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Effects of Growth Hormone on the *in Vitro* Maturation of Fetal Islets[1] (41913)

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Abstract. To study the effects of growth hormone (GH) on the *in vitro* maturation of fetal islets, the fetal islets were cultured for 7 days in RPMI 1640 containing 10% fetal bovine serum and 11.1 m*M* glucose with or without GH. Culture with 1 μg/ml of bovine GH increased the DNA content of the islets and [^{3}H]thymidine incorporation into DNA confirming results of other investigators. In addition, however, the insulin secretory dynamics and ultrastructural morphometrics were investigated. It was found that GH-treated islets demonstrated increased insulin release during acute glucose stimulation when expressed as microunits per islet per minute. However, when insulin release during acute glucose stimulation was expressed as microunits per microgram of DNA per minute to compensate for the increased DNA content of GH-treated islets, no change in insulin release was observed compared to control islets. When GH-treated islets were perifused with a linear glucose gradient, the insulin secretory response was suppressed as indicated by changes in the threshold level, plateau level, and half-maximal response. Ultrastructural morphometric data showed that the average β-cell volume in control and GH-treated islets was the same, eliminating the possibility that β-cell hypertrophy occurred. Similarly, the nuclear volumes of the β cells in control and GH-treated islets remained unchanged. This finding coupled with the observed increased DNA content and [^{3}H]thymidine incorporation suggests that GH functions by increasing cell multiplication within the islets and not by inducing polyploidy. Finally, the volumes of cytoplasmic organelles in control and GH-treated islets were the same indicating that cytodifferentiation did not occur.

The role that glucose plays in the *in vitro* maturation of 21.5-day-old fetal islets has been reported (1, 2). In addition, we have reported that *in vitro* exposure to 11.1 m*M* glucose for 7 days affects the maturation of fetal islets and that this involves increased insulin secretion and content, polyploidy, and hypertrophy of β cells (3). Although insulin secretion was increased by exposure to 11.1 m*M* glucose for 7 days, full secretory competence as compared to adult islets on a quantitative basis was not achieved. Consequently, exposure to other factors must be involved for complete *in vitro* development of fetal islets. Because of the long recognized relationship between growth hormone (GH) and the islets of Langerhans (4), we chose to study the effects of GH on the maturation of fetal islets in culture. The relationship between GH and islets has been supported by a number of studies in hypophysectomized rats (5, 6), in ateliotic dwarfs (7), and in patients with panhypopituitarism (8). In addition, studies of rats bearing GH-secreting tumors demonstrate the occurrence of increased islet mass, insulin content, and biosynthetic and secretory activity of islet tissue (9–11). To investigate whether GH affects islets directly, a number of *in vitro* studies have been performed. In recent studies, it has been shown that GH significantly increases cellular replication or DNA synthesis in the RIN-r islet cell line (12), neonatal islet monolayer cultures (13), and isolated adult and newborn pancreatic islets (14). Pierluissi *et al.* (15) reported that the insulin secretory response to acute glucose stimulation of adult islets cultured for 4 days with 10 μg/ml bGH was significantly elevated in comparison to untreated control islets. However, they expressed insulin release as microunits per islet per minute and even though islet size was

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matched using an eyepiece micrometer their data did not correct for the fact that GH increases cellular replication as indicated above. In contrast, Kawabe and Morgan (16) reported that the insulin secretory response to acute glucose stimulation of adult islets exposed to 1 or 10 μg/ml of bGH for 120 min was significantly inhibited. Our study sheds further light on the *in vitro* maturation of fetal islets by investigating the role of GH.

Materials and Methods. *Animals.* Sprague–Dawley pregnant rats, mated at 41–57 days of age, were obtained from Harlan Industries (Madison, Wisc.) at least 1 week before each experiment. The time of mating was assessed with an accuracy of ±12 hr and was considered Day 0 of pregnancy. In this strain, the length of gestation is 22–22.5 days, and all experiments with fetal rats were performed on Day 21.5 of pregnancy. For experiments with adult islets, male Sprague–Dawley rats weighing 200–250 g were purchased. All animals were allowed free access to standard laboratory chow and drinking water until they were killed.

Tissue culture of fetal islets. Tissue culture of fetal islets was performed as described by Hellerstrom *et al.* (2). Pancreata were partially digested with collagenase (Sigma, St. Louis, Mo.) and cultured in RPMI 1640 (Gibco, Grand Island, N.Y.) containing 11.1 m*M* glucose, 10% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, Va.), 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 100 U penicillin/ml, and 0.1 mg streptomycin/ml (Gibco). For experimental groups, 1 μg/ml of bovine GH (NIH-GH-B18, kindly provided by the Hormone Distribution Office of the National Pituitary Agency, NIAMDD) was added to the medium. The medium was changed daily.

DNA content and [^{3}H]thymidine incorporation. After 7 days in culture, 100 fetal islets were removed from the culture dish and placed into a new culture dish with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass., sp act 6.7 Ci/mmol) per milliliter of RPMI 1640 tissue culture medium with or without 1 μg/ml of GH. The islets were labeled with [^{3}H]thymidine for 24 hr. After labeling, the islets were washed three times with nonradioactive tissue culture medium followed by three washes with Hanks' balanced salt solution (HBSS, Gibco). Under a Wild dark-field stereomicroscope, 30–40 islets were picked with a finely drawn pipet and placed in a 12 × 75-mm plastic test tube. The excess HBSS was drawn off with a pipet and replaced with a 150 μl of distilled water. The islets were then disrupted for 3 min using a sonicator (Heat System-Ultrasonic Inc., Model W-225R). Twenty-five microliters of the sonicate was removed and filtered through glass-fiber disks (GF/A 2.5 cm; Whatman, England). After the disks were thoroughly dried they were placed in 10 ml of Scintiverse (Fisher, Raleigh, N.C.) for scintillation counting of the tritium. The remaining 125 μl of sonicate was assayed for DNA content (17). The incorporation of [^{3}H]thymidine was expressed as (disintegrations per minute × 10^{-3}) per microgram of islet DNA.

Perifusion of fetal islets. On Day 8 of culture, the islets were gently blown free from the culture dish and washed three times with Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4, modified to contain 0.5 m*M* HPO_4^{2-}, 1.7 m*M* Ca^{2+}, 2.8 m*M* glucose, 0.5 mg/ml bovine serum albumin (BSA fatty acid free, Sigma), and 10 m*M* Hepes. Groups of 40 islets were placed on polyester membranes (5-μm pore size, 13-mm diameter, Nuclepore, Pleasanton, Calif.) and perifused in chambers (Millipore, Bedford, Mass.). Islets were selected by a randomized sampling procedure. From the pool of fetal islets in each treatment group, we picked 10 islets at a time and repeated this procedure (10 islets/pick) until a total of 40 islets were on the polyester membrane. After 30 min of stabilizing perifusion with KRB containing 2.8 m*M* glucose, the islets were stimulated by either a constant, square wave of 16.7 m*M* glucose or a linear glucose gradient (from 2.8 to 20 m*M*). Linearity of the glucose gradients was determined by assaying an aliquot from each fraction collected using a glucose analyzer (Technicon, Tarrytown, N.Y.). The perifusion was performed in the absence of GH. Flow rate in perifusion chambers was 1 ml/min, and the gas phase was 95% O_2 and 5% CO_2. Perifusate samples were collected every minute or every 5 min and frozen at −20°C until insulin assay. Insulin concentrations were determined by radioimmunoassay (18) using a rat insulin

standard (Novo Research Institute). After perifusion, the islets that were placed on the filter paper and perifused were collected for the DNA determination.

Perifusion of adult islets. Adult islets were isolated by collagenase digestion (19), and collected under a microscope using a finely drawn pipet. About 700 islets were incubated for 120 min in RPMI 1640 containing 2.8 m*M* glucose and 1 mg/ml BSA. After the incubation, groups of 40 adult islets were perifused by the same method as the fetal islets.

Insulin content of islets. After 7 days in culture, the islets were washed vigorously three times with HBSS to remove any extracellular insulin. The islets were then placed in a Petri dish under a Wild dark-field microscope and 10 islets were picked with a finely drawn pipet and placed in a 12 × 75-mm plastic test tube. The excess HBSS was removed and replaced with 1 ml of acid ethanol (95% ethanol, H_2O, and 12 *N* HCl in volume proportions of 79:21:1.5). The islets were then sonically disrupted as mentioned above. The sonicate was appropriately diluted with 0.13 *M* borate buffer, pH 7.5, and assayed for insulin by radioimmunoassay.

Morphometric analysis of β cells. Morphometric analysis was carried out as previously described (3). Briefly, groups of 20 islets were flooded with ice cold 2% paraformaldehyde, 2.5% glutaraldehyde in 75 m*M* cacodylate buffer and fixed for 1 hr. After routine osmication, en bloc staining, dehydration, and infiltration, groups of five islets were layered onto fresh Araldite 502 in BEEM capsules and pelleted by centrifugation. Pale gold sections of islets were mounted on 200-mesh grids and photographed with a Philips 201 electron microscope using 35-mm film. A calibration grid (2160 lines/mm) was photographed at the sampling magnifications for each roll of film. Three grids of sections were collected from each of the blocks with embedded islets. One section which contained islet tissue and covered an entire grid square was randomly selected from each grid for analysis. From each chosen section, five nonoverlapping low magnification micrographs were taken at 1000×. These five micrographs would usually be sufficient to photograph the whole section; however, if the section extended into adjacent grid squares, additional nonoverlapping low magnification micrographs were taken until the entire section was sampled. Each section would usually include two to three islets within the viewing field. Therefore there were usually 5–8 micrographs from each grid, 15–24 micrographs from each block, and 45–72 micrographs from each experimental group. The low magnification micrographs were enlarged to 9000× and quantified using an acetate overlay of a 1-cm^2 lattice. The intersect points falling on the β cells, their nuclei, and the remaining islet tissue were counted. The volume densities and numerical densities, and ultimately the cell volume of the "average" β cell per group, were calculated using a revised computer program of Poole and Costoff (20). This program is based on the relation which has been well documented by Weibel and Bolender (21),

$$N_v = \frac{1}{\beta} \times \frac{N_a^{3/2}}{V_v^{1/2}},$$

where N_v equals the numerical density, N_a equals numerical density of profiles on area, V_v equals volume density, and β is a dimensionless relationship between the volume and its means cross-sectional area. In these studies since the axial ratio of the β-cell nuclei was found to be less than 1.5, a "β" coefficient of 1.38 was used in the above equation.

Organelle volumes were estimated using higher magnification micrographs (3300×) in which one β-cell profile was photographed from each area chosen for a low magnification micrograph. Therefore there were 5–8 β cells analyzed from each grid, 15–24 β cells analyzed from each block, and 45–72 β cells analyzed from each experimental group. These micrographs were enlarged to 29,700× and analyzed using a 1-cm multipurpose test system patterned after Weibel (21). The volume density of the organelles was determined by counting the number of test points falling on the respective organelles and the β-cell profiles. The organelle volumes were determined by multiplying their respective volume densities by the calculated "average" β-cell volume. A value for the cell and organelle volumes was determined for each block, and a mean value was obtained from all of the blocks within an experimental group.

Two points regarding the identification of organelles are noteworthy. The Golgi apparatus included stacked cisternae, trans-face vesicles, and GERL. Secretory granules were divided into "dark" and "pale" granule populations. Dark granules consisted of an electron-dense core and a clear halo within a membranous sac. Pale granules consisted of a flocculent material that completely filled the membranous sac.

Statistical analysis. Data were analyzed according to Student's t test. A P value less than 0.05 was considered statistically significant.

Results. *DNA content and [^{3}H]thymidine incorporation into DNA.* When fetal islets were cultured for 7 days in the presence or absence of GH, the DNA content ($P < 0.001$) and [^{3}H]thymidine incorporation ($P < 0.02$) increased in the GH-treated islets by approximately 50% when compared to control islets (Table I).

Insulin content. GH increased the insulin content in the fetal islets (Table I). Using the values of insulin content and total insulin released during a 60-min perifusion with acute glucose stimulus, we calculated the released/stored ratio (Table I). Both groups of the fetal islets released the same ratio of insulin, approximately 5.6%.

Insulin release. When fetal islets were cultured in 11.1 mM glucose, insulin release evoked by an acute glucose (16.7 mM) stimulus was biphasic (Fig. 1). When fetal islets were cultured in 11.1 mM glucose with the addition of GH, both phases of insulin release increased when expressed as microunits per islet per minute (Fig. 1), thereby raising the total insulin released during a 60-min stimulation period by 48.5% (Table I). However, since the islets cultured with GH contained more DNA, insulin release was expressed as microunits of insulin per microgram of DNA per minute (Fig. 2). Using this expression, GH had no effect on the maximal rate of secretion or the total amount of insulin released during an acute stimulus.

However, differences in the insulin secretory response were observed during perifusion with a linear 2.8–20 mM glucose gradient. Insulin release from fetal islets cultured in the absence of GH, was triggered at 4.4 ± 0.1 mM glucose and plateaued at 11.6 mM glucose (Fig. 3). However, when islets were cultured in the presence of GH, the threshold increased ($P < 0.001$) to 5.8 ± 0.2 mM glucose and plateaued at 14.6 mM glucose (Fig. 3). In addition, the insulin secretory response from GH-treated islets was significantly delayed at a number of other points (Fig. 3, lowercase letters) during the glucose gradient including the point of half-maximal stimulation (Fig. 3, see X).

Morphometric analysis of β cells. After 7 days of culture, β cells in the islets were morphometrically analyzed at the ultrastructural level (Table II). In control and GH-treated islets, the volume of the individual β cells and nucleus remained the same. In addition, GH had no effect on the volume

TABLE I. INSULIN CONTENT, RELEASE, DNA CONTENT, AND [^{3}H]THYMIDINE INCORPORATION

	11.1 mM Glucose	11.1 mM Glucose + GH	P
Insulin content, A (mU insulin/islet)	1.14 ± 0.10 (n = 29)	1.70 ± 0.12 (n = 39)	<0.001
Total insulin released, B (μU insulin/islet/60 min)	64.1 ± 6.2 (n = 7)	95.2 ± 8.0 (n = 8)	<0.01
Estimated ratio of released/stored insulin B/A (%)	5.62	5.60	
(ng DNA/islet)	11.1 ± 6.5 (n = 14)	16.9 ± 0.93 (n = 14)	<0.001
(dpm × 10^{-3}/μg DNA)	15.0 ± 2.1 (n = 8)	23.2 ± 1.8 (n = 6)	<0.02

Note. All values are means ± SE.

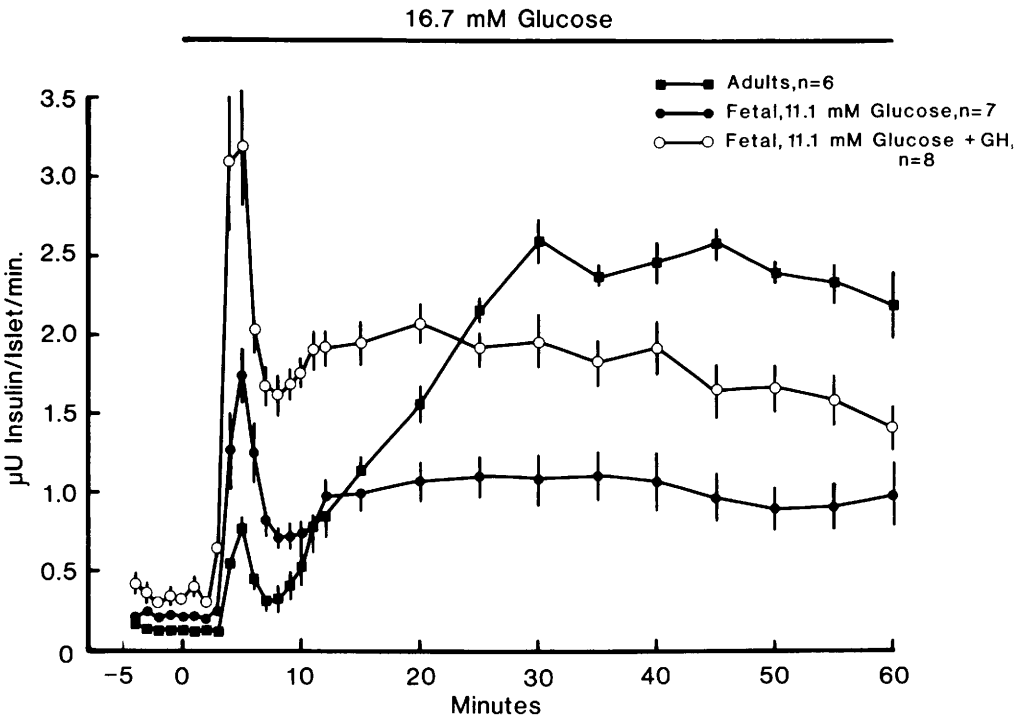

FIG. 1. Effect of GH on the dynamics of insulin release. Fetal islets were cultured for 7 days with or without GH. Groups of 40 islets were perifused for 60 min with a square wave of 16.7 m*M* glucose in the absence of GH. The insulin secretory curve for adult islets is shown only to indicate the relative pattern and quantity of insulin release from fetal islets as compared to adult islets. The values are given in μU insulin/islet/min. Points indicate means ± SE.

of cytoplasmic organelles such as mitochondria, rough endoplasmic reticulum (RER), Golgi, and secretory granules.

Discussion. The present study demonstrates that *in vitro* exposure of fetal islets to GH over a 7-day period results in increased DNA content, [^{3}H]thymidine incorporation, and insulin content. The results concerning DNA content and [^{3}H]thymidine incorporation are compatible with previous studies of Rabinovitch *et al.* (13) and Nielsen (14). In addition, this study expands our knowledge in two important areas.

First of all, we studied the insulin secretory dynamics during acute and gradient glucose stimulation. During acute glucose stimulation with 16.7 m*M* glucose, both the GH-treated and control islets secreted insulin in a similar fashion when expressed as μU insulin/μg DNA/min. This indicates that *in vitro* exposure to GH over a 7 day period does not "prime" or mature the insulin secretory mechanism in fetal islets. Indeed, our data from the linear glucose gradient perifusion indicate that the sensitivity of the insulin secretory mechanism was significantly decreased in the GH-treated islets since the following were altered: (a) threshold level, (b) plateau level, and (c) the half-maximal response. Although we do not know the exact significance of the response to the glucose gradient, we suggest that there may be (a) a suppression of insulin release in GH-treated fetal islets at low glucose concentrations or (b) a suppression of insulin release such as previously reported with short-term GH exposure (120 min) in adult islets (16).

One could raise the possibility that islet size plays a role in the decreased insulin secretory dynamics. Islet size is highly variable with a normal distribution ranging from approximately 100 to 500 μm in diameter. Since a 7-day exposure to GH increases the mitotic activity, the GH-treated islets were

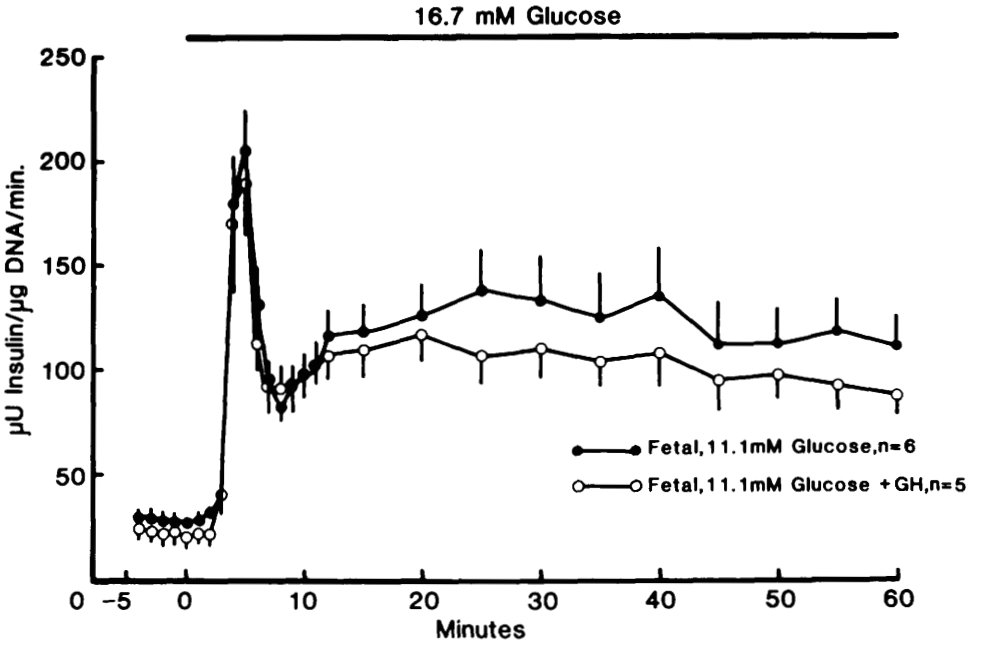

FIG. 2. Effect of GH on the dynamics of insulin release. Fetal islets were cultured for 7 days with or without GH. Groups of 40 islets were perifused for 60 min with a square wave of 16.7 m*M* glucose in the absence of GH. After perifusion, the islets were collected and DNA contents were determined. Insulin release was expressed as μU insulin/μg DNA/min. Points indicate means ± SE.

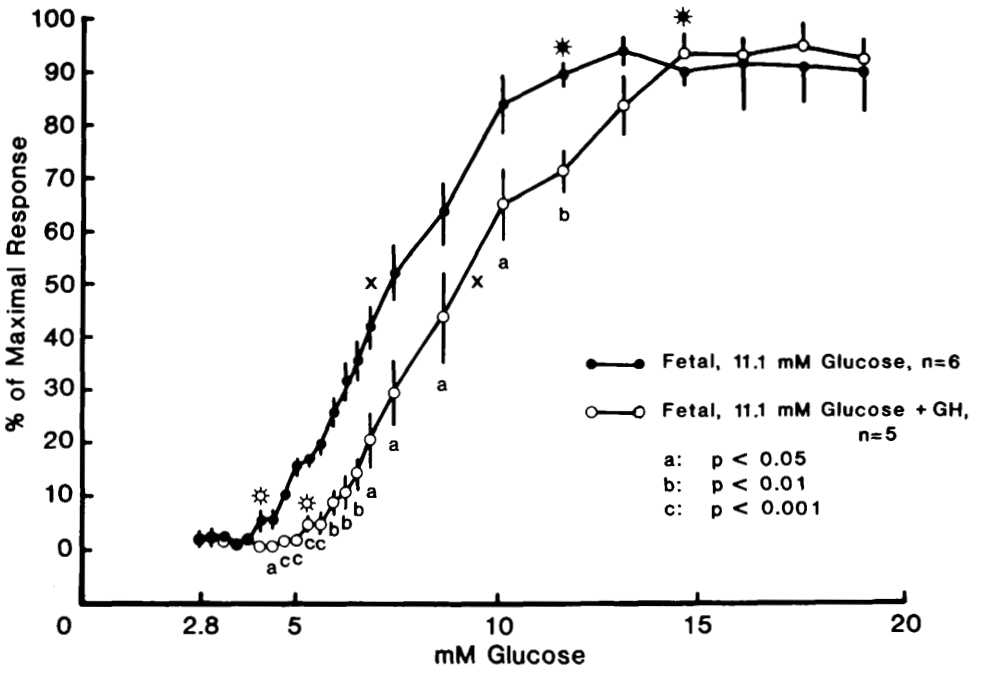

FIG. 3. Effect of GH on insulin release during a linear glucose gradient. The data are plotted as glucose concentration in perifusate versus percentage of maximal insulin response. Threshold point is indicated by open asterisk and plateau level is indicated by black asterisk for both curves. Lowercase letters a, b, c indicate the level of significance for various points during the gradient perifusion. Half-maximal point is indicated by the X. Points indicate means ± SE.

TABLE II. MORPHOMETRIC ANALYSIS OF β CELLS IN ISLETS CULTURED IN THE PRESENCE OR ABSENCE OF GH

	11.1 m*M* Glucose	11.1 m*M* Glucose + GH
β-Cell volume (μm^3)	1027 ± 100 (100)	911 ± 46 (100)
Nucleus	179 ± 42 (16.9 ± 2.7)	149 ± 18 (16.5 ± 1.9)
Mitochondria	56 ± 7 (5.4 ± 0.3)	36 ± 6 (3.8 ± 0.5)
RER[a]	240 ± 37 (23.1 ± 2.7)	212 ± 41 (23.2 ± 3.1)
Golgi apparatus	42 ± 9 (3.9 ± 0.6)	36 ± 4 (4.0 ± 0.3)
Secretory granules	112 ± 12 (11.3 ± 1.2)	104 ± 13 (11.7 ± 1.7)
Pale	34 ± 4 (3.7 ± 0.4)	29 ± 6 (3.3 ± 0.8)
Dark	75 ± 10 (7.5 ± 1.2)	70 ± 10 (7.8 ± 1.1)

Note. All values are means ± SE. Organelle volumes are given in μm^3. Numbers in parentheses indicate percentages of β-cell volume.

[a] Rough endoplasmic reticulum.

enlarged. In addition, it must be remembered that even small changes in the diameter of an islet are reflected by large changes in the volume since the radius is cubed. Consequently, could the changes in insulin secretion be accounted for by differences in perifusion of normal versus enlarged islets? Indeed, this is a difficult question. In fact, the question of whether *in vivo* all the β cells or only a subpopulation of β cells within islets responds to a particular secretagogue has not been adequately resolved (22–24). Although we cannot totally explain away this possibility in our experiments, we can indicate in our defense that (a) there was no blunting in the first phase peak of insulin secretion in response to an acute glucose stimulus as shown in Fig 1. A blunting in the first phase of secretion could indicate a problem of the KRB buffer adequately penetrating the entire islet, (b) the islets were cultured for 7 days in the presence of GH. Most likely, during the last 48 hr of exposure the islets had undergone considerable mitosis. If there was a penetration problem concerning the KRB buffer during perifusion, then we should have also witnessed a penetration problem of the tissue culture media into the center of the enlarged islets which would have been reflected in necrotic centers. In this regard, we did not observe any necrotic centers by light or electron microscopy, and (c) we used a randomized procedure for selecting the islets to be perifused (see Materials and Methods).

Second, we quantitatively analyzed the islets at the ultrastructural level. We found that GH treatment had no effect on the volume of the individual β cell eliminating the possibility of hypertrophy. In addition, the nuclear volumes of the β cells in GH-treated and control islets remained the same suggesting that the increased DNA content and [^{3}H]thymidine incorporation in the GH-treated islets were indicative of cell multiplication and not polyploidy since nuclear volume accurately indicates polyploidy (20). Finally, we observed that GH treatment had no effect on the volume of cytoplasmic organelles within the β cell indicating that, at least on a quantitative basis, GH had no effect on the cytodifferentiation of the β cell.

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