The β-cell biochemical mechanisms that account for the compensatory hyperfunction with insulin resistance (so-called β-cell adaptation) are unknown. We investigated glucose metabolism in isolated islets from 10–12-week-old Zucker fatty (ZF) and Zucker lean (ZL) rats (results expressed per mg/islet of protein). ZF rats were obese, hyperlipidemic, and normoglycemic. They had a 3.8-fold increased β-cell mass along with 3–10-fold increases in insulin secretion to various stimuli during pancreas perfusion despite insulin content per milligram of β-cells being only one-third that of ZL rats. Islet glucose metabolism (utilization and oxidation) was 1.5–2-fold increased in the ZF islets despite pyruvate dehydrogenase activity being 30% lowered compared with the ZL islets. The reason was increased flux through pyruvate carboxylase (PC) and the malate-pyruvate shuttles based on the following observations (% ZL islets): increased V_{max} of PC (160%), malate dehydrogenase (170%), and malic enzyme (275%); elevated concentrations of oxaloacetate (150%), malate (250%), citrate (140%), and pyruvate (250%); and 2-fold increased release of malate from isolated mitochondria. Inhibition of PC by 5 mM phenylacetic acid markedly lowered glucose-induced insulin secretion in ZF and ZL islets. Thus, our results suggest that PC and the pyruvate shuttles are increased in ZF islets, and this accounts for glucose mitochondrial metabolism being increased when pyruvate dehydrogenase activity is reduced. As the anaplerosis pathways are implicated in glucose-induced insulin secretion and the synthesis of glucose-derived lipid and amino acids, our results highlight the potential importance of PC and the anaplerosis pathways in the enhanced insulin secretion and β-cell growth that characterize β-cell adaptation to insulin resistance.

Insulin resistance is tissue insensitivity to the regulatory effects of insulin on glucose and fatty acid metabolism. Insulin resistance is a risk factor for type 2 diabetes (1). However, most affected individuals do not develop diabetes because of a compensatory increase in insulin secretion (2). The mechanism of the β-cell adaptation is poorly understood. Particularly unclear is the dichotomy that insulin resistance is typically accompanied by elevated blood and tissue triglyceride and fatty acid (FA) levels (3) when multiple studies of isolated islets and β-cell lines cultured with FA have shown detrimental effects on β-cell function and viability (4–7).

A well known mechanism for altered cellular function from excess FA is the glucose-fatty acid cycle of Randle, part of which is impaired activation of pyruvate dehydrogenase (PDH) (8). This effect is reported to occur in FA-cultured islets (9). PDH supplies pyruvate-derived acetyl-CoA to the citrate cycle so that in most tissues excess FA results in lowered ATP production. Intact β-cell mitochondrial function is required for glucose-induced insulin secretion (10, 11), leading to speculation that this is a mechanism of FA-induced impaired β-cell function (9). However, lowering and raising PDH activity in clonal β-cells through adenoviral overexpression of PDH kinase and the catalytic subunit of PDH phosphatase, respectively, had little effect on glucose-induced insulin secretion (12). This may reflect a second pathway for mitochondrial pyruvate metabolism in β-cells through pyruvate carboxylase (PC). Downstream of PC are pyruvate shuttles (13, 14) and malonyl-CoA (15, 16) which are proposed to provide coupling factors for glucose-induced insulin secretion. The effect of FA on these pathways is uncertain.

We investigated rat islets cultured for 4 days with 0.25 mM oleate/5.5 mM glucose (17). PDH activity was 35% lowered, but glucose oxidation and insulin secretion were normal to supernormal depending on the studied glucose concentration. We proposed the reason was the observed unaffected PC activity and enhanced flux through the malate-pyruvate shuttle; the latter is proposed to be an effector of insulin secretion through the malic enzyme production of cytosolic NADPH (13, 18, 19). Support for our conclusion is the lowered PC expression resulting in defective malate-pyruvate shuttle activity reported in FA-cultured islets with impaired glucose-induced insulin secretion (20). Also, PC expression and activity are lowered in islets of diabetic rodents with defective glucose-induced insulin secretion (21, 22).

These results focus on PC and the malate-pyruvate shuttle as components of the β-cell adaptation in states of excess FA such as insulin resistance. However, all of the cited studies were performed in vitro. The current study investigated Zucker fa/fa fatty rats. These rats are obese, insulin resistant, and hyperlipidemic because of mutated leptin receptors (23). Moreover, they are not diabetes-prone (24, 25), making them an

β-Cell Adaptation to Insulin Resistance

INCREASED PYRUVATE CARBOXYLASE AND MALATE-PYRUVATE SHUTTLE ACTIVITY IN ISLETS OF NONDIABETIC ZUCKER FATTY RATS

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The β-cell biochemical mechanisms that account for the compensatory hyperfunction with insulin resistance (so-called β-cell adaptation) are unknown. We investigated glucose metabolism in isolated islets from 10–12-week-old Zucker fatty (ZF) and Zucker lean (ZL) rats (results expressed per mg/islet of protein). ZF rats were obese, hyperlipidemic, and normoglycemic. They had a 3.8-fold increased β-cell mass along with 3–10-fold increases in insulin secretion to various stimuli during pancreas perfusion despite insulin content per milligram of β-cells being only one-third that of ZL rats. Islet glucose metabolism (utilization and oxidation) was 1.5–2-fold increased in the ZF islets despite pyruvate dehydrogenase activity being 30% lowered compared with the ZL islets. The reason was increased flux through pyruvate carboxylase (PC) and the malate-pyruvate shuttles based on the following observations (% ZL islets): increased V_{max} of PC (160%), malate dehydrogenase (170%), and malic enzyme (275%); elevated concentrations of oxaloacetate (150%), malate (250%), citrate (140%), and pyruvate (250%); and 2-fold increased release of malate from isolated mitochondria. Inhibition of PC by 5 mM phenylacetic acid markedly lowered glucose-induced insulin secretion in ZF and ZL islets. Thus, our results suggest that PC and the pyruvate shuttles are increased in ZF islets, and this accounts for glucose mitochondrial metabolism being increased when pyruvate dehydrogenase activity is reduced. As the anaplerosis pathways are implicated in glucose-induced insulin secretion and the synthesis of glucose-derived lipid and amino acids, our results highlight the potential importance of PC and the anaplerosis pathways in the enhanced insulin secretion and β-cell growth that characterize β-cell adaptation to insulin resistance.

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A well known mechanism for altered cellular function from excess FA is the glucose-fatty acid cycle of Randle, part of which is impaired activation of pyruvate dehydrogenase (PDH) (8). This effect is reported to occur in FA-cultured islets (9). PDH supplies pyruvate-derived acetyl-CoA to the citrate cycle so that in most tissues excess FA results in lowered ATP production. Intact β-cell mitochondrial function is required for glucose-induced insulin secretion (10, 11), leading to speculation that this is a mechanism of FA-induced impaired β-cell function (9). However, lowering and raising PDH activity in clonal β-cells through adenoviral overexpression of PDH kinase and the catalytic subunit of PDH phosphatase, respectively, had little effect on glucose-induced insulin secretion (12). This may reflect a second pathway for mitochondrial pyruvate metabolism in β-cells through pyruvate carboxylase (PC). Downstream of PC are pyruvate shuttles (13, 14) and malonyl-CoA (15, 16) which are proposed to provide coupling factors for glucose-induced insulin secretion. The effect of FA on these pathways is uncertain.

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These results focus on PC and the malate-pyruvate shuttle as components of the β-cell adaptation in states of excess FA such as insulin resistance. However, all of the cited studies were performed in vitro. The current study investigated Zucker fa/fa fatty rats. These rats are obese, insulin resistant, and hyperlipidemic because of mutated leptin receptors (23). Moreover, they are not diabetes-prone (24, 25), making them an

The abbreviations used are: FA, fatty acids; ZF, Zucker fatty rats; ZL, Zucker lean rats; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; BSA, bovine serum albumin; RIA, radioimmunoassay; PAA, phenylacetic acid; KRB, Krebs-Ringer bicarbonate; PFK, phosphofructokinase; MDH, malate dehydrogenase; mGPD, mitochondrial glycerol-3-phosphate dehydrogenase; G6P, glucose 6-phosphate.
excellent model to investigate \( \beta \)-cell adaptation to insulin resistance.

**MATERIALS AND METHODS**

**Animals and Isolated Islets—** Zucker fatty (fa/fa, ZF) and Zucker lean (fa/+, ZL) rats were studied at 10–12 weeks of age (Harlan, Indianapolis, IN) except for \( \beta \)-cell mass measurements that were performed in 10-week-old ZF and ZL rats. Tail vein blood was obtained from normally fed rats at 9:00 a.m.; glucose concentration was measured using a glucose analyzer II (Beckman, Fullerton, CA), and plasma insulin, triglycerides, and FA were measured by commercial kits (Alpco; Winham, NH; Sigma; Wakó; Richmond, VA, respectively). Islets were isolated by an adaptation of the Gotoh method (26): pancreas ducts were perfused with 0.1 M Tris-HCl buffer, pH 7.6, 40 mM glucose, and 0.07% BSA unless stated otherwise. Because of the insulin content post acid ethanol extraction using an insulin RIA (28), samples were collected in tubes on ice that contained 8 mg of EDTA and half the number of islets from obese Zucker fatty (ZF) rats, methods utilized half the number of islets from obese versus lean rats and results are expressed per milligram of islet protein.

**Pancreas \( \beta \)-Cell Mass—** Rats were euthanized, and pancreata dissected and immersion-fixed in 4.0% paraformaldehyde/100 mM phosphate-buffered saline. Following paraffin embedding, 5-\mu m sections throughout the entire pancreas were mounted as ribbons on microscope slides to facilitate section counting. At 200-\mu m intervals, (20 slides/pancreas), sections were rehydrated and immunostained with guinea pig anti-insulin IgG (Linco, St. Charles, MO) followed by donkey anti-guinea pig IgG-peroxidase (Jackson ImmunoResearch, West Grove, PA). Following development with diaminobenzidine/H\(_4\)O\(_2\), sections were counterstained with hematoxylin, cleared, and mounted in Permount. The proportion of \( \beta \)-cell surface area versus surface area of the whole pancreas was determined by digitally imaging at least three non-overlapping 3.89 mm\(^2\) fields per section on a Zeiss Universal microscope coupled to a Spot RT color charge-coupled device camera (Diagnostics Instruments, Sterling Heights, MI). \( \beta \)-Cell mass was calculated for each animal as the average proportional \( \beta \)-cell surface area multiplied by pancreatic weight.

**In Situ Perfused Pancreas and Pancreas Insulin Content—** The perfusion technique has been described elsewhere (29). The perfusate was oxygenated KRB buffer, pH 7.4, that contained 4% dextran T70 and 0.2% BSA fraction V (Sigma). The perfusion protocol is shown at the top of Fig. 1. Following 17 min of equilibration at 7.8 mM glucose, 1-min samples were collected in tubes on ice that contained 8 mg of EDTA and stored at \(-20^\circ\)C pending analysis by insulin RIA. Immediately after the perfusion, the pancreas was excised, blotted, and stored at \(-20^\circ\)C in acid ethanol pending homogenization and insulin RIA.

**Plasma Insulin, Triglycerides, and Free Fatty Acids—** Plasma insulin (ng/ml) and triglycerides (mg/dl) were determined using a commercial kit (Randox, Crumlin, U.K.). Plasma free fatty acids (mmol/l) were determined using a specific kit (Wako Chemicals, Richmond, VA). Insulin was measured using a kit with an antibody raised against Wistar rat insulin (DiaMetra, Minneapolis, MN). Plasma glucose was measured as described (30). 6-Phosphogluconate dehydrogenase activity was measured as described (31). Islets were transferred to a UV cuvette containing 1 ml of citrate lyase solution with the addition of reagent (9 units/ml of type II glutamate dehydrogenase from yeast; Boehringer Mannheim). Fluorescence was determined with a dual excitation-multiplex instrument (PerkinElmer, Norwalk, CT). NADPH was amplified by the cycling method by adding 100 \( \mu \)l of substrate (glucose-6-phosphate) and 10 \( \mu \)l of 6-phosphogluconate dehydrogenase (Fisher Scientific, Fair Lawn, NJ) to the cuvette. The reaction was initiated by adding 10 \( \mu \)l of citrate lyase solution to the cuvette with the Δ absorbance of 540 nm (excitation) and 465 nm (emission) monitored over a period of 5 min. NADPH was determined using a single excitation wavelength with the Δ absorbance representing the citrate content.

**Insulin Degradation in Zucker Fatty Rats**

**TABLE I**

| ZL | ZF | \( p \) value |
|----|----|---------------|
| \( n \) | \( n \) | \( \chi^2 \) |
| Body weight (g) | 245 ± 10 (13) | 357 ± 10 (13) | 0.001 |
| Blood glucose (mM) | 7.8 ± 0.1 (7) | 7.7 ± 0.2 (4) | NS |
| Plasma insulin (ng/ml) | 1.9 ± 0.1 (7) | 18.0 ± 0.2 (6) | NS |
| Plasma triglycerides (mg/dl) | 69 ± 4 (6) | 545 ± 39 (6) | 0.001 |
| Plasma free fatty acids (mmol/l) | 0.68 ± 0.04 (6) | 1.01 ± 0.04 (6) | 0.001 |

**Pancreas**

| ZL | ZF | \( p \) value |
|----|----|---------------|
| Weight (g) | 1.58 ± 0.08 (5) | 1.86 ± 0.37 (5) | NS |
| \( \beta \)-Cell mass (mg/pancreas) | 22 ± 0.5 (5) | 85 ± 3 (5) | 0.001 |
| Insulin content (mg/g pancreas) | 58 ± 3 (5) | 76 ± 8 (5) | NS |

**Isolated Islet**

| ZL | ZF | \( p \) value |
|----|----|---------------|
| DNA (ng/islet) | 44 ± 1 (4) | 118 ± 7 (4) | 0.001 |
| Protein (\( \mu \)g/islet) | 0.56 ± 0.05 (4) | 1.76 ± 0.13 (4) | 0.001 |
| Insulin content (ng/islet) | 31 ± 3 (6) | 41 ± 4 (6) | NS |

**FIG. 1. In situ perfusion of 10–12-week-old ZL and ZF rats.** Following a 17-min equilibration at 7.8 mM glucose, 1-min samples were collected and analyzed by insulin RIA. Data points are mean ± S.E. of each group.

- **General**
  - **ZL** weight: 245 ± 10 (13) g
  - **ZF** weight: 357 ± 10 (13) g
  - **Blood glucose**: ZL 7.8 ± 0.1 (7) mM, ZF 7.7 ± 0.2 (4) mM
  - **Plasma insulin**: ZL 1.9 ± 0.1 (7) ng/ml, ZF 18.0 ± 0.2 (6) ng/ml
  - **Plasma triglycerides**: ZL 69 ± 4 (6) mg/dl, ZF 545 ± 39 (6) mg/dl
  - **Plasma free fatty acids**: ZL 0.68 ± 0.04 (6) mmol/l, ZF 1.01 ± 0.04 (6) mmol/l

- **Pancreas**
  - **Weight**: ZL 1.58 ± 0.08 (5) g, ZF 1.86 ± 0.37 (5) g
  - **\( \beta \)-Cell mass**: ZL 22 ± 0.5 (5) mg, ZF 85 ± 3 (5) mg
  - **Insulin content**: ZL 58 ± 3 (5) mg/g pancreas, ZF 76 ± 8 (5) mg/g pancreas

- **Isolated Islet**
  - **DNA**: ZL 44 ± 1 (4) ng/islet, ZF 118 ± 7 (4) ng/islet
  - **Protein**: ZL 0.56 ± 0.05 (4) \( \mu \)g/islet, ZF 1.76 ± 0.13 (4) \( \mu \)g/islet
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and centrifuged 10 min at 12,000 × g. Supernatant was neutralized with 3 M KHCO₃ and centrifuged at 12,000 × g. Malate and pyruvate standards were prepared in perchloric acid. Malate: 50 µl of extract or malate standard (0.1–1 nmol) was added to 250 µl of reaction buffer (20 mM 2-aminooxy-methyl propanol, pH 9.9, 2 mM glutamate, 50 µM NAD, 1 µg of aspartate aminotransferase, 2.5 µg of malate dehydrogenase) for 20 min at 30 °C. Nonmetabolized NADH was removed by addition of 0.25 ml of potassium phosphate, pH 11.9, and incubation at 60 °C for 15 min, followed by addition of 12 µl of 1 M imidazole and another 15 min at 60 °C. Fluorescence was measured at 340 nm excitation and 465 nm emission. Pyruvate: 30 µl of extract or pyruvate standard (20–200 pmol of sodium pyruvate) was added to 100 µl of reaction buffer (50 mM imidazole, pH 7, 0.6 mM ascorbate, 0.2 mg/ml BSA, 6 µM NADH, 0.125 units/ml lactate dehydrogenase) at room temperature for 20 min. Nonmetabolized NADH was removed by addition of 20 µl of 2 M HCl at room temperature for 30 min followed by 1 ml of 6 N NaOH for 10 min at 60 °C. Fluorescence was measured at 380 nm excitation and 460 nm emission.

Oxaloacetate was measured as described (17). Islets (50 ZL, 25 ZF) were lysed 20 min in 40 µl of 0.25 M perchloric acid at −20 °C followed by sonication and addition of 20 µl of 0.94 M KOH. Extract (30 µl) or oxaloacetate standard in perchloric acid (0.2–2.0 µmol) was added to 200 µl of reaction buffer (75 mM K₂PO₄, pH 7.4, 80 nM acetyl-[3H]CoA, 50 µM/ml citrate synthase) at room temperature for 60 min. The reaction was stopped by 600 µl of charcoal mixture (8 g of charcoal, 38 g of citric acid monohydrate, 120 ml of 95% ethanol) followed by centrifugation at 12,000 × g and liquid scintillation counting of the supernatant.

Enzyme Activities—Phosphofructokinase (PFK; Vₚₕₚₖₓ was measured as described (32). Islets (100 ZL or 50 ZF per 0.1 ml of extraction buffer) were sonicated on ice in 15 mM K₂PO₄, pH 7.0, 100 mM KC1, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 50 µM/ml aprotinin and then centrifuged at 12,000 × g for 15 min at 4 °C. Supernatant (20 µl) was added to 1 ml of reaction buffer containing 50 mM Tris/HCl, pH 9.0, 1 mM EDTA, 2.5 mM dithiothreitol, 2 mM MgCl₂, 5 mM aminomethyl sulfonate, 1 mM ATP, 1 mM fructose-6-P, 0.16 mM NA DH, 0.4 units/ml aldolase, 2.4 units/ml triosephosphate isomerase, 0.8 units/ml glyceraldehyde-3-phosphate dehydrogenase mixture in a quartz cuvette and the NADH metabolized over 15 min was assessed by spectrophotometer at 340 nm. PFK activity was calculated based on 1 µmol of fructose-1,6-P₂ produced per min of NADH consumed.

Citrate synthase was measured as described (31). Homogenized islet extract (100 ZL, 50 ZF) in HEPES 10 mM, pH 7.4, 250 mM sucrose, 2.5 mM EDTA, 2 mM cysteine, and 0.02% BSA (40 µl) was added to 0.55 ml of 100 mM HEPES, pH 7.4, 0.1 mM oxaloacetate, 0.1 mM 5,5′-dithiobis (nitrobenzoic acid), 0.05 mM acetyl-CoA in a UV cuvette. CoA production was assessed spectrophotometrically at 412 nm.

Active PDH was measured as described (17). Islets (300 ZL, 150 ZF) were homogenized on ice in 0.3 ml of 50 mM HEPES, pH 7.5, 0.2 mM KC1, 3 mM EDTA, 5 mM dithiothreitol, 0.1 mM N₅⁰-p-tosyl-l-lysine chloromethyl ketone, 0.1 mM trypsin inhibitor, 0.02 trypsin inhibitory units/ml aprotinin, 2% rat serum, and 0.25% (v/v) Triton X-100 and then freeze-thawed three times and passed through a 0.5-ml insulin syringe 10 times. Extract (50 µl) and 50 µl of reaction buffer (50 mM HEPES, pH 7.5, 1 mM MgCl₂, 3 mM NAD, 0.4 mM thiamine pyrophosphate, 0.4 mM CoA, 2 mM dithiothreitol, 0.1% Triton X-100, 7.5 units/ml lipopamide dehydrogenase, 1 mM pyruvate, 0.1 µCi of [1-1⁴C]pyruvate) were added to a cup inside a rubber-stoppered 20-ml scintillation vial that contained a center well with filter paper. Following incubation at 37 °C for 20 min, the reaction was stopped by injecting 200 µl of 1 N HCl into the cup. CO₂ was trapped in the filter paper by injecting 100 µl of 1 N KOH into the center well. PDH activity 1 unit = 1 µmol of CO₂/min.

PC and malate dehydrogenase (MDH) were measured as described (17). Islets (100 ZL, 50 ZF) were homogenized in 10 mM HEPES, pH 7.4, 250 mM sucrose, 2.5 mM EDTA, 2 mM cysteine, and 0.02% BSA. PC: 80 µl of extract was added to 0.92 ml of reaction buffer (80 mM Tris/HCl, pH 8, 5.2 mM ATP, 8 mM potassium pyruvate, 21 mM KHCO₃, 9 mM MgSO₄, 0.16 mM acetyl-CoA, 0.16 mM NADH, 5 units/ml malate dehydrogenase). Absorbance at 340 nm was measured for 10 min. MDH: 10 µl of extract was added to 0.82 ml of 0.12 mM glycine, pH 10, 100 µl of 0.85 M l-malate, pH 7, and 67 µl of 37.5 mM NaOH. Absorbance at 340 nm was measured for 20 min.

Malate enzyme was measured as described (17). Islets (100 ZL, 50 ZF) were sonicated in 100 µl of 50 mM triethanolamine, pH 7.4, 3 mM MnCl₂, and 0.02% BSA. Extract (50 µl) or NADPH standard (1–12 nmol) was added to 0.95 ml of prewarmed reaction buffer (50 mM triethanolamine, pH 7.4, 3 mM MnCl₂, 0.02% BSA, 0.1 mM NADP, 1 mM l-malate).
Enzyme activities in freshly isolated islets of 10–12-week-old ZL and ZF rats. A, pyruvate dehydrogenase (n = 5); B, pyruvate carboxylase (n = 7); C, malate dehydrogenase (n = 4); and D, malic enzyme (n = 4). Data are mean ± S.E.

Fluorescence was measured 20 min at 340 nm excitation and 420 nm emission.

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPD) was measured as described (17). Islets (100 ZL, 50 ZF) were sonicated in 0.1 ml of 5 mM HEPES, pH 7.5, 230 mM mannitol, and 70 mM sucrose. Extract (10 μl) was added to 200 μl of reaction buffer (50 mM Bisce buffer, pH 8, 1 mM KCN, 50 mM t-glycerol-3-phosphate, 4 mM iodonitrotetrazolium violet) for 10 min at 37°C. The reaction was stopped by adding 1 ml of ethyl acetate followed by centrifugation and absorbance determination of the ethyl acetate layer at 490 nm. Results were compared with the standard curve made with 0–10 nmol of iodonitrotetrazolium violet in reaction mixture with or without (as blank) islet extract.

Malate Release from Isolated Islets—The method is previously described (17). Mitochondrial isolation: islets (400 ZL, 200 ZF) were homogenized in 0.4 ml of 5 mM potassium HEPES, pH 7.5, 230 mM mannitol, and 70 mM sucrose and centrifuged at 600 × g for 5 min to sediment the nuclear and cell debris, followed by recentrifugation of the supernatant at 5,500 × g for 10 min to sediment the mitochondria. The sedimented mitochondria were resuspended in 120 μl of ice cold buffer (5 mM potassium HEPES, pH 7.3, 5 mM K2PO4, 5 mM KHCO3, 2 mM Na2ADP, 230 mM mannitol, and 70 mM sucrose) and centrifuged at 14,000 rpm for 2 min followed by addition of 15 μl of 0.92 N perchloric acid to the supernatant, return of pH to 7.0 with 1 M KOH, and recentrifugation at 14,000 rpm for 2 min. Malate was measured using the method of MacDonald (13). The mixture was placed at 37°C, and 50-μl samples were taken at 0 and 10 min. Samples were centrifuged at 14,000 rpm for 2 min followed by addition of 15 μl of 0.92 N perchloric acid to the supernatant, return of pH to 7.0 with 1 M KOH, and recentrifugation at 14,000 rpm for 2 min. Malate was measured using the method of Sener et al. (33). Supernatant (40 μl) or malate standard (0–30 pmol) was added to 200 μl of reaction buffer (100 mM Tris/KCl, pH 8.0, 1 mM NAD, 0.2 mM [3H]acetyl-CoA, 20 μg/ml malate dehydrogenase from pig heart, 60 μg/ml citrate synthase from pig heart) at room temperature for 60 min. The final product of [3H]citrate was separated with 600 μl of charcoal mixture (120 ml of 95% ethanol, 8 g of charcoal, 38 g of citric acid monohydrate) and centrifuged at 14,000 rpm for 5 min followed by liquid scintillation counting of the supernatant.

Data Presentation—All data are expressed as mean ± S.E. For protocols that used isolated islets from a single rat, the n values are the number of rats studied. If pooled islets from more than one rat were needed, the n values are the number of experiments performed. Statistical significance was determined by the unpaired Student’s t test.

RESULTS

General Characteristics of ZF and ZL Rats and Isolated Islets—The 10–12 week-old ZF rats were obese, normoglycemic, hyperinsulinemic, and hyperlipidemic versus the lean controls (Table 1). ZF rats had a 3.8-fold increased mass of islet β-cells. Pancreas insulin content was 150% of the ZL rats so that insulin content per mg of β-cells in the ZF rats was only 35% of the ZL rats (0.9 ± 0.1 μg/mg β-cells ZF versus 2.6 ± 0.1 μg/mg β-cells ZL, p < 0.001). Isolated islets were similar: ZF islets were hypercellular compared with ZL islets (3-fold increased DNA and protein contents), and insulin content per islet protein was 40% of the ZL islets (23 ± 3 ng of insulin/μg of protein ZF versus 56 ± 9 ng of insulin/μg of protein ZL, p < 0.001). Pancreas perfusions carried out at 7.8, 16.7, and 16.7 mM glucose plus 10 mM arginine (Fig. 1) showed a 10-fold increase of insulin secretion at 7.8 mM glucose, and 3-fold increases at both high glucose conditions in the ZF rats (all p < 0.001).

Islet Glucose Metabolism—Glucose utilization (Fig. 2A) and oxidation (Fig. 2B) per mg of islet protein were 1.5–2-fold increased in the ZF islets at 2.8 and 16.7 mM glucose (utilization 2.8 mM glucose p < 0.025, 16.7 mM glucose p < 0.001; oxidation 2.8 mM glucose p < 0.015, 16.7 mM glucose p < 0.005). We next investigated potential mechanisms for the increased glucose metabolism.

We previously studied rat islets cultured for 1 day with palmitate and noted increased basal glucose metabolism and insulin secretion (30), possibly because of a lowered concentration of G6P that diminished end-product inhibition of hexokinase. We proposed that the mechanism was up-regulation of PFK activity through a dual effect, increased PFK expression and lowered production of the PFK allosteric inhibitor citrate by citrate synthase, so that flux through glycolysis was increased (31, 32). However, we found no difference in G6P level between the ZF and ZL islets at 2.8 mM glucose (0.99 ± 0.08 nmol/mg protein ZF versus 1.26 ± 0.14 nmol/mg protein ZL, n = 4, p = NS), Vmax values for PFK (52 ± 2 nmol/min/mg protein ZL versus 68 ± 14 nmol/min/mg protein ZF, n = 6, p = NS), or citrate synthase (44 ± 3 nmol/min/mg protein ZL versus 43 ± 5 nmol/min/mg protein ZF, n = 4, p = NS). Rather, the citrate concentration after 60 min of incubation at 8.3 mM glucose was increased in the ZF islets (Fig. 3A).

Islet Pyruvate Metabolism—An alternate mechanism based on our study of 4-day oleate-cultured islets (17) was impaired PDH activity with diversion of pyruvate metabolism through PC. That study noted increased flux through the malate-pyruvate shuttle, which entails pyruvate transport into the mitochondria, conversion to oxaloacetate by PC, malate production by MDH, and export of malate to the cytoplasm where it is converted back to pyruvate by malic enzyme. We proposed that the observed normal glucose oxidation in the FA-cultured islets stemmed from the pyruvate concentration being twice normal,

![Fig. 4](image1.png)

![Fig. 5](image2.png)
overcoming the observed 35% reduction in PDH activity through a mass action effect.

Similar findings were observed in the ZF islets. Active PDH $V_{\text{max}}$ was lowered 30% (Fig. 4A), whereas PC $V_{\text{max}}$ was increased 60% (Fig. 4B). Enhanced flux through the malate-pyruvate shuttle was supported by increased $V_{\text{max}}$ values of MDH (170% of ZL, Fig. 4C) and malic enzyme (275% of ZL, Fig. 4D), increased concentrations of pyruvate (250% of ZL, Fig. 3B), oxaloacetate (150% of ZL, Fig. 3C), and malate (250% of ZL, Fig. 3D) following a 60-min incubation at 8.3 mM glucose, and a 2-fold increase in malate release from isolated mitochondria of ZF islets compared with ZL islets (Fig. 5).

**Islet Glycerol Phosphate Shuttle**—The glycerol phosphate shuttle is another pathway in β-cells that links cytosolic glucose metabolism to mitochondrial regulation of insulin secretion (34). We investigated this pathway by studying activity of the rate-limiting enzyme, mGPD. mGPD $V_{\text{max}}$ was 50% lowered in the ZF islets ($34.1 \pm 0.8$ nmol/min/mg protein ZL versus $14.6 \pm 1.5$ nmol/min/mg protein ZF, $n = 7, p = 0.001$).

**Inhibition of PC Activity by Phenylacetic Acid**—We studied the effect of inhibition of PC activity by 5 mM PAA (14) on islet glucose metabolism and insulin secretion. In both the ZL and ZF islets, glucose utilization and oxidation were unaffected by PAA at 2.8 mM glucose and lowered 30% at 16.7 mM glucose (Fig. 2). Also, PAA markedly lowered glucose-induced insulin secretion in the ZL and ZF islets (Fig. 6).

**DISCUSSION**

The glucose homeostasis system to insulin resistance entails compensatory increases in β-cell mass and function so that glycemia is unaltered (2). A confounding issue is how the excess FA that accompanies insulin resistance affects the glucose metabolism to mitochondrial regulation of insulin secretion (42). Enhanced trafficking through these shuttles likely explains how glucose oxidation is not impaired in the ZF islets by providing a loop for pyruvate to rise to supernormal levels that overcome the lowered PDH activity by a mass action effect; the pyruvate concentration was nearly 3-fold increased in the ZF islets.

What potential insight do these findings provide into the β-cell adaptation of the ZF rats? By showing that there is a mitochondrial pyruvate metabolism pathway in β-cells that is not impaired by FA, they provide a mechanism for β-cells to escape the glucose dysmetabolism that occurs with excess FA in other tissues. A more speculative possibility is that the link cytosolic and mitochondrial metabolism. One that has attracted considerable attention is metabolism of pyruvate through PC, the anaplerosis pathway (13–16, 35). PC is unusually active in β-cells so that half the pyruvate is normally metabolized through PC, the other half through PDH (36–38). The reason for the atypically high PC activity is under investigation, with regulatory roles proposed for glucose-induced insulin secretion through downstream coupling factors: reducing equivalents produced by the malate-pyruvate (13, 18) or citrate-pyruvate (14, 39) shuttles, glutamate (40), and malonyl-CoA that inhibits fatty acid oxidation so that cytoplasmic levels of long chain acyl-CoAs and complex lipids rise (15, 16). The importance of this pathway is supported by studies that show rises and falls in β-cell PC activity closely correlate with glucose-induced insulin secretion (13, 20–22, 41) and also by the observation in this study and by others (14, 18) that glucose-induced insulin secretion is impaired by the PC inhibitor, PAA.

In contrast to the lowered PDH activity in the ZF islets was increased PC activity. Also, our results are compatible with enhanced flux through the malate-pyruvate shuttle based on increased $V_{\text{max}}$ values for MDH and malic enzyme, raised concentrations of pyruvate, malate, and oxaloacetate, and 2-fold increased release of malate from isolated mitochondria. A second shuttle, the citrate-pyruvate shuttle, is similar except that mitochondrial oxaloacetate proceeds to citrate by citrate synthase, and the citrate is exported to the cytoplasm for conversion to oxaloacetate by citrate lyase, then to malate by cytoplasmic MDH with conversion of NADH to NAD$^+$, and then to pyruvate by malic enzyme (14). Thus, this shuttle not only produces NADPH through the malic enzyme reaction as occurs in the malate-pyruvate shuttle but also maintains a high cytoplasmic NAD$^+$ to NADH ratio. Our finding an increased citrate concentration in the ZF islets may suggest increased flux through this shuttle; this would be particularly advantageous to the ZF islets due to the lowered mGPD activity possibly causing impairment of the glycerol phosphate shuttle, which is normally an important source in β-cells for the cytoplasmic NAD$^+$ that is required for glycolysis (42). Enhanced trafficking through these shuttles likely explains how glucose oxidation is not impaired in the ZF islets by providing a loop for pyruvate to rise to supernormal levels that overcome the lowered PDH activity by a mass action effect; the pyruvate concentration was nearly 3-fold increased in the ZF islets.
diversion of pyruvate metabolism through PC plays a direct role in the β-cell adaptive hyperfunction and/or expansion. This possibility is based on the growing evidence for downstream pathways from PC directly influencing insulin secretion, in particular the pyruvate shuttles (13, 14, 18). Furthermore, a facilitative role in the compensatory increase in β-cell mass may be played by providing glucose-derived amino acids and lipids for the needed structural macromolecules (38). How then to reconcile the pancreas perfusion and static incubation findings in this study that find that insulin secretion was not increased in the ZF rats after correcting for their larger β-cell mass? This result must be viewed in the context of the markedly reduced β-cell insulin stores in the ZF rats that is reported to stem from impaired proinsulin biosynthesis (43). We and others have shown a regulatory role for pancreas insulin content over insulin secretory responses in rats (44, 45). With that understanding, the observed insulin secretion in the ZF rats is in considerable excess to what would be expected for their level of stored insulin, which is more compatible with the observed increase in islet glucose metabolism.

An unexpected finding in the ZF islets was the lowered activity of mGPD, which is the rate-limiting enzyme of the glycerol phosphate shuttle. mGPD is 50 times more active in β-cells than other tissues (46). Interest in this enzyme was spurred by the observation that its activity is lowered in islets of diabetic rodents (21, 22), leading to the suggestion that it spurs by the observation that its activity is lowered in islets of diabetic rats or functional benefit from an impaired glycerol phosphate shuttle alone is not sufficient for impaired secretion that characterizes diabetic states. However, null mice of diabetic rodents (21, 22), leading to the suggestion that it

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REFERENCES

1. Reaven, G. M. (1993) Annu. Rev. Med. 44, 121–131
2. Kahn, S. E., Prieve, R. L., McCulloch, D. B., Boyko, K. J., Bergman, R. N., Schwartz, M. W., Neffing, J. L., Ward, W. K., Beard, J. C., Palmer, J. P., and Porte, D. Jr. (1993) Diabetes 42, 1663–1672
3. Brunzell, J. D., and Hokanson, J. E. (1999) Diabetes Care 22, C10–C13
4. Zhou, Y-P., and Grill, V. E. (1994) J. Clin. Invest. 93, 870–876
5. Milburn, J. L., Jr., Hirase, H., Lee, Y. H., Nagasawa, Y., Ogawa, A., Ohmura, M., Beltran-Rio, H., Newgard, C. B., Johnson, J. H., and Unger, R. H. (1995) J. Biol. Chem. 270, 1295–1299
6. Grenchick, S., Bonny, C., Waeger, G., and Thorens, B. (1997) J. Biol. Chem. 272, 36261–36269
7. Unger, R. H., and Zhou, Y. T. (2001) Diabetes 50, S118–S121
8. Randle, P. J. (1998) Diabetes Metab. Rev. 14, 263–283
9. Zhou, Y. P., and Grill, V. E. (1995) Diabetes 44, 394–399
10. Wollheim, C. B. (1998) Diabetes 47, 374–380
11. Silva, J. P., Kohler, M., Graff, C., Oldfors, A., Magnuson, M. A., Berggren, P. O., and Larsson, N. G. (2000) Nat. Genet. 26, 336–340
12. Nicholls, L. I., Ainscow, E. K., and Rutter, G. A. (2002) Biochem. Biophys. Res. Commun. 291, 1081–1088
13. MacDonald, M. J. (1995) J. Biol. Chem. 270, 20551–20558
14. Farfari, S., Schulz, V., Corkey, B., and Prentki, M. (2000) Diabetes 49, 718–726
15. Prentki, M., and Corkey, B. E. (1996) Diabetes 45, 273–283
16. Corkey, B. E., Deeney, J. T., Yaney, G. C., Tornehim, K., and Prentki, M. (2000) J. Nutr. 130, 2968–3045
17. Liu, Y. Q., Tornehim, K., and Lebelt, Y. L. (1999) Diabetes 48, 1747–1753
18. Lu, D., Mulder, H., Zhao, P., Burgeese, S. C., Jensen, M. V., Kamalsova, S., Newgard, C. B., and Sherry, A. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2707–2713
19. Laclau, M., Lu, F., and MacDonald, M. J. (2001) Mol. Cell. Biochem. 225, 151–160
20. Iizuka, K., Nakajima, H., Namba, M., Miyagawa, J., Miyazaki, J., Hanafusa, T., and Matsuzawa, Y. (2002) Biochim. Biophys. Acta 1586, 23–31
21. MacDonald, M. J., Efendic, S., and Ostenson C. G. (1996) Diabetes 45, 886–890
22. MacDonald, M. J., Tang, J., and Polonsky, K. S. (1996) Diabetes 45, 1626–1630
23. Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J., and Hess, J. F. (1996) Nat. Genet. 13, 18–19
24. Cockburn, B. N., Ostrega, D. M., Sturis, J., Kubstrup, C., Polonsky, K. S., and Bell, G. I. (1997) Diabetes 46, 1434–1439
25. Pick, A., Clark, J., Kubstrup, C., Levitski, M., Pugh, W., Bonner-Weir, S., and Polonsky, K. S. (1998) Diabetes 47, 358–364
26. Gotot, M., Maki, T., Satomi, S., Porter, J., Bonner-Weir, S., O’Hara, C. J., and Monaco, A. P. (1987) Transplantation 43, 725–730
27. Labacars, C., and Paigen, K. (1980) Anal. Biochem. 102, 344–352
28. Albano, D. J., Ekins, R. P., Maritz, G., and Turner, R. C. (1972) Acts Endo-cri- nal. 70, 487–509
29. Weir, G. C., Knowlton, S. D., and Martin, D. B. (1974) J. Clin. Invest. 54, 1403–1412
30. Hosokawa, H., Corkey, B. E., and Lebell, J. L. (1997) Diabetologia 40, 392–397
31. Liu, Y. Q., Tornehim, K., and Lebell, J. L. (1998) Diabetes 47, 1889–1893
32. Liu, Y. Q., Tornehim, K., and Lebell, J. L. (1998) J. Clin. Invest. 101, 1870–1873
33. Sievers, A., Malaisse-Lagee, F., Dufrane, S. P., and Malaise, W. J. (1984) Biochem. J. 220, 433–440
34. Eto, K., Tsuhimoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., Yamauchi, N., Kobota, N., Murayama, S., Aizawa, T., Akanuma, Y., Aizawa, S., Kasai, H., Yazaki, Y., and Kadowaki, T. (1999) Science 283, 981–985
35. Prentki, M. (1996) Eur. J. Endocrinol. 134, 272–286
36. MacDonald, M. J. (1993) Metabolism 42, 1229–1231
37. Khan, A., Ling, Z. C., and Landau, B. R. (1996) J. Biol. Chem. 271, 2539–2542
38. Schuit, F. C., De Vos, A., Farfari, S., Moens, K., Pipeleers, D., Bran, T., and Prentki, M. (1997) J. Biol. Chem. 272, 18572–18579
39. Flamez, D., Berger, V., Kruhoffer, M., Orntoft, T., Pipeleers, D., and Schuit, F. C. (2002) Diabetes 51, 2016–2024
40. Maechler, P., and Wollheim, C. B. (1999) Nature 402, 685–689
41. MacDonald, M. J. (1995) Arch. Biochem. Biophys. 319, 128–132
42. MacDonald, M. J., Kelley, P. C., and Laclau, M. (2002) Metabolism 51, 318–321
43. Zhou, Y-P., Cockburn, B. N., Pugh, W., and Polonsky, K. S. (1999) Metabolism 48, 857–864
44. Balasch, L. C., Skelly, R. H., Chester, M. W., and McGarry, J. D., Rhodes, C. J. (1998) J. Clin. Invest. 101, 1094–1101
45. Hosokawa, Y., A., and Lebell, J. L. (1997) Diabetes 46, 808–813
46. MacDonald, M. J. (1981) J. Biol. Chem. 256, 8287–8290
47. Ueda, K., Tonizawa, Y., Ishii, H., Kiraki, N., Ohita, Y., Matsutani, A., and Oka, Y. (1998) Diabetologia 41, 649–653