol/l 5TG, 0.40 ± 0.08 mmol/mg; n = 6) and partially counteracted the decrease in the EC<sub>50</sub> for CP-91149 caused by 25 mmol/l glucose (Fig. 7), consistent with the roles for both glucose and G6P in synergizing with CP-91149.

We tested other substrate conditions that are associated with an increase in G6P (12,13). Incubation with dihydroxyacetone (1 mmol/l), which caused a greater than twofold increase in G6P (0.22 ± 0.03 to 0.60 ± 0.07 nmol/mg protein), decreased the EC<sub>50</sub> for CP-91149 (2.5 ± 0.5 to 1.5 ± 0.1 μmol/l; n = 6; P < 0.03), and incubation with 0.2 mmol/l octanoate, which increased G6P by 60% (0.22 ± 0.03 to 0.35 ± 0.03 mmol/mg protein), also decreased the EC<sub>50</sub> for CP-91149 (2.0 ± 0.4 to 1.7 ± 0.4 μmol/l; n = 6; P < 0.02), consistent with the synergism between G6P and the indole carboxamide.
DISCUSSION

The concept of combination therapy for type 2 diabetes has been widely explored with drugs that target different organs such as insulin secretagogues and insulin sensitizers or metformin (33–35). In this study, we tested the hypothesis that combination therapy for targets within the same organ and metabolic pathway has the potential advantage of increased efficacy and potency, thereby enabling the usage of lower drug concentrations. We tested the combined effects of upregulation of glucokinase and downregulation of phosphorylase-α on hepatocyte glycogen metabolism because both enzymes have a high control strength on glycogen metabolism (4,5) and both glucokinase activators (6) and phosphorylase inhibitors (14–16,36) lower blood glucose in animal diabetes when used separately.

Glucokinase is regulated by changes in enzyme expression (37,38) and subcellular compartmentation (39). Pharmacological activators of glucokinase increase the enzyme affinity for glucose and cause enzyme translocation from the nucleus (6,7), thus mimicking the effects of physiological activators. In this study, we expressed glucokinase by 30–500% above endogenous to mimic the changes induced by nutritional state or pharmacological activators. These activities of glucokinase increase the G6P content, which causes both activation of glycogen synthase (10,11,32) and inactivation of phosphorylase (12,13). The combined effects of glucokinase activation and phosphorylase inactivation might be expected to be less than additive because phosphorylase inactivation is downstream of glucokinase activation (Fig. 8). However, glucokinase expression caused increased efficacy and potency of the phosphorylase inhibitor on glycogen synthesis. This result is partially explained by the synergy between G6P and the indole carboxamide in promoting dephosphorylation (inactivation) of phosphorylase-α, but it may also involve the synergy between the elevated G6P levels and depletion of phosphorylase-α in promoting activation of glycogen synthase (Fig. 8).

The partial counteraction by the phosphorylase inhibitor of the G6P increase caused by glucokinase overexpression indicates partitioning of G6P toward glycogen
synthesis in conditions of elevated glucokinase flux and activation of glycogen synthase. This result is consistent with the hypothesis of Schafer et al. (40) that the phosphorylation state of glycogen synthase determines the G6P concentration and suggests that this mechanism operates in hepatocytes in conditions of elevated glucokinase activity. Synergy between the indole carboxamide and G6P on dephosphorylation of phosphorylase-\(\alpha\) was observed despite the partial lowering of G6P by the inhibitor. Evidence for the synergy between G6P and the phosphorylase inhibitor is supported by the decrease in the EC\(_{50}\) for CP-91149 in cells overexpressing glucokinase or incubated with octanoate or dihydroxyacetone, both of which raise the cell content of G6P, and by the partial reversal of the effects of 25 mmol/l glucose with the glucokinase inhibitor 5TG, which counteracts the rise in G6P. This indicates that synergy between CP-91149 and high glucose is due in part to the elevated G6P and in part to a direct effect of glucose. The converse effect of AICAR, which is metabolized to an AMP analog, in decreasing the potency for CP-91149 and 

FIG. 5. Effects of glucokinase overexpression on CP-91149 affinity. A: Hepatocytes were either untreated or treated with adenovirus for twofold expression of glucokinase. Glycogen synthesis was determined in the presence of variable CP-91149 concentration. B: Comparison of the measured increment in glycogen synthesis above basal (nontransduced with no inhibitor) in cells overexpressing glucokinase and incubated with 1–10 \(\mu\)mol/l inhibitor (■) relative to the sum of the measured increments by glucokinase expression alone plus CP-91149 alone (□). Data are means ± SE, \(n = 4\). *\(P < 0.05\) and **\(P < 0.005\) for the measured increment vs. the sum of individual increments.

FIG. 6. Converse effects of AICAR and glucokinase overexpression on the dephosphorylation of phosphorylase-\(\alpha\) by CP-91149. Hepatocytes were untreated or treated (■ and ○) for overexpression of glucokinase by 1.8-fold. Phosphorylase-\(\alpha\) activity was determined after a 60-min incubation with varying amounts of CP-91149 in the absence or presence of 100 \(\mu\)mol/l AICAR. A: Phosphorylase-\(\alpha\) activity. B: EC\(_{50}\) for CP-91149. Data are means ± SE, \(n = 4\). *\(P < 0.05\) and **\(P < 0.005\) for effects of glucokinase overexpression; #\(P < 0.05\) for effect of AICAR.

FIG. 7. 5TG partially counteracts the effects of glucose on the inactivation of phosphorylase-\(\alpha\) by CP-91149. Hepatocytes were incubated for 1 h with varying concentrations of CP-91149 (0.5–10 \(\mu\)mol/l) at 5 or 10 mmol/glucose or 25 mmol/l glucose without or with 3 mmol/l 5TG. The EC\(_{50}\) for CP-91149 was determined as in Fig. 6. Data are means ± SE, \(n = 4\). *\(P < 0.05\) and **\(P < 0.005\) vs. 25 mmol/l glucose.
counteracting the effect of glucokinase expression is further support for the role for G6P, as AMP and G6P compete for the same site, but promote the R and T conformations, respectively (20) (Fig. 8).

The synergy between G6P and CP-91149 in causing dephosphorylation of phosphorylase-α is unlikely to be a unique property of these ligands. Studies on the purified enzyme have shown synergy between glucose analogs and purine-site (41) or AMP-site (42,43) ligands and between indole carboxamides and glucose or purine-site ligands (14) in causing allosteric inhibition. Studies on hepatocytes have shown synergy between nonmetabolizable glucose analogs and indole carboxamides on the inhibition of glycogenolysis (44) and between glucose and glucose analogs on the inactivation of phosphorylase-α (45). The latter can be explained by synergy between the G6P derived from glucose and the glucose analog (13). Synergy with glucokinase activators is therefore predicted for phosphorylase inhibitors that favor the T conformation.

The lack of correlation between rates of glycogen synthesis and the activity of phosphorylase-α in cells overexpressing glucokinase supports a fundamental role for G6P in promoting stimulation of glycogen synthesis by mechanisms other than inactivation of phosphorylase, such as activation and translocation of glycogen synthase (10,11,46). It is noteworthy that conditions that cause activation of glycogen synthase in the absence of inactivation of phosphorylase, such as inhibitors of glycogen synthase kinase-3, have a negligible effect on glycogen synthesis (19). This observation highlights a role for mechanisms downstream of phosphorylase-α depletion that are essential for the stimulation of glycogen synthesis (19).

The synergistic effects of glucokinase activation and phosphorylase inactivation are relevant for understanding insulin action. Insulin signaling involves multiple pathways with convergent effects on metabolism (47). In hepatocytes, insulin-induced inactivation of phosphorylase is part of the mechanism leading to activation of glycogen synthase (19). Nonetheless, the fractional inactivation of phosphorylase is modest compared with the increase in glycogen synthesis. Stimulation of glucokinase tranlocation by insulin (48), with a consequent increase in G6P, may cause both inactivation of phosphorylase and activation of synthase, and synergy between convergent pathways may explain the large effect of insulin on glycogenic flux, despite modest effects on individual enzyme activities.

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REFERENCES

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