Evidence of Enhancement of Malate-Aspartate Shuttle Activity in β Cells of Streptozotocin-Induced Non–Insulin-Dependent Diabetic Rats

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Glucose-induced insulin secretion is selectively impaired in β cells from animals with non–insulin-dependent diabetes mellitus (NIDDM). This study was performed to clarify whether the malate-aspartate shuttle among the glucose metabolic pathways is intact in β cells of NIDDM rats. The insulin secretory capacity of the islets and the K_{ATP} channel activity in single β cells were measured in control and NIDDM rats injected with streptozotocin (STZ) during the neonatal period, using a radioimmunoassay and patch-clamp technique. The increase of insulin secretion induced by 11.1 mmol/L glucose or 10 mmol/L dihydroxyacetone (DHA) was significantly reduced in NIDDM islets, suggesting an impaired glycerol-phosphate shuttle. The application of glyceraldehyde (10 mmol/L) in NIDDM or control islets elicited an increase in insulin secretion, but the difference between the 2 groups was indistinguishable. On the contrary, the increase of insulin secretion and the inhibition of K_{ATP} channel activity induced by aspartate, which preferentially participates in the malate-aspartate shuttle, were significantly greater in NIDDM versus the control. However, intracellularly applied aspartate in the inside-out mode did not inhibit K_{ATP} channel activity. These findings show that malate-aspartate shuttle activity is potentiated in pancreatic β cells of NIDDM rats, suggesting the development of a compensatory mechanism for the reduced activity of the glycerol-phosphate shuttle in NIDDM.

A MAJOR INSULIN secretagogue in the pancreatic β cell is glucose,[1] which induces an increase in adenosine triphosphate (ATP) within the β cell. ATP-sensitive potassium channels (K_{ATP} channels) are the targets of ATP[2-4] and of hypoglycemic sulfonylureas[5] such as tolbutamide and glibenclamide. It is well known that the inhibition of K_{ATP} channels by one of these agents and the resultant depolarization[6] are essential to the mechanism leading to insulin secretion. Glucose-induced insulin secretion is selectively impaired in non–insulin-dependent diabetes mellitus (NIDDM) patients[7] and in animal models of NIDDM.[8,9] The β cells of neonatally streptozotocin (STZ)-induced diabetic rats (NIDDM rats) have also demonstrated the similar selective impairment and a reduced sensitivity of K_{ATP} channels to glucose.[10,11] This animal model is characterized by remarkable defect in insulin secretion in response to glucose, which might be due to a reduced number of β cells. However, other secretagogues, eg, arginine, cause insulin secretion comparable to control levels,[12] implying that some intracellular steps within glucose metabolism are related to the abnormality of this model. Because glucose does not act directly on K_{ATP} channels, the change of K_{ATP} channel activity by glucose in an intact β cell may be modulated by the product(s) of intracellular glucose metabolism, for instance, ATP. Some reports[11,13,14] have shown that the intracellular site responsible for disturbed glucose metabolism in the NIDDM β cell is located within the glycerol-phosphate shuttle. However, it has not been fully investigated as to whether another major pathway for ATP production, the malate-aspartate shuttle, is also impaired in the NIDDM β cell.

In the present study, we investigated the possible impairment of the malate-aspartate shuttle in β cells of NIDDM rats, by testing the effect of aspartate on insulin secretion and on K_{ATP} channels using a radioimmunoassay and patch-clamp technique. The results showed that ATP production by aspartate is not impaired but is instead upregulated in NIDDM, suggesting the presence of a compensatory mechanism in response to the impairment of the glycerol-phosphate shuttle.

MATERIALS AND METHODS

Induction of NIDDM in Rats

Male Sprague-Dawley neonatal rats, 1.5 days old, received a subcutaneous injection of 70 to 90 mg/kg STZ in 0.05 mol/L citrate buffer, pH 4.3 (NIDDM rats) or an equivalent volume of citrate buffer alone (control rats). NIDDM and control rats between 8 and 12 weeks of age were used for the experiments.

Measurement of Blood Glucose and Serum Insulin

Blood was collected via the tail capillary and abdominal aorta for measurement of glucose and insulin levels, respectively. The capillary blood glucose level was determined by the glucose oxidase method (Medisense2; Medisense, Waltham, MA), and the serum insulin level was measured by radioimmunoassay using the polyethylene glycol method (Amersham, Little Chalfont, Buckinghamshire, UK).

Preparation of Single β Cells

Islets of Langerhans were isolated from the pancreas by a collagenase digestion technique. Briefly, under ether anesthesia, neutral red solution (3 mL, 1:1,000 wt/vol in 0.9% saline) was injected through the abdominal aorta after cutting the inferior vena cava, since this stain is selective for islet tissue.[15] Collagenase (1.2 mg/mL Hanks balanced salt solution) solution was perfused into the pancreatic ducts retrogradely through the common bile duct. The dissected pancreas was incubated in a 37°C water bath for 33 minutes. Dispersion of islet cells was accomplished with the modified method of Gray et al.[15] Separated single cells were then placed in RPMI 1640 medium (glucose 11.1 mmol/L) supplemented with fetal calf serum (10%), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). They were cultured on small cover glasses (10 × 3 mm) at 37°C in a humidified incubator supplied

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with 5% CO_2 and balanced air. Individual cover glasses were transferred into the bath chamber on an inverted microscope (Zeiss, Jena, Germany) for patch-clamp experiments.

Measurement of Insulin Secretory Capacity

Insulin secretory capacity was measured by the batch incubation method as previously reported with acutely isolated islets from NIDDM and control rats.[8] The islets were preincubated with standard external medium (in mmol/L: 135 NaCl, 5 KCl, 5 $CaCl_2$, 2 $MgSO_4$, 5 HEPES, and 5.5 glucose, pH 7.4 with NaOH) supplemented with 0.2% bovine serum albumin (BSA) at 37°C for 30 minutes. Batches of 5 to 10 islets were then incubated in standard external medium with testing materials and 0.2% BSA at 37°C for 30 minutes. After slight mixing of the supernatant of each batch with a 100-μL pipette, an aliquot was taken from the supernatant for later measurement of insulin by radioimmunoassay.

Electrophysiological Study

This study used the cell-attached and inside-out modes of the patch-clamp technique.[16] A pipette was pulled from borosilicate glass, coated with Sylgard resin (Dow Corning, Midland, MI) near the tip, and fire-polished, and it had resistance between 5 and 10 MΩ. Single-channel currents were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster, CA). During the experiments, current and voltage signals were stored on videotape via a VR-10B pulse code modulator (Instrutech, Great Neck, NY) and later analyzed by pClamp 6.04 software (Axon Instruments). The channel activity was expressed as the mean patch current ($I = N \times P_0 \times i$),[10] where N, P_0, and i represent the number of channels in the patch membrane, the open probability of the channel, and the unit amplitude of the single-channel current, respectively. The relative channel activity in the presence of testing materials was expressed as I/Ic, where Ic is the mean patch current recorded in control solution.

In the cell-attached experiment, cells were bathed in a solution composed of (in mmol/L) 135 NaCl, 5 KCl, 5 $CaCl_2$, 5 $MgSO_4$, and 5 HEPES (pH 7.4 with NaOH). The pipette solution consisted of (in mmol/L) 140 KCl, 5 $CaCl_2$, 5 $MgSO_4$, and 10 HEPES (pH 7.4 with KOH). The bath solution used for the inside-out mode of patch-clamp experiments contained 135 mmol/L KCl, 10 mmol/L NaCl, 0.1 mmol/L $CaCl_2$, 2 mmol/L $MgSO_4$, 1 mmol/L EGTA, 0.1 μmol/L ATP, and 5 mmol/L HEPES (pH 7.2 with KOH). Electrophysiological experiments were performed at room temperature (22° to 25°C). All chemicals were from Sigma Chemical (St Louis, MO).

Statistical Analysis

Data are expressed as the mean ±SE. Statistical significance was evaluated by the Student t test.

RESULTS

Insulin Secretory Capacity of Pancreatic Islets

Fasting blood glucose and serum insulin levels were examined in control and NIDDM rats. The results are summarized in Table 1, showing no statistical differences between the 2 groups.

The insulin secretory capacity of islets in response to various secretagogues was measured in control and NIDDM rats. Basal insulin secretion for islets incubated with 5.5 mmol/L glucose was 202 ± 30 pg/islet/30 min in control rats and 167 ± 51 pg/islet/30 min in NIDDM rats. No significant difference in basal insulin secretion was found in the 2 groups. Insulin secretion was augmented by increasing glucose and by various secretagogues such as dihydroxyacetone (DHA), glyceraldehyde, and aspartate. The increase is presented as a percent increase with respect to basal secretion (Table 2). The increase of insulin secretion in response to 11.1 mmol/L glucose and to 10 mmol/L DHA was significantly lower in NIDDM islets than in control islets. On the contrary, the increase of insulin secretion in response to aspartate, a substrate passing through the malate-aspartate shuttle, was significantly greater in NIDDM islets than in control islets. The response to 10 mmol/L glyceraldehyde showed no significant difference in control islets and NIDDM islets.

Table 1. Fasting Blood Glucose and Serum Insulin Levels in Control and NIDDM Rats

Parameter	Control (n = 10)	NIDDM (n = 10)
Blood glucose (mg/dL)	85.6 ± 5.25	84.7 ± 8.64
Serum insulin (ng/mL)	1.03 ± 0.327	0.96 ± 0.495

NOTE. Data are the mean ± SE.

Table 2. Insulin Secretory Capacity (%) of Cultured Islets in Response to Nutrient Secretagogues in Control and NIDDM Rats

Secretagogue	Control (n = 7)	NIDDM (n = 10)
5.5 mmol/L glucose	100	100
11.1 mmol/L glucose	451 ± 35	259 ± 56*
10 mmol/L DHA	279 ± 41	109 ± 24*
10 mmol/L glyceraldehyde	167 ± 58	181 ± 63
5 mmol/L aspartate	203 ± 42	264 ± 29
10 mmol/L aspartate	265 ± 36	530 ± 62*

NOTE. Data are the mean ± SE. Basal insulin secretion (pg/islet/30 min) was 202 ± 30 in control and 167 ± 51 in NIDDM.

*$P < .05$ v control.

Effects of Glucose and Aspartate on K_{ATP} Channels

It is well known that the increase of insulin secretion by the nutrient secretagogues is mediated by membrane depolarization caused by the decrease of K_{ATP} channel activity.[1,2,6] To investigate the mechanism of the high sensitivity of aspartate for insulin secretion in NIDDM rats, we recorded K_{ATP} channel activity in pancreatic β cells using the patch-clamp technique in a cell-attached configuration. When the concentration of glucose in bath solution was increased to 10 mmol/L, K_{ATP} channel activity was inhibited gradually in both the control and NIDDM groups (Fig 1A). However, the time course and extent of inhibition were significantly different between the 2 groups. Glucose-induced suppression of the channel activity (I/Ic) in control and NIDDM cells was about 0.0 and 0.7 after reaching a steady state, respectively (Fig 1B). The channel activity in NIDDM was not reduced further even after a long exposure to 10 mmol/L glucose, but it was completely abolished by the K_{ATP} channel blocker glibenclamide (1 μmol/L), confirming that the channel activity originated from the K_{ATP} channel (Fig 1A). This result is consistent with a previous report[13] suggesting that ATP production induced by a high concentration of glucose is impaired in NIDDM.

The effect of aspartate on K_{ATP} channel activity was tested (Fig 2). An increase in the concentration of aspartate in the bath solution induced a suppression of K_{ATP} channel activity concentration-dependently in both groups (Fig 2A). Contrary to the effect of glucose, the inhibition of channel activity was much

more pronounced in NIDDM versus the control at a given concentration of aspartate (Fig 2B). The time course of the inhibitory effect of 10 mmol/L aspartate on K_{ATP} channel activity is illustrated in Fig 3. In the control, the inhibitory effect of aspartate on K_{ATP} channel activity was smaller and slower than in NIDDM. After 2 minutes of 10 mmol/L aspartate application, I/Ic was about 0.5 in control, whereas it was less than 0.05 in NIDDM. All of these inhibitory effects of aspartate were reversible within 5 minutes after washout (data not shown). The difference in the 10-mmol/L aspartate effect on K_{ATP} channels between the 2 groups was statistically significant, and this result is consistent with the greater effect of aspartate on the insulin secretory capacity in NIDDM shown in Table 2. Under the assumption that K_{ATP} channel activity indicates the level of ATP inside the cell, it could be suggested from these results that ATP production induced by aspartate is facilitated in NIDDM compared with the control.

To exclude the possibility that the inhibitory effect of aspartate was due to a direct action on the K_{ATP} channel, the effect of aspartate was tested in the inside-out configuration (Fig 4). In contrast to the result in the cell-attached configuration, bath application of 10 mmol/L aspartate did not suppress K_{ATP} channel activity in the inside-out patch. ATP 1 mmol/L

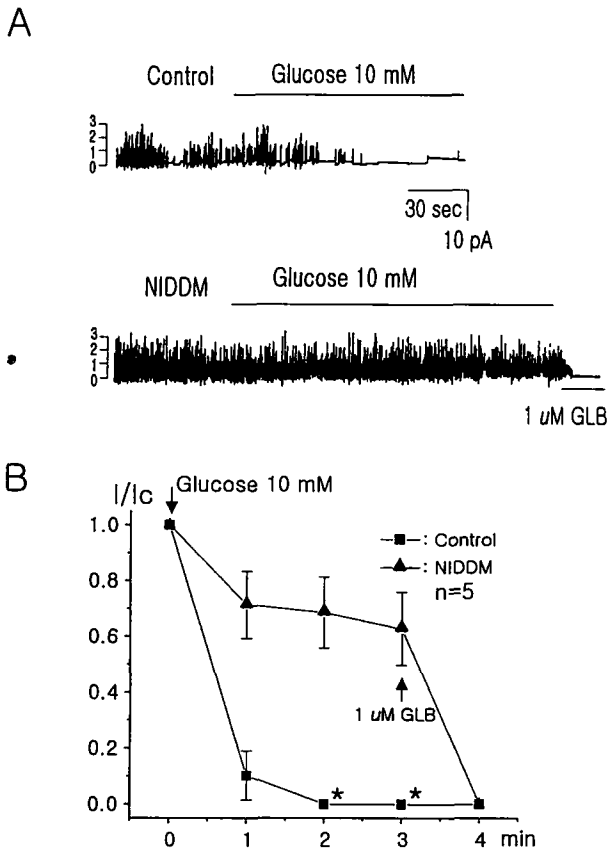

Fig 1. Effects of glucose on K_{ATP} channel activity on single β cells of control and NIDDM rats. Cell-attached mode, Vp = 0 mV. GLB, glibenclamide. (A) Representative traces showing the inhibition of K_{ATP} channel activity by glucose. (B) Time course for glucose effects on the normalized mean patch currents. *$P < .05$ *v* NIDDM (mean ± SE).

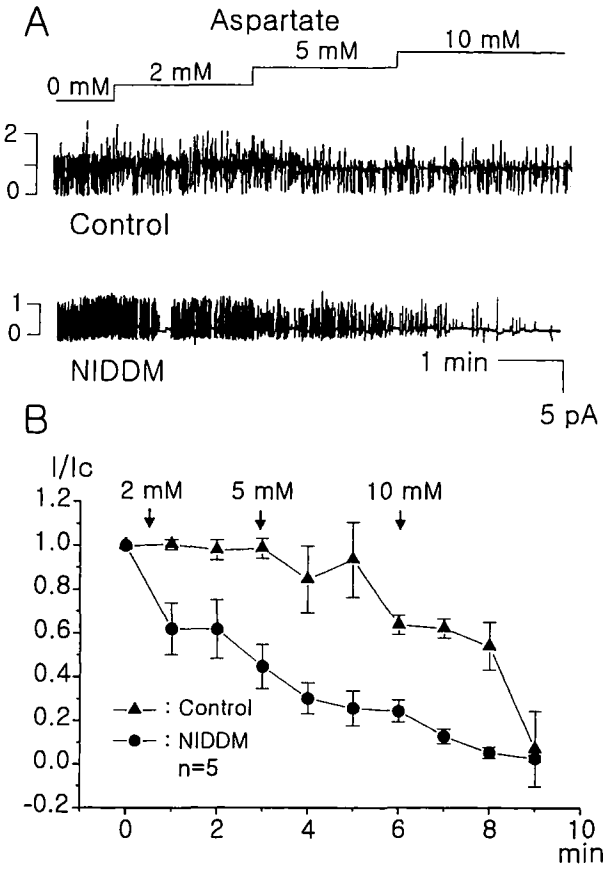

Fig 2. Effects of aspartate concentration on K_{ATP} channel activity recorded on single β cells of control and NIDDM rats. Cell-attached mode, Vp = 0 mV. (A) Representative traces showing the inhibition of K_{ATP} channel activity by aspartate dose-dependently. (B) Time course for aspartate effects on the normalized mean patch currents (mean ± SE).

acutely and reversibly inhibited the channel activity. This result implies that the aspartate effect observed in the cell-attached mode is not the result of its direct action on the K_{ATP} channel.

DISCUSSION

This study shows that insulin secretion and the inhibition of K_{ATP} channel activity by glucose were markedly impaired in β cells of NIDDM rats. In addition, the current-voltage relationship of single-K_{ATP} channel amplitude in NIDDM rat was almost identical to that in the control (data not shown), and the channels were readily blocked by glibenclamide or ATP, indicating that the function of the K_{ATP} channel is normal in NIDDM.[9,11] These findings suggest that the reduced sensitivity to glucose of NIDDM β cells should be due to impaired glucose metabolism, not to a malfunction of the K_{ATP} channel itself.[10]

A potent intracellular inhibitor of the K_{ATP} channel is ATP, which can be produced through glucose metabolism in the β cell. Three different pathways are well known to be involved in the glucose metabolism linking glycolysis to the mitochondrial oxidation system[17]: first, by the action of glucokinase providing ADP for the ATP synthetase; second, by providing $FADH_2$ through the glycerol-phosphate shuttle; and third, by NADH

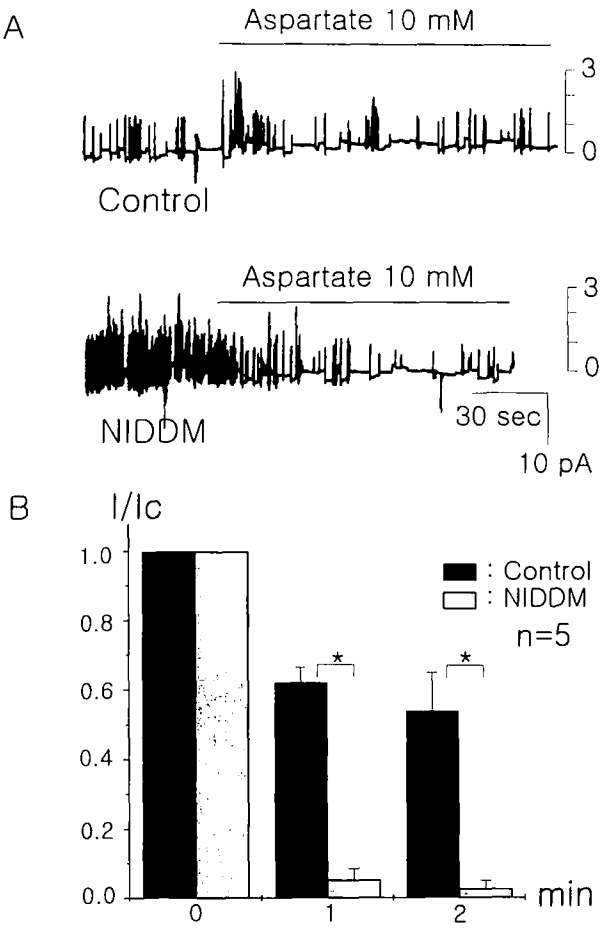

Fig 3. Effects of 10 mmol/L aspartate on K_{ATP} channel activity recorded on single β cells of control and NIDDM rats. Cell-attached mode, Vp = 0 mV. (A) Representative traces showing the inhibition of K_{ATP} channel activity by aspartate. (B) Time course for inhibitory effects of aspartate on the normalized mean patch currents. *$P < .05$ *v* control (mean ± SE).

transfer to the respiratory chain complex I via the malate-aspartate shuttle.

Glyceraldehyde is thought to diminish K_{ATP} channel activity through ATP production by entering into the distal portion of glycolysis via glyceraldehyde-3-phosphate dehydrogenase, and by the mitochondrial oxidation system. DHA is able to accumulate intracellular ATP via the glycerol-phosphate shuttle and glycolysis. In the present study, insulin secretion in response to DHA was significantly lower in NIDDM islets. The diminution of the inhibitory effect of DHA on K_{ATP} channels in the NIDDM β cell was observed in our previous study[11] and is consistent with another report.[14] There is also evidence that the activity of mitochondrial glycerol-phosphate dehydrogenase, thought to be the key enzyme in this shuttle, is lower in the NIDDM β cell than in the control.[18,19] Taken together, it can be confirmed that an impairment in the glycerol-phosphate shuttle is the primary cause of impairment in glucose-induced insulin release in this model of NIDDM. This metabolic disturbance may be linked or related to various further point[20,21] or length[22] mutations of mitochondrial DNAs and/or the glucokinase gene mutation (MODY 2)[23] in hereditary NIDDM patients.

ATP from aspartate can be produced through the malate-aspartate shuttle on the mitochondrial membrane in β cells. The present study shows that aspartate cannot act directly on K_{ATP} channels (Fig 4), and the function of the malate-aspartate shuttle in NIDDM β cells may be normal. Moreover, the inhibitory effect of aspartate on K_{ATP} channel activity and insulin secretory capacity in NIDDM β cells in response to aspartate was significantly greater than in the controls. So, it is probable that the enzymatic activity involved in the malate-aspartate shuttle of the NIDDM β cell may be increased by an excess of reducing equivalents that could not enter the mitochondria through the glycerol-phosphate shuttle. There have been many studies using enzymatic assay[13,17,24-26] to determine whether the enzymatic activities related to the malate-aspartate shuttle are actually changed in various tissues of NIDDM animals. In the liver and cerebellum, the activities of malate dehydrogenase (MDH) and aspartate aminotransferase (Asp-AT), which are the key enzymes in this shuttle, appear to be increased in alloxan-diabetic rats.[25] In contrast, these activities are lower in diabetic heart than in control.[25,26] It should be noted that the high NADH/NAD ratio caused by fatty acid oxidation during diabetes would tend to increase the activity of mitochondrial MDH and Asp-AT in the cerebellum.[27,28] In the liver, an increase in gluconeogenesis and urogenesis could induce the synthesis of these enzymes.[25] However, only a few studies have been performed on the change in malate-aspartate shuttle activity in the NIDDM β cell, even though the β cell should be preferentially investigated in NIDDM. In addition, the present study was performed electrophysiologically for the first time to observe the overall enzymatic activities involved in the malate-aspartate shuttle. The enzymatic study by Sener et al[13] revealed that the activities of several enzymes in islet homogenates of adult rats were affected by the injection of STZ during the neonatal period, but it was not clear which pathway was impaired. They concluded with metabolic data instead that there was an apparent defect in

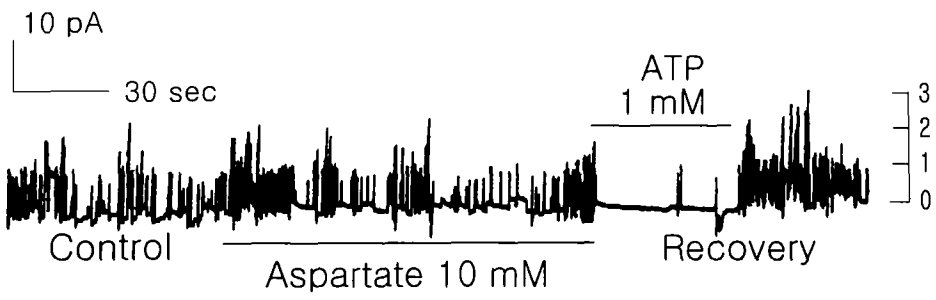

Fig 4. Effects of intracellularly applied aspartate and ATP on K_{ATP} channel activity on single β cell of NIDDM rat. Inside-out mode, Vp = 70 mV.

the glycerol-phosphate shuttle in β cells of NIDDM rats that was undetectable by enzymatic studies only, and this was not the case for the malate-aspartate shuttle.

Although the physiological significance of the upregulation of malate-aspartate shuttle activity in diabetic β cells is not clearly known, this study strongly suggests that the enhanced activity of the malate-aspartate shuttle in the β cells of this NIDDM model is probably a compensatory mechanism for a defect in the glycerol-phosphate shuttle to enable the metabolism to continue and to restore the intracellular ATP level.

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