

Long-Term Exposure of β -INS Cells to High Glucose Concentrations Increases Anaplerosis, Lipogenesis, and Lipogenic Gene Expression

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Chronic exposure of pancreatic β -cells to high glucose has pleiotropic action on β -cell function. In particular, it induces key glycolytic genes, promotes glycogen deposition, and causes β -cell proliferation and altered insulin secretion characterized by sensitization to low glucose. Postglycolytic events, in particular, anaplerosis and lipid signaling, are thought to be implicated in β -cell activation by glucose. To understand the biochemical nature of the β -cell adaptive process to hyperglycemia, we studied the regulation by glucose of lipogenic genes in the β -cell line INS-1. A 3-day exposure of cells to elevated glucose (5–25 mmol/l) increased the enzymatic activities of fatty acid synthase 3-fold, acetyl-CoA carboxylase 30-fold, and malic enzyme 1.3-fold. Pyruvate carboxylase and citrate lyase expression remained constant. Similar observations were made at the protein and mRNA levels except for malic enzyme mRNA, which did not vary. Metabolic gene expression changes were associated with chronically elevated levels of citrate, malate, malonyl-CoA, and conversion of glucose carbon into lipids, even in cells that were subsequently exposed to low glucose. Similarly, fatty acid oxidation was suppressed and phospholipid and triglyceride synthesis was enhanced independently of the external glucose concentration in cells preexposed to high glucose. The results suggest that a coordinated induction of glycolytic and lipogenic genes in conjunction with glycogen and triglyceride deposition, as well as increased anaplerosis and altered lipid partitioning, contribute to the adaptive process to hyperglycemia and glucose sensitization of the β -cell. *Diabetes* 47:1086–1094, 1998

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ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; BSA, bovine serum albumin; CPT, carnitine palmitoyl-transferase; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GK, glucokinase; K_{ATP} channel, ATP-sensitive potassium channel; KRBB, Krebs-Ringer bicarbonate buffer; ME, malic enzyme; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PFK, phosphofruktokinase; ZDF, Zucker diabetic fatty.

Chronic exposure of pancreatic β -cells to elevated concentrations of glucose causes β -cell hypertrophy and hyperplasia and enhanced proinsulin biosynthesis and results in altered secretory function, in particular, sensitization to low concentrations of sugar (1–4). The mechanisms that underlie these adaptations to hyperglycemia are poorly understood; they likely involve changes in the expression level of glucose-regulated genes, which must be identified.

Activation of intermediary metabolism by nutrients is linked to signal transduction in the β -cell (5,6). Therefore, the possibility should be considered that changes in the expression level of metabolic genes by glucose are causally implicated in these pleiotropic long-term actions of sugar. Previous work has shown that an important component of the β -cell adaptation process to hyperglycemia is an increase in both glucokinase (GK) activity (4) and GLUT2 expression (7,8). Other key glycolytic enzymes are also induced by glucose in the β -cell, and enzymes downstream of GK may play important regulatory roles in insulin secretion, particularly under hyperglycemic conditions (9,10). Thus, elevated glucose causes a coordinated induction of phosphofruktokinase (PFK) isozymes (C, M, and L), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and L-pyruvate kinase in β -INS-1 cells (11). These actions of glucose are largely mediated via transcriptional activation of these genes (11,12).

Because it is generally accepted that metabolic pathways other than glycolysis play a key role in coupling factor production, enzymes in other pathways should also be investigated. In this respect, anaplerotic and lipogenic enzymes should be considered. Thus the hypothesis was proposed that an anaplerotic/malonyl-CoA pathway, in conjunction with the ATP-sensitive potassium (K_{ATP}) channel pathway, is implicated in both the short- and long-term regulation of insulin secretion (5,6). In addition, the malonyl-CoA/carnitine palmitoyl-transferase (CPT)-1 interaction has emerged as a key component of a fuel “cross-talk” metabolic signaling system in a number of tissues (13–16). Alternatively, changes in anaplerotic influx in the citric acid cycle could facilitate variations in the concentration of the cytosolic ADP/ATP ratio, which controls the K_{ATP} channels (17,18) and NADPH, a candidate coupling factor (19). Thus, in addition to metabolic genes, the identification of the pathways affected by long-term exposure to glucose should provide insight regarding the long-sought mechanism by which prolonged exposure of β -cells to glucose causes sensitization to sugar (2,20).

Little is known about the long-term effects of glucose on β -cell anaplerosis and lipid metabolism. For the following reasons it is, however, attractive to hypothesize that changes in anaplerosis, lipogenesis, and lipid esterification processes play important roles in β -cell glucose sensitization (2,21). With respect to anaplerosis, the recent evidence indicates that glucose-induced insulin release tightly correlates with anaplerotic input into the citric acid cycle in both purified β -cells and INS-1 cells (22). Furthermore, direct support for the view that anaplerosis plays an essential role in β -cell nutrient signaling has recently been provided by the observation that the cycle intermediate succinate stimulates the exocytotic release of insulin in permeabilized INS cells (23). Concerning lipid metabolism, it is noteworthy that the antidiabetic effect of leptin in obese Zucker diabetic fatty (ZDF) rats is associated with reduced acetyl-CoA carboxylase (ACC) expression and triglyceride content of islet tissue, as well as an induction of enzymes of fatty acid oxidation (24–27). In addition, ZDF rats show increased islet lipogenesis and massive triglyceride deposition (27). Fatty acids are also known to amplify insulin secretion (5,27), cause a rise in cytosolic Ca^{2+} (28), and play a permissive role in glucose-induced insulin release (24,29).

Results from the present study provide support for the view that changes in anaplerosis and lipid metabolism play a role in the process whereby hyperglycemia modifies insulin secretion. We report that a 3-day exposure of INS cells to elevated glucose induces fatty acid synthase (FAS) and malic enzyme (ME) and causes transcriptional activation of the ACC gene while barely affecting the expression level of other enzymes in the pathway of malonyl-CoA formation from pyruvate. The increased expression of glycolytic enzymes (11), ME, FAS, and ACC is associated with exaggerated anaplerosis, lipogenesis, and malonyl-CoA levels and a chronic inhibition of fatty acid oxidation, even in cells subsequently exposed to low glucose. In addition, hyperglycemia causes a marked stimulation of lipid esterification and triglyceride deposition in the β -cell.

RESEARCH DESIGN AND METHODS

Cell culture and incubation conditions. INS-1 cells were seeded in 21-cm² petri dishes (1.4×10^6 cells/dish) and grown as described previously (30). When cells reached 80% confluence after approximately 7 days, they were washed twice with phosphate-buffered saline (PBS) and preincubated at 37°C for 2 days in culture medium containing 5 mmol/l glucose. Cells were then washed with PBS and incubated for 3 days in culture medium at various glucose concentrations.

mRNA analysis. Cells were washed twice with cold PBS, and total RNA was extracted by the guanidium isothiocyanate method (31). RNA samples (12 μ g) were denatured using glyoxal or formaldehyde, subjected to electrophoresis in 1% agarose gels, transferred by capillarity to a nylon membrane, and fixed by UV exposure. mRNA analysis was carried out by the Northern blotting hybridization method using the following ³²P-labeled cDNA probes: 0.77 kb *EcoRI-BamHI* fragment (positions 1–1780) of mouse rRNA 18S cDNA subcloned in pUC830; 1.4 kb *EcoRI-EcoRI* fragment of human E1 α subunit of pyruvate dehydrogenase (PDH) cDNA subcloned in pBSKS (provided by Dr. J. Maury, CNRS, Meudon, France); 2.316 kb *EcoRI-EcoRI* fragment (positions 1731–4067) of mouse pyruvate carboxylase (PC) cDNA subcloned in pBSKS; 1.1 kb *EcoRI-EcoRI* fragment of rat ME cDNA subcloned in pTZ18R (Pharmacia; provided by Dr. C.N. Mariash, University of Minnesota, Minneapolis, MN); and 0.921 kb *EcoRI-BamHI* fragment of a rat ATP-citrate lyase (ACL) fragment subcloned in pBSSK (provided by Dr. N. Iritani, Tezukayama Gakuin College, Osaka, Japan). ACC and FAS mRNAs were detected using [³²P]-cRNA probes obtained as follows: the linearized pGEM3-ACC containing the 0.509 kb *EcoRI-EcoRI* fragment of rat ACC, clone P181–6 (provided by Dr. K.-H. Kim, Purdue University, Purdue, IN) was transcribed with SP6 RNA polymerase, and the linearized pBSKS-FAS18 containing the 0.66 kb *PstI-PstI* fragment of rat FAS (provided by Dr. A.G. Goodridge, University of Iowa, Iowa City, IA) was transcribed with T3 RNA polymerase. Alternatively, the FAS fragment was used to synthesize a ³²P-cDNA probe. Membranes were exposed for autoradiography

at –70°C using pre-flashed X-ray films (Fuji). The autoradiograms were analyzed by laser densitometer scanning, and the mRNA values were normalized to those of the 18S rRNA, which was used as an unvariant control.

Protein analysis and enzymatic activities measurements. Following two washes of cells with an ice-cold Krebs-Ringer bicarbonate buffer (KRBB), 0.5 ml of extraction buffer (50 mmol/l Tris [pH 7.5], 5 mmol/l EDTA, 5 mmol/l EGTA, 0.5 mmol/l phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 5 μ g/ml antipain, and 10 mmol/l mercaptoethanol) was added to the cells. Cells were scraped from the dishes, transferred to an Eppendorf tube, and disrupted by sonication. Extracts (25 μ g) of total cellular proteins were resolved on 10% SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). E3 and E1 α subunits of PDH, ACL, ACC, and FAS proteins were detected by immunoblotting with specific antibodies. Streptavidin blotting (32) was used to monitor the abundance of the biotin-containing enzymes ACC and PC.

For enzymatic activity measurements (ME, ACL, ACC, and FAS), sonicated extracts were centrifuged at 10,000g for 30 min at 4°C. The resulting supernatants were used for the assays. PC activity was measured by a ¹⁴C fixation method (19) on the total homogenate of the sonicated extracts. ME activity was assessed as described elsewhere (33). ACL enzymatic activity was determined using an ACL/chloramphenicol acetyl-transferase (CAT)-coupled assay, which determines the amount of radiolabeled acetylated chloramphenicol formed by the CAT reaction from radiolabeled acetyl-CoA synthesized by ACL from [¹⁴C]citrate (34). ACC and FAS (35) were determined as described before. CPT-1 measurements were determined in a mitochondrial fraction of INS cells as described elsewhere (36). **In vitro transcription assay.** Nuclei isolation and nuclear run-on transcription assays were performed as indicated (37). Briefly, nascent transcripts were elongated in vitro in the presence of [³²P]UTP and heparin. The obtained ³²P-labeled RNAs were subjected to mild alkaline hydrolysis (30 min, 50°C, 50 mmol/l Na₂CO₃) and hybridized to 4 μ g/dot of the following DNA constructions immobilized on nitrocellulose membranes: 0.77 kb *EcoRI-BamHI* fragment (positions 1–1780) of mouse rRNA 18S cDNA subcloned in pUC830 and 1.688 kb *EcoRI-EcoRI* fragment (positions 4177–5865) of rat ACC cDNA subcloned in pUC18.

Malate, citrate, and malonyl-CoA measurements. Malate and citrate levels were determined as follows. After a culture period of 3 days at 5 or 25 mmol/l glucose, cells were washed with PBS and preincubated at 37°C for 30 min in KRBB-HEPES (pH 7.4) medium, at 5 mmol/l glucose, containing 0.07% of bovine serum albumin (BSA) (Fraction V; Sigma, St. Louis, MO). Cells were then incubated for 30 min in a fresh KRBB-HEPES medium at 5 or 25 mmol/l glucose. Incubation media were discarded or collected for insulin measurement, and 0.5 ml of 13% perchloric acid was added to the cells. Precipitated proteins were removed by centrifugation, and the supernatants were neutralized by adding 2 mol/l KH₂CO₃. The precipitated potassium perchlorate salt was eliminated by centrifugation, and the resulting supernatants were used for metabolite measurements. Total cellular proteins were measured by the Bradford (Bio-Rad) assay after dissolution of the protein pellets in 0.5 N NaOH. Malate was determined using the malate dehydrogenase (Boehringer Mannheim, Mannheim, Germany) reaction (38). Citrate was measured by coupling the citrate lyase/malate dehydrogenase (Boehringer Mannheim) reactions as described elsewhere (38). Malonyl-CoA was extracted from cells with 10% trichloroacetic acid. After centrifugation of precipitated proteins, cell extracts were brought to pH 5–6 by successive ether extractions. Samples were lyophilized and stored at –70°C. Malonyl-CoA was assayed as described elsewhere (39).

Fatty acid metabolism and triglyceride measurements. Fatty acid oxidation was measured in INS-1 cells cultured in 21-cm² petri dishes. After preexposure for 3 days at 5 or 25 mmol/l glucose, cells were washed with PBS and preincubated at 37°C for 30 min in KRBB-HEPES (pH 7.4) medium at 5 mmol/l glucose containing 0.07% BSA. Cells were then incubated for 1 h at 37°C in 5 ml of a fresh KRBB-HEPES medium at 5 or 25 mmol/l glucose, in the presence of 0.1 mmol/l palmitate, 0.5% defatted BSA, 1 mmol/l carnitine, and 0.11 μ Ci of [¹⁴C]palmitate (55 mCi/mmol) (Amersham). At the end of the incubations, media were collected and transferred to 25-ml Erlenmeyer flasks covered with septa caps. Media were acidified by injecting perchloric acid (6% final concentration) with a syringe. The liberated CO₂ was trapped in a plastic well and suspended from the septa caps containing 0.4 ml of methanolic benzethonium hydroxide. After a 1-h incubation at 37°C, the wells were removed, and the trapped ¹⁴CO₂ was measured by liquid scintillation counting. Cells were scraped in cold PBS, pelleted by centrifugation, and resuspended in 4 ml of Folch reagent (40). Total lipids were extracted as previously described (40) and separated by thin-layer chromatography to measure the incorporation of labeled palmitate into triglycerides and phospholipids (40).

The de novo synthesis of lipids from glucose (lipogenesis) was assessed in 78-cm² petri dishes as follows. After preexposure for 3 days at 5 or 25 mmol/l glucose, cells were washed with PBS and preincubated at 37°C for 30 min in KRBB-HEPES (pH 7.4) medium, at 5 mmol/l glucose containing 0.07% BSA. Cells were then incubated for 3 h at 37°C in 8 ml of a fresh KRBB-HEPES medium at 5 or 25 mmol/l glucose containing 100 μ Ci/mmol of [¹⁴C]glucose. Cells were then washed twice with cold PBS, scraped from the dishes, and sonicated in 2 ml of PBS. The radioactiv-

ity in the fatty acid moiety of lipids was measured after chloroform-methanol extraction, saponification with 0.5 mol/l KOH in 90% ethanol at 70°C for 90 min, and heptane extraction of the fatty acids after acidification of the solution (41). The cellular triglyceride content was measured as described previously (42).

Statistical analysis. Data are means \pm SE for the number of experiments stated. The statistical significance of the differences between experimental groups was assessed by the unpaired Student's *t* test.

RESULTS

Effect of glucose on the expression level of PDH, PC, ME, and lipogenic enzyme mRNAs in β -INS-1 cells. The studied nonglycolytic enzymes thought to be implicated in nutrient signaling can be classified in three groups: 1) PDH, a central regulatory enzyme of energy production; 2) the two anaplerotic enzymes of glucose metabolism—PC and ME; and 3) two key lipogenic enzymes—ACC and FAS—and two ancillary enzymes—ME and ACL. The reversible carboxylating reaction of ME allows this enzyme to function for both anaplerosis (pyruvate \rightarrow malate) and cytosolic NADPH generation (malate \rightarrow pyruvate).

ACC mRNA and protein induction by glucose in β -INS cells has been reported before (43). Nonetheless, ACC measurements are also shown in the present study to allow for the comparison of the magnitude of the effect of glucose on other genes encoding anaplerotic/lipogenic enzymes in the same set of experiments. Figure 1 shows that a 24-h incubation of INS cells at 25 mmol/l glucose, in comparison with cells cultured at 5 mmol/l glucose, did not change PDH-E1 α mRNA abundance. The transcripts encoding the anaplerotic enzymes PC and ME were slightly, although not significantly (see below), induced by high glucose. With regard to the transcripts encoding lipogenic enzymes, the ACL mRNA level remained constant whereas the ACC and FAS mRNAs were markedly induced by sugar. As previously described in other cell types (44), the FAS probe recognized two transcripts of 9.5–10 kb that were induced to a similar extent by glucose (Fig. 1). The effects of glucose on the expression level of the ACC and FAS transcripts were dosage dependent and maximal at 24 h. At 48 h, the effects were similar to those at 24 h (not shown). Quantification by densitometric scanning of the inductive action of glucose at 24 h indicated the following values (expressed as *x*-fold induction by 25 mmol/l glucose over 5 mmol/l glucose): PDH, 1.04 \pm 0.16 (NS); PC, 1.64 \pm 0.22 (NS);

ME, 1.43 \pm 0.19 (NS); ACC, 22.5 \pm 2.5 (*P* < 0.001); and FAS, 4.38 \pm 0.38 (*P* < 0.01). Data are means \pm SE of three experiments.

Glucose causes transcriptional activation of the ACC gene. We previously reported that glucose increases the expression of the ACC transcript and protein in β -INS cells (43) but did not conclusively identify the level at which this gene-inductive process occurs. Nuclei from INS cells incubated at 5 and 25 mmol/l glucose were isolated to perform a run-on transcriptional assay of the ACC gene. Figure 2 shows that glucose caused a marked transcriptional activation of the ACC gene, whereas it did not modify transcription of the 18S rRNA gene used as an invariant control. The run-on technique was not sensitive enough to assess FAS gene transcription at either high or low glucose. Thus, glucose causes a similar (~15-fold) transcriptional activation of the ACC gene (Fig. 2) and rise of ACC mRNA (Fig. 1) (43) and protein (Fig. 4). Because the sugar does not modify the half-life of the ACC transcripts (43), it can be concluded that the action of glucose on ACC gene expression occurs mainly at the transcriptional level.

Glucose increases the level of ME, ACC, and FAS enzymatic activities. Experiments were carried out to determine whether the accumulation of the inducible transcripts was associated with a similar increased expression of the corresponding enzymes. To have more information about the regulation by glucose of the selected enzymes, both activity and protein expression measurements were carried out. Indeed, ACL and ACC are known to be regulated by covalent modifications (34,45). Furthermore, glucose modulates the expression of a number of β -cell proteins (46), including proinsulin and its convertases (47), at the translational level. Because enzymes of intermediary metabolism generally have a long half-life (>24 h), enzyme expression was measured after 3–4 days of incubation at either low (5 mmol/l) or high (25 mmol/l) glucose.

Streptavidin blotting of INS cell proteins indicated that the slight inductive action of glucose on PC mRNA was not associated with a similar effect on PC protein expression, since the latter was identical at low and high glucose (Fig. 3). The expression level of the two PDH subunits E1 α and E3 was

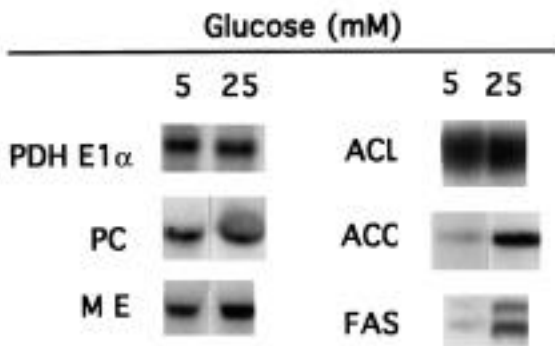


FIG. 1. Effect of glucose on the expression level of PDH-E1 α , PC, ME, ACL, ACC, and FAS mRNAs. After a preincubation period of 48 h in RPMI medium at 5 mmol/l glucose, INS cells were incubated for 24 h in the presence of 5 or 25 mmol/l glucose. Total RNA was extracted, and the different transcripts were detected by Northern blot hybridization. Results were obtained from representative experiments repeated at least three times.

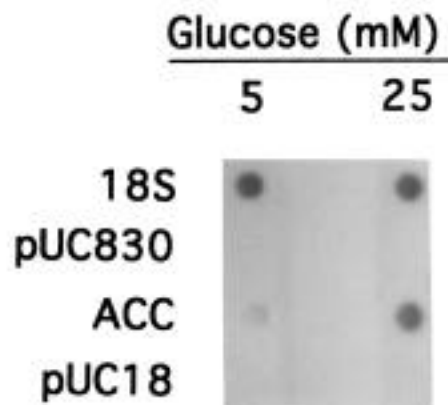


FIG. 2. Nuclear run-on transcriptional analysis of the ACC gene in glucose-stimulated INS cells. Nuclei from INS cells incubated for 24 h at 5 and 25 mmol/l glucose were isolated and incubated in the presence of [³²P]UTP. The ³²P-labeled nascent transcripts were hybridized to nitrocellulose-fixed plasmid pUC18 with or without an inserted ACC cDNA probe, as well as to pUC830 with or without an 18S rRNA cDNA insert used as an invariant control.



FIG. 3. Effect of glucose on PC, PDH-E1 α , and PDH-E3 protein accumulation. Cells were incubated for 72 h in the presence of 5 and 25 mmol/l glucose. PC was detected by streptavidin blotting, and the PDH subunits E1 α and E3 were detected by immunoblotting. Figure 3 shows blots from representative experiments.

assessed using a polyclonal antibody. The immunoblot shown in Fig. 3 indicates that high glucose did not affect PDH protein expression in β -INS cells.

ACC protein was assessed using streptavidin, which recognizes both the ACC265 and ACC280 isozymes, and specific antibodies. Thus ACC280 is abundant in a number of tissues, including the heart (48), and is expressed in rat islets (35,45), although at a very low level in comparison with the ACC265 isoform (35). Figure 4 indicates that only ACC265 is expressed in INS cells (one band in this range of molecular weight is apparent) and that glucose induced only this isoform. No ACC280 could be detected with an isoform-specific antibody (results not shown). The abundance of the FAS protein increased after glucose stimulation, whereas ACL protein expression remained constant (Fig. 4). Hence the expression pattern of lipogenic enzymes in β -INS cells at high and low glucose was similar to that of their corresponding mRNAs.

Table 1 details the activities of the three enzymes in the lipogenic pathway, CPT-1, and ME. It is interesting to note that at 5 mmol/l glucose, the lowest activities among the tested enzymes were those of ACC and CPT-1, which are limiting for malonyl-CoA synthesis and fat oxidation, respectively (49). Long-term exposure of INS cells to elevated glucose caused a considerable (~30-fold) augmentation of ACC enzymatic

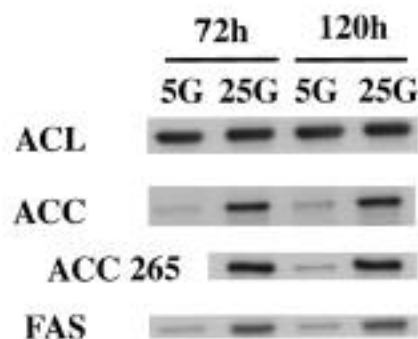


FIG. 4. Effect of glucose on ACL, ACC, and FAS protein accumulation. Cells were incubated for 72 and 120 h in the presence of 5 and 25 mmol/l glucose (G). ACL and FAS were detected by immunoblotting with specific antibodies. ACC was detected either by streptavidin blotting (ACC) or by immunoblot using an antibody specific for the 265 kD ACC isoform (ACC 265). Figure 4 shows blots from representative experiments.

activity. The magnitude of the effect, which is much larger than that examined at the mRNA and protein levels, is likely due to the fact that glucose, in addition to causing transcriptional induction of the ACC gene, also acutely activates the ACC enzyme, as indicated in the β -cell lines HIT (35,45) and INS (50). Glucose increased FAS enzymatic activity by three- to fourfold, an effect similar in magnitude to that observed at the protein and transcript levels. A modest (~30%) but significant rise in ME activity occurred at high glucose. ACL and CPT-1 enzymatic activities remained constant, indicating that glucose causes a selective augmentation of only some of the metabolic enzymes thought to be implicated in the nutrient-sensing process.

Long-term exposure of INS-1 cells to high glucose causes sustained anaplerotic influx in the citric acid cycle and elevated malonyl-CoA content. INS cells exposed for a long period of time to high glucose displayed an elevated metabolic activity and oxidative metabolism at low concentrations of sugar (11). This observation was made by measuring the reduction of the artificial electron acceptor 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), which provides an index of reducing equivalent production and the rate of glucose oxidation in the β -cell (51,52). Thus cells incubated for 3 days at 25 mmol/l glucose showed a threefold

TABLE 1

Effect of long-term exposure of INS-1 cells to elevated glucose on the enzymatic activities of PC, ME, ACL, ACC, FAS, and CPT-1

Glucose (mmol/l)	Enzymatic activities (mU/mg protein)					
	PC	ME	ACL	ACC	FAS	CPT-1
5	6.39 \pm 0.42	25.0 \pm 2.0	2.3 \pm 0.1	0.56 \pm 0.1	1.4 \pm 0.2	0.61 \pm 0.48
25	7.25 \pm 0.10	31.8 \pm 1.8	2.6 \pm 0.2	17.8 \pm 1.8	3.9 \pm 0.3	0.67 \pm 0.47
P value	NS	<0.025	NS	<0.0025	<0.001	NS

Data are means \pm SE of four to six independent experiments. Cells were incubated for 3 days at the indicated glucose concentrations, after which the expression level of the indicated enzymes was assessed. One milliunit of PC is equivalent to 1 nmol CO₂ fixed per minute, 1 mU of ME is equivalent to 1 nmol of NADPH formed per minute, and 1 mU of ACL is equivalent to 1 nmol of citrate cleaved per minute. ACC enzymatic activity was determined in the presence of 10 mmol/l citrate (total activity); 1 mU of ACC is equivalent to 1 nmol of malonyl-CoA formed per minute. One milliunit of FAS is equivalent to 1 nmol of NADPH oxidized per minute, and 1 mU of CPT-1 is equivalent to 1 nmol of palmitoyl-carnitine formed per minute.

higher MTT reduction than at low (2.5–5 mmol/l) glucose (11). To define the consequences of the induction by glucose of glycolytic (11), anaplerotic, and lipogenic (present study) enzymes on β -cell metabolic signaling, we measured key intermediates of the anaplerotic/malonyl-CoA pathway (5). Measurements of citrate were carried out because this intermediate results from the condensation of acetyl-CoA with oxaloacetate, the metabolic product of the PC reaction. Furthermore, citrate is the direct carbon precursor of malonyl-CoA and fatty acids as well an allosteric activator of ACC. Malate determinations were also made because malate is the product of the anaplerotic reaction carried out by ME, which is expressed at very high levels in both rat islets (53,54) and INS-1 cells (Table 1).

The exact experimental protocol was as follows and is also depicted in the upper part of Fig. 5. INS cells were preincubated for 2 days at 5 mmol/l glucose and subsequently exposed for 3 days to 5 or 25 mmol/l glucose. Cells from the two groups were then washed and incubated for 30 min in the presence of 5 and 25 mmol/l glucose (four final conditions). Acute stimulation of INS cells with elevated glucose resulted in a two- and sevenfold increase in citrate and malate, respectively (A versus B in Fig. 5). However, cells preexposed to high glucose for 3 days and subsequently incubated at low glucose showed the same elevated cellular content of the two metabolites as cells acutely stimulated by high glucose (B versus C in Fig. 5). Elevated glucose caused a threefold rise of malonyl-CoA (B versus A). Cells preexposed to high glucose for 3 days displayed a markedly elevated concentration of the metabolite at low glucose (C) (approximately eightfold above the basal [A] value) that could not be further increased by high glucose (D). The fact that the malonyl-CoA content of INS cells preexposed to high glucose (C) was higher than in cells acutely stimulated with glucose

(B) was likely due to the marked induction of the ACC enzyme by glucose (Table 1).

The data indicate that long-term exposure of β -INS cells to glucose results in chronically accelerated anaplerosis and elevated malonyl-CoA levels, even in cells that are subsequently exposed to a low basal concentration of the carbohydrate.

Long-term exposure of β -INS cells to elevated glucose causes a sustained alteration of lipid partitioning. The fate of fatty acids in cells incubated for a long period of time at high or low glucose was examined using a similar experimental protocol. [14 C] O_2 production from [14 C]-palmitate was determined as an index of the capacity of INS cells to oxidize fatty acids. The incorporation of labeled palmitate into phospholipids, triglycerides, and total lipids was measured to assess lipid esterification processes that are thought to be causally implicated in insulin secretion (55,56).

Acute glucose stimulation of INS cells caused inhibition of fatty acid oxidation and promoted phospholipid and triglyceride synthesis (Fig. 6). Cells preexposed to high glucose for 3 days showed an inhibition of fatty acid oxidation and stimulation of lipid esterification processes independently of the ambient glucose. Thus, changes in lipid metabolism (Fig. 6) paralleled those of malonyl-CoA (Fig. 5). This was consistent with the view that malonyl-CoA, the physiological inhibitor of CPT-1 (49), is a key metabolite regulating fatty acid partitioning in the β -cell.

Glucose acutely stimulates lipogenesis and promotes triglyceride deposition. Glucose (5–25 mmol/l) promoted an approximate eightfold stimulation of de novo synthesis of fatty acids from glucose carbon over a 3-h period (Fig. 7). Consistent with ACC and FAS induction, long-term (3-day) exposure to high glucose resulted in a fourfold elevation of lipogenesis, even in cells subsequently incubated at low glucose. The triglyceride mass of cells incubated for 3 days at

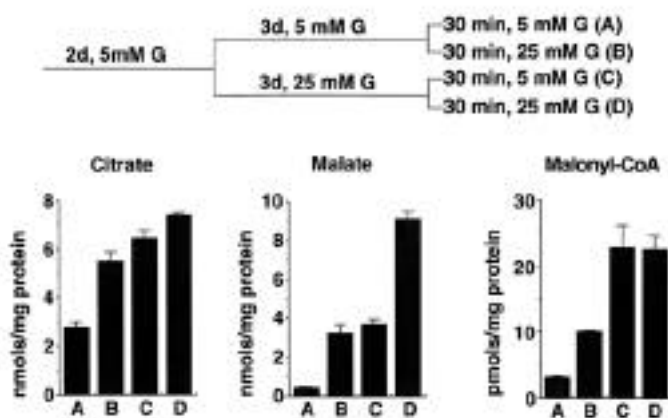


FIG. 5. Long-term exposure of INS-1 cells to elevated glucose results in a sustained augmentation of the intracellular levels of citrate, malate, and malonyl-CoA. Upper panel shows the protocol used in this series of experiments. INS cells were preincubated for 2 days in an RPMI medium at 5 mmol/l glucose (G) and subsequently exposed for 3 days to 5 or 25 mmol/l glucose. Cells were then washed and preincubated for 30 min in a KRBB-HEPES medium containing 0.07% defatted BSA and 5 mmol/l glucose. Afterwards, cells were washed and incubated for 30 min in the presence of 5 and 25 mmol/l glucose. Metabolites were extracted and measured as described in METHODS. Data are means \pm SE of four experiments.

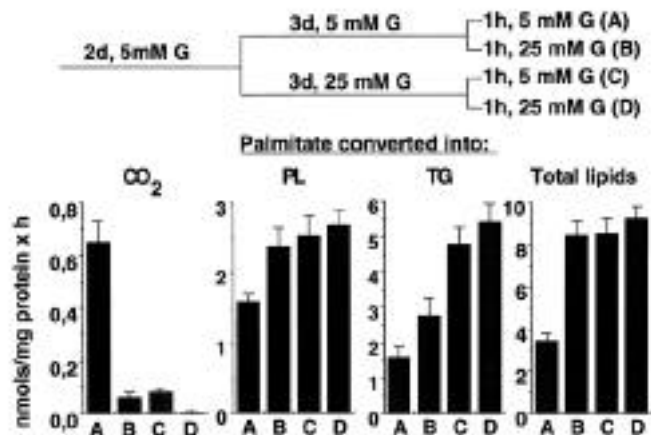


FIG. 6. Long-term exposure of INS-1 cells to elevated glucose causes a sustained alteration in β -cell fatty acid partitioning processes. An experimental protocol similar to that described in Fig. 5 was used except that the final incubation time was 1 h. During this 1-h period, cells were incubated at 5 or 25 mmol/l glucose (G) in the presence of 0.1 mmol/l palmitate, 0.5% defatted BSA, 1 mmol/l L-carnitine, and 0.11 μ Ci of [14 C]palmitate. $^{14}CO_2$ release (left panel) was determined as an index of fatty acid oxidation. The fatty acid esterification processes were determined by measuring the incorporation of [14 C]palmitate in phospholipids (PL), triglycerides (TG), and total lipids. Data are means \pm SE of four experiments.

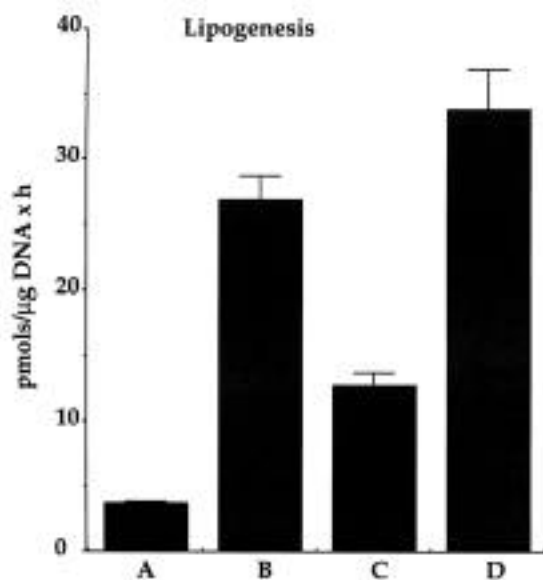


FIG. 7. Effect of short- and long-term exposure of cells to elevated glucose on the de novo synthesis of fatty acids from glucose. An experimental protocol similar to that described in Fig. 5 was used except that the final incubation time was 3 h. Cells were incubated as described in METHODS at 5 (conditions A and C) or 25 mmol/l (conditions B and D) glucose in the presence of [14 C-U]glucose. A and B: 3-day preexposure of cells at 5 mmol/l glucose. C and D: 3-day preexposure of cells at 25 mmol/l glucose. Data are means \pm SE of three experiments.

25 mmol/l glucose was about five times that of cells exposed to 5 mmol/l glucose (352 ± 4 vs. 74 ± 12 pg/ μ g DNA; means \pm SE of three experiments). The results indicate that the glucose carbon is rapidly transformed to malonyl-CoA and fatty acids in β -INS cells, particularly at high glucose, and that increased lipogenesis is associated with triglyceride accumulation. Importantly, the data indicate that malonyl-CoA not only is a regulator of fat oxidation in the β -cell (35), but, chiefly at high glucose, also serves as a substrate for lipogenesis.

With respect to insulin secretion, it should be mentioned that our previous work carried out under identical experimental conditions as the present study indicated that long-term exposure of INS cells to high glucose reproduced the observations made in islet tissue (1,2). It resulted in a sensitization of the β -cell to glucose and a suppression of glucose-induced insulin secretion such that insulin release at 5 mmol/l glucose was maximal and not further increased by higher concentrations of the sugar (11).

DISCUSSION

Important components of the adaptation process of the β -cell to hyperglycemia include massive glycogen deposition (11,21,57), increased glucokinase activity (4), and the induction of several glycolytic genes, in particular those encoding PFK-1 and glyceraldehyde-3-phosphate dehydrogenase (11), L-pyruvate kinase (11,12), and GLUT2 (8). The resulting accelerated glucose utilization (4), oxidation (3), and β -cell redox state (11) likely contribute to the glucose "sensitization" process that is a characteristic feature displayed by the β -cell after its long-term exposure to high glucose (2,58). However, other glucose responsive genes/enzymes should be considered because postglycolytic events, in particular increased mitochondrial oxidative metabolism (59), anaplerosis

(5,19,60), and the malonyl-CoA/CPT-1 fuel cross-talk signaling system (6,14), are all thought to be implicated in nutrient-regulated insulin secretion.

The present study showed that long-term exposure of INS cells to high glucose causes a sustained anaplerosis, as reflected by the chronically elevated levels of citrate and malate remaining in INS cells subsequently exposed to low glucose. Similarly, islets from obese hyperglycemic mice (*ob/ob*) have an elevated citrate content in comparison with islets obtained from lean control animals (61). Because PC protein expression barely changes and the ME content is only slightly increased in cells incubated for 3 days at high glucose, it appears that enhanced glycolytic flux (4) accounts for this phenomenon. What are the possible consequences of increased anaplerotic input into the citric acid cycle in terms of β -cell glucose sensing, insulin secretion, and other late phenotypic actions of glucose (i.e., insulin biosynthesis and β -cell growth)? As far as metabolic coupling factors are concerned, a rise in citrate and malate should favor cytosolic malonyl-CoA (62) and NADPH (19) formation via increased fluxes through the pyruvate/citrate and pyruvate/malate shuttles, respectively (5,19,63). In addition, it can be hypothesized that increased anaplerosis would also favor ATP production and, consequently, a decrease in cytosolic ADP, which may act as a signal on K_{ATP} channels (17). Indeed, studies in the liver and heart indicate that small changes in cycle intermediate concentrations greatly affect the capacity of the Krebs cycle (64). With respect to total protein and insulin biosynthesis as well as cell growth, an increased availability of carbon precursors provided via anaplerosis should favor the biosynthesis of some amino acids, various lipids, and other complex molecules such as porphyrin. Accordingly, ~25% of glucose carbon entering the Krebs cycle via anaplerosis is channeled into protein synthesis in normal purified rat β -cells (22).

Elevated glucose (5–20 mmol/l) caused a twofold rise in PC protein expression in rat islets in a previous study (65), whereas the sugar caused a modest rise in PC mRNA in INS cells that was not accompanied by an induction of the PC protein in the present study. The reason for this discrepancy is not known. Perhaps PC expression is differentially regulated in β -INS cells versus normal rat β -cells. Our recent work indicated that PC is expressed at very high levels and to the same extent in both purified rat β -cells and β -INS cells, whereas the abundance of the enzyme in non- β -cells is about 10% of that present in β -cells (22). Possibly the induction of PC in rat islets occurs only in non- β -cells. Nonetheless, a rise in PC protein per se would not be expected to contribute in a major way to accelerated anaplerosis because the enzyme is expressed at extremely high levels in rat islet (0.4% of total proteins) and is tightly regulated by its allosteric activator acetyl-CoA (35,65,66). The same reasoning applies to ME, whose activity is increased by 30% in INS cells exposed to high glucose.

The results also indicate that long-term exposure of INS cells to elevated glucose causes a coordinated induction of the three strategic enzymes of lipid biosynthesis—FAS, ACC, and ME. The question arises as to how the augmented expression of these proteins relates to the late phenotypic alterations of β -cell function caused by hyperglycemia. With respect to cell signaling, increased expression of the ACC, FAS, and ME genes would favor accelerated production of the candidate metabolic coupling factors malonyl-CoA (55,56,60,67),

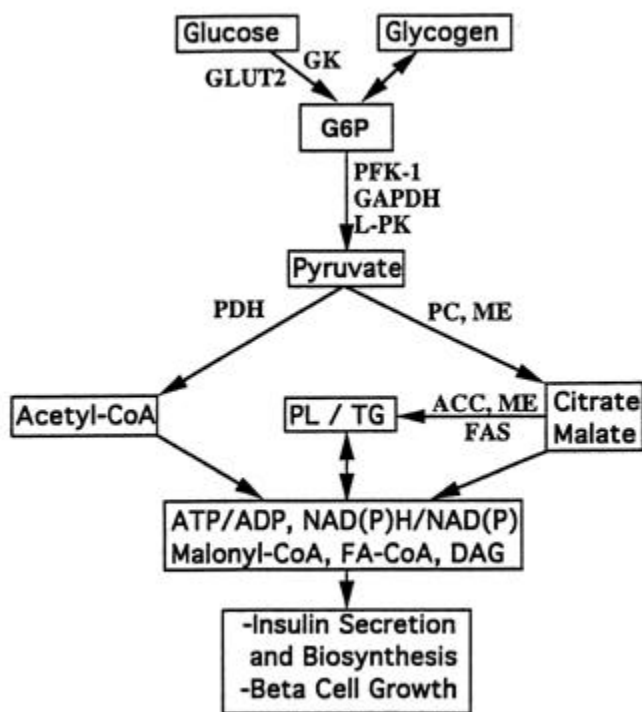


FIG. 8. Biochemical basis of the pancreatic β -cell adaptive processes to chronic hyperglycemia. The model illustrates the metabolic events and gene inductive processes contributing to the late phenotypic changes caused by sustained exposure of the β -cell to high glucose: glucose sensitization, increased insulin biosynthesis, β -cell hypertrophy, and hyperplasia (see text for description). Only the glucose-induced genes coding for glycolytic, anaplerotic, and lipogenic enzymes are shown as well as GLUT2 and glucokinase, whose activity increases by a posttranscriptional mechanism. DAG, diacylglycerol; FA-CoA, long-chain acyl-CoA esters; G6P, glucose-6-phosphate; PL, phospholipids; TG, triglycerides.

fatty acid-CoA (6,14), and NADPH (19), respectively. In addition, the chronic shift from fatty acid oxidation to esterification processes would promote synthesis of other putative coupling factors, such as diacylglycerol and phosphatidic acid (55). This view is entirely consistent with biochemical measurements showing sustained elevation in malonyl-CoA and reduced equivalent production (11) as well as phospholipid synthesis and triglyceride deposition in INS cells exposed to high glucose. Thus the accelerated production of these coupling factors may contribute to the β -cell glucose "sensitization" process caused by hyperglycemia. If extended to the *in vivo* situation, increased fatty acid synthesis and triglyceride deposition might subsequently allow for a high rate of insulin secretion, even during fasting. Thus Stein et al. (29) recently demonstrated the essentiality of fatty acids for glucose-stimulated insulin secretion in the fasted rat. In addition, lipogenesis and triglycerides are elevated in islets from diabetic ZDF rats (68). It is noteworthy that FAS is expressed at low levels in normal rat islets (35) and INS cells cultured at 5 mmol/l glucose (Table 1). It would therefore be of interest to assess whether the FAS gene is induced in rat islets by hyperglycemia as it is in β -INS cells.

The expression level of the PDH gene is not regulated by glucose in β -INS cells within the physiopathological range of concentrations of the sugar (5–25 mmol/l). In cultured rat

islets, a modest (30%) induction by glucose (from 5 to 20 mmol/l) of the PDH-E1 α subunit mRNA has been reported (54). However flux through the enzyme is considerably increased at normal (5 mmol/l) glucose in both INS cells and rat islets previously exposed to elevated glucose, because the oxidation of the sugar is markedly enhanced and insulin secretion is exaggerated (1,11). Glucose acutely activates islet PDH by a mechanism that remains to be defined (69). Thus it is attractive to hypothesize that a β -cell chronically exposed to glucose might display permanent PDH activation due to enhanced glycolytic flux providing a signal to the PDH complex. Hence in hyperglycemia there probably is no need to induce the PDH gene to match the enhanced glycolytic flux because the enzyme is activated by glucose.

Together, the results support the concept that at least six factors contribute to the late phenotypic changes of β -cell functions caused by hyperglycemia. A proposed mechanism for the β -cell "memory" to glucose and adaptation process to hyperglycemia is outlined in Fig. 8; only the glucose-induced genes and glucose-activated enzymes are shown. The six factors are as follows: 1) massive glycogen deposition (11,21) that subsequently allows fuel channeling to the glycolytic pathway when external glucose is low; 2) increased glucokinase activity by a posttranscriptional mechanism (4,11,70); 3) the coordinated induction of genes encoding strategic enzymes in the glycolytic (PFK-1, GAPDH, L-PK, and GLUT2) (7,8,11,12), anaplerotic (PC and ME), and lipogenic (ACC, FAS, and ME) pathways (although PC protein expression is not changed by glucose in β -INS cells, it is listed in the figure because it is increased by the sugar in cultured rat islets [65]); 4) accelerated glycolytic flux (11,70), and consequently enhanced acetyl-CoA production and anaplerosis for energy generation, signal transduction, and biosynthetic purposes (PDH activation by glucose may contribute to this process [69]); 5) ACC activation by glucose (35,45,50) and a sustained elevation of malonyl-CoA to allow for a) full glucose usage due to inhibition of fatty acid oxidation and b) cytosolic FA-CoA accumulation and the shift to esterification processes, which may be linked to coupling factor production (6,14,55); and 6) triglyceride deposition that subsequently, via lipolysis, provides FFA or FA-CoA, which appear to be necessary for efficient glucose-induced insulin secretion (29).

Hence the biochemical basis of the β -cell adaptation processes to hyperglycemia apparently involves, at least in part, the induction and activation of key enzymes of glucose metabolism and the deposition of the two major forms of energy stores: glycogen and triglycerides. The detailed molecular mechanisms that underlie these actions of glucose remain to be defined. The fact that metabolic changes, at least *in vivo*, do not solely account for the long-term action of the sugar is exemplified by the demonstration of an involvement of the autonomic nervous system in the *in vivo* "memory" to glucose in the endocrine pancreas (71).

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