Exhibition of Specific Alterations in Activities and mRNA Levels of Rat Islet Glycolytic and Mitochondrial Enzymes in Three Different In Vitro Model Systems for Attenuated Insulin Release

CARINA SVENSSON, NILS WELSH, STEPHEN A. KRAWETZ, AND MICHAEL WELSH

We studied the possible relationships between the functional status of the β-cell and activities or mRNA contents of enzymes involved in the catabolism of glucose. Three different in vitro models with attenuated insulin response were used: rat islets cultured at a low glucose concentration, rat islets incubated in vitro with streptozocin, and fetal rat islets. The fetal and streptozocin-administered islets were compared with adult islets cultured in RPMI-1640 containing 11 mM glucose, and the effects of the in vitro glucose concentrations (3.3, 11, and 28 mM) were assessed on adult islets only. Cellular mRNA levels for the mitochondrial DNA-encoded cytochrome b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by Northern-blot analysis. Enzymatic activities of high-K_m (glucokinase) and low-K_m (hexokinase) glucose-phosphorylating enzymes and succinate–cytochrome c reductase were also determined. Islets cultured at 3.3 mM glucose displayed a decreased activity of glucokinase compared with islets cultured at 28 mM glucose (23.3 ± 12%), whereas there was no difference in hexokinase activity or the level of GAPDH mRNA. The activity of succinate–cytochrome c reductase was similar in islets cultured at the different glucose concentrations. The level of cytochrome b mRNA increased at 28 mM glucose compared with islets cultured at 11 mM glucose (140 ± 14%). Islets incubated with streptozocin and subsequently cultured for 7 days at 11 mM glucose exhibited a decreased level of cytochrome b mRNA (65 ± 5%) and no differences in the activities of glucokinase, hexokinase, succinate–cytochrome c reductase, or the level of GAPDH mRNA. Fetal islets displayed increased activity of hexokinase (570 ± 130%) and succinate–cytochrome c reductase (185 ± 42%) and a decreased level of cytochrome b mRNA (62 ± 11%) in comparison with adult islets cultured at similar conditions. No differences of glucokinase activity or the level of GAPDH mRNA were detected in these islets. The results suggest that the degree of expression of certain genes coding for enzymes in the oxidative metabolism of glucose may influence the ability of the β-cell to synthesize and secrete insulin.
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The link between glucose metabolism and the cellular events involved in the exocytosis of the insulin secretory granule has not been identified. It has been emphasized that certain steps in both the glycolytic pathway (1,2) and mitochondrial processes (3–8) play an important role in generating a signal that controls insulin secretion. To define possible mechanisms involved in creating a defective secretory response to glucose in diabetes, certain in vitro models can be utilized. Impaired glucose-sensitive insulin release has been demonstrated in islets cultured at a low glucose concentration (9,10). Numerous studies have previously shown that these low-glucose–cultured islets also display decreased rates of glucose oxidation, oxygen consumption, insulin and total protein biosynthesis, and decreased contents of insulin mRNA and total RNA compared with islets cultured at a high glucose concentration (9–12). This suppression of islet activity resembles in vivo fasting of rats (13–16), in which depression of key glycolytic enzymes (glucokinase, hexokinase, and phosphofructokinase) has also been shown (17). Similarly, islets incubated in vitro with streptozocin (STZ) and then cultured for 6 days show an impaired insulin response to glucose (18). Analysis of such islets reveals decreased rates of glucose oxidation and oxygen consumption, decreased levels of ATP, and decreased activities of certain mitochondrial enzymes and the mRNA contents of insulin and adenine nucleotide translocator compared with control islets without changes in the activity of glucokinase or the rate of glucose

From the Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden; and the Department of Molecular Biology and Genetics, Wayne State University, Detroit, Michigan.

Address correspondence and reprint requests to Dr. Carina Svensson, Department of Medical Cell Biology, Biomedicum, PO Box 571, S-751 23 Uppsala, Sweden.

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utilization (8,18–20). The combined findings lead to the suggestion that these islets display a perturbation of glucose metabolism apparently at the mitochondrial level (8). Compared with adult islets (21), fetal islets also exhibit an attenuated insulin response to glucose, presumably because of an immature glucose metabolism judged from measurements of both glucose oxidation and glucose utilization (22). Curiously, fetal islets display elevated rates of oxygen consumption without glucose stimulation (23). Therefore, in this study, we compared the metabolic activity of islets from these three in vitro models of attenuated insulin release with corresponding controls. The models were evaluated with respect to enzymatic activities or mRNA levels of the glycolytic enzymes glucokinase, hexokinase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The gene expression of the GAPDH gene was studied rather than the activity of the enzyme, because enzyme activity determinations were used in previous studies dealing with similar models (8,24). Furthermore, the enzymatic activity of the mitochondrial succinate–cytochrome *c* reductase and the mRNA levels of mitochondrial encoded cytochrome b were examined.

RESEARCH DESIGN AND METHODS

Collagenase (E.C. 3.4.24.3) was obtained from Boehringer Mannheim (Mannheim, Germany), and Hanks' solution and calf serum were purchased from Statens Bakteriologiska (Stockholm, Sweden). Benzylpenicillin was from Astra Läkemedel (Södertälje, Sweden), and streptomycin was obtained from Glaxo (Greenford, UK). RPMI-1640 and fetal bovine serum were from Flow (Irvine, CA). An oligonucleotide labeling kit and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham (Aylesburg, UK). Ficoll 400 was from Pharmacia (Uppsala, Sweden). Glyoxal, yeast tRNA, poly(A), polyvinylpyrrolidone, salmon sperm DNA, HEPES, and formamide were from Sigma (St. Louis, MO). ECONOFLUOR and GeneScreen hybridization transfer membrane were obtained from Du Pont-NEN (Boston, MA). Quickscint 1 was purchased from Zinsser (Maidenhead, UK). All other analytical grade chemicals were from Merck (Darmstadt, Germany).

Islets of adult rats were partially purified on Ficoll gradients (25) from collagenase-digested pancreases (26) of male Sprague-Dawley rats weighing ~300 g. The islets were then picked manually and maintained free-floating in tissue culture at 37°C in air/5% CO_2. The culture medium was RPMI-1640 (11 mM glucose) containing 10% calf serum, 100 U/ml benzylpenicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine (27). The islets were cultured for 7 days, and the culture media were changed every 2nd day. For experiments with different glucose concentrations, groups of islets were cultured in the presence of 3.3 or 28 mM glucose for 7 days. Fetal rat islets at day 21 were prepared from mothers killed by cervical dislocation. Pancreases were excised from decapitated fetuses, minced, and then partially digested with collagenase as described previously (21). The digests were then seeded in attachment dishes and cultured in RPMI-1640 (11 mM glucose) supplemented with 10% fetal calf serum for 3–7 days. After this culture period, the fetal islets were detached from the bottom of the dishes and treated as follows.

The islets were incubated with STZ essentially as described by Sandler et al. (28). Groups of 200 adult rat islets were preincubated in RPMI-1640 containing 5.6 mM glucose for 30 min at 37°C. STZ was dissolved in cold citrate buffer (10 mM, pH 4.5) immediately before use, and this solution was added to the culture medium to a final concentration of 0.55 mM. Islets were then incubated with STZ for 30 min at 37°C in air and CO_2. The incubation was terminated by the removal of the STZ-containing medium and subsequent addition of culture medium (RPMI-1640 supplemented with 10% bovine serum and 11 mM glucose). Control islets (treated with citrate buffer only) and STZ-administered islets were maintained in culture for an additional 6 days.

Total RNA was prepared by the guanidinium method essentially as described by Maniatis et al. (29). Groups of 1000–2000 adult or fetal rat islets were briefly washed in cold phosphate-buffered saline (PBS; 154 mM Na^+, 4 mM K^+, 140 mM Cl^-, 10 mM HPO_4^{2-}, pH 7.4) and subsequently transferred to 3 ml of 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7), and 0.1 M 2-mercaptoethanol. The guanidinium solution was placed on a 1.5-ml cushion of 5.7 M CsCl and was centrifuged at 40,000 rpm for 16–20 h in an SW-50 rotor. After removal of the supernatant, the RNA pellet was suspended in 1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 10 mM Tris (pH 7.5), and extracted with phenol/chloroform/isopentyl alcohol (25:24:1 vol/vol). After extraction, the RNA was precipitated overnight at −20°C in 70 mM potassium acetate and 70% ethanol. The remaining precipitates were washed with 70% ethanol.

For Northern-blot analysis, the RNA was treated with 1 M glyoxal for 1 h at 50°C. Samples containing 10–30 μg of total RNA were applied to 1% agarose gels and electrophoresed as described by Thomas (30). The RNA was transferred to GeneScreen membranes and attached by means of a 2-h incubation at 80°C. After prehybridization for 6 h at 42°C in 50% (vol/vol) deionized formamide, 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate); 50 mM sodium phosphate (pH 6.5); sonicated salmon sperm DNA at 25 μg/ml; 100 μg/ml of poly(A); and 0.02% each of bovine serum albumin, Ficoll, polyvinylpyrrolidone (30), and 1% SDS, the samples were hybridized at 42°C in the same solution with 10^6 counts per min/ml of either the GAPDH or the cytochrome b probe (31,32) labeled with an oligonucleotide-labeling kit. After hybridization, the filters were washed for 30 min at 50°C three times in 0.1 × SSC + 0.1% SDS and then exposed at −70°C to Hyperfilm-MP (Amersham) with an intensifying screen. The optical densities of the bands thus obtained were determined by densitometry and normalized to the total amount of RNA applied. All values are given as a percentage of the density obtained with mRNA isolated from islets cultured at 11 mM glucose.

In our study of enzyme activities, glucose phosphorylation was measured according to Giroix et al. (33) but modified as follows. Approximately 200–300 islets were homogenized in 300 μl of 50 mM HEPES buffer (pH 7.4), 4 mM $MgCl_2$, 100 mM KCl, 10 mM NaF, 2 mM cysteine, 1 mM EDTA, 6.7 mM benzamidine, and 100 KIU/ml aprotinin (Trasylol). Afterward, 30 μl of this homogenate was mixed with 40 μl of a reaction mixture consisting of 50 mM HEPES, D-[U-^{14}C]glucose (sp act 270 mCi/mmol), 2 mM ATP, 0.5 or 10 mM glucose, and 10 μl of 12 mM glucose-6-phosphate

(G6P) or 10 μl of the homogenization buffer was added. After a 30-min incubation, the reaction was halted with the addition of 300 μl iced ethanol, whereafter 500 μl ice-cold H_2O was added. The diluted reaction medium was then passed through a 0.5-ml Dowex 1-X8 anion-exchange column to separate the hexose phosphates. The column was rinsed three times with 2 ml of water, then the hexose phosphates were eluted with 2.3 ml of 1.5 M ammonium formate–0.1 M formic acid. The eluate was mixed with 20 ml of scintillation fluid (Quickszint 1) and the radioactivity determined by liquid-scintillation spectrometry.

Succinate–cytochrome *c* reductase activity was measured in islets (150–250) homogenized in 250 μl citrate buffer (50 mM citrate, 250 mM sucrose, 10 mM benzamide, 1 mM EDTA). The homogenate was centrifuged for 10 s at 11,000 × *g*. The nuclei were rehomogenized in another 250 μl of the buffer and recentrifuged. A 50-μl aliquot of the pooled supernatants was analyzed for protein content (34), and the remaining aliquot was centrifuged for 45 min at 11,000 × *g* at 4°C. The pellet was resuspended in 20 μl of 100 mM KH_2PO_4 and the enzyme activity measured according to Fleischer and Fleischer (35).

Values are means ± SE. The data were analyzed by analysis of variance followed by multiple comparisons with the Student's paired *t* test. The *P* values were corrected for multiple comparisons with the Bonferroni method (36).

RESULTS

When the glucose-phosphorylating reactions were carried out at 0.5 mM glucose, where hexokinase activity predominates, the reaction velocity was identical in 3.3 and 28 mM glucose–cultured islets and in the control and STZ-administered islets (Table 1). The activity in fetal rat islets was elevated compared with that of adult control islets. In all cases, the phosphorylating activity was suppressed by 1.5 mM G6P ($P < 0.05$). Islets cultured at 3.3 mM glucose exhibited significantly lower glucose phosphorylation under these conditions than the islets cultured at 28 mM glucose. When the phosphorylating reactions were carried out at a glucose concentration of 10 mM and the hexokinase and glucokinase activity was measured, control islets, STZ-administered islets, and islets cultured at 28 mM glucose showed increased rates of glucose phosphorylation compared with the values for the 0.5-mM reactions ($P < 0.05$). This reaction velocity increased significantly when the reactions were carried out in the presence of G6P, indicating glucokinase activity ($P < 0.05$). When the glucose concentration during the reaction was increased from 0.5 to 10 mM, islets cultured at 3.3 mM glucose did not show increased glucose phosphorylation but a decreased activity compared with the activity of 28 mM glucose–cultured islets in the presence of G6P. Fetal islets did not increase their already high phosphorylation rate at 10 mM glucose. Calculating the activity of glucokinase by subtracting the activity at 0.5 mM glucose + 1.5 mM G6P from the activity at 10 mM glucose + 1.5 mM G6P gave no difference between control, STZ-administered, or fetal islets (22.1 ± 5.81 vs. 22.9 ± 8.73 and 33.1 ± 16.23 pmol · min^{-1} · μg^{-1} DNA, respectively, $P > 0.05$; Table 1). This is in the same range as in previously published data (8). However, there was a significant decrease in islets cultured at low glucose concentrations compared with islets cultured at high glucose (5.83 ± 7.75 vs. 42.44 ± 15.07 pmol · min^{-1} · μg^{-1} DNA, respectively, $P < 0.05$). Note that, to observe maximal activity of glucokinase, a higher concentration of glucose in the reaction is needed (1).

Northern-blot analysis revealed no difference in the level of GAPDH mRNA of islets cultured at either 3.3 or 28 mM glucose when normalized for the amount of total RNA applied (Table 2). Similarly, there was no difference between STZ-administered, control, or fetal islets in the amount of GAPDH mRNA.

On one hand, the level of cytochrome b mRNA decreased after incubation of islets with STZ compared with control islets (Table 2; Fig 1). On the other hand, the 28 mM glucose–cultured islets possessed a larger quantity of cytochrome b mRNA than the 11 mM glucose–cultured islets in parallel experiments (Table 2). The quantity of islet mRNA from 3.3 mM glucose–cultured islets was not significantly different from that of islets at 11 mM glucose when expressed per total RNA applied (Table 2). The level of fetal islet cytochrome b mRNA was less than that of adult islets (Table 2).

Islets cultured at 3.3 mM glucose showed the same activity of succinate–cytochrome *c* reductase compared with islets cultured at 28 mM glucose (0.52 ± 0.05 vs. 0.37 ± 0.08 nmol · μg^{-1} protein · 30 min^{-1}, respectively; $P > 0.05$). Moreover, there was no difference between STZ-incubated islets and control islets (0.46 ± 0.1 vs. 0.45 ± 0.1 nmol · μg^{-1}

TABLE 1
Glucose phosphorylation (pmol · min^{-1} · μg^{-1} DNA) in rat pancreatic islet homogenates after culture at different glucose concentrations and streptozocin (STZ) administration

		Adult				
Glucose (mM)	Glucose-6-phosphate (mM)	At 11 mM glucose	At 3.3 mM glucose	At 28 mM glucose	STZ at 11 mM glucose	Fetal at 11 mM glucose
0.5		71.2 ± 12.9	91.8 ± 18.7	110 ± 30	96.8 ± 13.7	336 ± 42*
0.5	1.5	8.8 ± 2.5	4.6 ± 1.8	13.7 ± 3.5†	7.3 ± 3.8	16.5 ± 3.1
10		83.3 ± 22.1	71.2 ± 20.4	158 ± 43	123 ± 23	440 ± 66*
10	1.5	31.6 ± 5.6	5.1 ± 7.8	56.4 ± 16.8†	30.2 ± 8.9	41.4 ± 14.2

Glucose phosphorylation values are means ± SE of 6–7 experiments. The phosphorylation of D-[U-^{14}C]glucose at 0.5 and 10 mM glucose and in the presence or absence of glucose-6-phosphate was measured in islet homogenates.
*$P < 0.05$ vs. adult islets cultured at 11 mM glucose.
†$P < 0.05$ vs. islets cultured at 3.3 mM glucose.

TABLE 2
Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cytochrome b mRNA in islets at different glucose concentrations and after streptozocin (STZ) administration

mRNA	Adult				Fetal at 11 mM glucose
	At 11 mM glucose	At 3 mM glucose	At 28 mM glucose	STZ at 11 mM glucose	
GAPDH	100%	100 ± 6% (2)	81 ± 6% (2)	113 ± 25% (2)	94% (1)
Cytochrome b	100%	112 ± 14% (5)	140 ± 14%* (5)	65 ± 5%* (3)	62 ± 11%* (4)

Values are means ± SE for (*n*) experiments. The level of GAPDH and cytochrome b mRNA in islets was determined by densitometric scanning of Northern blots after hybridization to the probe. After normalization for total RNA applied, the values were expressed in percentage of adult islets cultured at 11 mM glucose.
*$P < 0.05$ when probability of chance differences between experimental control groups were calculated in a paired Student's *t* test.

protein · 30 min^{-1}, respectively; $P > 0.05$). However, fetal islets displayed significantly higher activity of succinate–cytochrome *c* reductase compared with adult islets (1.28 ± 0.17 vs. 0.45 ± 0.1 nmol · μg^{-1} protein · 30 min^{-1}, respectively; $P < 0.05$).

DISCUSSION

Previous studies showed that islets exposed to STZ in tissue culture and then cultured for another 6 days had an impaired glucose-sensitive insulin release (18). In our studies with this model, we observed that such islets exhibit no apparent changes in the activities of the glycolytic enzymes hexokinase and glucokinase or in the level of GAPDH mRNA. These observations are in accord with previous findings by Eizirik et al. (8) suggesting unaltered glycolysis in these islets as assessed by glucose utilization and glucokinase measurements. Contrary to this, STZ incubation decreased the level of cytochrome b mRNA compared with controls. This does not reflect a generalized mitochondrial impairment, because the enzymatic activity of succinate–cytochrome *c* reductase was unchanged after STZ-incubation. Together with previous observations of decreased rates of glucose oxidation and oxygen consumption, contents of ATP, and adenine nucleotide translocator mRNA and enzymatic activities of certain mitochondrial enzymes, it suggests localized mitochondrial damage by STZ exposure (8,18–20). Also, in islets isolated from rats injected neonatally with STZ, glycolysis was not altered, yet mitochondrial activity was impaired (37). The pattern of localized mitochondrial damage may be a direct and nonreversible consequence of STZ treatment, because STZ is known to alkylate macromolecules such as DNA (38). Furthermore, the DNA of the mitochondria is considered more vulnerable to damage than nuclear DNA (39,40), which could explain the specificity of the STZ action. Alternatively, it may be secondary to other events of the cell, including disturbances in the regulation of the expression of the nuclear-encoded mitochondrial enzymes of the oxidative metabolism.

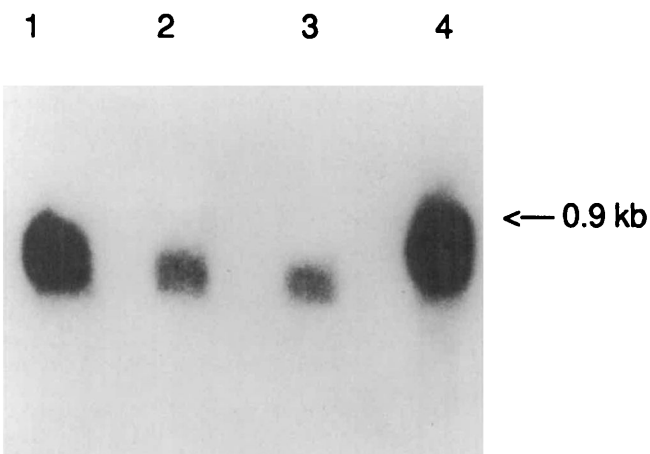

FIG. 1. Northern-blot analysis of RNA isolated from adult islets cultured at 28 mM glucose (*lane 1*), and at 3.3 mM glucose (*lane 2*), streptozocin-exposed islets cultured at 11 mM glucose (*lane 3*), and islets cultured at 11 mM glucose (*lane 4*). Total cellular RNA was isolated and treated with formaldehyde before electrophoresis on 1% agarose gel. Note that amount of RNA applied in 11-mM group was twice that of others. RNA was blotted and hybridized to pMTal probe (32). Migration of 0.9-kilobase (kb) molecular-weight standard is indicated.

Islets cultured at 3.3 mM glucose responded poorly to glucose with insulin release compared with islets cultured at 11 or 28 mM glucose (9–11). This is reminiscent of the impairment in glucose-stimulated insulin release observed after starvation and has been related to in vivo inhibition of glucose metabolism and in the activities of glycolytic enzymes (13–17). We observed a lower glucokinase activity after culture at 3.3 mM glucose, whereas the level of GAPDH mRNA was unaltered. It is unknown whether the observed decreased glucokinase activity was paralleled by diminished expression of the glucokinase gene. However, it has been shown that the glucokinase mRNA is expressed at a constant level in islets during the fasting-refeeding cycle of the rat (41). However, this discrepancy can be explained by the much smaller differences in glucose concentrations in vivo during such a fasting-refeeding cycle. Contrasting the above, the level of cytochrome b mRNA increased at concentrations of glucose >11 mM. The specific effect at 28 mM glucose may be explained by the fact that cytochrome b is a product of a gene transcribed from the mitochondrial DNA (32) and therefore may exhibit different glucose responsiveness than nuclear genes in the β-cell (20,42). Interestingly, it has been shown that the mitochondrial genome of other cells can be specifically regulated by different physiological modulators (43–45). The succinate–cytochrome *c* reductase activity was not modified by glucose in culture, suggesting that this enzymatic complex, like GAPDH and hexokinase, serves a constitutive function in the adult islet cell.

The maturation of fetal islets during tissue culture enhances the insulin secretory ability in response to glucose. This is paralleled by a large increase in mitochondrial glu-

cose oxidation without a significant alteration in the rates of glycolysis (6). These findings correlate with the decrease in the level of cytochrome b mRNA that we observed in fetal islets and the previous observation of decreased levels of fetal mitochondrial adenine nucleotide translocator mRNA (20). In addition, these data are also congruent with decreased enzyme activities of alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase observed in fetal rat islets (46). However, our finding that the activity of succinate–cytochrome c reductase was markedly increased contradicts the notion that, in general, mitochondrial function is lower under these conditions. Note the finding of elevated oxygen consumption in fetal islets, which did not respond to stimulation by glucose (23). In fact, the elevated levels of succinate–cytochrome c reductase and hexokinase in fetal islets raise the possibility that increased levels of certain enzymes may be associated with the state of differentiation and functional activity characteristic of the fetal β-cell just as well as low levels of other enzymes. The rapidly replicating β-cell requires high levels of cytosolic NADPH for the synthesis of nucleic acids. This demand is satisfied by constitutively enhanced glucose phosphorylation rates (hexokinase) and substitution of NADH with $FADH_2$ in the mitochondria (succinate dehydrogenase). This notion is supported by the findings that, in general, embryonic tissue shows high levels of succinate reductase activity (47).

In summary, different in vitro models exhibiting a diminished capacity to secrete insulin show a complex altered pattern of enzyme activities. In low-glucose–cultured islets, decreased glucokinase activity, in essence, may explain the low rates of glucose metabolism in these cells. A suppressed rate of glycolysis may be paralleled with a decrease in certain mitochondrial functions, such as the expression of the adenine nucleotide translocator gene (20). In STZ-incubated islets, no evidence for glycolytic impairment has been observed, whereas the activities of several mitochondrial enzymes are decreased (8), suggesting that this may be the most common and sensitive discriminator for impaired β-cell function. Finally, in fetal islets, findings similar to those of STZ-exposed islets were observed with respect to certain key mitochondrial enzymes. However, the enzymatic activities of certain other enzymes were greatly elevated, raising the possibility that elevation of certain key enzymes may also characterize the immature insulin response to glucose of the fetal β-cell.

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