Muscarinic Stimulation and Antagonism and Glucoregulation in Nondiabetic and Obese Hyperglycemic Mice

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Plasma glucose and insulin responses to a muscarinic agonist (bethanechol chloride) and a muscarinic antagonist (atropine) were evaluated in obese C57BL/6J *ob/ob* mice and in lean C57BL/6J *+/?* mice. In lean *+/?* mice, plasma glucose decreased in response to 1 and 2 μg/g bethanechol chloride, whereas insulin increased significantly. In *ob/ob* mice, insulin increased remarkably in response to bethanechol administration (saline, 632 ± 80 μU/ml; 2 μg/g bethanechol chloride, 1794 ± 97 μU/ml; *n* = 10), but surprisingly, plasma glucose also rose significantly (saline, 230 ± 14 mg/dl; 2 μg/g bethanechol chloride, 363 ± 18 mg/dl, *n* = 10). This exaggerated hyperglycemia in *ob/ob* mice was not associated with significant changes in plasma glucagon. Furthermore, administration of propranolol hydrochloride did not diminish bethanechol chloride–induced hyperglycemia in *ob/ob* mice. Administration of atropine (2.5, 5, and 10 mg/kg body wt) induced a significant decrease in plasma insulin without changes in plasma glucose in *ob/ob* mice, whereas neither plasma insulin nor plasma glucose changed in lean mice. Finally, conversion of [^{14}C]alanine to glucose was increased in *ob/ob* mice after bethanechol chloride administration, indicating that muscarinic stimulation increases gluconeogenesis in an animal model of type II (non-insulin-dependent) diabetes.
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There is considerable evidence that the sympathetic nervous system plays a major role in glucose metabolism in nondiabetic and diabetic subjects (1–3). However, the role of the parasympathetic nervous system in glucoregulation has not been widely studied. Several reports have suggested a link between the parasympathetic nervous system and glucose homeostasis. Investigators have reported the presence of abundant cholinergic innervation in pancreatic islet cells (4,5). Electrical stimulation of the dorsal motor nucleus of the vagus nerve (6,7) and of the nerve itself (8–13) has been shown to stimulate insulin secretion from the β-cells in the pancreatic islets in several species of animals. Finally, parasympathetic agents (muscarinic agonists) are known to stimulate insulin secretion in mice (14), in dogs (15), and most important, in humans (16).

Nevertheless, it is not known to what extent the parasympathetic nervous system plays a role in diabetes mellitus. The genetically obese C57BL/6J *ob/ob* mouse is characterized by a syndrome of obesity, hyperinsulinemia, insulin resistance, hyperglycemia, and glucose intolerance and is regarded as a model for type II (non-insulin-dependent) diabetes mellitus. Previous reports from our laboratory clearly showed evidence of altered sympathetic nervous system function in these animals (2,3). Although other early reports suggested that basal hyperinsulinemia in *ob/ob* mice is caused by vagal hyperactivity (17,18), direct evidence of a cholinergic mechanism that could explain hyperglycemia in *ob/ob* mice is lacking. The purpose of this study is to investigate possible parasympathetic involvement in this animal mode of type II diabetes by evaluating the effects of muscarinic agonists on blood glucose and insulin.

RESEARCH DESIGN AND METHODS

Obese mice (C57BL/6J *ob/ob*) and their lean littermates (C57BL/6J *+/?*) were obtained from The Jackson Laboratory (Bar Harbor, ME) at 4 wk of age. Animals between 8 and 24 wk of age (body wt 40–60 g for *ob/ob*, 20–30 g for lean) were used for experiments, but the mean age of each strain was similar and not statistically significantly different.

Glucagon	1 ng/L = 1 pg/ml	Insulin	1 pM = 0.167 μU/ml
Glucose	1 mM = 18 mg/dl		

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Animals were housed in group cages with four or five animals per cage in an animal room with a 12-h light-dark day-night cycle. They were provided with water and laboratory chow (Ralston-Purina, St. Louis, MO) ad libitum; food was available to all animals before experimentation. Animals were accustomed to handling and blood sampling by retro-orbital sinus puncture for 1 mo before the start of the experiment to minimize distress. Bethanechol (carbamyl-β-methylcholine) chloride, atropine sulfate, propranolol hydrochloride, and regular insulin were dissolved in 0.9% NaCl.

DRUG TREATMENTS

Dose response. Different doses (0.5, 1, and 2 μg/g body wt) of bethanechol chloride were administered subcutaneously to both strains (n = 12–15), and blood was withdrawn 10 min after injection. Similarly, different doses (2.5, 5, and 10 mg/kg body wt) of atropine were given subcutaneously to both strains (n = 5–10), and samples were obtained 30 min after injection.

β-blockade. If bethanechol chloride induced a reflexive sympathetic excitation via cardiovascular reflex, administration of the adrenergic antagonist propranolol hydrochloride would prevent the bethanechol chloride–induced hyperglycemia seen in the *ob/ob* mice. Therefore, an experiment was conducted in which *ob/ob* mice (n = 5) were given an injection of 100 mg/10 g body wt i.p. saline or propranolol, followed 30 min later by injection of 2 μg/g body wt s.c. saline or bethanechol chloride. Blood was collected 10 min after saline or bethanechol chloride injection.

Effect of bethanechol chloride on glucagon. To test the hypothesis that bethanechol chloride induces a release of the counterregulatory hormone glucagon, animals were given a subcutaneous injection of saline (control), epinephrine (3 mg/10 g body wt [positive control]), or bethanechol chloride (2 μg/g body wt), and blood was collected 10 min later for analysis.

Hepatic glucose output. An experiment was conducted to investigate the possibility that administration of bethanechol chloride induces a direct increase in hepatic glucose output as approximated by an increase in gluconeogenesis. Most studies have focused on alanine as a substrate for gluconeogenesis because it has been shown to be the major amino acid converted to glucose in vitro (19) and the major amino acid extracted by the liver in vivo (20). Furthermore, previous experiments by Mobley et al. (21) have shown that *ob/ob* mice converted significantly more [^{14}C]alanine to [^{14}C]glucose after injection of [^{14}C]alanine than lean mice. Animals were given a simultaneous injection of [^{14}C]alanine (5 μCi/30 g body wt) and either saline or bethanechol (2 μg/g body wt) at time 0. Animals were then bled at 5, 10, 15, and 60 min postinjection.

Samples obtained from the hepatic glucose output experiment were analyzed for whole-blood glucose immediately after collection with a One-Touch glucose meter (Lifescan, Mountain View, CA). The remaining blood samples were centrifuged, and the plasma was analyzed for [^{14}C]glucose and for [^{14}C]alanine. Samples of plasma were deproteinized with perchloric acid (13%) and then applied to cation-exchange columns (AG 50W-X8, 50 mesh, H^+ form; Bio-Rad, Rockville Centre, NY) to remove unreacted [^{14}C]alanine. Samples were next applied to anion-exchange columns (AG1-X8, 50–100 mesh, HCo^{3-} form; Bio-Rad), producing an eluant that contained the [^{14}C]glucose. The glucose in the column eluant was then converted to gluconic acid with glucose oxidase and applied again to the anion-exchange columns, which trapped the gluconic acid. The gluconic acid was eluated with ammonium bicarbonate (1 M), and the resulting samples were counted.

At the indicated time, blood was sampled by retro-orbital sinus puncture into heparinized pipettes or by decapitation into K-EDTA blood tubes. Plasma was separated by centrifugation at 1000 × g for 3 min and stored at −40°C for later analysis.

Plasma glucose was analyzed with a Beckman glucose analyzer (Beckman, Brea, CA). Insulin was determined by double-antibody immunoassay with commercially available kits (Cambridge Medical Diagnostics, Billerica, MA). Glucagon was assayed by radioimmunoassay with a kit from Radioassay Systems (Carson City, CO). The antibody used in this kit is directed against porcine glucagon-HSA. The antibody does not cross-react with gut glucagon (0.0013%), porcine insulin (0.0005%), porcine gastrin (0.0005), or human ACTH (0.0002). In this assay as in many other glucagon assays, an additional large plasma protein, possibly a glucagon precursor, cross-reacts with the antibody, elevating basal levels above those typically observed in humans (18). In addition, elevated glucagon levels have been observed in plasma of fed *ob/ob* mice in several studies (22–24). To control for these problems, we demonstrated that epinephrine-induced increases in glucagon could be detected (RESULTS). Inter- and intra-assay coefficients of variation were 5 and 9% for insulin and 11 and 8% for glucagon.

All values are means ± SE. Results were analyzed by two- or three-way analysis of variance (ANOVA) and post hoc paired t test for the change in the same group, Student's t test for the different groups with equal variance, and Welch's t test for the different groups with unequal variance. Dose-response data for bethanechol chloride and atropine were analyzed by two-way ANOVA (dose × strain). Propranolol hydrochloride's effects were analyzed by three-way ANOVA (propranolol treatment × bethanechol chloride treatment × strain). Glucose-output data were analyzed by three-way ANOVA (bethanechol chloride treatment × strain × time).

RESULTS

Dose response to bethanechol chloride. The dose-related effects of bethanechol chloride on plasma glucose and insulin are shown in Fig. 1. In lean mice, plasma glucose decreased from 169 ± 3 mg/dl to 166 ± 6 (NS), 150 ± 4 (P < .01), and 140 ± 7 (P < .001) mg/dl in response to 0.5, 1, and 2 μg/g bethanechol chloride, respectively, whereas insulin increased from 27 ± 3 μU/ml to 39 ± 5 (P < .05), 33.4 ± 3.0 (NS), and 66 ± 13 (P < .001) μU/ml in response to 0.5, 1, and 2 μg/g bethanechol chloride. In contrast, plasma glucose in *ob/ob* mice showed a dose-related increase from 214 ± 11 mg/dl to 264 ± 8 (P < .01), 290 ± 10 (P < .001), and 336 ± 16 (P < .001) mg/dl in response to 0.5, 1, and 2 μg/g body wt bethanechol chloride, respectively, despite a striking increase in plasma insulin at each dose from 625 ± 60 μU/ml to 1061 ± 77 (P < .001), 1406 ± 115 (P < .001), and 1693 ± 90 (P < .001) μU/ml

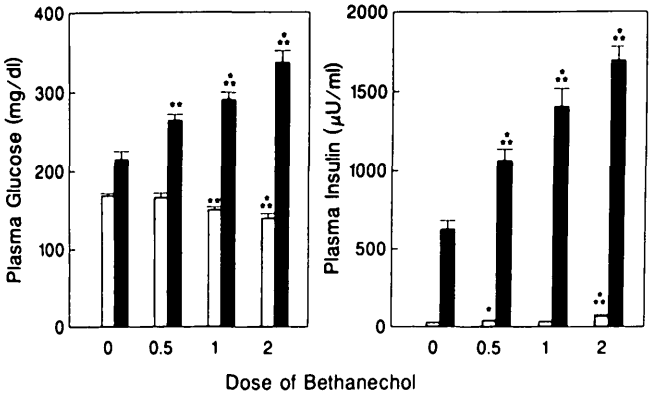

FIG. 1. Effect of various doses of bethanechol chloride (μg/g body wt) on plasma glucose and insulin in lean (*open bars*, *n* = 12–15) and *ob/ob* (*solid bars*, *n* = 12–15) mice. Values are means ± SE. **P* < .05, ***P* < .01, ****P* < .001, vs. controls (saline).

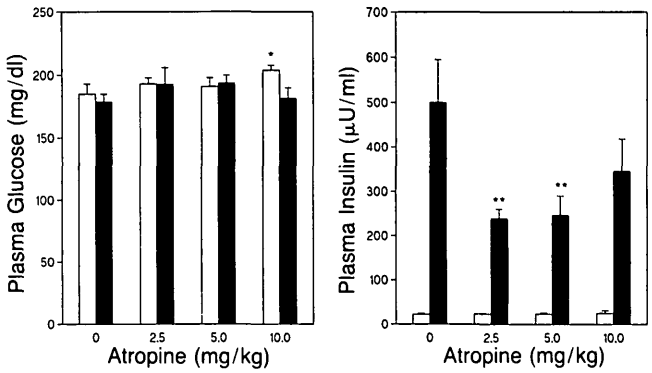

FIG. 2. Effect of various doses of atropine on plasma glucose and insulin in lean (*solid bars*, *n* = 5–10) and *ob/ob* (*open bars*, *n* = 5–10) mice. Values are means ± SE. **P* < .01, ***P* < .05, vs. controls (saline).

in response to 0.5, 1, and 2 μg/g of bethanechol chloride, respectively. The responses of plasma glucagon in *ob/ob* mice to epinephrine (3 mg/10 g body wt) and various doses of bethanechol chloride are shown in Table 1. Bethanechol chloride (2 μg/g) had no effect on plasma glucagon in *ob/ob* mice. A control study of epinephrine administration produced a significant rise in plasma glucagon from 741 ± 171 to 1224 ± 37 pg/ml ($P < .05$).

Dose response to atropine. The dose-related effects of atropine on plasma glucose and insulin are shown in Fig. 2. In lean mice, the highest dose of atropine (10 mg/kg body wt) was associated with a slight but significant increase in plasma glucose ($P < .05$), whereas in *ob/ob* mice, atropine did not change plasma glucose at any dose. However, a significant decrease in plasma insulin was produced in the *ob/ob* mice by the two lower doses (2.5 and 5 mg/kg) of atropine from 500 ± 95 μU/ml to 241 ± 21 ($P < .05$) and 247 ± 43 ($P < .05$) μU/ml, respectively. Despite changes in plasma glucose, lean mice did not show a significant change in plasma insulin.

Interaction of muscarinic stimulation and β-adrenergic blockade. Propranolol hydrochloride did not block the hyperglycemic effects of bethanechol chloride seen in the *ob/ob* mice. As shown in Fig. 3, propranolol hydrochloride (10 μg/g) caused a fall in plasma insulin from 453 ± 53 to 125 ± 30 μU/ml ($P < .001$) but did not change plasma glucose. Although there was a significant effect of propranolol hydrochloride alone, there was no significant interaction with the effects of bethanechol chloride in a two-way ANOVA, demonstrating that β-adrenergic blockade does not affect the glucose response to bethanechol chloride.

TABLE 1
Effect of epinephrine and various doses of bethanechol on plasma glucagon in *ob/ob* mice

	Plasma glucagon (pg/ml)
Control (saline)	741.0 ± 170.8
Epinephrine (3 μg/10 g body wt)	1224.1 ± 36.8 *
Bethanechol (μg/g body wt)	
0.5	926.7 ± 86.3
1	775.1 ± 76.7
2	871.2 ± 91.7

Values are means ± SE. $n = 5$.
*$P < .05$ vs. control.

Effect of bethanechol chloride on hepatic glucose output. The effect of bethanechol chloride on hepatic glucose output in lean and *ob/ob* mice is shown in Fig. 4. Bethanechol chloride increased conversion of alanine to glucose at 10, 15, and 60 min in *ob/ob* mice. The increased conversion was most pronounced after 15 min (saline, 127 ± 15% increase from initial value; 2 μg/g bethanechol chloride, 230 ± 34% increase from initial value; $n = 5$). In contrast, bethanechol chloride administration slightly decreased conversion of alanine to glucose in the lean mice. Obese (*ob/ob*) controls that received saline showed a significantly greater rate of conversion than did the lean controls at 15 min.

DISCUSSION

The major finding of this study is that *ob/ob* mice show altered responsiveness to muscarinic stimulation in the liver and β-cell. This implies that a dysfunction of the parasympathetic nervous system may play an important role in the etiology of hyperglycemia and hyperinsulinemia in this model of type II diabetes. Bethanechol chloride produced a profound hyperglycemia accompanied by an equally remarkable hyperinsulinemia, and atropine significantiy attenuated the baseline hyperinsulinemia seen in the *ob/ob* mouse. Lean euglycemic controls also showed an increase in plasma insulin in response to bethanechol chloride, but this was associated with a fall in blood glucose, and they displayed no response to atropine. These findings suggest that the *ob/ob* mouse has an exaggerated muscarinic receptor sensitivity and/or a tonic elevation in vagal activity that contribute to the persistent hyperinsulinemia seen in this animal. This study also shows that the paradoxical hyperglycemia seen in the *ob/ob* mouse in response to bethanechol chloride may be explained by an increase in hepatic glucose output.

The increased responsiveness to parasympathetic stimulation is supported by several previous experiments. Several studies have shown direct enhancement of insulin secretion by vagal (cholinergic-muscarinic) stimulation (6–13, 15,16). Moreover, Ahren and Lundquist (18) examined the effects of various agents that modify basal insulin secretion and found that only mannoheptulose and cholinergic blockade produced a significant difference between *ob/ob* and lean mice. They concluded that hyperinsulinemia in *ob/ob* mice is largely governed by enhanced responsiveness to

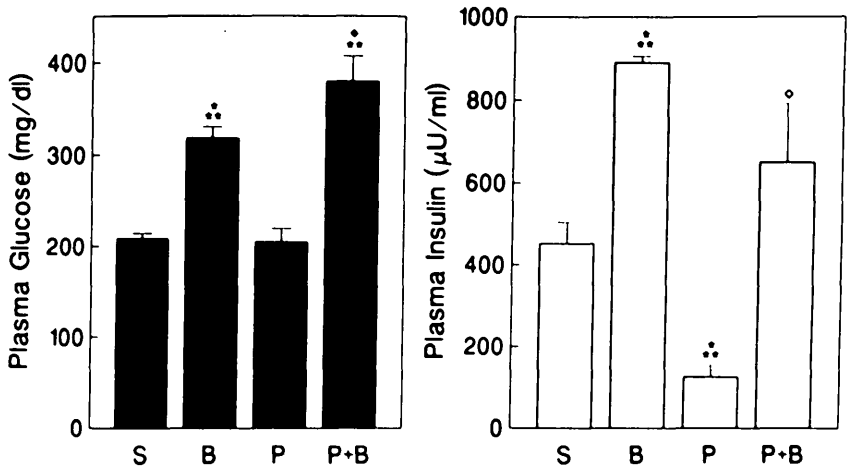

FIG. 3. Effect of bethanechol chloride (2 μg/g body wt) with propranolol hydrochloride ([P] 100 mg/10 g body wt) on plasma glucose and insulin in *ob/ob* mice (n = 5). S, saline; B, bethanechol chloride; P + B, propranolol hydrochloride + bethanechol hydrochloride. Values are means ± SE. **$P < .01$, ***$P < .001$, vs. controls (saline). ◇$P < .05$, ♦$P < .001$, vs. P.

normal and/or increased vagal activity. These experiments provide additional support for the idea that increased vagal activity contributes to the basal hyperinsulinemia in *ob/ob* mice.

However, previous experiments do not provide an explanation for the differential glucose response produced by bethanechol chloride in the *ob/ob* mice compared with their lean littermates. Although bethanechol chloride increased glucose markedly in *ob/ob* mice, it produced the expected fall in glucose of lean euglycemic controls. The latter results from the lean mice are consistent with a preliminary report by Lundquist (14), who administered carbachol to mice and noted an increase in plasma insulin levels, accompanied by a fall in blood glucose.

There are several potential explanations for the bethanechol chloride–induced hyperglycemia seen in the *ob/ob* mice. First, hyperglycemia in the *ob/ob* mice could result from the differentially induced release of counterregulatory hormones by bethanechol chloride. However, the rapidity with which bethanechol chloride induced a rise in plasma glucose (within 5 min) suggests that this phenomenon was not mediated by release of corticosterone or growth hormone because these substances take much longer to produce a hyperglycemic effect. In contrast, catecholamines and glucagon are plausible candidates, because both hormones antagonize the effect of insulin acutely (25).

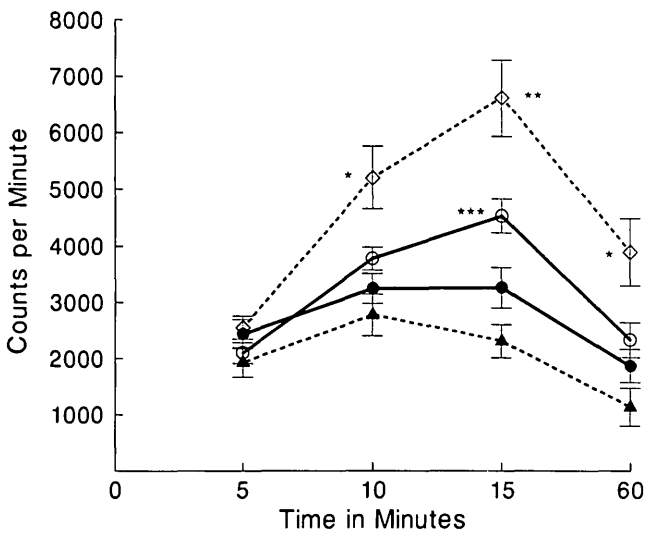

FIG. 4. Effect of bethanechol chloride (2 μg/g body wt) on gluconeogenesis (conversion of [^{14}C]alanine to [^{14}C]glucose) in lean (▲) and *ob/ob* (◇) mice (n = 5–10). Values are means ± SE. *$P < .05$, **$P < .02$, vs. *ob/ob* controls (○). ***$P < .05$ vs. lean controls (●).

The effect of bethanechol could be mediated by catecholamines in two different ways: via direct counterregulatory-hormone release or indirectly via sympathetic excitation induced by cardiovascular reflex. Administration of cholinergic muscarinic agonists into the cerebral ventricle of normoglycemic mice is known to increase hepatic glucose output via an increased epinephrine release (26). However, in our experiment, β-adrenergic blockade by propranolol hydrochloride failed to suppress bethanechol chloride–induced hyperglycemia in *ob/ob* mice, and previous work has shown that the α-adrenergic antagonist phentolamine also failed to suppress cholinergic hyperglycemia (8). Therefore, it is unlikely that bethanechol chloride produced its effect through some direct or indirect action on sympathetic activity.

The possibility that glucagon contributes to hyperglycemia was also investigated in this experiment. Several authors have reported that glucagon is released by electrical stimulation of the dorsal motor nucleus (7) of the vagus nerve or the vagus nerve itself (10,12,13,27) and by pharmacological muscarinic stimulation (28). Moreover, Shull and Mayer (29) have reported that comparable doses of glucagon produced a greater plasma glucose increase in *ob/ob* mice than in lean mice. Therefore, differentially induced secretion of glucagon by bethanechol chloride could explain the hyperglycemia in the *ob/ob* mice. However, this study showed no changes in glucagon after bethanechol chloride administration. In contrast, glucagon and glucose increased in response to epinephrine in the *ob/ob* mice. These results are not surprising, because glucagon is just one of many potential mediators of changes in plasma glucose, and previous studies have reported changes in plasma glucose independent of changes in plasma glucagon (24,30).

A third possible explanation for the bethanechol chloride–induced hyperglycemia seen in the *ob/ob* mice would be a direct effect of bethanechol chloride on insulin sensitivity. Because pharmacological agents are known to alter insulin sensitivity (31), it is possible that bethanechol chloride changes the degree of insulin sensitivity, thereby contrib-

uting to differential glycemic effects for lean and *ob/ob* mice. Although we have not directly examined this possibility, the *ob/ob* mice already have a very high level of insulin resistance (32). It is therefore unlikely that the hyperglycemic effect of bethanechol chloride is caused by a further increase of insulin resistance substantial enough to produce the profound hyperglycemia seen in this study.

A final possibility is that the discrepancy in glucose response to bethanechol chloride between the *ob/ob* and lean mice reflects a relative increase in hepatic glucose output in the *ob/ob* mice. The *ob/ob* mice have previously been shown to have increased levels of gluconeogenic enzymes (33) and a greater capacity to synthesize blood glucose by gluconeogenesis (21,34). Our results showed that, consistent with previous findings (21), *ob/ob* mice displayed significantly greater rates of gluconeogenesis than their lean littermates. Furthermore, bethanechol chloride actually accelerated glucose production in *ob/ob* mice, whereas it suppressed conversion of alanine to glucose in lean mice. The latter findings are consistent with recent studies showing that direct cholinergic muscarinic inhibition of hepatic glucose production occurs in nondiabetic humans (35). The likelihood that glycogenolysis is also a contributing factor must be considered. However, the possibility that glycogenolysis could produce a rapid and dramatic rise in blood glucose in *ob/ob* mice is quite remote considering the substantial resistance to liver glycogen mobilization in these animals (36).

Consequently, the most plausible explanation is that the hyperglycemic effect of bethanechol chloride is due to a direct increase in hepatic glucose output. This study suggests that increased muscarinic sensitivity creates both an increase in insulin secretion and an increase in gluconeogenesis that together in turn contribute to the insulin resistance and hyperglycemia in this animal model of type II diabetes. The failure of atropine to change blood glucose supports a stronger role for postsynaptic responsivity in bethanechol chloride–induced hyperglycemia. This is consistent with our previous demonstration that blood glucose is not tonically elevated in undisturbed *ob/ob* mice, although it is extremely responsive to stress (2). The model of diet-induced type II diabetes we recently developed (37) might prove to be a better model of human type II diabetes in this regard. The ability of low doses of atropine to decrease insulin levels implies however that vagal stimulation of insulin secretion might contribute to the persistent hyperinsulinemia that characterizes *ob/ob* mice. (The reason that high doses of atropine did not change insulin is not clear, but sympathetic reflexes could potentially play a role.) The lack of change in glucose despite this decrease in insulin suggests that atropine simultaneously blocked parasympathetic stimulation of hepatic glucose output as well. The changes induced by atropine indicate the possibility that complex, tissue-specific changes in presynaptic activity and postsynaptic receptor sensitivity could occur during the gradual development of insulin resistance in the *ob/ob* mouse and suggest that parallel evaluation of atropine's effects on hepatic glucose output and insulin secretion are vital to elucidate the potential role of tonic vagal activity in the glucoregulatory defects observed in these animals.

We have previously demonstrated that enhanced adrenergic sensitivity in *ob/ob* mice contributes to hyperglycemia, and we speculated that this defect contributes to the etiology of the disease (37,38). These data suggest that the hyperinsulinemia and elevated hepatic glucose output that also characterize this animal are partly due to abnormal responses to parasympathetic nervous system activity as well. These findings raise the possibility that the etiology of the diabetic syndrome of the *ob/ob* mouse may be related to a problem in central autonomic regulation of glucose metabolism.

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