Anomeric Specificity of the Stimulatory Effect of D-Glucose on D-Fructose Phosphorylation by Human Liver Glucokinase

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D-Glucose was recently reported to stimulate D-fructose phosphorylation by human B-cell glucokinase. The present study aims at investigating the anomeric specificity of such a positive cooperativity. The a-anomer of D-glucose was found to increase much more markedly than β-D-glucose the phosphorylation of D-fructose by human liver glucokinase. Such an anomeric preference diminished at high concentrations of the D-glucose anomers, i.e. when the effect of the aldohexose upon D-fructose phosphorylation became progressively less marked. A comparison between the effects of the two anomers of D-glucose and those of equilibrated D-glucose upon D-fructose phosphorylation by human liver glucokinase indicated that the results obtained with the equilibrated aldohexose were not significantly different from those expected from the combined effects of each anomer of D-glucose. In isolated rat islets incubated for 60 min at 4 °C, a-D-glucose (5.6 mM), but not β-D-glucose (also 5.6 mM), augmented significantly the conversion of D-[U-14C]fructose (5.0 mM) to acidic radioactive metabolites. Likewise, in islets prelabelled with 46Ca and perfused at 37 °C, D-fructose (20.0 mM) augmented 46Ca efflux and provoked a biphasic stimulation of insulin release from islets exposed to α-D-glucose (5.6 mM), while inhibiting 46Ca efflux and causing only a sluggish and modest increase in insulin output from islets exposed to β-D-glucose (also 5.6 mM). The enhancing action of D-glucose upon D-fructose phosphorylation by glucokinase thus displays an obvious anomeric preference for α-D-glucose, and such an anomeric specificity remains operative in intact pancreatic islets.

In 1997, Scruel et al. (1) first revealed a glucose-induced positive cooperativity of D-fructose phosphorylation by human B-cell glucokinase. In 2001, Moukil and Van Schafftengen (2) analyzed the cooperativity of human B-cell glucokinase through such a stimulatory effect of D-glucose on D-fructose phosphorylation. They concluded that the effect of the aldohexose on D-fructose phosphorylation indeed reflects the positive cooperativity for D-glucose, as mediated by its binding to the catalytic site. Further experiments conducted in isolated rat pancreatic islets have documented that D-glucose also causes a concentration-related increase in the oxidation of D-[U-14C]fructose (3). A comparable situation was observed in pancreatic islets prepared from either Goto-Kakizaki rats or adult rats that had been injected with streptozotocin during the neonatal period, i.e. in two animal models of non-insulin-dependent diabetes mellitus (4, 5).

The major aim of the present study was to investigate whether the stimulatory effect of D-glucose on D-fructose phosphorylation by human liver glucokinase displays anomeric specificity. The effects of the two anomers of D-glucose upon D-fructose conversion to 14CO2 and 14C-labeled acidic metabolites and upon the cationic and insulin secretory responses to D-fructose were also examined in isolated rat pancreatic islets. The experiments were conducted over 10 min of incubation at 25 °C (D-fructose phosphorylation), 60 min of incubation at 4 °C (D-fructose metabolism in islets) or with D-glucose anomers maintained for 90 min or less at 4 °C (perfused islets) to minimize the interconversion of the glucose anomers (6). Under these conditions, the fraction of a-D-glucose converted to β-D-glucose, expressed relative to the equilibrium value, is close to 5.4 and 9.0% after 60 and 90 min of incubation at 4 °C and to 17.8% after 10 min incubation at 25 °C (6). The mean value for the fractional conversion of each anomer during the incubation period used for the measurement of biological variables is close to only half of these percentages.

EXPERIMENTAL PROCEDURES

Materials—D-Fructose and the anomers of D-glucose were purchased from Sigma. D-[U-14C]fructose was prepared from D-[U-14C]glucose (PerkinElmer Life Sciences), and its purity assessed as previously reported (7). Recombinant liver glucokinase was kindly provided by Prof. E. Van Schafftengen (Universite´ Catholique de Louvain, Brussels, Belgium).

D-Fructose Phosphorylation—The phosphorylation of D-fructose (10 mM; mixed with a tracer amount of D-[U-14C]fructose) was conducted over 10 min of incubation at 25 °C in a reaction mixture (0.1 ml) consisting of a Hepes-NaOH buffer (50 mM, pH 7.5) containing 6 mM MgCl2, 60 mM KCl, 10 mM KH2PO4, 0.2 mg/ml bovine serum albumin, 5 mM ATP, human liver glucokinase (about 25 U/ml or 0.3 units/ml) and, when required, freshly dissolved α- or β-D-glucose. Labeled D-fructose 6-phosphate was then separated from its precursor by ion exchange chromatography (8). Blank values were measured in the absence of glucokinase.

Metabolism of D-[U-14C]Fructose in Pancreatic Islets—Groups of 30 pancreatic islets each, prepared by the collagenase procedure from fdd Brachyplus rats (9), were incubated for 60 min at 4 °C in 0.1 ml of a Hepes- and bicarbonate-buffered medium (10) containing bovine serum albumin (5 mg/ml), d-fructose (5 mM, mixed with a tracer amount of D-[U-14C]fructose) and, when required, freshly dissolved α- or β-D-glucose. The production of 14CO2 and 14C-labeled acidic metabolites was measured as described elsewhere (11, 12).

Ca Efflux and Insulin Release from Perfused Pancreatic Islets—Groups of 110 pancreatic islets each were preincubated for 60 min at 37 °C in the same Hepes- and bicarbonate-buffered medium as mentioned above in the presence of D-glucose (16.7 mM), this medium being enriched with a tracer amount of 46CaCl2. The islets were then placed in a perfusion chamber. The perfusion media containing either α-D-glucose or β-D-glucose (5.6 mM each) and, when required, D-fructose (20.0 mM) were maintained at 4 °C in a container placed on ice and warmed to 37 °C just before reaching the perfusion chamber. The efflux

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Anomeric Specificity of Glucokinase

Fig. 1. Effect of increasing concentrations of D-glucose, tested at anomeric equilibrium, upon the phosphorylation of D-[U-14C]fructose (10.0 mM) by human liver glucokinase. Mean values (± range of individual variations) are expressed relative to the mean reaction velocity recorded in the absence of D-glucose, after normalization of the data (14) within each of two independent experiments. The inset refers to the results obtained in the low range of D-glucose concentrations (0.25 to 2.0 mM).

Presentation of Results—All results are presented as mean values (± S.E.) together with the number of individual observations (n). The statistical significance of differences between mean values was assessed by use of Student’s t test.

RESULTS

Enzymatic Data—Two batches of recombinant human liver glucokinase were used. Over 10 min of incubation at 25 °C, they yielded for the phosphorylation of D-[U-14C]fructose (10.0 mM) mean reaction velocities of 122 ± 3 (n = 3) and 163 ± 11 (n = 8) nmol/min per mg of protein.

In two pilot experiments, D-glucose, tested at anomeric equilibrium, caused a concentration-related increase in D-[U-14C]fructose phosphorylation (Fig. 1). As little as 0.25 mM D-glucose increased the reaction velocity by 43 ± 1%. The peak value for D-[U-14C]fructose phosphorylation was reached at a 5.0 mM concentration of D-glucose and corresponded to a 6-fold increase in reaction velocity. At higher concentrations of D-glucose, the reaction velocity progressively decreased. The value reached at 20.0 mM D-glucose averaged 63 ± 4% of that recorded, within the same experiment(s), at 5.0 mM D-glucose.

In further experiments, the effects of α-D-glucose and β-D-glucose upon D-[U-14C]fructose phosphorylation were compared, distinct ranges of the anomer concentrations being explored in each case. In a first series of five experiments conducted at anomer concentrations of 0.25–2.0 mM, a progressive increase in reaction velocity was observed with each anomer. The stimulation of D-[U-14C]fructose phosphorylation was much more pronounced, however, with α-D-glucose than β-D-glucose (Fig. 2, left panel). For instance, at the lowest anomer concentration tested in these experiments (i.e. 0.25 mM), the reaction velocity, expressed relative to the basal value (no D-glucose present) recorded within the same experiment(s), averaged 189 ± 9 and 121 ± 7% (n = 5 in both cases; p < 0.001) with α-D-glucose and β-D-glucose, respectively. At an anomer concentration of 2.0 mM, these values were increased to 557 ± 24 and 271 ± 14% (n = 8 in both cases; p < 0.001) with α-D-glucose and β-D-glucose.

In a second series of four experiments conducted at higher concentrations of the two anomers (2.0–2.5 to 10.0–20.0 mM), the peak value for D-[U-14C]fructose phosphorylation was reached at a concentration of α-D-glucose of 4.7 ± 0.3 mM, as distinct (p < 0.001) from 9.4 ± 0.6 mM in the case of β-D-glucose (n = 4 in both cases). The peak value reached with the α-anomer was no greater than 68 ± 3% of the peak value recorded with the β-anomer but at half the concentration within the same experiments. In these and further experiments, the phosphorylation of D-[U-14C]fructose was virtually the same with α- and β-D-glucose when the anomers were tested in the 10.0–20.0 mM range (Fig. 2, right panel).

In the last set of experiments, the effects of α-D-glucose, β-D-glucose and D-glucose at anomeric equilibrium upon D-[U-14C]fructose phosphorylation were compared at three concentrations of the aldohexose (Table I). At D-glucose concentrations of 2.0 and 5.0 mM, the results obtained with equilibrated D-glucose were always in between those recorded with the pure anomers. At 10.0 mM, however, the reaction velocity measured in the presence of equilibrated D-glucose always exceeded that found with each anomer. The experimental values found with equilibrated D-glucose were about the same as the corresponding theoretical values calculated from both the relative abundance of α-D-glucose (36.5%) and β-D-glucose (63.5%) at anomeric equilibrium, and the reaction velocities measured in the presence of each anomer. In calculating these theoretical values, allowance was made for the increase or decrease in reaction velocity found at increasing concentrations of α-D-glucose (as illustrated in Fig. 2), the measurements made in the presence of β-D-glucose being converted to corresponding molar amounts of α-D-glucose. The ratio between theoretical and experimental values for D-[U-14C]fructose phosphorylation in the presence of equilibrated D-glucose averaged 103 ± 2% (n = 9) and, as such, was not significantly different from unity (p > 0.2). Fig. 3 illustrates the comparison between these theoretical and experimental values. These findings document that the results recorded with equilibrated D-glucose were not significantly different from those expected from the combined effects of each anomer upon D-[U-14C]fructose phosphorylation.
Anomeric Specificity of Glucokinase

TABLE I

| Glucose          | α-Glucose     | β-Glucose     | Equilibrated d-glucose | Mean Values (±S.E.) |
|------------------|---------------|---------------|------------------------|---------------------|
| 2.0 mM           | 557.4 ± 24.2  | 277.2 ± 20.5  | 397.1 ± 19.7           | 453.4 ± 22.9        |
| 5.0 mM           | 606.7 ± 22.9  | 405.8 ± 22.1  | 573.4 ± 30.9           | 512.0 ± 34.3        |
| 10.0 mM          | 476.2 ± 43.4  | 432.4 ± 26.3  | 512.0 ± 34.3           | 453.4 ± 22.9        |

* Reaction velocity expressed as percent of the paired value found in the absence of d-glucose.
x Reaction velocity expressed as percent of the paired value found in the presence of 2.0 mM α-d-glucose.

**Metabolic Data**—When groups of 30 isolated pancreatic islets each were incubated for 60 min at 4 °C, α-d-glucose (5.6 mM) and, to a lesser extent, β-d-glucose (also 5.6 mM) increased the mean value for the conversion of d-[U-14C]fructose (5.0 mM) to 14CO2 (Fig. 4, upper panel). None of the mean values for d-[U-14C]fructose oxidation illustrated in Fig. 4 were significantly different from one another, however. Such was also the case when groups of 50 islets each were incubated for only 30 min at 25 °C under otherwise the same experimental conditions (data not shown).

In the experiments conducted at 4 °C, no significant production of 14C-labeled acidic metabolites could be detected from islets incubated in the sole presence of d-[U-14C]fructose (Fig. 4, lower panel). Once again, α-d-glucose and, to a lesser extent, β-d-glucose augmented the mean value for the conversion of d-[U-14C]fructose to radioactive acidic metabolites. Such an increase failed to achieve statistical significance in the case of β-d-glucose but was highly significant (p < 0.01) in the case of α-d-glucose. The increment in the net generation of 14C-labeled acid metabolites caused by β-d-glucose represented 37.9 ± 18.2% (n = 15; p < 0.07) of the mean corresponding value found within the same experiment(s) in the case of α-d-glucose (100.0 ± 27.6%; n = 14).

**Secretory and Cationic Data**—In pilot experiments conducted over 30 min of incubation at 37 °C, neither equilibrated d-glucose (5.6 mM) nor d-fructose (20.0 mM), when tested separately from one another, augmented significantly (p > 0.5 or more) insulin output above basal value (7.3 ± 1.2 microunit/islet per 30 min; n = 29). Thus, the secretory rate averaged 7.8 ± 1.5 and 8.3 ± 1.2 microunit/islet per 30 min (n = 27–28) in the presence of 5.6 mM d-glucose and 20.0 mM d-fructose, respectively. In the concomitant presence of the aldohexose and ketohexose, however, the output of insulin (12.7 ± 1.5 microunit/islet per 30 min; n = 29) reached a value significantly higher than the basal insulin release (p < 0.01) or that recorded in the sole presence of either d-glucose (p < 0.025) or d-fructose (p < 0.05).

To investigate the functional response of isolated islets to d-fructose in the presence of either α-d-glucose or β-d-glucose, groups of 110 islets each were first preincubated for 60 min at 37 °C in the presence of 16.7 mM equilibrated d-glucose in a medium enriched with 45CaCl2 and then placed in a perifusion chamber. The perifusion media containing either α-d-glucose or β-d-glucose (5.6 mM each) and, when required, d-fructose (20 mM) were maintained at about 4 °C (in a container placed in ice) and warmed to 37 °C just before reaching the perifusion chamber.

As documented in the upper panel of Fig. 5, the mean value for 45Ca fractional output rate before administration of d-fructose (min 31 to 45) was higher in the islets exposed to α-d-glucose (0.62 ± 0.09 10−2/min; n = 4) as compared with β-d-glucose (0.49 ± 0.04 10−2/min; n = 4); this difference failed, however, to achieve statistical significance. d-fructose exerted opposite effects upon 45Ca outflow in the islets exposed to either α-d-glucose or β-d-glucose. In the former case, d-fructose

**Fig. 3.** Comparison between theoretical and experimental values for the phosphorylation of d-[U-14C]fructose in the presence of increasing concentrations (2.0, 5.0, and 10.0 mM) of equilibrated d-glucose. The experimental values were collected in the experiments summarized in Table I. The solid and dashed lines correspond, respectively, to the calculated regression line and a line defining identity between theoretical and experimental values. All results are expressed relative to the reaction velocity found in the absence of d-glucose.

**Fig. 4.** Effects of α-d-glucose and β-d-glucose (5.6 mM each) upon the conversion of d-[U-14C]fructose (5.0 mM) to 14CO2 (upper panel) and 14C-labeled acidic metabolites (lower panel) in islets incubated for 60 min at 4 °C. Mean values (± S.E.) are expressed as pmol of d-[U-14C]fructose equivalent per islet and refer to the number of individual determinations indicated at the bottom of each column.
provoked a sustained increase in $^{45}\text{Ca}$ outflow. Thus, the integrated value for $^{45}\text{Ca}$ fractional output rate was, during the period of $d$-fructose administration (min 46–70) $814 \pm 321$ $10^{-6}\text{min}$ higher ($n = 4; p < 0.08$) than the theoretical values calculated by exponential extrapolation (see below) between the 15 measurements made before introduction of the ketohexose (min 31–45) and the five last measurements made 16–20 min after halting its administration (min 86–90). This corresponded to a $17.0 \pm 6.9\%$ increase in effluent radioactivity, relative to the paired theoretical value. On the contrary, in the presence of $\beta$-$d$-glucose, the integrated value for $^{45}\text{Ca}$ fractional output rate was, over the same period (min 46–70), $787 \pm 174$ $10^{-6}\text{min}$ lower ($n = 4; p < 0.025$) than the theoretical values, this corresponding to a $18.5 \pm 3.5\%$ relative decrease in effluent radioactivity. The difference between the response to $d$-fructose in the presence of $\alpha$-$d$-glucose versus $\beta$-$d$-glucose was thus highly significant ($p < 0.005$), whether judged from the absolute or relative magnitude of the changes in effluent radioactivity.

As illustrated in the lower panel of Fig. 5, the output of insulin before introduction of $d$-fructose (min 31–45) was slightly higher ($p < 0.05$) in the islets exposed to $\alpha$-$d$-glucose ($490 \pm 83$ nanounit/min per islet; $n = 12$) rather than $\beta$-$d$-glucose ($291 \pm 38$ nanounit/min per islet; $n = 12$). The increment in insulin output provoked by $d$-fructose (min 46–70) above the control values (min 42–45) was much higher ($p < 0.01$) in the experiments conducted in the presence of $\alpha$-$d$-glucose ($607 \pm 102$ nanounit/min per islet) rather than $\beta$-$d$-glucose ($264 \pm 63$ nanounit/min per islet). As a matter of fact, $d$-fructose provoked, in the presence of $\alpha$-$d$-glucose, a typical biphasic secretory response (early peak followed by a later reascension in insulin output), this contrasting with a modest and sluggish increase in insulin release in the presence of $\beta$-$d$-glucose. As already observed in a prior study (14), the stimulation of insulin release by $d$-fructose was apparently not rapidly reversible. Thus, between min 71 and 90, the output of insulin remained significantly higher ($p < 0.001$) than the control value (min 42–45), the increment above such a control value averaging $889 \pm 196$ and $444 \pm 86$ nanounit/min per islet ($n = 12$ in both cases; $p < 0.05$) in the case of $\alpha$- and $\beta$-$d$-glucose, respectively. Incidentally, even when the theoretical control curve for insulin output was calculated according to an exponential equation ($y = b \times mx^n$, equation in which $y$ and $x$ refer to the output of insulin and time, respectively, and $b$ and $m$ to constants) and took into account both the eight first measurements made between min 31–45 and the last four measurements made between min 84–90, the increment in insulin output attributable to the administration of $d$-fructose (min 46–70) remained higher ($p < 0.05$) in the presence of $\alpha$-$d$-glucose ($448 \pm 119$ nanounit/min per islet) than in the presence of $\beta$-$d$-glucose ($152 \pm 62$ nanounit/min per islet).

**DISCUSSION**

The experimental conditions used in this study to assess the anomeric specificity of the effect of $d$-glucose upon the phosphorylation, metabolism, and insulinotropic action of $d$-fructose were selected to minimize the interconversion of the $d$-glucose anomers (6).

In the absence of $d$-glucose, the phosphorylation of $d$-fructose by glucokinase does not display positive cooperativity (1), the apparent $K_m$ for the ketohexose being close to 160 $\text{mM}$ (15). The present data extend to human liver glucokinase the knowledge that $d$-glucose stimulates the phosphorylation of $d$-fructose, as previously documented in the case of human B-cell glucokinase (1, 2). Such a stimulation is much more marked in the case of $\alpha$-$d$-glucose than $\beta$-$d$-glucose. This anomeric specificity disappeared, however, at high concentrations of the $d$-glucose anomers, i.e. when the glucose-induced increase in $d$-fructose phosphorylation became itself progressively less marked.

The metabolic data collected from islets incubated for 60 min at 4°C are compatible with the view that the anomeric specificity of the enhancing action of $d$-fructose upon $d$-fructose phosphorylation, as documented in the experiments conducted in the presence of human liver glucokinase, is also operative in intact pancreatic islets. More convincingly, the present results indicate that the effect of $d$-fructose upon both $^{45}\text{Ca}$ efflux and insulin release from prelabeled and perfused islets is different at 37°C in the islets exposed to $\alpha$-$d$-glucose versus $\beta$-$d$-glucose. In this respect, the cationic and secretory response to $d$-fructose recorded in islets exposed to $\alpha$-$d$-glucose are comparable to those otherwise observed when the concentration of $d$-glucose is raised from a level close to the threshold value for stimulation of insulin release (i.e. close to 5 $\text{mM}$) to a much higher concentration. They indeed consisted in an increase in $^{45}\text{Ca}$ efflux corresponding to the stimulation of $^{45}\text{Ca}^{2+}$ influx into the islets and subsequent increase in $^{45}\text{Ca}$ efflux and a biphasic stimulation of insulin release. In the islets exposed to $\beta$-$d$-glucose, however, the prevailing cationic effect of $d$-fructose consisted in a decrease in $^{45}\text{Ca}$ efflux, as otherwise observed in response to the administration of equilibrated $d$-glucose in a concentration not exceeding 7 $\text{mM}$ (16) and as attributable to the effects of the hexose upon both the sequestration of $\text{Ca}^{2+}$ by intracellular organelles and $\text{Na}^+\text{-Ca}^{2+}$ countertransport at the level of the B-cell plasma membrane (17, 18). This coincided with a delayed and quite modest increase in insulin output. This interpretation of the experiment results is further supported by the fact that, before introduction of $d$-fructose, the release of insulin was somewhat higher in the presence of $\alpha$-$d$-glucose rather than $\beta$-$d$-glucose.

In conclusion, therefore, the present data unambiguously...
document that the enhancing effect of D-glucose upon D-fructose phosphorylation by glucokinase displays anomeric preference toward α-D-glucose and that such an anomeric specificity remains operative in intact rat pancreatic islets.

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