A distinct difference in the metabolic stimulus–response coupling pathways for regulating proinsulin biosynthesis and insulin secretion that lies at the level of a requirement for fatty acyl moieties

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The regulation of proinsulin biosynthesis in pancreatic β -cells is vital for maintaining optimal insulin stores for glucose-induced insulin release. The majority of nutrient fuels that induce insulin release also stimulate proinsulin biosynthesis, but since insulin exocytosis and proinsulin synthesis involve different cellular mechanisms, a point of divergence in the respective metabolic stimulus–response coupling pathways must exist. A parallel examination of the metabolic regulation of proinsulin biosynthesis and insulin secretion was undertaken in the same β -cells. In MIN6 cells, glucose-induced proinsulin biosynthesis and insulin release shared a requirement for glycolysis to generate stimulus-coupling signals. Pyruvate stimulated both proinsulin synthesis (threshold 0.13–0.2 mM) and insulin release (threshold 0.2–0.3 mM) in MIN6 cells, which was eliminated by an inhibitor of pyruvate transport (1 mM α -cyano-4-hydroxycinnamate). A combination of α -oxoisohexanoate and glutamine also stimulated proinsulin biosynthesis and insulin release in MIN6 cells, which, together with the effect of pyruvate, indicated that anaplerosis was necessary for instigating secondary metabolic stimulus-coupling signals in the β -cell. A consequence of increased anaplerosis in β -cells is a marked increase in malonyl-CoA, which in turn inhibits β -oxidation and elevates cytosolic fatty acyl-CoA levels. In the β -cell, long-chain fatty acyl moieties have been strongly implicated as metabolic stimulus-coupling signals for regulating insulin exocytosis. Indeed, it was found in MIN6 cells and isolated rat pancreatic islets that exogenous

oleate, palmitate and 2-bromopalmitate all markedly potentiated glucose-induced insulin release. However, in the very same β -cells, these fatty acids in contrast inhibited glucose-induced proinsulin biosynthesis. This implies that neither fatty acyl moieties nor β -oxidation are required for the metabolic stimulus–response coupling pathway specific for proinsulin biosynthesis, and represent an early point of divergence of the two signalling pathways for metabolic regulation of proinsulin biosynthesis and insulin release. Therefore alternative metabolic stimulus-coupling factors for the specific control of proinsulin biosynthesis at the translational level were considered. One possibility examined was an increase in glycerophosphate shuttle activity and change in cytosolic redox state of the β -cell, as reflected by changes in the ratio of α -glycerophosphate to dihydroxyacetone phosphate. Although 16.7 mM glucose produced a significant rise in the α -glycerophosphate/dihydroxyacetone phosphate ratio, 1 mM pyruvate did not. It follows that the cytosolic redox state and fatty acyl moieties are not necessarily involved as secondary metabolic stimulus-coupling factors for regulation of proinsulin biosynthesis. However, the results indicate that glycolysis and the subsequent increase in anaplerosis are indeed necessary for this signalling pathway, and therefore an extramitochondrial product of β -cell pyruvate metabolism (that is upstream of the increased cytosolic fatty acyl-CoA) acts as a key intracellular secondary signal for specific control of proinsulin biosynthesis by glucose at the level of translation.

INTRODUCTION

The major function of the pancreatic β -cell is production, storage and regulated secretion of insulin [1]. Under normal physiological circumstances, it maintains intracellular insulin stores at a constant level, so that there is a readily available pool for secretion [2]. This is mainly achieved by co-ordinated regulation of proinsulin biosynthesis, which replenishes insulin secreted from the β -cell in response to a stimulus. In the short term (< 2 h), glucose specifically stimulates proinsulin biosynthesis above that of total β -cell protein synthesis, which is predominantly controlled at the translational level [2–6]. However, the molecular mechanism behind the specific translational regulation of proinsulin biosynthesis essentially remains undefined. Furthermore, the specific metabolic stimulus–response coupling mechanism that transduces a ‘glucose signal’ to specifically increase the rate of preproinsulin mRNA translation in the β -cell has yet to be elucidated.

Control of proinsulin biosynthesis can be mediated by many factors including certain nutrients, of which glucose is the most physiologically relevant [2]. In general, nutrients that stimulate insulin secretion also specifically stimulate proinsulin biosynthesis [2,7]. Glucose metabolism is required for the stimulus–response coupling mechanisms of both insulin secretion and proinsulin biosynthesis [7], but the threshold glucose concentration required to trigger insulin secretion (4–6 mM glucose) is higher than that to stimulate proinsulin biosynthesis (2–4 mM glucose) [7]. Common metabolic regulation of proinsulin biosynthesis and insulin secretion is further indicated in that they are both stimulated by nutrient compounds that feed into intermediate stages of glycolysis, such as glyceraldehyde and dihydroxyacetone, as well as the mitochondrial fuels leucine and α -oxoisohexanoate (α -KIC) [2,7]. However, although nutrient regulation of proinsulin biosynthesis and insulin secretion can share common metabolic requirements, there must be a point of divergence of these stimulus–response coupling pathways, since

Abbreviations used: α -CHC, α -cyano-4-hydroxycinnamate; α -KIC, α -oxoisohexanoate; α -GP, α -glycerophosphate (glycerol 3-phosphate); DHAP, dihydroxyacetone phosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration.

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the distal targets of the β -cell's insulin exocytotic apparatus and protein synthesis machinery are distinctly different cellular mechanisms.

The early events in the β -cell's metabolic stimulus-response coupling pathway of glucose-induced insulin release have been relatively well defined [8–10]. Essentially, an increased rate of β -cell glycolytic metabolism increases the intracellular ATP/ADP ratio leading to closure of ATP-sensitive K^+ channels, depolarization across the β -cell plasma membrane for opening of L-type voltage-gated Ca^{2+} channels and rapid elevation of cytosolic Ca^{2+} concentrations $[Ca^{2+}]_i$. Elevation of $[Ca^{2+}]_i$ has long been thought to be a key intracellular signal for inducing exocytosis [8,10,11]. In contrast, the stimulus-response coupling pathway for glucose-regulated proinsulin biosynthesis at the translational level is apparently independent of either extracellular or intracellular Ca^{2+} [2]. Furthermore, in the β -cell, ATP-sensitive K^+ -channel antagonists (e.g. diazoxide [12]) or agonists (e.g. sulphonylureas [13]), which inhibit or stimulate insulin secretion respectively, have no effect on glucose-stimulated proinsulin biosynthesis [2]. It should be noted that $[Ca^{2+}]_i$ is not the only secondary signal required to trigger insulin release [14]. For example, it is becoming evident that elevated levels of cytosolic long-chain acyl-CoA esters in the β -cell are additional metabolic coupling factors in nutrient-induced insulin release [9,15,16]. However, the role of fatty acids in regulating proinsulin biosynthesis at the translational level has yet to be defined. Furthermore downstream of the elevated $[Ca^{2+}]_i$ signal, Ca^{2+} /calmodulin-dependent protein kinases [17] and protein kinase C [18] have been implicated in insulin release, but neither of these protein kinase activities appear to be involved in the translational regulation of proinsulin biosynthesis [6,19]. Therefore, although there are some common metabolic requirements, downstream differences in the stimulus-response coupling requirements for glucose-induced proinsulin biosynthesis from those for insulin release indicate that a point of divergence lies before an effect on the ATP-sensitive K^+ channel, probably at the distal stages of β -cell metabolism. In this study, we have examined in parallel the metabolic regulation of proinsulin biosynthesis and insulin secretion in the same β -cells, as stimulated by a glycolytic fuel (e.g. glucose), mitochondrial fuels (i.e. pyruvate and α -KIC) and long-chain non-esterified fatty acids, in order to find the point of divergence in β -cell glycolytic flux that leads to differential regulation of insulin secretion and proinsulin biosynthesis.

EXPERIMENTAL

Reagents and materials

L- $[^{35}S]$ Methionine (1200 Ci/mmol) was from Du Pont–New England Nuclear. ^{125}I -insulin (374 mCi/mg) was a gift from Eli Lilly (Indianapolis, In, U.S.A.). Pansorbin[®] was from Calbiochem Corp. (La Jolla, CA, U.S.A.). Oleic acid and 2-bromopalmitate were from Aldrich Biochemicals (Milwaukee, WI, U.S.A.). Palmitic acid and all other chemicals were from Sigma (St. Louis, MO, U.S.A.) and of the highest grade and purity available. Insulin was measured by RIA using a kit from Linco (St. Louis, MO, U.S.A.).

Cell culture and preparation

Low-passage MIN6 cells (passage 16–30) have been previously characterized as a suitable model for islets in terms of glucose-regulated proinsulin biosynthesis at the translational level [6]. MIN6 cells were maintained in culture as previously described [6]. Pancreatic islets of Langerhans were isolated from male 150 g Sprague–Dawley rats by collagenase digestion and Histopaque-

Ficoll[®] density-gradient centrifugation as previously described [20]. In some experiments, pancreatic islets were isolated from rats deprived of food for 20 h to lower intracellular fatty acids and triacylglycerol [16].

Parallel analysis of proinsulin biosynthesis and insulin secretion in the same β -cells

Incubation of MIN6 cells in 300 μ l of Krebs–Ringer bicarbonate buffer/16 mM Hepes/0.1 % BSA to monitor proinsulin biosynthesis and insulin secretion was performed in 24-well plates as previously described [6]. Essentially, cells were preincubated for 1 h at 37 °C at basal 2.8 mM glucose, then for a further 1 h in the same buffer containing insulin secretagogues as indicated. The last 20 min of this latter incubation period was in the presence of 100 μ Ci/ml $[^{35}S]$ methionine to monitor protein biosynthesis [6,20]. To assess insulin secretion in the same cells, a 200 μ l aliquot of this second incubation medium was collected and centrifuged at 1500 *g* for 2 min to remove debris; the supernatant was saved and stored at –80 °C pending analysis by RIA of insulin secreted. The cells were then washed, resuspended in 300 μ l of lysis buffer and lysed as previously described [6,20]. A 5 μ l aliquot of the resulting lysate was taken and stored at –80 °C pending analysis of intracellular insulin content by RIA. Another 5 μ l aliquot of the lysate was taken for assessment of total protein synthesis by trichloroacetic acid precipitation as previously described [6,20]. Subsequently, all cell lysates were equilibrated for equivalent levels of protein synthesis before analysis of proinsulin synthesis, so that specific regulation of proinsulin synthesis above that of general protein synthesis in the β -cell could be assessed [6,20]. The remaining equilibrated cell lysate was subjected to specific (pro)insulin immunoprecipitation for assessment of proinsulin biosynthesis, with analysis by alkaline-gel electrophoresis, fluorography and densitometric scanning as described [6,20].

For the islet experiments, groups of 50 islets per observation were preincubated in 1.5 ml microcentrifuge tubes in 300 μ l of Krebs–Ringer bicarbonate buffer/16 mM Hepes/0.1 % BSA containing 2.8 mM glucose for 1 h at 37 °C. Cells were centrifuged at 800 *g* for 2 min, resuspended, then incubated for a 1 h in 300 μ l of the same buffer containing insulin secretagogues as indicated. At the end of this period, islets were centrifuged down at 800 *g* for 2 min, and 200 μ l of the supernatant was removed for storage at –80 °C pending analysis by RIA of insulin secreted. The islets were then resuspended in the remaining 100 μ l of buffer with 10 μ Ci of $[^{35}S]$ methionine added, and then incubated for a further 20 min. After this radiolabelling period, the islets were centrifuged down at 800 *g* for 2 min, then resuspended in 300 μ l of lysis buffer, and analysed for intracellular insulin content, total protein synthesis and specific proinsulin biosynthesis as described above for MIN6 cells [6,20].

When MIN6 cells or isolated islets were incubated with long-chain fatty acids, oleic acid and palmitic acid, solutions were first made up at stock concentrations of 15 mM complexed to 15 % (w/v) fatty acid-free albumin (Sigma fraction V) and used in 0.5 mM final concentration as previously described [16]. BSA (0.5 %, w/v) was used in all control experiments.

Measurement of α -glycerophosphate (α -GP) and dihydroxyacetone phosphate (DHAP)

MIN6 cells were used for these experiments as opposed to isolated pancreatic islets because of the large amount of material required for these measurements. MIN6 cells at 50 % confluence in 6 cm diameter plates were preincubated at 37 °C for 1 h in 1 ml

of Krebs–Ringer bicarbonate buffer/16 mM Hepes/0.1 % BSA containing a basal 2.8 mM glucose. The cells were then subjected to a second incubation at 37 °C for 2 h in the same buffer containing various secretagogues as indicated. At the end of this latter incubation, the medium was collected, centrifuged at 1500 *g* for 2 min, and the supernatant stored at –80 °C pending analysis of insulin secretion by RIA and lactate output. The cells were scraped off the dish in 400 μ l of ice-cold 5 mM Hepes/NaOH buffer, pH 7.2, containing 60 mM sucrose, 190 mM mannitol, 15 mM KCl, 3 mM KH_2PO_4 , 1 mM MgCl_2 and 0.5 mM EGTA. The cells were then homogenized on ice by 20 strokes of a Dounce homogenizer. The resulting homogenate was then centrifuged at 1000 *g* for 10 min at 4 °C, the supernatant removed and the pellet rehomogenized in 400 μ l of the buffer described above. These two post-nuclear supernatants were then pooled, and a 50 μ l aliquot was removed and stored at –80 °C pending assay of total protein concentration. The remainder of the supernatant was deproteinized with 6 M HClO_4 as described [21], and the extract stored at –80 °C. Before analysis, the samples were neutralized to pH 7.2 with 5 M K_2CO_3 , and the protein precipitate removed by centrifugation for 10 min at 10000 *g* at 4 °C. The levels of DHAP and α -GP in the supernatant were measured using specific fluorimetric enzyme assays as previously described [21], and the concentration determined against appropriate α -GP/DHAP standard curves prepared in the same buffer as the sample.

Measurement of lactate output

A 50–75 μ l aliquot of the incubation medium of MIN6 cells used in studies to analyse cytosolic α -GP and DHAP levels was used to assess lactate output from MIN6 cells. Secreted lactate levels were measured as previously described [22].

Other methods

Protein was assayed by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.). Data are presented as means $\pm$ S.E.M. Statistically significant differences between groups were analysed using Student's *t* test; $P < 0.05$ was considered significant.

RESULTS

Glucose metabolism is required for both insulin release and proinsulin biosynthesis in MIN6 cells

Mannoheptulose, an inhibitor of glucokinase activity [7], inhibited glucose-stimulated proinsulin biosynthesis and insulin release in MIN6 cells ($P \leq 0.001$) (Figure 1). In addition, elevated concentrations of the non-metabolizable glucose analogues 2-deoxyglucose or 3-*O*-methylglucose did not stimulate proinsulin biosynthesis or insulin release in MIN6 cells above the basal rate at 2.8 mM glucose ($P \leq 0.01$) (Figure 1). This indicated that glycolysis was required to stimulate proinsulin biosynthesis at the translational level as well as insulin release in MIN6 cells, as seen in pancreatic islets [7].

Pyruvate stimulates both insulin secretion and proinsulin biosynthesis in MIN6 cells

Pyruvate, the final product of glycolysis, is transported into mitochondria for oxidative metabolism. In MIN6 cells, proinsulin biosynthesis was specifically stimulated by pyruvate at a threshold concentration of between 0.13 and 0.20 mM pyruvate and reached a significant maximum at 1.0 mM (3.5-fold; $P \leq 0.05$), over the basal rate at 2.8 mM glucose (Figure 2A). In the

Figure 1 Effect of mannoheptulose on proinsulin biosynthesis and insulin secretion in MIN6 cells

MIN6 cells were incubated for 60 min in 2.8 mM or 16.7 mM glucose in the presence or absence of 20 mM mannoheptulose (MH), or in the presence of 16.7 mM 2-deoxyglucose (2-DOG) or 16.7 mM 3-*O*-methylglucose (3-OMeG). Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ^{35}S -proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least four individual experiments, and an example fluorogram is shown. (B) Results for insulin secretion (means $\pm$ S.E.M. for at least four experiments).

same MIN6 cells, pyruvate stimulated insulin secretion at a threshold between 0.2 and 0.3 mM pyruvate, which reached a significant maximum of 13–14-fold at 1.0 mM ($P \leq 0.01$) (Figure 2B). Lactate (10 mM) elicited no stimulation of insulin release or proinsulin biosynthesis in MIN6 cells (results not shown). Pyruvate entry into cells and mitochondria can be inhibited by α -cyano-4-hydroxycinnamic acid (α -CHC) [23–25]. Pyruvate (1 mM)-induced stimulation of proinsulin biosynthesis and insulin secretion was completely inhibited by 1 mM α -CHC ($P \leq 0.001$) (Figure 3). However, although inhibition of pyruvate entry into mitochondria by α -CHC resulted in a significant 60 % reduction in glucose-induced insulin secretion ($P \leq 0.001$; Figure 3B), there was little effect of α -CHC on glucose-induced proinsulin biosynthesis in the same MIN6 cells (Figure 3A).

Mitochondrial fuels generate metabolic signals to stimulate both insulin secretion and proinsulin biosynthesis

The mitochondrial fuel α -KIC alone [26] produced a slight stimulation of insulin secretion but had little effect on proinsulin

Figure 2 Dose-response curve for pyruvate-stimulated proinsulin biosynthesis and insulin secretion in MIN6 cells

MIN6 cells were incubated in buffer with the addition of the indicated pyruvate and glucose concentrations for 60 min. Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ^{35}S -proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least five individual experiments, and an example fluorogram is shown. (B) Results for insulin secretion (means $\pm$ S.E.M. for at least four experiments).

biosynthesis (Figure 4). However, when 10 mM α -KIC was combined with 10 mM L-glutamine (to activate glutamate dehydrogenase and increase intramitochondrial α -oxoglutarate [27]), significant stimulation of insulin secretion ($P \leq 0.001$) and proinsulin biosynthesis ($P \leq 0.05$) was observed, reminiscent of that previously seen in pancreatic islets [2]. The addition of glutamine alone (10 mM) failed to provide a stimulus for insulin secretion or proinsulin biosynthesis (Figure 4).

Long-chain fatty-acid potentiated glucose-induced insulin secretion but inhibited glucose-induced proinsulin biosynthesis

In MIN6 cells, oleate, palmitate and 2-bromopalmitate (0.5 mM) all increased basal insulin secretion at 2.8 mM glucose and potentiated 16.7 mM glucose-induced insulin secretion 2-fold ($P \leq 0.02$) (Figure 5B). However, in contrast, oleate and palmitate modestly inhibited glucose-induced proinsulin biosynthesis by 25–30% in the same MIN6 cells ($P \leq 0.05$; Figure 5A), whereas bromopalmitate had no significant effect. In isolated pancreatic

Figure 3 Effect of α -CHC on proinsulin biosynthesis and insulin secretion in MIN6 cells

MIN6 cells were incubated in buffer with the addition of the indicated glucose or pyruvate concentrations in the presence or absence of α -CHC for 60 min. Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ^{35}S -proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least four individual experiments, and an example fluorogram is shown. (B) Results for insulin secretion (means $\pm$ S.E.M. for at least four experiments).

islets, palmitate and 2-bromopalmitate significantly potentiated basal and 16.7 mM glucose-induced insulin secretion ($P \leq 0.05$; Figure 6B), similar to that in MIN6 cells. On the other hand, in

Figure 4 Effect of α -KIC on proinsulin biosynthesis and insulin secretion in MIN6 cells

MIN6 cells were incubated in buffer for 60 min with the addition of the indicated glucose concentrations or 10 mM α -KIC in the presence or absence of 10 mM L-glutamine, or with 10 mM L-glutamine alone. Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ^{35}S -proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least three individual experiments, and an example fluorogram is shown. (B) Results for insulin secretion (mean $\pm$ S.E.M. for at least three experiments).

the same islets palmitate and 2-bromopalmitate inhibited glucose-induced proinsulin biosynthesis by 35–45% ($P \leq 0.05$; Figure 6A).

Figure 5 Effect of fatty acid on proinsulin biosynthesis and insulin secretion in MIN6 cells

MIN6 cells were incubated for 60 min in 2.8 mM or 16.7 mM glucose in the presence or absence of oleate, palmitate or 2-bromopalmitate [0.5 mM complexed with 0.5% (w/v) BSA]. Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ^{35}S -proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least four individual experiments, and an example fluorogram is shown. (B) Results for insulin secretion (means $\pm$ S.E.M. for at least four experiments).

In pancreatic islets isolated from rats that had free access to food, oleic acid (0.5 mM) significantly potentiated 16.7 mM glucose-induced insulin secretion 2-fold ($P \leq 0.05$; Figure 7B). However, as in MIN6 cells, there was a partial inhibition of glucose-induced proinsulin biosynthesis in the presence of oleate ($P \leq 0.05$; Figure 7A). In an effort to lower intracellular fatty acid and triacylglycerol levels [16], rats were starved for 20 h and the islets isolated. In these islets, a reduction of glucose-induced insulin secretion was noted in the presence of glucose alone ($P \leq 0.05$), as previously observed [16,28]. In contrast, no effect of 20 h starvation on glucose-induced proinsulin biosynthesis was observed (Figure 7A). Addition of oleic acid in the presence of glucose restored glucose-induced insulin secretion to normal control levels (Figure 7B), in agreement with previous observations [16]. However, with the stimulatory effect of fatty acids on insulin secretion, in the same islets isolated from starved animals, 16.7 mM glucose-induced proinsulin biosynthesis was significantly inhibited in the presence of oleate, similar to that observed in islets isolated from fed animals ($P \leq 0.05$; Figure 7A). We have also observed similar effects to these

Figure 6 Effect of palmitate on proinsulin biosynthesis and insulin secretion in isolated pancreatic islets

Isolated rat pancreatic islets were incubated for 60 min in 2.8 mM or 16.7 mM glucose in the presence or absence of palmitate or 2-bromopalmitate [0.5 mM complexed with 0.5% (w/v) BSA]. Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ³⁵S-proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least four individual experiments and an example fluorogram is shown. (B) Results for insulin secretion (means $\pm$ S.E.M. for at least three experiments).

of oleate by palmitate on glucose-regulated insulin secretion and proinsulin biosynthesis in isolated islets from 20 h starved and fed animals (results not shown).

Fatty acid-induced decrease in pancreatic β -cell α -GP and DHAP levels without changing cytosolic redox state

The α -GP and DHAP couple has been used as an indication of α -GP shuttle activity and cytosolic redox state in mammalian cells [29–31]. In MIN6 cells incubated with a stimulatory 16.7 mM concentration of glucose compared with the basal 2.8 mM glucose, there was an increase in DHAP levels, whereas α -GP remained unchanged (Table 1). This, in turn, resulted in a significant increase in the DHAP/ α -GP ratio ($P \leq 0.05$; Table 1) induced by glucose, as previously observed [31]. Pyruvate (1 mM) did not alter DHAP or α -GP levels or their relative ratio compared with that with 2.8 mM glucose. In the presence of oleate (0.5 mM) at either 2.8 mM or 16.7 mM glucose, MIN6 cell α -GP levels decreased by about 50% and DHAP by about 30%

Figure 7 Effect of oleate on proinsulin biosynthesis and insulin secretion in isolated pancreatic islets from fed and starved rats

Rat pancreatic islets, isolated from either fed or 20 h starved animals, were incubated for 80 min in 2.8 mM or 16.7 mM glucose in the presence or absence of oleate [0.5 mM complexed with 0.5% (w/v) BSA]. Insulin secretion was analysed by RIA. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography and densitometric scanning as described in the Experimental section. The relative increase in scanning volume density of the band on the autoradiograph equivalent to ³⁵S-proinsulin above that at a basal 2.8 mM glucose was calculated for each analysis as described [20]. Results for proinsulin biosynthesis are indicated in (A) as means $\pm$ S.E.M. for at least four individual experiments, and an example fluorogram is shown. (B) Results for insulin secretion (means $\pm$ S.E.M. for at least four experiments). Note that experiments on islets isolated from starved rats were performed on fresh not cultured islets.

Table 1 Oleate-induced decrease in α -GP and DHAP levels in MIN6 cell extracts irrespective of glucose concentration

MIN6 cells were incubated for 2 h in 2.8 mM or 16.7 mM glucose in the presence or absence of oleate [0.5 mM complexed with 0.5% (w/v) BSA] and 1 mM pyruvate. The concentrations of α -GP and DHAP were then measured in cell lysates as described in the Experimental section. Results are a means $\pm$ S.E.M. for at least three independent determinations. *Significant difference from 2.8 mM glucose alone ($P \leq 0.05$).

	α -GP (nmol/mg of protein)	DHAP (nmol/mg of protein)	DHAP/ α -GP ratio
2.8 mM glucose	40 $\pm$ 6	65 $\pm$ 6	1.6
16.7 mM glucose	43 $\pm$ 4	96 $\pm$ 11*	2.2*
1 mM pyruvate	43 $\pm$ 5	64 $\pm$ 10	1.5
2.8 mM glucose + oleate (0.5 mM)	20 $\pm$ 2*	43 $\pm$ 4*	2.2*
16.7 mM glucose + oleate (0.5 mM)	22 $\pm$ 2*	47 $\pm$ 5	2.1*

Figure 8 Effect of glucose, pyruvate and oleate on lactate output from MIN6 cells

MIN6 cells were incubated for 2 h in 2.8 mM or 16.7 mM glucose in the presence or absence of oleate [0.5 mM complexed with 0.5% (w/v) BSA], or in 1 mM pyruvate. The cells were then collected for analysis of cytosolic α -GP and DHAP levels, and the medium analysed for lactate output as described in the Experimental section. Results are means $\pm$ S.E.M. for at least four individual experiments.

compared with basal 2.8 mM glucose controls ($P \leq 0.02$; Table 1). In spite of this oleate-induced decrease in α -GP and DHAP levels, the DHAP/ α -GP ratio remained increased in the presence of oleate at both 2.8 mM and 16.7 mM glucose compared with control MIN6 cells incubated at a basal 2.8 mM glucose only ($P \leq 0.05$; Table 1). In the same MIN6 cells, insulin release increased in the presence of 16.7 mM glucose, 1 mM pyruvate and 0.5 mM oleate as previously observed (Figures 2, 3, and 5).

Fatty acid increases lactate output in MIN6 cells

Lactate production was assessed in the MIN6 cells in which α -GP and DHAP levels were measured. Glucose induced a 2-fold increase in lactate output as previously observed [32] (Figure 8). Pyruvate did not alter the basal lactate output from that observed in MIN6 cells incubated at 2.8 mM glucose (Figure 8). However, in the presence of oleate (0.5 mM), lactate output in MIN6 cells increased 2-fold at both 2.8 mM and 16.7 mM glucose compared with the respective controls in the absence of oleate ($P \leq 0.05$) (Figure 8).

DISCUSSION

It is unlikely that the β -cell stimulus–response coupling pathways generated by increased nutrient metabolism for regulating both insulin secretion and proinsulin biosynthesis are the same. Similar early metabolic events may be commonly required, but downstream steps probably diverge since the molecular mechanisms for insulin exocytosis and preproinsulin mRNA translation have little in common. However, whereas the secondary signalling elements emanating from glucose metabolism required to stimulate glucose-induced insulin release have been relatively well

defined [8–10], those that induce the specific translational control of proinsulin biosynthesis remain undefined [2]. In this study, in the exact same β -cells, we have examined parallel nutrient-regulated proinsulin biosynthesis and insulin secretion in order to gain a better idea of the differential metabolic requirements for these characteristic β -cell functions.

Glucose-induced insulin secretion and proinsulin biosynthesis share a common requirement for glycolysis [7], as indicated by inhibition with mannoheptulose and lack of any effect of non-metabolizable glucose analogues (Figure 1) [5,7]. Pyruvate, the end product of glycolysis and a substrate for the citric acid cycle and oxidative mitochondrial metabolism [9,25], modestly increases proinsulin biosynthesis and insulin secretion in cultured pancreatic islets [33]. In MIN6 cells, pyruvate stimulated both proinsulin biosynthesis and insulin secretion as effectively as glucose in MIN6 cells. It was interesting to note that the threshold concentration for pyruvate-stimulated proinsulin biosynthesis was lower (0.13–0.2 mM) than that for stimulation of insulin secretion (0.2–0.3 mM; Figure 2), which was reflective of a lower threshold glucose concentration required to stimulate proinsulin biosynthesis (2–4 mM) compared with insulin secretion (4–6 mM) [7]. It follows that the different concentration thresholds observed for glucose regulation of proinsulin biosynthesis compared with insulin release do not necessarily lie at the level of glucose sensing, as previously proposed [7], but further downstream in the generation of secondary metabolic stimulus–response coupling signals. It should be further noted that total intracellular pyruvate concentration in islets has been found to be 0.5 mM [34], thus the concentration of pyruvate required to stimulate proinsulin biosynthesis and insulin secretion in MIN6 cells is probably within the physiologically relevant range. Inhibition of pyruvate transport into cells and mitochondria by α -CHC [23], and consequential inhibition of pyruvate-stimulated proinsulin biosynthesis and insulin secretion, underline the specific effect of pyruvate on MIN6 cells. However, when glucose was used as the nutrient stimulus in MIN6 cells, α -CHC only partially inhibited glucose-induced insulin secretion, and had no effect on glucose-induced proinsulin biosynthesis in the same β -cells. This probably reflects the lower threshold pyruvate concentration required to stimulate proinsulin biosynthesis compared with insulin secretion. In the presence of α -CHC, pyruvate (the product of glycolysis when glucose is used as the primary stimulus) would not be efficiently transported into β -cell mitochondria [23], which in turn would decrease pyruvate oxidation [25] and consequently the generation of secondary stimulus–coupling signals for insulin release [8]. However, glucose-regulated insulin release was not fully inhibited in the presence of α -CHC, suggesting that at least some pyruvate was being transported into β -cell mitochondria to generate a stimulus for insulin release. Moreover, sufficient pyruvate appeared to be getting into β -cell mitochondria in the presence of α -CHC to generate secondary stimulus–coupling signals to stimulate proinsulin biosynthesis at the translational level, probably because of the lower threshold pyruvate concentration required.

The effect of pyruvate and α -CHC on MIN6 cells suggests that mitochondrial metabolism of pyruvate is required to generate the metabolic secondary signal that increases the rate of both proinsulin biosynthesis and insulin secretion. Pyruvate entry into the mitochondria provides increased levels of intermediates for the citric acid cycle (i.e. anaplerosis) via pyruvate carboxylase production of oxaloacetate and production of acetyl-CoA by pyruvate dehydrogenase [8,33]. The notion that increased anaplerosis leads to generation of secondary signals for stimulation of insulin release and biosynthesis [8] is supported by the observation that α -KIC, in the presence of glutamine, was as effective as

glucose and pyruvate in providing a stimulus for both proinsulin biosynthesis and insulin secretion in MIN6 cells, as found in islets [8,26].

A consequence of increased glycolysis and subsequent anaplerosis is that cytosolic malonyl-CoA levels rise, which in turn inhibit β -oxidation, resulting in an increase in cytosolic long-chain fatty acyl-CoA [8,16,35]. There is growing evidence to suggest that elevated levels of cytosolic long-chain fatty acid moieties in the β -cell are a critical stimulus–response coupling factor for metabolic regulation of insulin secretion [8,35–38], but experimental evidence indicated that cytosolic long-chain fatty acyl-CoA levels probably do not play any stimulus–coupling role in metabolic regulation of proinsulin biosynthesis. Oleate and palmitate have been shown to either have no effect on the basal rate of proinsulin biosynthesis [39] or partly inhibit proinsulin biosynthesis under normoglycaemic conditions [40]. The results of this study show that addition of exogenous fatty acids to MIN6 cells or islets potentiated glucose-induced insulin release, as previously noted [16,41], but, in contrast, partially inhibited glucose-induced proinsulin biosynthesis in the same β -cells. Furthermore exogenous 2-bromopalmitate, which is not oxidized in β -cells [15], also potentiated insulin release but inhibited glucose-induced proinsulin biosynthesis. This indicated that β -oxidation was not required for stimulation of proinsulin biosynthesis, as found for insulin release [8]. The role that intracellular lipid in β -cells might play in metabolic regulation of proinsulin biosynthesis was also examined. Intracellular lipid can be depleted in β -cells by a 20 h period of starvation; this inhibits glucose-induced insulin secretion [16,41], which can be fully restored by the addition of exogenous oleate [16,41]. In contrast, glucose-induced proinsulin biosynthesis in islets isolated from 20 h starved rats was similar to that in islets isolated from control fed animals. Although addition of oleate or palmitate markedly potentiated glucose-induced insulin secretion in both ‘fed’ and ‘starved’ islets, there was a contrasting 50% inhibition of glucose-stimulated proinsulin biosynthesis by fatty acid in the same islet β -cells. The means by which fatty acid induces partial inhibition of glucose-induced proinsulin biosynthesis is not certain. However, it might be due to inhibition of glycolysis [40], perhaps by adversely affecting glucokinase activity [42].

The results of this study indicate that, unlike metabolic regulation of insulin release [8], intracellular fatty acyl moieties are not required for the metabolic stimulus–response coupling pathway that controls proinsulin biosynthesis at the translational level. For the metabolic stimulus–response coupling pathway that controls insulin release, fatty acids are thought to mediate their effects downstream of metabolism at the level of acylation of a protein involved in the mechanism of exocytosis [35], and/or activation of a protein kinase C isoform [8,35]. It follows that the lack of any effect of exogenous or endogenous fatty acid on up-regulation of proinsulin biosynthesis agrees with previous observations that this mechanism is independent of protein kinase C activation and elevated $[Ca^{2+}]_i$ [2,5,6,20,43].

The question remains what is an appropriate stimulus–coupling factor for glucose regulation of proinsulin biosynthesis. It has been suggested that the redox state of the β -cell may be a metabolic stimulus–response coupling factor [7,44], and this is supported by the observation that isolated β -cells that display a higher than normal mitochondrial redox state also have a higher rate of proinsulin biosynthesis [45]. An increased rate of glycolysis can increase the NADH/NAD⁺ ratio in the β -cells via increased glyceraldehyde-3-phosphate dehydrogenase activity [9]. However this is usually compensated for by increased NAD⁺ production either by increased lactate production and/or α -GP shuttle activity, so that glycolysis does not become hindered [9,46]. In

the pancreatic β -cell, lactate dehydrogenase activity is relatively low, so that NAD⁺ production for glycolysis is mostly mediated by α -GP shuttle activity [46]. In this study, we have measured the ratio between DHAP and α -GP, which reflects the cytosolic redox state as well as α -GP shuttle activity [47]. An increase in glucose concentration results in an increase in the DHAP/ α -GP ratio, concordant with an increase in β -cell cytosolic redox state, as previously observed [44,47]. However, 1 mM pyruvate (a concentration that can evoke both insulin release and proinsulin biosynthesis from MIN6 cells) did not change the DHAP/ α -GP ratio, which suggests that the cytosolic redox state is not a β -cell metabolic stimulus–response coupling factor, in agreement with most of the previous experimental evidence [8]. Notwithstanding this, in the presence of oleate at either a basal (2.8 mM) or stimulatory (16.7 mM) concentration of glucose, the DHAP/ α -GP ratio was increased above that in the presence of 2.8 mM glucose alone. Although this appeared to correlate with oleate-induced potentiation of insulin release, it did not correlate with regulation of proinsulin biosynthesis, substantiating the notion that cytosolic redox is not a metabolic coupling factor for stimulation of proinsulin biosynthesis. However, one should notice that DHAP and α -GP levels were decreased in the presence of oleate. This was probably due to syphoning of α -GP away from the α -GP shuttle activity for synthesis of diacylglycerol and triacylglycerol in the presence of increased fatty acid [9]. Such an apparent lowering of α -GP shuttle activity could result in decreased cytosolic NAD⁺ levels, which in turn would impair glycolysis [9]. However, any potential reduction in NAD⁺ levels was probably compensated for by increased lactate dehydrogenase activity in the presence of oleate, as indicated by increased lactate output (Figure 8). Therefore glycolysis was not necessarily compromised in the presence of fatty acid by diminished NAD⁺ levels. Notwithstanding this, it has been suggested that increased α -GP shuttle activity in MIN6 cells as a consequence of increased glycolysis could represent a means whereby more reducing equivalents can be delivered to mitochondria for oxidative phosphorylation and increased ATP production [8]. However, although increased ATP in the β -cell is a key metabolic coupling factor for instigating insulin release [8,9], it is unlikely to be one for specific regulation of proinsulin biosynthesis, as general protein synthesis has a requirement for ATP [48,49].

Unlike that for insulin release [8,9], the specific metabolic stimulus–response coupling factors for glucose-induced proinsulin biosynthesis remain relatively undefined [2]. The results of this study indicate that they cannot be attributed to a change in cytosolic redox state or elevation of cytosolic fatty acyl moieties. However, the effects of pyruvate and a combination of α -KIC and glutamine in stimulating proinsulin biosynthesis indicate that anaplerosis is important, in addition to glycolysis, for generating metabolic secondary coupling signals. Thus it follows that products of mitochondrial pyruvate metabolism, which increase before any increase in cytosolic fatty acyl-CoA, are necessary to stimulate proinsulin biosynthesis. As control of translation of preproinsulin mRNA takes place in the cytosolic compartment of β -cells [2], products of pyruvate metabolism as candidate secondary stimulus–coupling factors for proinsulin biosynthesis must be exported from the mitochondria. This raises the possibility of oxaloacetate, citrate, malate, acetyl-CoA and/or malonyl-CoA being potential metabolic coupling factors for the regulation of proinsulin biosynthesis. However, further experiments will be required to substantiate such a hypothesis, and it is likely that other post-metabolic steps in a signal-transduction pathway for specific translational control of proinsulin biosynthesis are necessary. Nonetheless, a branch point in the metabolic stimulus–response coupling pathways for proinsulin biosynthesis

and insulin release can now be placed between anaplerosis and extramitochondrial production of malonyl-CoA. This early point of divergence in β -cell metabolic signalling pathways precedes cytosolic increases in fatty acyl-CoA and/or closure of ATP-sensitive K^+ channels and elevation of $[Ca^{2+}]_i$.

This work was supported by grants from the National Institute of Health Grants DK 47919 to (C.J.R.), DK50610 (to C.J.R.), DK 35914 (to B.E.C.), DK 50662 (to B.E.C.) and DERC DK 36836, at the Joslin Diabetes Center, Boston. We are grateful to the Eli Lilly company for providing us with ^{125}I -labelled insulin.

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