Mechanisms of leucine- and theophylline-stimulated insulin biosynthesis in isolated rat pancreatic islets

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To extend previous observations on the mechanisms of translational regulation of insulin biosynthesis [Welsh, Scherberg, Gilmore & Steiner (1986) *Biochem. J.* 235, 459–467], we have now compared the intracellular distributions of insulin mRNA after stimulation of insulin biosynthesis by glucose, leucine or theophylline. In comparison with low glucose (3.3 mM) only, the presence of 10 mM-leucine + 3.3 mM-glucose resulted in the transfer of insulin mRNA from the pool of the uninitiated mRNA to the free polysome/monosome fraction and an increase in the amount of insulin mRNA associated with the microsomal fraction. Islets exposed to 5 mM-theophylline + 3.3 mM-glucose also showed a decreased content of uninitiated insulin mRNA in the cytosol, but these islets showed no increase in insulin mRNA in the microsomal fraction. These results suggest that leucine, a nutrient stimulant of insulin biosynthesis, acts essentially by the same mechanisms as those of glucose, whereas theophylline acts only to stimulate initiation rates.

INTRODUCTION

Insulin biosynthesis in pancreatic β -cells is regulated by a multitude of factors, of which glucose is the pre-eminent stimulus (Permutt, 1981). Aspects of the translational control exerted by glucose on insulin biosynthesis have been described by Welsh *et al.* (1986a). It was found that glucose-stimulated insulin biosynthesis is mediated via a combination of at least three mechanisms. (a) A stimulation of initiation rates by glucose at concentrations above 3.3 mM, as demonstrated by the effects of various glucose concentrations on the subcellular distribution of insulin mRNA. High-glucose challenge decreased the amounts of free uninitiated insulin mRNA as compared with insulin mRNA bound to ribosomes. (b) Glucose increased the transfer of initiated, ribosome-bound, insulin mRNA from the cytosol to the RER (rough endoplasmic reticulum), as demonstrated by increased amounts of insulin mRNA associated with membranes. This stimulatory effect of glucose could be rationalized in terms of the SRP (signal-recognition particle)–SRP-receptor interaction, a process which directs nascent secretory proteins into the lumen of the endoplasmic reticulum (Meyer *et al.*, 1982). (c) Glucose also caused a specific stimulation of (pro)insulin biosynthesis at concentrations below 5 mM. This effect was not essential with any changes in the intracellular distribution of insulin mRNA, and thus was interpreted to result from increased elongation of nascent preproinsulin.

The metabolism of glucose, a process which leads to the production of energy and formation of substrate intermediates of importance for protein biosynthesis (Jagus & Safer, 1981; Jackson *et al.*, 1983), is believed to be essential for stimulation of insulin biosynthesis (Ashcroft *et al.*, 1978). However, agents which increase the content of cyclic AMP in the β -cell, such as the phosphodiesterase inhibitor theophylline, also stimulate insulin biosynthesis (Sandler *et al.*, 1983). Previous

studies have shown a close agreement between effects of theophylline and other cyclic-AMP-increasing compounds such as dibutyryl cyclic AMP on insulin biosynthesis (Schatz *et al.*, 1973). Cyclic AMP at supraphysiological concentrations of about 10 mM has been shown to modulate protein synthesis studied in a translational system *in vitro* (Suzuki & Mukoyama, 1985). Furthermore, it has been reported that the presence of cyclic AMP leads to the activation of cyclic-AMP-dependent protein kinases, which phosphorylate ribosomal subunits (Burkhard & Traugh, 1983). To elucidate further the role of the cyclic AMP system in relation to that of the β -cell nutrients in the stimulation of insulin biosynthesis, we have here compared the effects, on the subcellular distribution of insulin mRNA, of glucose and leucine with that of theophylline, which inhibits degradation of cyclic AMP. The results obtained suggest that accumulation of cyclic AMP results in increased initiation rates only, whereas the nutrient-stimulated insulin biosynthesis is mediated by a combination of increased initiation rates and a relief from the SRP-induced arrest.

EXPERIMENTAL

Materials

[2,4,6-³H]Phenylalanine (40 Ci/mmol), [α -³²P]dCTP (3000 Ci/mmol), ¹²⁵I, ACS and NCS were from Amersham, Arlington Heights, IL, U.S.A. A nick-translation system from BRL, Rockville, MD, U.S.A., was used. RNasin was from Promega Biotech, Madison, WI, U.S.A. Glyoxal, yeast tRNA, poly(A), polyvinylpyrrolidone, salmon sperm DNA, dithiothreitol, Dowex MR-3, Hepes and formamide were from Sigma Chemical Co., St. Louis, MO, U.S.A. GeneScreen Hybridization Transfer Membrane was obtained from New England Nuclear, Boston, MA, U.S.A. Ficoll 400 was from Pharmacia,

Uppsala, Sweden. Collagenase (CLS IV) was from Worthington, Freehold, NJ, U.S.A. Formalin-fixed *Staphylococcus aureus* was from the Enzyme Centre, Boston, MA, U.S.A.

Preparation of isolated islets

Islets were isolated on Ficoll gradients (Lernmark *et al.*, 1976) from collagenase-digested pancreata (Howell & Taylor, 1968) of male Sprague-Dawley rats weighing 150 g. The islets were picked free from other pancreatic tissue and maintained in Hanks salt solution until further use.

Insulin biosynthesis

Freshly isolated islets were incubated in groups of 20 at 37 °C for 60 min in 100 µl of Hanks salt solution containing 0.5 mg of bovine serum albumin/ml, 10 mM-Hepes, pH 7.4, and 10 µCi of [2,4,6-³H]phenylalanine, with further additions as stated in Table 1. The islets were then washed once with ice-cold buffer (containing 0.5 mg of bovine serum albumin/ml), and sonicated in 1 ml of redistilled water. Samples (0.1 ml) were precipitated by addition of 1 ml of 10% (w/v) trichloroacetic acid and counted for radioactivity by liquid scintillation after dispersion in NCS (0.5 ml) and ACS (5 ml). The remaining volumes were immunoprecipitated with guinea-pig anti-insulin serum (Welsh *et al.*, 1986a). Normal guinea-pig serum served as a control. The immunoprecipitates were pelleted by adding formalin-fixed *Staph. aureus* and subsequently dissolved in NCS and ACS before liquid-scintillation counting.

Subcellular fractionation

The procedure used for subcellular fractionation was similar to that described and carefully characterized by Welsh *et al.* (1986a), Bag & Sarkar (1975) and Ramsey & Steele (1976). Groups of 200–300 freshly isolated islets were incubated for 90 min in 200 µl of Hanks salt solution containing 10 mM-Hepes, pH 7.4, 0.5 mg of bovine serum albumin/ml and glucose, L-leucine or theophylline as stated in Table 2. After the incubation period, the islets were washed and homogenized in 2 ml of the standard homogenization buffer (250 mM-sucrose, 250 mM-KCl, 10 mM-MgCl₂, 10 mM-Tris, pH 7.5, 1 mM-EDTA, 2 mM-dithiothreitol and 5–20 units of RNasin/

ml) with 12 strokes in a glass/Teflon-pestle Potter-Elvehjem homogenizer at 4 °C. After an initial 10 s centrifugation at 12000 g to pellet nuclei, the supernatant was centrifuged for 12 min at 130000 g. The pellet thus obtained contained insulin mRNA associated with membranes. The 130000 g supernatant was further centrifuged for 2 h at 255000 g to pellet the 80 S monoribosomes. The supernatant obtained after 2 h at 255000 g was placed on a 10–40% (w/v) sucrose gradient in homogenization buffer and centrifuged for 20 h at 100000 g to determine the sedimentation coefficient of non-ribosome-bound cytosolic insulin mRNA. RNA was extracted from all fractions with phenol/chloroform/3-methylbutan-1-ol/2-mercaptoethanol (240:240:10:1, by vol.), equilibrated with 0.1 M-Tris/HCl, pH 7.5, and was then precipitated with ethanol from the aqueous phase after addition of 30 µg of yeast tRNA to ensure efficient precipitation of RNA.

Quantification of insulin mRNA

Insulin mRNA was quantified by Northern-blot analysis (Thomas, 1980) or dot-blot analysis (White & Bancroft, 1982). The RNA precipitates were treated with 1 M-glyoxal and electrophoresed on 2%-agarose gels before being transferred to GeneScreen membranes as described by Thomas (1980). Alternatively, glyoxylated samples were diluted with 4 parts of 20 × SSC (SSC = 0.15 M-NaCl/0.015 M-sodium citrate) and bound to GeneScreen membranes with a suction apparatus. After pre-hybridization, the samples were hybridized at 42 °C with the appropriate nick-translated probe (pRI-7; Chan *et al.*, 1979). After hybridization, the filters were washed and then exposed at –70 °C to Kodak XAR-5 film (Kodak, Rochester, NY, U.S.A.) with an intensifying screen. The intensities of the spots thus obtained were determined by densitometry with an LKB densitometer (LKB, Bromma, Sweden).

RESULTS AND DISCUSSION

Several mechanisms underlying the stimulation of insulin biosynthesis by glucose have previously been described by Welsh *et al.* (1986a). Regulation of insulin synthesis was found to occur at three major steps: initiation of protein synthesis, transfer of the SRP-

Table 1. Effects of glucose, leucine and theophylline on biosynthesis of (pro)insulin and total protein in freshly isolated rat islets

Rates of (pro)insulin (PI-I) biosynthesis and total protein biosynthesis were estimated by measuring the incorporation of [2,4,6-³H]phenylalanine into the PI-I and trichloroacetic acid (TCA)-precipitable proteins (total protein biosynthesis) of freshly isolated rat islets incubated for 1 h in Hanks salt solution containing glucose, L-leucine or theophylline as denoted below. (PI-I/TCA) × 100 denotes the percentage contribution of labelled (pro)insulin to the total pool of labelled islet proteins. Labelled islet (pro)insulin was separated from other labelled islet proteins by an immunoabsorption technique (Welsh *et al.*, 1986a). Values are means ± S.E.M. for five experiments. **P* < 0.05 and ***P* < 0.01 respectively for chance differences versus islets incubated at 3.3 mM-glucose (Student's paired *t* test).

Islet incubation	PI-I (c.p.m./h per islet)	TCA (c.p.m./h per islet)	(PI-I/TCA) × 100 (%)
3.3 mM-Glucose	280 ± 62	3200 ± 550	8.8 ± 2.2
16.7 mM-Glucose	1550 ± 340**	8400 ± 1900*	19.3 ± 2.0**
3.3 mM-Glucose + 10 mM- L-leucine	700 ± 150**	5200 ± 960*	15.4 ± 3.5*
3.3 mM-Glucose + 5 mM- theophylline	580 ± 200*	5300 ± 1700	10.8 ± 1.0

Table 2. Effects of glucose, leucine and theophylline on the subcellular distribution of insulin mRNA

Freshly isolated rat islets were incubated for 90 min in the presence of the additives listed in the Table. After homogenization and subcellular fractionation of the islets, the subcellular distribution of insulin mRNA between the microsomal, free polysome/monosome and cytosolic fractions obtained was determined by dot-blot analysis. The values are absorbance units of the insulin mRNA in each fraction determined by densitometry, and are given as means $\pm$ S.E.M. for seven experiments.

Islet incubation	Microsomal fraction	Polysome/monosome fraction	Free insulin mRNA
3.3 mM-Glucose	5.7 $\pm$ 0.9	28.9 $\pm$ 3.8	57.7 $\pm$ 24.9
16.7 mM-Glucose	9.8 $\pm$ 2.0	29.6 $\pm$ 4.3	29.0 $\pm$ 15.7
3.3 mM-Glucose + 10 mM-L-leucine	9.6 $\pm$ 2.3	31.6 $\pm$ 3.6	34.5 $\pm$ 15.8
3.3 mM-Glucose + 5 mM-theophylline	6.5 $\pm$ 1.6	32.3 $\pm$ 4.0	44.0 $\pm$ 19.2

arrested monosome–insulin mRNA complexes to the RER (SRP–SRP-receptor interaction) and, thirdly, continued elongation at the RER. Regulation of the third step of the translational process has not been addressed in this study, owing to the prior finding that glucose mainly stimulates nascent proinsulin-elongation rates at concentrations below 3 mM (Welsh *et al.*, 1986a). The first two steps of proinsulin synthesis discussed above can be investigated by subcellular localization of the islet insulin mRNA content. Most of the insulin mRNA is observed in the non-ribosome-bound cytosolic fraction, in the non-membrane-associated polysome fraction (mainly as monosomes) and in the microsomal fraction (Welsh *et al.*, 1986a). In the present study, the insulin mRNA content in the different fractions was determined by dot-blot analysis. To exclude experimental artifacts due to mRNA degradation or non-specific hybridization with the 32 P-labelled insulin cDNA probe, Northern-blot analyses were also performed. These revealed specific hybridization to a 550 bp mRNA and no detectable degradation of insulin mRNA (results not shown). Finally, the non-ribosome-bound cytosolic insulin mRNA had a sedimentation coefficient of 11 S when determined by sucrose-gradient ultracentrifugation (results not shown), a value similar to that observed previously (Itoh & Okamoto, 1980).

Initiation of protein biosynthesis is a multistep process, which leads to the binding of the ribosome to the mRNA. We have chosen to study regulation of this process by comparing the relative amounts of uninitiated cytosolic insulin mRNA and free polysome/monosome-associated insulin mRNA after incubation in the presence of glucose, leucine or theophylline, since these compounds were all stimulators of (pro)insulin biosynthesis (Table 1). As previously observed (Welsh *et al.*, 1986a), when the experiments were performed on freshly isolated islets, the variations in the intracellular distribution of insulin mRNA between different experiments were somewhat large. In spite of this, the redistributions exerted by the additives within each individual experiment were consistent. When islets were incubated with 16.7 mM-glucose, 10 mM-leucine or 5 mM-theophylline, the fraction of free cytosolic insulin mRNA was decreased, as compared with incubation in a low glucose concentration only (Table 2). The relative decreases of uninitiated cytosolic insulin mRNA evoked by glucose, leucine or theophylline under paired conditions were

44 $\pm$ 17%, 43 $\pm$ 16% and 25 $\pm$ 8% respectively ($P < 0.05$ each). These results suggest that both the β -cell nutrients and theophylline increase initiation rates of insulin mRNA. Like glucose, leucine is a metabolizable β -cell substrate, which generates high-energy compounds such as metabolic intermediates, ATP, GTP and reducing equivalents (Hutton & Malaisse, 1980; Malaisse *et al.*, 1980). Various cell-free studies on the regulation of protein-synthesis initiation have implicated all of these compounds as stimulatory agents (Hunt *et al.*, 1983; Jackson *et al.*, 1983). The possibility that nutrient stimulation of initiation rates is mediated via the cyclic AMP system also must be considered, since it has been shown that glucose increases the β -cell content of cyclic AMP (Sharp, 1979). Cyclic AMP may augment initiation of protein synthesis in the β -cell via cyclic-AMP-dependent protein kinases (Christie & Ashcroft, 1984, 1985), which have been shown to phosphorylate the ribosomal subunit S6 *in vitro* (Burkhard & Traugh, 1983), possibly also in islets (Christie & Ashcroft, 1985). In addition, cyclic AMP may stimulate protein synthesis by inhibiting phosphorylation of eIF-2 (Suzuki & Mukoyama, 1985). However, the physiological significance of cyclic-AMP-dependent phosphorylation of ribosomal protein and initiation factors is still unclear, since glucagon-induced accumulation of cyclic AMP in hepatocytes, which leads to increased phosphorylation of ribosomal proteins, decreases protein-biosynthesis rates (Ayuso *et al.*, 1986).

The transfer of initiated insulin mRNA from the cytosol to the RER is directed by the SRP and its receptor (Gilmore & Blobel, 1983). This process was reported to be stimulated by glucose, as judged by increased amounts of insulin mRNA associated with membranes relative to the free polysomes/monosomes (Welsh *et al.*, 1986a). This hypothesis is substantiated by the finding that glucose specifically increased the synthesis of growth hormone, another RER-associated protein, in pancreatic islets isolated from transgenic mice carrying the metallothionein–rat growth hormone gene (Welsh *et al.*, 1986b). In the present investigation it was found that leucine, similarly to glucose, increased the relative insulin mRNA content of the microsomal fraction to 189 $\pm$ 32% ($P < 0.05$) for leucine and 203 $\pm$ 40% ($P < 0.05$) for glucose, whereas theophylline exerted no such effect (117 $\pm$ 17%) as compared with low glucose (Table 2). Interestingly, the insulin/total-protein biosynthesis ratio

was increased by both leucine and glucose, whereas incubation with theophylline did not affect this ratio (Table 1). We therefore propose that leucine increases initiation rates, leading to increased insulin and total protein synthesis and, in addition, specifically promotes the transfer of insulin-mRNA-loaded polyribosomes from the cytosol to the endoplasmic reticulum (SRP-SRP-receptor interaction).

Prolonged exposure of pancreatic islets *in vitro* to leucine results in a morphological and functional state which resembles that of high-glucose culture (Andersson *et al.*, 1977). More specifically, islets cultured with leucine display both stimulated insulin biosynthesis and ultrastructural signs of increased biosynthesis, such as a well-developed RER and a prominent Golgi apparatus (Andersson *et al.*, 1977). The specific effect of theophylline on insulin biosynthesis during short-term incubations was shown to be only slightly stimulatory, as reported by Malaisse *et al.* (1974). Neonatal-rat islets cultured for 1 day in the presence of theophylline released more insulin in the media than did islets cultured without theophylline (Nielsen, 1985). However, in islets cultured with theophylline this increase in secretion was accompanied by a corresponding decrease in islet insulin content, indicating failure of the islets to adapt well to the state of high insulin secretion evoked by cyclic AMP accumulation. The present observations agree with that investigation and support the view that β -cell nutrients stimulate insulin biosynthesis by enhancing both the SRP-SRP-receptor interactions and initiation rates of protein synthesis, whereas cyclic AMP acts only on initiation rates.

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