# Development of Insulin Secretory Function in Young Obese Hyperglycemic Mice (Umeå *ob/ob*)

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**The obese-hyperglycemic syndrome is well characterized in adult mice. However, little is known about islet morphology and function at an early age when obese mice islets start to proliferate. We have now studied islet morphology and functional development in obese-hyperglycemic mice (Umeå *ob/ob*) and their lean littermates at ages ≤38 days. The weight of obese mice began to increase more than that of the lean littermates at days 8 to 12. At day 18, clinical diagnosis of the *ob/ob* syndrome could be made with 100% certainty. Islets from obese mice started to show enhanced growth rate during week 4, coinciding with the time of onset of hyperglycemia. $^3$H-thymidine labeling index is enhanced in *ob/ob* mice from day 22. Insulin secretion in islets from mice aged 18 to 21 days was the same in obese and lean mice from the same litter. At days 30 to 33, second-phase release and islet insulin content were decreased in islets from obese animals, but were restored after an overnight fast. It is likely that the hyperglycemia rather than increased insulin demand triggers increased β-cell growth.**


MICE WITH A TRAIT for obesity and hyperglycemia were originally found at the Roscoe B. Jackson Memorial Laboratory (Bar Harbor, ME).[1] The strain was called *ob/ob*, and the syndrome is autosomally recessive. In adult animals, *ob/ob* mice are characterized by hyperphagia, obesity, hyperinsulinemia, and hyperglycemia.[2,3] These changes reportedly occur when the mice are aged between 25 and 35 days.[4] The animals do not normally develop diabetes. Islet volume in these mice is ≤10 times greater than in normal mice.[5] The islet hyperplasia is probably not caused by an abnormality in the islets; instead, it is rather the consequence of increased demand for insulin. Hyperglycemia may be the most important trigger of islet growth.[6,7] The primary defect of the syndrome is thought to affect the hypothalamus/pituitary system.[8]

Characterization of function and properties of the islets of Langerhans at early stages of the syndrome is scanty. This may be because investigators have been interested in the full-fledged syndrome, but also because of difficulties in differentiating obese siblings from lean littermates. However, experienced staff at our animal facility can now distinguish between the two genotypes at day 18. This enables us to investigate young *ob/ob* mice and their siblings.

Most islet endocrine cells (>90%) in adult animals are normally arrested in the $G^o$ phase of the cell cycle and therefore have a limited capacity for regenerative growth.[9] The reason *ob/ob* mouse islets grow may be that they are stimulated at an early age when β cells are still proliferating. The aim of this study was to investigate the correlation between islet growth and the early development of the syndrome.

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## MATERIALS AND METHODS

### *Animals*

Non-inbred *ob/ob* mice from the Umeå colony (Umeå *ob/ob*) and their lean littermates were used throughout. In addition to these mice, six litters of C57BL/6J mice with both lean and obese siblings (Bomhuldtgaard Breeding and Research Centre, Ry, Denmark) were used as a control strain to the Umeå mice. Although Umeå *ob/ob* mice are metabolically abnormal with hyperglycemia and insulinemia, islets from these animals respond adequately to stimulators and inhibitors of insulin release.[10,11] The animals were fed R3 Rat and Mouse Breeding Food pellets from Lactamine (Vadstena, Sweden). Water and food were given ad libitum. In experiments using overnight-fasted animals, food was taken away at 3 PM the day before the experiment.

Comparisons were always made between obese and lean animals from the same litter, and both male and female mice were used. No distinction is made between +/+ and *ob*/+ mice—both groups are named "lean." Mice were kept at 22°C, and lights were on between 6 AM and 5 PM.

### *Characterization/Selection of Animals*

The *ob/ob* syndrome is manifest only in homozygotes. To test at what age clinical diagnosis of the syndrome can be made, 16 litters were evaluated continuously from birth. At the age of 1 week, the pups were marked in the ear. From this time and every 4 to 6 days, the mice were weighed on a balance. Two experienced staff members of the animal facility made separate judgments of the animals. The diagnosis was always certain at day 18. To confirm the diagnosis, animals were allowed to grow for another 20 days, after which the syndrome was manifest. Blood samples were taken from the retro-orbital plexus at days 21 and 30 from animals included in perifusion experiments, and at days 18, 24, 28, and 38 from mice included in morphometric experiments. Blood glucose level was then measured.

The clinical investigation is based on the width of the mouse's belly when letting it stretch out in the air while being held by the tail. In pronounced cases of obesity, the skin of the belly also has a slight pink color. Older obese mice show an increased neck size.

### *Islet Morphology*

Pancreases were removed from 85 animals at ages 21, 24, 28, or 38 days. The pancreas was fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. The pancreas was cut transversely from head to tail in slices of 5 μm, with a microtome. Every 40th slice was placed on a microscope slide and stained with hematoxylin-eosin. Slide sections were analyzed on a light microscope linked to a

digitizing table connected to a computer-aided image-analysis device (MOP-Videoplan; Kontron Bildanalyse, Eching/Munich, Germany). All data obtained from slides from the same pancreas were combined. The data included the total area of the stained pancreas section, total islet area, and number of islets. To investigate whether there are any differences between Umeå *ob/ob* and other strains of obese mice during the early stages of the syndrome, eight C57BL/6J mice were also investigated.

### *Studies of In Vitro Islet Function*

Insulin secretory capacity was compared in islets isolated from obese and lean littermates at ages 18 to 21 and 30 to 33 days. Before animals were killed by decapitation, blood samples were obtained from the retro-orbital plexus. Ten microliters of blood was placed in 1,600 μL 1-mmol/L EDTA for later analysis of blood glucose. After decapitation under ether anesthesia, blood was collected for assay of serum hormones.

### *Collagenase Isolation*

The pancreas was removed, immersed in Hanks solution, and minced with scissors. Hanks solution contained (in mmol/L) NaCl 137.0, KCl 5.3, $Na_2HPO_4 \cdot 7H_2O$ 0.33, $KH_2PO_4$ 0.44, $MgSO_4$ 0.81, $CaCl_2$ 0.78, and glucose 5.6. pH was adjusted to 7.4 with 8.4% $NaHCO_3$. During isolation, 4.5 mg Collagenase P (Boehringer, Mannheim, Germany) was added to 5 mL Hanks solution. The collagenase digestion proceeded for 10 to 12 minutes in a water bath at 36°C. The reaction was stopped by placing the vial on ice, and islets were rinsed in Hanks solution containing 1 mg bovine serum albumin (fraction V)/mL. Islets were picked under a stereomicroscope and counted.

### *Measurement of Labeling Index*

Pancreata of lean and obese littermates in 20 litters aged 19 to 32 days, were removed from the animals and placed in Hanks solution with collagenase as described earlier. Islets from obese and lean mice from each litter were pooled into two groups: lean and obese. There were approximately 100 to 150 islets in each group. Islets were then preincubated for 30 minutes in Hanks buffer at 37°C. Then islets were incubated for 2 hours in KRBH solution, which is described later, containing $^3$H-thymidine at a concentration of 1 μCi/mL with a specific activity of 126 GBq/mmol. $^3$H-thymidine was obtained from the Radiochemical Centre (Amersham, UK). Islets were then rinsed in Hanks solution with 1 mg/mL albumin several times and placed directly in Bouin's solution. Then islets were dehydrated, embedded in paraffin, sectioned at 5 μm, and mounted on microscope slides. After removal of paraffin, the sections were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 1 week at +6°C. Autoradiographs were developed for 5 minutes in Kodak D-19 and fixed for 10 minutes in a solution from Stena Scanfors (Gothenburg, Sweden). The sections were dried overnight and were counterstained with hematoxylin and eosin. To determine the labeling index, an oil-immersion lens with magnification 1,000× was used. In each experiment, ≥2,500 islet cells per group were counted from lean and obese littermates, respectively. Each litter contained eight to 10 mice.

### *Perifusion of Isolated Islets*

Insulin secretion was measured in perifusion studies in which 40 to 60 isolated islets from both lean and obese mice from the same litter were transferred to perifusion chambers and perifused in parallel. Islets were preperifused for 30 minutes with KRBH medium with 3 mmol/L glucose at 37°C in 1 mL modified Krebs-Ringer bicarbonate buffer containing (in mmol/L) NaCl 115, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.56, $NaHCO_3$ 20, and HEPES 20, pH 7.40 (KRBH). It also contained 3 mmol/L D-glucose and 1 mg/mL bovine serum albumin (Miles Laboratories, Stoke Poges, UK). The medium was gassed with $O_2$:$CO_2$ (95:5), pH 7.40. Glucose concentration was then increased to 20 mmol/L for 30 minutes, after which it was again decreased to 3 mmol/L. The perifusion device was entirely enclosed in an infant incubator (Isolette; Air Shields, Hatboro, PA) maintained at 37°C, as described previously.[12] A perifusion pump (Ismatec ms-reglo; Isamatec, Glattbrygg-Zurich, Switzerland) pumped the medium through the perifusion chamber at a speed of 1 mL/min. The filter in the chamber is of Nuclepore type (Pleasanton, CA) with 8-μm pore size and was produced by Costar (Cambridge, MA). Samples of media were saved for later insulin radioimmunoassay using mouse insulin as reference. Islets were placed in acid ethanol (3 mL concentrated HCl in 200 mL 70% ethanol vol/vol) for later measurement of insulin content.

All chemicals were commercially available reagents of analytic grade. Data are presented as the mean ± SEM for the number of experiments indicated. Statistical analyses were made using Student's *t* test for paired samples.

### *Blood Glucose Analysis*

Ten microliters of blood was mixed with 1,600 μL 1 mmol/L EDTA adjusted to pH 7.4 by KOH. These samples were later assayed by a single-step assay using the luciferin/luciferase system and a liquid scintillation spectrometer.[13] This method was originally developed for serum. Control measurements showed that the method also has good reliability for measurements on blood (data not shown). Luciferin and luciferase were obtained from Boehringer.

### *Insulin Assay*

All serum samples and effluent aliquots of perifusions were assayed by radioimmunoassay using crystalline mouse insulin as standard. Free and antibody-bound insulin was separated by precipitation with ethanol.[14] $^{125}$I-labeled insulin was supplied by Hoechst (Frankfurt, Germany).

### *Protein Analysis*

Protein was assayed with the absolute method reported by Whitaker and Granum.[15] Isolated islets from 20- and 30-day-old lean and obese animals were counted and cleaned in albumin-free Hanks solution. The samples were ultrasonicated for 3 seconds repeatedly with a Sonifier (Branson Sonic Power, Danbury, CT). A solution containing 10 mg/mL bovine albumin (Fraction 5; Sigma Chemical, St Louis, MO) was used as standard. Standards and samples were read in a Varian DMS 100 UV/Visible spectrophotometer (Varian Techtron, Springvale, Australia).

## RESULTS

### *Characterization of Animals*

When the status of each animal as obese/lean had become apparent, results from clinical observations were compared with results of the weighing of animals. Clinical diagnosis could be made with 100% certainty at 18 days. This is at least as early as could be detected with certainty by weighing of individual animals. Figure 1 shows the weight of animals from all litters. When lean and obese mice were compared as groups, there was a significant difference in weight at day 12. Table 1 lists blood glucose data from mice between 16 and 38 days of age. Blood sugar

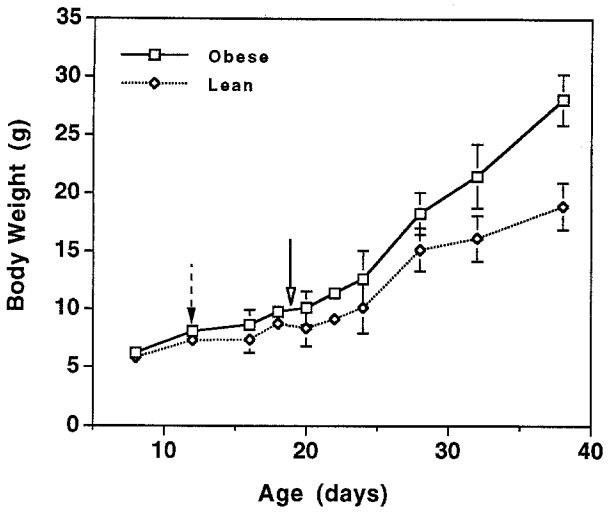


**Fig 1. Increase in body weight for animals from all 16 litters tested. Mean ± SD. Number of animals varied from 6 to 18 obese animals per group and 12 to 48 lean animals per group. The white arrow indicates when clinical diagnosis can be made with 100% certainty; black arrow indicates earliest time of a significant difference ($P < .01$) when comparing mean weights of obese and lean animals in individual litters (Student's $t$ test for paired data).**

increased in all obese animals during week 4, but did not change in the lean group. There was no significant increase in blood glucose until day 22, but after that nonfasted obese animals had higher blood glucose values than the lean littermates. At day 21, only occasional high blood glucose values were observed in the obese group. Serum insulin level was measured from samples obtained in connection with the perfusion experiments at days 20 and 30. Already at day 20, there were significantly higher insulin levels in the obese group (4.64 ± 0.34 ng/mL [n = 7] *v* 0.59 ± 0.06 [n = 8], $P < .005$). At day 30, serum insulin in obese animals was 11.4 ± 0.59 ng/mL (n = 8) versus 0.61 ± 0.06 (n = 8; $P < .0005$).

*Islet Morphology*

Table 2 lists the findings of stereologic analysis of pancreata from animals at ages 21, 24, 28, and 38 days. There was no difference between obese and lean animals at day 21. At day 24, there was a tendency toward increased islet size in obese animals, but the difference in mean values did not attain statistical significance when six obese and 13 lean pancreata were compared. At days 28 and 38, islets from obese animals are considerably larger. Already from day 21, there was a tendency for islets from obese mice to comprise a larger proportion of the pancreas. ANOVA for days 21, 24, and 28 shows a $P$ value of .053 for the comparison between lean and obese mice. At day 38, there was a significant difference ($P < .05$) using Student's $t$ test. No significant difference could be seen in islet number per pancreas area. At day 38, there was an increase in the number of islets in obese mice.

Table 2 also lists morphometric results from C57BL/6J mice at day 38. Significant differences in islet size, density, and number at day 38 are analogous to the morphometric data from the Umeå strain. Protein and insulin contents were measured in collagenase-isolated islets. Protein content in islets from 30-day-old lean mice was 4.34 ± 0.54 μg/islet (n = 12), and in obese mice, 4.36 ± 0.54 (n = 12; nonsignificant). Figure 2 shows that at day 20, there was a small but statistically significant difference between the two groups. However, at day 30, insulin content in obese mice was only 40% of that found in the lean littermates. After an overnight fast, insulin content in islets from obese mice increased to values not different from those found in islets of starved lean mice (Fig 2). In islets from lean mice, starvation caused a slight reduction in insulin content.

*Autoradiographic Labeling Indices*

Autoradiographic $^3$H-thymidine labeling indices in islet sections from 19- to 32-day-old lean and obese littermates are shown in Fig 3. At days 19 to 20, there is no significant difference between lean and obese mice, but at days 22 to 23, the labeling index is higher in obese than in lean mice (3.2% ± 0.7% *v* 1.7% ± 0.2%); the difference is significant ($P < .05$ for paired data). The labeling index is higher in islets from obese mice also at days 24 and 26 to 32.

*Insulin Secretion*

Basal insulin release was not different between islets from lean and obese mice from the same litter either at days 18 to 21 or 30 to 33. At days 18 to 21, there was no difference between islets from lean and obese mice in terms of glucose-stimulated secretion (Fig 4). At days 30 to 33 (Fig 5), first-phase stimulated insulin release was the same in islets from the two groups, but second-phase release was higher in islets from lean animals (1.80 ± 0.37 ng insulin/min *v* 1.11 ± 0.27) when measured over 28 to 58 minutes ($P < .01$, Student's $t$ test for paired data). If 30- to 33-day-old mice were fasted overnight, the secretory response was higher in islets from *ob/ob* mice ($P < .05$ when compared over 28 to 58 minutes; Fig 6). After an overnight fast, the secretory response was increased in islets from obese mice, but was slightly decreased in islets from lean mice.

The secretory response in C57BL/6J mice was tested in three parallel perifusions. The same tendency toward a

**Table 1. Blood Glucose Values in Mice From Day 16 to Day 38 (mean ± SEM)**

| | Day of Blood Glucose (mmol/L) Measurement | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 16 | 18 | 21 | 22* | 24* | 28* | 31* | 38* |
| Obese | 3.6 ± 1.0 | 4.2 ± 1.1 | 5.7 ± 0.6 | 7.0 ± 0.7 | 8.5 ± 1.8 | 13.7 ± 1.6 | 14.5 ± 1.9 | 18.1 ± 1.7 |
| Lean | 3.9 ± 0.3 | 4.9 ± 0.8 | 4.6 ± 0.3 | 4.9 ± 0.4 | 4.7 ± 0.3 | 4.9 ± 0.7 | 6.7 ± 0.4 | 6.3 ± 0.4 |
| No. of obese/lean | 3/8 | 5/10 | 15/36 | 6/8 | 8/16 | 8/16 | 8/8 | 12/15 |

*$P < .05$, obese *v* lean mice (Student's $t$ test for independent data).

**Table 2. Morphometric Analysis of Pancreata From Umea *ob/ob* and C57BL/6J Mice (mean ± SEM)**

| | Umea *ob/ob* | | | | C57BL/6J |
|---|---|---|---|---|---|
| | Day 21 | Day 24 | Day 28 | Day 38 | Day 38 |
| Islet number per pancreas section area | | | | | |
| Obese | 1.5 ± 0.13 | 1.1 ± 0.17 | 0.8 ± 0.03 | 1.1 ± 0.17 | 1.4 ± 0.11* |
| Lean | 1.3 ± 0.07 | 1.3 ± 0.13 | 1.0 ± 0.07 | 0.8 ± 0.06 | 1.0 ± 0.08 |
| Islet area per pancreas section area | | | | | |
| Obese | 9.7 ± 0.94 | 9.1 ± 1.18 | 7.5 ± 2.20 | 13.9 ± 4.05* | 19.5 ± 4.52* |
| Lean | 7.7 ± 1.23 | 6.2 ± 0.35 | 5.8 ± 0.46 | 4.6 ± 0.57 | 5.0 ± 0.80 |
| Islet mean size | | | | | |
| Obese | 6.1 ± 0.31 | 6.1 ± 1.54 | 8.2 ± 1.29* | 10.8 ± 1.62* | 13.1 ± 1.01* |
| Lean | 6.2 ± 0.62 | 4.8 ± 0.38 | 5.6 ± 0.35 | 5.6 ± 0.42 | 4.8 ± 0.07 |

NOTE. Calculations were made on mean values from obese and lean animals, respectively, from each litter. Islet number per pancreas section area is given as number per $mm^2$. Islet area per pancreas section area is given as $K\mu m^2/mm^2$. Islet mean size is given as $K\mu m^2$.

*Significant difference in islet volume ($P < .01$) between lean and obese animals (Student's *t* test for independent data).

decreased second-phase release as in Umeå *ob/ob* mice was observed in 30-day-old obese 6J mice (Fig 7).

## DISCUSSION

When the obese-hyperglycemic syndrome was first described, a number of reports were published showing growth characteristics of the animals and islet morphology of adult animals.[16,17] The early stages of the *ob/ob* syndrome are not as well explored as the later, but still a number of early signs of the syndrome have been recognized, such as abnormal thermoregulation,[18] congenital hypothyreodism,[19] decreased oxygen consumption,[20] and increased food intake.[21,22] Earlier studies have also found increased serum insulin at days 17 to 20,[23] marked degranulation in islets together with a reduced insulin content in the pancreas from 5-week-old obese animals,[24] and an increase in serum glucose in obese animals at approximately 4 weeks of age.[23] An increased output of insulin could be explained by several factors, such as peripheral insulin resistance, persistent hyperglycemia, and increased levels of β-endorphin.[3,25] There are many insulin secretion studies in adult animals that show an increased insulin response to glucose in obese mice.[26,27] However, in Umeå *ob/ob* mouse islets, response characteristics to stimulators and inhibitors of insulin release are normal.[10,11]

In adult animals, islets from obese mice are both enlarged and increased in number.[16,17,28,29] Little has been done to characterize islet function and morphology at early stages (< 2 months) of the syndrome, although such knowledge is important for understanding the mechanisms regulating islet growth. To our knowledge, this is the first description of the development of insulin secretion, islet proliferation, and morphology in which *ob/ob* and lean mice have been compared at these early ages. We find that islets from *ob/ob* mice show an increased growth rate early during week 4 and that changes in islet morphology can be

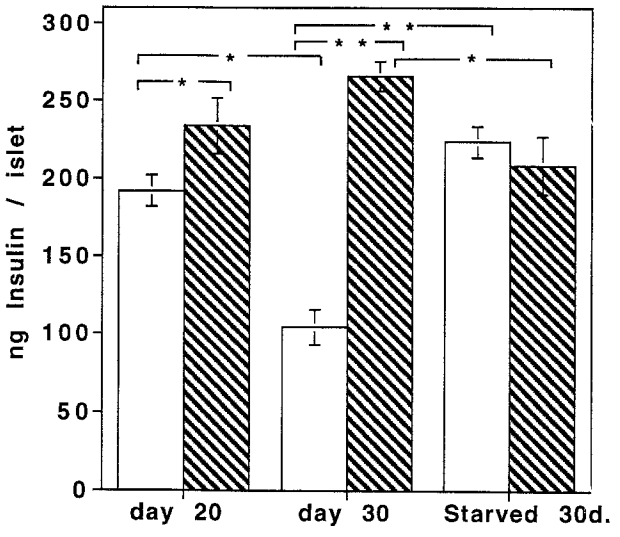


**Fig 2. Islet insulin content in (▨) lean and (□) obese mice. Insulin content was measured in islets isolated from 20- or 30-day-old mice. At day 30, islet insulin content from starved lean and obese animals is compared with that in unstarved animals. *$P < .05$, **$P < .001$ (Student's *t* test for independent data). Mean ± SEM for 25 observations.**

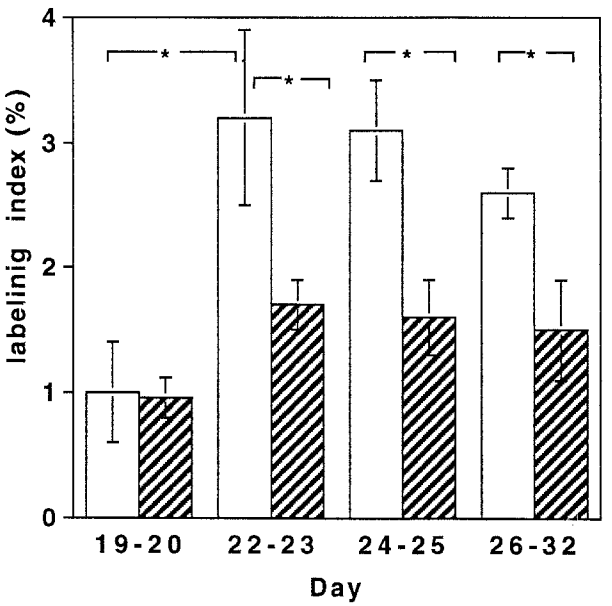


**Fig 3. Labeling index from islets in (▨) lean and (□) obese mice (8 to 10 per group) at 19 to 32 days. Mean ± SEM. *$P < .005$ (Student's *t* test for independent data).**

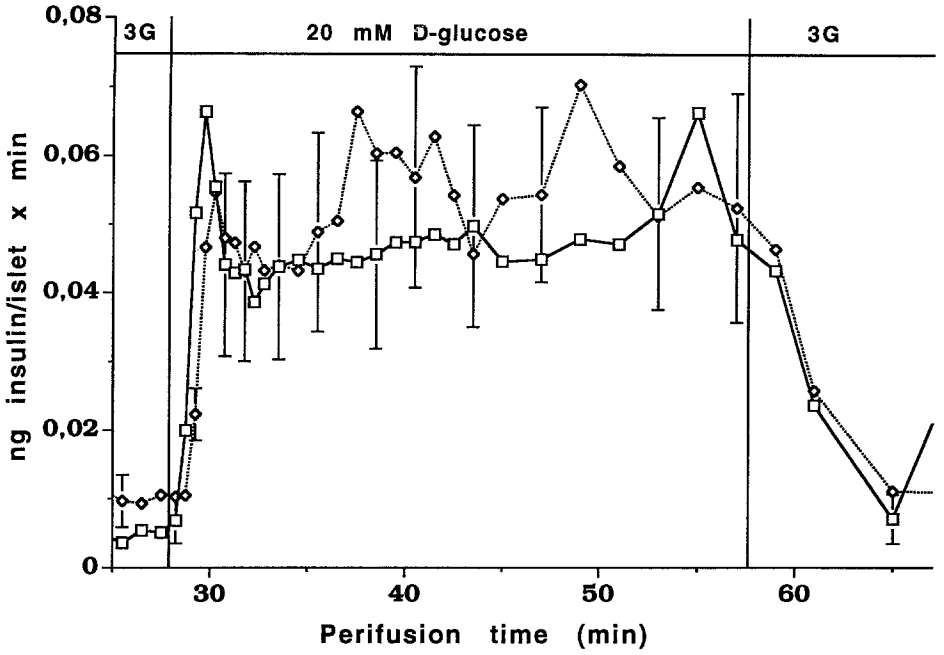


**Fig 4. Insulin release in perifused mouse pancreatic islets isolated by collagenase digestion from 18- to 21-day-old (◇) normal (n = 6) and (□) *ob/ob* (n = 7) nonfasted mice taken from the same litter. Islets were transferred to perifusion chambers and perifused in parallel with Krebs-Ringer medium. D-Glucose (20 mmol/L) was introduced during 30 minutes as indicated by vertical bars. Fractions of effluent were analyzed for insulin by radioimmunoassay. Mean ± SEM.**

seen already at day 28. It is probable that the increased islet growth reflects β-cell proliferation, because β-cells comprise a large proportion (>90%) of all islet cells in adult obese mice.[29] No differences in morphologic characteristics were found between islets from Umeå *ob/ob* and C57BL/6J mice at day 38. This suggests that an increased islet growth during week 4 is not confined to the Umeå strain. Blood glucose values and body weight gain were also similar in the two strains (data not shown). To examine more precisely when the increased islet proliferation begins, $^{3}$H-thymidine labeling indices were measured from days 19 to 20. The labeling index was higher in obese littermates at days 22 to 23, which is at the time of the increase in blood glucose. However, insulin content in obese mouse islets is lower at day 21[25] (and this report) and serum insulin levels are elevated at day 20[23] (and this report). Early islet growth and insulin secretory response have been characterized in the obese rat model Zucker (*fa/fa*).[30] In these animals, islet

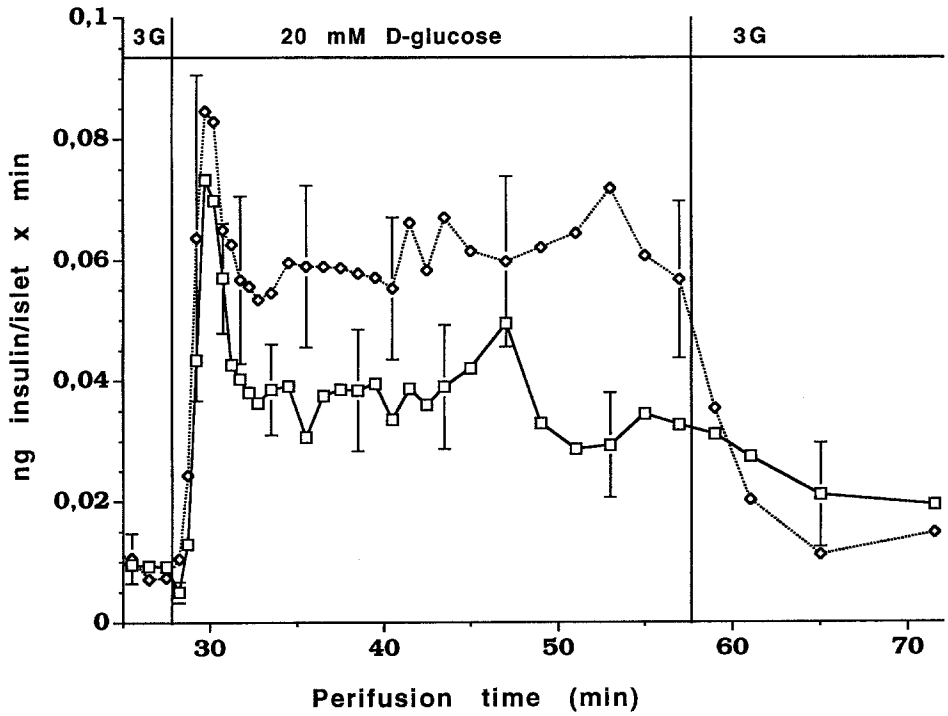


**Fig 5. Insulin release in perifused mouse pancreatic islets of 30- to 33-day-old (◇) lean (n = 7) and (□) obese (n = 7) mice. Mean ± SEM. See Fig 4 legend.**

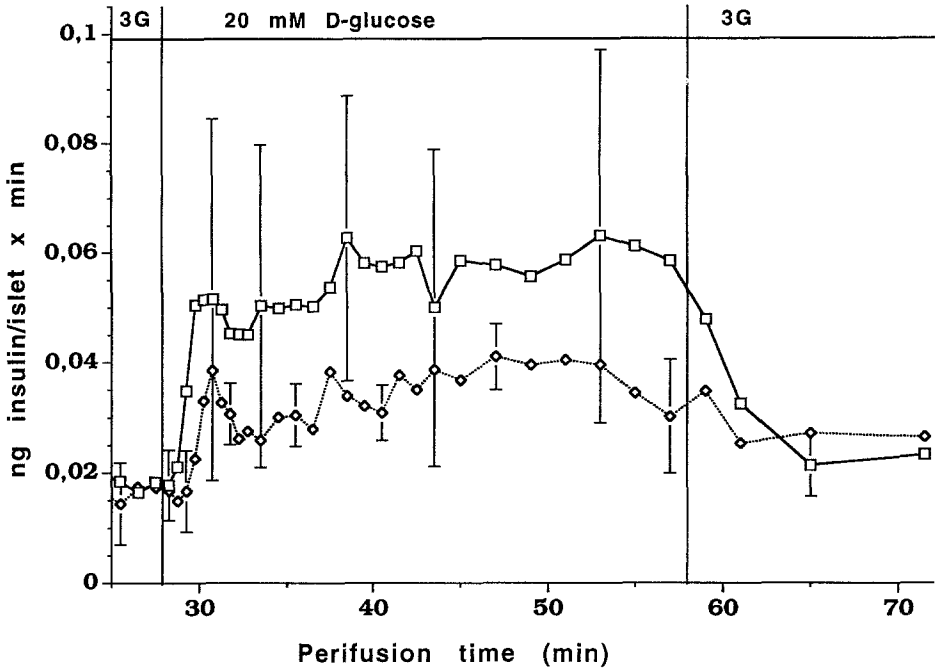


**Fig 6. Insulin release in perifused mouse pancreatic islets of 30- to 33-day-old overnight-fasted (◇) lean (n = 5) and (□) obese (n = 5) mice. Mean ± SEM. See Fig 4 legend.**

growth had started already at 7 days of age and fasting hyperinsulinemia and increased glucose-induced insulin release in vitro were seen at day 35.[30]

There was no difference in the in vitro insulin secretion between islets from lean and obese mice at days 18 to 20. This suggests that the secretory machinery is intact in *ob/ob* mouse islets with no oversecretion of insulin at this age. Blood glucose was 8.5 mmol/L in obese mice at day 24. This concentration is at the lower end of the range required to obtain an efficient stimulation of β-cell replication in neonatal rat β cells.[31] Regarding the timing of events leading to an altered islet morphology in obese mice, we see a difference in weight at day 12, decreased islet insulin content and increased serum insulin at day 20, and an enhanced labeling index at day 22, at the time blood glucose increases. This suggests that islet proliferation is probably triggered primarily by increased blood glucose, but may also be a consequence of increased insulin demand. It is possible

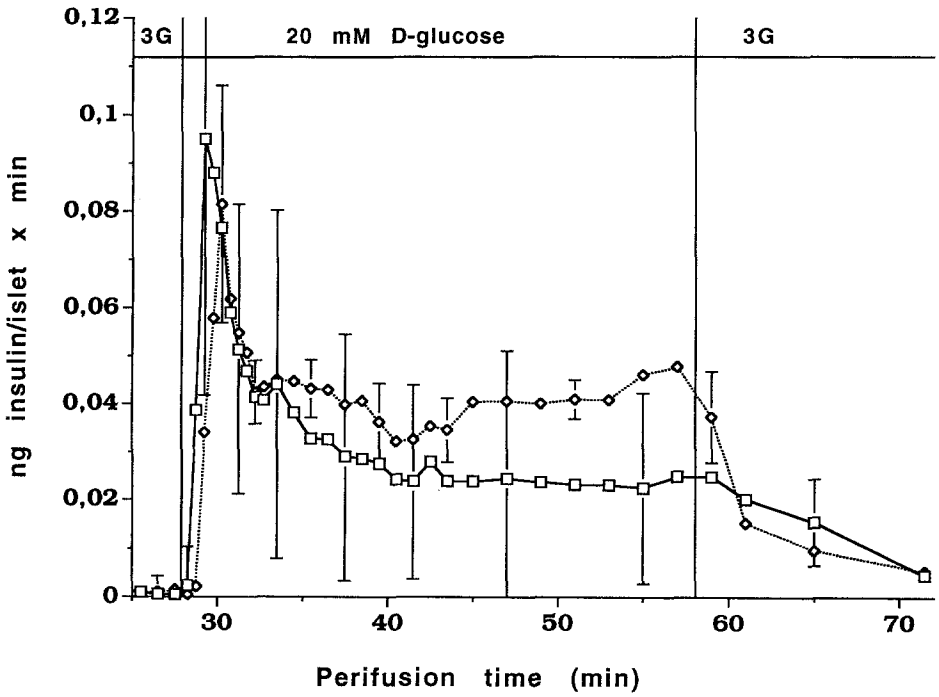


**Fig 7. Insulin release in perifused mouse pancreatic islets of 30- to 33-day-old nonfasted C57BL/6J (◇) lean (n = 3) and (□) obese (n = 3) mice. Mean ± SEM. See Fig 4 legend.**

that at constant insulin secretory pressure, a cell signal is generated in β cells that stimulates islet growth in cells that have the capacity for proliferation. Future experiments will aim to identify those signals. β cells produce and respond to a number of growth factors that may be involved,[32,33] but the change can also be in glucose-induced intracellular signals.[34] Our findings suggest that week 4 is the period of greatest interest in this matter.

Second-phase insulin was reduced in islets from unstarved obese mice at days 30 to 33. Islets from these animals had a reduced insulin content, probably explained by an incapability of the islets to produce sufficient amounts of insulin in this expansive period. To test whether reduced insulin release could be a consequence of the decreased insulin content, we studied insulin release in starved animals. When 30- to 33-day-old *ob/ob* mice were fasted overnight, islet insulin content increased to the levels found in islets of lean mice. Islets from obese starved animals also released more insulin as compared with islets from unstarved obese and lean animals at this time. This suggests that β cells from obese animals are basically well-functioning also during this period of rapid growth. A consistent finding was that at both days 18 to 21 and 30 to 33, islets from obese animals became more optically dense (opaque) after overnight starvation (data not shown). This occurs to a much lesser degree in islets from lean mice. The phenomenon is well known in islets from adult *ob/ob* mice[17] and may reflect replenishing of insulin stores.

Protein content was not measurably higher in islets from obese mice at day 30, although the average size of islets is larger at that age. This apparent discrepancy between islet morphology and protein content may be explained by the fact that both collagense digestion and selection of islets may discriminate in favor of medium- to large-sized islets.

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