Normalization by Insulin Treatment of Low Mitochondrial Glycerol Phosphate Dehydrogenase and Pyruvate Carboxylase in Pancreatic Islets of the GK Rat

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The enzyme activity of the mitochondrial glycerol phosphate dehydrogenase (mGPD) in the pancreatic islet has been reported to be less than one-half of normal in the Goto-Kakizaki (GK) rat, a genetic model of NIDDM. In the current study, mGPD enzyme activity and the amount of mGPD protein, as judged by Western analysis, were 35–40% of normal in the islets of these animals. With the exception of pyruvate carboxylase, the activities of other enzymes were not abnormal. The assayable activity and amount of pyruvate carboxylase protein were decreased ~50% in the islets of the GK rats. Because mGPD, which is the key enzyme of the glycerol phosphate shuttle, and pyruvate carboxylase, which is the key component of the pyruvate malate shuttle, have been proposed to be essential for stimulus-secretion coupling in the pancreatic β-cell, an important question is whether the decreases in these enzymes have a causal role in the hyperglycemia or whether the diabetic syndrome caused the decreases. To attempt to differentiate between these two possibilities, GK rats were treated with insulin to normalize their blood sugars. The activities of both mGPD and pyruvate carboxylase were also normalized by insulin treatment. An incidental discovery of this study was the identification of a high level of propionyl-CoA carboxylase activity and a lesser amount of methylcrotonyl-CoA carboxylase activity in pancreatic islets. These enzymes were normal in the islets of the GK rats. This is the first report on the presence of these two carboxylases in the islet and of low pyruvate carboxylase activity in the islet in NIDDM. We conclude that the decreased mGPD and pyruvate carboxylase in the pancreatic islet of the GK rat result from the diabetic syndrome. *Diabetes* 45:886–890, 1996

The Goto-Kakizaki (GK) rat is a model of inherited NIDDM. In rats younger than 3 months, islet morphology is virtually intact and β-cell density is preserved (1,2). Glucose-induced insulin secretion is impaired, possibly because of abnormalities of glucose metabolism in the pancreatic islet (3–5). Mitochondrial glycerol phosphate dehydrogenase (mGPD), the key enzyme of the glycerol phosphate shuttle, a shuttle that oxidizes NADH formed during glycolysis, is abundant in the pancreatic β-cell of both rodents and humans (6–10). This suggests that mGPD and its shuttle are important for glucose-induced insulin secretion. The enzyme activity of mGPD has been found to be ~40% of normal in the islets of the GK rat (11), as has the activity of the glycerol phosphate shuttle (4). It is not clear whether this finding reflects an inherited defect in pancreatic islet mGPD or is secondary to metabolic disturbances in the GK rat. In this context, it is of interest that the activity of the glycerol phosphate shuttle is also decreased in islets of animals in which diabetes has been experimentally induced, such as the neonatal streptozotocin rat (12), as well as in other hereditary models of NIDDM, such as the diabetic obese *db/db* mouse (13) and the fatty *fa/fa* rat (14) but not in the obese hyperglycemic *ob/ob* mouse (15).

To attempt to discern if the low islet mGPD activity is a primary or secondary feature of diabetes, GK rats were treated with insulin for 14 days to find out if correction of diabetes corrected the enzyme level. For comparison, other mitochondrial enzyme activities, such as pyruvate carboxylase, succinate dehydrogenase, fumarase, quinone reductase, cytochrome C reductase, malate dehydrogenase, propionyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase, were estimated in islets of GK and Wistar control rats.

RESEARCH DESIGN AND METHODS

GK rat islets. Male diabetic GK rats, 2–3 weeks old, were bred and housed at the Karolinska Institute, Stockholm, Sweden. Age- and sex-matched Wistar rats from a commercial breeder (B & K Universal, Sollentuna, Sweden) were used as controls. All animals were given free access to water and food. One group of GK rats was treated once daily (5:00 P.M.) for 14 days with insulin (4–6 IU subcutaneously of heat-treated Ultralente insulin, Novo-Nordisk A/S, Bagsvaerd, Denmark). Glucose levels were measured at 9:00 A.M. and 4:00 P.M in these animals, as well as in untreated GK and control rats fed ad libitum, from blood obtained by tail snipping on 2 consecutive days before the start of the treatment and then every 3rd day during the 14 days of treatment. At the killing of the rats, blood was collected for determination of glucose and radioimmunological assay of insulin. Islets were isolated by collagenase digestion (16), washed carefully in Hanks' buffer solution, and shipped as frozen pellets on dry ice to Madison, Wisconsin, where enzyme assays and Western analysis were performed within 2 weeks of receipt of the islets.

Estimates of enzyme activities and Western analysis. Islets from one to three rats (200–500 islets per batch) were homogenized in 100–200 μl of a solution of 230 mmol/l mannitol, 70 mmol/l sucrose, and 5 mmol/l potassium HEPES, pH 7.5 (~40 islets/20 μl). Homogenate (5–20 μl) was used for each enzyme assay, Western analysis, or estimate

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GK, Goto-Kakizaki; mGPD, mitochondrial glycerol phosphate dehydrogenase.

TABLE 1
Activities of enzymes in homogenates of pancreatic islets of GK and normal Wistar rats

Enzyme	Enzyme activity	
	GK rats	Wistar rats
mGPD	17 ± 2* (9)	44 ± 5 (9)
Succinate dehydrogenase	25 ± 6 (8)	20 ± 2 (9)
Fumarase	1,093 ± 65 (7)	1,382 ± 141 (8)
Quinone reductase	198 ± 19 (5)	178 ± 16 (5)
Cytochrome C oxidase	19 ± 3 (3)	16 ± 4 (4)
Malate dehydrogenase	1,085 ± 152 (5)	1,133 ± 240 (5)
Propionyl-CoA carboxylase	6.7 ± 0.3 (3)	ND
Methylcrotonyl-CoA carboxylase	1.47 ± 0.32 (6)	1.41 ± 0.32 (7)

Data are means ± SE and the number of batches of islets studied. Enzyme rates are expressed as nmol · min^{-1} · mg^{-1} homogenate protein, except for malate dehydrogenase, which is expressed as μmol · min^{-1} · mg^{-1} homogenate protein. *P < 0.01 vs. Wistar controls. ND, not done because of insufficient sample.

of proteins. Enzyme activities were assayed under V_{max} conditions by standard methods, as previously described. The activity of all enzymes, except mGPD, was estimated in continuous spectrophotometric assays at 37°C in 1 ml of enzyme reaction mixture. mGPD activity was estimated in a 0.2-ml volume in a timed and stopped assay. In each assay, changes in absorbance in blanks without substrate was subtracted from the rates with substrate to give rates attributable to catalysis. Briefly, mGPD activity was estimated in a reaction mixture containing 50 mmol/l L-glycerol-3-phosphate as its D-L isomers, 4 mmol/l iodonitrotetrazolium violet, 1 mmol/l KCN, and 50 mmol/l Bicine buffer, pH 8.0, maintained at 37°C. The reaction was stopped by adding 1 ml of ethyl acetate, and the ethyl acetate layer was separated from the aqueous layer by centrifugation for 3 min. The absorbance of the reduced iodonitrotetrazolium violet in the ethyl acetate extract was measured at 490 nm (6,17). Succinate dehydrogenase activity was estimated in a reaction mixture containing 20 mmol/l succinate, 100 μmol/l phenazine methosulfate, 50 μmol/l dichloroindophenol, 1 mmol/l KCN, 20 μg/ml antimycin A, and 80 mmol/l potassium phosphate buffer, pH 7. The reduction of dichloroindophenol was monitored at 600 nm. Succinate dehydrogenase was activated by adding succinate to the reaction mixture containing islet homogenate 5–10 min before phenazine methosulfate and dichloroindophenol were added (8). Cytochrome C oxidase activity was estimated in a reaction mixture containing 10 μmol/l reduced cytochrome C, 10 μmol/l EDTA, 1 mg/ml bovine serum albumin, and 50 mmol/l MES (2-[N-Morpholino]ethanesulfonic acid) buffer, pH 6.0. Oxidation of cytochrome C was monitored at 550 nm (18). Quinone reductase activity was estimated in a reaction mixture of 50 μmol/l coenzyme Q_0, 200 μmol/l NADH, and 50 mmol/l Tris chloride buffer, pH 7.6. Oxidation of NADH was monitored at 340 nm (19). Fumarase activity was estimated in a reaction mixture containing 20 mmol/l malate and 50 mmol/l potassium phosphate buffer, pH 7.5. The production of fumarate was monitored at 250 nm (20). Malate dehydrogenase activity was estimated in a reaction mixture of 100 μmol/l oxaloacetate, 100 μmol/l NADH, and 80 mmol/l triethanolamine buffer, pH 7.5. The oxidation of NADH was monitored at 340 nm (21).

Pyruvate, propionyl-CoA, and methylcrotonyl-CoA carboxylase activities were estimated by $^{14}CO_2$ fixation in 50 μl of a reaction mixture containing 10 μl of homogenate, 2 mmol/l Na_3ATP, 10 mmol/l $MgCl_2$, 100 mmol/l KCL, 1 mmol/l dithiothreitol, and 8 mmol/l pyruvate (plus 0.2 mmol/l acetyl-CoA), 2 mmol/l propionyl-CoA, or 2 mmol/l β-methylcrotonyl-CoA, respectively. The reaction mixture was maintained at 37°C, and the reaction was stopped after 30 min by adding 50 μl of 10% trichloroacetic acid, as previously described (22–25). Acidified reaction mixture (80 μl) was transferred to a scintillation vial where unreacted $^{14}CO_2$ was permitted to evolve for 3 h. Radioactivity was then estimated by liquid scintillation spectrometry. Radioactivity in blanks (20–25 cpm) containing only acetyl-CoA and/or no substrate was subtracted from the total radioactivity to give the counts attributable to catalysis.

The amount of mGPD protein was estimated by Western analysis as previously described (26) and the amount of pyruvate carboxylase and propionyl-CoA carboxylase plus methylcrotonyl-CoA carboxylase was estimated by probing the nitrocellulose blots with ^{125}I-labeled streptavidin before they were used for Western analysis, also as previously described (26,27).

Enzyme activities were expressed on the basis of protein in homogenates of whole islets estimated by the method of Lowry et al. (28) after proteins were precipitated and washed in 10% trichloroacetic acid.

RESULTS

At killing, the body weights of untreated GK and Wistar control rats were 233 ± 4 (n = 21) and 252 ± 4 (n = 14) g, respectively (P < 0.01). Nonfasting blood glucose concentrations were 11.6 ± 1.1 mmol/l in GK and 5.7 ± 0.3 mmol/l in Wistar rats (P < 0.001), and plasma insulin levels were 270 ± 42 and 288 ± 42 pmol/l, respectively. Before treatment, insulin-treated GK rats (n = 5) had mean nonfasting blood glucose concentrations of 9.1 ± 0.5 mmol/l at 9:00 A.M. and 7.9 ± 0.6 mmol/l at 4:00 P.M. They achieved blood glucose levels during insulin therapy of 3.4 ± 0.3 mmol/l at 9:00 A.M. and 3.7 ± 0.5 mmol/l at 4:00 P.M., as compared with 4.4 ± 0.5 and 4.9 ± 0.5 mmol/l, respectively, in Wistar rats (n = 5) receiving saline (NS). A small group of untreated GK rats (n = 3) with blood glucose concentrations of 8.9 ± 0.4 and 7.2 ± 0.7 mmol/l at the morning and afternoon time points was also included in this experiment. During the 2-week period of insulin treatment, GK rats increased in weight from 219 ± 5 to 267 ± 6 g, while nontreated GK rats increased from 223 ± 11 to 253 ± 9 g.

The mGPD enzyme activity in pancreatic islets of GK rats was 35–38% of that in normal Wistar rats (Tables 1 and 2). Western analysis showed that the amount of mGPD protein in islets of GK rats was also about one-half, or less, of that in islets of Wistar rats (Fig. 1). In islets from GK rats treated with insulin for 2 weeks, the mGPD enzyme activity was completely normalized to the level found in Wistar rat islets (Table 2).

The activities of succinate dehydrogenase, fumarase, quinone reductase, cytochrome C oxidase, and malate dehydrogenase were similar in GK and Wistar rat islets (Tables 1 and 2), whereas pyruvate carboxylase activity was 45% of that in the GK rat islets (P < 0.01) (Table 2). Nitrocellulose blots of

TABLE 2
Effect of treating GK rats with insulin on activities of mGPD, pyruvate carboxylase, and other enzymes in pancreatic islets

Enzyme	Enzyme activity (nmol · min^{-1} · mg^{-1} homogenate protein)		
	Wistar	GK untreated	GK insulin-treated
mGPD	26 ± 2.7	9.1 ± 1.6*	25 ± 3.4
Succinate dehydrogenase	33 ± 4.4	28 ± 6.9	32 ± 4.1
Pyruvate carboxylase	5.3 ± 0.8	2.4 ± 0.2*	6.4 ± 1.2
Propionyl-CoA carboxylase	4.9 ± 0.5	6.3 ± 0.5	8.0 ± 1.4
Methylcrotonyl-CoA carboxylase	1.6 ± 0.2	1.2 ± 0.2	1.6 ± 0.5

Data are means ± SE and are from the five normal Wistar rats, three untreated GK rats, and five insulin-treated GK rats. *P < 0.01 vs. normal Wistar and GK insulin-treated rats.

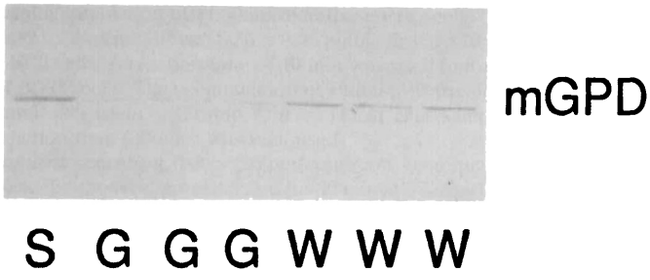

FIG. 1. Western analysis of mGPD in pancreatic islets of GK, normal Wistar, and normal Sprague-Dawley rats. There was 30 μg of protein per lane. G, Goto-Kakizaki; W, Wistar; SD, Sprague-Dawley.

PAGE-separated islet proteins, probed with ^{125}I-streptavidin that binds to biotin-containing enzymes, showed that the amount of pyruvate carboxylase protein (M_r of 116,000) was also low in GK rat islets (Fig. 2). It was also evident from the streptavidin-probed blot that islets contain a biotin-containing enzyme(s) with an M_r of ~75,000–80,000 (Fig. 2). The density of this protein band in islets from the GK rats was the same as in that of the Wistar and Sprague-Dawley rats. By a CO_2 fixation assay, this band was shown to probably represent propionyl-CoA carboxylase (M_r of 74,000) alone or propionyl-CoA carboxylase plus methylcrotonyl-CoA carboxylase (M_r of 79,000) (29,30). This is because the enzyme activity of propionyl-CoA carboxylase was four- to fivefold higher than the activity of methylcrotonyl-CoA carboxylase (Tables 1 and 2). Insulin treatment of GK rats normalized the activity of pyruvate carboxylase, while the activities of succinate dehydrogenase, propionyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase remained similar to those of untreated GK rats (Table 2).

DISCUSSION

The high enzyme activity (6–10) and amount of mGPD protein (31) in the islet β-cell in comparison with other tissues, as well as functional studies (32), suggests that the glycerol phosphate shuttle has a role in glucose-induced insulin secretion. In agreement with previous reports (4,5,11), mGPD enzyme activity in pancreatic islets of GK rats was shown to be markedly decreased (Tables 1 and 2). Because the amount of mGPD protein, as judged from Western analysis, was also reduced in islets of GK rats (Fig. 1), the decreased assayable enzyme activity is most likely due to decreased net synthesis of mGPD rather than a result of decreased activity of a normal amount of enzyme. A new finding of the current study was the low pyruvate carboxylase enzyme activity (Table 2) and protein (Fig. 2) in the islets of the GK rat. Pyruvate carboxylase is as abundant in the normal islet as in the gluconeogenic tissues, such as liver and kidney (25). However, the islet cannot perform gluconeogenesis because it lacks phosphoenolpyruvate carboxykinase (33–35). Pyruvate carboxylase likely plays a key role in glucose-induced insulin secretion by participating in a pyruvate malate shuttle that transports reducing equivalents out of the mitochondria as malate, which then provides NADPH via the malic enzyme reaction in the cytosol (25). The enzyme activity of both mGPD and pyruvate carboxylase was corrected by insulin treatment (Table 2).

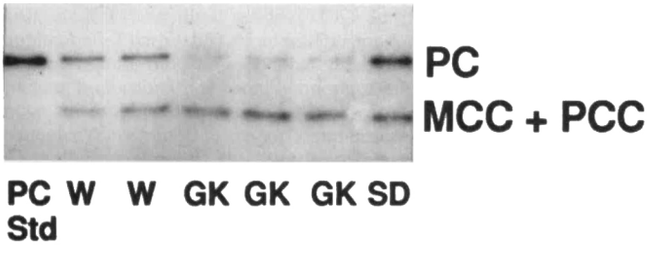

FIG. 2. Pyruvate carboxylase and propionyl-CoA carboxylase plus methylcrotonyl-CoA carboxylase in pancreatic islets of GK, normal Wistar, and Sprague-Dawley rats. Pancreatic islet proteins were separated by PAGE and transferred to nitrocellulose, which was probed with islet ^{125}I-streptavidin. There was 30 μg of protein per lane. PC Std, lane with 0.3 μg of authentic pyruvate carboxylase; W, Wistar; GK, Goto-Kakizaki; SD, Sprague-Dawley; PC, the pyruvate carboxylase band; MCC + PCC, the band of methylcrotonyl-CoA carboxylase and/or propionyl-CoA carboxylase.

The major glucose transporter in the pancreatic β-cell is GLUT2, and it is decreased by ~50% in the β-cell in several rodent models of NIDDM, including the GK rat (1), the Zucker diabetic fatty rat (36,37), the *db/db* diabetic mouse (38), and the partially pancreatectomized model (39). The decrease in GLUT2 appears to be localized to the islet, is proportional to the severity of hyperglycemia, and is reversible (38,40). It thus appears that at least three proteins that are relatively preferentially expressed in the β-cell and that play key roles in β-cell glucose metabolism are also most affected by diabetes. Because glucose transporter activity is 100-fold higher than the rate of glucose metabolism in the β-cell, it is unlikely that a 50% decrease in GLUT2 in GK islets is sufficient to impair glucose metabolism and insulin secretion. Similarly, because mGPD (6,10,31) and pyruvate carboxylase (25,27) are quite abundant in the islet, it seems unlikely that a 40–60% reduction in the levels of these proteins would severely impede glucose metabolism and insulin secretion. Like GLUT2, both mGPD and pyruvate carboxylase are low in the islet in more than one model of NIDDM. Islet mGPD is decreased both in genetic models, such as the Zucker diabetic rat (14; M.J.M., J. Tang, K.S. Polonsky, unpublished observations) and the *db/db* mouse (13) and in experimentally induced models of NIDDM, such as adult rats that received streptozotocin in the neonatal period (5,12) and islets of partially pancreatectomized rats (J.L. Leahy, M.J.M., unpublished observations). Pyruvate carboxylase has not been as extensively studied. However, it is low in islets of the Zucker diabetic fatty rat (M.J.M., J. Tang, K.S. Polonsky, unpublished observations). Low enzyme levels in both genetic and experimentally induced models of diabetes and the reversibility with correction of diabetes and/or hyperglycemia suggest that the low levels of GLUT2, mGPD, and pyruvate carboxylase may well be a concomitant, rather than a cause, of diabetes.

The activities of several other enzymes assayed in the islet of the GK rat were normal. This included succinate dehydrogenase, which is a particularly good control for mGPD because, like mGPD, it is an FAD-containing enzyme that is located in the inner mitochondrial membrane and also transfers electrons directly to ubiquinone. A secondary finding of the current study is that it was the first to identify propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase enzyme activities in pancreatic islets from healthy rats. Propionyl-CoA carboxylase activity approximated that of pyruvate carboxylase, whereas methylcrotonyl-CoA carboxylase activity was about one-fifth that of the other two carboxylases. Both propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase activities were normal in GK rat islets, and insulin treatment did not significantly affect their

activities. It is of interest that methylcrotonyl-CoA carboxylase catalyzes a reaction in the metabolic pathway of leucine, which is a potent nutrient insulin secretagogue. This reaction is the carboxylation of β-methylcrotonyl–CoA to β-methylglutaconyl–CoA. It is noteworthy that leucine-induced insulin release is also impaired in GK rats (41). However, this defect cannot be accounted for by a decreased methylcrotonyl-CoA carboxylase activity. Propionyl-CoA carboxylase catalyzes a reaction in the pathways for metabolism of valine, isoleucine, and for fatty acids with odd numbers of carbons. Because neither of these branched chain amino acids nor these types of fatty acids are known to stimulate insulin secretion, the purpose of propionyl-CoA carboxylase in the islet, as well as its location in the various types of cells present in the islet, are unknown.

It is interesting to speculate that the low levels of GLUT2, mGPD, and pyruvate carboxylase in the β-cell in NIDDM are an adaptive response by the cell to attempt to protect itself from a high concentration of glucose by modulating glucose metabolism. In the case of mGPD, however, it is less likely that a high concentration of glucose itself downregulates mGPD because islets cultured for 1 day in the presence of a high concentration of glucose exhibit normal mGPD enzyme activity (26). Although it cannot be ruled out that culture for only 1 day was insufficient to cause a change in the amount of the enzyme, culture of islets for 1 day in the presence of various concentrations of glucose causes profound changes in their ability to release insulin in response to glucose and other secretagogues (42), as well as changes in activities and mRNAs encoding various enzymes of glucose metabolism (27,43,44). It is possible that something other than hyperglycemia in the diabetic syndrome causes the downregulation of mGPD. It has been suggested that a high concentration of fatty acids in the islet may be in part responsible for the metabolic abnormalities in the β-cell in NIDDM (45,46).

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