Glucose–Fatty Acid Cycle to Inhibit Glucose Utilization and Oxidation Is Not Operative in Fatty Acid–Cultured Islets

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The glucose–fatty acid cycle of Randle entails two elements: decreased pyruvate dehydrogenase (PDH) activity, which inhibits glucose oxidation, and inhibition of phosphofructokinase (PFK) by a rise in citrate so that glucose-6-phosphate (G-6-P) levels increase, thereby inhibiting hexokinase activity and hence glucose utilization. Chronic exposure of islets to long-chain fatty acids (FA) is reported to lower PDH activity, but the effect on glucose oxidation and glucose-induced insulin secretion is uncertain. We investigated rat islets that were cultured for 4 days with 0.25 mmol/l oleate/5.5 mmol/l glucose. Glucose oxidation was doubled at 2.8 mmol/l glucose and unchanged at 27.7 mmol/l glucose in the FA-cultured islets despite a 35% decrease in assayed PDH activity. Pyruvate content was increased 60%, which may well compensate for the decreased PDH activity and maintain flux through the citric acid cycle. However, a greater diversion of pyruvate metabolism through the pyruvate-malate shuttle is suggested by unchanged pyruvate carboxylase V_{max} and a fourfold higher release of malate from isolated mitochondria. The FA-cultured islets also showed increased basal glucose usage and insulin secretion together with a lowered level of G-6-P and 50% reductions in citrate synthase V_{max} and the citrate content. Thus, the effects of chronic FA exposure on islet glucose metabolism differ from the glucose–fatty acid interactions reported in some other tissues. *Diabetes* 48:1747–1753, 1999

Islet β-cells secrete insulin in response to circulating nutrients and cellular fuels. Best studied is the plasma glucose concentration: a rise in glycemia induces insulin secretion through a process that is dependent on increased flux through β-cell glucose metabolism (1). Chronic exposure of islets to long-chain fatty acids (FA) alters β-cell glucose metabolism and glucose-stimulated insulin secretion. A 6-h FA exposure lowers the glucose concentration for half maximal glucose utilization (EC_{50}) for glucose utilization and insulin secretion (2,3), and a longer exposure (48 h) is reported to inhibit maximal insulin release (3), although not all studies have made that observation (4). These findings are of interest because animal models of type 2 diabetes are characterized by a similarly altered β-cell function (5–7) and a raised plasma level and/or islet content of triglyceride, the precursor of FA (8–10).

The lowered maximal glucose-induced insulin response after a multiday exposure to FA is reported to stem from reduced oxidative glucose metabolism because of inhibition of pyruvate dehydrogenase (PDH) (11). However, the importance of this finding is uncertain, since β-cells contain other active pathways that link cytosol and mitochondrial glucose metabolism—notably the glycerol phosphate and malate-aspartate NADH shuttles (12,13) and pyruvate carboxylase (14). This study investigated glucose oxidation and pyruvate metabolism in 4-day FA-cultured islets.

A related issue concerns the glucose hypersensitivity (lowered EC_{50}) of FA-cultured islets. We previously studied 24-h oleate-cultured islets and found phosphofructokinase (PFK) activity was upregulated through a dual mechanism: increased PFK V_{max} (15) and a lowered production/content of the PFK inhibitor citrate (16). The result was a 60% lowered glucose-6-phosphate (G-6-P) content that increased basal glucose usage and insulin secretion because of deinhibited hexokinase (15). However, the correctness of this schema for a longer exposure to FA is uncertain because of the inhibition of glucose metabolism by FA, commonly known as the "glucose-fatty cycle" of Randle (17–19). (As discussed in more detail in DISCUSSION, the Randle cycle was originally proposed based on acute effects of FA, but longer-term mechanisms were subsequently discovered, and this may lead to some confusion over the use of the term.) One facet is decreased PDH activity, which, as stated above, is found in islets after a multiday exposure to FA (11). However, a second element is inhibition of PFK because of high citrate production, so that G-6-P levels rise and inhibit hexokinase (18,19). If that also developed after prolonged FA culture, the β-cell hypersensitivity to glucose would be reversed. We therefore also investigated G-6-P levels in 4-day oleate-cultured islets.

RESEARCH DESIGN AND METHODS

Islet culture. Freshly isolated islets from Sprague-Dawley rats (Taconic, Germantown, NY) were cultured overnight at 37°C in humidified air and 5% CO_2 in RPMI 1640 supplemented with 5.5 mmol/l glucose, 2 mmol/l glutamine, 10% newborn calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Gibco, Grand Island, NY). Thereafter, the medium was supplemented with 0.25 mmol/l oleate

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BSA, bovine serum albumin; DTT, dithiothreitol; G-6-P, glucose-6-phosphate; FA, fatty acids; KRPB, Krebs-Ringer phosphate buffer; mGPD, mitochondrial glycerol-3-phosphate dehydrogenase; PDH, decreased pyruvate dehydrogenase; PFK, phosphofructokinase.

(sodium salt) in 2% ethanol or 2% ethanol alone for 4 days. Islet protein was measured by a commercial kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as standard. DNA was measured by the method of Labarca and Paigen (20).

Insulin secretion and content. Insulin secretion was measured as previously described (15). Triplicate glass vials containing 10 islets were incubated 60 min in Krebs-Ringer phosphate buffer (KRPB), 10 mmol/l HEPES, 0.5% BSA, and 5.5 or 27.7 mmol/l glucose in a 37°C shaking water bath. Insulin content was measured in triplicate groups of five islets in 1 ml acid ethanol. The insulin radioimmunoassay (21) used charcoal separation and rat insulin standards (gift of Eli Lilly, Indianapolis, IN).

Islet glucose metabolism. Glucose utilization and oxidation were determined with D-[5-^{3}H]glucose and [U-^{14}C]glucose, as previously described (2).

G-6-P content. After culture, islets (20 per tube) were incubated 60 min in KRPB/glucose (1.0, 8.3, and 27.7 mmol/l) with or without 0.25 mmol/l oleate to replicate the culture conditions. They were lysed with 10 µl 40 mmol/l NaOH and placed on ice for 10 min, followed by addition of 3 µl 0.15 mol/l HCl and being kept for 20 min at 75°C to destroy the cellular enzymes. G-6-P was measured as previously described (15) by the cycling method of Lowry and Passonneau (22).

PFK activity. PFK V_{max} was measured as previously described (14) using a method based on the study by Narabayashi et al. (23).

Mitochondrial glycerol-3-phosphate dehydrogenase activity. Mitochon-drial glycerol-3-phosphate dehydrogenase (mGPD) V_{max} was measured using the method of MacDonald et al. (24). Islets (100) were sonicated in 0.1 ml 5 mmol/l HEPES, pH 7.5, 230 mmol/l mannitol, and 70 mmol/l sucrose. Extract (10 µl) was added to 200 µl reaction buffer (50 mmol/l Bicine buffer, pH 8, 1 mmol/l potassium cyanide, 50 mmol/l L-glycerol-3-phosphate, and 4 mmol/l iodonitrotetrazolium violet) for 10 min at 37°C. The reaction was stopped by adding 1 ml 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 iodo-nitrotetrazolium violet in reaction mixture with or without (as blank) islet extract.

Pyruvate dehydrogenase activity. Islets (300) were homogenized on ice in 0.3 ml 50 mmol/l HEPES, pH 7.5, 0.2 mmol/l KCl, 3 mmol/l EDTA, 5 mmol/l dithiothreitol (DTT), 0.1 mmol/l Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mmol/l trypsin inhibitor, 0.02 trypsin inhibiting units per milliliter aprotinin, 2% rat serum, and 0.25% (vol/vol) Triton X-100, then freeze-thawed three times and passed through a 0.5-ml insulin syringe 10 times. Active PDH activity was measured using the method of Zhou and Grill (11). Extract (50 µl) and 50 µl reaction buffer (50 mmol/l HEPES, pH 7.5, 1 mmol/l $MgCl_2$, 3 mmol/l NAD, 0.4 mmol/l thiamine pyrophosphate, 0.4 mmol/l CoA, 2 mmol/l DTT, 0.1% Triton X-100, 7.5 U/ml lipoamide dehydrogenase, 1 mmol/l pyruvate, and 0.1 µCi [1-^{14}C]pyruvate) were added to a cup inside a rubber stoppered 20-ml scintillation vial that contained a center well with filter paper. After incubation at 37°C for 20 min, the reaction was stopped by injecting 200 µl 1 N HCl into the cup. CO_2 was trapped in the filter paper by injecting 100 µl 1 N KOH into the center well. PDH activity (1 U) equals 1 µmol CO_2/min.

Pyruvate carboxylase and malate dehydrogenase activities. Islets were homogenized in 10 mmol/l HEPES, pH 7.4, 250 mmol/l sucrose, 2.5 mmol/l EDTA, 2 mmol/l cysteine, and 0.02% BSA. Pyruvate carboxylase V_{max} was measured by the method of McClure et al. (25). Extract (80 µl) was added to 0.92 ml reaction buffer (80 mmol/l Tris/HCl, pH 8, 2 mmol/l ATP, 8 mmol/l potassium pyruvate, 21 mmol/l $KHCO_3$, 9 mmol/l $MgSO_4$, 0.16 mmol/l acetyl CoA, 0.16 mmol/l NADH, and 5 U/ml malate dehydrogenase). Absorbance at 340 nm was measured for 10 min.

Malate dehydrogenase V_{max} was measured by the method of Englard and Siegel (26). Extract (10 µl) was added to 0.82 ml 0.12 mol/l glycine, pH 10, 100 µl 0.85 mol/l L-malate, pH 7, and 67 µl 37.5 mmol/l NAD. Absorbance at 340 nm was measured for 20 min at 30°C.

Malic enzyme activity. Islets (100) were sonicated in 100 µl 50 mmol/l triethanolamine, pH 7.4, 3 mmol/l $MnCl_2$, and 0.02% BSA. Malic enzyme V_{max} was measured by the method of Sener et al. (27). Extract (50 µl) or NADPH standard (1–12 nmol) was added to 0.95 ml of prewarmed reaction buffer (50 mmol/l triethanolamine, pH 7.4, 3 mmol/l $MnCl_2$, 0.02% BSA, 0.1 mmol/l NADP, and 1 mmol/l L-malate). Fluorescence was measured for 20 min at 37°C at excitation 340 nm and emission 420 nm.

Malate and pyruvate contents. Islets (300) were lysed in 150 µl 2 mol/l perchloric acid on ice for 20 min, then centrifuged 10 min at 12,000*g*. Supernatant was neutralized with 3 mol/l $KHCO_3$ and recentrifuged at 12,000g. Malate and pyruvate standards were prepared in perchloric acid.

Malate was measured by the method of MacDonald (14). Extract (50 µl) or malate standard (0.1–1 nmol) was added to 250 µl reaction buffer (20 mmol/l 2-amino-2-methyl propanol, pH 9.9, 2 mmol/l glutamate, 50 µmol/l NAD, 1 µg aspartate aminotransferase, and 2.5 µg malate dehydrogenase) for 20 min at 30°C. Nonmetabolized NAD was removed by addition of 0.25 ml potassium phosphate, pH 11.9, and incubation at 60°C for 15 min, followed by addition of 12 µl 1 mol/l imidazole and another 15 min at 60°C. Fluorescence was measured at excitation 340 nm and emission 465 nm.

Pyruvate was measured by the method of Malaisse et al. (28). Extract (30 µl) or pyruvate standard (20–200 pmol sodium pyruvate) was added to 100 µl reaction buffer (50 mmol/l imidazole, pH 7, 0.6 mmol/l ascorbate, 0.2 mg/ml BSA, 6 µmol/l NADH, and 0.125 U/ml lactate dehydrogenase) at room temperature for 20 min. Nonmetabolized NADH was removed by addition of 20 µl 2 N HCl at room temperature for 30 min followed by 1 ml 6 N NaOH for 10 min at 60°C. Fluorescence was measured at excitation 360 nm and emission 460 nm.

Oxaloacetate content. Islets (50) were lysed 20 min in 40 µl 0.25 mol/l perchloric acid at –20°C followed by sonication and addition of 20 µl 0.94 mol/l KOH. Oxaloacetate was measured by the method of Parvin et al. (29). Extract (30 µl) or oxaloacetate standard in perchloric acid (0.2–2.0 pmol) was added to 200 µl reaction buffer (75 mmol/l K_2PO_4, pH 7.4, 80 nmol/l [acetyl-^{3}H]acetyl-CoA, and 50 µg/ml citrate synthase) at room temperature for 60 min. The reaction was stopped by 600 µl of charcoal mixture (8 g charcoal, 38 g citric acid monohydrate, and 120 ml 95% ethanol) followed by centrifugation at 12,000*g* and liquid scintillation counting of the supernatant.

Citrate content and citrate synthase activity. Citrate content and citrate synthase V_{max} were measured as previously described (16) by the methods of Lowry and Passonneau (30) and Eizirik et al. (31), respectively.

Malate release from isolated islets. Mitochondria were isolated from oleate-cultured and control islets using the method of MacDonald (32): islets (400) were homogenized in 0.4 ml of 5 mmol/l potassium HEPES, pH 7.5, 230 mmol/l mannitol, and 70 mmol/l 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. We confirmed that the oleate-cultured and control islets were separated in an identical fashion (n = 4): 72 ± 3% of the original protein content of the control islets was present in the cytosol and 7 ± 0% in the mitochondrial fractions versus 70 ± 2% in the cytosol and 8 ± 0% in the mitochondrial fractions for the oleate-cultured islets (the remaining 20% sedimented with the cellular and nuclear debris in the first centrifugation).

The sedimented mitochondria were resuspended in 120 µl of ice-cold buffer (5 mmol/l potassium HEPES, pH 7.3, 5 mmol/l K_2PO_4, 5 mmol/l $KHCO_3$, 2 mmol/l Na_2ADP, 230 mmol/l mannitol, 70 mmol/l sucrose, and with or without 200 µmol/l pyruvate) and kept on ice. Malate release was measured using the method of MacDonald (14). The mixture was placed at 37°C, and samples (50 µl) were taken at 0 and 10 min. Sample processing was centrifugation at 14,000 rpm for 2 min, addition of 15 µl 0.92 mol/l perchloric acid to the supernatant, return of pH to 7.0 with 1 mol/l KOH, and recentrifugation at 14,000 rpm for 2 min. Malate was measured using the method of Sener et al. (27). Supernatant (40 µl) or malate standard (0–30 pmol) was added to 200 µl reaction buffer (100 mmol/l Tris/KCl, pH 8.0, 1 mmol/l NAD, 0.2 mmol/l [^{3}H]acetyl-CoA, 20 µg/ml malate dehydrogenase from pig heart, and 60 µg/ml citrate synthase from pig heart) at room temperature for 60 min. The final product of [^{3}H]citrate was separated with 600 µl charcoal mixture (120 ml 95% ethanol, 8 g charcoal, and 38 g citrate acid monohydrate) and centrifugation at 14,000 rpm for 5 min followed by liquid scintillation counting of the supernatant.

Data presentation and statistical methods. All data are expressed as means ± SE. Unless otherwise stated, the listed n values represent the number of experiments performed using islets from separate isolation and culture days. Statistical significance was determined by the unpaired Student's t test unless stated otherwise.

RESULTS

Glucose-induced insulin secretion in 4-day oleate-cultured islets. Islet DNA (22 ± 1 vs. 21 ± 2 ng/islet, control versus oleate-cultured, n = 4) and protein levels (0.78 ± 0.01 vs. 0.76 ± 0.01 µg/islet, control versus oleate-cultured, n = 3) were unaffected by the 4-day oleate culture in contrast to the insulin content, which was lowered by 15% (18.7 ± 0.6 vs. 16.0 ± 0.7 ng/islet, control versus oleate-cultured, n = 3, P < 0.03). Insulin secretion was twice normal at both 5.5 and 27.7 mmol/l glucose in the oleate-cultured islets (Table 1). We thus failed to observe the lowered maximal glucose-induced insulin response that has been observed in some (3) but not all (4) studies of islets or clonal β-cells after long-term exposure to FA.

Islet glucose metabolism. Glucose utilization and oxidation were increased at 1.0 and 2.8 mmol/l glucose in the oleate-cultured islets (Fig. 1*A* and *B*). There was a dichotomy at higher glucose values in that glucose usage in the oleate-cultured islets doubled between 2.8 and 27.7 mmol/l glucose (P < 0.01 by paired t test), whereas glucose oxidation leveled off at 2.8 mmol/l glucose. Thus, there was a biphasic effect of the oleate culture on glucose oxidation: increased at a subphys-

TABLE 1
Insulin secretion

	Insulin secretion (ng · 10 islets^{-1} · 60 min^{-1})	
	5.5 mmol/l glucose	27.7 mmol/l glucose
Control islets	4.8 ± 0.7	9.2 ± 1.9
0.25 mmol/l Oleate	9.3 ± 0.4	20 ± 1.2
P value	0.002	0.003

Nonpooled islets from eight rats were cultured in a single experiment over 4 days with 0.25 mmol/l oleate in 2% ethanol (n = 4) or 2% ethanol alone (n = 4) added to the culture medium. Islets were preincubated for 30 min in KRPB/2.8 mmol/l glucose, followed by a 60-min incubation at 5.5 or 27.7 mmol/l glucose. Statistical significance was determined by the Student's t test.

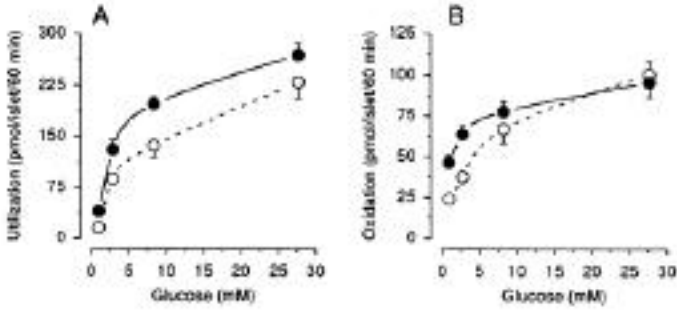

FIG. 1. Islet glucose utilization (*A*) and oxidation (*B*). Islets were cultured 4 days with 0.25 mmol/l oleate in 2% ethanol (●) or 2% ethanol alone (○) added to the culture medium. Glucose utilization (n = 4) was determined with D-[5-^{3}H]glucose and glucose oxidation (n = 7) with [U-^{14}C]glucose.

iologic glucose level and a failure to rise as expected thereafter. However, in absolute terms, glucose oxidation was not subnormal at any glucose level in the oleate-cultured islets. One note of caution is that islets contain both β-cells and non–β-cells so that we cannot rule out an effect of oleate to impair β-cell glucose oxidation, which was not apparent from studying the whole islet.

Defective PDH activation is reported to be the basis for impaired glucose oxidation after long-term exposure of islets to FA (11). We wondered if our culture system failed to induce this effect. However, this possibility was eliminated by measuring PDH activity and finding it was 35% lower in the 4-day oleate-cultured islets (P < 0.015, Table 2), analogous to the report of Zhou and Grill (11). A second point concerned our previous observation in 1-day oleate-cultured islets of substantial reductions in citrate synthase V_{max} and the citrate content (16). The current study noted identical findings in the 4-day oleate-cultured islets (Tables 2 and 3). Our results thus gave the seemingly paradoxical finding of no overt reduction of glucose oxidation despite the advent of decreased PDH and citrate synthase activities, which are key sites of regulation of substrate entry into the citric acid cycle. Hence, we investigated glucose oxidative pathways in the oleate-cultured islets.

Mitochondrial glycerol-3-phosphate dehydrogenase. The glycerol phosphate shuttle is an important pathway in β-cells that links cytosolic and mitochondrial glucose metabolism (13,14) with the major regulatory enzyme mGPD (33). This enzyme's activity was unchanged by the oleate culture (Table 2).

Pyruvate content and metabolism. We next investigated pyruvate metabolism; islet enzyme V_{max} and metabolite levels are shown in Tables 2 and 3, respectively. Pyruvate content of the oleate-cultured islets was 60% greater than in the control islets. In contrast to the decreased PDH activity, pyruvate carboxylase V_{max} was unchanged. That reaction generates oxaloacetate, which normally proceeds through the citric acid cycle by way of citrate synthase. However, as already stated, there were substantial reductions in citrate synthase V_{max} and the citrate content in the oleate-cultured islets. An alternate pathway for oxaloacetate is to regenerate pyruvate by way of malate dehydrogenase and malic enzyme, the so-called pyruvate-malate shuttle that is highly active in β-cells under normal conditions (14,34). This involves conversion of mitochondrial oxaloacetate to malate, transfer of the malate out of the mitochondria, and oxidative decarboxylation of malate to pyruvate by cytosolic malic enzyme. Malate dehydrogenase V_{max} was unchanged in the oleate-cultured islets, and malic enzyme V_{max} was reduced a minor extent (15%). Also, oxaloacetate and malate contents were unchanged as opposed to the lowered citrate. (These are total contents. There are mitochondrial membrane carriers for citrate and malate, and their mitochondrial and cytosolic concentrations are likely to move in parallel. However, there is no carrier for oxaloacetate; thus, it is possible that the mitochondrial concentration could rise and not be detected in this measurement.) Finally, malate release from isolated mitochondria was increased fourfold in the oleate-cultured islets, both with and without the addition of exogenous pyruvate (Fig. 2, P < 0.015 oleate versus control islets both with and without 200 µmol/l pyruvate). Pyruvate content of isolated mitochondria from oleate-cultured islets was increased by 50% (10.8 ± 1.2 vs. 6.9 ± 0.9 nmol/mg mitochondrial protein, oleate-cultured versus control islets, P < 0.05, n = 4). Together these data suggest enhanced flux through the pyruvate-malate shuttle.

Islet G-6-P content. Our finding increased basal glucose metabolism in the oleate-cultured islets (Fig. 1) suggested that the lowered G-6-P content we had noted in 1-day oleate-cultured islets (15) was still present after the 4-day culture. We tested this idea by incubating 4-day cultured islets for 60 min in KRPB with 1.0, 8.3, or 27.7 mmol/l glucose followed by measurement of G-6-P (Fig. 3). The G-6-P content of the oleate-cultured islets was 70% reduced at 1 mmol/l glucose (P < 0.002). Also, PFK V_{max} was increased twofold (P < 0.02) in the oleate-cultured islets (Table 2)—perhaps even more than in 1-day oleate-cultured islets (15).

DISCUSSION

Glucose metabolism of islets was investigated after chronic oleate exposure. This subject has attracted much attention because of the suggestion that chronic FA exposure impairs β-cell glucose oxidation and that this effect explains the impaired insulin secretion to high glucose that characterizes type 2 diabetes (3). Moreover, the well-known glucose–fatty acid cycle that links tissue exposure to excess FA and impaired glucose oxidation seems consistent with this idea, since defective activation of PDH is known to be causative in other tissues (18), and impaired PDH activation has been reported in FA-cultured islets (11). However, there have been several reports with results different from those above, so it remains uncertain how chronic FA exposure affects β-cell glucose oxidation and insulin secretion and what is the signifi-

TABLE 2
Islet enzyme activities

Enzyme	Experiment number	Control islets	Oleate-cultured islets	*P* value
Active pyruvate dehydrogenase (pmol · min^{-1} · $islet^{-1}$)	5	4.1 ± 0.4	2.7 ± 0.2	0.015
Citrate synthase (pmol · min^{-1} · $islet^{-1}$)	4	23.7 ± 1.4	12.7 ± 0.8	0.001
Mitochondrial glycerol-3-phosphate dehydrogenase (nmol · min^{-1} · mg^{-1} homogenate protein)	4	37.0 ± 2.6	32.9 ± 2.8	NS
Pyruvate carboxylase (pmol · min^{-1} · $islet^{-1}$)	5	31.5 ± 3.2	30.3 ± 1.6	NS
Malate dehydrogenase (pmol · min^{-1} · $islet^{-1}$)	5	390 ± 14	370 ± 19	NS
Malic enzyme (pmol · min^{-1} · $islet^{-1}$)	5	6.9 ± 0.2	5.9 ± 0.2	0.015
Phosphofructokinase (pmol · min^{-1} · $islet^{-1}$)	4	11.9 ± 0.8	22.0 ± 2.9	0.02

Data are means ± SE. Islets were cultured 4 days with 0.25 mmol/l oleate in 2% ethanol or 2% ethanol alone added to the culture medium. Enzyme rates were calculated as described in the text. Statistical significance was determined by the Student's unpaired *t* test.

cance of the lowered PDH activity. Brun et al. (4) cultured INS-1 cells for 3 days with various FA and found preserved maximal glucose-induced insulin secretion. Liang et al. (35) observed reduced maximal glucose-induced insulin secretion in βHC9 cells after a 4-day culture with oleate in tandem with increased oxygen consumption and preserved glucose-derived ATP generation. Bollheimer et al. (36) suggested that the inhibitory effect of FA on glucose-induced insulin secretion stemmed from a lowered storage pool of insulin because of impaired proinsulin synthesis combined with the FA insulinotropic effect at basal glucose levels, since adding somatostatin to the FA culture to inhibit insulin secretion prevented the lowered insulin content and restored the insulin secretory capacity.

The present study was undertaken in 4-day oleate-cultured islets to clarify these issues; that time point was chosen to allow sufficient time for development of the inhibitory effect of FA on islet glucose oxidation and PDH activity reported by Zhou and Grill (3,11) after a 48-h FA exposure and to encompass the somewhat longer times used by others as noted above. No reduction of glucose-induced insulin secretion or glucose oxidation (in an absolute sense) was observed despite confirmation of a reduction in PDH activity, although this result should perhaps be viewed with caution because islets contain multiple cell types. These findings suggested that countermechanisms exist in the β-cells to compensate for the decreased PDH activity and preserve glucose signaling pathways for insulin secretion. Of particular interest in that regard was the supernormal islet glucose oxidation under basal glucose conditions, and we investigated mitochondrial glucose metabolism in islets that were cultured 4 days with 0.25 mmol/l oleate/5.5 mmol/l glucose.

The glycerol phosphate shuttle is an important pathway in β-cells that links cytosolic and mitochondrial glucose metabolism (13,14). Furthermore, the activity of mitochondrial glycerol-3-phosphate dehydrogenase is decreased in islets of animals with type 2 diabetes (24,37). However, no effect of oleate was found on this enzyme's activity. We next focused on pyruvate metabolism. β-Cells present special characteristics in this regard. Gluconeogenesis is not possible because of the absence of phosphoenolpyruvate carboxykinase (38). Also, lactate dehydrogenase has a low level of expression and is a minor metabolic pathway (12). Instead, pyruvate is metabolized by PDH or pyruvate carboxylase. The latter is unusually active in β-cells so that normally half the pyruvate enters each pathway (34,39,40). Moreover, both enhance citric acid cycle activity by providing the two substrates for citrate synthase, acetyl-CoA and oxaloacetate. The reason for the atypically high pyruvate carboxylase activity in β-cells is unknown, but regulatory roles for insulin secretion have been proposed to replenish citric acid cycle intermediates to generate citrate for export to the cytosol to raise malonyl-CoA (41) and to increase cytoplasmic NADPH through the pyruvate-malate shuttle (14).

Pyruvate metabolism was perturbed in several ways by the oleate culture. Pyruvate content was increased 60%. One likely reason was increased production from the upregulated glycolysis. Another may have been diminished metabolism due to the lowered PDH activity. In addition, at the higher pyruvate concentration, the flux through pyruvate carboxylase was likely increased because its V_{max} was unaffected in contrast to PDH; then, perhaps in part because of the decreased citrate synthase activity, the resultant oxaloacetate would tend to be recycled back to pyruvate through

TABLE 3
Islet metabolite content

Metabolite	Experiment number	Control islets	Oleate-cultured islets	*P* value
Citrate (pmol/islet)	5	3.4 ± 0.2	1.4 ± 0.1	0.001
Pyruvate (pmol/islet)	5	1.0 ± 0.1	1.6 ± 0.1	0.01
Oxaloacetate (fmol/islet)	4	31.9 ± 1.1	28.7 ± 1.6	NS
Malate (pmol/islet)	8	2.6 ± 0.2	2.9 ± 0.3	NS

Data are means ± SE. Islets were cultured 4 days with 0.25 mmol/l oleate in 2% ethanol or 2% ethanol alone added to the culture medium. Metabolite content values were calculated as described in the text. Statistical significance was determined by the Student's unpaired *t* test.

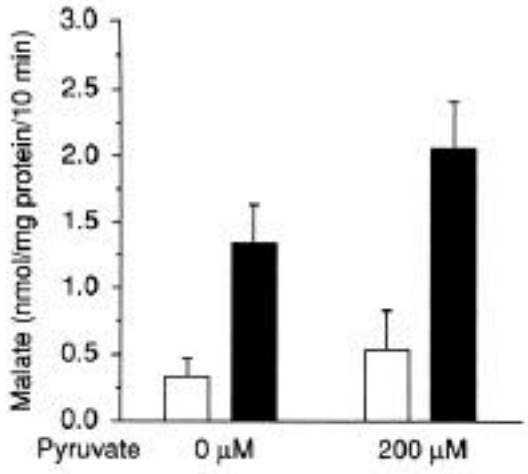

FIG. 2. Malate production from isolated mitochondria. Islets were cultured 4 days with 0.25 mmol/l oleate in 2% ethanol (■) or 2% ethanol alone (□) added to the culture medium. Malate efflux from isolated mitochondria was measured over 10 min with (n = 5) or without (n = 5) 200 μmol/l pyruvate in the incubation buffer.

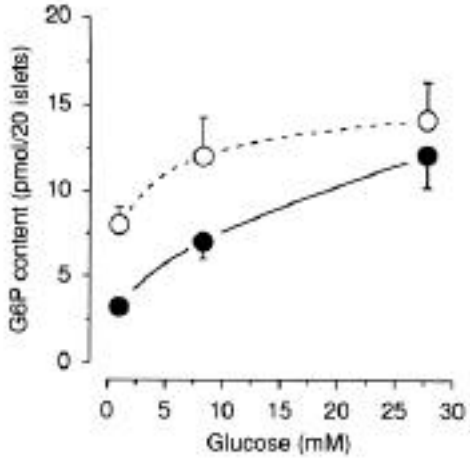

FIG. 3. Islet G-6-P content. Islets were cultured 4 days with 0.25 mmol/l oleate in 2% ethanol (●, n = 5) or 2% ethanol alone (○, n = 5) added to the culture medium. At the end of the culture period, islets were incubated 60 min in KRPB and glucose (1.0, 8.3, and 27.7 mmol/l) with or without 0.25 mmol/l oleate, followed by measurement of G-6-P by the cycling method of Lowry and Passonneau (22).

malate dehydrogenase and malic enzyme (so-called pyruvate-malate shuttle), which also were generally unaffected. Further, malate dehydrogenase is allosterically inhibited by citrate (42) and the lowered citrate content should promote flux through this enzyme if the reaction is not at equilibrium. Our demonstration that mitochondrial malate efflux was increased fourfold from the oleate-cultured islets supports enhanced flux through this pathway. Two issues regarding this latter measurement need comment. First, in developing the method to measure malate release from isolated mitochondria, MacDonald (32) showed that malate, but not citrate or isocitrate, exited the mitochondria in a time-dependent fashion that eliminated leakage of mitochondrial contents as the basis for the malate release. Second, we observed malate release in the absence of exogenous pyruvate, with that effect being pronounced in the oleate-cultured tissue. We interpret this finding as being due to the presence of intramitochondrial endogenous pyruvate (which was increased in the oleate-cultured islets) and citric acid cycle intermediates acting as substrate for malate production, coupled with the short duration of the experiment (10 min).

Although glucose oxidation was preserved and even enhanced at a low glucose concentration in the oleate-cultured islets, the further increase at higher glucose concentrations was blunted (Fig. 1). A likely explanation is limitation of PDH flux under those conditions. This is consistent with the assayed level of active PDH after oleate culture (2.7 pmol · min^{-1} · $islet^{-1}$) being similar to twice the maximum rate of glucose oxidation (~1.5 pmol · min^{-1} · $islet^{-1}$, calculated from Fig. 1) and the stoichiometry of two pyruvates per glucose molecule. The values of active PDH measured here were if anything somewhat greater than that reported by Zhou and Grill (11) (2.1 and 1.1 pmol · min^{-1} · $islet^{-1}$, control and palmitate-cultured, respectively). As such, their decrease in [U-^{14}C]glucose oxidation with palmitate culture, from 0.9 to 0.7 pmol · min^{-1} · $islet^{-1}$ (3), may be due to the greater restriction in active PDH that they observed (11).

Zhou and Grill (3) also reported a 30–50% decrease in glucose-stimulated insulin secretion with fatty acid culture and attributed this to the inhibition of glucose oxidation through a glucose–fatty acid cycle; however, as suggested by Bollheimer et al. (36), the explanation may instead be the large decrease in insulin content and proinsulin biosynthesis they observed. Our finding no inhibition of insulin secretion in this study with oleate culture seems more consistent with the latter suggestion because the difference in insulin content with our experimental conditions was much smaller (15%). Zhou and Grill cultured islets at a supernormal glucose concentration (11 mmol/l) as opposed to the 5.5 mmol/l glucose of the current study, which may account for this difference.

Insulin secretion doubled between 5.5 and 27.7 mmol/l glucose in the oleate-cultured islets despite a blunted increase in glucose oxidation. This result seems counter to the generally accepted concept that metabolism of pyruvate through the citric acid cycle is necessary for glucose-induced insulin secretion, as suggested by studies showing lowered glucose-induced insulin secretion when β-cell mitochondrial metabolism was made defective (43). Challenging this idea is the observation that exogenous pyruvate can be oxidized by islets but does not elicit insulin secretion (44). These findings suggest a permissive role for mitochondrial glucose oxidation in glucose-induced insulin secretion, but that other signals must be involved. One possibility is reducing equivalents in the form of NADH that are directly generated during glycolysis, the mitochondrial use of which would not be reflected in $^{14}CO_2$ production. Indeed, a recent study indicated a crucial role of β-cells in shuttling of cytosolic NADH into the mitochondria through the glycerol phosphate shuttle and the malate-aspartate shuttle to promote ATP generation and glucose-induced insulin secretion (13). It has also been suggested that there is a special role for glycolytically generated ATP in closing ATP-sensitive K^+ channels and causing insulin secretion (45,46). The parallel rises of insulin secretion and glucose utilization (hence glycolysis) with elevated glucose in the oleate-cultured islets are consistent with preserved activity of such glycolytic-based mechanisms for insulin secretion. Another issue is that citric acid cycle operation and mitochondrial ATP production are also supported from fatty acid oxidation; hence, radio-labeled glucose oxi-

dation should not be taken as indicative of total mitochondrial metabolism. Finally, MacDonald (14) postulated that the malate-pyruvate shuttle is normally highly active in β-cells to generate cytosolic NADPH as a signal for insulin secretion, and the greater diversion of pyruvate through this shuttle seen here in the oleate-cultured islets might account for the increased insulin secretion.

The use of the term "glucose–fatty acid cycle" in the context of chronic effects of FA on glucose metabolism potentially may cause confusion, since the original mechanisms were described as acute effects, the inhibition of PFK by citrate, and the direct inhibition of PDH by acetyl-CoA/CoA. The indirect effect of acetyl-CoA/CoA to activate PDH kinase was then discovered; the measurements of active PDH reported here and elsewhere are essentially of the nonphosphorylated PDH. It is now known that diabetes and nutritional effects to inhibit glucose oxidation, and their reversal, can take many hours—even days—and that this involves slowly developing effects on a PDH kinase (akin to the suggestions of Grill and colleagues in their studies of islets), which brings this into the time realm of changes in gene expression. The more recent reviews of the glucose–fatty acid cycle by Randle (17–19) discuss and even focus on these chronic mechanisms. With regard to the pancreatic β-cell, FA do not have acute negative effects, but instead potentiate glucose-stimulated secretion. Furthermore, glucose itself acutely causes a rise in citrate, which is considered a part of the signaling process, raising malonyl-CoA and then cytosolic FA-CoA (8). One day of oleate culture caused a decrease in citrate and G-6-P and a corresponding increase in basal glucose utilization and oxidation (15,16), and here we have shown that these changes are maintained and not reversed after 4 days of oleate culture; it should be noted that this process does take hours and involves changes in citrate synthase and PFK V_{max} that may reflect changes in gene expression. These changes in citrate and G-6-P are opposite to those expected from the glucose–fatty acid cycle. The inhibitory effect of FA on citrate synthase is not unique to islets, but has been reported in brain and lymphoid tissue (47,48); hence, some other tissues may be resistant to a glucose–fatty acid cycle mechanism in a similar manner. The most important consequence of these enzyme and metabolite changes caused by chronic FA exposure in the pancreatic β-cell is probably the increased basal secretion of insulin.

In summary, our results show complex effects of chronic oleate culture on islet glucose metabolism that vary substantially from the glucose–fatty acid cycle seen in some other tissues. At basal glucose values, glucose oxidation and overall glucose usage were increased. The operative mechanism appeared to be the augmenting effect of FA on PFK activity and thus G-6-P catabolism so that hexokinase activity was increased. Additional rises in glucose concentration further increased glucose utilization, whereas glucose oxidation plateaued, which suggested a limited capacity of one or multiple enzymes in the glucose oxidative pathway and perhaps the demonstrated lowering of PDH activity. The result was a lowered proportion of the metabolized glucose undergoing oxidative metabolism. However, the absolute oxidative rate was not lowered by the FA culture, probably because of increased pyruvate levels to offset the decrease in PDH activity. Thus, the oleate-cultured islets showed neither of the defining elements of the glucose–fatty acid cycle, a lowered glucose oxidation, nor enhanced citrate production inhibiting PFK activity and glucose usage.

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