Regulation of Pancreatic β -Cell Glycogen through Cyclic-3,5-AMP*

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Summary. Glycogen was measured in isolated pancreatic islets from obese-hyperglycaemic mice. The recordings were made on crude tissue extracts using a highly specific enzymatic assay. The amount of glycogen depended on the extracellular glucose concentration. Dibutyl cyclic-3,5-AMP as well as compounds (glucagon and theophylline) known to increase the islet level of cyclic-3,5-AMP reduced the content of glycogen. Epinephrine, which has been reported to inhibit the synthesis of cyclic-3,5-AMP, increased the level of islet glycogen in the presence of 3.0 mg/ml of glucose. The results suggest that the level of cyclic-3,5-AMP is an important regulator of glycogen metabolism in the insulin-producing β -cells.

Regulation du glycogène des cellules β pancréatiques par le 3',5':AMP cyclique

Résumé. Le glycogène a été mesuré dans des îlots pancréatiques isolés de souris obèses-hyperglycémiques. Les enregistrements ont été faits sur des extraits bruts de tissus, à l'aide d'un dosage enzymatique hautement spécifique. La quantité de glycogène dépendait de la concentration extracellulaire de glucose. Le 3',5'-AMP cyclique dibutyl ainsi que des composés (glucagon et théophylline) connus pour augmenter le taux insulaire de 3',5'-AMP cyclique, diminuaient le contenu en glycogène. L'adrénaline, dont l'effet inhibiteur sur la synthèse du

3',5'-AMP cyclique a été rapporté, augmentait le taux de glycogène des îlots en présence de 3,0 mg/ml de glucose. Les résultats suggèrent que le taux de 3',5'-AMP cyclique est un régulateur important du métabolisme du glycogène dans les cellules β insulino-sécrétrices.

Die Regulation des Glykogens der β -Zelle des Pankreas durch zyklisches 3,5-AMP

Zusammenfassung. An isolierten Pankreasinseln von fettstüchtigen, hyperglykämischen Mäusen wurde der Glykogengehalt gemessen. Die Untersuchungen wurden an rohen Gewebsextrakten mit Hilfe einer hoch spezifischen enzymatischen Methode durchgeführt. Der Glykogengehalt hing von der extrazellulären Glukosekonzentration ab. Dibutyl-zyklisches 3,5-AMP als auch Substanzen (wie Glukagon und Theophyllin), welche bekannt sind, den zyklischen 3,5-AMP-Spiegel der Inseln zu heben, verminderten den Glykogengehalt. Epinephrin, das die Synthese von zyklischem AMP hemmen soll, erhöhte den Glykogenspiegel der Inseln in Anwesenheit von 3,0 mg/ml Glukose. Die Ergebnisse deuten darauf hin, daß der zyklische 3,5-AMP-Spiegel ein wichtiger Regulator des Glykogen-Metabolismus in der Insulin produzierenden Zelle ist.

Key-words: Pancreatic β -cell, glycogen, cyclic-3,5-AMP, obese-hyperglycaemic mice.

It is well established that the synthesis and release of insulin is regulated by the glucose levels to which the pancreatic β -cells are exposed. Evidence has been presented for the view that glucose has to be metabolized in the β -cells for it to be effective as a trigger for insulin release (Grodsky *et al.*, 1963; Coore and Randle, 1964). The β -cells are not only equipped with the enzymes necessary for synthesis and degradation of glycogen (Brolin and Berne, 1970), but it has also been shown that they normally contain glycogen (Matschinsky and Ellerman, 1968; Hellman and Idahl, 1969). The glucose metabolite supposed to be involved in the regulation of the β -cell function might thus be derived from glycogen (Samols *et al.*, 1966; Täljedal, 1970).

The observation that hypoglycaemic sulphonylurea compounds induce an impressive reduction of the β -cell content of glycogen suggests that this substrate may be a sensitive indicator of metabolic changes

associated with insulin release (Hellman *et al.*, 1969). In view of the proposed role of the adenylyl cyclase system for hormonal regulation of insulin release (Kipnis, 1970; Malaisse and Malaisse-Lagae, 1970), it should furthermore be noted that cyclic-3,5-AMP activates glycogen phosphorylase in various tissues (Haugaard and Hess, 1965). The present study deals with the influence of the cyclic-3,5-AMP level on the net result of glycogen synthesis and degradation, i.e. the glycogen content, in microdissected islets from obese-hyperglycaemic mice.

Materials and Methods

Animals and experimental design. Forty-four 7–9 months old female obese-hyperglycaemic mice were taken from a colony originating from R.B. Jackson Memorial Laboratories, Bar Harbor, Maine, U.S.A. (Hellman, 1965). All animals were starved for 18–24 h before being killed by decapitation. Pancreatic islets were dissected under a stereomicroscope (Hellerström, 1964), and incubated at 37°C in single glass vessels

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containing 1 ml Krebs-Ringer bicarbonate buffer with 0.5% albumin (pH 7.4) under a gas phase of 95% O₂ and 5% CO₂. Two series of experiments were performed: 1) The effect of glucagon on the islet content of glycogen was studied in the presence of high and low glucose concentrations. Ten islets were microdissected from each of 9 mice and incubated for 60 min either in the presence or absence of 15 μ g/ml of porcine glucagon (lot B 66, Novo Industri A/S, Copenhagen) with 0.6 or 3.0 mg/ml glucose. Furthermore, the initial glycogen content was recorded in 2 islets taken immediately after the dissection.

2) The second series of experiments was designed to give further information on how the islet level of glycogen is affected by drugs supposed to influence insulin release by altering the level of cyclic-3,5-AMP. A total of 35 mice were used for these experiments. After 10 min of preincubation with 0.6 mg/ml glucose, 9 islets from each animal were equally distributed between one control medium and two test media supplemented according to one of the following four possibilities: 1 mM theophylline (Mann Research Laboratories Inc., New York), 1 mM theophylline + 15 μ g/ml porcine glucagon, 250 μ g/ml dibutyryl cyclic-3,5-AMP (Boehringer, Mannheim) or 2 μ g/ml L-epinephrine (Sigma Chemical Co., St. Louis). Each compound or combination of compounds was tested by incubating islets from 10–14 animals for 30 min with a low (0.6 mg/ml) or high (3.0 mg/ml) concentration of glucose. Before exposure to the various drugs the initial glycogen content was recorded by analyses of 3 preincubated islets from each mouse.

Preparation of tissue. Pieces of pancreas were rapidly removed and placed in Krebs-Ringer bicarbonate buffer at +2°C. The buffer was equilibrated with 95% O₂ and 5% CO₂ and contained 0.6 mg/ml glucose and 0.5% albumin. Intact and metabolically active islets were carefully dissected. After the islets had been incubated under the desired conditions, they were dropped into melting isopentane (–165°C). The islets were dried at –40°C and 0.001 mm Hg for 12 h and weighed on a quartz-fibre balance, before being transferred to micro test tubes of polythene for analysis. In some cases the large islets were divided into pieces, which were individually weighed and extracted.

Chemical methods. The islet content of glycogen was recorded by enzymatic fluorimetric technique essentially as described by Passonneau *et al.* (1967). The extraction of glycogen was performed by heating the islets for 10 min at 100°C in 0.03 M HCl (1.5–2.0 μ g tissue/ μ l of acid). This treatment also destroys interfering enzymes, glucose-1-phosphate and native NADPH₂. Aliquots (3 μ l) of the extracts were then mixed with 15 μ l of a reagent composed as previously described (Hellman and Idahl, 1969). The time of incubation was 40 minutes. After destruction of excess NADP⁺ by heating with 0.05 M NaOH, the NADPH₂ formed was measured by enzymatic cycling (Lowry *et al.* 1961). The cycling medium contained 25 μ g

glucose-6-phosphate dehydrogenase (centrifuged and resuspended in 2 M ammonium acetate to reduce the sulphate concentration) and 200 μ g glutamic dehydrogenase (free from sulphate) per ml. The cycling reaction was carried out in a total volume of 115 μ l for 60 min at 38°C, giving an approximate cycling amplification of 8.000X. The final fluorimetric readings were performed in an Aminco-Bowman spectrophotofluorimeter. Phosphorylase *a* (Sigma Chemical Co.) was activated daily and “debrancher” enzyme activity checked monthly as described by Passonneau *et al.* (1967). Reagent blanks and standards of glycogen (rabbit liver glycogen, grade III, Sigma Chemical Co.) covering the expected range were carried through the entire procedure. The glycogen stock solution was analyzed for glucose before and after hydrolysis in HCl according to Passonneau *et al.* (1967). Before use the stock solution was heated for 10 min at 60°C in 0.02 M NaOH to ensure its reactivity to phosphorylase. The amounts of glycogen were calculated as glucosyl units, i.e. 1 mmol is equal to 162 mg.

All enzymes and biochemicals, except phosphorylase *a* and glycogen, were obtained from Boehringer, Mannheim.

Results

The glycogen level after exposing microdissected islets to glucagon are shown in Table 1. Before incubation, the islet content of glycogen was equivalent to 5.68 ± 0.88 mmol per kg dry weight (mean values $\pm$ S.E.M. for 9 animals). Incubation of the islets in the

Table 1. Effect of glucagon (15 μ g/ml) on the glycogen levels (mmol glucosyl units per kg dry weight) in islets incubated for 60 min in media containing either 0.6 or 3.0 mg glucose per ml. The glycogen content before incubation was 5.68 ± 0.88 mmol per kg dry weight. The effect of treatment was calculated with Student's *t*-test for paired samples. Mean values $\pm$ S.E.M.

Glucose concn. mg/ml	Test substance	Glycogen content	Changes from controls
0.6	None	3.97 ± 0.82	
0.6	Glucagon	2.67 ± 0.68	-1.30 ± 0.45 ($P < 0.025$)
3.0	None	9.14 ± 2.15	
3.0	Glucagon	8.36 ± 1.27	-0.78 ± 2.10 ($P > 0.10$)

presence of 0.6 mg/ml glucose resulted in lower glycogen levels. In the presence of glucagon this decrease was significantly accelerated. Glucagon, on the other hand, had no effect when the glycogen levels were enhanced by incubating the islets with 3.0 mg/ml glucose. Addition of theophylline to a medium containing 0.6 mg/ml of glucose resulted in a glycogen decrease similar to that observed in the presence of

glucagon (Table 2). Combination of theophylline with glucagon did not produce further glycogen depletion compared with that obtained in the presence of theophylline alone; the difference between these two groups was 0.54 ± 0.33 mmol per kg dry weight.

Table 2. Glycogen content (mmol glucosyl units per kg dry weight) in microdissected islets exposed for 30 min either to theophylline (1 mM) or theophylline combined with glucagon (15 μ g/ml) in the presence of 0.6 mg glucose per ml. The glycogen level after 10 min of preincubation amounted to 4.75 ± 0.67 mmol per kg dry weight. Mean values $\pm$ S.E.M. for 11 animals.

Test substance	Glycogen content	Changes from controls
None	4.20 ± 0.40	
Theophylline	3.14 ± 0.39	-1.06 ± 0.30 ($P < 0.01$)
Theophylline + glucagon	2.60 ± 0.49	-1.60 ± 0.33 ($P < 0.001$)

Table 3. The effects of dibutyryl cyclic-3,5-AMP (250 μ g/ml) and epinephrine (2 μ g/ml) on the glycogen levels (mmol glucosyl units per kg dry weight) in islets incubated for 30 min in the presence of 0.6 mg/ml glucose (10 animals) or 3.0 mg/ml glucose (14 animals). Mean values $\pm$ S.E.M.

Treatment	0.6 mg/ml glucose			3.0 mg/ml glucose		
	Glycogen content	Changes from control values	Changes from preincubation values	Glycogen content	Changes from control values	Changes from preincubation values
Preincubation	4.69 ± 0.50			3.60 ± 0.28		
Control	3.45 ± 0.39		-1.24 ± 0.37 ($P < 0.01$)	5.25 ± 0.59		1.65 ± 0.43 ($P < 0.005$)
DBcAMP	2.38 ± 0.29	-1.07 ± 0.30 ($P < 0.01$)	-2.31 ± 0.53 ($P < 0.005$)	3.54 ± 0.37	-1.71 ± 0.46 ($P < 0.005$)	-0.06 ± 0.28 ($P > 0.10$)
Epinephrine	3.36 ± 0.58	-0.09 ± 0.50 ($P > 0.10$)	-1.33 ± 0.33 ($P < 0.005$)	7.20 ± 0.48	1.95 ± 0.57 ($P < 0.005$)	3.60 ± 0.42 ($P < 0.001$)

The dibutyryl derivative of cyclic-3,5-AMP was found to give lower islet glycogen levels compared with the controls both in the presence of low and high glucose concentrations (Table 3). It is evident from the Table that dibutyryl cyclic-3,5-AMP actually prevents the accumulation of glycogen normally caused by the high glucose concentration. Epinephrine had no effect on the islet glycogen levels in the low glucose medium. However, epinephrine induced a glycogen increase of no less than 3.60 ± 0.42 mmol per kg dry weight when the islets were incubated for 30 min in the presence of 3.0 mg/ml of glucose.

Discussion

In view of the high proportion of β -cells in the islets from obese-hyperglycaemic mice, the results should be considered as representative for these cells. It should be noted that the glycogen assay used actually measures the sum of glycogen and glucose-

6-phosphate. The islet level of glucose-6-phosphate is linearly related to extracellular glucose, increasing from 0.1 mmol to 0.3 mmol per kg dry weight when the glucose concentration is raised from 0.6 to 3.0 mg/ml (Idahl *et al.*, 1971). Furthermore, the concentration of epinephrine used increases the glucose-6-phosphate level in islets exposed to 3.0 mg/ml of glucose by about 50% (Hellman, 1970). These variations in glucose-6-phosphate are, however, too small to contribute significantly to the changes in the glycogen content recorded in the present study.

Nothing is known of the properties of islet glycogen phosphorylase and synthetase apart from recordings *in vitro* of their maximal activity (Brolin and Berne, 1970). Our discussion is also hampered by the fact that the observed changes of the glycogen levels represent the net result of the synthesis and degradation of this compound. We have no information about the turnover rate of glycogen, and are therefore unable to evaluate whether glycogen is essential for

the β -cells by transferring glucose molecules from one intracellular glucose-6-phosphate pool to another as suggested by Samols *et al.* (1966).

The extracellular glucose concentration is obviously of great importance for regulation of the levels of β -cell glycogen. Whereas the β -cells are capable of building up or maintaining significant glycogen stores in the presence of 3.0 mg/ml of glucose, exposure to low glucose levels diminishes the glycogen content. These effects would be expected from what is known from other tissues about the allosteric effects of various substrates (glucose, glucose-6-phosphate, adenine nucleotides and inorganic phosphate) on glycogen synthetase and phosphorylase (Smith *et al.*, 1968; Helmreich, 1969). In the case of the islets from the obese-hyperglycaemic mice, a correlation between extracellular glucose concentration and intracellular glucose-6-phosphate has been observed under both *in vivo* and *in vitro* conditions (Matschinsky and Ellerman, 1968; Idahl *et al.*, 1971). There is also an elevated ATP level in glucose-stimulated β -cells (Hellman *et al.*,

1969). This glucose-induced increase of ATP is likely to be coupled to a decrease in the concentrations of AMP and inorganic phosphate, i.e. an alteration of the "phosphate potential" that might be expected to decrease glycogenolysis. An altered "phosphate potential" would also explain the depletion of β -cell glycogen evoked by hypoglycaemic sulphonylurea compounds (Hellman *et al.*, 1969).

The levels of cyclic-3,5-AMP have been reported to increase after addition of glucagon or theophylline to an *in vitro* system with isolated rat islets (Kipnis, 1970). This is consistent with the previously established role of glucagon as an activator of adenylyl cyclase (Sutherland and Rall, 1960) and theophylline as an inhibitor of cyclic-3,5-AMP phosphodiesterase (Butcher and Sutherland, 1962). When microdissected islets from obese-hyperglycaemic mice were exposed to one of these compounds or to dibutyryl cyclic-3,5-AMP, there was a significant acceleration of the glycogen disappearance in the presence of 0.6 mg/ml of glucose. The depressive effect of the adenylyl-cyclase system on the β -cell content of glycogen compares favourably with the cyclic-3,5-AMP activation of phosphorylase in liver and muscle (Haugaard and Hess, 1965). The failure of glucagon to enhance the effect obtained with theophylline alone might reflect that the level of cyclic-3,5-AMP was already high enough for maximal activation of glycogenolysis. Glucagon and dibutyryl cyclic-3,5-AMP were also tested at a glucose concentration of 3.0 mg/ml. Under these conditions the latter compound prevented the accumulation of glycogen, whereas no effect was encountered with glucagon. There is a general need to use very high concentrations of glucagon to show effects *in vitro*, which stresses the importance of being cautious when discussing results obtained with this hormone.

It has been reported that epinephrine inhibits the synthesis of cyclic-3,5-AMP in the pancreatic islets by stimulating the α -adrenergic receptor sites (Kipnis, 1970). When epinephrine was included in our system with isolated islets, there was no effect in a medium with low glucose, whereas a significantly accelerated increase of glycogen was noted in the high glucose medium. Epinephrine has been found to interfere with glucose metabolism below fructose-1,6-diphosphate, resulting in a striking accumulation of early glycolytic intermediates including glucose-6-phosphate (Hellman, 1970). This effect of epinephrine might be more important in the control of β -cell glycogen than decreased phosphorylase activity due to the lowered cyclic-3,5-AMP level.

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