Long-Term Effects of Glucose on Insulin Release and Glucose Oxidation by Mouse Pancreatic Islets Maintained in Tissue Culture

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Rates of glucose oxidation and insulin release in response to a wide range of glucose concentrations were studied in short-term experiments in isolated mouse pancreatic islets maintained in tissue culture for 6 days at either a physiological glucose concentration (6.7 mM) or at a high glucose concentration (28 mM). The curves relating glucose oxidation or insulin release to the extracellular glucose concentration obtained with islets cultured in 6.7 mM-glucose displayed a sigmoid shape similar to that observed for freshly isolated non-cultured islets. By contrast islets that had been cultured in 28 mM-glucose showed a linear relationship between the rate of glucose oxidation and the extracellular glucose concentration up to about 8 mM-glucose. The maximal oxidative rate was twice that of the non-cultured islets and the glucose concentration associated with the half-maximal rate considerably decreased. In islets cultured at 28 mM-glucose there was only a small increase in the insulin release in response to glucose, probably due to a depletion of stored insulin in those B cells that had been cultured in a high-glucose medium. It is concluded that exposure of B cells for 6 days to a glucose concentration comparable with that found in diabetic individuals causes adaptive metabolic alterations rather than degeneration of these cells.

We have reported that isolated mouse islets maintained in tissue culture for up to 12 days showed a satisfactory survival in terms of the oxygen uptake, glucose oxidation and insulin release (Andersson & Hellerström, 1972). Throughout the culture period there was a good preservation of the islet structures, without any obvious signs of degeneration. This was true for islets cultured in widely different glucose concentrations, and microscopical damage of the B cells was not evident even if the glucose concentration during the culture period was comparable with that in the blood of diabetic individuals. Nevertheless the B-cell function and metabolism were both altered by the glucose concentration of the culture medium; when islets were incubated in short-term experiments after culture in a high glucose concentration (28 mM) the rates of both oxygen uptake and glucose oxidation were increased, whereas the insulin release was slightly decreased. The lower insulin release was most likely due to the marked B-cell degranulation and a decrease in the insulin content of the cultured islets.

It appears from the above observations that this culture system for isolated islets is a suitable technique for studies of the long-term effects of glucose on the B-cells. Also, the results suggest that the B cells of mice respond to prolonged elevations of the extracellular glucose concentration with a metabolic adaptation rather than with degeneration. In the present study we have attempted to elucidate further

the nature of these changes by measuring in short-term experiments the rates of glucose oxidation and insulin release in relation to the extracellular glucose concentration of isolated islets after a 6-day culture period in either 6.7 mM- or 28 mM-glucose. For comparison, the corresponding dose-response curves of freshly isolated islets have also been determined.

Experimental

Materials

Collagenase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Tissue-culture medium (TCM 199), Hanks solution, calf serum and trypsin were supplied by Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Penicillin and streptomycin were from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Antimycin A was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Hyamine hydroxide, PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] were from Packard Instrument Co., Downers Grove, Ill., U.S.A. D-[U-¹⁴C]Glucose (3 mCi/mmol) and kits for radioimmunoassay of insulin (code IM.39) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of the purest grade commercially

available. The tissue-culture dishes were supplied by A/S NUNC, Roskilde, Denmark.

Methods

Tissue-culture technique. Adult male NMRI mice (Anticimex, Stockholm, Sweden) were starved overnight before the experiments. The islets were isolated under aseptic conditions by collagenase treatment (Howell & Taylor, 1968), washed in Hanks solution (Hanks & Wallace, 1949) and then transferred to plastic Petri dishes. The culture medium consisted of TCM 199, supplemented with 20% (v/v) calf serum, 100 units of penicillin/ml and 0.1 mg of streptomycin/ml, and with the glucose concentration adjusted to either 6.7 or 28 mM. After 6 days culture the islets were harvested from the bottom of the plastic dish by treatment for 15 min with a Ca^{2+} - and Mg^{2+} -free 0.25% (w/v) trypsin solution (1:300) and washed twice in the relevant incubation medium. Further details of the culture technique are given by Andersson & Hellerström (1972). Groups of islets were also studied directly after isolation, without being cultured (see below).

Assay of glucose oxidation. Groups of about ten cultured or non-cultured islets were incubated in vials similar to those described by Keen *et al.* (1963). The samples were incubated for 90 min at 37°C with continuous shaking in a water bath. The gas phase consisted of $\text{O}_2 + \text{CO}_2$ (95:5). The bicarbonate-buffered incubation medium (Gey & Gey, 1936), pH 7.4, contained D-[U- ^{14}C]glucose with carrier glucose, the total glucose concentrations varying between 0.55 mM and 16.7 mM (final sp. radioactivity 0.35–2.4 mCi/mmol). At the end of each experiment the islet metabolism was arrested by injection into the inner vessel of 100 μl of 0.05 mM-antimycin A dissolved in 70% (v/v) ethanol, followed immediately by an injection of 250 μl of hyamine into the outer vessel (Edwards *et al.*, 1972). CO_2 was liberated from the incubation medium by another injection of 100 μl of 0.4 M-sodium phosphate buffer, pH 6.0. After 2 h shaking at 37°C the radioactivity trapped by the hyamine was determined in a liquid-scintillation spectrometer (Packard Tri-Carb model 3380) by using a scintillation fluid composed of toluene (1 litre), PPO (5 g) and dimethyl-POPOP (0.05 g).

The islets were recovered from the inner vessel and weighed on an ultramicro-balance (Mettler UM7) after drying on preweighed platinum foil for 20 min at 80°C as described by Hellerström (1967). Blank incubations without islets were taken through each experiment and the blank values were subtracted from the experimental values.

Determination of insulin release. Isolated islets, either cultured or non-cultured, were incubated in groups of ten for 30 min at 37°C with continuous shaking in a water bath in glass vials as described

above. Before the experiment each vial was gassed for 1 min with $\text{O}_2 + \text{CO}_2$ (95:5). The non-cultured islets were preincubated in a medium containing 3.3 mM-glucose for 30 min before the final incubation. The incubation medium consisted of a bicarbonate-buffered medium (Gey & Gey, 1936) supplemented with 2 mg of bovine albumin/ml and glucose in concentrations given below. In each incubation 5 mM-theophylline was also added to enhance the rate of insulin release. Immediately after the end of the incubation period samples of the incubation medium were frozen and then stored at -20°C until the insulin assay, which was done as described by Heding (1972), with crystalline mouse insulin as a standard. The error of a single determination was $\pm 12\%$.

Expression of results. Glucose-oxidation rates were calculated from the formation of $^{14}\text{CO}_2$ from D-[U- ^{14}C]glucose and are given as $\mu\text{mol/h}$ per g dry wt. of islets. Corrections were not made for possible differences in the intracellular pools of unlabelled glucose and glucose metabolites of the various groups of islets at the beginning of each experiment. The calculated rates should therefore be regarded as approximations to the true rates of glucose oxidation. The insulin release is given as ng/30 min per ten islets.

Results

Glucose oxidation

As shown in Fig. 1, glucose oxidation by cultured islets, after an initial lag, proceeded at a constant

Fig. 1. Time-course of glucose oxidation by cultured pancreatic islets

Eight to ten islets previously cultured for 6 days at 28 mM-glucose were incubated at 37°C in bicarbonate medium containing 5.5 mM-glucose. The glucose oxidation was calculated from the formation of $^{14}\text{CO}_2$ from [U- ^{14}C]glucose (1.5 mCi/mmol). Each point is the mean $\pm$ s.e.m. for three batches of islets.

rate for at least 2 h. An incubation time of 90 min was therefore used in all experiments. To test the possible effect of the trypsin treatment used for harvesting the cultured islets the glucose oxidation by islets cultured for 6 days in 28 mM-glucose was measured. The rates of glucose oxidation by islets harvested from the culture dishes by means of free-hand dissection were not significantly different from those obtained with normally trypsin-treated islets (Fig. 2).

Rates of glucose oxidation in different groups of islets are given in Fig. 3. In non-cultured islets the curve relating the rate of glucose oxidation to the glucose concentration of the incubation medium was

Fig. 2. Effect of harvesting by trypsin treatment versus dissection on glucose oxidation by cultured pancreatic islets

Islets cultured for 6 days at 28 mM-glucose were harvested from the culture dish by treatment for 15 min with a 0.25% (w/v) Ca^{2+} - and Mg^{2+} -free trypsin solution ($\blacktriangle$) or directly by freehand dissection ($\triangle$). The effect of various concentrations of glucose on the rates of glucose oxidation was studied by incubating batches of about ten islets for 90 min at 37°C in bicarbonate medium containing [$\text{U}-^{14}\text{C}$]-glucose (1.0–1.5 mCi/mmol) at the concentrations indicated. Glucose-oxidation rates were calculated from the formation of $^{14}\text{CO}_2$. Each point is the mean $\pm$ S.E.M. for four or more batches of islets; for clarity the S.E.M. is shown on only one side of each mean.

Fig. 3. Effect of extracellular glucose concentration on rates of glucose oxidation

Batches of islets were incubated for 90 min at 37°C in bicarbonate medium containing [$\text{U}-^{14}\text{C}$]-glucose (0.35–2.4 mCi/mmol) at the concentrations given in the Figure. Glucose-oxidation rates were calculated from the formation of $^{14}\text{CO}_2$. Each point is the mean $\pm$ S.E.M. for four or more batches of islets. (a) Non-cultured islets ($\bullet$). (b) Islets cultured for 6 days at 6.7 mM-glucose ($\blacksquare$). (c) Islets cultured for 6 days at 28 mM-glucose ($\blacktriangle$).

Fig. 4. Effect of extracellular glucose concentration on insulin release

Batches of ten islets were incubated for 30 min at 37°C in bicarbonate medium containing 2 mg of albumin/ml, 5 mM-theophylline and glucose at the concentrations given in the Figure. Insulin released into the medium was assayed by radioimmunoassay. Each point is the mean $\pm$ S.E.M. for 11 to 24 batches of islets, each assayed in duplicate. (a) Non-cultured islets ($\circ$). (b) Islets cultured for 6 days at 6.7 mM-glucose ($\square$). (c) Islets cultured for 6 days at 28 mM-glucose ($\triangle$).

sigmoid and approached a maximum at about 11 mM-glucose (Fig. 3a). There was a threshold at about 4.5 mM-glucose and the half-maximum rate was reached at about 6 mM. Relative rates of oxidation were about $7 \mu\text{mol/h}$ per g dry wt. of islets at 3 mM-glucose and $38 \mu\text{mol/h}$ per g above 11 mM-glucose. The corresponding curve obtained with islets cultured for 6 days in a standard medium (6.7 mM-glucose; Fig. 3b) differed from the above mainly with regard to the maximal oxidative rate, which was higher, i.e. about $55 \mu\text{mol/h}$ per g. Also, the curve was less sigmoidal and the half-maximum rate was attained already at about 5 mM-glucose. Islets cultured for 6 days in a high-glucose medium (28 mM-glucose; Fig. 3c) showed a much more pronounced change of the dose-response curve. Thus there was no lower threshold, but a nearly linear increase of the oxidative rate up to 8 mM-glucose with an apparent maximal rate of about $70 \mu\text{mol/h}$ per g, which was reached at a glucose concentration of about 11 mM. The glucose concentration at which the half-maximum rate occurred was not more than about 3 mM.

Insulin release

The relationship between the insulin release and the glucose concentration of non-cultured and cultured islets is given in Fig. 4. In other experiments the trypsin-treatment procedure did not affect insulin release from cultured islets. For the freshly isolated islets (Fig. 4a) there was a sigmoid dose-response curve with a maximal stimulation at about 20 mM-glucose. This maximal stimulation was about eight times the insulin release observed when glucose was absent from the incubation medium. A qualitatively similar but quantitatively somewhat different relationship was obtained with islets cultured for 6 days in 6.7 mM-glucose (Fig. 4b). Although the basal insulin release was equal to that of freshly isolated islets the maximal degree of stimulation appeared at a somewhat lower glucose concentration and was only three times the basal value of insulin secretion. A distinctly different form of curve was seen for islets cultured for 6 days in 28 mM-glucose (Fig. 4c). Although the basal value of insulin secretion remained unchanged there was only a small, though significant ($P < 0.01$), increase with the glucose concentration up to an apparent maximum at about 5 mM-glucose. This was only twice the basal value and there was no tendency for the curve to be sigmoidal.

Discussion

The present data together with previous biochemical and ultrastructural findings indicate that the cultured islets represent a viable system with a cellular composition closely resembling that of the endocrine pancreas *in vivo* (Andersson & Heller-

ström, 1972; Andersson *et al.*, 1973; A. Andersson, J. Westman & C. Hellerström, unpublished work). This does not mean, however, that islets kept in culture remain entirely 'normal' (i.e. similar to freshly isolated islets) in every respect. Thus it has been observed that even when culture is performed at a physiological (6.7 mM) glucose concentration there is a raised respiratory rate and a gradual decrease in the insulin content, despite an apparently normal rate of insulin biosynthesis (Andersson & Hellerström, 1972; Andersson, 1973). These alterations most probably represent adaptations to the highly artificial environment *in vitro*. In fact, the specific functions of the islet cells seem remarkably well preserved, which is particularly well illustrated by the present observation of the sigmoid relationship between the insulin release and the extracellular glucose concentration of the islets cultured in a physiological (6.7 mM) glucose concentration. It therefore seems reasonable to conclude that the data obtained with islets maintained in the present culture system are as valid as those derived from short-term incubations of freshly isolated islets.

The sigmoid nature of the curve relating rates of glucose oxidation by freshly isolated mouse islets to glucose concentration agree well with those previously described (Ashcroft *et al.*, 1970; Hellman *et al.*, 1974). A comparison between the glucose oxidation curve of non-cultured islets and that of islets cultured at a physiological (6.7 mM) glucose concentration displayed an increase in glucose sensitivity and a higher maximal rate of oxidation in the cultured islets. At least in part, these differences may be explained on the assumption that the cultured islets represent a generally more viable cell population than in freshly isolated preparations. In support of this view we have observed in the electron microscope a small but consistent proportion of obviously damaged cells in freshly isolated islets, whereas such cells have been only rarely seen after culture (A. Andersson, J. Westman & C. Hellerström, unpublished work). This means that a fraction of the non-cultured islet cells, which are injured by the collagenase treatment, contribute to the weight of the islets but not to their glucose oxidation and therefore calculated values for the oxidative metabolism are an underestimate of the true metabolic rates.

When comparing the glucose oxidation of the two groups of cultured islets it was found that the glucose sensitivity of the islets was remarkably increased by culture in a high-glucose medium. This increase may reflect marked changes of the rate-limiting steps of the pathways for glucose degradation in the islet cells. So far only one of these steps has been studied in cultured islets and the results indicated that 7 days culture in a high glucose concentration induced a significantly raised activity of a high- K_m enzyme for glucose phosphorylation, whereas a low- K_m hexo-

kinase system remained unaffected by the glucose concentration of the culture medium (Andersson *et al.*, 1974). It cannot be excluded that this enzymic adaptation contributed to the increased glucose sensitivity, which was manifest at glucose concentrations rather close to the estimated K_m value of the high- K_m enzyme. Other studies have shown that islet ATP concentrations remained unaffected by the glucose concentration during the culture period (Andersson *et al.*, 1974). This suggests that regulation of glycolytic flow by an altered steady-state concentration of ATP is not of significance for the differences in glucose sensitivity between the two groups of cultured islets.

There are a few previous studies of the glucose metabolism in islets subjected to long-lasting elevations of the extracellular glucose concentrations. Smith & Lacy (1962) noted only minor alterations in the enzyme pattern of the glycolytic pathway in glucose-injected rabbits. Similar results were obtained by Brolin *et al.* (1964) using glucose-injected NZO mice. However, as yet it cannot be decided to what extent the islet enzymes *in vivo* depend on factors other than glucose. We have so far been unable to find any previous information in the literature on the long-term effects of glucose *in vitro* on the islet glucose metabolism.

The sigmoid shape of the dose-response curve for insulin release by freshly isolated islets agrees well with previous reports (Löffler *et al.*, 1969; Green & Taylor, 1972; Ashcroft *et al.*, 1972; Hellman *et al.*, 1974). The same shape was also characteristic of the curve from islets cultured at a physiological (6.7 mM) glucose concentration, although there was a somewhat decreased maximal release. This finding was in marked contrast with the small increase of insulin release obtained with islets cultured for 6 days in 28 mM-glucose. Such islets have, however, been found to contain only about 5% of the insulin concentration in non-cultured islets (Andersson & Hellerström, 1972), and an obvious explanation for the low glucose sensitivity would therefore be a lack of readily mobilized insulin stores. An alternative explanation, that the prolonged glucose-stimulation period might have damaged the releasing mechanism itself, seems less likely, since a constant high insulin-secretion rate from islets after culture in a high glucose concentration has been reported during the culture period (A. Andersson, J. Westman & C. Hellerström, unpublished work). Moreover, it was found in short-term incubations that not less than about 50% of the total insulin store was released during 30 min of maximal stimulation. The latter observations also support the view that the decreased glucose response and insulin storage do not signify a real degenerative process in the islet cells, but rather that prolonged functional stimulation by glucose causes depletion of the secretory-granule pools.

It is noteworthy in this context that a corresponding loss of stored insulin has been observed in severely diabetic individuals, in which the islets have been subjected to prolonged hyperglycaemia (Gepts *et al.*, 1970). Further, these patients showed a poor increase in the insulin release in response to an acute glucose challenge (Cerasi & Luft, 1967) similar to that seen in the present islets after culture in a high glucose concentration. However, the diabetic pancreas also shows clear morphological signs of deterioration of the B-cells, which is at variance with the findings in the present study. Therefore the prolonged effects of high glucose *in vitro* should not as yet be regarded as entirely representative of the situation *in vivo* in the diabetic patient. Nevertheless, it cannot be excluded that a longer exposure to high glucose concentrations in culture might have caused similar degenerative lesions in the B-cells.

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