Acute Glucose Intolerance in Insulinoma Cells with Unbalanced Overexpression of Glucokinase

Haiyan Wang and Patrick B. Iynedjian
From the Division of Clinical Biochemistry and Diabetes Research, University of Geneva School of Medicine, CH-1211 Geneva 4, Switzerland

The INS-r3-GK27 insulinoma cells are endowed with artificially inducible glucokinase under control of the reverse tetracycline-dependent transcriptional activator. Moderate induction of glucokinase has been shown to result in proportionate increases in glycolytic flux and in potentiation of glucose effects on insulin secretion and pyruvate kinase gene expression. In cells with 20-fold overexpression of glucokinase, however, glucose activation of secretion and gene expression was severely impaired. Measurements of the glycolytic flux in cells with 7- and 21-fold increases in glucokinase activity and determination of the flux control coefficient of this enzyme showed that control of glycolysis at the glucokinase step was lost in the cells at the higher level of overexpression. Challenging the cells with glucose above 6 mM resulted in massive accumulation of glucose 6-phosphate and caused a rapid and sustained depletion of cellular ATP, in contrast with the glucose-induced rise in ATP in cells with wild-type glucokinase levels. Loss of cell viability ensued upon prolonged culture in high glucose. In summary, in insulinoma beta cells overexpression of glucokinase, an imbalance between glucose phosphorylation and turnover of glucose 6-phosphate resulted in acute glucose intolerance due to trapping of cellular orthophosphate in dead-end product and severe paralysis of energy metabolism.

The major function of the endocrine beta cell in the pancreas is to sense variations of the glucose concentration in the extracellular fluid and to increase its rate of insulin secretion when the sugar concentration rises above the normoglycemic level of 5–6 mM, or to curtail secretion when glucose falls. Physiological glucose concentration sensing is a property of the intracellular glucose phosphorylating enzyme glucokinase. Glucokinase is half-saturated with glucose at a glucose concentration of about 6 mM (1, 2). This means that the enzyme reaction rate is highly responsive to changes in glucose concentration around 6 mM. Glucokinase can therefore act as a glucose sensor if supplied with such glucose levels. This requisite is fulfilled by the beta cell very high capacity for glucose transport across the plasma membrane, which allows moment to moment equilibration of the glucose concentration between extracellular and intracellular spaces (3).

The role of glucokinase in controlling the rate of metabolism along the entire glycolytic pathway in the beta cell is an important issue, because the rate of glycolysis appears to be the major determinant for physiological processes such as insulin secretion (4, 5) or the transcriptional regulation of specific genes. If glucokinase has high control strength in the pathway, glycolysis as a whole (and the subsidiary physiological processes) will be made directly responsive to fluctuations of the extracellular glucose concentration. Although much evidence suggests a regulatory role for glucokinase (6), attempts to provide a quantitative estimate of this regulatory function have been limited. Using computer modeling of metabolism and experiments with the hexokinase inhibitor mannoheptulose, Sweet et al. (7) have shown that glycolysis, ATP production, and insulin secretion in pancreatic islets are strongly correlated with glucokinase activity.

We have used genetic engineering as an alternative approach to analyze the regulatory impact of glucokinase in the beta cell. By placing glucokinase cDNA downstream of a tetracycline operator-controlled promoter and transferring this construct into established insulinoma cells expressing the reverse tetracycline-dependent transactivator, we have generated the INS-r3-GK27 cell line, in which precise increments in glucokinase activity could be imposed by culture with the tetracycline doxycycline (8). Effects of graded glucokinase induction on glycolytic flux as well as on insulin secretion or specific gene expression were measured. The experiments have demonstrated that moderate overexpression of glucokinase results in proportional increase in glycolytic flux and in potentiation of glucose-induced insulin secretion and stimulation of gene expression (8). These results were in apparent conflict with the study of Becker et al. (9), who overexpressed glucokinase at very high levels in cultured pancreatic islets using adenovirus-mediated gene transduction but failed to observe parallel effects on glycolysis or insulin release. In an attempt to understand the conflicting conclusions of the two studies, we have examined the metabolic and physiologic consequences of forced overexpression of glucokinase at levels comparable to those in the adenovirus-transduced islets using the tetracycline-inducible INS-r3-GK27 cells.

EXPERIMENTAL PROCEDURES

Cells—The creation of the INS-r3-GK27 cell line from INS-1 rat insulinoma cells by two-stage transfection of plasmid PUH17-1 (10) followed by the liver glucokinase cDNA plasmid derived from PUHD10-3 has been described (8). The cells were cultured in 10% fetal bovine serum-enriched RPMI 1640 medium supplemented with sodium pyruvate, mercaptoethanol, and antibacterial antibiotics as described (11). Selection pressure for the foreign genes was maintained with 150 μg/ml G418 and 100 μg/ml hygromycin. For induction of glucokinase, cells were transferred to the same medium as above but containing 2.5 mM glucose instead of the maintenance concentration of 11 mM. Doxycycline was added in doses specified for each experiment 2 h after the change of medium. The pre-experimental period of culture with doxycycline was between 14 and 48 h, as indicated in individual experiments.

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† To whom correspondence should be addressed: Division of Clinical Biochemistry, University of Geneva School of Medicine, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland. Tel.: 41 22 702 55 51; Fax: 41 22 702 55 43; E-mail: iynedjian@medecine.unige.ch.
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Glucokinase Activity—The procedure for fluorometric enzyme assay was exactly as described (12). For the enzyme kinetics experiment of Fig. 4, a spectrophotometric assay (13) was used.

Analysis of Pyruvate Kinase mRNA—Total cell RNA was extracted by a phenol-guanidinium thiocyanate method as in previous work (14). The techniques for RNA electrophoresis and Northern blotting and the L-type pyruvate kinase cDNA probe were as published (15).

Measurement of Glycolysis—At the end of the pre-experimental glucokinase induction period, cells in 48-well dishes were washed twice in a Hepes-supplemented Krebs-Henseleit buffer described previously (8) and incubated for 30 min in the same buffer containing various glucose concentrations and [3-14C]glucose at a specific activity of 0.313 nCi/nmol. The formation of [14C]lactate from [3-14C]glucose in the triose-phosphate isomerase and enolase reactions was used to estimate the glycolytic flux. The medium was collected to measure the radioactivity of [14C]lactate, using a water diffusion procedure essentially as described (16). The measured recovery of radioactive water amounted to 62%, and this factor was used for correction of crude data. The specific activity of glucose and the amount of DNA/wells were used to calculate the rate of glycolytic flux in nmol of glucose metabolized/µg of DNA, which was assayed as previously (8).

Assay of Intracellular Metabolites—Cell monolayers in 6-cm dishes were washed twice with ice-cold phosphate-buffered saline and scraped with rubber spatulas in 1 ml of ice-cold 10% trichloroacetic acid. The extract was sonicated in ice and centrifuged at 4 °C for 6 min. The resulting supernatant was extracted four times with 4 volumes of ether. After evaporation of residual ether at 45 °C for 10 min, the final aqueous phase was lyophilized and stored at −75 °C. For the assay of glucose 6-phosphate, lyophilizates were dissolved in 50 µl of water. Samples of 20 µl were added to 1 ml of reaction mixture containing 50 nM Hepes, pH 7.7, 7.5 mM MgCl₂, and 40 µM NADP. The amount of NADPH generated after the addition of 350 milliunits of glucose 6-phosphate dehydrogenase from yeast was measured fluorometrically. Fluorescence data were converted into glucose 6-phosphate concentrations with the help of a calibration curve obtained with glucose 6-phosphate standards. For the assay of ATP, lyophilizates were dissolved in 200 µl of a buffer containing 20 mM Hepes, pH 7.3, 5 mM MgCl₂, and 40 µM NADP. The specific activity of ATP was determined using a luciferin-luciferase reaction cocktail (Boehringer Mannheim), and luminescence was measured in a luminometer (Turner Design TD-20/20). Amounts of metabolites were normalized to the amount of DNA measured in each experiment in repared as previously (8).

Analysis of Pyruvate Kinase mRNA— Northern blot analysis of specific mRNA. In agreement with earlier results, the inductive effect of glucose was potentiated in cells with glucokinase activity raised 9-fold above basal (Fig. 1A). More unexpectedly, we found the glucose response to be severely blunted in cells with a 17-fold enhancement of glucokinase activity. In these cells, the amount of pyruvate kinase mRNA was maximal at 6 mM glucose and was strikingly reduced during culture with higher glucose concentrations. Quantification of these effects by phosphor imaging of Northern blots from two separate experiments is presented in Fig. 1B.

RESULTS

Impairment of Physiological Responses to Glucose in Insulinoma Beta Cells with Strong Overexpression of Glucokinase—A rise in the extracellular glucose concentration results in transcriptional stimulation of the L-type pyruvate kinase gene in the beta cell type (15). A regulatory role of glucokinase in this process was suggested by our earlier finding of a leftward shift in glucose dose response in insulinoma INS-r3-GK27 beta cells overexpressing glucokinase 3–8-fold above basal level after doxycycline treatment (8). We have now investigated the glucose effect on pyruvate kinase mRNA in cells at higher levels of overexpressed glucokinase activity. Cells were pretreated for 28 h with 150 or 500 ng/ml doxycycline (dox) or left untreated in culture medium containing 2.5 mM glucose. The cells were then exposed for 8 h to glucose concentrations as indicated and were harvested thereafter for RNA isolation. A, autoradiograph of a Northern blot (16 µg of total RNA/gel lane) hybridized with a pyruvate kinase cDNA probe. Numbers below the brackets indicate the medium glucose concentration during the 8-h experimental period. The left-most and right-most lanes under each bracket represent RNA from cells at 2.5 mM glucose at the beginning and end, respectively, of the experimental period. Arrows on the right designate the specific 3.1-, 2.2-, and 2.0-kilobase pair mRNA species, due to multiple polyadenylation sites. B, phosphor imaging quantification of 3.1-kilobase pair mRNA band in two distinct experiments. Symbols and vertical lines represent the average and range of two values from independent experiments. The average glucokinase (GK) activity in the absence (top) and presence of 150 (middle) and 500 ng/ml doxycycline (bottom) is given in the inset. mU/mg, milliunits/mg.

The data show a 50% reduction in message level between 6 and 24 mM glucose in cells with the highest glucokinase activity. We have previously shown that glucose-dependent insulin release was potentiated in INS-r3-GK27 cells overexpressing glucokinase 2.5–6-fold above basal (8). The effect of a larger increase in glucokinase activity was investigated. Cells were cultured in basal medium or in the presence of 500 ng/ml doxycycline for 28 h, which resulted in 24-fold overexpression of glucokinase. Thereafter, insulin secretion was estimated during a 30-min incubation at 2.5 and 24 mM glucose by radiomunological assay of the hormone released in the medium (Fig. 2). High glucose caused a 2.2-fold stimulation of insulin secretion in cells with wild-type level of glucokinase. In marked contrast, insulin release was depressed rather than activated by high glucose in cells with strong overexpression of glucokinase. Studies described next were performed in an attempt to provide a metabolic explanation for these unexpected results.

Response of Glycolytic Flux to Increasing Glucose Concentration in Cells with Forced Expression of Glucokinase—The rate of glycolysis was measured in INS-r3-GK27 cells that had been cultured for 28 h with 150 or 500 ng/ml doxycycline. The first doxycycline dose increased glucokinase activity from 9.5 to 65 milliunits/mg of protein, a 7-fold increment similar to the highest level of induction previously investigated in our laboratory.
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Glucose Dependence of Glucokinase Reaction Rate and Glycolytic Flux—The rate of glucose phosphorylation by glucokinase as a function of the glucose concentration was measured in cell-free extracts from doxycycline-induced INS-r3-GK27 cells (Fig. 4). The half-saturation concentration of glucose (\(K_{m}\)) calculated from a Hill plot of the data in Fig. 4 was 6.0 mM, and the Hill coefficient (h) was 1.85, in agreement with published enzyme kinetics data for mammalian glucokinase (1). It is interesting to compare the effects of glucose on enzyme reaction rate on the one hand and on glycolytic flux in intact cells on the other hand. For instance, the glucokinase reaction rate increases 2-fold between 6 and 24 mM glucose (Fig. 4). This was accompanied by a 2.8-fold increase in glycolysis in cells with basal glucokinase activity of 9.5 milliunits/mg of protein and only by a 28% increase in glycolysis in cells with glucokinase activity set at 180 milliunits/mg of protein. Thus, the change in glycolytic flux in response to glucose became small in comparison with the change in glucokinase reaction rate when glucokinase was strongly overexpressed. This is consistent with the notion that the glycolytic flux becomes limited at one or several steps distinct from glucokinase in strong glucokinase overexpressor cells.

Accumulation of Glucose 6-Phosphate in INS-r3-GK27 Cells Overexpressing Glucokinase—If glycolysis is limited downstream of glucokinase in high glucokinase cells, challenging these cells with increasing glucose may lead to unchecked accumulation of glucose 6-phosphate (and its conversion prod-
exposure to 24 mM glucose, ATP in cells with glucokinase set at content on longer incubation in high glucose. After 3 and 6 h of incubations, we found there was no recovery of the cellular ATP shift-up from 2.5 to 6 mM. The glucose effect was more displayed a small (20%) and transient (5 min) rise of ATP after investigated next (Fig. 6). Cells with basal level of glucokinase concentration of ATP during a glucose challenge was inves-
tigated for the DNA content measured in replicate dishes and expressed in metabolite content/10^7 cells using an equivalence of 71.3 μg of DNA/10^7 cells. Data are the means ± S.E. from four separate experiments. Glucokinase activity (GK) given in the insets was measured in replicate dishes in each experiment. mU/mg, milliunits/mg.

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FIG. 4. Rate of glucose phosphorylation by glucokinase in cell-
free extract from insulinoma cells. Cells were induced for glucoki-
nase by culture with 500 ng/ml doxycycline for 28 h in the presence of medium containing 2.5 mM glucose. The cell monolayers were then harvested for preparation of a soluble protein extract. The rate of glucose phosphorylation was measured from the production of NADH in a glucose 6-phosphate dehydrogenase-coupled spectrophotometric as-
say. Base-line production of NADH in cuvettes containing 0.5 mM glu-
cose was subtracted from the rates measured at the glucose concentra-
tions shown. This base line amounted to 2.7% of the rate recorded at 100 mM glucose. Assays were performed using 75–140 μg of protein. Rates were normalized to 100 μg of protein. Values are means ± S.E. of five determinations.

FIG. 5. Level of glucose 6-phosphate in insulinoma cells with basal or overexpressed glucokinase activity. Cells were cultured for 14 h at 2.5 mM glucose without doxycycline (left panel) or with 150 mg/ml (middle panel) or 500 mg/ml doxycycline (right panel). They were transferred to Krebs-Henseleit solution containing glucose at the indicated concentrations and incubated for 60 min before the addition of trichloroacetic acid to the cell monolayers. Glucose 6-phosphate was assayed in cell extracts using an enzymic method. Values were normalized for the DNA content measured in replicate dishes and expressed in metabolite content/10^7 cells using an equivalence of 71.3 μg of DNA/10^7 cells. Data are the means ± S.E. from four separate experiments. Glucokinase activity (GK) given in the insets was measured in replicate dishes in each experiment. mU/mg, milliunits/mg.

FIG. 6. Time course of glucose effect on cellular concentration of ATP in insulinoma cells with basal or overexpressed glucoki-
nase activity. The experimental protocol was as described in the legend to Fig. 5, except that doxycycline was used at a single dose of 500 mg/ml. Cells were preincubated in Krebs-Henseleit solution containing 2.5 mM glucose for 30 min before time 0, which is the time of glucose shift-up to the glucose concentrations indicated in the inset. Thereafter, cell monolayers were harvested in trichloroacetic acid at indicated time points to follow the ATP time-course. Data are means ± S.E. of five distinct experiments. dox, doxycycline.

Compromise of Cellular Energy Metabolism—The intracellular concentration of ATP during a glucose challenge was inves-
tigated next (Fig. 6). Cells with basal level of glucokinase displayed a small (20%) and transient (5 min) rise of ATP after glucose shift-up from 2.5 to 6 mM. The glucose effect was more conspicuous at 24 mM. Compared with the control level in cells maintained at 2.5 mM glucose, ATP was increased by 107% at the 5-min time point and by 43% at 20 and 60 min. A striking reversal of the glucose effect was noted in cells preinduced for strong glucokinase overexpression. Challenging these cells with 24 mM glucose resulted in 37, 56, and 65% decreases of ATP at 5, 20, and 60 min respectively. In additional experiments, we found there was no recovery of the cellular ATP content on longer incubation in high glucose. After 3 and 6 h of exposure to 24 mM glucose, ATP in cells with glucokinase set at 191 ± 6.5 milliunits/mg of protein, was depressed by 82 ± 2 and 83 ± 1%, respectively, compared with the content of control cells maintained at 2.5 mM glucose. It is noteworthy that the latter experiments were performed with cells continuously maintained in culture medium rich in inorganic phosphate (5.6 mM Na_2HPO_4).

Loss of Cell Viability on Continuous Culture in High Glu-
cose—The collapse of cellular ATP in the high glucokinase cells could be expected to result in a severe compromise of biosynthetic processes and might eventually lead to the demise of the cells. This prediction was confirmed by microscopic examina-
tion of INS-r3-GK27 that had been cultured with 500 mg/ml doxycycline for 48 h in the presence of 2.5 mM glucose and subsequently shifted to 24 mM glucose for 16 h. At this time, large numbers of rounded, detached cells were visible (Fig. 7D). This effect was glucose-dependent, because INS-r3-GK27 cells
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DISCUSSION

The INS-r3-GK27 clonal line of endocrine beta cell type, when cultured in absence of tetracycline, expresses glucokinase at a level similar to the parental INS-1 insulinoma cells (8). Importantly, in both INS-r3-GK27 and INS-1 cells, glucokinase is by far the preponderant hexokinase isoenzyme, representing 90% of total hexokinase activity in soluble cell extract. Data presented here and earlier (8) demonstrate that glucokinase exerts strong control over glycolysis in these insulinoma cells and by inference in non-tumoral pancreatic beta cells. In the formulation of Metabolic Control Analysis, the flux control and by inference in non-tumoral pancreatic beta cells. In the exerts strong control over glycolysis in these insulinoma cells. Data Importantly, in both INS-r3-GK27 and INS-1 cells, glucokinase at a level similar to the parental INS-1 insulinoma cells (8). It can be calculated from the data in Fig. 5 that the concentration of glucose 6-phosphate in cells with the highest glucokinase activity amounted to 3.5 and 11.3 mM at 6 and 24 mM extracellular glucose respectively. Thus, the build-up of glucose 6-phosphate between these two glucose concentrations corresponded to an 8 mM increase in concentration. Glucose is phosphorylated from ATP, which is itself regenerated at the expense of inorganic phosphate. The estimated intracellular concentration of inorganic phosphates is -5 mM (20). It is therefore apparent that a major fraction of the intracellular pool of inorganic phosphates could be rapidly sequestered in glucose 6-phosphate when cells with unbalanced overexpression of glucokinase are challenged with glucose. As a consequence, oxidative phosphorylation would be compromised, explaining the glucose-induced drop in intracellular ATP.

The above scenario is reminiscent of metabolic disturbances occurring in a recessive human genetic disorder called hereditary fructose intolerance (21). This disease is caused by mutations of a tissue-specific form of the enzyme fructose 1,6-phosphate aldolase (aldolase B) expressed in liver, kidney, and intestine. The enzyme is necessary for the conversion of fructose 1-phosphate generated by fructokinase to trioses that can be further metabolized in the lower glycolytic pathway. With a less active, mutated aldolase B, fructose ingestion results in protracted hepatic accumulation of fructose 1-phosphate, trapping of inorganic phosphate, and ATP depletion. This situation has been mimicked in yeast cells, which contain no aldolase B, by transfection of a plasmid encoding fructokinase (22). We use the term “glucose intolerance” to stress the metabolic similarities between our insulinoma cells with unbalanced overexpression of glucokinase and fructose-intolerant cells.

A rise in the beta cell ATP level triggered by the acceleration of glycolysis and resulting in the closure of plasma membrane K+ channels appears to be the metabolic switch for glucose-induced insulin secretion (23). The suppression, indeed reversal, of this signal in the INS-r3-GK27 cells with highest glucokinase activity can explain the abolition of glucose-induced insulin secretion. An additional contributing factor may be the depletion of the cellular content of insulin that was noted in these cells, probably as a result of chronic stimulation of hormone release at basal glucose concentration during the pre-experimental glucokinase induction phase. The link between glucose metabolism and activation of L-type pyruvate kinase gene expression remains elusive, but indirect evidence sug-
gests that accumulation of a metabolic intermediate, perhaps glucose 6-phosphate, may serve as the signal (24). The biphase effect of glucose on pyruvate kinase mRNA in the high glucokinase cells might be explained on one hand by the strong accumulation of glucose 6-phosphate (positive factor) and on the other hand by depressed biosynthetic activity due to ATP depletion above 6 mM glucose (negative factor).

The question of whether or not glucokinase is a rate-limiting step of glucose metabolism in the beta cell has been the subject of intensive inquiries. From the experiments presented here, this feature appears indeed to be a biological necessity. Cells expressing glucokinase at a high level relative to the capacity of the enzymes responsible for the turnover of glucose 6-phosphate would be at risk of acute glucose intolerance. How much biological safety margin does the beta cell possess to protect against this? In the INS-r3-GK 27 cells, overexpression of glucokinase by slightly more than an order of magnitude over normal is sufficient to create a major glucose-induced disorder of energy metabolism, incompatible with prolonged survival at glucose concentration of 12 mM or higher.

In summary, two major conclusions emerge from this and our earlier work. Firstly, glucose metabolism in the beta cell represents an unusual example (19) where metabolic control is exerted predominantly at a single enzyme step. Therefore, targeting glucokinase in the design of novel anti-diabetic compounds aimed at potentiating insulin secretion represents a rational approach. Secondly, unbalanced overexpression of glucokinase, as made possible by experimental gene transfer techniques, can result in glucose intolerance. It is possible that the phenomenon of glucose intolerance has been a confounding factor in the experiments of Becker et al. (9), who have used an adenovirus vector to transduce glucokinase cDNA into organ cultures of pancreatic islets, since the reported increase in glucokinase activity of islet homogenate was 30–50-fold. However, the failure of these authors to observe any enhancement of glycolysis at low glucose concentration in transduced islets remain puzzling. Whole islets are constituted by a mixture of diverse cell types, including (exocrine) cells with high affinity hexokinases and little or no glucokinase. In such cells, presumed accumulation of glucose 6-phosphate from overexpressed glucokinase would hinder glucose phosphorylation by hexokinases, which are strongly product-inhibitable. It is possible that this mechanism may have offset the increase in glycolysis from the transduced glucokinase.

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