Role of NADH Shuttles in Glucose-Induced Insulin Secretion From Fetal β-Cells

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The NADH shuttle system, which transports the substrate for oxidative metabolism directly from the cytosol to the mitochondrial electron transport chain, has been shown to be essential for glucose-induced activation of mitochondrial metabolism and insulin secretion in adult β-cells. We examined the role of these shuttles in the fetal β-cell, which is immature in being unable to secrete insulin in response to glucose. The activity and concentration of the two key enzymes of the NADH shuttles, mitochondrial glycerol phosphate dehydrogenase (mGPDH) and mitochondrial malate dehydrogenase (mMDH), were eight- and threefold lower, respectively, in fetal compared with adult rat islets. Likewise, mGPDH and mMDH activity was fivefold lower in islet-like cell clusters (ICCs) and sevenfold lower in purified β-cells compared with adult islets in the pig. The low level of enzyme activity was a result of low gene expression of the mitochondrial enzymes in the fetal β-cells. Increasing NADH shuttle activity by transduction of fetal rat islets with mGPDH cDNA enabled the fetal islets to secrete insulin when stimulated with glucose. We concluded that the immaturity of the NADH shuttles contributes to the inability of fetal β-cells to secrete insulin in response to glucose. *Diabetes* 51:2989–2996, 2002

Glucose is the major physiological stimulus for insulin secretion in adult β-cells (1–3) but is unable to cause a similar response in fetal β-cells (4,5). The secretion of insulin from an adult β-cell involves the transport of glucose into the β-cell via the GLUT2 transporter, followed by the production of ATP, as a result of glucose metabolism in the glycolytic pathway, the tricarboxylic acid (TCA) cycle, and the mitochondrial electron transport chain. The resulting increase in ATP-to-ADP ratio leads to the closure of the ATP-dependent K^+ channels on the cell surface, which results in the depolarization of the cell membrane and the opening of the voltage-activated Ca^{2+} channels on the cell surface to allow the influx of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} triggers insulin secretion. It is hypothesized that the failure of fetal β-cells to respond to glucose is due to an immaturity in one or more of the above steps (4).

Glucose transport into the fetal β-cell does not appear to be as defective as GLUT2 and has been identified in human fetal β-cells at 13 weeks gestation (6) and in fetal rat by 19 days gestation (7). The glycolytic pathway in the fetal β-cell appears to be intact (5). This is shown by normal glucose utilization and normal activity of the rate-limiting enzyme, glucokinase (5). A reduction of glucose oxidation has been observed more distally (8). Several factors may be responsible for this reduction: *1*) immaturity in the TCA cycle; *2*) the lack of substrate for the TCA cycle, e.g., the accumulation of lactate due to high lactate dehydrogenase activity, which results in the unavailability of pyruvate for glucose oxidation; or *3*) reduced NADH shuttle activity. Immaturity of the TCA cycle in fetal β-cells is suggested because this is the pathway in which most ATP required for glucose-stimulated insulin secretion is produced. Against this hypothesis is the fact that leucine is able to cause insulin secretion from fetal rat β-cells (9). Leucine acts via two different intramitochodrial pathways, one involving the degradation of ketoisocaproic acid to acetyl Co-A, which enter the TCA cycle and the other by acting as an allosteric activator of glutamate dehydrogenase, resulting in the formation of 2-ketoglutarate from endogenous glutamate, which is further oxidized in the TCA cycle (10). It is unlikely that lack of substrate for the TCA cycle occurs in fetal β-cells, as lactate dehydrogenase is low in adult β-cells (11). Furthermore, the amount of pyruvate entering the TCA cycle appears to not be pivotal to glucose-stimulated insulin secretion, since pyruvate alone is unable to stimulate insulin secretion, even though it is readily metabolized by β-cells (2,12). The final steps required for insulin secretion, i.e., the closure of the ATP-dependent K^+ channels and the opening of the Ca^{2+} channels have been shown to be normal in fetal β-cells (9).

The NADH shuttles, which transport NADH, the substrate for oxidative metabolism and ATP production, from the cytosol to the mitochondria are crucial to glucose-induced activation of mitochondrial metabolism and insulin secretion in the adult β-cell (13). Two NADH shuttles have been identified: the glycerol phosphate shuttle and the malate-aspartate shuttle. The glycerol phosphate shuttle enables the transfer of electrons from NADH generated from glycolysis to coenzyme Q (complex II) in the electron transport chain. The malate-aspartate shuttle results in the regeneration of NADH utilized in the mitochondria and its subsequent transfer to complex I in the electron

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cGPDH, cytosolic glycerol phosphate dehydrogenase; cMDH, cytosolic malate dehydrogenase; EH, HEPES-buffered Earle's media; ICC, islet-like cell cluster; mGPDH, mitochondrial glycerol phosphate dehydrogenase; mMDH, mitochondrial malate dehydrogenase; MOI, multiplicity of infection; RIA, radioimmunoassay; TCA, tricarboxylic acid.

transport chain, contributing to the production of ATP in the mitochondria (14,15).

The activity of mitochondrial glycerol phosphate dehydrogenase (mGPDH), the key enzyme of the glycerol-phosphate shuttle is 40- to 70-fold higher in islets relative to other tissues, indicating that mitochondrial oxidation of metabolized glucose is favored in β-cells (1). A reduction in the activity of this enzyme theoretically should result in diminished ATP production in the mitochondria and hence a reduction in glucose-induced insulin secretion. Indeed this is true for both animals models (16,17) and some humans with type 2 diabetes (18). In contrast, insulin secretion is normal in transgenic mice with no functional mGPDH (13). When the second NADH shuttle, the malate aspartate shuttle, was inhibited in these transgenic mice, insulin secretion in response to glucose was adversely affected (13). Such studies show the importance of the NADH shuttles in glucose-stimulated insulin secretion.

The current study was undertaken to investigate the role of the NADH shuttles, in particular the role of the mitochondrial enzymes that are rate limiting, in glucose-stimulated insulin secretion from fetal β-cells.

RESEARCH DESIGN AND METHODS

Unless indicated otherwise, all reagents were analytical grade and were purchased from Sigma Chemical (St Louis, MO).

Islets, islet-like cell clusters, and β-cell preparation

Rat islets. Fetal rat pancreata were obtained from Wistar pregnant rats at gestational age 20–21 days (crown-rump length 45 ± 1 mm). Approval to conduct the experiments was obtained from the Animal Care and Ethics Committee of the University of New South Wales, Australia. Pancreata from within a litter were pooled, and islets were isolated by digestion with Collagenase A (Roche Diagnostics, Mannheim, Germany) as described previously (19,20). Adult rat islets were obtained from 8- to 10-week-old male Wistar rats by Collagenase P (Roche Diagnostics) digestion of the pancreas (5). The isolated islets (both fetal and adult) were cultured for 24 h before any enzyme, protein, or mRNA measurements were carried out.

Pigs islets and islet-like cell clusters. Large White Landrace fetal pigs were obtained from Bunge Meat (Corowa, New South Wales, Australia) at 70–100 days gestation, and islet-like cell clusters (ICCs) were isolated based on the method described previously (21,22). Fetal pig pancreata were diced into 1-mm^3 explants and digested for 20–30 min in Collagenase P, washed, and cultured for 48–72 h in RPMI/10% FCS. During that time, the ICCs rounded up and were then used in the experiments described below. Islets from Pure Landrace adult pigs were kindly provided by Dr. Bernhard Hering and his team (Minneapolis, MN) (23). The adult pig islets were cultured for 24 h in RPMI/10% pig serum after isolation before use in experiments, which were conducted in Minneapolis (by C.T.).

After the 24- or 72-h culture after isolation, some islets were cultured for a further 24 h in different glucose concentrations (2–20 mmol/l glucose) to study the effects of glucose on the activity, concentration, and gene expression of the different enzymes.

Fetal pig β-cells. Fetal pig β-cells were included in this study because the percentage of β-cells in fetal pig ICCs is significantly lower than that in adult pig islets (24). The method for the separation of fetal pig β-cells is an adaptation of a technique for isolating β-cells from adult rat islets by one of the authors (J.T.) (25). Large white Landrace fetal pigs were obtained from Bunge Meat (Corowa, New South Wales, Australia) at 70–100 days of gestation, and the pancreata were removed, minced finely, and digested in 0.8 mg/ml Collagenase A (20 ml/pancreas) for 15 min at 37°C in a shaking water bath. The digest was then washed and filtered through a 500-μm nylon mesh. The cell clusters obtained after the collagenase digestion were then washed in calcium-free Hank's Balanced Salt Solution followed by HEPES-buffered Earle's media (EH), supplemented with 1 mmol/l EGTA. The cells clusters were then resuspended in Earle's Media/1 mmol/l EGTA and dissociated by gentle repeated pipeting of the cell clusters. When ~80% of the cell clusters had dissociated to single cells, an isotonic Percoll solution (Pharmacia, Uppsala, Sweden) with a density of 1.04 g/ml was layered underneath the cell suspension in order to remove cellular debris by centrifugation at 1,500 rpm for 6 min. The pelleted cells consisted of ~80% β-cells and were used for measurement of enzyme activity.

Enzyme activity. Islets were homogenized in 0.23 mol/l mannitol, 0.07 mol/l sucrose, 20 mmol/l HEPES (pH 7.5), and mGPDH enzyme activity in islet homogenate measured by reduction of 2-*p*-iodo-3-*p*-nitro-5-phenyltetrezolium as described by Gardner (26). Cytosolic glycerol phosphate dehydrogenase (cGPDH) activity was measured by the reduction of NADH in the presence of the enzyme substrate dihydroxyacetone phosphate (DHAP) (27). Mitochondrial malate dehydrogenase (mMDH) was measured by the oxidation of NADH in the mitochondria in the presence of the enzyme substrate malate, and cytosolic malate dehydrogenase (cMDH) activity was measured by the reduction of NADH in the presence of enzyme substrate oxaloacetate (28).

Western analysis. The islet homogenate was electrophoresed on a 10% SDS-polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. The signal for mGPDH and mMDH were detected with an enhanced chemiluminescence system (Amersham). The anti-mGPDH (rat) antibody was raised in rabbit and was a gift from Dr. M.J. MacDonald (Madison, WI), while the mMDH antibody (pig) was raised in rabbit by the Institute of Medical & Veterinary Science, Adelaide, Australia, to pig mMDH. The density of the Western blots was quantified using the Biorad Gel Documentation System and Quantity One software.

RT-PCR. Total RNA was extracted from the islets using Trizol reagent from Gibco Invitrogen (Carlsbad, CA). The RNA (1 μg) was reverse-transcribed with a reverse transcription kit from Life Technologies and PCR was amplified following the reverse transcription reaction. Four pairs of primers were designed based on the published sequence of rat mGPDH and mMDH. 5′ agaagctcagctcatgactc 3′ and 5′ agaggactgaaaacagccct 3′ were used for amplification of mGPDH gene transcripts, and 5′ agctcgagtcaatgtgcctgtcatt 3′ and 5′ tattcggtacctgtctcaccgtct 3′ for mMDH. Actin or glyceraldehyde-3-phosphate dehdydrogenase (GAPDH) was used as the internal control.

NAD(P)H measurement. Single adult or fetal rat islets were placed under an inverted microscope (Nikon, ×40 UV objective) and constantly perfused with Krebs-Ringer buffer at 37°C, bubbled with 95% O_2 and 5% CO_2 containing 2, 5, 11, or 20 mmol/l glucose. NAD(P)H auto fluorescence was measured at 360 nm (excitation) and 470 nm (emission) (Xenon light source) using a photomultiplier system.

Adenovirus-mediated gene transduction. The recombinant adenoviruses, Adex1CAlacZ and Adex1CAmGPDH (a gift from Prof. Yoshitomo Oka) (29), which encode *E. coli* lacZ and murine mGPDH, respectively, were incubated with fetal rat islets for 1 h at 37°C. The islets were then washed to remove the viral particles and cultured overnight (between 16 and 24 h) to allow for expression of the above genes. The adenoviruses were added at a multiplicity of infection (MOI) of 1,000 plaque-forming units per cell. LacZ expression was observed to be between 50 and 60% at this MOI. An increase of MOI beyond 1,000 did not increase transduction efficiency.

Insulin secretion and content. Following overnight culture after adenovirus transduction, groups of 100 fetal islets were washed twice in EH containing 2 mmol/l glucose and 0.5% BSA. The islets were resuspended in 200 μl EH media/0.5% BSA containing 2.0 mmol/l glucose (basal) or 20 mmol/l glucose or 20 mmol/l KCl and incubated at 37°C for 1 h. The media was then removed and insulin content measured by radioimmunoassay (RIA) with a rat insulin standard (Novo Lab, Bagsvaerd, Denmark). To determine the insulin content of islets, they were incubated in acid-ethanol at 4°C for at least 16 h to extract the insulin in the cells and the insulin content in the supernatant measured by RIA.

Statistical analysis. Statistical analysis was carried out using Number Cruncher Statistical System (NCSS) statistical software (Version 5.01). ANOVA (Scheffe's test) was used to analyze the data for significance.

RESULTS

Enzyme activity. Activity of both mitochondrial enzymes involved in the NADH shuttles was lower in fetal than adult rat islets. The magnitude of this difference was eightfold for GPDH and threefold for MDH (Table 1). A similar pattern was observed in pigs with the magnitude of the difference between fetal and adult being fivefold for both adult GPDH and MDH (Table 2). Activity of the non–rate-limiting component of the shuttles, cGPDH and cMDH, was different. cGPDH was similar in fetal and adult rat islets (Table 1) and in fetal pig ICCs and adult pig islets (Table 2). cMDH was also similar in fetal and adult rat islets (Table 1), but higher in fetal pig ICCs versus adult pig islets (Table 2). Enzyme activity was also measured in fetal pig β-cells, because the percentage of β-cells in fetal pig

TABLE 1
NADH shuttle enzyme activities in fetal and adult rat islets

Enzyme	*n*	Fetal rat	Adult rat	*P*
mGPDH	8–10	1.2 ± 0.3	9.3 ± 2.9	<0.001
cGPDH	3	113 ± 32	61 ± 1	0.29
mMDH	3–4	262 ± 65	762 ± 59	0.002
cMDH	4–7	1106 ± 100	1472 ± 200	0.07

Data are means ± SE. Enzyme activities were measured after allowing the islets to recover overnight from the isolation procedure. Units for mGPDH enzyme activity are nanomoles of substrate (2-p-iodo-3-nitro-5-phenyltetrazolium) reduced per minute per milligram protein; for cGPDH and cMDH nanomoles of NADH reduced per minute per milligram protein; and for mMDH nanomoles of NADH oxidized per minute per milligram protein. *n*, number of experiments/islet preparations. Duplicate samples were taken for each experiment/islet preparation.

ICCs is significantly lower than in adult pig islets (9 and 63%, respectively) (24). The activity of mitochondrial enzymes, GPDH and MDH, was lower in fetal pig β-cells than in adult islets. Similarly, the activity of the non–rate-limiting enzymes, cGPDH and cMDH, was lower or undetectable in fetal pig β-cells. In comparison with fetal pig β-cells (day 0), we also measured enzyme activity in fetal pig ICCs on day 0, i.e., immediately after collagenase digestion, before the cell clusters roundup (during days 1–3). The activity of all enzymes was significantly lower compared with fetal pig β-cells as well as fetal pig ICCs (day 3) (Table 2).

Enzyme level. The level of the mitochondrial enzymes involved in the NADH shuttles was lower in both fetal rat islets and fetal pig ICCs. mGPDH was lower by a factor of five in fetal rat islets as compared with adult islets (Fig. 1). Levels in fetal pig ICCs appeared to be lower than those in adult pig islets but were difficult to quantify, as the antibodies used were raised against rat mGPDH and did not bind well to pig mGPDH. The relative differences in enzyme levels between fetal and adult islets found in the glycerol phosphate shuttle was also reflected in the malate aspartate shuttle. The level of the enzyme, mMDH, was significantly lower in fetal rat islets (3-fold lower) (Fig. 1*B*) and in fetal pig ICCs (10-fold lower) (Fig. 1*C*).

mRNA levels. Expression of the gene for mGPDH and mMDH was studied by RT-PCR analysis, as shown in Fig. 2. The levels of expression of mRNA for mGPDH and mMDH were significantly lower in fetal rat islets. Quantitation of the PCR products after correction for differences in quality of RNA with the house-keeping gene, GAPDH, showed that the level of expression of mGPDH in fetal rat islets was 41% of that observed in adult rat islets ($P < 0.005$) (Fig. 2*A*). The level of expression of mMDH in fetal rat islets was 40% of that in adult rat islets ($P < 0.001$) (Fig. 2*B*).

Effect of glucose on NADH shuttle enzymes. The effect of glucose on activity, protein content, and gene expression of mGPDH and mMDH was studied in islets cultured for 24 h in medium containing 2, 5, 11, or 20 mmol/l glucose. These experiments were conducted as it was hypothesized that enzymes intimately involved in glucose metabolism would be regulated by the concentration of glucose. mGPDH enzyme activity in neither fetal nor adult rat islets was influenced by the amount of glucose in the culture medium (Fig. 3*A*). Similar results were observed in fetal and adult pigs (Fig. 3*A*). Likewise, the amount of mGPDH enzyme was affected by glucose in neither fetal nor adult islets (Figs. 3*B*). However, the number of mGPDH transcripts in fetal rats increased when the glucose concentration was increased to 5 mmol/l, and no further increase was observed when the glucose concentration was raised (Fig. 3*C*).

The response of mMDH enzyme activity and levels in fetal and adult islets to low and high concentrations of glucose after 24 h culture is shown in Fig. 3*D* and *E*. The enzyme activity in neither fetal nor adult rat increased with rising glucose concentrations (Fig. 3*D*). These changes in enzyme activity were mirrored by similar changes in the Western blots (Fig. 3*E*). A similar lack of effect of glucose on mMDH was observed in fetal pig ICCs. With adult pig islets, activity (Fig. 3*D*) but not protein levels (Fig. 3*E*) increased when the glucose concentration was raised from 2 to 5 mmol/l ($P < 0.05$); the activity (Fig. 3*D*) did not increase further with glucose concentrations >5 mmol/l.

NAD(P)H levels. As expected, NAD(P)H levels increased with increasing glucose concentration in adult rat islets, but a similar response was not observed in fetal rat islets indicating a much lower rate of glucose metabolism compared with adult islets (Fig. 4*A*). We also observed a rapid increase in NAD(P)H autofluorescence in response to increasing glucose concentrations and an equally rapid

TABLE 2
NADH shuttle enzyme activities in fetal pig ICCs and adult islets

Enzyme	*n*	Fetal pig	Adult pig	*P*
mGPDH, ICCs (day 3)	4–8	7.2 ± 1.2	33.1 ± 1.1	<0.001
mGPDH, ICCs (day 0)	6	1.7 ± 0.2	—	—
mGPDH, β-cells	15	4.7 ± 0.8	—	—
cGPDH (day 3)	3–8	186 ± 62	106 ± 11	0.07
cGPDH, ICCs (day 0)	6	5.3 ± 0.7	—	—
cGPDH, β-cells	8	47.6 ± 8.1	—	—
mMDH, ICCs (day 3)	6	462 ± 72	2525 ± 124	<0.001
mMDH, β-cells	8	284 ± 38.5	—	—
cMDH, ICCs (day 3)	5	2126 ± 345	1172 ± 115	0.03
cMDH, β-cells	8	650 ± 73	—	—

Data are means ± SE. Enzyme activities were measured after allowing the islets to recover overnight from the isolation procedure. Units for mGPDH enzyme activity are nanomoles of substrate (2-p-iodo-3-nitro-5-phenyltetrazolium) reduced per minute per milligram protein; for cGPDH and cMDH nanomoles of NADH reduced per minute per milligram protein; and for mMDH nanomoles of NADH oxidized per minute per milligram protein. *n*, number of experiments/islet preparations. Duplicate samples were taken for each experiment/islet preparation.

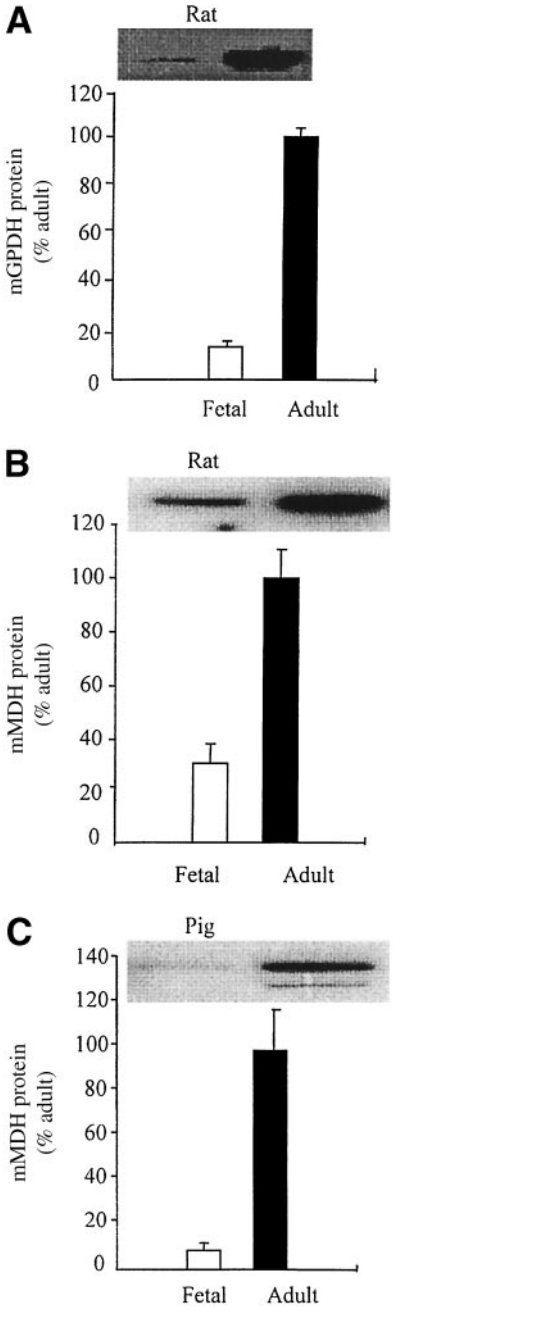

FIG. 1. mGPDH and mMDH enzyme levels in adult and fetal islets/ICCs quantitated by Western analysis. Fetal enzyme level is expressed as a percentage of adult enzyme level (adult = 100%). The data are expressed as means ± SE. *A*: mGPDH protein levels in fetal ($n = 3$) and adult ($n = 5$) rat islets, $P < 0.001$. *B*: mMDH protein levels in fetal ($n = 3$) and adult ($n = 5$) rat islets, $P < 0.001$. *C*: mMDH protein levels in fetal pig ICCs ($n = 4$) and adult pig islets ($n = 4$), $P < 0.001$. n = the number of experiments/islet preparations. Duplicate samples were taken for each experiment/islet preparation.

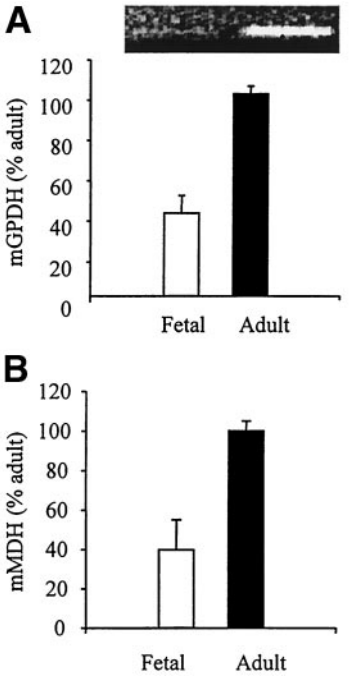

FIG. 2. mGPDH and mMDH gene expression in adult and fetal islets assessed by RT-PCR. Fetal mRNA level is expressed as a percentage of adult mRNA level (adult = 100%). The data are expressed as means ± SE. *A*: mGPDH mRNA level in fetal and adult rat islets ($n = 4$), $P < 0.001$. *B*: mMDH mRNA levels in fetal and adult rat islets ($n = 4$), $P < 0.001$. n = the number of experiments/islet preparations. Duplicate samples were taken for each experiment/islet preparation.

decrease in NAD(P)H autofluorescence when the glucose stimulus was removed, indicating that NAD(P)H is rapidly used (probably due to the high NADH shuttle activity) in adult rat islets (Fig. 4*B*). These changes were not observed in fetal islets (Fig. 4*B*).

Effect of adenoviral transduction with mGPDH cDNA. mGPDH activity in fetal rat islets in control islets and after transduction with Adex1-mouse-mGPDH or negative control Adex1-lacZ is shown in Fig. 5. The enzyme activity in islets transduced with lacZ was similar to normal islets, whereas the islets infected with Adex1-mouse-mGPDH were twofold higher compared with control islets. The transduction efficiency was 50–60% (data not shown), which probably explains why the level of enzyme activity was lower than that of adult rat islets (Fig. 5).

Insulin secretion in untreated fetal rat islets and fetal rats transduced with ADex1CAmGPDH and ADex1CAlacZ in response to 2.8 mmol/l glucose (basal), 20 mmol/l glucose, and 20 mmol/l KCl is shown in Fig. 6. The untreated islets and islets transduced with lacZ were not able to secrete insulin in response to glucose, but the islets transduced with mGPDH secreted insulin (57% increase from basal) in response to stimulation with 20 mmol/l glucose. All islets released insulin (a twofold increase) when exposed to 20 mmol/l KCl.

DISCUSSION

Comparison of two of the key enzymes involved in both NADH shuttles in fetal and adult islets/ICCs showed that the activity of both enzymes was significantly reduced in fetal islets/ICCs in both species studied. Although the enzyme activities in fetal and adult pigs were much higher than those of rats, a similar magnitude of difference between the enzyme activity in fetal versus adult islets in both species indicates that the lower enzyme activity observed in fetal islets is likely a developmental feature. Previous studies in our laboratories have shown that the percentage of β-cells in fetal and rat islets are the same (63 vs. 62% of islets consist of β-cells) (5), hence we can compare the results in fetal islets with adult islets without the possibility that the differences observed are due to the difference in the percentage of cell types. However, the

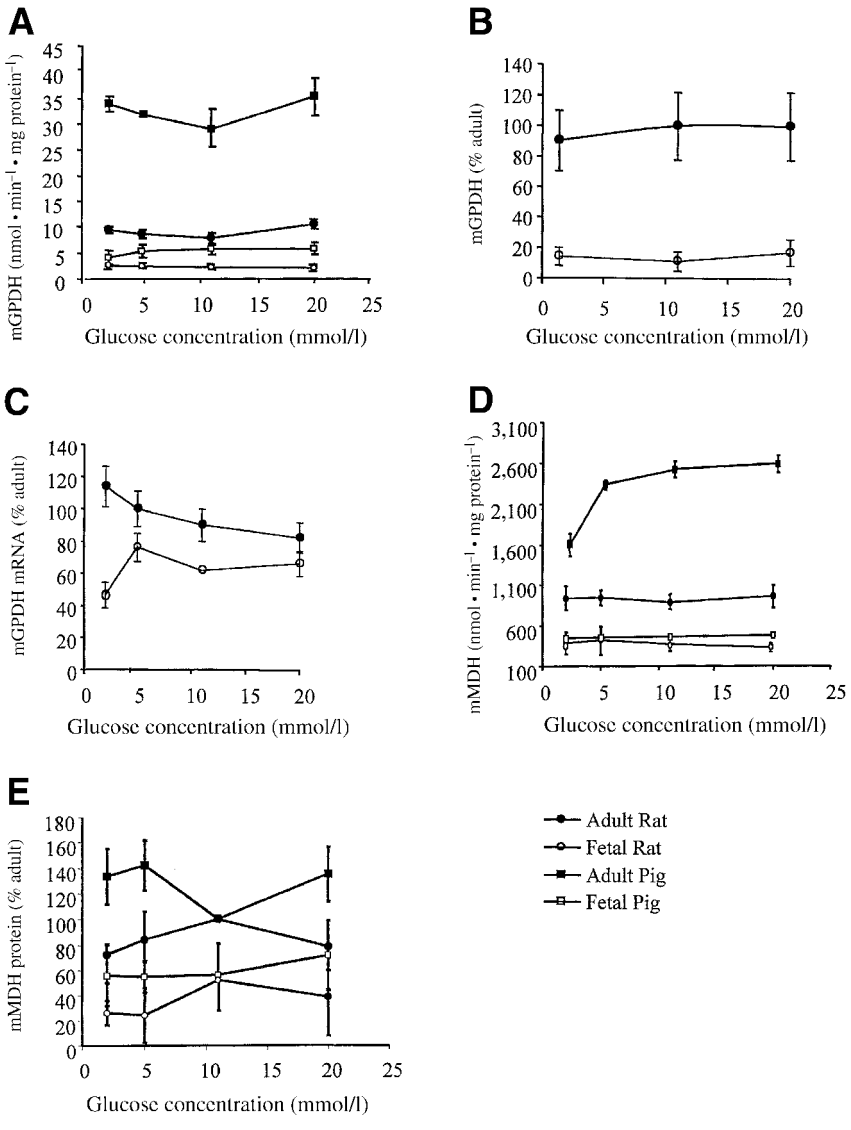

FIG. 3. Effect of glucose on enzyme activity, protein level, and gene expression of mGPDH and mMDH in fetal and adult islets. Fetal enzyme level is expressed as a percentage of adult enzyme level (adult = 100%). The data are expressed as means ± SE. *A*: mGPDH activity in fetal and adult rat islets (n = 4) and fetal pig ICCs and adult islets (n = 4–6). *B*: mGPDH enzyme levels in fetal and adult rats (n = 4). *C*: mGPDH mRNA levels in fetal and adult rat islets (n = 3). *D*: mMDH activity in fetal and adult rat islets (n = 4) and fetal pig ICCs and adult islets (n = 4–6). *E*: mMDH protein levels in fetal and adult rat islets (n = 3) and fetal pig ICCs and adult islets (n = 4–6). n = the number of experiments/islet preparations. Duplicate samples were taken for each experiment/islet preparation.

same cannot be said for fetal pig ICCs. Only 9% of fetal pig ICCs consist of β-cells, while 63% of adult islets are made up of β-cells. Hence, we also measured enzyme activities in purified fetal pig β-cells (80% β-cells), and we observed that all enzyme activities measured were lower in fetal pig β-cells than in adult pig islets. Furthermore, enzyme activities in fetal pig β-cells were also lower compared with ICCs, and we speculate that this is probably due to the "maturation" or recovery of ICCs from the isolation procedure during culture (3 days). Measurement of enzyme activities in cell clusters immediately after collagenase digestion, i.e., before they were cultured and allowed to form ICCs, showed lower enzyme activities compared with both ICCs (day 3) and β-cells (day 0). Therefore, the activities of these enzymes are higher in fetal pig β-cells than in other cells in the fetal pig ICCs. These data are similar to the adult, in which enzyme activity in the β-cell is higher than that in other cells (2).

In contrast to our results, Rasschaert et al. (30) reported that the activity of mGPDH was higher in fetal than adult rat islets. In support of our data showing lower enzyme activity in fetal rat islets, we also found that the islets also had significantly lower levels of mRNA and protein. In their studies, Rasschaert et al. compared fetal rat islets that were cultured for 7 days in RPMI (11 mmol/l glucose) with freshly isolated adult rat islets. In our study, we used freshly isolated islets that had been allowed to recover overnight from the isolation procedure. It is possible the differences Rasschaert et al. observed in mGPDH activity was due to an effect of culture. Another possibility is that the size of their fetal islets was different from ours. In our hands, islets from both fetal and adult islets were of similar size and had similar protein content (31). Rasschaert et al. found a lower protein content in fetal islets than adult islets.

We have shown that the low enzyme activity observed is due to a lower level of gene expression and perhaps a reduced synthesis of the enzyme in the fetal islets, rather than the result of an inactive or immature enzyme. This is demonstrated by the lower number of mRNA copies of both mGPDH and mMDH in fetal islets, and the ratio of fetal-to-adult enzyme levels was the same as the ratio of fetal-to-adult enzyme activity.

NAD(P)H autofluorescence was measured in both adult and fetal rat islets to observe the flux of NAD(P)H during glucose stimulation. The levels of NAD(P)H were respon-

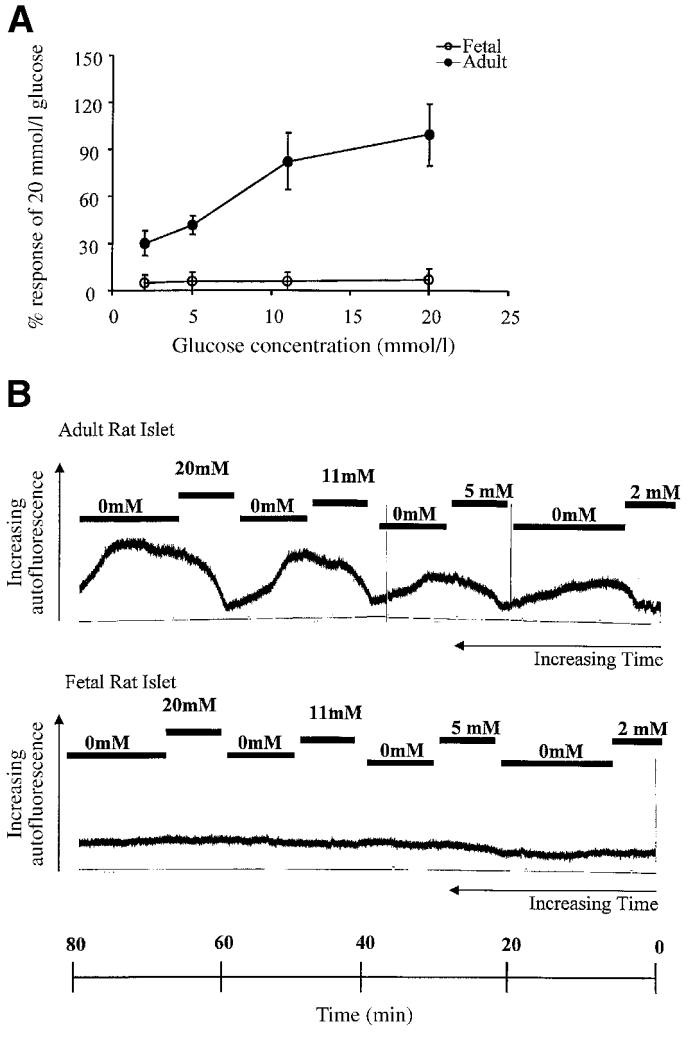

FIG. 4. *A*: Effect of glucose on NAD(P)H auto fluorescence in adult and fetal rat islets. Data are expressed as a percentage of the NAD(P)H auto fluorescence stimulated by 20 mmol/l glucose (20 mmol/l glucose = 100%). *B*: Results from one representative experiment with adult and fetal rat islets. Data should be read from right to left. The number of experiments/islet preparations = 10. Duplicate samples were taken for each experiment/islet preparation.

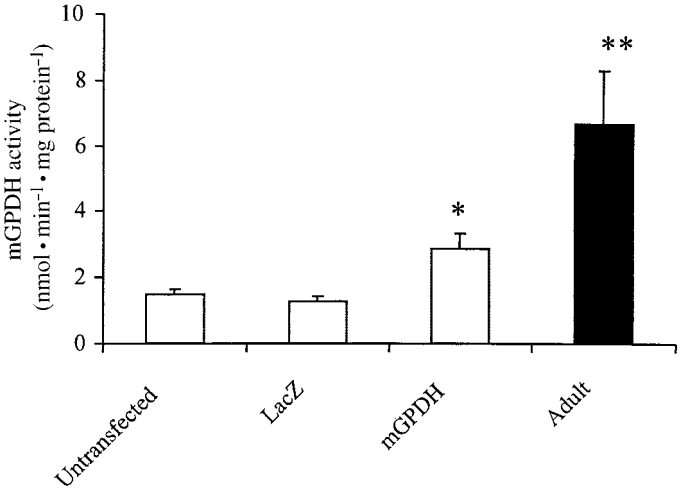

FIG. 5. mGPDH enzyme activity from untreated fetal rat islets (n = 6), fetal rat islets infected with Adex1CAlacZ (n = 6), and Adex1CAmGPDH (n = 6) and adult rat islets (n = 6). n = the number of experiments/islet preparations. Duplicate samples were taken for each experiment/islet preparation. *P < 0.05 compared with lacZ, **P < 0.001 compared with all three groups (untransfected, Lac Z, and mGPDH).

sive to glucose, i.e., within minutes of glucose stimulation, NAD(P)H activity rapidly increased and when glucose was removed the levels dropped very rapidly to basal levels, indicating that NAD(P)H was rapidly being utilized or shuttled into the mitochondria to generate an electrochemical gradient, which results in ATP synthesis and insulin secretion. Such a response was not observed in fetal rat islets, suggesting that glucose metabolism in the mitochondria is impaired and that the NADH shuttles are not functioning at the same capacity as the adult rat islets.

Fetal islets are immature with regards to insulin secretion in response to glucose. By using adenoviral transduction to increase the activity of mGPDH, a rate-limiting enzyme in the GPDH shuttle, we were able to induce insulin secretion in response to glucose, even though the enzyme activity was increased only twofold. This indicates that a reason for the inability of fetal islets to secrete insulin in response to glucose is the inability of the NADH shuttles in fetal islets to contribute to mitochondrial metabolism and ATP synthesis. NADH shuttle activity, which results in the regeneration of cytosolic NAD^+ and the transfer of reducing equivalents produced during glycolysis to the mitochondria (electrons to complex I and II) for oxidative metabolism/ATP synthesis, is crucial for glucose-stimulated insulin secretion. Eto et al. (13) showed that when both shuttles are turned off, glucose-stimulated insulin secretion was abrogated and ATP-to-ADP ratio was reduced to 38% of control islets, even though glucose utilization and glucose oxidation remained normal. Previously, we have shown that glucose utilization and glucokinase (the glucose sensor) activity is similar in fetal and adult rat islets and that the immaturity appears to be in glucose oxidation or ATP synthesis (5). Furthermore, culturing fetal rat islets for 7 days in high glucose concentrations did not restore glucose-stimulated insulin secre-

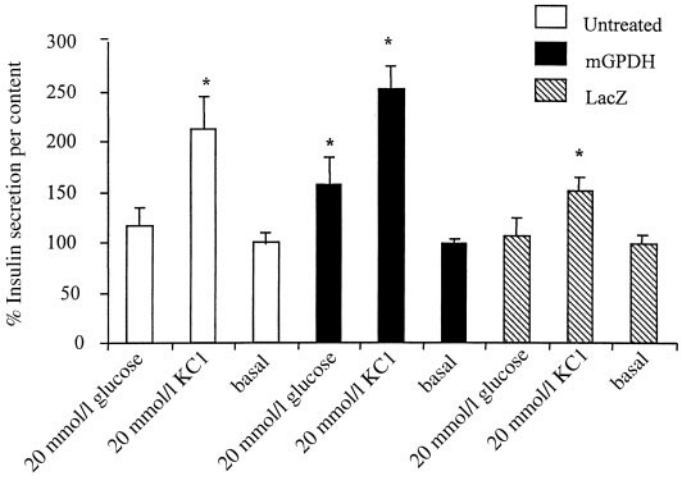

FIG. 6. Insulin secretion from untreated fetal rat islets (n = 18), fetal rat islets infected with Adex1-lacZ (n = 18), and Adex1-mouse-mGPDH (n = 18) in response to basal glucose (2.8 mmol/l), 20 mmol/l glucose, and 20 mmol/l KCl. The results are expressed as a percentage of basal insulin secretion/content, basal insulin secretion/content = 100%. n = the number of experiments from six litters of fetal rats/islet preparations. Duplicate samples were taken for each experiment. *P < 0.05 compared with the basal in each group.

tion, even though the V_{max} of glucokinase increased and hence glucose utilization was also significantly increased (5). Our results are in agreement with those from Rorsman et al. (8) but disputed by those of Hughes (4). However, as shown by Eto et al. (13), the supply of NADH to the mitochondria is more crucial to ATP synthesis and glucose-stimulated insulin secretion than total glucose utilization and oxidation. They hypothesized that mitochondrial metabolism in β-cells is triggered by the transfer of NADH generated by the metabolism of glucose via glycolysis. Our findings of lowered activity of NADH shuttles in immature β-cells and their "maturation" by enhancing such activity supports the hypothesis of Eto et al.

It is interesting to note that the activity and expression of mGPDH and mMDH were regulated by glucose in neither fetal nor adult islets. This is contrary to what might be expected of an enzyme, which is intimately involved in glucose metabolism. This may be due to the fact that mGPDH, in particular, is already present in such high levels in pancreatic islets. A study by Gong et al. (32) suggests that mGPDH activity in adult β-cells is reduced after prolonged exposure (7 days) to high glucose concentrations but remains normal in non–β-cells. This is consistent with reports that mGPDH activity is decreased in islets of humans (18) and animals with type 2 diabetes (17,33) when blood glucose is normalized (34).

In summary, we have shown that activity of the key enzymes of both NADH shuttles, mGPDH and mMDH, is lower in fetal islets/ICCs than in adult islets. Increasing shuttle activity by transduction of fetal rat islets with the key enzyme of one of the NADH shuttles allowed the fetal islets to secrete insulin in response to glucose. We suggest that the immaturity of the NADH shuttles is one of the reasons for the inability of fetal β-cells to secrete insulin in response to glucose.

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