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Function of Microdissected Pancreatic Islets Cultured in a Chemically Defined Medium. I. Insulin Content and Release

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Summary. Microdissected pancreatic islets from non-inbred *ob/ob*-mice were cultured for 6 or 7 days in serum-free tissue culture medium 199. The insulin content of the islets decreased 60% during culture in 17 mM or 28 mM glucose and about 70% in the presence of 3.3 mM or 5.6 mM glucose. At the end of a culture period in high glucose, the sum of the insulin in the islet plus that in the culture medium was almost twice as high as the insulin content of fresh islets, indicating an active insulin biosynthesis. The maximal insulin response to glucose after culture in 17 mM or 28 mM glucose was about 40% of that in fresh islets; after culture in 3.3 mM glucose it was 10%. Half-maximal stimulation was observed at a glucose concentration of 5 mM for islets cultured with high glucose as compared to 9 mM for fresh islets. Like glucose, glibenclamide was a more effective insulin stimulator after culture with a high glucose concentration than with a low one. However, leucine-induced insulin release was not affected by the glucose concentration in the preceding culture medium. Whereas potentiation of glucose-stimulated release by arginine or dibutyryl-cAMP was independent of glucose concentration during the culture, theophylline released three times more insulin when the islets had been cultured with high glucose.

Key words: Microdissected pancreatic islets, *ob/ob*-mouse islets, tissue culture, insulin release, insulin content.

Isolated pancreatic islets can be maintained *in vitro* for considerable periods of time [1, 2, 3]. It has been shown that culturing induces changes in every investigated parameter, *i. e.* respiration, glucose oxidation, insulin content and release [3, 4, 5]. Common to all previously reported studies is the use of collagenase-isolated islets. A characteristic feature of these islets, when cultured in media containing calf serum, is that they attach firmly to adjacent surfaces. In order to remove these islets from their support, further enzymatic digestion or mechanical means have to be used [6].

The present study demonstrates the possibility of maintaining B-cell-rich pancreatic islets isolated by microdissection for seven days in a serum-free medium. This technique prevents the attachment of the islets to the incubation vials during culture. Furthermore, it will be reported how the glucose concentration in the culture medium affects the islets with regard to content of insulin, insulin release into the culture medium, dose-response relationship for glucose, and the response to other stimulators and potentiators of insulin release.

Material and Methods

Chemicals

Reagents of analytical grade and distilled, deionized water were used. N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (Hepes), D-glucose, L-leucine, L-arginine, theophylline, and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., St. Louis, Mo., USA. N^6-2′-0-dibutyryl-adenosine-3′:5′-monophosphate (dibutyryl-cAMP) came from Boehringer Mannheim GmbH, Mannheim, FRG; and N-4-[2-(5-chloro-2-methoxybenzamide)ethyl]benzenesulfonyl-N′-cyclohexylurea (glibenclamide) from Farbwerke Hoechst A. G., Frankfurt/Main, FRG. Tissue culture medium 199 (TCM 199; 10X concentrated) [7] was obtained from National Bacteriological Laboratories, Stockholm, Sweden and gentamycin from Bio-Cult Laboratories Ltd., Glasgow, Scotland. ^{125}I-labelled insulin was from Farbwerke Hoechst A. G. and insulin antibodies from Wellcome Reagents Ltd., Beckenham, England. Crystalline mouse insulin was prepared by Novo A/S, Copenhagen, Denmark.

Composition of the Medium

The same basal medium was used for isolation, culture and incubation of the islets. It consisted of a modified TCM 199 lacking glucose and phenol red and buffered with 20 mM Hepes and 15 mM $NaHCO_3$. Bovine serum albumin (1 mg/ml) and gentamycin (50 μg/ml) were also included. The medium was adjusted to pH 7.35 (37° C) with NaOH. The medium was sterilized by filtration through a Millex

Filter Unit (0.22 μm; Millipore S. A., Buc, France). Additions to the basal medium are described in the appropriate context. During the course of this study it became evident that the pH increased to about 8.5 during culture for one week, probably due to loss of CO_2. Therefore, a series of control experiments was performed using a modified basal medium buffered with 25 mM Hepes and 4.2 mM $NaHCO_3$. After sterile filtration this medium was kept overnight at 37° C in an open bottle. It was then adjusted to pH 7.35 (37° C) with NaOH and sterilized before use. The pH of the latter medium was stable throughout the entire culture period. Similar results were obtained with the two types of media (see Tables 1, 2 and 3). Thus, the increase in pH encountered during the culture in the present study seems to be of minor importance for the B-cell function. These results are in accordance with observations concerning the effect of pH on cultured rat islets [5] and *in vitro* incubated fresh mouse islets [8].

Animals and Isolation of Islets

Adult, female mice, homozygotic for the gene *ob*, were taken from the non-inbred stock in Umeå [9]. Fresh pancreatic islets were isolated from animals fasted overnight by free-hand microdissection [10] at room temperature in basal medium containing 3.3 mM glucose.

Table 1. *Culture of islets for 7 days in medium buffered with 25 mM Hepes and 4.2 mM $NaHCO_3$*

Glucose concentration in the culture medium (mM)	Insulin content		Insulin release		
	Islets	Culture medium	1 mM glucose	18 mM glucose	Difference
3.3	25.9 ± 3.6 (24)	48.9 ± 5.1 (24)	0.07 ± 0.01 (6)	0.26 ± 0.04 (6)	0.19 ± 0.04* (6)
16.7	50.8 ± 3.6 (31)	233.9 ± 13.2 (32)	0.10 ± 0.02 (7)	1.02 ± 0.13 (7)	0.92 ± 0.12** (7)

The experiments were performed as described in the legends to tables 2 and 3. The results are expressed as ng insulin/μg dry islet and shown as mean values ± S. E. M. The number of observations are given within brackets.
* $P < 0.01$; ** $P < 0.001$

Table 2. *Insulin content in islets and culture media after incubation for 6 days*

Glucose concentration in the culture medium (mM)	Insulin content (ng/μg dry islet)		
	Islets	Culture medium	Islets plus culture medium
3.3	46.9 ± 3.5 (20)	59.2 ± 3.5 (51)	107.5 ± 5.9 (20)
5.6	44.3 ± 2.7 (48)	67.8 ± 3.5 (46)	111.8 ± 5.3 (46)
16.7	64.9 ± 4.7* (20)	218.8 ± 10.1** (51)	281.0 ± 19.6* (20)
27.8	65.3 ± 3.9* (48)	188.3 ± 8.1** (46)	253.2 ± 10.7* (46)
Fresh islets	158.0 ± 3.9 (40)		158.0 ± 3.9 (40)

Two islets were cultured in each dish containing 4 ml medium supplemented with glucose as indicated. The mean insulin content of the two islets is entered as a single observation. The values denote mean ± S. E. M. The number of observations are given within brackets. Statistical significances were calculated according to Student's test using the observations from islets cultured at 3.3 mM glucose as control values.
* $P < 0.005$; ** $P < 0.001$

Table 3. *Effect of various secretagogues on the release of insulin from islets cultured for 7 days*

Additive to incubation medium	Glucose concentration in the culture medium			
	3.3 mM		16.7 mM	
	Insulin released	Difference from control	Insulin released	Difference from control
D-glucose (control) (1 mM)	0.29 ± 0.05	–	0.19 ± 0.05	–
D-glucose (18 mM)	0.64 ± 0.14	+ 0.35 ± 0.12**	1.67 ± 0.35	+ 1.49 ± 0.37***
L-leucine (20 mM)	0.59 ± 0.13	+ 0.30 ± 0.11*	0.53 ± 0.10	+ 0.34 ± 0.09***
Glibenclamide (20 μM)	0.53 ± 0.13	+ 0.24 ± 0.09*	0.75 ± 0.08	+ 0.57 ± 0.09***

After a culture period of 7 days in basal medium containing either 3.3 mM or 17 mM glucose, the islets were incubated in fresh medium supplemented as indicated. Insulin release is expressed as ng/μg dry islet and shown as mean value ± S. E. M. for 9 separate experiments.
* $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$

Incubation of Islets

Isolated islets were transferred to sterile plastic Petri dishes with lids (5 cm diameter, Heger Plastics AB, Stallarholmen, Sweden). Each dish contained either 2 islets in 4 ml or 16 islets in 12 ml of basal medium, supplemented with glucose as indicated in the Tables and Figures. The dishes were then stored for 6 or 7 days at 37° C in humidified air. In experiments where only two islets were cultured in each Petri dish, the amount of insulin was determined in the medium at the end of the culture period. After culture, the islets were rinsed and preincubated for 60 min in 1 ml basal medium containing 1 mM glucose. Insulin release was studied during a subsequent 60 min period of incubation of 2 islets in 300 μl medium supplemented as described in the Tables and Figure. Both preincubation and incubation were performed in closed vials at 37° C using ambient air as gas phase.

Weighing of Islets and Assay of Insulin

After incubation the islets were freeze-dried (−40° C; 0.1 Pa) and weighed on a quartz-fiber balance. Islets used for determination of insulin content were then subjected to ultrasonic disintegration in 1 mM HCl (Sonifier B-12. Branson Sonic Power Co., Danbury, Conn., USA, equipped with a microtip and run at a meter reading of 50 W). Insulin in islet extracts and media was assayed radioimmunologically [11] using crystalline mouse insulin as reference.

Presentation of Results

Insulin values were expressed per unit dry weight of islets. Unless otherwise stated, statistical significance of effects was judged by Student's test from the mean ± S. E. M. of differences between paired test and control incubations in a series of repeated experiments.

Results

The dose-response relationship for glucose-induced insulin release in fresh and cultured islets is shown in Fig. 1. The maximal response was depressed in all cultured islets. In fact, islets cultured with 3.3 mM glucose almost lost their ability to release insulin in response to a glucose challenge. In fresh islets maximal stimulation of insulin release was reached at a glucose concentration of 12 mM, but at slightly lower concentrations in islets cultured with 17 or 28 mM glucose. The corresponding half-maximal response occurred at a glucose concentration of 9 mM in the fresh islets and at about 5 mM in the cultured islets. The basal release tended to be depressed in the islets cultured at the high glucose concentrations, resulting in a high ratio between maximal response and baseline release.

Table 2 shows the islet content of insulin as well as the amount of insulin in the incubation medium at the end of the culture period. The content of insulin within the B-cells decreased in all islets during the culture period ($P < 0.001$); islets cultured at 3.3 mM or 5.6 mM contained significantly less insulin than those cultured at 17 mM or 28 mM glucose. Substantially more insulin was recovered in the culture media with 17 mM or 28 mM glucose than in culture media containing 3.3 mM or 5.6 mM glucose. When comparing the insulin

content of fresh islets with the amount of insulin present in cultured islets plus their culture medium, it is obvious that high glucose concentrations in the culture medium stimulated insulin biosynthesis. In the latter case the total amount of insulin recovered was almost twice as high as found in fresh islets.

In addition to glucose, leucine and glibenclamide stimulated insulin release from cultured islets (Table 3). Glucose and glibenclamide also seemed to be more effective after the islets had been maintained in a milieu with high glucose. Whereas the potentiation of glucose by arginine and dibutyryl-cAMP was independent of the glucose concentration during the preceding culture period, theophylline released three times more insulin when the islets had been cultured in high glucose (Table 4).

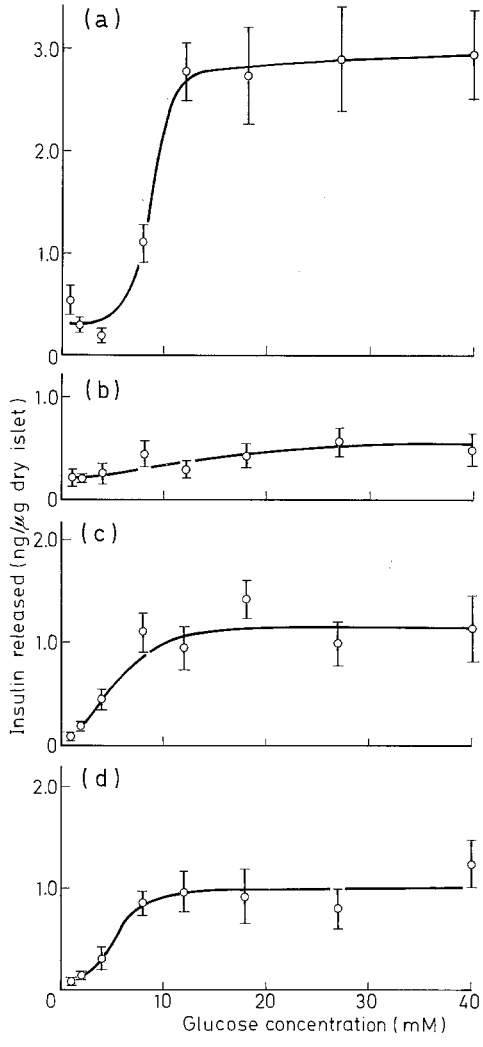

Fig. 1. Fresh islets (a) or islets cultured for 6 days in basal medium containing 3.3 mM glucose (b), 17 mM glucose (c) or 28 mM glucose (d) were incubated for 60 min in basal medium supplemented with various concentrations of glucose. Amounts of insulin released are presented as mean values ± S. E. M. for 4–7 different experiments

Discussion

In principle, there are two ways of isolating pancreatic islets – the mechanical microdissection technique [10] and the enzymatic isolation procedure employing crude collagenase [12]. Although both techniques have been in use for several years, only a little is known about possible effects on the B-cells of the isolation procedure [8, 13]. The purpose of the present study was to establish a culture technique for isolated islets using a chemically defined (*i. e.* serum-free) medium. In preliminary experiments collagenase isolation was used to increase the yield of islets. Although Boder *et al.* [14] reported a successful culture of collagenase-isolated rabbit islets in a chemically defined medium, all islets disintegrated within a few days of culture in our experiments. A possible explanation for this discrepancy is the fact that Boder *et al.* used a very mild collagenase treatment, leaving the islets surrounded by acinar tissue [14]. Disintegration of the islets can be prevented by the addition of serum to the culture medium [6]. However, when present in the culture medium, serum induces an outgrowth of cells from the islet periphery, leading to a firm attachment of the islets to adjacent surfaces [6]. The attached islets can be removed by scraping or trypsination. Although trypsin treatment was reported not to affect the insulin-releasing capacity of cultured islets isolated by collagenase [6], freshly isolated islets displayed decreased secretory and metabolic responses to glucose after treatment with trypsin or collagenase [6, 8, 15]. These observations point to the advantage of isolating islets without using enzymes. A further reason for restricting the use of collagenase in the isolation of pancreatic islets is that some commercial preparations contain a β-cytotoxic factor [16].

Most mammalian cells require serum in order to grow *in vitro* [17]. However, it is evident from the present study that microdissected islets cultured in a serum-free medium maintain their insulin-releasing capacity and content of insulin equally well or even better than previously found for collagenase-isolated islets cultured in serum-supplemented media. The omission of serum has the obvious advantage of a less complex medium more suited in determining the factors which influence the functional state of the B-cells.

In keeping with earlier reports on cultured islets [4, 18], a high glucose concentration during the culture period resulted in a better preservation of the secretory response to glucose. Furthermore, active biosyn-

Table 4. *Effect of different potentiators of glucose-induced insulin release on islets cultured for 7 days*

Additive to incubation medium containing 18 mM glucose	Glucose concentration in the culture medium			
	3.3 mM		16.7 mM	
	Insulin released	Difference from control	Insulin released	Difference from control
Nil (control)	0.64 ± 0.14	–	1.67 ± 0.35	–
L-arginine (20 mM)	1.25 ± 0.21	+ 0.60 ± 0.26*	2.38 ± 0.45	+ 0.71 ± 0.31*
Dibutyryl-cAMP (1 mM)	1.92 ± 0.43	+ 1.34 ± 0.29***	2.88 ± 0.67	+ 1.48 ± 0.44**
Theophylline (5 mM)	1.79 ± 0.20	+ 1.14 ± 0.28***	6.63 ± 1.28	+ 4.96 ± 1.00***

After culture for 7 days, islets were incubated for 60 min in basal medium containing 18 mM glucose and further additives as indicated. Insulin release is expressed as ng/μg dry islet. Mean values ± S. E. M. are given for 9 separate experiments except for the islets exposed to dibutyryl-cAMP (7 experiments).
* $P < 0.05$; ** $P < 0.02$; *** $P < 0.005$

thesis of insulin is indicated by a net increase of insulin during culture in high glucose media. Nevertheless, the islet content of insulin decreased even in high-glucose media. It is well established that different endocrine cells have to be exposed to certain stimulatory factors or hormones in order to maintain their function. The present study indicates that glucose alone is not sufficient to sustain an optimal B-cell function.

Prolonged glucose loading has been shown to increase adenylate cyclase activity in the B-cells [19]. Whereas the insulin-releasing effect of dibutyryl-cAMP was unaffected by the glucose concentration in the culture medium, the phosphodiesterase inhibitor theophylline was much more effective in potentiating glucose-induced insulin release in islets cultured with high glucose. This difference could be taken as an indirect evidence for a higher adenylate cyclase activity during culture in high glucose. A better preservation of the cyclic AMP system in islets cultured with high glucose might explain the greater insulin response to glucose or glibenclamide. On the other hand, such an explanation would imply that neither arginine nor leucine depend on the cyclic AMP system for their stimulation of insulin release.

A striking correlation between the rates of insulin release and glucose metabolism in non-cultured islets has previously been shown [20, 21, 22]. After culture of islets in the presence of high or low glucose concentrations, Andersson [2] found increased rates of glucose oxidation in the high-glucose cultured islets, an observation which provides an additional explanation of the greater insulin-releasing effect of glucose in these islets. Furthermore, islets cultured with high glucose contain ten times more glycogen than islets from low-glucose media [23]. The high glycogen content might cause a substantial production of glucose intermediates in the presence of sulfonylureas, which are known to exert a marked glycogenolytic effect on the B-cells [24]. This effect may consequently explain the larger insulin response to glibenclamide in the high-glucose cultured islets.

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