Fundamental Metabolic Differences between Hepatocytes and Islet β-cells Revealed by Glucokinase Overexpression[†]

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ABSTRACT: Adenovirus-mediated overexpression of the glucose phosphorylating enzyme glucokinase causes large changes in glycolytic flux and glucose storage in isolated rat hepatocytes, but not in pancreatic islets. We have used the well-differentiated insulinoma cell line INS-1 to investigate the basis for these apparent cell-type specific differences. We find that 2- or 5-[^{3}H]glucose usage is increased at low ($\leq$5 mM) but not high glucose concentrations in INS-1 cells treated with a recombinant adenovirus containing the glucokinase cDNA (AdCMV-GKI), while glucose usage is increased at both low and high glucose concentrations in similarly treated hepatocytes. Utilization of 2-[^{3}H]glucose in INS-1 cells is suppressed in glucokinase overexpressing INS-1 cells in a rapid, glucose concentration-dependent, and reversible fashion, while such regulation is largely absent in hepatocytes. Levels of hexose phosphates (glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate) were profoundly and rapidly elevated following the switch to high glucose in either AdCMV-GKI-treated INS-1 cells or hepatocytes relative to controls. In contrast, triose phosphate levels (glyceraldehyde-3-phosphate + dihydroxyacetone phosphate) were much higher in AdCMV-GKI-treated INS-1 cells than in similarly treated hepatocytes, suggesting limited flux throught the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) step in the former cells. Hepatocytes were found to contain approximately 62 times more lactate dehydrogenase (LDH) activity than INS-1 cells, and this was reflected in a 3-fold increase in lactate production in AdCMV-GKI-treated hepatocytes relative to similarly treated INS-1 cells. Since the amounts of G3PDH activity in INS-1 and hepatocyte extracts are similar, we suggest that flux through this step in INS-1 cells is limited by failure to regenerate NAD in the LDH reaction and that a fundamental difference between hepatocytes and islet β-cells is the limited capacity of the latter to metabolize glycolytic intermediates beyond the G3PDH step.

Hepatocytes are efficient producers of glucose in the fasted state when levels of the hexose are low, but convert to active glucose storage and utilization when concentrations are raised. Similarly, glycolysis is rapidly activated in islet β-cells in response to increases in external glucose, and the rate of glycolytic flux is proportional to the amount of insulin secreted (for review, see refs *1*, *2*). In keeping with their capacity to "sense" glucose, hepatocytes and β-cells contain certain specialized proteins involved in glucose metabolism, particularly the GLUT-2-facilitated glucose transporter and the glucose phosphorylating enzyme glucokinase (*3*). Both proteins have a K_m for glucose that is higher than that of other members of their respective gene families, and that would appear to allow their activity to be modulated in response to increments in glucose concentration over the physiological range (*4*, *5*). However, the weight of biochemical evidence suggests that glucokinase plays a particularly prominent role in controlling the rate of glucose utilization in both cell types (*1*, *6*). The importance of glucokinase in control of glucose homeostasis has been recently underscored by studies in which genetic deficiencies in the enzyme have been linked to maturity-onset diabetes of the young (MODY) (*7*). Further, experimental reduction in glucokinase expression in liver or islets has been achieved in transgenic mice, resulting in reduced glucose-stimulated insulin secretion and less efficient suppression of hepatic glucose output during glucose clamping (*8*−*10*).

On the basis of these studies of the effects of underexpression of glucokinase, one might have predicted that the overexpression of the enzyme would enhance glucose utilization in liver and islet cells. Consistent with this model, treatment of rat hepatocytes with a recombinant adenovirus containing the glucokinase cDNA caused a large increment in glycolytic flux and glycogen accumulation relative to control cells (*11*). In contrast, use of the same recombinant virus to overexpress glucokinase in isolated rat islets resulted in no detectable increase in glucose usage, lactate production, or glycogen synthesis (*12*, *13*).

The current study was undertaken to provide a biochemical explanation for the differential metabolic impact of overexpressed glucokinase in liver and islet cells. Motivation for performing this work also came from our interest in defining metabolic enzymes other than glucokinase that may

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participate in limiting glycolytic flux and insulin secretion in β-cells. The well-differentiated INS-1 β-cell line (*14*) was used as an accessible model system, allowing an in-depth comparison between the fate of overexpressed glucokinase in these cells and primary hepatocytes. We demonstrate that overexpressed glucokinase is rapidly and reversibly suppressed by high glucose in INS-1 cells and that such inactivation occurs only to a limited extent in hepatocytes. By measurement of specific intermediary metabolites and enzyme activities we provide evidence for vastly different metabolic environments in these two cell types that could explain the differential regulation of overexpressed glucokinase activity.

MATERIALS AND METHODS

Preparation and Purification of Recombinant Adenovirus. Crude stocks of recombinant adenovirus containing the cDNA encoding human islet glucokinase (AdCMV-GKI) (*12*) were prepared by lysis of virus-infected 293 cells in 0.5% NP-40 (*15*). As a control, we also prepared a recombinant adenovirus containing a human islet glucokinase cDNA with a point mutation (G261R) that renders the enzyme catalytically inactive (*16*). The cDNA was a generous gift of Dr. Jun Takeda and Dr. Graeme Bell, University of Chicago; it was cloned as a 2.4 kb *Bam*HI fragment into the pACCMV.pLpA adenovirus vector (*17*), which was in turn used to generate a recombinant adenovirus (AdCMV-GKI_{261}) as previously described (*15*). Viral stocks were then purified by precipitation in 2.5 M NaCl/20% PEG 8000 followed by two rounds of CsCl centrifugation. Purified virus was desalted through Sephadex G-25 (Pharmacia Biotech) and stored at 4 °C in D-PBS (GibcoBRL) with 10% glycerol, 0.2% BSA, and 1% penicillin/streptomycin (Biowhittaker).

Cell Culture/Isolation and Treatment with Recombinant Adenovirus. INS-1 cells (*14*), a gift from Dr. C. B. Wollheim (Geneva, Switzerland), were cultured in INS-1 media, consisting of RPMI 1640 (Gibco) containing10% fetal bovine serum (Sigma), 500 mM Hepes, 100 mM glutamine, 50 mM sodium pyruvate, 2.5 mM β-mercaptoethanol, and 1% penicillin/streptomycin (Sigma) (*18*). Primary hepatocytes were prepared from overnight fasted male Wistar rats as described previously (*19*) and cultured in hepatocyte media consisting of DMEM (Gibco) with 0.2% BSA, 10 nM dexamethasone, 1 mM insulin, and 2% penicillin/streptomycin. Recombinant adenovirus was added to INS-1 cells or hepatocytes in their respective media containing 11 mM glucose for 60 min at 37 °C. Viral stocks were characterized such that a titer of approximately 20 pfu/cell of AdCMV-GKI virus provided a consistent 12−15-fold increase in glucose phosphorylating activity when assayed at 20 mM glucose, with lower levels of activity attained as a near linear function of the amount of virus added. After addition of virus, the virus-containing medium was replaced with fresh INS-1 or hepatocyte medium containing 11 mM glucose, and cells were incubated for 20 h at 37 °C. This medium was then removed and replaced with INS-1 or hepatocyte medium containing 3 mM glucose. Experiments were performed after an additional 20 h at 37 °C.

Assay of Glucokinase Expression. Glucokinase protein was measured by immunoblot analysis using a polyclonal antisera raised against whole GK protein fused to glutathione *S*-transferase (*12*). Glucose phosphorylating activity was measured from whole cell extracts at varying glucose concentrations using a previously described radioisoptic assay (*12*, *20*).

Measurement of Glucose Usage. Glucose usage was measured by conversion of 2- or 5-[^{3}H]glucose to ^3H_2O as described previously (*12*). All incubations and reactions were carried out in Hanks Balanced Salt Solution (HBSS; 1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4) containing various concentrations of glucose, as described in the text and figure legends.

Measurement of Intracellular Concentrations of Glycolytic Intermediate. Six centimeter plates of INS-1 cells or hepatocytes were flash frozen in liquid nitrogen for 10 min. Thawed cells were extracted in 100 μL of 10% perchloric acid and then titrated to neutral pH using 5 M potassium carbonate and phenol red as an indicator. Neutralized samples were assayed for glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) levels by the method of Lang and for fructose-1,6-bisphosphate (F1,6BP), dihydroyxacetone phosphate (DHAP), and glyceraldehyde-3-phosphate (GA3P) levels by the method of Michal, as described in Bergmeyer (*21*), except that the protocols were scaled down from a 2 mL final volume to a 100 μL final volume to allow 96-well spectrophotometric analysis. To calculate the cellular concentration of these intermediates, cell space was determined by incubating INS-1 cells or hepatocytes with 0.1 mM [^{3}H]-3-*O*-methyl glucose (3-O-MG) or 0.1 mM ^{3}H L-glucose for 30 min at 37 °C. The medium was collected, and the cells were lysed in 2 mL of 2 M NaOH. The cell space was calculated as the percentage of [^{3}H]-3-O-MG counts in the cell lysate, corrected for [^{3}H]-L-glucose counts, multiplied by the total volume. All reagents for these determinations were obtained from Sigma.

RESULTS

Glucokinase Overexpression in INS-1 Cells and Rat Primary Hepatocytes. Our previous work on glucokinase overexpression has been carried out in primary islets and hepatocytes (*11*, *12*, *22*, *23*). Here we have used the more available INS-1 insulinoma cell line, which has a ratio of high K_m glucose phosphorylating activity (glucokinase) to low K_m activity (hexokinase) similar to that of normal islets (*24*) and which secretes insulin in response to glucose over a similar range of concentrations (*14*, *18*).

INS-1 cells or hepatocytes were treated with recombinant adenoviruses expressing normal glucokinase (AdCMV-GKI) or a mutant, enzymatically inactive glucokinase (AdCMV-GKI_{261}) or were left untreated. Treatment of either INS-1 cells (Figure 1A) or hepatocytes (Figure 1B) with AdCMV-GKI increased glucose phosphorylating capacity in fresh cellular extracts at glucose concentrations $\geq$2 mM, with a maximal 15.5-fold increase measured at 20 mM glucose in INS-1 cells, and a 13.5-fold increase at 20 mM glucose in hepatoyctes, relative to untreated control cells (Figure 1). Note that our experiments were performed to achieve the same fold-increase in glucokinase activity in the two cell types. Because hepatocytes have more endogenous glucokinase activity (*13*), total activity in AdCMV-GKI-treated

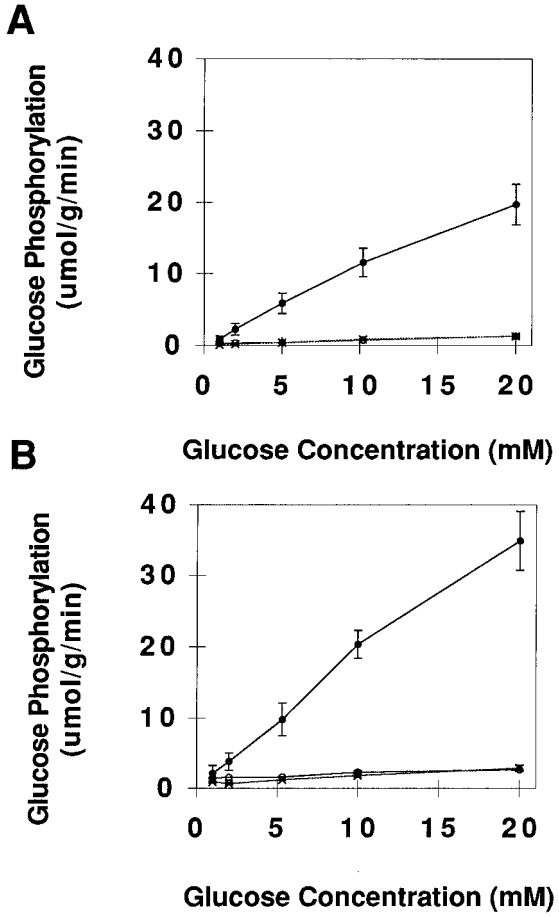

FIGURE 1: Glucose phosphorylating capacity in INS-1 cells and heptocytes. INS-1 cells (panel A) or cultured rat hepatocytes (panel B) were treated with AdCMV-GKI, encoding wild-type islet glucokinase (filled circles) or AdCMV-GKI$_{261}$, encoding catalytically inactive glucokinase (crosses), or were left untreated (open circles). Glucose phosphorylation was measured in extracts from each of these groups of cells, over a range of glucose concentrations from 1 to 20 mM. Data represent the mean ± SEM for four independent measurements, each performed in triplicate.

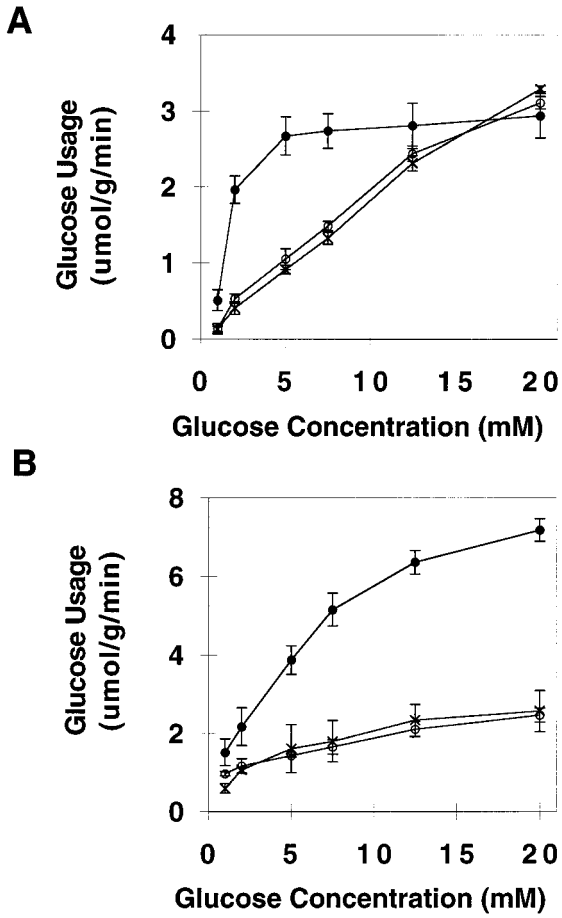

FIGURE 2: 2-[^{3}H]Glucose usage in INS-1 cells and hepatocytes. INS-1 cells (panel A) or cultured rat hepatocytes (panel B) were treated with AdCMV-GKI, encoding wild-type islet glucokinase (filled circles) or AdCMV-GKI$_{261}$, encoding catalytically inactive glucokinase (crosses), or were left untreated (open circles). The rate of 2-[^{3}H]glucose usage was then measured in each group of cells as a function of glucose concentration, as described in Materials and Methods. Data represent the mean ± SEM for four independent experiments, each performed in triplicate.

hepatocytes was 1.8-fold higher than in similarly treated INS-1 cells. We have previously shown that increases in immunodetectable protein correlate with increases in enzyme activity measured in AdCMV-GKI-treated islet extracts (*12*). Similar findings were obtained in INS-1 cells in the current study, and AdCMV-GKI or AdCMV-GKI$_{261}$ treatment was found to increase immunodetectable glucokinase protein in cellular extracts to a similar degree (data not shown). Note that for both INS-1 cells (Figure 1A) and hepatocytes (Figure 1B), the rate of glucose phosphorylation in extracts from untreated cells was the same as that in extracts from cells treated with AdCMV-GKI$_{261}$ at all glucose concentrations tested.

Metabolic Impact of Glucokinase Overexpression in Hepatocytes and INS-1 Cells. Despite the almost identical effect of AdCMV-GKI treatment on glucose phosphorylating capacity in INS-1 and hepatocyte extracts, the overexpressed enzyme had a clear differential effect on glucose metabolism in the two cell types (Figure 2). In INS-1 cells, overexpression of GK increased 2-[^{3}H]glucose usage at low but not high glucose concentrations. Results nearly identical to those shown in Figure 2 were obtained with 5-[^{3}H]glucose as the tracer (data not shown). We chose 2-[^{3}H]glucose for measurement of glucose usage in Figure 2 and all subsequent experiments because it provides the most direct measure of in situ glucose phosphorylation, since the ^{3}H label is transferred to water by rapid equilibration in the hexose phosphate isomerase reaction that immediately follows glucokinase. Glucose usage increased in AdCMV-GKI-treated INS-1 cells, reaching a maximal value at approximately 5 mM (approximately 3-fold greater than the rate of glucose flux in control cells at 5 mM glucose) and then exhibited no further increase at glucose levels between 5 and 20 mM. In contrast, the rate of glucose metabolism in either INS-1 cell control group continued to rise with increasing glucose, such that at the highest concentrations used for the assays (15 and 20 mM) there was no difference in glucose utilization between the AdCMV-GKI-treated, AdCMV-GKI$_{261}$-treated, and untreated cells (Figure 2A). Overexpression of glucokinase in cultured rat hepatocytes increased glucose usage at both low and high concentrations of glucose, such that glucose usage in AdCMV-GKI-treated cells was 3-fold higher at 15 or 20 mM glucose than in either control group (Figure 2B).

The differential metabolic impact of overexpressed glucokinase in INS-1 cells and hepatocytes at high glucose was not due to the choice of glucokinase isoform or the extent

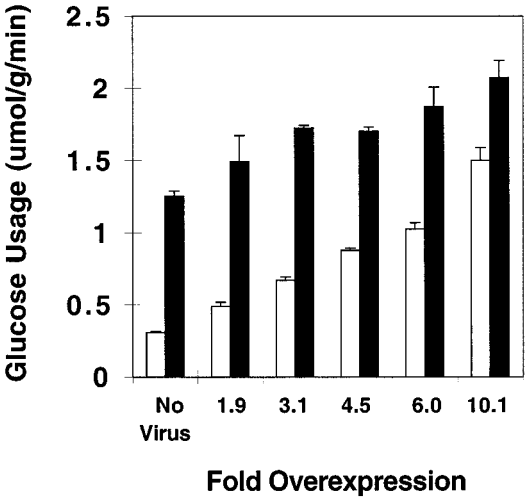

FIGURE 3: 2-[^{3}H]Glucose usage as a function of extent of glucokinase overexpression in INS-1 cells. INS-1 cells were treated with increasing titers of AdCMV-GKI to achieve the extent of glucokinase overexpression noted on the horizontal axis, measured as enzyme activity at 20 mM glucose in INS-1 cell extracts. 2-[^{3}H]-glucose usage was then measured at 2 mM glucose (open bars) or 10 mM glucose (black bars) as described in Materials and Methods. Data represent the mean ± SEM for four independent determinations per group.

of overexpression. Thus, results similar to those shown in Figure 2 were achieved by overexpression of the liver isoform of glucokinase (data not shown, and ref *12*). Figure 3 shows data for 2-[^{3}H]glucose usage as a function of extent of overexpression of glucokinase in INS-1 cells. When glucose usage was measured at 2 mM glucose, a steady increase occurred such that usage at 10.1-fold overexpression was about 5 times that in untreated cells, compared to only a 1.7-fold increase when usage was measured for the same range of overexpression at 10 mM glucose. These data are thus consistent with the data of Figure 2. All subsequent experiments were performed with the AdCMV-GKI virus at a titer of approximately 10 pfu/cell that consistently produced 12−15-fold overexpression of the enzyme in either INS-1 cells or hepatocytes.

Rapid and Reversible Regulation of Glycolytic Flux by Glucose in INS-1 Cells. The foregoing data show that glycolytic flux is suppressed in INS-1 cells, particularly at high glucose concentrations. The relationship between glucose concentration and glycolytic flux in the intact cell was determined by preincubating INS-1 cells or hepatocytes in either low (1 mM) or high (20 mM) glucose for 20 min, followed by assay of glucose usage for 90 s in the presence of 20 mM 2-[^{3}H] glucose. In INS-1 cells preincubated at 1 mM glucose for 20 min, a 7.4-fold increase in glucose usage was observed in cells overexpressing glucokinase compared to controls cells (Figure 4A). However, preincubation of AdCMV-GKI-treated INS-1 cells at 1 mM glucose for 20 min, followed by a second 20 min preincubation at 20 mM glucose, resulted in a reduction in glucose usage to levels observed in untreated or AdCMV-GKI$_{261}$-treated control cells. This reduction was reversible, since glucokinase-mediated increases in glucose usage could be restored by following the preincubation period at 20 mM glucose with an additional 20 min at 1 mM glucose. Thus, the data of Figure 4A show rapid, glucose concentration-dependent, and reversible suppression of increases in glucose usage caused by overexpression of glucokinase in INS-1 cells.

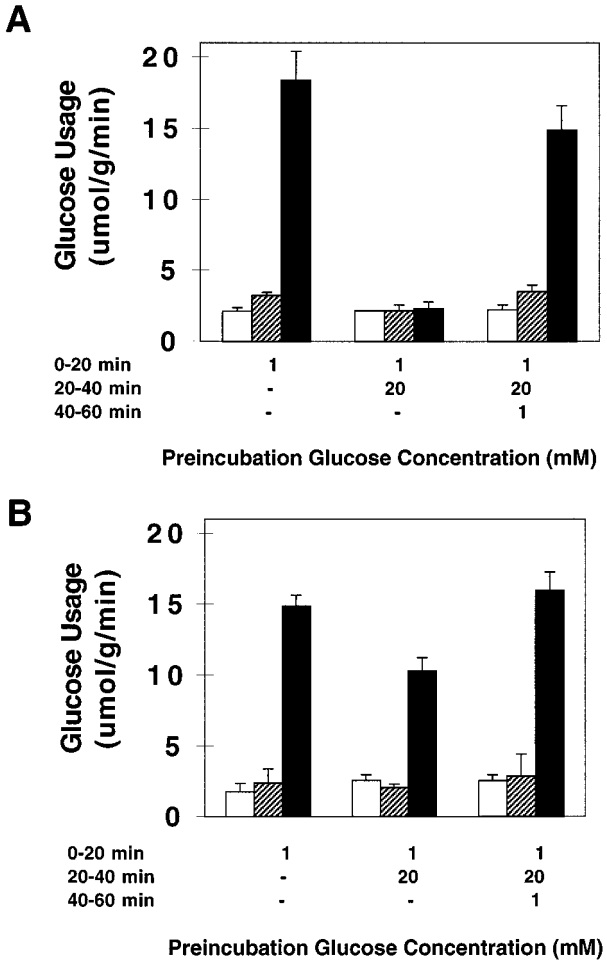

FIGURE 4: Rapid suppression of overexpressed glucokinase in INS-1 cells but not hepatocytes by high glucose. INS-1 cells (panel A) or cultured rat hepatocytes (panel B) were treated with AdCMV-GKI, encoding wild-type islet glucokinase (black bars), or AdCMV-GKI$_{261}$, encoding catalytically inactive glucokinase (hatched bars), or were left untreated (white bars). Cells were cultured overnight in media containing 3 mM glucose, washed, and then preincubated at the glucose concentrations and for the time periods indicated under the figure. Immediately following the indicated preincubations, the conversion of 20 mM 2-[^{3}H]glucose to ^3H_2O was assayed for 90 s. Data represent the mean ± SEM for three independent experiments, each performed in triplicate.

Similar to INS-1 cells, overexpression of glucokinase in hepatocytes (Figure 4B) increased 2-[^{3}H]glucose usage 7.2-fold when cells were preincubated at 1 mM glucose for 20 min. However, when hepatocytes were incubated at 1 mM glucose for 20 min and switched to 20 mM glucose for 20 min, a 4-fold increase in glucose usage was sustained in hepatocytes treated with AdCMV-GKI relative to controls. Thus, in contrast to INS-1 cells, hepatocytes appear to be capable of maintaining enhanced rates of glucose metabolism in response to glucokinase overexpression, even at high glucose concentrations.

The results in Figures 2-4 suggest that glycolytic flux is reduced in a glucose concentration-dependent manner in glucokinase-overexpressing INS-1 cells. The results in Figure 5 define the glucose concentration (Figure 5A) and time (Figure 5B) dependence of this inactivation. INS-1 cells were preincubated at 1 mM glucose for 20 min and then switched into media containing a wide range of glucose concentrations for an additional 20 min preincubation period

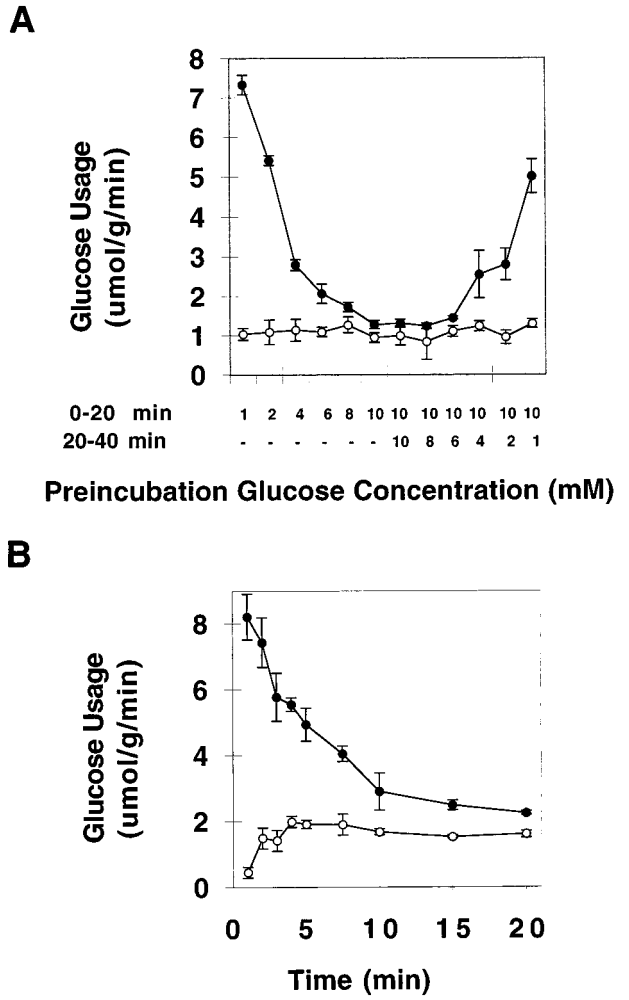

FIGURE 5: Time course and glucose concentration dependence for suppression of overexpressed glucokinase in INS-1 cells. Panel A: INS-1 cells were left untreated (open circles) or were treated with AdCMV-GKI, encoding wild-type islet glucokinase (closed circles), and preincubated at the glucose concentrations and for the time periods indicated in the legend under the figure. After preincubation, the conversion of 20 mM 2-[^{3}H]glucose to ^3H_2O was assayed for 90 s. Note that complete suppression of overexpressed glucokinase is achieved at 8 mM glucose, and that suppression of glucokinase achieved by preincubation at 10 mM glucose begins to be reversed by a subsequent preincubation period at glucose concentrations $\leq$4 mM. Data represent the mean $\pm$ SEM for three independent experiments, each performed in triplicate. Panel B. INS-1 cells were left untreated (open circles) or were treated with AdCMV-GKL, encoding wild-type liver glucokinase (closed circles). Cells were preincubated in 1 mM glucose for 20 min and then switched to preincubation at 20 mM glucose for the time periods indicated on the horizontal axis of the graph. Glucose usage was measured after the indicated periods of preincubation by measuring the conversion of 20 mM 2-[^{3}H]glucose to ^3H_2O for 90 s. Data represent the mean $\pm$ SEM of four independent samples per data point.

(Figure 5A). There was a progressive enhancement in the suppression of the metabolic impact of overexpressed glucokinase in INS-1 cells as a function of glucose concentration, with complete inactivation achieved at 8 mM glucose. There is evidence that suppression of glycolytic flux may occur even at the lowest glucose concentration tested (1 mM), since glucose usage is increased by 7.3-fold in cells incubated at this concentration of the hexose, while in comparison, an increase in GK enzymatic activity of 15-fold was measured in cellular extracts in these cells (Figure 1A).

After switching from 1 to 20 mM glucose, suppression of glycolysis ensues within seconds (Figure 5B). At the earliest time point measured, 1 min after the switch to high glucose, glucokinase overexpression results in an 8-fold increase in usage, or approximately 60% of the increase in GK activity measured in cell extracts. The increase in 2-[^{3}H]glucose usage in glucokinase overexpressing cells is completely suppressed after 10 min of incubation at 20 mM glucose.

Levels of Hexose Phosphates in GK Overexpressing INS-1 Cells and Hepatocytes. To gain further insight into the differential metabolic impact of overexpressed glucokinase in INS-1 cells and hepatocytes, we measured the levels of several hexose phosphates following a switch from 1 to 20 mM glucose (Figure 6). Treatment of AdCMV-GKI-treated INS-1 cells or hepatocytes in this way caused large increases (maximum of 29-fold in hepatocytes, 110-fold in INS-1 cells) in the levels of glucose-6-phosphate (G6P). The final intracellular concentrations of G6P obtained due to glucokinase overexpression in these cell types are quite comparable: 1.91 $\pm$ 0.24 mM in INS-1 cells and 2.15 $\pm$ 0.08 mM in hepatocytes incubated for 20 min at 20 mM glucose (Figure 6A,B). The accumulation of G6P followed a different pattern in AdCMV-GKI-treated INS-1 cells and hepatocytes, however, in that the maximal level was attained at the earliest time point (1 min) in INS-1 cells incubated at high glucose and then declined to a lower plateau level between 5 and 20 min. In AdCMV-GKI-treated hepatocytes, a large rise in G6P was also observed at 1 min, but levels then continued to rise during the entire 20 min of incubation at 20 mM glucose. Untreated INS-1 cells and hepatocytes also differed in their G6P profiles. Levels of G6P were very low in control INS-1 cells at the early time points but then increased steadily throughout the 20 min incubation at 20 mM glucose, attaining levels nearly matching those in AdCMV-GKI-treated cells. In contrast, G6P levels in control hepatocytes did not increase following the switch from low to high glucose.

Changes in intracellular concentrations of fructose-6-phosphate (F6P; Figure 6C,D) and fructose-1,6-bisphosphate (F1,6BP; Figure 6E,F) followed a pattern very similar to that described for G6P. Thus, large increases in F6P and F 1,-6BP were noted in both glucokinase overexpressing INS-1 cells and hepatocytes upon switching from 1 to 20 mM glucose, and the maximal levels of these intermediates were similar in the two cell types, but with different patterns of accumulation. In control cells not containing overexpressed glucokinase, F6P and F 1,6BP increased in INS-1 cells but not in hepatocytes, although the accumulation of F 1,6BP was less than that observed for G6P or F6P.

Evidence for Reduced Flux through Glyceraldehyde-3-phosphate Dehydrogenase in AdCMV-GKI-Treated INS-1 Cells. In contrast to the results shown in Figure 6 concerning the various hexose phosphates, striking differences were observed in the levels of triose phosphates (dihydroxyacetone phosphate + glyceraldehyde-3-P) in AdCMV-GKI-treated INS-1 cells and hepatocytes (Figure 7A,B). While glucokinase overexpression in either cell type caused an enhanced accumulation of triose phosphates relative to control cells in response to a switch to high glucose, the levels attained were 7 times higher in INS-1 cells (1308 $\pm$ 127 μM in INS-1 cells versus 186 $\pm$ 20 μM in hepatocytes). Also, triose

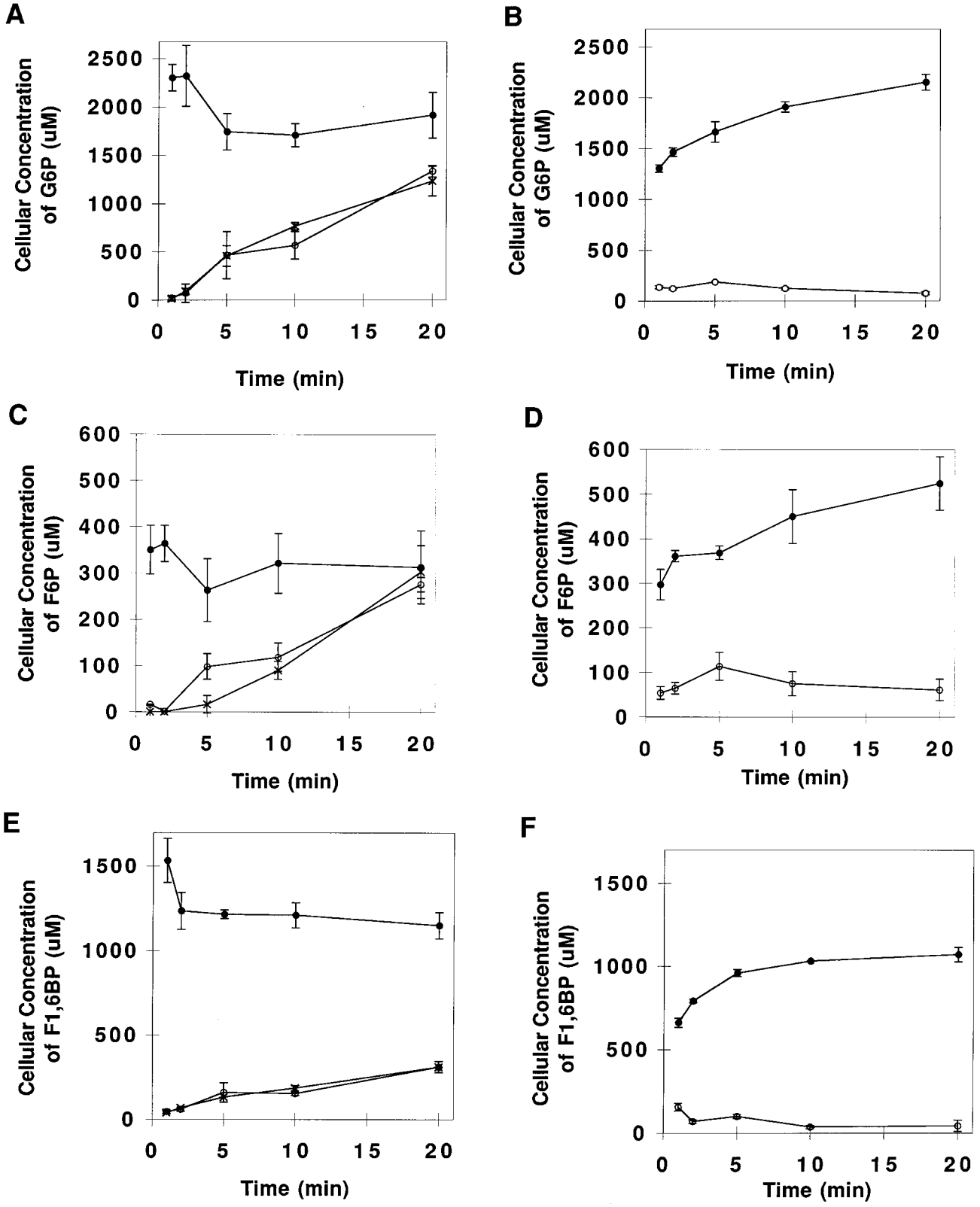

FIGURE 6: Changes in levels of hexose phosphates as a consequence of glucokinase overexpression in INS-1 cells and hepatocytes. Panels A, C, and E. INS-1 cells were treated with AdCMV-GKI, encoding wild-type islet glucokinase (filled circles), or AdCMV-GKI$_{261}$, encoding catalytically inactive glucokinase (crosses), or were left untreated (open circles). Cells were preincubated in 1 mM glucose for 30 min and then switched to 20 mM glucose; times along the horizontal axis of each graph refer to the time in minutes after the switch from 1 to 20 mM glucose. Levels of glucose-6-phosphate (G6P) (panel A), fructose-6-phosphate (F6P) (panel C), and fructose-1,6-bisphosphate (F 1,6 BP) (panel E) were measured as described in Materials and Methods. Panels B, D, and F. Hepatocytes were treated as described for INS-1 cells and levels of G6P (panel B), F6P (panel D), and F1,6BP (panel F) were measured. For all panels, data represent the mean ± SEM for four independent experiments, each performed in triplicate.

phosphates showed a gradual increase in glucokinase-overexpressing INS-1 cells during the 20 min incubation in high glucose rather than an initial rapid rise followed by a decline as observed for the hexose phosphates.

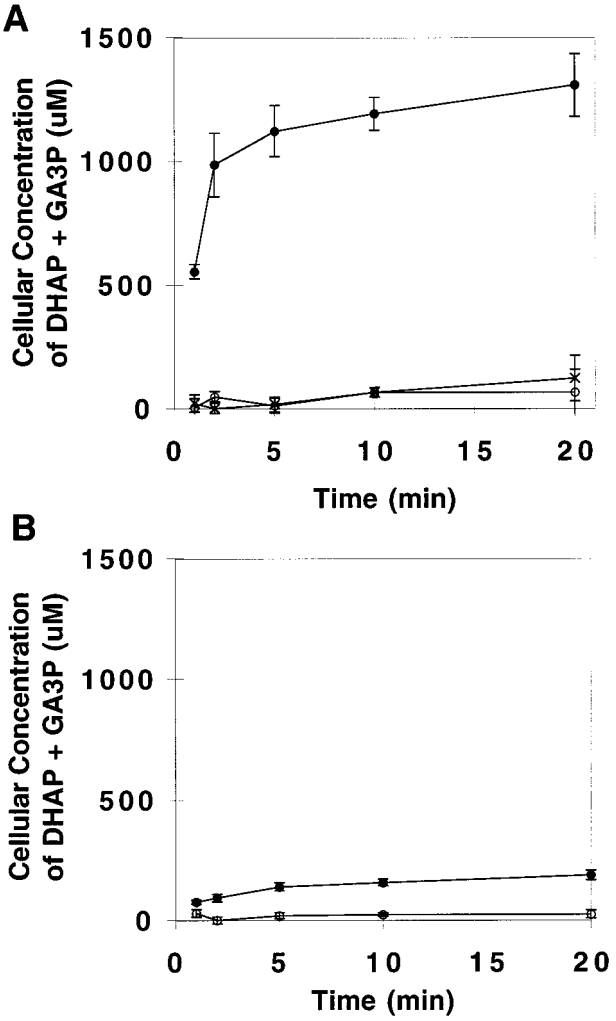

FIGURE 7: Changes in levels of triose phosphates (glyceraldehyde-3-P + dihydroxyacetone phosphate) as a consequence of glucokinase overexpression in INS-1 cells and hepatocytes. INS-1 cells (panel A) or cultured rat hepatocytes (panel B) were treated with AdCMV-GKI, encoding wild-type islet glucokinase (filled circles), or AdCMV-GKI$_{261}$, encoding catalytically inactive glucokinase (crosses), or were left untreated (open circles). Cells were preincubated in 1 mM glucose for 30 min and then switched to 20 mM glucose; the horizontal axis of each graph refers to the time in minutes after the switch from 1 to 20 mM glucose. Levels of glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetone phosphate (DHAP) were measured as described in Materials and Methods, and the values were added together to provide a measure of the total triose phosphate pool. For both panels, data represent the mean ± SEM for four independent experiments, each performed in triplicate.

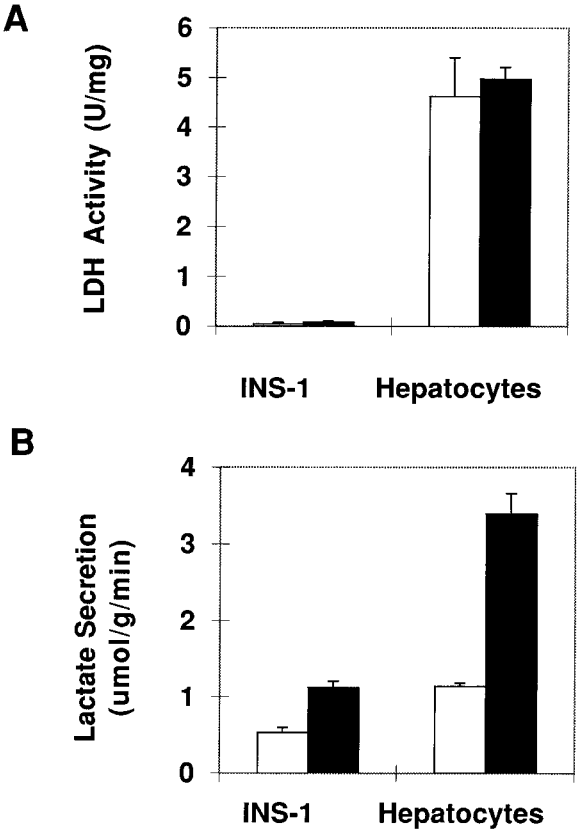

FIGURE 8: Lactate dehydrogenase and lactate production in INS-1 cells and hepatocytes. Panel A: Lactate dehydrogenase activity was measured in extracts of INS-1 cells or hepatocytes that were left untreated (open bars) or were treated with AdCMV-GKI (black bars). Data represent the mean ± SEM for four independent determinations. Panel B: Lactate levels were measured in media samples from INS-1 cells or hepatocytes that were left untreated (open bars) or treated with AdCMV-GKI (black bars). All samples were collected 10 min after a switch from 1 to 20 mM glucose. Data represent the mean ± SEM for four independent determinations.

Lactate Dehydrogenase and Lactate Production in Hepatocytes and INS-1 Cells. The foregoing results suggest that flux through the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) reaction is limited in INS-1 cells relative to hepatocytes. Measurement of total G3PDH enzyme activity in INS-1 and hepatocyte extracts revealed that INS-1 cells actually contain approximately 3 times more activity than hepatocytes (data not shown). An alternative explanation for reduced flux through G3PDH in glucokinase-overexpressing INS-1 cells could be limitation in supply of NAD, an essential cofactor for the reaction. During active glycolysis, one mechanism for regenerating NAD is flux through the lactate dehydrogenase (LDH) step. We therefore investigated the relative levels of LDH in INS-1 cells and hepatocytes. We found that AdCMV-GKI-treated hepatocytes have 4.97 ± 0.24 units/mg protein of LDH activity, 62 times more than AdCMV-GKI-treated INS-1 cells, which contain 0.08 ± 0.02 units/mg protein (Figure 8A). To determine whether this large difference in enzyme activity is reflected in reduced metabolic flux, we measured lactate secretion from glucokinase overexpressing INS-1 cells and hepatocytes following a switch from low to high glucose. As shown in Figure 8B, AdCMV-GKI-treated hepatocytes secreted approximately 3 times as much lactate as AdCMV-GKI-treated INS-1 cells into the medium during the first 10 min after a switch from low to high glucose. We also measured intracellular lactate and pyruvate concentrations in AdCMV-GKI and control INS-1 cells and hepatocytes. This allowed us to determine a pyruvate:lactate ratio in these cell types as an indirect measurement of the NADH:NAD ratio. As shown in Table 1, the pyruvate:lactate ratio was 3.2-fold higher in untreated INS-1 cells than in untreated hepatocytes, and 8.8-fold higher in AdCMV-GKI-treated INS-1 cells than in similarly treated hepatocytes. These results are consistent with a model in which intrinsically low levels of LDH activity in INS-1 cells impact flux through G3PDH by limiting the capacity to regenerate the cofactor NAD.

Table 1: Intracellular Lactate and Pyruvate Levels in INS-1 Cells and Hepatocytes[a]

	lactate (μM)	pyruvate (μM)	pyruvate: lactate ratio
INS-1 Cells			
untreated	77.3 ± 44.3	345.1 ± 27.9	4.5
AdCMV-GKI-treated	172.3 ± 29.9	610.2 ± 51.8	3.5
hepatocytes			
untreated	167.8 ± 48.0	242.5 ± 35.0	1.4
AdCMV-GKI-treated	618.6 ± 43.6	254.8 ± 31.7	0.4

[a] INS-1 cells or hepatocytes were treated with AdCMV-GKI or were left untreated. Cells were preincubated in 1 mM glucose for 30 min and then switched to 20 mM glucose for 10 min. Extracts were treated with ice-cold TCA, and neutralized supernatants were used for assay of cellular lactate and pyruvate levels. For columns 2−4, data represent the mean ± SE for three independent groups of cells.

DISCUSSION

Using the recombinant adenovirus system, our previous studies have shown that overexpression of glucokinase has no impact on 2- or 5-[^{3}H]glucose usage, lactate production, glucose oxidation, or glycogen content in isolated rat islets, while in cultured rat hepatocytes, overexpression of the enzyme results in potent enhancement of glycolytic flux and glycogen accumulation (*11*, *12*). The purpose of the current study was to provide a biochemical explanation for the differential metabolic impact of the overexpressed enzyme in the two cell types.

In keeping with our earlier findings in rat islets (*12*), glucokinase overexpression in INS-1 cells did not enhance 2- or 5-[^{3}H]glucose usage at 20 mM glucose relative to untreated cells or cells treated with a recombinant virus encoding a catalytically inactive glucokinase (AdCMV-GK_{261}), while a clear increase in glucose usage was observed at 20 mM glucose in glucokinase-overexpressing hepatoyctes. Thus, a fundamental difference between the metabolic impact of glucokinase overexpression in liver and islet cells was confirmed. Distinct from our earlier findings, however, was the observation that glucokinase overexpression caused a substantial increase in glycolytic flux at low glucose concentrations (1−5 mM). Our data in INS-1 cells are consistent with recent work of Wang and Iynedjian, who studied INS-1 cells stably transfected with a tetracyclin regulatable glucokinase construct and found that 2-fold overexpression of the enzyme caused a proportional increase in 5-[^{3}H]glucose usage at both low and high glucose, but that further increases in expression resulted in little additional increase in glycolysis at higher glucose concentrations (*25*). The failure to obtain increases in high K_m glucose usage commensurate with the extent of glucokinase overexpression is generally consistent with our earlier observations (*12*) and those reported herein. Wang and Iynedjian interpreted their findings to indicate that steps distal to glucokinase in glycolysis become saturated in response to small increments in glucokinase expression and that further increases in enzyme overexpression fail to exert a metabolic impact because these distal events become rate-limiting, but no insight into specific mechanisms was provided (*25*). In the current study, we have defined enzymatic steps distal to glucokinase that become rate-limiting upon overexpression of glucokinase in INS-1 cells but not hepatocytes that can explain the observed differences between the two cell types.

Important metabolic differences between hepatocytes and INS-1 cells that could explain the differential metabolic impact of overexpressed glucokinase were uncovered in the course of these studies. First, glucose-6-phosphate, fructose-6-phosphate, and to a lesser extent fructose-1,6-bisphosphate all accumulate during incubation with high glucose in control INS-1 cells that do not overexpress glucokinase, with G6P and F6P attaining the same high levels as in glucokinase-overexpressing cells, while none of these metabolites accumulates in control hepatocytes (Figure 7), suggesting that a reduced capacity to metabolize hexose phosphates is intrinsic to INS-1 cells even before glucokinase is overexpressed. Second, while G6P, F6P, and F1,6BP levels accumulate to similarly high levels in AdCMV-GKI-treated INS-1 cells or hepatocytes, maximum levels of these intermediates are achieved at the earliest time point sampled in INS-1 cells (2 min), after which levels begin to decline, while in hepatocytes, levels are also elevated at 2 min but then continue to climb throughout the continuing 20 min of exposure to high glucose. Finally, we found much lower flux through the glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase steps in AdCMV-GKI-treated INS-1 cells than in similarly treated hepatocytes. We suggest that the low LDH activity in INS-1 cells limits the capacity to regenerate NAD required for the G3PDH reaction, thereby limiting glycolytic flux and preventing efficient metabolism of hexose phosphates. Consistent with this idea was our finding of a much higher pyruvate:lacate ratio in AdCMV-GKI-treated INS-1 cells than in similarly treated hepatocytes (Table 1). Our findings concerning LDH activity are consistent with those of Sekine et al., who showed that the well-differentiated INS-1 cell line contains relatively low levels of LDH, while the poorly differentiated insulinoma cell line RINm5F contains much higher levels of activity (*26*). Interestingly, isolated islets contain even lower levels of LDH than INS-1 cells (*26*), suggesting a possible explanation for the finding that overexpressed glucokinase has a measurable metabolic impact at low glucose in INS-1 cells but not in islets (refs *12*, *25*; this study).

An alternate pathway for regeneration of NAD in mammalian cells is metabolism of DHAP to glycerol phosphate via the cytosolic glycerol phosphate dehydrogenase step. We found that glycerol phosphate dehydrogenase activity was essentially identical in INS-1 and hepatocyte extracts (data not shown). Interestingly, glycerol phosphate levels were at or below the limit of detection in both INS-1 cells and hepatocytes, independent of the glucokinase level, while DHAP levels were 3-fold higher in AdCMV-GKI-treated INS-1 cells than in hepatocytes. Thus, it appears that the DHAP:glycerol phosphate ratio is significantly higher in INS-1 cells than in hepatocytes, which may indicate limited capacity to metabolize DHAP via the glycerol phosphate dehydrogenase step in the former cells. An alternate explanation would be that INS-1 cells contain a higher level of mitochondrial glycerol phosphate dehydrogenase, allowing regeneration of DHAP via this pathway more effectively than in hepatocytes. A third possible explanation for the low levels of glycerol phosphate might be utilization of the glycerol phosphate pool for esterification with fatty acyl CoAs, but this is unlikely on the basis of our recent finding of a very low rate of glycerol phosphate incorporation into cellular lipids in INS-1 cells (*18*). Thus, while it remains

possible that the glycerol phosphate dehydrogenase reaction could represent a compensatory mechanism for regeneration of NAD in the face of low LDH activity in INS-1 cells, the elevated DHAP:glycerol phosphate ratio in these cells, coupled with an equilibrium constant for glycerol phosphate dehydrogenase reacton that favors formation of DHAP (*21*), suggests that this mechanism may be insufficient to allow active flux through G3PDH.

Our data are consistent with a mechanism in which overexpressed glucokinase is somehow suppressed or inhibited at high glucose in INS-1 cells. Several possible mechanisms by which suppression of overexpressed glucokinase can occur in islet cells can be eliminated. For example, previous reports have suggested that glucokinase can be regulated by covalent modification, either by phosphorylation by protein kinase A (*27*) or by reaction with long chain acyl CoA (*28*). Such mechanisms, which remain to be confirmed by other laboratories, are unlikely to be operative in our experiments, since the overexpressed enzyme was fully active in fresh extracts prepared from hepatocytes or INS-1 cells (Figure 1). Furthermore, triacsin C, an inhibitor of long chain acyl CoA synthase and an efficient blocker of long chain acyl CoA synthesis in INS-1 cells (*18*), does not prevent the suppression of overexpressed glucokinase by high glucose in this β-cell line (data not shown). Another potential means of suppressing glucokinase activity might be its interaction with the glucokinase regulatory protein (GRP), which binds to and inhibits glucokinase in response to changes in cellular levels of certain hexose phosphates (*29*). Again, this is highly unlikely, given that we and others have demonstrated that islets contain extremely low levels of glucokinase regulatory protein activity (*12*, *30*) or mRNA (*12*). Further, GRP protein is not detectable in INS-1 cells by immunoblot analysis, using an antibody which readily detects GRP in hepatocytes (H. K. Berman, J. Grippo, and C. B. Newgard, unpublished observations). Finally, strong overexpression of glucokinase could have caused ATP depletion and general metabolic paralysis in our INS-1 cell experiments. Indeed, we observed an approximate 40% fall in ATP levels in the period from 1 to 10 min after a switch from low to high glucose, but this occurred in both AdCMV-GKI-treated and untreated INS-1 cells. Further, ATP levels returned to normal by 2 h after the switch in both groups of cells, but overexpressed glucokinase remained suppressed at this time point (data not shown). While these data suggest that ATP levels are not the critical factor, formal elimination of fluctuations in ATP as contributory to glucokinase suppression in INS-1 cells will require more extensive biochemical analysis.

Nevertheless, differential regulation of overexpressed glucokinase in INS-1 cells and hepatocytes is still a tenable explanation for our results. In support of this idea, we find that 3-*O*-methyl glucose, an analogue which is transported into cells but not phosphorylated, fails to suppress glycolytic flux in INS-1 cells, while 2-deoxyglucose, which is transported and phosphorylated but then not metabolized further, is a potent inhibitor (data not shown). While these data could suggest that accumulation of G6P could suppress glucokinase in INS-1 cells, it should be noted that G6P levels rise to a similar extent in AdCMV-GKI-treated INS-1 cells and hepatocytes exposed to 20 mM glucose, with little inhibition of glycolytic flux in the latter cells. Further, in vitro studies with glucokinase reveal the enzyme to be insensitive to allosteric inhibition by G6P, even at concentrations as high as 10 mM (*11, 12*), while our estimate of intracellular G6P levels in AdCMV-GKL-treated INS-1 cells or hepatocytes is in the range of 1−3 mM. Assuming that the G6P pool in INS-1 cells is turning over more slowly than that in hepatocytes, it is nevertheless possible that local concentrations of the intermediate could attain much higher levels in INS-1 cells, possibly so high as to inhibit glucokinase via a product inhibition mechanism. It has been clearly established that in hepatocytes cultured at low glucose levels, glucokinase protein is localized mainly to the nucleus via its interaction with GRP (*31*−*33*). Upon stimulation with glucose or other hexoses, hepatic glucokinase dissociates from GRP and translocates from the nucleus to the cytoplasm. Activation of glycogen synthesis in response to glucose seems to coincide with the translocation of glucokinase (*23*, *31*). It remains unclear whether glucokinase translocates in islet cells in the same manner as it does in hepatocytes in response to glucose. There has been one report of translocation of glucokinase from a "perinuclear" site to the cytoplasm of islets in response to glucose (*34*), but the enzyme does not appear to be sequestered inside the nucleus, consistent with the very low levels of GRP in islet cells. Thus, a lesser efficiency of G6P clearance in INS-1 cells due to limitations in distal metabolic steps coupled with islet cell-specific localization of glucokinase that allows the intermediate to build up to very high concentrations around the enzyme could lead to suppression of its activity. It should be noted that we have recently overexpressed glucokinase in isolated human muscle cells, and as in hepatocytes, we have observed large increases in glycogen synthesis and glucose metabolism (S. Baque, E. Montell, J. J. Guinovart, C. B. Newgard, and A. M. Gomez-Foix, manuscript submitted for publication), further arguing that glucokinase is uniquely regulated in the islet cell environment. Future studies will be directed at learning more about the intracellular localization of endogenous and overexpressed glucokinases in islet cells.

These studies have implications for investigators interested in using molecular approaches for improving the performance of insulinoma cells lines. Because of its kinetic features and the fact that its activity appears to be rate-limiting for glucose flux in normal β-cells, glucokinase has been ascribed the role of "glucose sensor" in regulation of glucose-stimulated insulin secretion. Indeed, reduced expression of glucokinase in β-cells, whether achieved by transgenic approaches or occurring spontaneously in genetic diseases, is correlated with reduced capacity of islets to respond to glucose (*7*−*10*). In contrast, the current and previous studies show that the relationship between the extent of glucokinase overexpression and glucose metabolism is nonlinear in islet β-cells (*12*, *25*; this study). Furthermore, we find that insulin secretion is increased in GK overexpressing cells relative to controls at low glucose, consistent with the elevated glycolytic rate at the low glucose concentrations, while no increase in insulin secretion is observed at high glucose concentrations, consistent with the lack of metabolic impact at high concentrations of the sugar (data not shown). Similar findings have been reported by Wang and Iynedjian (*25*). The findings of the current study indicating that limited metabolic impact of overexpressed glucokinase in the islet

cell environment is secondary to limiting flux through distal steps in glycolysis, particularly glyceraldehyde-3-phosphate dehydrogenase and LDH, provides a potential solution to the problem. Recently, we have constructed a virus containing LDH and have coexpressed this enzyme and glucokinase in INS-1 cells. We find a large (6-fold) increase in lactate output in co-transduced cells relative to cells treated with GK or LDH virus alone (H. Berman, and C. B. Newgard, unpublished observations). These results encourage us to investigate whether engineering of islets or INS-1 cells for simultaneous overexpression of glucokinase and LDH will be an approach to unveiling the full metabolic impact of glucokinase overexpression, and if so, whether such a manuever will enhance glucose-stimulated insulin secretion.

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