Insulin and Multiplication Stimulating Activity (an Insulin-like Growth Factor) Stimulate Islet β-Cell Replication in Neonatal Rat Pancreatic Monolayer Cultures

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SUMMARY

A possible role for insulin in stimulating islet β-cell replication was examined in neonatal rat pancreatic monolayer cultures. Addition of insulin to serum-free medium increased the mitotic index and stimulated dose-dependent increases in [^{3}H]-thymidine incorporation in nuclei of islet β-cells in aldehyde-thionine-stained autoradiographs. The effects of insulin were not associated with any significant changes in glucagon or somatostatin levels in the culture media. Multiplication stimulating activity (MSA), an insulin-like growth factor, was about 100-fold more potent than insulin: 3 ng/ml MSA stimulated a half-maximal increase in thymidine labeling of β-cells (+63%, $P < 0.005$), whereas 300 ng/ml insulin was required for a similar effect. The maximal effects of insulin and MSA were similar, and the combination of maximal stimulatory concentrations of MSA (30 ng/ml) and insulin (3000 ng/ml) was not more effective than either substance added alone, suggesting that both peptides act on the same mechanism(s) regulating β-cell replication. Furthermore, an antibody to the insulin receptor did not prevent the stimulatory effects of either insulin or MSA on thymidine labeling of β-cells. These results demonstrate that insulin can stimulate islet β-cell replication directly, possibly through a receptor for MSA or another insulin-like growth factor. DIABETES *31*: 160–164, February 1982.

Recent observations indicate that insulin can stimulate pancreatic islet β-cell growth in vivo. McEvoy and Hegre[1] reported that administration of insulin to diabetic rats implanted with syngeneic fetal pancreases resulted in a threefold greater β-cell mass in some of the pancreatic recipients. Brown et al.[2] recently confirmed these results and demonstrated that, with prolonged insulin treatment, complete reversal of diabetes can be achieved by transplanting a single fetal pancreas in an adult rat. It is not clear from these studies,[1,2] however, whether islet β-cell growth was the result of a direct action of insulin. The present study was undertaken to determine whether insulin can directly stimulate β-cell replication. In view of recent evidence[3] that the growth-promoting activities of insulin in human fibroblasts may be mediated by a receptor for the insulin-like growth factor, multiplication stimulating activity (MSA), we also examined MSA for possible effects on β-cell replication.

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MATERIALS AND METHODS

Preparation of islet cell monolayer cultures. Pancreases were removed aseptically from 3-day-old Lewis inbred rats (LEW/CrlBR, Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts), and dissociated with a solution of trypsin (2 mg/ml) and collagenase (0.2 mg/ml), as previously described.[4,5] The cells were washed and diluted in culture medium 199 with modified Earle's salts, L-glutamine and phenol red (Gibco Laboratories, Grand Island, New York), buffered with 25 mM sodium bicarbonate, and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal calf serum, and 16.7 mM glucose (standard culture medium). About 10^6 cells/ml were transferred into 100-mm plastic culture dishes (Falcon Labware, Div. Becton Dickinson and Co., Oxnard, California) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The primary cultures were decanted into 35-mm dishes after 15 h of incubation and again 5 h later, in order to reduce fibroblast contamination. Three days later, the standard culture medium was changed and fresh medium containing 2 μg/ml iodoacetic acid was added for 5–7 h to kill fibroblasts.[6] Standard culture medium was then replaced and changed each 2–3 days for 7 days, at which time the islet cells had spread out in monolayer clusters. The cultures were washed three times with phosphate-buffered saline, and test and control media additions to the cultures were

begun at this time. We have previously found[5] that approximately 80% of the cells in these cultures are β-cells (aldehyde-thionin positive).

Hormones, growth factors, and antisera. These were added to the cultures as concentrated sterile aqueous solutions. Crystalline bovine insulin was supplied by Dr. R. Chance, Lilly Research Laboratories (Eli Lilly and Co., Indianapolis, Indiana). Multiplication stimulating activity (MSA) was purchased from Collaborative Research, Inc. (Waltham, Massachusetts). Anti-insulin receptor immunoglobulin (Ig)G was a gift from Dr. George L. King (Section on Cellular and Molecular Physiology, Diabetes Branch, National Institutes of Health, Bethesda, Maryland). Control cultures were incubated in medium 199 containing 16.7 mM glucose and 0, 0.1%, 1%, or 10% fetal calf serum without test agents.

Islet β-cell replication studies. After 24 h of incubation in test or control media, the cultures were incubated for 18 h in fresh test or control media containing [methyl-^{3}H]-thymidine (10 μCi/ml, 40–60 Ci/mmol, Amersham Corp., Arlington Heights, Illinois). The cultures were then washed three times with phosphate-buffered saline, pH 7.4, fixed in Bouin's solution, and stained with aldehyde-thionine.[7] Autoradiographs were prepared[8] with Ilford L-4 photographic emulsion (Polysciences, Inc., Warrington, Pennsylvania). The percentage of β-cells with [^{3}H]thymidine-labeled nuclei was determined by counting at least 500–1000 aldehyde-thionine-stained β-cells in each dish. To determine that the [^{3}H]-thymidine incorporation index represented β-cell replication, N-desacetyl-N-methylcolchicine (Colcemid, 1 μg/ml; Gibco Laboratories) was added to the cultures for 18 h, in place of [^{3}H]-thymidine, in order to arrest dividing cells in metaphase. The cells were then swollen in hypotonic solution (0.075 M KCl) to improve identification of mitotic figures,[9] fixed, stained, and the percentage of β-cells in metaphase was determined by counting at least 500–1000 β-cells in each dish.

Islet hormone release into the culture media. After 24 h of incubation in test or control media, the cultures were incubated for an additional 18 h in fresh test or control media. The 18 h media were collected, centrifuged at 250 × *g* for 10 min to remove floating cells, and the supernatants were stored at −20°C for subsequent assay. Insulin was measured by a charcoal separation method of radioimmunoassay,[10] using purified rat insulin standards (Lilly Research Laboratories). Glucagon was measured by a radioimmunoassay[11] using antiserum 30K (gift from Dr. R. H. Unger, Dallas, Texas). Somatostatin was measured by a previously described radioimmunoassay with a mean sensitivity of 1 pg.[12]

Statistical calculations. Statistical significance was analyzed by Student's *t* test for paired or unpaired data.

RESULTS

The morphologic appearance of the islet cultures and the effects of insulin on [^{3}H]-thymidine labeling of β-cells in serum-free medium are shown in Figure 1. It is apparent that β-cell labeling is increased by insulin. The magnitude of the

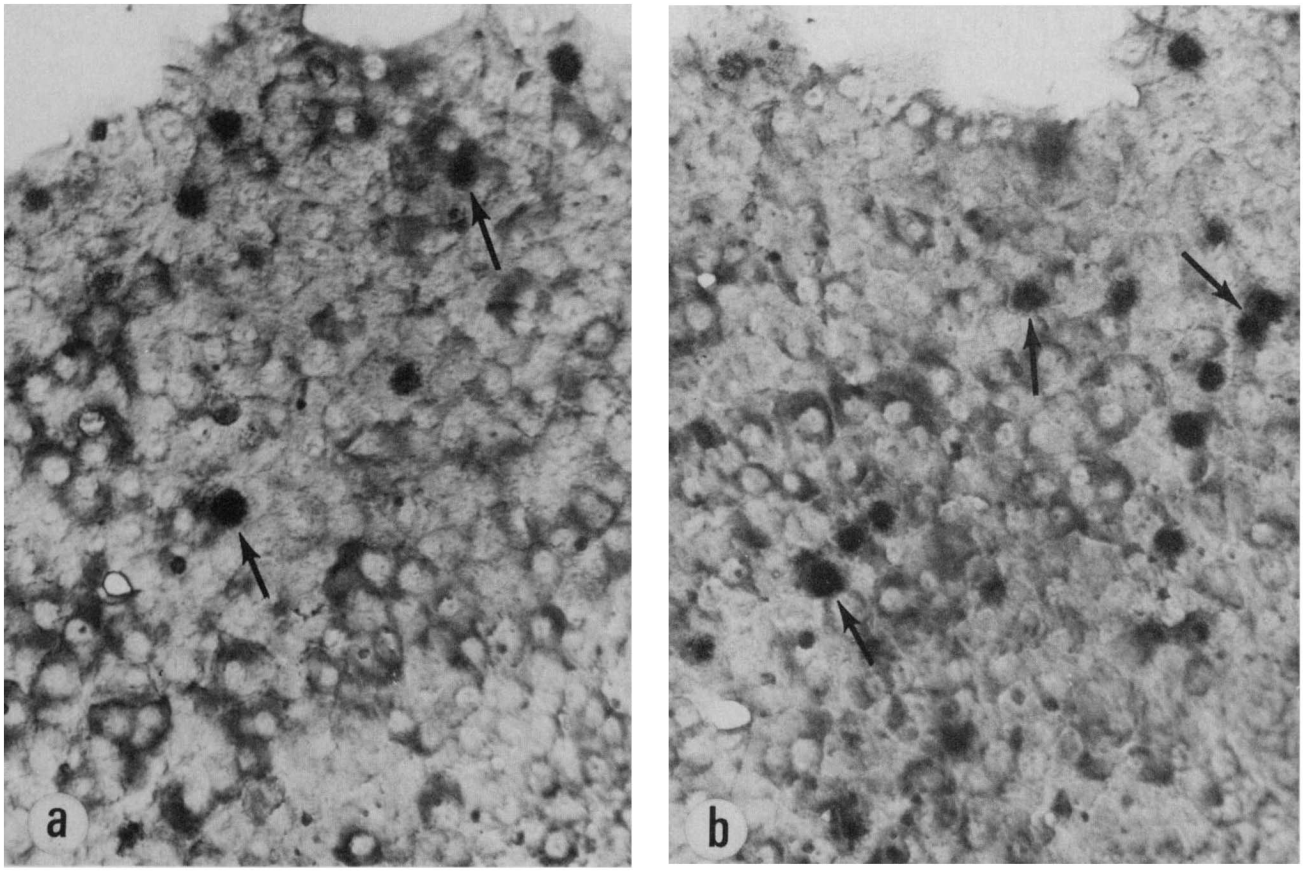


FIGURE 1. Radioautographs of islet cells cultured for 2 days in serum-free medium (a) without and (b) with 10 μg/ml insulin, and labeled with [^{3}H]-thymidine for the last 18 h of the 2-day culture. β-cells are identified by the presence of dark staining (aldehyde-thionine positive) cytoplasmic granulation. Some of the labeled β-cells are indicated by arrows (×400).

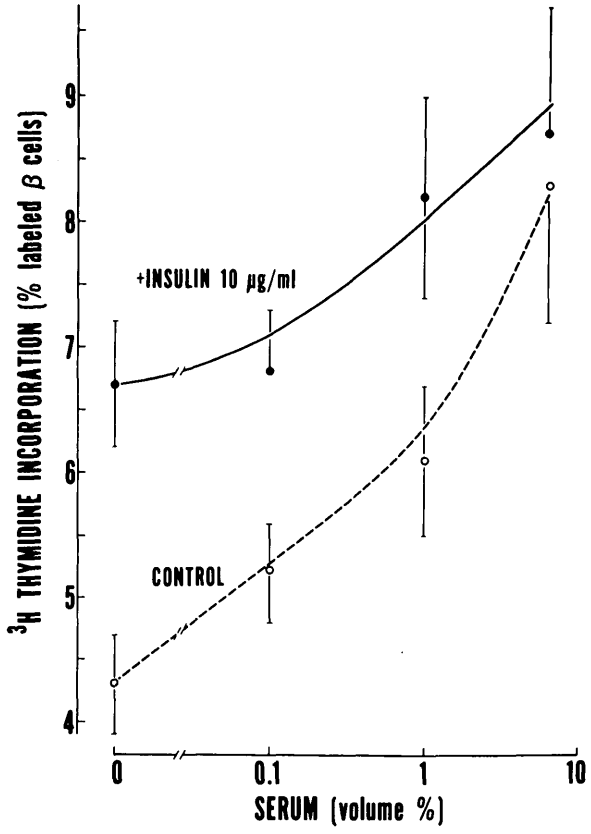


FIGURE 2. Effects of insulin (10 μg/ml) on [^{3}H]-thymidine incorporation in β-cells cultured for 2 days in medium 199 containing 16.7 mM glucose, and either serum-free or supplemented with 0.1%, 1%, or 10% serum. Mean values ± SEM are shown for three experiments (three dishes per condition).

effects are shown in Figure 2. Addition of insulin (10 μg/ml) to serum-free medium significantly increased [^{3}H]-thymidine incorporation in β-cells (+56%, $P < 0.01$, for paired data). The effect of insulin was not as great in the presence of 0.1% serum (+31%, $P < 0.05$) or 1% serum (+34%, $P < 0.02$), and not significant in medium supplemented with 10% serum. Table 1 shows that the stimulatory effects of insulin

TABLE 1
Effects of insulin on islet β-cell replication

Additions to culture medium*			
Serum (vol. %)	Insulin (μg/ml)	[^{3}H]-thymidine incorporation† (% labeled β-cells)	Mitotic index† (% β-cells in mitosis)
0	0	4.8 ± 0.8	2.1 ± 0.1
0	10	7.6 ± 1.2 (+58 ± 2%)‡	3.4 ± 0.6 (+56% ± 19%)‡
10	0	9.5 ± 1.8 (+100 ± 38%)	5.4 ± 0.6 (+153 ± 15%)
10	10	11.3 ± 1.8 (+142 ± 45%)	6.1 ± 0.2 (+191 ± 26%)

* Cultures were incubated for 24 h in medium containing 16.7 mM glucose and the additions indicated, then for 18 h in fresh test or control media containing either [^{3}H]-thymidine (10 μCi/ml) to measure β-cell labeling or Colcemid (1 μg/ml) to measure β-cell mitosis.
† Mean values ± SEM for three experiments (four dishes per condition).
‡ Percent change from the mean value in the corresponding control culture in serum-free medium.

TABLE 2
Insulin, glucagon, and somatostatin concentrations in culture media

Additions to culture medium*		Hormone concentrations in media†		
Serum (vol. %)	Insulin (ng/ml)	Insulin‡ (ng/ml)	Glucagon (pg/ml)	Somatostatin (pg/ml)
0	0	34 ± 4	271 ± 14	42.6 ± 3.0
0	1000	1023 ± 89	273 ± 48	36.8 ± 6.0
10	0	55 ± 4	178 ± 9	64.6 ± 2.4
10	1000	974 ± 57	179 ± 18	72.2 ± 1.2

* Cultures were incubated for 24 h in medium containing 16.7 mM glucose and the additions indicated, then for 18 h in fresh test or control media that were collected and assayed for hormone concentrations.
† Mean values ± SEM for 10 dishes.
‡ Samples were diluted with insulin assay buffer to read between 0.125 and 0.5 ng/ml on a standard curve constructed with rat insulin, since bovine insulin (added to culture medium) and rat insulin (released into medium from cultures) reacted identically over this range of concentrations in the insulin radioimmunoassay used.

and serum, on [^{3}H]-thymidine incorporation in β-cells were accompanied by similar increases in the β-cell mitotic indices, indicating that changes in thymidine labeling reflected effects on mitosis. Table 2 shows that the stimulatory effects of insulin on thymidine labeling of β-cells were not accompanied by any significant changes in glucagon or somatostatin levels in the culture media. Thus, it does not appear that insulin affected β-cell replication by acting on α- or δ-cells in the islet culture.

The dose-dependent effects of insulin on [^{3}H]-thymidine incorporation in β-cells are shown in Figure 3. The minimally effective concentration of insulin was 100 ng/ml (+37%, $P < 0.05$, for paired data), a half-maximal effect was seen with 300 ng/ml (+60%, $P < 0.02$), and a maximal effect was seen with 10,000 ng/ml (+86%, $P < 0.01$). By using MSA in place of insulin, the dose-reponse curve was displaced to the left. Thus, addition of as little as 1 ng/ml MSA to serum-free medium significantly increased labeling of β-cells (+19%, $P < 0.05$), a half-maximal effect was observed with 3 ng/ml (+63%, $P < 0.005$), and a maximal effect with 30 ng/ml (+94%, $P < 0.01$). Figure 3 also shows that the combination of maximal stimulatory concentrations of MSA and insulin was not more effective than either agent added alone

TABLE 3
Effects of MSA and insulin on thymidine incorporation in β-cells

Additions to culture medium*		
MSA (ng/ml)	Insulin (ng/ml)	[^{3}H]-thymidine incorporation† (% labeled β-cells)
0	0	5.8 ± 0.3
30	0	10.3 ± 1.0‡
0	3000	10.7 ± 0.6§
30	3000	9.5 ± 0.2§

* Cultures were incubated for 24 h in serum-free medium containing 16.7 mM glucose and the additions indicated, then for 18 h in fresh test or control media containing [^{3}H]-thymidine (10 μCi/ml).
† Mean values ± SEM for five dishes.
‡ $P < 0.02$, compared with serum-free medium.
§ $P < 0.001$.

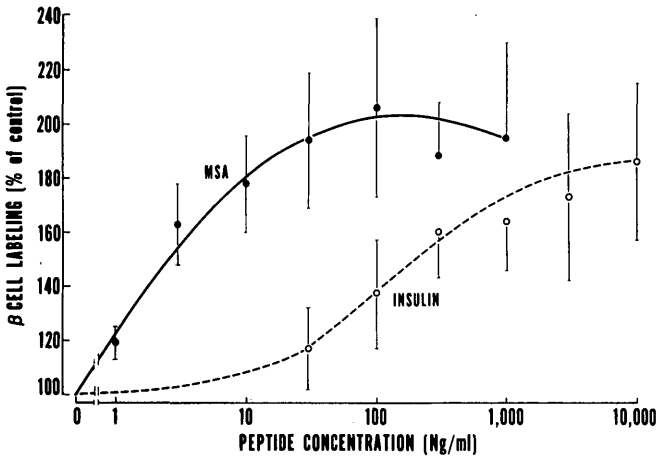


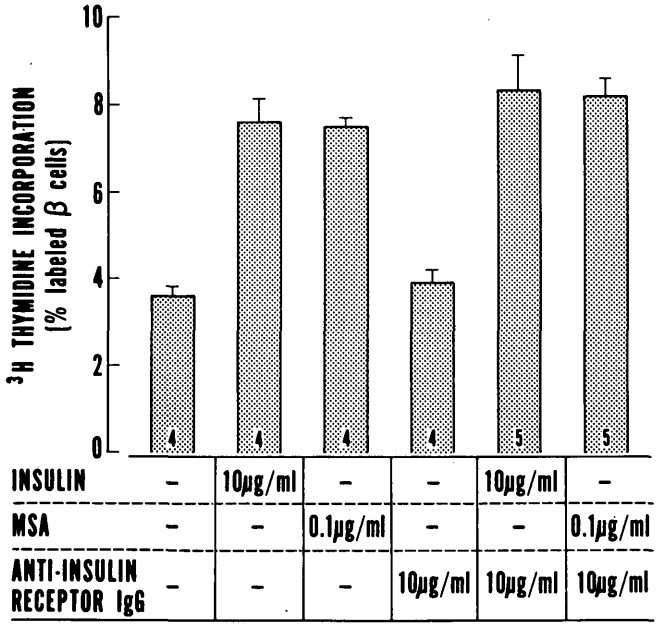
FIGURE 3. Dose-dependent effects of insulin and MSA on [^{3}H]-thymidine incorporation in β-cells cultured for 2 days in serum-free medium. Mean values ± SEM for five experiments (four dishes per condition) are shown as percentages of the value in the corresponding control culture in serum-free medium.

to serum-free medium, suggesting that both peptides acted on the same mechanism(s) regulating β-cell replication. The stimulatory effects of insulin and MSA on β-cell replication did not appear to be mediated through the insulin receptor, since a concentration of anti-insulin receptor IgG (10 μg/ml) with fairly selective binding to the insulin receptor and capable of exerting agonist effects[3] did not stimulate [^{3}H]-thymidine incorporation in β-cells, and did not prevent the stimulatory effects of either insulin (10 μg/ml)or MSA (0.1 μg/ml) on [^{3}H]-thymidine incorporation in β-cells (Figure 4).

DISCUSSION

In the last few years, it has been demonstrated that normal differentiated mammalian cells can survive and grow in serum-free medium supplemented with nanogram quantities of purified growth factors in various combinations with hormones.[13–15] Insulin, in particular, has been found to be stimulatory for the growth of most, if not all, cell types examined.[16] The present study extends these observations to cultured pancreatic islet β-cells. Thus, insulin stimulated significant increases in [^{3}H]-thymidine incorporation, as well as increases in mitotic activity, in β-cells of the neonatal rat pancreas maintained in monolayer culture. Since the effects of insulin were not associated with any significant changes in glucagon or somatostatin levels in the culture media, it appears that insulin was acting directly on β-cells in the islet cultures.

FIGURE 4. Effects of insulin (10 μg/ml), MSA (0.1 μg/ml), and anti-insulin receptor immunoglobulin (Ig)G (10 μg/ml) on [^{3}H]-thymidine incorporation in β-cells cultured for 2 days in serum-free medium. Mean values ± SEM are shown for the number of dishes indicated at the base of each column.



The concentrations of insulin that stimulated significant increases in [^{3}H]-thymidine incorporation in β-cells were quite high (>100 ng/ml), and this is usually the case when the effects of insulin on cell growth in tissue culture are examined.[13–16] Since β-cells themselves produce insulin, however, intra-islet concentrations of insulin may be much higher (~100–1000 ng/ml)[17] than in peripheral blood (<10 ng/ml), and similar to the concentrations we found to stimulate β-cell replication (>100 ng/ml).

Verspohl and Ammon[18] have recently provided evidence for the presence of insulin receptors in rat islets. The "high affinity" insulin binding sites exhibited a dissociation constant of approximately 0.5 nM (~3 ng/ml), and this corresponds to the mean effective dose for certain "metabolic" effects of insulin on islet cell function, i.e., inhibition of insulin secretion[19,20] and decrease in the pentosephosphate shunt activity.[20] There also existed "low affinity" insulin binding sites in the islets,[18] with a dissociation constant of approximately 44 nM (~250 ng/ml). This corresponds to the concentration of insulin that stimulated half-maximal increases in [^{3}H]-thymidine incorporation in β-cells in the present study (Figure 3). This suggests that insulin stimulated β-cell replication through a "low affinity" insulin receptor on the β-cell. However, since an anti-insulin receptor antibody did not stimulate [^{3}H]-thymidine incorporation in β-cells and did not inhibit insulin-stimulated [^{3}H]-thymidine incorporation in β-cells, it did not appear that insulin was acting through the "classic" insulin receptor. The findings that (1) the insulin-like growth factor, MSA, was a potent stimulator of β-cell replication, (2) the maximal effects of insulin and MSA were similar, and (3) the combination of maximal stimulatory concentrations of MSA and insulin was not more effective than either substance added alone, suggest that both peptides act on the same mechanism(s) regulating β-cell replication. Indeed, King et al.[3] recently provided direct evidence that insulin receptors may mediate the metabolic effects of insulin and MSA in human fibroblasts, whereas the growth-promoting action of both peptides in these cells may be mediated by a receptor(s) for MSA or other insulin-like growth factors. Similarly, direct evidence for separate receptors on β-cells for metabolic and growth-promoting activities of insulin (and MSA) will require examination of the binding kinetics of radiolabeled insulin and MSA in these islet cell cultures.

In conclusion, we have demonstrated that insulin can stimulate β-cell replication directly, at least in neonatal rat islet cells in monolayer culture. Taken together with the stimulatory effects of insulin on islet β-cell growth in vivo,[1,2] the present results may explain why early, intensive insulin

therapy may increase β-cell reserve and decrease subsequent insulin requirements in subjects with insulin-dependent diabetes mellitus.[21]

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