Glucokinase activity in isolated islets from obese *fa/fa* Zucker rats

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Glucokinase (EC 2.7.1.2) activity of B-cells was measured in extracted pancreatic islets isolated from lean and obese *fa/fa* Zucker rats and maintained in primary culture overnight. Formation of [^{14}C]glucose phosphoric esters from D-[U- ^{14}C]glucose was measured in the presence of unlabelled glucose from 0.05 to 0.50 mM for hexokinase (EC 2.7.1.1) activity, and 8.0–16.0 mM unlabelled glucose for glucokinase activity. Eadie–Hofstee analysis revealed that hexokinase kinetic parameters (V_{max} and K_m) for [^{14}C]glucose phosphoric ester formation were similar in lean- and *fa/fa*-rat islets. For glucokinase, there was no difference in V_{max} between phenotypes. A non-significant tendency to increased sensitivity to glucose was noted in the *fa/fa*-rat islets

($P = 0.13$). In lean-rat islets, the glucokinase inhibitor mannoheptulose (3 mM) decreased V_{max} by 80% and increased the apparent K_m from 3.3 ± 0.7 mM to 12.2 ± 2.0 mM ($P < 0.05$). There was no difference in K_m or V_{max} in mannoheptulose-treated versus control islets from *fa/fa* rats. This lack of effect was consistent with reported effects of mannoheptulose on insulin secretion from *fa/fa*-rat islets [Chan, MacPhail and Mitton (1993) *Can. J. Physiol. Pharmacol.* 71, 34–39]. The data from glucose and mannoheptulose experiments support the hypothesis that glucokinase function is altered in *fa/fa* Zucker rats and may contribute to fasting hyperinsulinaemia *in vivo* in these animals.

INTRODUCTION

Hyperinsulinaemia in obese humans was first described in 1967 (Perley and Kipnis, 1967) and has since been shown to be a common element of obesity in many species, including rodents (Jeanrenaud, 1979). The cause of this phenomenon is likely to be multifactorial, and dependent on the interplay between dietary, hormonal and nervous influences on the pancreatic B-cell. It is possible that either genetic or environmental changes in the internal milieu may result in persistent changes in the response of B-cells of obese animals to glucose or other secretagogues. Because glucose is the most important physiological stimulus of insulin secretion, a change in the responsiveness of the B-cell to this nutrient could have profound effects on glucose homeostasis.

Evidence has been accumulating that B-cells from *fa/fa* rats have increased sensitivity to glucose, contributing to hyperinsulinism *in vivo* and *in vitro* (Chan et al., 1984; Curry and Stern, 1985; Kuffert et al., 1988), but the mechanism of this phenomenon remains unsolved. In isolated islets from *fa/fa* rats, increased sensitivity to glucose, but not to other secretagogues, was demonstrated, suggesting a link to glucose metabolism (Chan et al., 1993). This increase in sensitivity to glucose may result in hypersecretion in response to other secretagogues, such as glucose-dependent insulinotropic polypeptide, which require a minimum glucose concentration in order to stimulate insulin release (Chan et al., 1984, 1985). Impaired ability to differentiate the α - and β -anomers of D-glucose has also been reported in *fa/fa* rats (Leclercq-Meyer et al., 1987).

Glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.2) has been proposed as the primary glucose sensor (Matschinsky, 1990) or signal-recognition enzyme (Lenzen and Panten, 1988) within the B-cell. Its function is regulated by events which change ambient glucose levels, such as fasting (Tiedge and Lenzen, 1991) or culture in low glucose (Liang et al., 1990, 1992; Svensson et al., 1991). Small changes in glucokinase activity in pancreatic islets or liver could cause significant changes

in glucose homeostasis (Meglasson and Matschinsky, 1984). Even if glucokinase is judged not to be the primary glucose sensor (Malaisse and Sener, 1985), changes to a rate-limiting step in glycolysis could result in altered insulin secretion rates. The hypothesis that glucokinase, the first rate-limiting enzyme of glycolysis, could be involved in the hyperinsulinaemia of obesity was supported by the observation that mannoheptulose inhibited insulin secretion from lean-, but not *fa/fa*-, rat islets (Chan et al., 1993).

Recently, the islet glucokinase genes for rats (Magnuson and Shelton, 1989) and humans (Koranyi et al., 1992) have been characterized, leading to identification of subgroups of non-insulin-dependent diabetes mellitus (NIDDM) patients having mutations in the glucokinase gene (Hattersley et al., 1992; Vionnet et al., 1992). Studies of mutated glucokinases revealed decreases in V_{max} and/or increases in K_m of the enzyme for glucose which could explain decreased B-cell responsiveness in NIDDM (Gidh-Jain et al., 1993). Such mutations have not been described in rodent models of obesity or NIDDM, but it might be expected that the increased sensitivity to glucose observed in obese *fa/fa* rats could be accompanied by an increase in V_{max} and/or a decrease in K_m of glucokinase. The evidence for increased glucose sensitivity of *fa/fa* Zucker rat B-cells (Chan et al., 1993) led to the hypothesis that this change is related to functional defects in glucokinase. In these studies, glucokinase activity of lean- and *fa/fa*-rat islets is assessed.

MATERIALS AND METHODS

Reagents

Tissue-culture medium and reagents were from Gibco Canada Ltd. (Burlington, Ont., Canada). All enzyme assay reagents and the Lowry protein assay kit were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Radiolabelled glucose (D-[U- ^{14}C]glucose) was from NEN Dupont (Mississauga, Ont., Canada) and the DEAE-cellulose filters (DE-81) were from Whatman (Terochem Scientific, Markham, Ont., Canada).

Figure 1 Formation of [^{14}C]glucose phosphoric esters as a function of glucose concentration in an islet preparation from an obese Zucker rat

Overnight-cultured isolated islets were suspended in Krebs–Ringer bicarbonate buffer plus glucose at the concentrations shown, disrupted by 3 freeze–thaw cycles, then incubated at 37 °C for 20 min in the presence of 0.5 μCi D-[^{14}C]glucose, as described in the Materials and methods section. The velocity of [^{14}C]glucose phosphoric ester formation was calculated as nmol/h per μg of protein. The data are from a single experiment, illustrating that [^{14}C]glucose phosphoric ester formation decreased with increasing unlabelled glucose concentration, as would be expected in a competitive type of assay. Hexokinase activity was estimated from velocities for glucose concentrations ≤ 0.5 mM, whereas glucokinase activity was calculated from velocities for glucose concentrations ≥ 6.0 mM.

Animals

Lean (*Fa/fa* or *Fa/Fa*) and obese (*fa/fa*) Zucker rats were obtained from Charles River Canada (St. Constant, Quebec, Canada) and were used in these experiments at 8–12 weeks of age. Animals were allowed access to water and commercial rat chow (Purina) *ad libitum*. All animals were fasted overnight before islet isolation. Before surgery, the rats were anaesthetized with 60 mg/kg sodium pentobarbital, intraperitoneally. They were killed by pneumothoracotomy before excision of the pancreas. These procedures were approved by the local Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care, Volumes 1 and 2.

A large body of work (for review see Bray et al., 1989) has established that the *fa/fa* Zucker rat is markedly hyperinsulinaemic, but not hyperglycaemic, at the age used in this study. These observations were confirmed by us in previous studies *in vivo* (Chan et al., 1984, 1993).

Glucokinase activity and insulin secretion

Islets from lean and *fa/fa* rats were isolated by using collagenase digestion and a dextran step-density gradient, and then cultured overnight as previously described (Cawthorn and Chan, 1991). The following day, batches of 30 islets were transferred to 1.5 ml microcentrifuge tubes, centrifuged to remove the culture medium, then washed with assay buffer containing 0.05 mM glucose. The medium for glucokinase assays was Krebs–Ringer bicarbonate buffer supplemented with 10 mM Hepes (pH 7.4), 5 mM MgATP and 10 mM NaF (Bedoya et al., 1984). Glycolysis was blocked distal to phosphoric ester formation by the addition of NaF (Feig et al., 1971). The islets were then resuspended in 225 μl of assay buffer, containing the indicated glucose and other reagents as specified in the Results section, vigorously vortex-mixed and subjected to three freeze–thaw cycles, with vortex-mixing between each cycle. At zero time, D-[^{14}C]glucose (0.5 μCi , 25 μl volume)

was added and the disrupted islets were incubated for 20 min at 37 °C. The reaction was terminated with 30 μl of 2 M glucose/0.25 M EDTA, and duplicate 60 μl samples were spotted on DEAE-cellulose filters as described by Bedoya et al. (1984). After washing with distilled water, the filters were dried, and the radioactivity (i.e. formation of [^{14}C]glucose phosphoric esters) was counted by liquid scintigraphy. Blank incubations without cells were included in each experiment. The protein content of the islets was measured in 120 μl samples, with BSA as the standard.

Insulin secretion from intact islets was measured in batches of 5 (lean) and 3 (*fa/fa*) islets, by previously described methods (Cawthorn and Chan, 1991).

Data analysis

For these experiments, an approach to the glucokinase assay which minimized the amount of radioisotope was used. Because this assay operated under the principles of competitive binding assays such as radioreceptor- or radioimmuno-assays, it was assumed that the actual rate of D-glucose phosphorylation was inversely proportional to the rate of tracer phosphorylation. As predicted, the rate of [^{14}C]glucose phosphoric ester formation decreased with increasing concentration of unlabelled D-glucose (Figure 1).

The velocity (v) of [^{14}C]glucose phosphorylation was calculated in nmol/h per μg of protein for each glucose concentration (s). Thus the actual velocity of formation of unlabelled glucose phosphoric esters would be proportional to the inverse of the value reported here. By plotting v versus s (direct linear) or v versus v/s (Eadie–Hofstee plot), values for V_{max} and K_m of [^{14}C]glucose phosphoric ester formation could be assessed. The Eadie–Hofstee plot was used in these studies because the transformation results in more uniform error distribution (Price and Stevens, 1989). Hexokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.1) contributes significantly to glucose phosphorylation at glucose concentrations above 5 mM (Trus et al., 1981), but glucokinase kinetic values were not corrected for that contribution because the results indicated no difference in hexokinase behaviour between the phenotypes (see Table 1). Involvement of glucokinase in glucose phosphorylation at low glucose concentrations was minimized by using glucose concentrations of 0.05–0.5 mM. Glucose concentrations of 0.5–6.0 mM are not suitable for hexokinase activity measurement (Trus et al., 1981).

Differences in slope (K_m) and ordinate intercept (V_{max}) were assessed by Student's unpaired t test after linear-regression analysis using the statistical package Minitab, version 7.1. Differences were considered significant when $P < 0.05$. Significant differences in insulin secretion were tested with Student's t test for paired data, with significance taken as $P < 0.05$. The n reported in the Results refers to the number of individual rat islet donors.

RESULTS

Hexokinase activity

Hexokinase activity was calculated after Eadie–Hofstee analysis on glucose concentrations of 0.05–0.5 mM. As shown in Figure 2, there was no significant difference in the slope or ordinate intercept of the regression of v versus v/s . Values for V_{max} and K_m of [^{14}C]glucose phosphoric ester formation are summarized in Table 1. An estimate of the V_{max} of unlabelled glucose phosphoric ester formation is given by the reciprocal of the calculated V_{max} , 0.98 and 1.1 arbitrary units/h for lean and *fa/fa* phenotypes

Figure 2 Eadie-Hofstee plot of hexokinase activity

[¹⁴C]Glucose phosphoric ester formation was measured in extracted islets in the presence of glucose concentrations (*s*) of 0.05–0.5 mM. Eadie-Hofstee analysis of the raw data was performed as described under 'Data analysis' in the Materials and methods section. Kinetic values derived from the plot are summarized in Table 1. Means $\pm$ S.E.M. are given for islet preparations from lean (▲, *n* = 12) and *fa/fa* (■, *n* = 8) rats.

Table 1 Summary of $V_{\max}$ and K_m values for hexokinase

Values (means $\pm$ S.E.M. for 12 lean- and 8 *fa/fa*-rat islet preparations) were calculated from linear regression analysis of Eadie-Hofstee plots as indicated in the text. There were no significant differences between phenotypes for either parameter (Student's unpaired *t* test).

	Lean	<i>fa/fa</i>
$V_{\max}$ (nmol/h per μ g of protein)	1.019 $\pm$ 0.088	0.888 $\pm$ 0.144
K_m (mM)	0.0284 $\pm$ 0.0064	0.0328 $\pm$ 0.0044

respectively. The observed K_m for hexokinase is similar to that reported by Trus et al. (1981) in freshly isolated islets (0.032 mM) and by Liang et al. (1992) in islets cultured for 48 h in 15 mM glucose (0.05 mM). Both studies cited used measurement of NADH fluorescence to quantify glucokinase activity, indicating that the radioisotopic method used in this study generated similar values for hexokinase sensitivity to glucose.

Glucokinase activity

Glucokinase activity was calculated by using glucose concentrations of 8.0–16.0 mM. Use of glucose concentrations greater

Figure 3 Eadie-Hofstee plot of glucokinase activity

[¹⁴C]Glucose phosphoric ester formation was measured in the presence of glucose concentrations (*s*) of 8.0–16.0 mM. Eadie-Hofstee analysis of the raw data was performed as described under 'Data analysis' in the Materials and methods section. Kinetic values derived from the plot are summarized in Table 2. Means $\pm$ S.E.M. are given for islet preparations from lean (▲, *n* = 20) and *fa/fa* (■, *n* = 16) rats.

than 16.0 mM was precluded by the blank values (i.e. non-specific binding of tracer to the filter), which were not reliably different from tracer binding in the presence of 22.0 mM glucose. As shown in Figure 3, there was no difference in the ordinate intercept ($V_{\max}$) of [¹⁴C]glucose phosphoric ester formation between phenotypes. Estimating unlabelled glucose phosphoric ester formation rate from the reciprocal of the reported $V_{\max}$ gives 4.4 and 4.5 arbitrary units/h for lean and obese phenotypes. The sensitivity to glucose tended to be lower in lean-rat than in *fa/fa*-rat islets, although this did not achieve statistical significance (Table 2; *P* = 0.13). The K_m for glucose of lean-rat islet glucokinase reported in this study was lower than many published values, which range from 8.5 to 10.5 mM in freshly isolated islets (Matschinsky and Ellerman, 1968; Trus et al., 1981; Burch et al., 1981; Purrello et al., 1993) and up to 51 mM in islets cultured under similar conditions (Purrello et al., 1993; Liang et al., 1992).

These experiments were repeated in disrupted islets in the presence of 10.0 mM glucose 6-phosphate, which inhibits hexokinase, but not glucokinase. Addition of glucose 6-phosphate did not alter $V_{\max}$ in either phenotype. Surprisingly, glucose 6-phosphate caused an apparent 2-fold increase in glucose sensitivity of lean rat islet glucokinase, but had no effect on K_m of obese-rat islet glucokinase (Table 2).

Table 2 Summary of $V_{\max}$ and K_m values for glucokinase

Values (means $\pm$ S.E.M. for the numbers of individual islet preparations indicated in parentheses after K_m values) were calculated from linear-regression analysis of Eadie-Hofstee plots as described in the Materials and methods section. **P* < 0.05 compared with phenotype control (Student's unpaired *t* test). There was no significant difference between the phenotype controls.

	Lean rats		<i>fa/fa</i> rats	
	$V_{\max}$ (nmol/h per μ g of protein)	K_m (mM)	$V_{\max}$ (nmol/h per μ g of protein)	K_m (mM)
Control	0.225 $\pm$ 0.031	6.3 $\pm$ 0.8 (20)	0.221 $\pm$ 0.034	4.8 $\pm$ 0.6 (16)
Glucose 6-phosphate	0.162 $\pm$ 0.036	3.3 $\pm$ 0.7 (4)*	0.200 $\pm$ 0.128	6.7 $\pm$ 2.1 (4)
Mannoheptulose	0.031 $\pm$ 0.011*	12.2 $\pm$ 2.0 (6)*	0.108 $\pm$ 0.021	5.0 $\pm$ 1.1 (8)

Effect of mannoheptulose

Glucokinase activity was measured in the presence of 3.0 mM mannoheptulose for glucose concentrations of 8.0–16.0 mM. This concentration caused approximately half-maximal inhibition of insulin secretion in earlier studies (Chan et al., 1993). In lean-rat islets, mannoheptulose significantly decreased sensitivity of glucokinase to glucose, and the velocity of [14 C]glucose phosphoric ester formation. In contrast, mannoheptulose had no effect on [14 C]glucose phosphoric ester formation by islets of *fa/fa* rats, although a non-significant ($P = 0.32$) decrease in $V_{\max}$ was noted (Table 2). These results are consistent with the effects of mannoheptulose on insulin secretion from lean- and *fa/fa*-rat islets previously documented (Chan et al., 1993).

Effects of other glycolytic inhibitors

Other agents have also been reported to inhibit insulin secretion by affecting glucokinase or other glycolytic-enzyme function. However, 5-amino-4-imidazolecarboxamide riboside ('AICARiboside'; 100 μ M), aluminium sulphate (10 μ g/ml) or alloxan (25 μ M) did not reliably inhibit glucose (8.0–16.0 mM)-stimulated insulin secretion from intact lean- or *fa/fa*-rat islets (results not shown). The effects of these compounds on glucokinase function were therefore not tested.

DISCUSSION

Glucokinase phosphorylates glucose to glucose 6-phosphate at physiological glucose concentrations and is in a prime position to act as the B-cell glucose sensor in the pancreas (Matschinsky, 1990; Lenzen and Panten, 1988), although this hypothesis is not universally accepted (Malaisse and Sener, 1985). Exciting new studies, which were possible once the human gene for glucokinase was identified (Koranyi et al., 1992), have identified subpopulations of NIDDM patients with genetic defects in glucokinase (Permutt et al., 1992), but few physiological correlations with the gene defects have been reported. Computer-modelling studies of one missense-mutation product suggested that glucose- and ATP-binding sites might both be altered (Stoffel et al., 1992). Missense mutations of glucokinase expressed in *Escherichia coli* did alter $V_{\max}$ and/or K_m for glucose (Gidh-Jain et al., 1993), strengthening the evidence that changes in glucokinase structure have the potential to cause pathological changes in insulin secretion. Even small changes in islet glucokinase function are hypothesized to affect glucose homeostasis (Meglasson and Matschinsky, 1984). In the present paper, alterations in glucokinase function of obese *fa/fa* Zucker rats which correlate with previous observations of changes in glucose-stimulated insulin release (Chan et al., 1993) are reported.

Insulin secretion from *fa/fa*-rat pancreas is stimulated by hypoglycaemic glucose concentrations. Fasting for prolonged periods, which decreases serum glucose concentrations to less than 6 mM, decreased insulin secretion *in vivo* in lean but not in *fa/fa* rats (Zucker and Antoniadis, 1972; Diersen-Schade et al., 1988; Chan et al., 1993). In the isolated perfused pancreas, glucose concentrations less than 5 mM stimulated insulin secretion in *fa/fa* rats (Chan et al., 1984; Curry and Stern, 1985; Kuffert et al., 1988). Finally, in isolated overnight-cultured islets, the EC_{50} for glucose, but not other secretagogues, was decreased in *fa/fa* rats compared with lean rats. Culture of *fa/fa*-rat islets overnight in low glucose did not ameliorate the glucose response (Chan et al., 1993). The assay of glucose-phosphorylating activity in the present study demonstrates that increased sensitivity to glucose could be due to altered function of glucokinase. Although

the differences in sensitivity to glucose did not achieve statistical significance, even the small changes observed might be enough to trigger inappropriate insulin secretion to hypoglycaemic levels because of the sigmoid nature of the concentration–response curve.

Mannoheptulose is a C₇ sugar isolated from avocado, which inhibits glucokinase, but not hexokinase, to decrease insulin secretion (Zawalich et al., 1978). Our previous finding that mannoheptulose was without effect on insulin secretion from *fa/fa*-rat islets (Chan et al., 1993) corresponds well with the present data showing a decreased effect of mannoheptulose on glucokinase activity. These studies provide strong evidence that glucokinase function is altered in *fa/fa* rats, perhaps due to altered sugar binding sites that preclude mannoheptulose effects. The decrease in $V_{\max}$ elicited in both phenotypes by mannoheptulose was not expected of a compound thought to be a competitive inhibitor, being more consistent with non-competitive-inhibitor properties. Further studies with multiple combinations of glucose and mannoheptulose concentrations, coupled with insulin-secretion studies, are required to resolve this unexpected finding. Unfortunately, these studies did not define whether the lack of inhibition by mannoheptulose was specific, because other putative inhibitors of glucokinase examined had little effect on insulin secretion in lean rats. These agents included the known glucokinase inhibitors alloxan (Lenzen and Mirzaie-Petri, 1991) and 5-amino-4-imidazolecarboxamide riboside (Vincent et al., 1992) and the phosphofructokinase inhibitor aluminium sulphate (Xu et al., 1990). At present we are unable to resolve this lack of effectiveness, although perhaps the compounds were not stable under the conditions tested. Alloxan's effectiveness may have been improved by preincubation in the absence of glucose, because glucose has been shown to protect islets from the inhibitory effects of this compound (Meglasson et al., 1986).

The implications for altered B-cell glucokinase function in obesity include fasting hyperinsulinism, a state first described 25 years ago (Perley and Kipnis, 1967) and common to obese humans and rodent models. Hyperinsulinaemia leads to tissue insulin resistance and glucose intolerance, creating a vicious cycle of increasing insulin secretion and resistance. Islets from adult LA/N-corpulent and SHR/N-corpulent rats have decreased sensitivity to mannoheptulose (Timmers et al., 1992), similar to previous observations in the *fa/fa* rat (Chan et al., 1993). Studies of glucokinase function in these and other obese models are necessary to determine whether defects in glucokinase are a common manifestation in hyperinsulinaemic obese rodents.

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