

Regulatory Effects of Glucose on the Catalytic Activity and Cellular Content of Glucokinase in the Pancreatic β Cell

Study Using Cultured Rat Islets

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Abstract

Glucose regulates the cellular content of glucokinase in the pancreatic β cell by altering the level of the enzyme. We investigated the existence of a second regulatory pathway, an alteration in the catalytic activity, by comparing V_{max} and protein levels of glucokinase in rat islets cultured under high glucose conditions (16.7 mM) for 6, 14, and 24 h. The V_{max} was increased by glucose at all time points. In contrast, glucokinase protein levels on Western blots were unchanged from the control value at 6 h but increased 40% at the later time points ($P < 0.0002$). Further evidence for a dual regulatory system was obtained with a reversal protocol. After a 6-h incubation at high glucose, an additional 3-h incubation at 5.5 mM glucose restored glucokinase V_{max} to normal, but failed to change the V_{max} after a 24-h incubation at high glucose. Finally, 10 μ M cycloheximide partially prevented the increase in glucokinase V_{max} induced by 24 h of high glucose, but had no effect at 6 h, suggesting the early increase in enzymatic activity did not require protein synthesis.

In summary, glucose regulates both the catalytic activity and cellular content of glucokinase in the β cell. Glucose-induced increases in glucokinase activity are an important element of the β cell adaptive response to hyperglycemia. (*J. Clin. Invest.* 1994; 94:1616–1620.) Key words: islets of Langerhans • insulin secretion • glucose phosphorylation • hexokinase • immunoblot

Introduction

In hepatocytes and pancreatic β cells, the high K_m glucose phosphorylation enzyme glucokinase is the rate-limiting step for glucose metabolism, and a key site of regulation for glucose-induced insulin secretion and hepatic glucose production (1, 2). The recent discovery that glucokinase gene mutations account for many of the cases of MODY (maturity-onset diabetes of youth) has pointed out the pivotal role played by this enzyme in glucose homeostasis (3, 4). Different isoforms with separate regulatory controls occur in the liver and β cell (5, 6). Interest

has focused primarily on glucose as a regulatory factor of the β cell isoform. Culturing rat islets in high glucose for 24 h causes the cellular content of this enzyme to increase without changing the mRNA level (7). Culture in low glucose has the opposite effect (7). This posttranscriptional regulation of the synthesis and/or degradation of glucokinase by glucose is to date the only known mode of regulation for the β cell isoform.

We recently postulated that a second type of regulation occurs at the level of the catalytic activity of the enzyme. This hypothesis was based on our findings in two hyperinsulinemic rat models of increased activity of islet glucokinase without any change in the level of its protein measured by Western blot (8, 9). The present study was designed to test if glucose regulates the activity of this enzyme independent of changes in its cellular content by comparing glucokinase V_{max} and protein levels in rat islets cultured at high concentrations of glucose for 6, 14, or 24 h. A dual regulatory system was confirmed, with glucose increasing both the catalytic activity and cellular content of glucokinase over different time courses and with different susceptibilities to reversal.

Methods

Isolation and culture of islets. Male Sprague-Dawley rats (150–200 g) were anesthetized with pentobarbital sodium (100 mg/kg i.p.). Islets were isolated using an adaption of the method of Gotoh et al. (10): pancreatic duct infiltration with collagenase, Histopaque gradient separation (Sigma Chemical Co., St. Louis, MO), and hand picking. Islets were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% newborn calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Gibco Laboratories, Grand Island, NY) at 37°C in humidified air/5% CO_2 .

Protocols. (a) 18 h of 5.5 mM glucose followed by 16.7 mM glucose \times 6 h; 16.7 mM glucose \times 24 h; 5.5 mM glucose \times 24 h. (b) 4 h of 5.5 mM glucose followed by 16.7 mM glucose \times 14 h; 5.5 mM glucose \times 18 h. (c) Groups of protocol a followed by an additional 3 h at 5.5 mM glucose. (d) 18 h of 5.5 mM glucose followed by 16.7 mM glucose \times 6 h with or without 10 μ M cycloheximide (Sigma Chemical Co.); 16.7 mM glucose \times 24 h with or without 10 μ M cycloheximide; 5.5 mM glucose \times 24 h.

Insulin secretion. Islets were washed three times in KRB, 10 mM Hepes, 2.8 mM glucose, 0.5% BSA fraction V (Sigma Chemical Co.), and preincubated in the same buffer for 90 min at 37°C. Triplicate batches of 10 islets were transferred to glass vials containing 1 ml KRB + 2.8–27.7 mM glucose, and incubated 60 min in a 37°C shaking water bath. The medium was separated from the islets by gentle centrifugation (500–750 rpm 5 min at 10°C) and stored at –20°C pending measurement of insulin with an RIA that used charcoal separation (11) and rat insulin standards (Eli Lilly and Company, Indianapolis, IN). The three replicates at each glucose concentration were combined to give a single data point.

Glucokinase enzymatic activity. Islets were preincubated 90 min at 2.8 mM glucose, homogenized, and centrifuged 10 min at 12,000 \times g to remove mitochondrial-bound hexokinase (12). DNA content was

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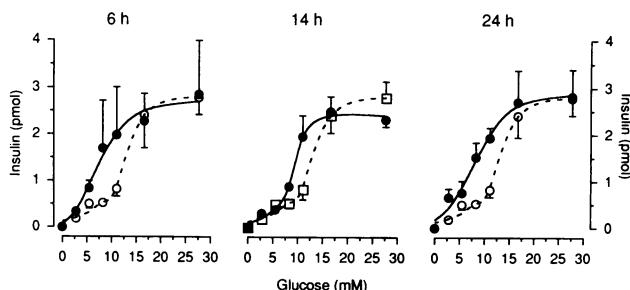


Figure 1. Glucose/insulin secretion dose response curves in cultured rat islets. (Left panel) Islets cultured 6 h at 16.7 mM glucose preceded by 18 h at 5.5 mM glucose (●—● $n = 3$) and 24 h at 5.5 mM glucose (○—○ $n = 3$). (Middle panel) Islets cultured 14 h at 16.7 mM glucose preceded by 4 h at 5.5 mM glucose (●—● $n = 3$) and 18 h at 5.5 mM glucose (□—□ $n = 3$). (Right panel) Islets cultured 24 h at 16.7 mM glucose (●—● $n = 3$) and 24 h at 5.5 mM glucose (○—○ $n = 3$). 10 islets per vial were incubated 60 min at the glucose concentrations shown.

measured in 10 μ l aliquots \times 3 (13). Glucose phosphorylation was measured as the conversion of NAD⁺ to NADH by exogenous glucose-6-phosphate dehydrogenase (7, 14). Extract (5 μ l) was added to 100 μ l of reaction buffer (50 mM Hepes/HCl pH 7.60, 100 mM KCl, 7.4 mM MgCl₂, 0.05% BSA, 5 mM ATP, 0.5 mM NAD⁺, 15 mM β -mercaptoethanol, 0.7 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim, Indianapolis, IN), 0.03–100 mM glucose), and incubated 90 min at 30°C. The reaction was stopped with 1 ml 500 mM NaHCO₃ pH 9.4. Triplicate samples were measured per glucose concentration (excitation 350 nm/emission 460/nm), and the mean value used as a single observation. The standard curve used glucose-6-phosphate standards (0.3–3.0 nmol) in reaction buffer that contained 100 mM glucose. Glucokinase and hexokinase V_{max} and K_m were calculated by linear regression from an Eadie-Scatchard plot ($v/[s]$ vs v) after extrapolating the data to 37°C assuming a Q_{10} of 2 (15) followed by 10 cycles of the method of Spears et al. (16).

Glucokinase Western blot. 300–500 islets were lysed in 5% SDS, 80 mM Tris/HCl pH 6.8, 1 mM phenylmethylsulfonyl fluoride, 10 μ g DNAase, 0.2 mM *N*-ethylmaleimide, and 5 mM EDTA. Protein content was measured by the BCA assay (Pierce, Rockford, IL) with BSA as standard. 20- μ g protein aliquots were resolved by electrophoresis on a 0.75-mm 10% polyacrylamide gel containing SDS, and were electroblotted onto nitrocellulose (Schleicher & Schuell Inc., Keene, NH) (17). After blocking overnight at 4°C in 5% nonfat dry milk/0.01% Tween 20 in 20 mM Tris/HCl pH 7.4, filters were incubated with a polyclonal sheep antiserum (1:500 dilution for 3 h at room temperature) raised against an *Escherichia coli*–derived B1 isoform of rat glucokinase (gift from Dr. Mark Magnuson, Vanderbilt University, Nashville, TN), then rabbit anti–sheep IgG antiserum (Sigma Chemical Co.) at 1:1500 dilution for 1 h. Bound antibody was detected by ¹²⁵I-conjugated protein A (ICN, Costa Mesa, CA), and quantified by densitometry using IMAGE 1.4 software (National Institutes of Health, Bethesda, MD).

Data presentation and statistical methods. Data are expressed as mean \pm SEM with the reported n always being the number of experiments. The high glucose culture times were 6 h (protocol *a*), 14 h (protocol *b*), and 24 h (protocol *a*). Statistical comparisons were made with the islets incubated at 5.5 mM glucose from that protocol (“time-matched controls”) using one-way ANOVA. The one-way Student’s *t* test was used for the Western blot results by assigning a densitometry reading of 100% to the 5.5-mM glucose islets on each gel.

Results

Insulin secretion. Glucose/insulin dose response curves were constructed over a glucose range of 2.8–27.7 mM (Fig. 1). As

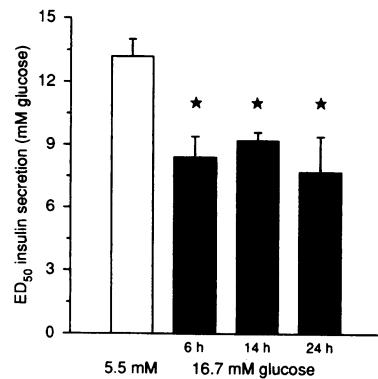


Figure 2. The ED₅₀ for glucose-induced insulin secretion in islets cultured for 6, 14, or 24 h at 16.7 mM glucose. The half-maximal glucose concentration (ED₅₀) was calculated from the curves in Fig. 1. *, statistically significant result vs the 5.5-mM glucose islets (*P* values are in the text).

expected, the curves for the control islets (cultured at 5.5 mM glucose) from protocols *a* and *b* were superimposable with half-maximal glucose concentrations (ED₅₀) of 12.5 ± 0.3 mM in the 18-h controls and 13.2 ± 0.8 mM in the 24-h controls. After 6 h of high glucose, the ED₅₀ for glucose was 8.4 ± 1.0 mM (*P* < 0.02) with no change in the maximal response. Extending the time to 14 h and then 24 h caused no additional left shift of the curve (Fig. 2). The ED₅₀ for glucose at 14 h was 9.2 ± 0.4 mM (*P* < 0.004 vs time-matched controls) and 7.7 ± 1.7 mM at 24 h (*P* < 0.043 vs time-matched controls).

Glucokinase enzymatic activity. The V_{max} and K_m values for glucokinase and hexokinase are listed in Table I. The phosphorylation data after 6- and 24-h incubations at high glucose are shown in Fig. 3. Hexokinase V_{max} and K_m were unaffected by the high glucose. In contrast, glucokinase V_{max} was increased at all time points: $149 \pm 4\%$ of control at 6 h (*P* < 0.018), $161 \pm 10\%$ of control at 14 h (*P* < 0.003), and $168 \pm 16\%$ of control at 24 h (*P* < 0.027). These values are not different from each other statistically. The K_m for glucokinase was unchanged at any time point.

Glucokinase Western blot. To investigate the etiology of the increased glucokinase V_{max} , a time course of glucokinase protein level was constructed by Western blotting of islets cultured for 6, 14, 24, and 48 h at 16.7 mM glucose. After 6 h at high glucose, the level of glucokinase was unchanged from the control islets. In contrast, at 14, 24, and 48 h, glucokinase protein levels were increased $\sim 40\%$. Finding divergent protein levels between 6 h and the later time points was surprising because the V_{max} measurements were equal up through 24 h. We retested the observation with additional groups of islets ($n = 5$) studied after 6 and 24 h of 16.7 mM glucose, and we obtained the same result (Fig. 4). Combined densitometry readings from the five experiments are shown in Fig. 5. Glucokinase protein levels were $101 \pm 8\%$ of control after 6 h of high glucose vs $144 \pm 3\%$ of control with the 24-h incubation (*P* < 0.0002).

Reversal of the high glucose-induced increase in glucokinase activity. We next tested the reversibility of the increased activity of glucokinase after 6 and 24 h of high glucose by incubating for another 3 h at 5.5 mM glucose (Table I). Glucokinase V_{max} decreased to basal control levels in the 6-h high glucose islets ($112 \pm 8\%$ of control, *P* = NS), as opposed to no change in the 24-h group ($141 \pm 7\%$ of control).

Prevention of the high glucose-induced increase in glucokinase activity by cycloheximide. A third difference between the 6- and 24-h time points pertained to the ability of cycloheximide, an inhibitor of protein synthesis, to prevent the increase in glucokinase activity. Islets were cultured for 6 and 24 h at

Table I. Kinetic Parameters for Glucokinase and Hexokinase in Cultured Islets

Culture conditions	Islet DNA	Hexokinase		Glucokinase		
		V_{max}	K_m	V_{max}	V_{max}	K_m
		ng/islet	mol G-6-P/kg DNA/60 min	mm glucose	mol G-6-P/kg DNA/60 min	percent of matched control
Protocol a: 24 h Total culture						
5.5 mM glucose (3)	19.3±1.9	1.32±0.13	0.09±0.13	3.59±0.44	100±12	18.3±1.96
6 h of 16.7 mM glucose (3)	18.8±3.2	1.49±0.31	0.08±0.03	5.37±0.15*	149±4*	14.4±1.31
24 h of 16.7 mM glucose (3)	20.1±3.7	1.33±0.09	0.10±0.04	6.03±0.57†	168±16†	17.3±2.65
Protocol b: 18 h Total culture						
5.5 mM glucose (4)	16.4±1.4	3.22±0.35	0.03±0.00	4.29±0.32	100±7	11.9±1.08
14 h at 16.7 mM glucose (4)	17.8±1.2	2.54±0.43	0.02±0.00	6.92±0.45§	161±10§	14.4±1.03
Protocol c: Reversal¹						
5.5 mM glucose (3)	13.6±1.8	4.47±0.31	0.02±0.00	5.04±0.12	100±2	16.6±2.28
6 h of 16.7 mM glucose (3)	13.8±1.6	4.70±0.57	0.03±0.01	5.65±0.42	112±8	14.4±2.40
24 h of 16.7 mM glucose (3)	15.3±2.9	3.90±0.28	0.02±0.00	7.09±0.34	141±7	12.1±2.09

Statistical significance was determined by comparing each group of islets cultured at 16.7 mM glucose against the time-matched group at 5.5 mM glucose. * $P < 0.018$, † $P < 0.027$, § $P < 0.003$, || $P < 0.0046$. V_{max} and K_m values for hexokinase and glucokinase were calculated as described in the text. ¹The reversal protocol entailed the culture conditions shown followed by an additional 3 h at 5.5 mM glucose.

16.7 mM glucose in the presence or absence of 10 μ M cycloheximide. The previous findings concerning the glucokinase V_{max} and protein levels after 6 and 24 h of high glucose were replicated in the islets not exposed to cycloheximide (Table II). Cycloheximide did not prevent the increase in glucokinase V_{max} that occurred with the 6-h incubation at high glucose (Fig. 6); the V_{max} was similar with (144±14% of control) and without (136±4% of control) cycloheximide. In contrast, with 24 h of high glucose, cycloheximide halved the increase in glucokinase V_{max} (126±8% of control with cycloheximide and 158±7% of control without cycloheximide, $P < 0.027$). However, the V_{max} was still significantly increased ($P < 0.035$ vs 5.5 mM glucose islets), even though the rise in the glucokinase protein level

had been totally blocked (102±10% of control) (Fig. 7). These data support the notion of dual regulatory effects of glucose to control the synthesis and catalytic activity of glucokinase. Protein synthesis is not required for the second effect.

Discussion

By mapping out a 24-h profile of glucokinase activity and protein levels plus the ED_{50} for glucose-induced insulin secretion in islets cultured at a high concentration of glucose, new insight was obtained into how β cells adapt to hyperglycemia. Two modes of regulation for glucokinase were noted, with relatively acute changes in the catalytic activity followed by a more chronic increase in the cellular content of the enzyme. Furthermore, the setpoint for insulin secretion was lowered in concert with the upregulated glucokinase activity, which fits with the well-known role of this enzyme in regulating the β cell sensitivity to glucose (2). Thus, our results revealed for the first time a multistep sequence of β cell adaptive responses that are initi-

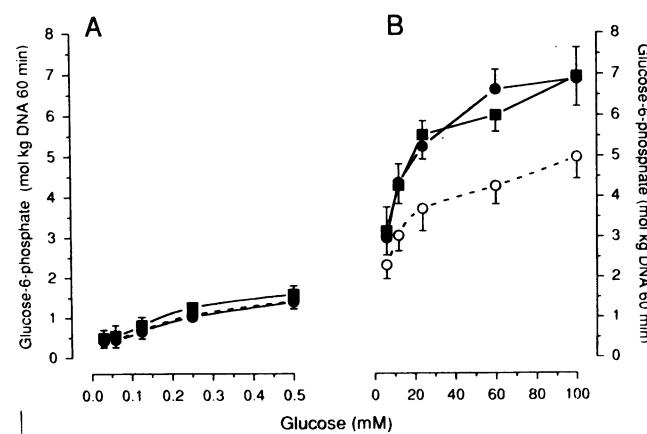


Figure 3. Glucose phosphorylation to glucose-6-phosphate in rat islets during 90-min incubations at the glucose concentrations shown. Islets were cultured 6 h at 16.7 mM glucose preceded by 18 h at 5.5 mM glucose (●—● $n = 3$), 24 h at 16.7 mM glucose (■—■ $n = 3$), or 24 h at 5.5 mM glucose (○—○ $n = 3$). (A) Incubations carried out at 0.03–0.5 mM glucose representing hexokinase activity. (B) Incubations carried out at 6–100 mM glucose representing glucokinase activity.

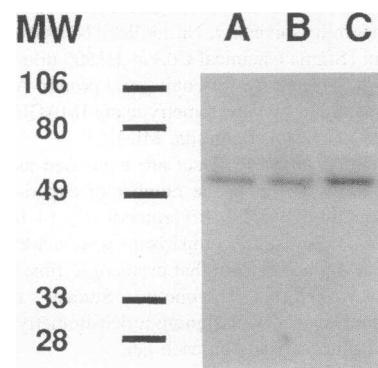


Figure 4. Western blot for glucokinase in cultured rat islets. Culture conditions: 6 h at 16.7 mM glucose preceded by 18 h at 5.5 mM glucose (A), 24 h at 5.5 mM glucose (B), 24 h at 16.7 mM glucose (C). Protein aliquots (20 μ g) were resolved by electrophoresis on a polyacrylamide gel and transferred onto nitrocellulose. Filters underwent sequential incubations with a polyclonal sheep antiserum raised against an *E. coli*-derived B1 isoform of rat glucokinase, then rabbit anti-sheep IgG. Bound antibody was detected with 125 I-conjugated protein A.

Table II. Kinetic Parameters for Glucokinase and Hexokinase in Cycloheximide-treated Cultured Islets

Culture conditions	Hexokinase		Glucokinase			Protein on Western blot
	V_{max} mol G-6-P/kg DNA/60 min	K_m mM glucose	V_{max} mol G-6-P/kg DNA/60 min	V_{max} percent of matched control	K_m mM glucose	
Protocol d: cycloheximide						
5.5 mM Glucose (3)	4.98±1.50	0.12±0.03	4.87±0.15	100±3	12.4±1.4	100
6 h of 16.7 mM (3)	4.91±1.27	0.12±0.03	6.66±0.18*	136±4*	14.8±0.9	100±3
6 h of 16.7 mM + 10 μ M Cycloheximide (3)	4.50±1.41	0.13±0.05	7.01±0.66†	144±14†	17.4±2.2	100±4
5.5 mM Glucose (4)	4.71±0.31	0.10±0.02	5.42±0.26	100±5	15.5±5.6	100
24 h of 16.7 mM (4)	4.81±0.46	0.11±0.5	8.54±0.40§	158±7§	12.5±2.4	141±9¶
24 h of 16.7 mM + 10 μ M Cycloheximide (4)	3.86±0.76	0.11±0.04	6.82±0.45	126±7	11.1±2.4	102±10

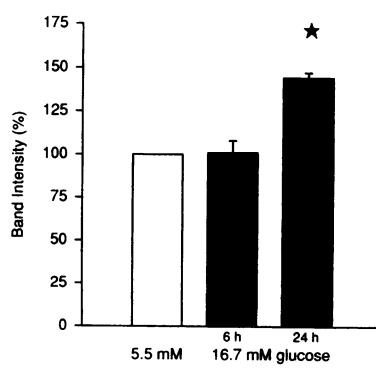
Statistical significance was determined by comparing each group of islets cultured at 16.7 mM glucose against the time-matched group at 5.5 mM glucose. * $P < 0.002$, † $P < 0.001$, || $P < 0.035$, ¶ $P < 0.02$.

ated by a high level of glucose. Almost immediately, insulin levels rise because of the direct stimulation of insulin secretion. If that degree of insulin release does not normalize the glucose concentration, a few hours later, the inductive effect on glucokinase catalytic activity causes a further increase in insulin output because of the increased β cell sensitivity to glucose. With persistent hyperglycemia, a third effect to augment the cellular content of glucokinase is added.

Glucose modulating the cellular content of glucokinase is a well-known regulatory mechanism for this enzyme (7, 8). In contrast, control of the catalytic activity of glucokinase separate from changes in the amount of enzyme is a new concept that is still controversial. Iynedjian et al. (18) proposed this possibility to explain the reduced glucokinase activity that occurs in islets from rats fasted for 72 h (19). We expanded the idea to insulin-resistant states by finding increased enzymatic activity of glucokinase without changes in the level of the protein measured by Western blotting in islets isolated from two hyperinsulinemic rat models (8, 9). The present study provided irrefutable evidence for the catalytic activity being a locus of regulation for this enzyme. The results with cycloheximide in the 6-

h high glucose islets were the most telling. The concentration used (10 μ M) is known to abolish islet protein synthesis (20). Still, the V_{max} increased as with high glucose alone, which can only indicate an increase in the ratio of enzyme activity to protein level. The stimulus for the increased glucokinase activity in this study was a high level of glucose. Our previous studies made similar findings in normoglycemic rats: normoglycemic/hyperinsulinemic rats from a 48-h glucose infusion (8), and in spontaneously hypertensive rats (9), a model of insulin resistance with normal glucose tolerance (21, 22). It remains to be determined whether multiple regulatory factors for glucokinase catalytic activity exist, one of which is glucose.

Therefore, adaptive changes of the catalytic activity of glucokinase have now been reported in such diverse situations as fasting and refeeding (18), insulin resistance (9), and this study with high glucose. Knowing the central role played by glucokinase in determining the β cell sensitivity for glucose and insulin output (2), these findings strongly suggest that the catalytic



densitometry and expressed in relative terms against the islets cultured at 5.5 mM glucose (designated as 100%). * $P < 0.0002$ vs the 5.5 mM glucose islets.

Figure 5. Combined data from five experiments for glucokinase protein level in cultured rat islets. Culture conditions: 24 h at 5.5 mM glucose, 6 h at 16.7 mM glucose preceded by 18 h at 5.5 mM glucose, and 24 h at 16.7 mM glucose. Glucokinase was quantified by Western blot. The glucokinase bands on each gel were measured by densitometry and expressed in relative terms against the islets cultured at 5.5 mM glucose (designated as 100%). * $P < 0.0002$ vs the 5.5 mM glucose islets.

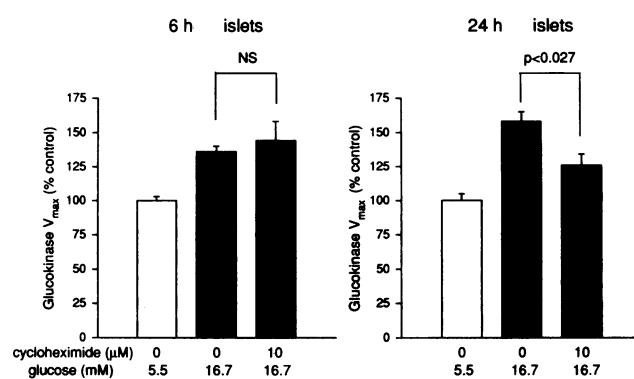


Figure 6. Effect of cycloheximide to prevent the high glucose-induced increase in glucokinase V_{max} . Islets were cultured for 6 h ($n = 3$) or 24 h ($n = 4$) at 16.7 mM glucose in the presence or absence of 10 μ M cycloheximide. Glucokinase V_{max} was measured as described in the text, and it was expressed as percentage of control islets (cultured at 5.5 mM glucose).

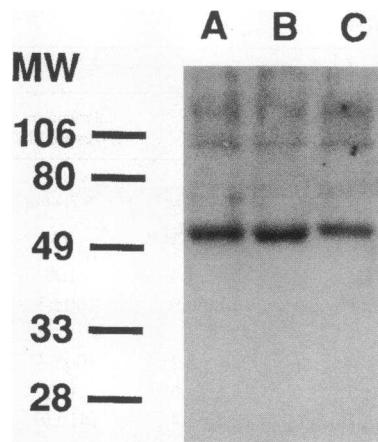


Figure 7. Western blot for glucokinase in rat islets cultured in the presence of cycloheximide. Culture conditions: 24 h at 5.5 mM glucose (A), 24 h at 16.7 mM glucose (B), and 24 h at 16.7 mM glucose + 10 μ M cycloheximide (C).

activity of glucokinase is a key locus of regulation in the β cell for mediating adaptive changes in insulin output. Potential mechanisms include the inhibitory protein for glucokinase that has been identified in the liver (23, 24). However, our results virtually eliminated this possibility because that protein is a competitive inhibitor of glucokinase that alters the affinity (ED_{50}) for glucose, not the V_{max} (24). An area of current speculation concerns a regulatory role for the β cell glucose transporter GLUT-2 on glucokinase (25–27). Consistent with this idea, we observed that GLUT-2 levels were increased in tandem with an increased activity of glucokinase in islets isolated from spontaneously hypertensive rats (9). Nonetheless, the current results seem incompatible with the level of GLUT-2 being the regulatory factor because the increase in glucokinase activity with 6 h of high glucose was unaffected by the presence of the protein synthesis inhibitor cycloheximide. An alternate possibility not addressed in this study is a regulatory role for GLUT-2 based on the flux of glucose uptake through the transporter.

In summary, we have confirmed the presence of a dual regulatory system for glucokinase in the β cell, with glucose-mediated control of the activity and cellular content of this enzyme. These results markedly strengthen the growing literature that suggests that the catalytic activity of glucokinase is a key site of β cell regulatory control, which, until recently, has gone unrecognized.

Acknowledgments

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