Effect of the Bonito Insulin on the Insulin Release from Monolayer Culture of Rat Pancreas

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Synopsis

The effect of bonito insulin on insulin release was examined in the monolayer culture of rat pancreatic β -cells. The β -cells were preincubated for 5 to 20 hr with or without a small dose (100 μ U/ml) of bonito insulin in the medium containing 100 mg% glucose. And then, they were incubated in 300 mg% glucose alone or together with bonito insulin for 5 hr. There was no significant difference between the IRI release from these β -cells with or without bonito insulin. The concentration of bonito insulin was augmented from 100 μ U/ml to 500, 1,000 and 2,000 μ U/ml. A significant inhibitory effect on the glucose-induced insulin release was observed only after the preincubation for 20 hr with 2,000 μ U/ml of bonito insulin.

Previously, we found that the infusion of a small dose of bonito insulin, which was ineffective on the blood sugar levels, resulted in an inhibition of glucose-induced insulin secretion in man and dogs (Ohgawara *et al.*, 1973 and 1974), but more than 20 hr of insulin infusion was necessary to inhibit the glucose induced insulin secretion. It still remains to be established whether a direct or indirect feedback mechanism is responsible for this inhibition. Evidence for a direct effect of insulin on insulin release was reported in several studies (Chu and Gooder, 1968; Hahn and Michael, 1971; Iversen and Miles, 1971; Sodoyez *et al.*, 1969; Ammon *et al.*, 1973). On the other hand, some investigators found no inhibition of insulin secretion by insulin (Grodsky *et al.*, 1968; Curry *et al.*, 1968; Malaisse *et al.*, 1967; Sando *et al.*, 1970).

The present studies were undertaken, therefore, to determine the effect of exo-

genous insulin on insulin release from the monolayer culture of rat pancreatic cells.

Materials and Methods

The procedures used to prepare the monolayer cultured cell of pancreas were based on the method described by Lambert *et al.* (Lambert *et al.*, 1974). Pancreases were removed from newborn rats aged 3 to 5 days and cut into small pieces. Then these fragments were dissociated in a enzyme solution containing 0.25% trypsin (1:250, Gifco) and 0.01% collagenase (Type DLS, Worthington Biochemical Corp.) at 37°C with stirring. This procedure was repeated until complete digestion of the tissue. The dissociated cells were collected by centrifugation and the pellet of cells was suspended in "culture medium" (TC 199 Earle's solution, Flow Lab. Inc. U.S.A., supplemented with fetal calf serum, Flow Lab. Inc. and 300 mg/100 ml of glucose). Aliquots were distributed in plastic culture dishes (Petri type, Falcon Plastic), and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The pancreatic cells were grown for 4 days in the culture medium indicated above. At the end of this 4-day period, they were incubated for 2 hr with the control medium that was consisted of TC 199, 100 mg/100 ml glucose, and 10% fetal calf serum (pre-preincubation).

In order to determine the effect of bonito insulin on insulin release from cultured β -cells, seven types of experiment were carried out (Fig. 1). The β -cells were incubated for 5 hr after 2 hr pre-preincubation with "control medium" with or without $100 \mu\text{U/ml}$ of bonito insulin (Group I). The same type of experiment was carried out with "culture medium" (Group II). From Group III to VI, the cultured cells were preincubated in control medium for 5, 10, 15, 20 hr with or without $100 \mu\text{U/ml}$ of bonito insulin after 2 hr pre-preincubation. The cells were washed twice with 1 ml of corresponding medium, then incubated for 5 hr with "culture medium" with or without $100 \mu\text{U/ml}$ of bonito insulin. In the group, the cells were preincubated in the control medium for 20 hr with or without 500, 1,000 or 2,000 $\mu\text{U/ml}$ of bonito insulin. Incubation was carried out in the culture medium for 5 hr containing the same concentrations of bonito insulin which were present in preincubation. After the incubation, each petri dish was extracted with acid ethanol (Scott and Fihers, 1938) to determine the insulin content. Since there were some variation of the number of β -cells from petri to petri, insulin content of each dish was used as an index of the amount of β -cells in that dish. The IRI release could be expressed as μU released in the incubation medium per mU content in the extract. Each aliquot of incubation medium was stored at -20°C until assay and was analyzed for insulin content by radioimmunoassay by the method of Morgan and Lazarow (Morgan and Lazarow, 1963) using purified rat insulin as a standard. IRI in the acid ethanol was directly measured after appropriate dilution with buffer used for radioimmunoassay.

The system of radioimmunoassay used here was the same as that described in the previous report (Ohgawara, 1974). The cross-reactivity of bonito insulin with rat insulin in our assay system is less than 5%, therefore that is almost negligible.

Results

Morphology of the cultured β -cells did not alter their appearance throughout the experiment. At the end of the experiment, the contents of some of these petri dishes were fixed by Bouin's solution and stained with aldehyde-fuchsin. In most of the clusters, aldehyde-fuchsin-positive cells were found presumably β -cells. Table 1 summarizes the results of experiments studying the effect of glucose alone or together with bonito insulin on IRI release from monolayer cultured pancreatic cells.

The IRI concentration of the media after incubation ranged from 1000 to 5000 $\mu\text{U/ml}$. The IRI release with 100 mg/100 ml glucose was $11.9 \pm 0.7 \mu\text{U/mU}$ per dish. Increasing the glucose concentration to 300 mg/100 ml, IRI release was markedly stimulated during the 5-hr incubation period ($33.5 \pm 4.6 \mu\text{U/mU}$ per dish) ($P < 0.001$).

Fig. 1. Schematic outline of experimental protocol.

The addition of 100 μ U/ml of bonito insulin to the preincubation and incubation medium did not inhibit IRI output under the condition of 100 mg/100 ml glucose and/or 300 mg/100 ml glucose (Table 1).

The duration of preincubation was prolonged from 5 hr to 20 hr, and no inhibitory effect on insulin release appeared by the addition of 100 μ U/ml bonito insulin (Table 1). Finally, the concentrations of bonito insulin were elevated to 500, 1,000 and 2,000 μ U/ml (Group VII), and the significant inhibition of insulin release appeared only in the presence of 2,000 μ U/ml of bonito insulin ($P < 0.01$).

Discussion

Previously, we found that the systemic infusion of a small dose of exogenous bonito insulin resulted in an inhibition of glucose-induced insulin secretion in man and in dogs. However, prolonged insulin infusion was necessary to obtain a signifi-

cant inhibition of glucose-induced insulin release (Ohgawara *et al.*, 1973 and 1974). The inhibition of insulin release was also observed in the *in vitro* incubation with cultured pancreatic β -cells only after preincubation for 20 hr with bonito insulin of 2,000 μ U/ml. The prolonged incubation with bonito insulin had no effect on the extractable insulin in cultured cells.

This result may support the possibility of the existence of a direct feedback mechanism of insulin on insulin release. From this viewpoint, our result is in keeping with some of the reports (Hahn, 1971; Iversen, 1971; Ammon *et al.*, 1973; Sodoyez *et al.*, 1969). While Grodsky (1968) reported that the inhibitory effect of insulin on insulin secretion could not be detected in the isolated rat pancreas perfused with rat insulin. He used rather small amount of rat insulin (100 μ U/ml) and perfused for a short period (30 min); therefore the inhibitory effect of rat insulin might not have appeared in his experiment. The necessity of prolonged exposure of the β -cells to fairly

Group No.	Glucose concentration (mg/100ml)	bonito Insulin (μ U/ml)	(n)	IRI release (μ U/mU $\cdot$ 5 hr $\cdot$ dish) mean $\pm$ SEM	P value
I	100	—	9	11.9 $\pm$ 0.7	N.S.
		100	9	11.3 $\pm$ 2.0	
II	300	—	10	33.5 $\pm$ 4.6	N.S.
		100	10	26.4 $\pm$ 4.2	
III	300	—	9	54.8 $\pm$ 7.5	N.S.
		100	9	50.9 $\pm$ 5.9	
IV	300	—	10	61.0 $\pm$ 6.3	N.S.
		100	10	64.2 $\pm$ 7.4	
V	300	—	10	46.0 $\pm$ 4.3	N.S.
		100	10	41.1 $\pm$ 6.5	
VI	300	—	10	57.1 $\pm$ 6.0	N.S.
		100	10	56.8 $\pm$ 5.4	
VII	300	500	10	41.3 $\pm$ 8.1	N.S.
		1000	10	43.6 $\pm$ 5.7	N.S.
		2000	10	38.3 $\pm$ 1.0	<0.01

Table 1. The IRI release by cultured rat pancreatic B-cells. See text for details.

Table 1. The IRI release by cultured rat pancreatic β -cells. Details are described in the text.

high dose of insulin might suggest that some metabolic changes in β -cell were important for the inhibition of insulin release.

In contrast to our previous experiment *in vivo*, the experiment presented here indicates that the relatively high concentration of bonito insulin was necessary to inhibit the glucose-stimulated insulin release. This discrepancy between the *in vivo* and *in vitro* experiment could be explained as follows; firstly, the non-significant blood sugar change by bonito insulin administration could have some effect on insulin release *in vivo*; secondly the extracellular environment of the cultured cell might be quite different from that of cells in the tissue, and such a micro-environment of β -cells especially in insulin concentration might have some effect on the sensitivity of β -cells to insulin. There is another possibility which cannot be ruled out that a high concentration of bonito insulin could have some protective effect and decrease the leakage of insulin from islet cells during the prolonged incubation.

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