Effects of Long-Term Restricted Insulin Production in Obese-Hyperglycemic (Genotype ob/ob) Mice

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Summary. Primary hypersecretion of insulin has been suggested as one possibility for the genetic fault of ob/ob mice. To test this hypothesis, streptozotocin (SZO) was used to reduce permanently insulin secretion in young lean and obese mice. After establishment of hyperglycaemia and weight reduction in treated obese mice (obese-SZO), daily insulin replacement was begun in some (obese-SZO-Ins). Obese-SZO mice maintained insulin levels and body weights similar to lean controls, though they were shorter and fatter, while food intake and blood sugar levels exceeded lean values. Obese-SZO-Ins mice with reduced islet hyperplasia, but great insulin resistance, gained more weight than obese-SZO mice; had high serum insulin and controlled blood glucose; and exhibited hyperphagia. These results suggest that primary hypersecretion of insulin cannot be the genetic defect, as ob/ob mice are hyperphagic, hyperglycaemic, insulin resistant, and “obese” even when insulin levels are restricted.

Key words: Mutation ob/ob, obese mouse, streptozotocin, insulin resistance, hyperphagia, spontaneous diabetes, diabetes in mice, obesity in mice, hereditary obesity, obese-hyperglycaemic mice, B-cells.

The obese-hyperglycaemic mutation in mice (ob/ob) has been the subject of a great number of studies because of its intriguing inherited syndrome characterized by hyperglycaemia, hyperinsulinaemia, hyperphagia, obesity and insulin resistance. Yet the fundamental question of the nature of the primary genetic fault is still unanswered.

From the earliest descriptions of this mutation,

insulin and the pancreas have been of great interest. Resistance to massive injections of insulin was shown in these mice in 1951 [1] and greatly enlarged islets of Langerhans were described in 1952 [2]. In 1955, increased extractable insulin from the pancreas was reported [3] and elevated circulating levels of insulin have been shown many times by bioassay or radioimmunoassay procedures [4, 5, 6, 7]. In more recent years, several authors have suggested that hypersecretion of insulin could be the primary pathogenetic mechanism leading to compensatory responses which result in the abnormalities characteristic of these animals [8, 9, 10].

The experiment reported here was designed to test this hypothesis and to study the long-term effects of reduced endogenous insulin secretion in ob/ob mice. To do this, a dose of streptozotocin, a drug specifically cytotoxic to pancreatic B-Cells, was found that would destroy sufficient B-cells in young ob/ob mice to result in insulin levels equivalent to those of lean animals. The consequence of controlled insulin levels was studied over a sixteen week period for effect on: blood glucose, glucosuria, obesity, resistance to exogenous insulin, food intake, body weight and body length. The permanence of streptozotocin damage to B-cells was determined by microscopic examination of the pancreas.

Materials and Methods

Animals

Male, weanling, ob/ob mice (strain C57 BL/6J-ob) and their lean littermates (ob/+ or +/+) were purchased from the Jackson Laboratory, Bar Harbor, Maine. They were housed individually in suspended stainless steel cages and fed *ad libitum* Purina Mouse Chow (Ralston Purina Co., St Louis, Missouri).

Treatment

Streptozotocin, provided by the Upjohn Company (Kalamazoo, Michigan), was dissolved in saline (10 mg/ml) and adjusted to pH 4.0 with HCl immediately before injection. Injections were given intraperitoneally at 9:00 A.M. in varying doses (125, 150, 175, or 200 mg/kg body weight) to mice that had been fasted overnight. Control mice were injected with equivalent volumes of saline. All mice were between 4 and 5 weeks of age at the time of injection.

Insulin replacement was initiated in some obese mice two weeks after streptozotocin treatment. NPH insulin, diluted in sterile saline to an appropriate concentration, was injected subcutaneously once per day at 4:00 P.M. Volumes injected were about 0.5 ml. Daily injections were continued in these mice in increasing doses for the remaining 14 weeks of the experiment. Initial doses were 2 U/100 g body weight. This was increased to a maximum of 12 U/100 g in one group and 30 U/100 g in another.

Daily subcutaneous injections of insulin were also given to 14 lean mice which had not been previously injected with streptozotocin. These mice were either 5 or 7 weeks of age at first injection.

Blood Glucose and Serum Insulin

Blood samples were taken from the sub-orbital sinus on the morning prior to streptozotocin injection and at biweekly intervals thereafter until the conclusion of the experiment 16 weeks later. These samples (about 150 λ of blood) were collected in small non-heparinized capillary tubes which were spun in a hematocrit centrifuge to separate serum, which was subsequently frozen in small plastic tubes. Serum samples were later analyzed for glucose by a micro-modification of the glucose oxidase technique [11] and for insulin by radioimmunoassay (Phadebas Insulin Test), using porcine insulin as a standard.

Body Weights, Urinary Glucose and Food Intake

Body weights and tests for urinary glucose (Clinistix) were recorded weekly. Quantitative determinations of urinary glucose were made at about 14 weeks past injection by using metabolism cages which allowed separation and collection of 24 hours urine samples. Urine was collected into a solution of 0.5% sodium benzoate and 0.5% sodium fluoride, with 2 drops of toluene, and was analyzed for glucose by the glucose oxidase method.

Food intake was measured at 5 different intervals of 3 to 6 days each. Amount of food consumed was ascertained by difference between the weights of the

Fig. 1. Body weight changes of ob/ob and lean control mice, and ob/ob mice injected with streptozotocin at 4 to 5 weeks of age, followed by either insulin injections (obese-SZO-Ins) or no insulin injections (obese-SZO). Values are means $\pm$ standard error of the mean. I < II and IV at $p \leq 0.005$, III < II at $p \leq 0.005$, IV < II at $p \leq 0.005$

Fig. 2. Changes in serum insulin values of lean and ob/ob mice, and ob/ob mice injected at 4 to 5 weeks of age with streptozotocin, followed by either insulin injections begun at 2 weeks past streptozotocin treatment (obese-SZO-Ins) or no insulin injections (obese-SZO). Dosage of insulin injected was a maximum of 12 U in the obese-SZO-Ins (low) group and 30 U in the Obese-SZO-Ins (high) group. Values are means $\pm$ standard error of the mean. I < II and IV at $p \leq 0.005$, I < V at $p \leq 0.0005$, II < IV at $p \leq 0.01$, II < V at $p \leq 0.005$, III < II at $p \leq 0.01$, III < IV at $p \leq 0.025$, III < V at $p \leq 0.005$

ground chow plus food cup on different days at the same hour, corrected for food spilled. All weights were taken to the nearest tenth of a gram.

Sixteen weeks after streptozotocin injection, the experiment was terminated. The mice were anesthetized with a CO₂:O₂ mixture (50:50) and measured for length before sacrifice by decapitation. Pancreata were removed and fixed in Bouin's solution. Paraffin-embedded sections were stained with H & E and aldehyde-fuchsin.

Statistical analyses were carried out using a one-sided Student's t-test.

Results

The doses of streptozotocin found to have a diabetic effect in obese mice, as tested by glucose spillage in the urine, were 175 and 200 mg/kg body weight, with the higher doses giving consistent and severe effects. Of the 13 obese mice injected at these doses, in only one did the drug seem ineffective. Few of the lean mice were injected with streptozotocin, but loss of weight and glucosuria did result from injections of 150 or 175 mg/kg body weight, with poor survival at both doses.

Growth Curves

Body weights during the course of the experiment are shown in Figure 1. All mice showed an initial loss of weight due to the overnight fast prior to streptozotocin or saline injection. Thereafter, the lean and obese controls displayed typical growth patterns with the obese gaining rapidly over the lean and reaching average weights of 47 and 27 g, respectively, at 16 weeks past injection (20 weeks of age).

It can be seen that streptozotocin had an immediate effect in restricting weight gain of obese mice to weights below even those of lean controls. After 7 weeks, the obese-streptozotocin (obese-SZO) mice had reached the weight of the lean controls and thereafter weights for the two groups were similar. Even at 16 weeks past injection these groups weighed 30 and 27 g, respectively, and were not significantly different.

At two weeks past injection, insulin replacement by daily subcutaneous injection was begun in some of the obese-SZO mice. Initially 2 U/100 g body weight were injected, and this dosage was gradually increased to 12 U/100 g body weight and in 3 of the mice to 30 U/100 g body weight. Insulin injections did result in greater weight gain in SZO-mice, but, at the end of the experiment, they still weighed significantly less than obese controls.

Two lean-SZO mice (not shown) were consistent-

ly lighter than their lean controls, with a final average weight of 21 g.

Serum Insulin

Values for serum insulin are plotted in Figure 2. Typical values for lean mice were about 20 μ U/ml, and remained fairly constant during the experiment. Values for untreated obese mice rose from 51 to 289 μ U, but fell again as the mice got older. Insulin levels of the obese-SZO mice were significantly reduced below obese controls and were similar to untreated lean mice throughout the experiment. Insulin values of obese-SZO mice given maximum insulin injections increased

Fig. 3. Comparison of survival rates of lean and obese-streptozotocin mice at various doses of exogenous insulin. Note that the amount of insulin injected is presented on a logarithmic scale

Fig. 4. Changes in serum glucose values of lean and ob/ob control mice and obese mice injected with streptozotocin, followed by insulin replacement (obese-SZO-Ins), or no insulin replacement (obese-SZO). Values are means $\pm$ standard error of the mean

steeply and reached the remarkable level of 1777 μ U/ml serum by the end of the experiment. Obese-SZO mice injected with lower doses of insulin reached an average of 516 μ U insulin/ml serum, while values for lean-SZO mice (not shown) average about 10 μ U/ml, only half the average for lean controls.

All of the 14 lean mice given daily injections of insulin died, most within a few days of the first injection (Fig. 3). Maximum dosage of insulin that could be given before resulting in death was 5 U/100 g body weight, with many of these lean mice unable to tolerate even 2 U/100 g body weight.

Serum Glucose

In Fig. 4, it can be seen that serum glucose values were higher for obese than lean controls, with the difference increasing during the experiment to 327 and 215 mg/100 ml serum respectively at 16 weeks. As expected, but in contrast to results reported by Batchelor et al. [12], streptozotocin injection of obese animals resulted in greatly elevated glucose values (above 500 mg/100 ml serum). These values remained high in the group not receiving insulin replacement (453 mg/100 ml at 16 weeks). The very high levels of insulin which were present in the obese-SZO-Ins group were effective in lowering the glucose levels of this group to 260 mg/100 ml at 16 weeks past streptozotocin injection. Lean-SZO mice had an average of 495 mg/100 ml serum at 16 weeks past injection of streptozotocin (not shown).

Food Intake

Average food intake data from five separate periods of measurement are shown in Fig. 5. Lean, control mice ate significantly less food than lean-SZO mice and all groups of obese mice ($p \leq 0.005$). There were no significant differences between the groups of obese mice, or between the lean-SZO mice and any obese group.

Urinary Glucose

Table 1 shows the results obtained from glucose analysis of 24 hour urine samples. As expected, there was almost no glucose in the urine of untreated lean mice. Obese mice, however, spilled a significant amount of glucose even with no treatment. Streptozotocin injection resulted in greater urinary loss in both lean and obese mice, while insulin-injected-SZO mice spilled less than obese controls. At this time (14 weeks past streptozotocin injection) serum insulin levels were quite high in these mice.

Body Lengths

Body lengths at the end of the experiment are shown in Table 2. Setting the lean control mice at 100%, it can be seen that obese controls are the same length whereas all mice treated with streptozotocin were stunted. Insulin replacement improved body length

Fig. 5. Food intake of lean mice, lean mice injected with streptozotocin (lean-SZO), ob/ob mice, ob/ob mice injected with streptozotocin (obese-SZO) and ob/ob mice given both streptozotocin and subsequent insulin injections (obese-SZO-Ins). Values are means $\pm$ standard error of the mean. I < II, III, IV, and V at $p \leq 0.005$, No significant differences between II, III, IV and V

Fig. 6. Weights of epididymal fat pads of control and treated lean and ob/ob mice 16 weeks after streptozotocin injection. Obese-SZO-Ins mice were given daily insulin injections starting 2 weeks after streptozotocin treatment. Values are means $\pm$ standard error of the mean. I < III, IV, V at $p \leq 0.005$, II < I at $p \leq 0.005$, IV < III at $p \leq 0.025$, V < III at $p \leq 0.01$

from 91% of lean in obese-SZO, to 96%. It is of interest also that in the few lean-SZO mice surviving, body length was not as severely reduced as in the obese-SZO mice.

Fat Pads

Weights of epididymal fat pads are presented in Fig. 6. The weights of fat pads of all obese mice were greater than those of lean mice, even though the body weights of obese-SZO mice were not significantly greater than lean. Streptozotocin injected mice had smaller fat pads than their controls, whether obese or lean, and insulin injections in SZO-mice did not increase these weights.

Because obese-SZO mice weighed less than obese controls and obese-SZO-Ins mice, their fat pads represented a greater proportion of total body weight (Table 3), 6.6%, compared to 5.9% for obese, 5.8% for obese-SZO-Ins, and 2.1% for lean. The lean-SZO mice, however, did not preserve their relative adiposity which fell to less than 0.5%.

Pancreatic Islet Morphology

Islets of untreated, non-obese mice were small, well-defined and had well granulated B-cells when stained with aldehyde fuchsin. The islets varied in the number of apparent A-cells, but these were always located at the periphery of the islet, with B-cells in the interior.

Islets of lean streptozotocin-diabetic mice were, in general, smaller in size than their controls, and contained markedly degranulated B-cells. Many islets also showed an admixture of A- and B-cells in the islet interior.

Obese untreated mice had islets that were characteristically very large, with degranulated B-cells. Islets from obese mice treated with streptozotocin were still larger than lean controls, but smaller than those from untreated obese mice, due to the destruction of B-cells. Like the islets from lean, treated mice, these were characterized by the presence of A-cells in the islet interior. Some islets from treated, obese mice had less defined configurations with acinar tissue intruding into the islets.

Some of these effects of streptozotocin on the islet are similar to those reported by others [13, 14] for adult obese mice treated with this drug. The presence of A-cells scattered within the islet interior has also been reported by Like and Chick [15] for diabetic mutant mice in the terminal stage of the syndrome.

Discussion

In this experiment, a dose of streptozotocin was found which was effective in destroying sufficient B-cells in

weanling ob/ob mice to result in insulin levels equivalent to those of lean mice. This effect was permanent, due to insufficient regeneration of B-cells following destruction by this drug.

Other investigators, using streptozotocin in older animals, have reported islet regeneration after initial B-cell necrosis [14]. The Birmingham ob/ob mice used in their study were 3 to 4 months old at treatment. Coleman [16] also found only transitory effects with streptozotocin in older ob/ob mice. The difference in permanence of effect may depend on the age at treatment and consequently the number of B-cells initially present. The background strain of the mice could also influence regeneration capability.

Table 1. Urinary glucose excretion of lean and obese mice 14 weeks after streptozotocin injection. Values are means $\pm$ the standard error of the mean

	n	Glucose (mg/24 h)
I Lean	3	< 0.02
II Lean-SZO	2	100 $\pm$ 50
III Obese	4	23 $\pm$ 11
IV Obese-SZO	4	78 $\pm$ 14
V Obese-SZO-INS	2	15 $\pm$ 3

I < II at $p \leq 0.05$

III < IV at $p \leq 0.025$

IV < V at $p \leq 0.025$

Table 2. Body lengths of control and streptozotocin-treated lean and obese mice 16 weeks after streptozotocin injection. Measurements were made from nose to anus. Values are means $\pm$ standard error of the mean

	n	Length (cm)
I Lean	5	9.3 $\pm$ 0.3
II Lean-SZO	2	9.0 $\pm$ 0.1
III Obese	5	9.5 $\pm$ 0.2
IV Obese-SZO	3	8.5 $\pm$ 0.4
V Obese-SZO-Ins	5	8.9 $\pm$ 0.1

IV < III at $p \leq 0.025$

V < III at $p \leq 0.01$

Table 3. Weights of epididymal fat pads as per cent body weight for control and streptozotocin-treated lean and obese mice. Values are means $\pm$ standard error of the mean

	n	Body weight (g)	Weight of fat pads (g)	Fat pads as % body weight
Lean	5	26.7 $\pm$ 0.4	0.56 $\pm$ 0.05	2.1
Lean-SZO	2	21.0 $\pm$ 0.7	0.10 $\pm$ 0.03	0.5
Obese	5	47.2 $\pm$ 1.9	2.82 $\pm$ 0.13	5.9
Obese-SZO	3	30.2 $\pm$ 2.4	2.00 $\pm$ 0.31	6.6
Obese-SZO-Ins	5	35.3 $\pm$ 2.0	2.06 $\pm$ 0.19	5.8

In this experiment, lean treated mice became diabetic, as their insulin levels averaged less than half normal values, and persistent hyperglycaemia and glucosuria resulted. The hyperphagia seen in these mice was undoubtedly a secondary result of the induced insulin insufficiency.

Obese mice treated with streptozotocin had insulin values that remained much lower than those of obese controls, yet were not lower than those of lean controls. Therefore, the hyperglycaemia and glucosuria seen in these mice is not a result of low levels of insulin, but rather of hyperphagia. That obese mice continue to overeat whether their insulin levels are high or low (comparable to lean mice), makes it apparent that their hyperphagia cannot be the result of hyperinsulinaemia. Rather, the hypersecretion of insulin normally seen in these obese mice must be an effect that is secondary to the increased food consumption and/or insulin resistance.

Marked insulin resistance is seen in these obese-streptozotocin mice without prior hyperinsulinaemia and without increased body weight. While lean, untreated mice could withstand no more than 5 U of exogenous insulin, obese-SZO mice were able to tolerate repeated injections of 30 U. It is not known what would be the maximum level these mice could survive, but clearly it is at least a factor of six times the lethal dose in normal lean mice.

Mahler and Szabo [17, 18] have reported that insulin sensitivity in the obese mouse can be restored by suppression of pancreatic islet cell hyperplasia. Their studies involved injection of alloxan in adult ob/ob mice after which they reported a reduction of immunoreactive insulin levels, but no increase in blood sugar, and no reduction in body weight. Perhaps this discrepancy between their results and those reported here is again due to the age of the mice treated, and to the fact that the older mice had already become obese and hyperinsulinaemic by the time of their study. Certainly in these younger treated mice the ability to tolerate huge amounts of injected insulin is evidence for persisting abnormal resistance to the hormone, even after islet hyperplasia has been considerably reduced.

While it is possible that this extreme resistance to exogenous insulin could be explained by greater development of insulin-binding antibodies in the ob/ob mice than in ob/+ or +/+ mice, this seems unlikely. More probable would be the explanation of insulin resistance due to the relative obesity of these mice.

It is impressive that the epididymal fat pads of these mice weighed almost four times as much as did those of lean mice, though body weights and insulin levels were not significantly different. That these mice were shorter, yet fatter, implies that adipose tissue is

more sensitive to insulin than is lean body mass. Stauffacher and Renold [19] have shown a relative resistance to insulin in ob/ob mice of muscle over adipose tissue when they compared glucose incorporation into adipose tissue lipids, following intraperitoneal injection of insulin and glucose ^{14}C .

The results of this long-term study clearly establish that hyperinsulinaemia is not necessary for insulin resistance or "obesity" in ob/ob mice. These conditions are most probably the result of early and persisting hyperphagia. The drive to overeat could lead to increased nursing and perhaps earlier and greater consumption of solid food in pre-weanling ob/ob mice. Overeating would result in greater deposition of fat and consequently resistance to endogenous insulin and hyperglycaemia and hyperinsulinaemia. Such a sequence is consistent with experiments suggesting that fat stores are increased in obese mice before circulating insulin levels are elevated [20].

References

1. Mayer, J., Bates, M. W., Dickie, M. M.: Hereditary diabetes in genetically obese mice. *Science* **113**, 746-747 (1951)
2. Bleisch, V. R., Mayer, J., Dickie, M. M.: Familial diabetes mellitus and insulin resistance associated with hyperplasia of the islands of Langerhans in mice. *Amer. J. Path.* **28**, 369-385 (1952)
3. Wrenshall, G. A., Andrus, S. B., Mayer, J.: High levels of pancreatic insulin coexistent with hyperplasia and degranulation of beta-cells in mice with the hereditary obese-hyperglycemic syndrome. *Endocrinology* **56**, 335-340 (1955)
4. Christophe, J., Dagenais, Y., Mayer, J.: Increased circulating insulin-like activity in obese-hyperglycemic mice. *Nature (Lond.)* **184**, 61-62 (1959)
5. Stauffacher, W., Lambert, E., Vecchio, D., Renold, A. E.: Measurements of insulin activities in pancreas and serum of mice with spontaneous ("obese" and "New Zealand obese") and induced (goldthioglucose) obesity and hyperglycemia, with consideration on the pathogenesis of the spontaneous syndrome. *Diabetologia* **3**, 230-237 (1967)
6. Genuth, S.: Hyperinsulinism in mice with genetically determined obesity. *Endocrinology* **84**, 386-391 (1969)
7. Abraham, R. R., Beloff-Chain, A.: Hormonal control of intermediary metabolism in obese hyperglycemic mice. I. The sensitivity and response to insulin in adipose tissue and muscle in vitro. *Diabetes* **20**, 522-534 (1971)
8. Bray, G. A., York, D. A.: Genetically transmitted obesity in rodents. *Physiol. Rev.* **51**, 598-646 (1971)
9. Stauffacher, W., Orci, L., Cameron, D. P., Burr, I. M., Renold, A. E.: Spontaneous hyperglycemia and/or obesity in laboratory rodents: An example of the possible usefulness of animal disease models with both genetic and environmental components. *Recent Progr. Hormone Res.* **27**, 41-95 (1971)
10. Stern, J. S., Hirsch, J.: Obesity and pancreatic function. In: *Handbook of Physiology*, Section 7 (eds. R. O. Greep, E. B. Aspmood), pp. 644-651. Washington: American Physiological Society 1972
11. Fales, F. W.: *Standard methods of clinical chemistry*, Vol. 4 (ed. D. Seligson), p. 101. New York: Academic Press 1963
12. Batchelor, B. R., Stern, J. S., Johnson, P. R., Mahler, R. J.: Ef-

- fects of streptozotocin on glucose metabolism, insulin response and adiposity in ob/ob mice. *Metabolism* **24**, 77–91 (1975)
13. Brosky, G., Logothetopoulos, J.: Streptozotocin diabetes in the mouse and guinea pig. *Diabetes* **18**, 606–611 (1969)
14. Findlay, J. A., Rookledge, K. A., Beloff-Chain, A., Lever, J. D.: A combined biochemical and histological study on the islets of Langerhans in the genetically obese hyperglycemic mouse and in the lean mouse, including observations on the effects of streptozotocin treatment. *J. Endocr.* **56**, 571–583 (1973)
15. Like, A. A., Chick, W. L.: Studies in the diabetic mutant mouse: I. Light microscopy and radioautography of pancreatic islets. *Diabetologia* **6**, 207–215 (1970)
16. Coleman, D. L.: Personal communication (1974)
17. Mahler, R. J., Szabo, O.: Restoration of insulin sensitivity in the obese mouse following suppression of pancreatic islet cell hyperplasia. *Israel J. med. Sci.*, **8**, 810–811 (1972)
18. Mahler, R. J., Szabo, O.: Amelioration of insulin resistance in obese mice. *Amer. J. Physiol.* **221**, 980–983 (1971)
19. Stauffacher, W., Renold, A. E.: Effect of insulin in vivo on diaphragm and adipose tissue of obese mice. *Amer. J. Physiol* **216**, 98–105 (1969)
20. Chlouverakis, E., Dade, F., Batt, R. A. L.: Glucose tolerance and time sequence of adiposity, hyperinsulinemia and hyperglycaemia in obese-hyperglycemic mice (ob/ob). *Metabolism* **19**, 687–693 (1970)

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