Hormonal and non-hormonal protein biosynthesis in the pancreatic beta cell of the intact rat after prolonged hyperglycaemia

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Abstract. We examined the relative changes in the rates of biosynthesis of (pro)insulin and of non-hormonal beta cell proteins in rats with pronounced hyperglycaemia for up to several days. Labelling of pancreatic cells in vivo eliminated certain pitfalls that we encountered when isolated pancreatic islets from these rats were labelled in vitro. Rats were infused with glucose or buffer solutions for 24 and 72 h. Glucose-infused rats had sustained hyperglycaemia throughout the infusion periods. [3H]leucine or [2,5,3H]tryptophan (an amino acid absent from proinsulin) was injected iv 30 min before the rats were killed. Pancreatic islets were isolated by enzymatic digestion of the pancreas. Pancreatic islets from the rats injected with [3H]leucine were processed for measurement of [3H]proinsulin and [3H]insulin by a double antibody immunoprecipitation procedure. Islets from rats injected with [3H]tryptophan were processed for autoradiography, in order to assess the incorporation of label into non-hormonal sedentary beta cell proteins. Incorporation of [3H]leucine into proinsulin and insulin per beta cell was estimated to be about 2–2.5 (24 h infusion) and 3.5–4 (72 h infusion) times greater in the hyperglycaemic than in normoglycaemic rats. Incorporation of [3H]tryptophan into non-hormonal beta cell proteins showed similar increments in the hyperglycaemic rats. Contrary to our expectation these results indicate that glucose does not exert a significant preferential effect on insulin biosynthesis even after sustained stimulation of the beta cells. Instead, glucose seems to increase equally the incorporation of labelled amino acids into proinsulin and into non-hormonal, beta cell proteins.

The stimulation of (pro)insulin (i.e. proinsulin + insulin) biosynthesis by glucose in a concentration-dependent manner has been clearly demonstrated by several groups of investigators using freshly isolated (Howell & Taylor 1966; Lin & Haist 1969; Permutt & Kipnis 1972; Pipeleers et al. 1973; Bone & Howell 1977; Ashcroft et al. 1978) or cultured pancreatic islets (Anderson et al. 1974) incubated with labelled amino acids. Because glucose affected to a much smaller extent the incorporation of labelled amino acids into total islet proteins it has been frequently implied that glucose exerts a strong preferential effect on proinsulin biosynthesis. More recently, however, autoradiographic studies from this laboratory (Logothetopoulos & Jain 1980a) have indicated that incorporation of labelled amino acids into non-hormonal beta cell proteins at low glucose concentrations is very likely overestimated when radioactivity in total non-proinsulin islet proteins is measured, for the following reasons: a) incorporation of amino acids into proteins of non-beta endocrine, endothelial and connective tissue islet cells is not as sensitive to glucose concentration as that of beta cells and b) non-beta endocrine cells in isolated pancreatic islets of rodents, being situated at the periphery of the islets, close to the stirred medium, incorporate label preferentially. Thus as glucose concentrations decrease an increasingly larger proportion of total non-proinsulin islet proteins is being contributed by non-beta cells.

By labelling isolated rat islet with [3H]tryptophan, an amino acid not present in rat proinsulin (Sundby & Markussen 1972) and by evaluating silver grain densities over beta cells in autoradio-
graphs of multiple islet cross-sections, we showed that in isolated rat islets incubated with 0, 5 and 20 mM glucose, biosynthesis of non-(pro)insulin beta cell proteins closely paralleled that of (pro)insulin (Logothetopoulos & Jain 1980a).

The autoradiographic studies, however, confirmed the observations reported by Sando et al. (1972). Beta cells especially in the centre of large-sized islets incorporated the label poorly and non-uniformly. Several beta cells in these areas, had undergone nuclear pycnosis. Measurements of silver grain densities in these in vitro experiments had therefore to be made in a non-random fashion over selected areas of uniformly labelled beta cells.

In order to ensure that islet beta cells were exposed to glucose in their physiological milieu and were labelled uniformly, we injected [3H]leucine and [3H]tryptophan iv to sets of intact rats which were killed ½ h later. Pronounced, short-term hyperglycaemia (induced by glucose infusion for 1 h) or hypoglycaemia (induced by insulin infusion) preceded the injection of the label. These short-term in vivo experiments resulted in uniformly labelled beta cells throughout all islet cross-sections. They confirmed that glucose exerted an effect on the biosynthesis of non-proinsulin beta cell proteins nearly as potent as that on proinsulin (Logothetopoulos & Jain 1980b). Incorporation of [3H]leucine into proinsulin and of [3H]tryptophan into non-hormonal beta cell proteins increased about 2-fold in rats subjected to short-term hyperglycaemia. Hypoglycaemic rats showed 3 to 4-fold decreases compared with their normoglycaemic pair-injected controls.

The elegant work of Giddings et al. (1982), clearly demonstrated that mRNA of pro-proinsulin is metabolically modulated in the pancreatic beta cell. It was therefore reasonable to expect that hyperglycaemia sustained for days, by inducing a progressive increase in pro-proinsulin mRNA without a coordinate increase of many other beta cell mRNAs might result in a marked preferential increment of (pro)insulin biosynthesis. Data previously reported from this laboratory (Zucker & Logothetopoulos 1975) showing that pancreatic islets isolated from rats infused with glucose for 24 h incorporated in vitro over 10 times more [3H]leucine into proinsulin than islets isolated from buffer infused rats were compatible with such a pattern of protein biosynthesis in the beta cells. The present study was designed to inquire more precisely into the question of modulation of pro-insulin and non-proinsulin protein biosynthesis in the beta cell and to evaluate the biosynthetic potential of this cell, following sustained hyperglycaemia in the rat, using the in vivo labelling procedure.

Materials and Methods

Animals and glucose infusions

Adult male Wistar rats, weighing 290 to 330 g were cannulated with a fine Silastic tubing (Dow Corning Co., Midland, Michigan) inserted into the superior vena cava 2 days before the infusion started. In each experiment one rat was infused with a 45 g/dl glucose solution in a modified Ringer buffer, while the control rat received the buffer solution only. The procedure has been previously described (Cole & Logothetopoulos 1974). Glucose infused rats were continuously glucosuric. At the end of an infusion period of 24 or 72 h the labelled amino acid was injected through the indwelling catheter. Two mCi of [4,5-3H]leucine (50 to 60 Ci/mmol) or 0.35 mCi L[2,3-3H]tryptophan (15 to 20 Ci/mmol) (New England Nuclear Corp., Boston, Mass.) in 0.5 ml of sterile normal saline were injected within 10 to 12 sec after transient interruption of the continuous infusions. The same radioactive solution was used for each set of infused rats, which were matched for similar body weights. Rats were killed 30 min after the injection of the labelled amino acid by bleeding from the aorta under Nembutal anaesthesia. Excess non-radioactive amino acid was added to the iv administered anaesthetic. The pancreas was removed and placed in cold (3 to 4°C) Hank's buffer for the isolation of the pancreatic islets. Duplicate slices from the brain cortex (grey matter) and the anterior pituitary were also removed, weighed and placed in tubes with cold buffer containing 3 mM of the injected non-labelled amino acid.

Isolation and processing of pancreatic islets

Pancreatic islets were isolated from the splenic portion of the pancreas by a modified collagenase procedure of Lacy & Kostianovsky (1967). Pancreatic islets from rats injected with [3H]leucine were collected in groups of 20 to 25 in 3 individual tubes containing Hank's buffer, after the relative volume of each individual islet was approximately measured by a method already described (Zucker & Logothetopoulos 1975). (Pro)Insulin was quantitatively extracted with acid ethanol (64 ml ethanol, 35 ml H2O, 1 ml HCl).

Incorporation of [3H]leucine into total (pro)insulin was measured by a validated double antibody procedure (Zucker & Logothetopoulos 1975). Briefly, guinea pig anti-insulin antibody was added to aliquots of the acid alcohol extract in phosphosaline buffer pH 7.5 (0.1 M sodium phosphate, 0.05 M sodium chloride). The binding
capacity of the antibody was at least 1.5 times that needed to completely bind the (pro)insulin present in the aliquot. The antibody-(pro)insulin complexes were quantitatively precipitated by rabbit anti-guinea pig (γ-globulin) antibody. The differences in radioactivity in immunoprecipitates formed in the absence or presence of excess exogenous insulin (40 μg) was taken as representing [3H](pro)insulin. The effectiveness of the procedure was monitored repeatedly by repeating the immunoprecipitation in the supernatants of the initial immunoprecipitates with additional antibody reagents. In all cases precipitation of [3H](pro)insulin was complete.

Pancreatic islets isolated from rats injected with [3H]tryptophan were fixed in a modified Bouin's solution, enmeshed in a small fibrin clot, according to the method of Furtado (1970), embedded in paraffin and sectioned. The preparation of radioautographs and the validation of the procedure as a reliable method to measure labelled cell proteins have been described previously (Logothetopoulos & Jain 1980b).

Trichloroacetic (TCA) precipitation of brain and pituitary proteins
Brain slices and the anterior pituitary were disrupted by ultrasonication and their proteins were precipitated by 12% cold TCA containing 3 mM of non-labelled leucine or tryptophan. Precipitates were sedimented by centrifugation, re-suspended in TCA and re-sedimented. They were finally suspended in 1 ml formic acid and fully dissoluted before transference into scintillation fluid for radioactive counting.

Glucose, leucine and tryptophan in plasma of infused rats
Glucose in serum obtained from blood removed at the time of killing was measured by a glucose oxidase method. Chemstrip reagent strips (Boehringer Mannheim Co., Germany) were used for estimating glucose in small blood samples taken during the infusion period from the cut tip of the tail of the rat. Plasma concentrations of leucine and tryptophan were measured in separate sets of rats infused with glucose or buffer solutions as above but not injected with labelled amino acids. Leucine was measured by the amino acid analyser and tryptophan by a modification of the fluorometric procedure of Bloxam & Warren (1974).

Measurement of DNA in pancreatic islets
DNA was measured by the method of Parman (1975) with highly polymerized calf thymus DNA (Sigma Co., St. Louis, Mo.) as a standard.

In vitro incorporation of [3H]leucine into (pro)insulin in islets isolated from rats infused with glucose or buffer for 24 h
The preparation and incubation of pancreatic islets followed exactly the protocol reported previously by Zucker & Logothetopoulos (1975). Digestion of pancreatic pieces lasted for about 15 min. Cold (3–4°C) Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 7.5 mM Heps and 4.5 mM glucose was used to wash and keep the pancreatic islets. Islets were transferred to siliconized tubes filled with cold buffer after recording two transverse diameters at 90° to each other and the average height of the islet. A pre-incubation period of 40 min at 37°C was followed by a further 45 min incubation in 150 μl of buffer containing 4.5 mM glucose, 60 mg/dl bovine albumin, 10 μCi [3H]leucine and 19 other naturally occurring amino acids. Extraction and immunoprecipitation of (pro)insulin from the incubated islets followed the procedures described (Zucker & Logothetopoulos 1975). The islets of three tubes from each rat were extracted and (pro)insulin was immunoprecipitated. Pancreatic islets in two additional tubes were washed with cold buffer containing 3 mM non-radioactive leucine, fixed in Bouin’s, enmeshed in fibrin and processed for autoradiography as described above.

Expression of results
The volume of each islet was estimated by measuring the two transverse diameters and the average height of the islet using a calibrated grid in the ocular of the stereomicroscope and assuming a disk-like shape and an elliptical cross-sectional area of the islets. The total volume of islets in each tube was then calculated. Incorporation of [3H]leucine was expressed per 0.1 mm³ of islet volume. This volume corresponds to approximately 8 to 12 medium sized islets of a non-glucose stimulated pancreas of a rat. According to Parman (1975), dimensions of rat pancreatic islets correlated very well with dry weight and their DNA content. In order to assess islet cell hyper trophy after prolonged stimulation by glucose, islet volumes were expressed per μg of islet DNA and values between buffer infused and glucose infused rats were compared. Radioactivity incorporated into brain tissue and anterior pituitary was expressed as counts/mg wet weight.

| Table 1. |
| Plasma leucine and tryptophan in rats infused with buffer or glucose solutions for 24 or 72 h. |
| Infusion | Plasma leucine | Plasma tryptophan |
| Buffer 24 h | 15.0 ± 0.6 | 6.4 ± 0.6 |
| Glucose 24 h | 9.5 ± 0.5 | 3.9 ± 0.3 |
| Buffer 72 h | 13.4 ± 0.8 | 7.2 ± 0.4 |
| Glucose 72 h | 8.2 ± 0.4 | 4.7 ± 0.2 |

μM/100 ml. Each value represents the mean ± SE from 5 rats. P < 0.01 for differences between glucose and buffer infused rats.
Results

All rats infused with glucose were consistently hyperglycaemic throughout the period of infusion. Serum glucose concentrations at killing ranged from 318 to 540 mg/dl. The serum concentrations of leucine and tryptophan measured in blood samples taken at killing are given in Table 1. Serum concentrations of both amino acids in hyperglycaemic rats were consistently lower by 30 to 35% than those in the buffer infused rats.

Radioactivity incorporated into [3H](pro)insulin, corresponding to approximately two pancreatic islets (aliquots of the total acid ethanol extract were immunoprecipitated), ranged from 150 to 350 and 70 to 135 counts per min for islets from glucose and buffer infused rats, respectively. Radioactivity in the immunoprecipitates formed in the presence of excess exogenous insulin was in all instances less than 10% of that obtained in the corresponding tubes without insulin. All samples were counted repeatedly for 30 min each time, after storing the scintillation vials for 24 h at 4°C in the dark, until stable counts within expected statistical variation were obtained.

Hyperglycaemic rats incorporated about 2 to 3 times more [3H]leucine into (pro)insulin than their normoglycaemic, buffer-infused controls (Fig. 1). Brain tissue and the anterior pituitary of the hyperglycaemic rats also showed slightly higher rates of incorporation of [3H]leucine than those of the normoglycaemic buffer infused controls (Fig. 1). Hyperglycaemia maintained for 72 h did not have a significantly greater effect on [3H]leucine incorporation into (pro)insulin (expressed per 0.1 mm³ islet volume) than hyperglycaemia maintained for 24 h (Fig. 1).

The density of silver grains over beta cells in autoradiographs of pancreatic islets from buffer and glucose infused rats injected with [3H]tryptophan, ½ h before killing are shown in Fig. 2. The density of silver grains in rats maintained hyperglycaemic for 24 or 72 h exceeded that found in
their corresponding buffer-infused controls by a factor of 2.5 and 2.9. Incorporation of [3H]tryptophan into TCA precipitable proteins of brain cortex and the anterior pituitary of the hyperglycaemic rats also exceeded by a factor of about 1.5 and 1.3 the incorporation of [3H]tryptophan into the corresponding tissue proteins of the buffer infused control rats.

The extent of islet cell hypertrophy occurring during protracted hyperglycaemia can be evaluated from results of the 10 separate experiments presented in Fig. 3. In these experiments 3 pairs of rats received buffer or glucose infusions for 24 or 72 h. No labelled amino acids were injected before killing. The total volume of approximately 30 and 60 islets from each rat were estimated from the individual volumes of islets. DNA content of these islets was measured and volumes/µg of DNA were plotted (Fig. 3).

Volumes of islets/µg of DNA were not significantly different in rats infused for 24 h with buffer or glucose solutions. Following 3 days of glucose infusion islet volumes/µg of DNA were about 60 to 70% greater in the hyperglycaemic rats compared to those of their buffer-infused controls. Because non-beta islet cells are 25 to 30% of the total islet cells and do not respond by hypertrophy to prolonged glucose exposure, increases in size of beta cells are likely to be underestimated by measurements of islet volume/DNA ratios.

Table 2 shows the incorporation of [3H]leucine in islets isolated from rats infused for 24 h with glucose or buffer solutions and incubated in vitro with the labelled amino acid. In contrast to results obtained by in vivo labelling and in agreement with the previously reported data (Zucker & Logothetopoulos 1975) isolated islets from hyperglycaemic rats incorporated in vitro about 10-fold greater amounts of [3H]leucine into proinsulin than those from buffer infused rats. Autoradiographic evaluation of the labelling pattern of these two groups of islets revealed that large areas of histologically well preserved beta cells had incorporated the label in the islets from the hyperglycaemic rats. In contrast most islets from the buffer infused rats had extensive patchy areas of centrally located necrotic beta cells: only narrow rims of beta cells close to the periphery having incorporated the label (Fig. 4).

**Discussion**

Without knowledge of the specific activity of tRNA<sub>leu</sub> within pancreatic beta cells during the labelling period a certain degree of uncertainty cannot be eliminated when rates of incorporation of labelled amino acids into proinsulin are taken as representing rates of net proinsulin biosynthesis. This applies to both in vitro and in vivo labelling experiments. The main advantage of labelling isolated islets, besides the small quantities of radioactivity needed, is that the extracellular specific activity of the amino acid can be determined and maintained during the labelling period. When labelling pancreatic beta cells in the intact animal information is required on the extracellular specific activity of the labelling amino acid in the groups of animals which are being compared. Labelling pancreatic beta cells in isolated islets in vitro, on the
other hand, has certain disadvantages. Islet cells are subjected to various injuries during the isolation and incubation procedures; labelled amino acids reach cells by diffusion from the islet periphery leading to non-uniform labelling; islet cells are not exposed to their physiological milieu. These limitations of the in vitro procedures may be inconsequential when relative effects of various treatments or stimuli on insulin secretion or biosynthesis are compared using groups of pancreatic islets from a pool which was exposed simultaneously to all steps of the isolation procedures. When, however, islets are isolated separately from experimental animals that had been subjected to different treatments (hyperglycaemic vs normoglycaemic, starved vs non-starved, young vs old) the isolation and/or incubation steps may have a markedly different effect on the functional integrity and viability of the islet beta cells of the two groups that are being compared. As the autoradiographic studies revealed the striking 10- to 12-fold difference in proinsulin biosynthesis between islets isolated from hyperglycaemic and normoglycaemic rats incubated in vitro with the labelled amino acids was partly due to a significantly greater number of non-labelled necrotic cells in the islets of the normoglycaemic rats. It appears that beta cells from islets isolated from rats exposed to prolonged hyperglycaemia show a decreased susceptibility to metabolic injury and necrosis during the isolation and/or incubation steps.

Labelling of the islet cells in the intact rat by an iv injection of the labelled amino acid resulted in uniform and even labelling of all beta cells in islets of all sizes. We therefore trust that the values obtained with islets labelled in vivo, indicating a smaller difference between hyperglycaemic and normoglycaemic rats, reflect more accurately the true relative rates of proinsulin biosynthesis in these two groups.

The incorporation of [3H]leucine into (pro)insulin in vivo after sustained hyperglycaemia for 24 and 72 h was about 200 to 300% greater than that found in the buffer-infused normoglycaemic rats. Because serum concentrations of both leucine and tryptophan in the rats infused with glucose for 24 and 72 h were about 70% of those in serum normoglycaemic rats, one may expect higher specific activities of the amino acids in the glucose infused rats during the brief incorporation period. It is of interest that brain cortex and the anterior pituitary of the hyperglycaemic rats showed a 25 to 35% increase in [3H]leucine incorporation. These two tissues were chosen for comparisons because they do not store large quantities of fuel macro-molecules and because they were considered less likely candidates to be targets for stimulation of protein biosynthesis by glucose. It is very improbable that incorporation of [3H]leucine into (pro)insulin in hyperglycaemic rats has been underestimated because of a fast transit time of newly synthesized (pro)insulin from the endoplasmic reticulum to the cell surface and into the circulation (Sando et al. 1972). Shortening the interval of killing after the injection of [3H]leucine from 30 to 15 min in two pairs of rats infused for 72 h
did neither affect significantly the radioactivity of [3H](pro)insulin extracted from the islets nor the relative incorporation of label between the two buffer and the two glucose infused rats (results not shown). Furthermore, in experiments with islets isolated from rats infused with glucose for 24 h, pulse labelled with [3H]leucine and chased for 45 min. [3H](pro)insulin which appeared in the medium was only about 10% of the total compared with 4% in control islets (Jain & Logothetopoulos 1977).

Evaluating the rates of [3H]leucine incorporation into (pro)insulin on a per beta cell basis would be more meaningful than on an islet volume basis. As discussed in the section on Results the islet volume/DNA ratios strongly indicated that the size of beta cells may slightly have increased after 24 h of glucose infusion and likely doubled after 72 h of infusion. Thus a given volume of islets from rats infused with glucose for 72 h may contain half the number of beta cells than the number of beta cells of an equivalent islet volume from buffer infused rats. The above considerations lead us to conclude that a 2- to 2.5- and 3.5- to 4-fold increase of (pro)insulin biosynthesis per beta cell in rats infused with glucose for 24 and 72 h may be a closer estimate of true responses. It would be of interest to measure cellular (pro)insulin mRNAs (Giddings et al. 1982) in identically glucose infused rats and compare mRNA levels with our estimated values of rates of (pro)insulin biosynthesis.

The autoradiographic data on the incorporation of [3H]tryptophan into non-(pro)insulin proteins of beta cells indicate that the overall rate of biosynthesis of these proteins increased to the same extent as (pro)insulin biosynthesis. The per cent increases in the density of silver grains over beta cells in glucose infused rats were within the upper range of those obtained for [3H]leucine incorporations into (pro)insulin; 250 and 300% for rats infused with glucose for 24 and 72 h, respectively. Considerations of beta cell hypertrophy as shown by the islet volume/DNA ratios in hyperglycaemic rats would raise these values, when expressed on a per beta cell basis, to levels close to those found for [3H]leucine incorporation into (pro)insulin. Thus prolonged stimulation of beta cells by glucose affected nearly equally the biosynthesis of exportable (pro)insulin and probably of most non-hormonal, non-exportable beta cell cellular proteins.

Our observations raise an important aspect in the biology of the pancreatic beta cell. A progressive, severe loss of pancreatic beta cells has been well documented in certain species (dog, cat) when beta cells are exposed to prolonged glucose or hormonal stimulation (Homans 1914; Lukens & Dohan 1942; Volk & Lazarus 1964). The underlying molecular mechanisms of this beta cell loss are not understood. One possible hypothesis arising from this work is that continuous anabolic beta cell growth which is not fully compensated by protein degradation or by cell replication may be a cause of beta cell lysis and degeneration.

References


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