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Expression of the 64 kDa/glutamic acid decarboxylase rat islet cell autoantigen is influenced by the rate of insulin secretion

E. Björk[1], O. Kämpe[1], A. Andersson[2] and F. A. Karlsson[1]

[1] Department of Internal Medicine, University Hospital, and
[2] Department of Medical Cell Biology, Biomedical Centre, Uppsala University, Uppsala, Sweden

Summary. This study examined the relationship between insulin secretion and expression of the 64 kDa/glutamic acid decarboxylase autoantigen in pancreatic islets. Islets isolated from Wistar rats were cultured for 3 days under different conditions: in 5.5 mmol/l glucose with or without α-ketoisocaproic acid or glipizide and in 28 mmol/l glucose with or without diazoxide. The 64 kDa/glutamic acid decarboxylase autoantigen was precipitated from lysates of [^{35}S]-methionine -labelled islets with sera from patients with Type 1 (insulin-dependent) diabetes mellitus and identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fluorography. In parallel, insulin contents of the islets and the media were determined as well as the rates of glucose-stimulated (pro)insulin biosynthesis. α-Ketoisocaproic acid and glipizide were found to stimulate the expression of the 64 kDa/glutamic acid decarboxylase autoantigen and also the rate of insulin secretion. Diazoxide on the other hand reduced the rate of the 64 kDa/glutamic acid decarboxylase autoantigen synthesis in parallel with an inhibition of glucose-stimulated insulin release. Under most of the conditions employed, (pro)insulin biosynthesis was not affected. The correlation found between the rate of insulin release and expression of the 64 kDa/glutamic acid decarboxylase autoantigen might provide an explanation for the earlier observed relationship between the functional demands on the Beta cells and their rate of destruction which may result in diabetes.

Key words: Autoantigen, glutamic acid decarboxylase, insulin secretion, diazoxide, islet cells.

Type 1 (insulin-dependent) diabetes mellitus is due to an autoimmune destruction directed against antigens in the islets of Langerhans. At the onset of the disease, autoantibodies that recognize islet antigens are present in the circulation in a majority of patients. Recently, one such islet antigen, the 64 kDa autoantigen was identified as glutamic acid decarboxylase (GAD) [1], the biosynthesizing enzyme of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). High levels of this enzyme are expressed in pancreatic Beta cells, in many central neurons, testis and retina.

In the present study, we explored our previous finding of a glucose-dependent expression of the 64 kDa/GAD autoantigen [2], to investigate whether a parallel between the functional activity of islets and autoantigen expression exists. Stimulation of insulin secretion was achieved by culturing islets for 3 days in the presence of α-ketoisocaproic acid or glipizide. In another set of experiments islets were exposed to diazoxide in order to inhibit glucose-stimulated insulin release [3]. For comparative purposes rates of glucose-stimulated (pro)insulin biosynthesis were estimated in islets that had been cultured with the different test substances.

Materials and methods

Preparation and culture of islets

Islets from 250 g male Wistar Furth rats were isolated by handpicking by means of a braking pipette from collagenase digests (Collagenase A; Boehringer Mannheim, Mannheim, FRG) of pancreata and cultured free floating in RPMI 1640 medium containing 10% (volume/volume, v/v) calf serum (Statens Bakteriologiska Laboratorium, Stockholm, Sweden), 100 U/ml penicillin, and 100 μg/ml streptomycin. All incubations were performed at 37 °C in humified air containing 5% CO_2. Islets were cultured in either 5.5 or 28 mmol/l glucose with or without the addition of 10 mmol/l α-ketoisocaproic acid (α-KIC; Sigma Chemical Co., St Louis, Mo., USA), 0.5 μg/ml glipizide (K4024-706156; Carlo Erba S.p.A., Milano, Italy) or 22.5 μg/ml diazoxide (Essex, Milano, Italy). Diazoxide (100 mg/ml) was dissolved in dimethylsulfoxide before being added to the culture medium. For control purposes dimethylsulfoxide was added at the same concentration to culture media

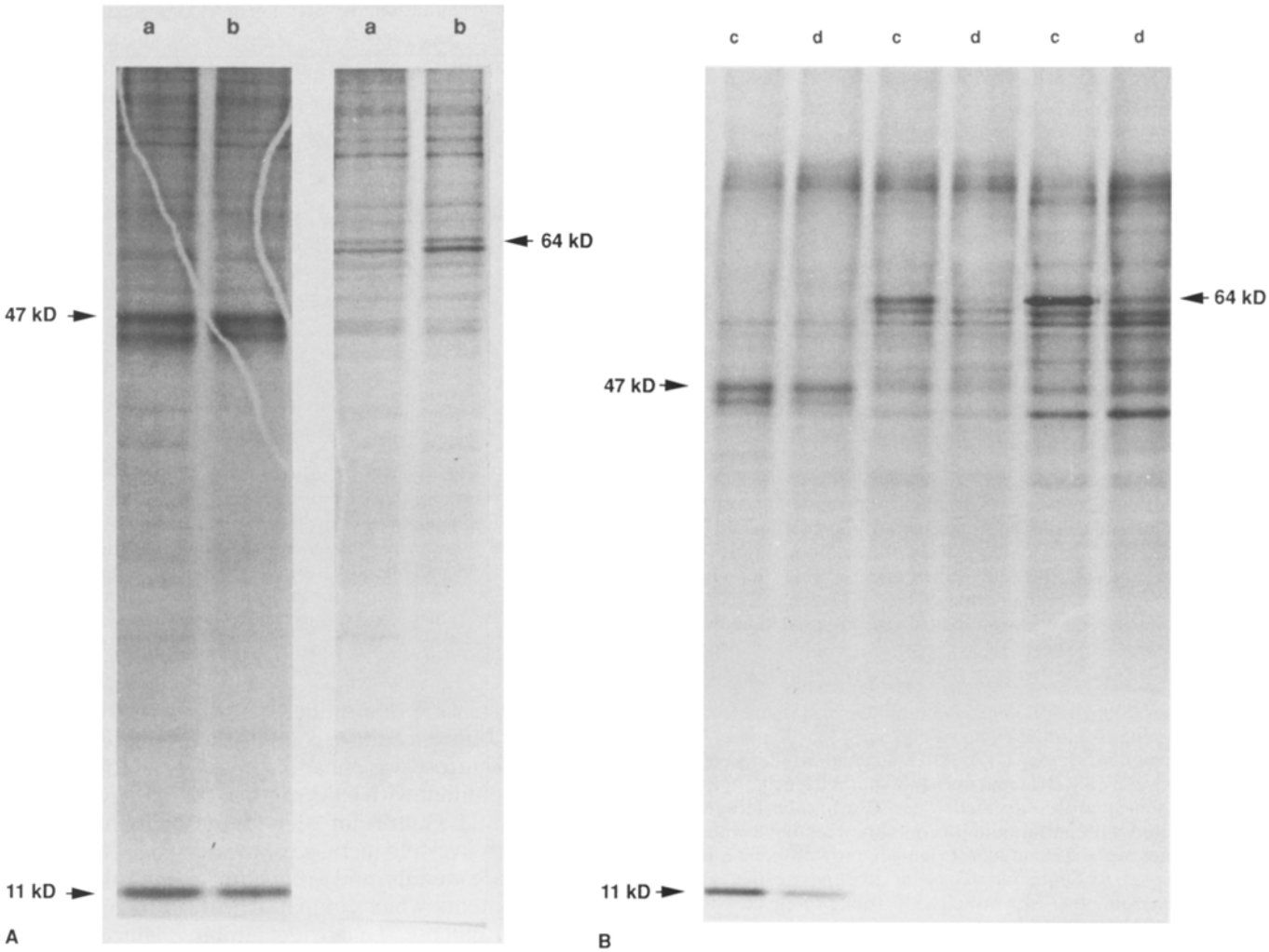

Fig. 1 A, B. Fluorograph of SDS-polyacrylamide gel electrophoresis of immunoprecipitates of [^{35}S]-methionine-labelled lysates of rat islets. **A.** Islets were incubated for 72 h in 5.5 mmol/l glucose (lane a) or 5.5 mmol/l glucose + α-ketoisocaproic acid (lane b), and precipitated with a polyclonal rabbit anti-MHC class I serum (left pair) and serum from a patient with Type 1 (insulin-dependent) diabetes mellitus containing antibodies against the 64 kDa/glutamic acid decarboxylase autoantigen (right pair). **B.** Islets were incubated for 72 h in 28 mmol/l glucose (lane c) or 28 mmol/l glucose + diazoxide (lane d), and precipitated with a polyclonal rabbit anti-MHC class I serum (left pair), a polyclonal sheep anti-GAD serum (middle pair) and serum from a patient with Type 1 diabetes containing antibodies against the 64 kDa/GAD autoantigen (right pair)

with 28 mmol/l glucose. α-KIC and glipizide were dissolved in Hanks' balanced salt solution and added to the media. Culture media were changed after 48 h and the incubations continued for another 24 h.

Immunoprecipitation

Subsequently, the media were changed and the islets labelled by incubation for 6 h with [^{35}S]-methionine (> 1000 Ci/mmol, New England Nuclear, Boston, Mass., USA). After labelling, the islets were lysed. Specific immunoprecipitates were obtained by incubating with a selected high titre serum from a patient with insulin-dependent diabetes mellitus of recent-onset and with a polyclonal sheep anti-GAD serum (1440, Laboratory of Clinical Science, NIMH, Bethesda, Washington DC, USA). For comparison, immunoprecipitates using a polyclonal rabbit anti-MHC class I serum were formed. The immunoprecipitates were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fluorography as described [2]. The bands of interest were scanned, and the autoradiographic intensities estimated from the paper weights of the scanned areas.

Insulin content of culture media and islet homogenates

Islets were incubated under various conditions in triplicates of 50 islets each. Following 48 h of culture, the media were changed and the incubations continued for 24 h. Aliquots of the different media were taken at the end of the latter incubation periods and kept frozen (− 20 °C) until analysed for their insulin contents. Subsequently, islets in triplicates of 10 each were employed for studies of insulin content and in triplicates of 20 each for biosynthesis experiments (see below). For determination of insulin contents the various three 10-islet samples were pooled and ultrasonically disrupted in 0.2 ml redistilled water. Fifty microlitre fractions of the aqueous islet homogenates were mixed with 125 µl acid ethanol (0.18 mol/l HCl in 96 % (v/v) ethanol) and insulin extracted overnight at 4 °C. Insulin was determined by radioimmunoassay.

(Pro)insulin and total protein biosynthesis

After 72 h of culture under different conditions, groups of 20 islets were incubated for 120 min in 100 µl Krebs-Ringer bicarbonate buffer (KRBH) containing 2 mg/ml bovine serum albumin (Miles

Table 1. Effects of stimulatory and inhibitory agents on insulin release, (pro)insulin biosynthesis and expression of 64 kDa/glutamic acid decarboxylase (GAD) autoantigen in cultured rat islets

	5.5 mmol/l glucose	5.5 mmol/l glucose + α-ketoisocaproic acid	5.5 mmol/l glucose + glipizide	28 mmol/l glucose	28 mmol/l glucose + diazoxide
Medium, insulin accumulation ($ng \cdot ml^{-1} \cdot 50\ islets^{-1}$)	89 ± 19 (9)	373 ± 32 (9)[c]	281 ± 15 (9)[c]	430 ± 55 (9)	227 ± 14 (9)[c]
Islet insulin content (ng/islet)	131 ± 3 (3)	56 ± 14 (3)[c]	64 ± 17 (3)[c]	49 ± 3 (3)	84 ± 14 (3)[b]
(Pro)insulin biosynthesis ($dpm \cdot 20\ islets^{-1} \cdot 2\ h$)	1270 ± 72 (9)	692 ± 35 (9)[c, d]	1072 ± 89 (9)	1273 ± 78 (9)	1380 ± 74 (9)
(Pro)insulin biosynthesis vs total protein biosynthesis (%)	22.7 ± 1.5 (9)	19.0 ± 1.8 (9)	16.0 ± 1.4 (9)[a]	19.4 ± 0.8 (9)	21.7 ± 1.7 (9)
Intensities[e] of 64 kDa/GAD bands given as percentage of that of the MHC class I heavy chain	33 27 23 29	49 36	37 39	74 48	17 7

Values for means ± SEM with numbers of experiments in parentheses. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ when compared with corresponding glucose concentration with no agent added. [d] The absolute value is falsely low due to dilution of the [^{3}H]-leucine precursor by the presence of α-ketoisocaproic acid (an amino acid analogue).

[e] Autoradiographic staining of protein bands was measured by a Biorad 1650 transmittance/reflectance scanning densitometer. The values represent individual sets of electrophoretic runs. Immunoprecipitates obtained using a serum from a patient with Type 1 (insulin-dependent) diabetes mellitus were scanned

Labaratories, Slough, UK) and 10 mmol/l Hepes (Sigma Chemical Co.) containing 16.7 mmol/l glucose and 5 μCi [^{3}H]-leucine (Amersham, Amersham, Bucks, UK). After incubation, the islets were washed twice in KRBH containing 10 mmol/l leucine and subsequently sonicated in redistilled water. (Pro)insulin biosynthesis was estimated with an immunoprecipitation technique where the homogenates were incubated with guinea-pig anti-bovine insulin serum coupled to Sepharose 4B beads to separate the labelled (pro)insulin from other islet proteins [4]. Total protein biosynthesis was estimated by measuring the amount of trichloroacetic acid precipitable tritium activity.

Statistical analysis

Experimental data were analysed by Student's unpaired *t*-test.

Results

The addition of 10 mmol/l α-KIC to islets cultured for 3 days in 5.5 mmol/l glucose enhanced the expression of the 64 kDa/GAD autoantigen (Fig. 1 A). The expression of the autoantigen was also enhanced by glipizide (0.5 μg/ml) (Table 1). In islets kept in culture for 3 days in 28 mmol/l glucose in the presence of diazoxide (22.5 μg/ml), there was a pronounced decrease in the expression of the 64 kDa/GAD autoantigen when compared with the high-glucose cultured control islets (Fig. 1 B). The amount of cell membrane-bound MHC class I proteins, consisting of a heavy 47 kDa protein and a light 11 kDa protein, were not altered by the additions of α-KIC, glipizide or diazoxide. The results were confirmed by scanning the autoradiographic intensities of the 64 kDa/GAD doublet in relation to the MHC class I bands obtained from immunoprecipitates of the corresponding lysates (Table 1).

Islet insulin contents and insulin concentrations in the media, as well as the ability of the islets to synthesize (pro)insulin, were determined following 72 h of culture under the different culture conditions. Insulin secretion at 5.5 mmol/l glucose was enhanced both by α-KIC and glipizide, concomitant with a decrease in the islet insulin content (Table 1). Culture of islets in the high-glucose medium gave a five-fold increase of the insulin accumulation in the culture medium and a reduction to one-third of their insulin contents when compared with islets cultured at 5.5 mmol/l glucose. Diazoxide inhibited glucose-stimulated insulin accumulation into media, paralleled by an increase of the islet insulin content (Table 1). Islets cultured in 5.5 mmol/l glucose in the presence of glipizide showed a small but significant inhibition of their rate of pro(insulin) biosynthesis when expressed as a percentage of the total protein biosynthesis, whereas no effects of α-KIC or diazoxide were detected (Table 1). Total protein biosynthesis, as measured by the amount of [^{3}H]-leucine incorporated into trichloroacetic acid precipitable material, was similar in the different groups of islets examined (data not shown).

Discussion

It is obvious from the present investigation that there is a close parallel between the expression of the islet 64 kDa/GAD antigen and the rate of insulin release. Thus, stimulation of insulin secretion with α-KIC and glipizide effected an increased 64 kDa/GAD expression, whereas inhibition of glucose-stimulated insulin secretion with diazoxide led to a drastically decreased islet antigen expression. Moreover, the present study confirms our previous demonstration of a marked glucose-dependence on the expression of the 64 kDa/GAD antigen in the islets [2]. In contrast to the effects on 64 kDa/GAD expression, the synthesis of the MHC class I proteins was not in-

fluenced by the drugs influencing the rate of insulin secretion. Diazoxide reduced insulin release but did not inhibit the biosynthesis of (pro)insulin. α-KIC and glipizide stimulated insulin release but not the production of (pro)insulin; glipizide may actually inhibit the biosynthesis. This suggests a relationship between the expression of the 64 kDa/GAD autoantigen and the secretion, rather than the biosynthesis, of insulin. In preliminary experiments the chemical amount of the 64 kDa/GAD antigen of islets incubated in different glucose concentrations has been determined, using a Western blot technique. It was found that the chemical amount of the antigen changes in parallel to the expression of antigen, as evidenced with the immunoprecipitation technique used in this study (unpublished data).

The catabolism of glucose and amino acids in Beta cells represents a critical step in the control of insulin secretion. Increases in ATP-content are thought to initiate insulin release by decreasing the activity of the ATP-dependent K^+ channel [5]. Properties of glucose and α-KIC which stimulate secretion are mediated through islet metabolism. The simultaneous stimulation of (pro)insulin biosynthesis is correlated to the energy turnover of the Beta cell. Our stimulatory experiments were performed at a glucose concentration of 5.5 mmol/l, at which near maximal rates of (pro)insulin biosynthesis, but not insulin secretion, are achieved [4]. Sulfonylureas, such as glipizide, bind to ATP-sensitive K^+ channels, or to closely associated proteins, and inhibit K^+ efflux through the channel, ultimately leading to stimulated insulin secretion. Thus, the increased expression of the 64 kDa/GAD autoantigen achieved by glucose, α-KIC and glipizide, suggests that their effect on antigen expression is coupled to a stimulation of insulin release or to an inhibition of the ATP-dependent K^+ channel in the plasma membrane, rather than to a non-specific stimulation of protein biosynthesis. Sulphonamide diazoxide, which inhibits glucose-stimulated insulin release via hyperpolarization of the Beta cell plasma membrane via opening of the ATP-dependent K^+ channels [3], decreased the expression of islet 64 kDa/GAD autoantigen which further supports this notion.

The presence of both GABA and GAD in rat pancreatic islets has previously been described [1]. The localization of GABA in synaptic-like microvesicles and the synthesizing enzyme GAD at their cytoplasmic surfaces [6], as well as the reported glucose-stimulated release of GABA and insulin [7], suggest a linkage between expression of the 64 kDa/GAD autoantigen and insulin release. It has been proposed that upon secretion from the Beta cells GABA regulates the function of Alpha and Delta cells [8]. To our knowledge there are no previous reports on the regulation of GAD in pancreatic islets.

Interest has been focused lately on the functional state of the Beta cell in studies of the pathogenesis of Type 1 diabetes. Pregnant women, with a high demand on their Beta cells and subsequently high production and secretion of insulin, have an increased incidence of Type 1 diabetes [9]. Moreover, in a number of studies prophylactic insulin treatment has been found to decrease the incidence of diabetes in experimental animals. In humans, Shah et al. have shown that extensive Beta cell 'rest', achieved by intensive insulin therapy for 2 weeks at the onset of Type 1 diabetes preserved a significantly higher endogenous insulin production 1 year later [10]. It is tempting to believe that these latter in vivo data could be explained by an inhibition of the functional activity of the Beta cells and their subsequent expression of autoantigens, which ultimately would reduce the risk of islet destruction. In this context it would be of interest to pursue other clinical interventions to minimize islet damage via inhibition of insulin secretion.

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Dr. E. Björk
Department of Internal Medicine
University Hospital
S-751 85 Uppsala
Sweden