Shared Biochemical Properties of Glucotoxicity and Lipotoxicity in Islets Decrease Citrate Synthase Activity and Increase Phosphofructokinase Activity

Ye Qi Liu, Keith Tornheim, and Jack L. Leahy

Diabetic states are characterized by a raised serum/islet level of triglycerides and a lowered EC_{50} (concentration at half-maximal stimulation) for glucose-induced insulin secretion. Culturing islets with long-chain fatty acids (FAs) replicates the basal insulin hypersecretion. In a previous study, we showed that the mechanism involved deinhibition of hexokinase by a 60% decrease in glucose-6-phosphate (G-6-P). The key event was proposed to be an increased phosphofructokinase (PFK) V_{max} secondary to an upregulatory effect of the FA metabolite, long-chain acyl–coenzyme A (LC-CoA). We now show another contributory factor, a lowered content of the PFK inhibitor citrate. Citrate synthase V_{max} and citrate levels were lowered 45% in rat islets cultured with 250 µmol/l oleate for 24 h. Both effects were reversed by triacsin C, an inhibitor of fatty acyl–CoA synthetase, the enzyme that generates LC-CoA. Culturing islets with high doses of glucose (16.7 mmol/l) for 48 h should also raise cytosolic LC-CoA. As predicted, citrate synthase V_{max} was lowered and PFK V_{max} was increased, both in a triacsin C–reversible fashion. These results show shared selected functional and biochemical properties in β-cells of so-called glucotoxicity and lipotoxicity. *Diabetes* 47:1889–1893, 1998

Long-term exposure (>6 h) of islets to long-chain fatty acids (FA) lowers the concentration at half-maximal effect (EC_{50}) of glucose-induced insulin secretion (1–3). This finding is of interest because animal models of type 2 diabetes have a similarly altered glucose–insulin secretion relationship (4–7) and a raised plasma and islet content of triglycerides, precursors of FA (8–11). Moreover, our studies in diabetic rats have shown that the β-cell hypersensitivity to glucose is an early, predisposing step for the β-cell failure that typifies overt hyperglycemia (12).

Flux through glycolysis is the process that is sensed for β-cell glucose responsiveness (13). β-Cell glucose metabolism is multienzyme regulated. Glucokinase determines the kinetics of glucose entry into glycolysis. Its high K_m (10–12 mmol/l glucose) confers an EC_{50} for glucose usage/insulin secretion that is in the physiologic glucose range (14,15). β-Cells also contain hexokinase, but its activity at physiologic glucose values is minimal because of allosteric inhibition by glucose-6-phosphate (G-6-P) (16). G-6-P is in equilibrium with fructose-6-phosphate, and therefore the steady-state level of G-6-P is governed by control of the outflow by phosphofructokinase (PFK).

We previously investigated the altered glucose sensing in rat islets cultured with FA for 24 h (17). The G-6-P content was lowered 60%, while the PFK V_{max} increased 50%. Triacsin C, an inhibitor of fatty acyl–CoA synthetase (18–20), reversed these changes (17) and the shifted secretion curve (3). Thus, long-chain fatty acids act through generation of long-chain acyl–coenzyme A (LC-CoA) to increase PFK activity, which lowers the cellular level of G-6-P and deinhibits hexokinase. Because the decrease in G-6-P exceeded the increase in PFK V_{max}, however, and because increased hexokinase activity would be expected to increase G-6-P production, another factor affecting PFK seemed likely.

Citrate is a potent inhibitor of PFK (21), and citrate production occurs exclusively through citrate synthase (22,23). A few studies in other tissues have shown an inhibitory effect of FA-enriched diets or LC-CoA (in vitro) on citrate synthase (24–26). The studies presented here examined citrate synthase activity in FA-cultured islets, since a substantial decrease in citrate would act in tandem with the raised PFK V_{max} to augment that enzyme's activity and its effect on the level of G-6-P.

A second issue concerns another aspect of the diabetes milieu, hyperglycemia. Reversal by triacsin C of the functional and biochemical changes of FA culture (3,17) indicated mediation by LC-CoA. Glucose also raises the β-cell cytosolic LC-CoA level in two ways: metabolism to malonyl-CoA, which inhibits LC-CoA catabolism (27,28), and increased expression of acetyl-CoA carboxylase, the enzyme that synthesizes malonyl-CoA (29). This shared biochemical action of glucose and FA to raise the cytosolic LC-CoA level is of interest in view of the tendency to separately consider detrimental effects of excessive glucose and FA (so-called glucotoxicity and lipotoxicity, respectively) in terms of pathogenic roles in type 2 diabetes. We tested the hypothesis that high glucose and FA would induce identical changes in PFK and citrate synthase activities.

RESEARCH DESIGN AND METHODS

Islet isolation and culture. Islets were isolated from Sprague-Dawley rats (Taconic, Germantown, NY) using an adaptation of the method of Gotoh et al. (30): pancreatic duct infiltration with collagenase (Serva, Heidelberg, Germany), Histopaque gradient separation, and hand picking. After isolation, islets were cul-

From the Division of Endocrinology, Diabetes and Metabolism (Y.Q.L., J.L.L.), University of Vermont, Burlington, Vermont; and the Diabetes and Metabolism Unit and Department of Biochemistry (K.T.), Boston University School of Medicine, Boston, Massachusetts.

Address correspondence and reprint requests to Dr. Jack Leahy, University of Vermont College of Medicine, Given C331, Burlington, VT 05405-0068. E-mail: jleahy@zoo.uvm.edu.

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BSA, bovine serum albumin; CoA-SH, coenzyme A; EC_{50}, concentration at half-maximal stimulation; FA, long-chain fatty acid; G-6-P, glucose-6-phosphate; KRBH, Krebs-Ringer bicarbonate HEPES; LC-CoA, long-chain acyl–coenzyme A; PFK, phosphofructokinase.

tured 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). According to the manufacturer, newborn calf serum contains 2.9–3.2 g albumin per 100 ml, so 0.3% albumin was present in the culture medium. FA culture entailed 24 h in this medium plus 250 µmol/l oleate (sodium salt) in 2% ethanol or 2% ethanol alone. Concentration studies tested multiple oleate concentrations (0–250 µmol/l) for 24 h. In time-course studies, islets were incubated for a total of 6 h in the culture medium, with 250 µmol/l oleate added to give exposure times of 30 min and 1, 2, and 6 h; those studies used oleate that had not been preexposed to BSA. An additional protocol used oleate from an 8 mmol/l stock solution that was prepared in Krebs-Ringer bicarbonate HEPES (KRBH), pH 7.4, that contained 10% fatty acid–free bovine serum albumin (BSA) as described by Gremlich et al. (31). Glucose culture entailed 48 h in the standard medium with 5.5 or 16.7 mmol/l glucose. Triacsin C (Biomol, Plymouth Meeting, PA) in DMSO to a final concentration of 0.5 µg/ml was included where stated below.

Citrate content. Citrate was measured by the method of Lowry and Passonneau (32). Islets were lysed with trichloroacetic acid, placed on ice, and centrifugated to remove precipitated proteins. The supernatant was neutralized by ether extraction × 5, lyophilized, and resolubilized in 100 µl H_2O. Extract or citrate standard (0.1–2 nmol) was added to 0.1 mol/l Tris-HCl buffer, pH 7.6, 40 µmol/l $ZnCl_2$, 3 µmol/l NADH, and 0.4 µg/ml malate dehydrogenase, final volume 0.5 ml. Fluorescence at 340 nm excitation and 465 nm emission was determined, then repeated 5 min after adding 10 µl citrate lyase solution, with the delta value representing the citrate content.

Citrate synthase activity. Citrate synthase V_{max} was measured using the method of Eizirik et al. (33). Homogenized islet extract (40 µl) was added to 0.55 ml of 100 mmol/l HEPES, pH 7.4, 0.1 mmol/l oxaloacetate, 0.1 mmol/l dithionitrobenzoic acid, and 0.05 mmol/l acetyl-CoA in an ultraviolet cuvette. Coenzyme A (CoA-SH) production was assessed spectrophotometrically at 412 nmol/l. Blanks contained no acetyl-CoA. Results were calculated from CoA-SH standards (1–10 nmol).

PFK activity. Islets were sonicated on ice in 0.15 ml extraction buffer containing 15 mmol/l K_2PO_4, pH 7.0, 100 mmol/l KCl, 2 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, 0.2 mg/ml leupeptin, and 50 µg/ml aprotinin. After centrifugation at 12,000*g* for 15 min at 4°C, PFK activity was measured by the method of Narabayashi et al. (34). Supernatant was added to 1 ml buffer that contained 50 mmol/l Tris/HCl, pH 8.0, 1 mmol/l EDTA, 2.5 mmol/l dithiothreitol, 5 mmol/l ammonium sulfate, 2 mmol/l $MgCl_2$, 1 mmol/l ATP, 1 mmol/l fructose-6-phosphate, 0.16 mmol/l NADH, 0.4 U/ml aldolase, and 2.4 U/ml triose phosphate isomerase plus 0.8 U/ml glycerophosphate dehydrogenase mixture (Boehringer Mannheim, Indianapolis, IN) in a quartz cuvette. The NADH metabolized was assessed at 340 nm by spectrophotometry. PFK activity was calculated based on 1 µmol fructose-1,6-diphosphate = 2 µmol NADH consumed.

Data presentation and statistical methods. All data are expressed as mean ± SE. The listed *n* values represent the number of experiments performed using islets from separate isolation and culture days. Statistical significance was determined by one-way analysis of variance unless stated otherwise.

RESULTS

Citrate content of FA-cultured islets. Culturing islets for 24 h with 250 µmol/l oleate lowered the citrate content by 45% ($P < 0.01$) (Fig. 1*A*). Adding 0.5 µg/ml triacsin C to the oleate culture normalized the citrate content. Our previous study used the same 24-h oleate culture protocol and showed that islet protein and DNA values were unaffected (17).

Citrate synthase activity of FA-cultured islets. Citrate synthase V_{max} paralleled the citrate results: 45% reduction in the oleate-cultured islets ($P < 0.001$) and full reversal by triacsin C (Fig. 1*B*).

Time course and oleate concentration effects. The time course of the oleate-induced decrease in citrate production/level was compared with the increased PFK V_{max} (found previously [17]). Citrate synthase V_{max} was maximally inhibited at the first time point tested, 30 min (Fig. 2*A*). Citrate content lagged slightly, with its level declining over 1 h (Table 1). In contrast, the increase in PFK V_{max} had not yet occurred by 6 h but was present when the study was extended to 24 h (Fig. 2*B*), in agreement with our previous results. A small fall in PFK activity during the first 6 h became significant at 1 h ($P < 0.03$ vs. control islets).

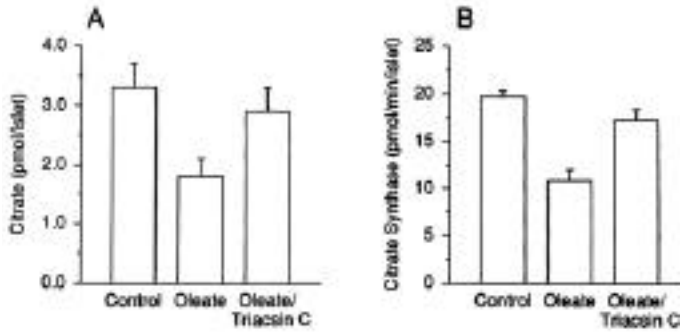

FIG. 1. Citrate content (*A*) (*n* = 5) and citrate synthase V_{max} (*B*) (*n* = 5) in oleate cultured islets. Rat islets were cultured for 24 h in medium with or without 250 µmol/l oleate. Additional islets were co-cultured with triacsin C (0.5 µg/ml) plus 250 µmol/l oleate. Citrate content and citrate synthase V_{max} were measured as described in METHODS.

Concentration studies were performed by culturing islets for 24 h in 0, 30, 60, 125, or 250 µmol/l oleate. There was a 35% reduction of citrate synthase V_{max} with 125 µmol/l oleate ($P < 0.003$ vs. control islets) and an additional 10% reduction with 250 µmol/l (Fig. 3*A*). The results for PFK V_{max} were slightly different (Fig. 3*B*): the first significant increase was noted with 60 µmol/l oleate ($P < 0.01$ vs. control islets) followed by concentration-dependent increases to 250 µmol/l.

The above protocols used oleate that had not been precomplexed to BSA. An additional 24 h study was performed with 250 and 600 µmol/l oleate from an 8 mmol/l oleate/10% fatty acid–free BSA stock (Table 2). Similar qualitative effects on citrate production/level and PFK were found, although as expected, higher oleate concentrations were required. Oleate at 600 µmol/l decreased citrate synthase V_{max} and the citrate level and increased PFK V_{max} by 30–35% ($P < 0.002$ for all three vs. the respective control values).

Citrate synthase and PFK activities of islets cultured with high doses of glucose. Reversal of the biochemical effects of oleate by triacsin C suggested that increased LC-CoA was the causative factor, and that similar changes would be expected with high glucose because it increases cytoplasmic LC-CoA in β-cells (27–29). We thus investigated islets cultured

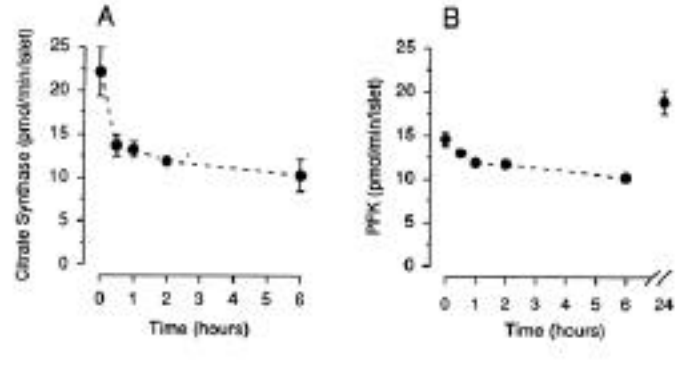

FIG. 2. Time course for oleate effects on citrate synthase V_{max} (*A*) (*n* = 4) and PFK V_{max} (*B*) (*n* = 4). Rat islets were incubated for a total of 6 h in medium with the addition of 250 µmol/l oleate to give exposure times of 30 min or 1, 2, or 6 h. The PFK protocol added a 24-h time point. V_{max} values for citrate synthase and PFK were measured as described in METHODS.

TABLE 1
Time course of the effect of oleate culture on islet citrate content

Time (h)	Citrate (pmol/islet)	P value
0	2.6 ± 0.1	—
0.5	2.2 ± 0.1	0.025
1	1.8 ± 0.1	0.005
2	1.7 ± 0.1	0.005
6	1.6 ± 0.1	0.001

Data are means ± SE. Islets were cultured with 250 µmol/l oleate for the times shown (n = 4). Citrate content was measured as described in METHODS. Statistical significance was calculated against the 0 time result using one-way analysis of variance.

for 48 h in 5.5 or 16.7 mmol/l glucose; the longer time period from the oleate protocol was chosen on the basis of preliminary studies that showed a slightly greater effect of 2 days versus 1 day of high glucose on PFK V_{max}. As predicted, high glucose lowered citrate synthase V_{max} by 34% ($P < 0.001$), and that effect was prevented by 0.5 µg/ml triacsin C (Fig. 4*A*). Similarly, PFK V_{max} was increased 60% by the high-dose glucose culture ($P < 0.001$), with reversal of the effect by triacsin C (Fig. 4*B*).

DISCUSSION

We have investigated the altered β-cell glucose sensing that occurs when islets are chronically exposed to FA. Our previous study identified a large decrease in the cellular level of G-6-P, and the resulting deinhibition of hexokinase, as the likely cause (17). A key factor, because of its regulatory influence over G-6-P removal, was an increased PFK V_{max}. The present study has provided further insight by showing that FA culture also reduced citrate synthase activity, and thus the production/cellular level of citrate.

This study measured citrate content of whole islet extracts, which included the mitochondrial and cytoplasmic pools, whereas only the cytoplasmic fraction influences PFK. Separately measuring the pools would require rapid cell disruption/fractionation and inactivation of enzymes/carriers, which is technically very difficult. Such studies have been done in isolated cells, such as hepatocytes, but are impractical for islets, which consist of several thousand cells. Thus, our conclusions assume that the two pools are interrelated and change in a parallel fashion, as was observed in hepatocytes with lowered citrate content after ammonia exposure (35).

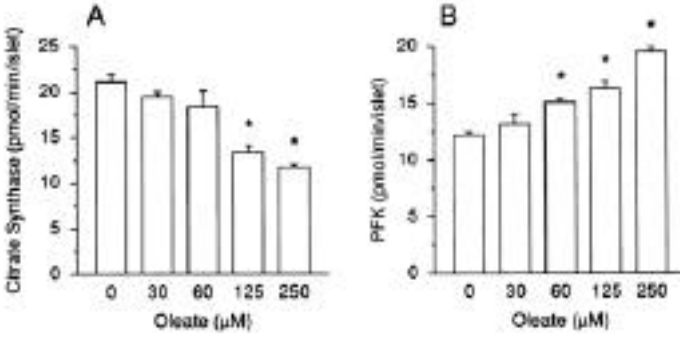

FIG. 3. Oleate concentration effects on citrate synthase V_{max} (*A*) (n = 4) and PFK V_{max} (*B*) (n = 4). Rat islets were cultured for 24 h in medium with the addition of 30, 60, 125, or 250 µmol/l oleate in 2% ethanol; control islets received 2% ethanol alone. V_{max} values for citrate synthase and PFK were measured as described in METHODS. *P < 0.05 vs. control islets.

Citrate is a potent inhibitor of PFK (21). The newly identified reduction in citrate would act in tandem with the increased PFK V_{max} to increase that enzyme's activity, and thus G-6-P catabolism. These effects were reversed by triacsin C, indicating that LC-CoA (or a subsequent metabolite such as diacylglycerol or phosphatidic acid) was the cellular mediator. Consistent with this conclusion, we observed that 2-day culture in high-dose glucose had similar effects on citrate synthase and PFK to those of FA culture, since high glucose raises cytosolic LC-CoA levels in β-cells through malonyl-CoA inhibition of carnitine palmitoyltransferase I (27,28). A final observation was the different timings for the decreased citrate synthase V_{max} (30 min) and the increased PFK V_{max} (>6 h), indicating that the actions of LC-CoA in the β-cell involve more than one mechanism. The multihour time for the increased PFK V_{max} is presumed to reflect induction of PFK gene expression, in agreement with the effect shown in clonal β-cells that were cultured for several days with high-dose glucose (36).

Our results suggest that FA induces complex effects on PFK in both expression and activity. Moreover, these actions were shared by two aspects of the diabetes syndrome, high glucose and high FA. This understanding is of particular interest in view of the common tendency to think of glucotoxicity and lipotoxicity separately in terms of the pathogenesis of type 2 diabetes. Prentki and Corkey (8) pointed out that high glucose and FA both increase cytosolic LC-CoA and thereby could similarly cause a variety of signaling and enzymatic/metabolic changes; they coined the term "glucolipoxia" in part to reflect the convergent effects of the two fuels. The studies here support and add to that hypothesis by showing similar changes in citrate synthase and PFK in response to high-dose glucose or FA. Further, reversal by triacsin C in both culture systems showed involvement of the shared effector, LC-CoA. It should not be concluded, however, that FA and glucose cause identical changes in β-cell glucose metabolism, and thus insulin secretion. It is well established that other enzymes are differently affected. Best known is glucokinase; its activity and cellular level are glucoregulated in β-cells (37,38), but FA culture induced no change (3,39). Another example is acetyl-CoA carboxylase: high glucose increased its gene expression (29) in contrast with inhibition by FA (40). Finally, glycolysis is dependent on the availability of its substrate (glucose). The key finding in our previous study in terms of the shifted insulin secretion curve was the lowered G-6-P level (17). It is not expected that the G-6-P level will necessarily be lowered in islets cultured with high levels of glucose, despite identical effects on PFK V_{max} and citrate production, because of the increased glucokinase activity and the mass action of glucose on glycolysis. The same argument can be made for citrate, since factors increasing its production from glucose (29) may counterbalance the subnormal citrate synthase activity. Indeed, recent preliminary studies have indicated that the citrate content of islets cultured for 2 days at 16.7 mmol/l glucose is somewhat greater (35%) than that of islets cultured at 5.5 mmol/l glucose (Y.Q.L., J.L.L., unpublished observations). A similar issue regards fructose-2,6-bisphosphate, a potent activator of PFK (21), which is synthesized

TABLE 2
Effects of oleate precomplexed to BSA

Parameter	Oleate			
	None	250 µmol/l	None	600 µmol/l
PFK V_{max} (pmol fructose-1,6-diphosphate · min^{-1} · $islet^{-1}$)	13.1 ± 0.5	14.9 ± 0.8*	13.9 ± 0.3	18.6 ± 0.2‡
Citrate synthase V_{max} (pmol · min^{-1} · $islet^{-1}$)	21.0 ± 0.6	18.5 ± 0.3†	20.5 ± 0.6	13.6 ± 0.6‡
Citrate content (pmol/islet)	2.7 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	1.7 ± 0.1‡

Data are means ± SE. Islets were cultured for 24 h (n = 4 for all parameters). Oleate was added to the culture medium from an 8 mmol/l stock solution prepared in KRB containing 10% fatty acid–free BSA. Two oleate concentrations (250 and 600 µmol/l) were used in separate experiments; the control values from both are shown. Statistical significance was calculated against the respective control islets using the unpaired Student's t test. *P < 0.05; †P < 0.01; ‡P < 0.002.

from fructose-6-phosphate and can be sensitive to mass action effects of glucose. Our preliminary studies have shown an increased content of this metabolite in high glucose–cultured islets (Y.Q.L., J.L.L., unpublished observations) in contrast to the lack of change with oleate culture (17). Thus, the chronic effects of high glucose and FA to alter β-cell glucose and citrate metabolism and insulin secretion are complex, with both similarities and differences.

A technical issue of this study regards our use of oleate that was not precomplexed to BSA, replicating the original study of Zhou and Grill (1). Some investigators advocate the use of precomplexed FA/BSA, ostensibly to avoid deleterious cellular effects of supraphysiologic FA concentrations. We also performed studies with oleate that was preabsorbed to BSA using the protocol of Gremlich et al. (31). Similar effects on citrate production/level and PFK activities were noted, although with higher oleate concentrations, as would be expected because of the FA-binding effect of BSA. Note that the 600 µmol/l oleate concentration exactly matches the concentration of palmitate/BSA that was used by Gremlich to study IDX-1 gene expression in FA-cultured islets (31).

Our results are in agreement with a previous study in lymphoid tissue that showed a negative effect of a FA-enriched diet on citrate synthase activity (24). Investigation of isolated mitochondria (26) and purified citrate synthase (25) attributed that effect to allosteric inhibition of citrate synthase by LC-CoA. The current study has expanded those results. First, inhibition of citrate synthase by FA, and subsequently high glucose, was shown in islets. Second, it was blocked by triacsin C, confirming a key role for LC-CoA. Third, the time course showed rapid development of the inhibition, consistent with a direct effect on enzyme activity rather than net synthesis. However, our protocol treated intact islets with FA and measured citrate synthase activity in extracts after a 15-fold dilution. Allosteric inhibition of citrate synthase would require sufficiently tight binding of LC-CoA to the enzyme to negate the dilution; that may be reasonable, since LC-CoA preferentially binds to proteins and membranes rather than dissolving in the aqueous medium. An alternative possibility concerns the heteroenzyme complexes in mitochondria that facilitate transfer of substrates between enzymes, so-called channeling (41,42). Glutamate dehydrogenase forms complexes with citrate synthase, malate dehydrogenase, and aspartate dehydrogenase to shunt metabolites into citric acid cycle reactions, the malate-pyruvate shunt, and amino acid catabolism, respectively. LC-CoA facilitates formation, thus activation, of these complexes, but with varying affinities; the affinity of LC-CoA for citrate synthase is the weakest (41). This mode may mediate the inhibitory effect of LC-CoA on citrate synthase, assuming stability of these complexes during the homogenization and assay conditions.

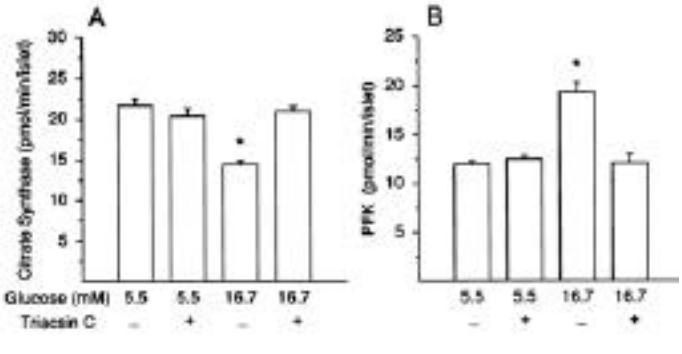

FIG. 4. Citrate synthase V_{max} (A) (n = 4) and PFK V_{max} (B) (n = 4) in high glucose–cultured islets. Rat islets were cultured for 48 h in medium with 5.5 or 16.7 mmol/l glucose. Groups of islets were co-cultured with triacsin C (0.5 µg/ml). V_{max} values for citrate synthase and phosphofructokinase were measured as described in METHODS. *P < 0.05 vs. islets cultured with 5.5 mmol/l glucose.

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Author Queries (please see Q in margin and underlined text)

Q1: Please check definition of EC_{50}

Q1a: Would you like to add “LC” to the abbreviation FA (long-chain fatty acid) to be consistent with the abbreviation LC-CoA?

Q2: Please define CoA-SH.

Q4: Was it Krebs-Ringer bicarbonate (KRBB) or bicarbonate HEPES (KRBH)?

Q6: *Diabetes* journal style does not permit footnotes. A footnote has been added to the text where it was cited.

Ref 24: Please check page range.

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