Leptin-Deficient Mice Commence Hypersecreting Insulin in Response to Acetylcholine between 1 and 2 Weeks of Age

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Leptin-deficient Lep^{ob}/Lep^{ob} mice develop hyperinsulinemia early in life, before they begin to overeat or develop insulin resistance. Pancreatic islets from these young mice do not yet hypersecrete insulin in response to glucose, but they hyperrespond to acetylcholine. Islets from 4-day, and 1-, 2-, and 4-week-old mice were used in the present study to determine when leptin-deficient mice first hypersecrete insulin in response to acetylcholine. This relative hypersecretion of insulin from islets of leptin-deficient mice occurred between 1 and 2 weeks of age. The divergence in insulin secretion occurred at this time because islets from lean, leptin-sufficient mice became relatively less responsive to acetylcholine between 1 and 2 weeks of age, whereas islets from leptin-deficient mice maintained a high responsiveness to acetylcholine during development. Leptin addition to islets isolated from 4-day, and 2-, and 4-week-old leptin-deficient mice rapidly (i.e., within 30 min) suppressed acetylcholine-induced insulin secretion at each stage of development. In contrast, islets from 4-day, and 2- and 4-week-old leptin-sufficient mice became progressively less responsive to leptin with development. Leptin targets pancreatic islets early in development to specifically constrain the overall capacity for acetylcholine-induced insulin secretion, and to acutely modulate this secretion. [Exp Biol Med Vol. 226(10):906–911, 2001]

Key words: insulin secretion; leptin; Lep^{ob}/Lep^{ob} mice; pancreatic islets

Hyperinsulinemia is detectable early in development of Lep^{ob}/Lep^{ob} mice before they begin to overeat or develop insulin resistance and observable obesity (1). Pancreatic islets from 2-week-old Lep^{ob}/Lep^{ob} mice, the earliest age examined, do not yet hypersecrete insulin in response to glucose, but are already hypersensitive and hyperresponsive to acetylcholine potentiation of glucose-induced insulin secretion (2). Presumably this acetylcholine-potentiated hypersecretion of insulin contributes to the early-onset hyperinsulinemia characteristic of these Lep^{ob}/Lep^{ob} mice.

Since an inability of Lep^{ob}/Lep^{ob} mice to synthesize the adipose tissue-derived polypeptide leptin is now known to be the primary cause of obesity in these animals (3), it has been speculated that leptin acts within pancreatic islets to inhibit insulin secretion (4–11). Leptin receptors are present in pancreatic islets and in insulin-secreting cell lines (5, 7). Longer-term exposure of islets or insulin-secreting cell lines to exogenous leptin lowers insulin mRNA abundance and insulin synthesis (7, 12). This action of leptin provides one potential mechanism to prevent hyperinsulinemia. Other studies have examined more acute effects of leptin on insulin secretion *per se*. Leptin has been shown to inhibit insulin secretion in some studies (4–11), but not in others (13, 14). Since leptin-deficient Lep^{ob}/Lep^{ob} mice as early as 2 weeks of age exhibit a specific enhancement in acetylcholine-induced insulin secretion (2, 15) this pathway might be a target for leptin action. Indeed, addition of leptin to islets from 4-week-old Lep^{ob}/Lep^{ob} mice rapidly abolishes their enhanced acetylcholine potentiation of insulin secretion (4).

A characterization of the temporal relationship between the initial development of enhanced acetylcholine-potentiated insulin secretion from islets of neonatal Lep^{ob}/Lep^{ob} mice and of the effects of leptin on this pathway should add to our understanding of how hyperinsulinemia develops in these mice. The present study was thus conducted to first determine when the enhanced insulin secretion response to acetylcholine initially appears in Lep^{ob}/Lep^{ob} mice by examining mice younger than 2 weeks of age. Comparisons of insulin secretion from islets of +/+ versus $Lep^{ob}/+$ mice were included to determine if a single copy of the mutated Lep^{ob} gene would enhance acetylcholine-induced insulin secretion. The second aim of this study

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was to examine the role of leptin in modulating acetylcholine-induced insulin secretion from islets of young Lep^{ob}/Lep^{ob} and lean mice. Neonatal (4-day-old), and 2-, and 4-week-old mice were used to determine if the insulin-secretion response of islets to leptin changes as neonatal mice develop.

Materials and Methods

Animals. Lep^{ob}/Lep^{ob} mice and lean (Lep^{ob}/+ and/or +/+) mice were obtained from our breeding colony (C57BL/6J-Lep^{ob}/+). The *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985) and local institutional guidelines were followed for the care and treatment of the mice. They were housed in solid-bottom cages with wood shavings for bedding in a room maintained at 25°C with a 12:12-hr light:dark cycle (lights on at 0700 hr). Mice were fed a nonpurified diet (Teklad Rodent Diet 8640; Harlan, Bartonville, IL). Litters were adjusted to six pups per litter within a few days after birth. Mice were weaned at 3 weeks of age. Approximately equal numbers of male and female mice were assigned to each group. Mice were used at 4 days, and 1, 2, and 4 to 5 weeks of age as noted in the experimental design. Littermate mice were compared in selected trials as noted in the table footnotes and figure legends.

Experimental Design. Experiment 1 involved glucose and acetylcholine-potentiated insulin secretion from islets of neonatal mice. Islets from 1- and 2-week-old +/+, Lep^{ob}/+, and Lep^{ob}/Lep^{ob} mice were incubated for three consecutive 30-min periods in Krebs-Ringer bicarbonate buffer (KRB, pH 7.4) with 0.1% bovine serum albumin (BSA, Amoresco, Solon, OH), and containing 0.5 mM glucose during the first 30-min period, 20 mM glucose during the second period, and finally 20 mM glucose + 10 μM acetylcholine (Sigma Chemical, St. Louis, MO) during the last period. Liver samples were obtained to genotype each pup to retrospectively separate pups into groups, i.e., +/+, Lep^{ob}/+, and Lep^{ob}/Lep^{ob}. Body weights and abdominal body fat pads were measured.

Experiment 2 questioned leptin effects on acetylcholine-potentiated insulin secretion. The role of leptin (murine leptin, a generous gift from Pfizer Central Research, Groton, CT) to regulate insulin secretion potentiated by the acetylcholine signaling pathway was examined. Islets from lean (+/+ or Lep^{ob}/+) and Lep^{ob}/Lep^{ob} mice at 4 days, and 2, and 4 to 5 weeks of age were incubated for three 30-min periods in 37°C KRB with 0.1% BSA, and containing 0.5 mM glucose, then 10 mM glucose, and finally 10 mM glucose + 10 μM acetylcholine ± 20 nM leptin during the last 30-min period, respectively. This dose of leptin was previously shown to maximally inhibit acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} (4). Lean and Lep^{ob}/Lep^{ob} mice at 4 days and 2 weeks of age were genotyped for identification. They were identified visually at 4 weeks of age.

Islet Isolation. Pancreatic islets were isolated by collagenase type V (Sigma Chemical) digestion (16). Pancreases from 4-day, and 1-, 2-, and from 4- to 5-week-old mice were injected in multiple sites with a total of 3 ml of 37°C KRB (pH 7.4) containing 0.5 mM glucose, 0.01% BSA, and 0.5, 1, and 2.5 mg collagenase per milliliter, respectively. Each pancreas was then quickly dissected and transferred into a small tube containing 0.5 ml of 37°C KRB and 0.5 mg collagenase per milliliter, and was incubated at 37°C with gentle shaking for about 2 to 3 min. Ice-cold KRB was then added to stop the digestion. After washing two to three times with ice-cold KRB to remove digested acinar tissue and collagenase, isolated islets were selected with the aid of a pipette under a stereoscopic microscope.

Insulin Secretion and Measurement of Insulin. Similar-sized islets (7–10 islets/mouse) at each age were selected and distributed into small black-bottom petri dishes. Islets were preincubated at 37°C for 30 min under a 95% O_2-5% CO_2 atmosphere in 1 ml of KRB containing 0.5 mM glucose and 0.1% BSA. This 30-min preincubation was followed by 30-min consecutive incubations in KRB containing various treatments.

We demonstrated in an earlier study that insulin secretion from islets of 2-week-old mice remained constant for 1 hr when islets were exposed to 20 mM glucose (2). We confirmed this observation in the present study. Islets from 2-week-old lean mice secreted 0.6 ± 0.1 fmol insulin/islet/min when maintained in 0.5 mM glucose for 30 min, and then 2.4 ± 0.2 fmol and 2.2 ± 0.4 fmol insulin/islet/min in the next two 30-min consecutive periods, respectively (n = 5 mice). To measure insulin secretion from islets stimulated by various secretogogues, 0.5 ml of incubation media was collected. Islets secreting more than 2 fmol insulin/islet/min in 0.5 mM glucose were considered damaged during isolation. Data from these islets were excluded.

Insulin was quantified by an enzyme-linked-immunosorbent assay (17). Rabbit anti-guinea pig IgG and guinea pig anti-rat insulin were purchased from EY Lab (San Medeo, CA) and Linco Research (St. Louis, MO), respectively. Rat insulin standard was purchased from Crystal Chemical (Chicago, IL). Peroxidase-labeled insulin was obtained from Sigma Chemical (St. Louis, MO).

Genotyping. DNA was extracted from livers of mice (4-day, and 1- and 2-week-old mice) by a modified phenol extraction method (18) and was used to distinguish the Lep^{ob}/Lep^{ob}, Lep^{ob}/+, and +/+ mice. Two different sense primers (i.e., the wild and mutant types) paired with same antisense primer were used (19). PCR products were electrophoresed on 3.5% Nuseive 3:1 agarose gels (FMC Bioproducts, Rockland, ME) and were stained with ethidium bromide (Sigma Chemical). DNA from known Lep^{ob}/+ mice was used as a control. Homozygous lean and Lep^{ob}/Lep^{ob} mice exhibit 100-bp bands amplified in the presence of wild-type and mutant-type primers, respectively. Heterozygous mice exhibit a 100-bp band am-

plified in the presence of both wild-type and mutant-type primers.

Statistical Analysis. Data were presented as means ± SE. Data in Experiment 1 for 1- and 2-week-old Lep^{ob}/Lep^{ob} versus lean littermates, and for 1-week-old +/+ versus Lep^{ob}/+ littermates were analyzed by the Student's paired t test. Comparisons of 2-week-old +/+ versus Lep^{ob}/+ mice were analyzed by the Student's unpaired t test because littermates were not always available. Effects of phenotype, leptin, and phenotype-leptin interactions on insulin secretion in Experiment 2 were analyzed by two-way analysis of variance (ANOVA) in conjunction with LSD adjustment. Differences were considered statistically significant at $P < 0.05$.

Results

A single copy of the mutated Lep^{ob} gene did not influence body weight and fat pad weights of 1- and 2-week-old pups (+/+ versus Lep^{ob}/+ pups) (Table I). Although 1- and 2-week-old Lep^{ob}/Lep^{ob} and lean (+/+ or Lep^{ob}/+) littermates had similar body weights, abdominal fat pads of Lep^{ob}/Lep^{ob} mice were already enlarged (Table I).

Glucose and Acetylcholine-Potentiated Insulin Secretion from Neonatal Mice. Islets from 1-week-old +/+ and Lep^{ob}/+ lean mice secreted similar amounts of insulin in response to 20 mM glucose, as well as in response to 20 mM glucose + 10 μM acetylcholine (Fig. 1). At 2 weeks of age, insulin secretion also remained unaffected by a single copy of the mutated Lep^{ob} gene. In subsequent trials, data from +/+ and Lep^{ob}/+ mice (i.e., lean mice) were combined for comparisons of lean versus Lep^{ob}/Lep^{ob} mice.

Enhanced insulin secretion in response to acetylcholine has been reported in 2-week-old Lep^{ob}/Lep^{ob} mice (2, 15). To determine whether this alteration in insulin secretion occurs in islets from younger Lep^{ob}/Lep^{ob} mice, 1-week-old mice were examined. Islets from Lep^{ob}/Lep^{ob} mice and their lean littermates secreted similar amounts of insulin in response to 20 mM glucose at either 1 or 2 weeks of age (Fig. 2). Addition of 10 μM acetylcholine similarly increased insulin secretion from islets of 1-week-old Lep^{ob}/Lep^{ob} mice and lean littermates. But as observed previously (2, 15), islets from 2-week-old Lep^{ob}/Lep^{ob} mice secreted more insulin in the presence of acetylcholine than did islets from lean littermates (Fig. 2). Thus, the enhanced acetylcholine-induced insulin secretion in Lep^{ob}/Lep^{ob} pups first occurs between 1 and 2 weeks of age.

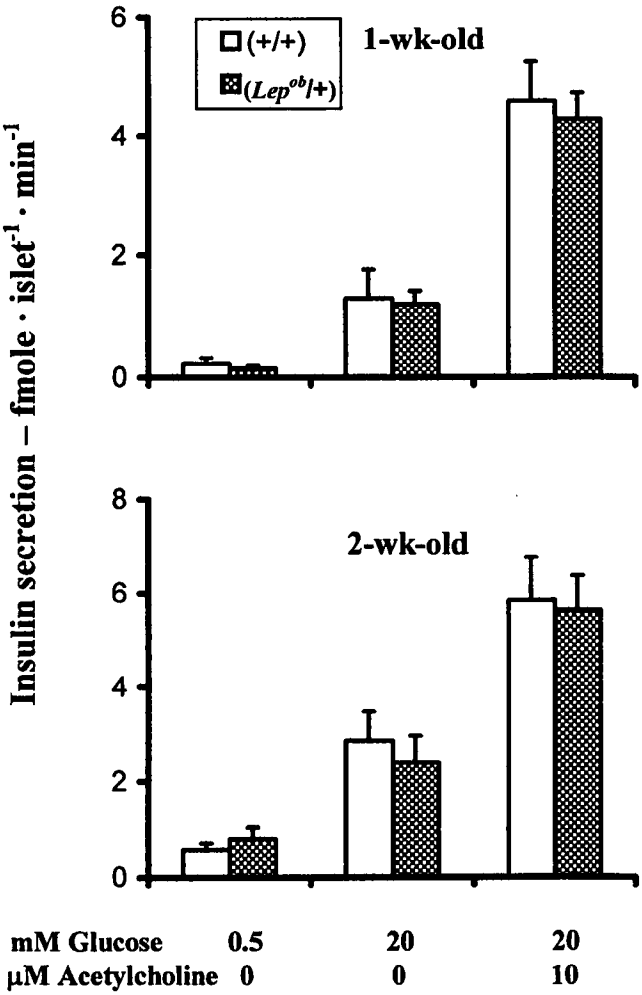

Figure 1. Insulin secretion from islets of homozygote (+/+) and heterozygote (Lep^{ob}/+) lean mice. Islets from 1-week-old (n = 7) and 2-week-old (n = 6) mice were incubated for 30 min in 0.5 mM glucose, then in 20 mM glucose for 30 min, and finally in 20 mM glucose + 10 μM acetylcholine for 30 min. Heterozygosity did not influence glucose or acetylcholine-potentiated insulin secretion at either age as determined by Student's t test ($P > 0.05$).

Table I. Comparisons of Body and Abdominal Fat Pad Weights of +/+, Lep^{ob}/+, and Lep^{ob}/Lep^{ob} Mice

Parameter	1-week-old		2-week-old	
	+/+ (7)	Lep^{ob}/+ (7)	+/+ (6)	Lep^{ob}/+ (6)
Body weight (g)	4.0 ± 0.2	4.0 ± 0.2	7.3 ± 0.2	7.0 ± 0.2
Fat pad (mg)	47 ± 5	54 ± 4	94 ± 9	94 ± 12
	lean (6)	Lep^{ob}/Lep^{ob} (6)	lean (5)	Lep^{ob}/Lep^{ob} (5)
Body weight (g)	3.7 ± 0.1	4.1 ± 0.1	7.0 ± 0.2	7.5 ± 0.3
Fat pad (mg)	48 ± 3	81 ± 7[a]	84 ± 9	235 ± 33[a]

Note. Values are means ± SE. Numbers of animals are indicated in parentheses. Data were analyzed by the Student's paired t test utilizing littermate lean (+/+ or Lep^{ob}/+) and Lep^{ob}/Lep^{ob} pairs, and +/+ and Lep^{ob}/+ pairs, except for the comparison between 2-week-old +/+ and Lep^{ob}/+ mice, which was made with the Student's unpaired t test.
[a] Indicates significant differences ($P < 0.05$).

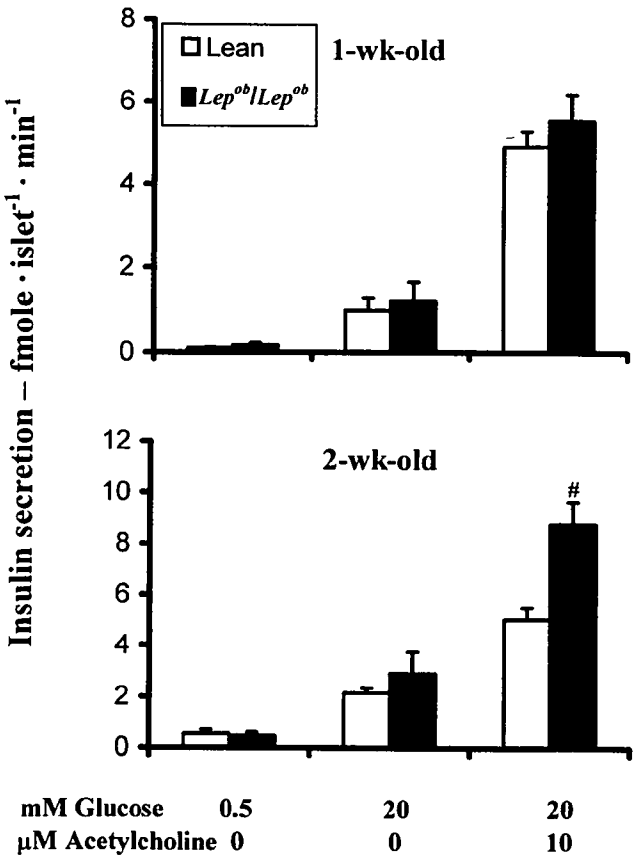

Figure 2. Insulin secretion from pancreatic islets of 1- and 2-week-old mice. Islets from 1- week-old (n = 6) and 2-week-old (n = 5) Lep^{ob}/Lep^{ob} and lean littermate mice were incubated in 0.5 mM glucose for 30 min, and then in 20 mM glucose for 30 min, followed by 20 mM glucose + 10 μM acetylcholine for 30 min. Diameters of the islets from 1-week-old Lep^{ob}/Lep^{ob} and lean littermates averaged 0.098 ± 0.004 mm and 0.095 ± 0.002 mm, respectively. Diameters of the islets from 2-week-old Lep^{ob}/Lep^{ob} and lean littermates averaged 0.111 ± 0.002 mm and 0.107 ± 0.001 mm, respectively. Data represent means ± SE. Phenotype effects on insulin secretion stimulated by 20 mM glucose and by 20 mM glucose plus 10 μM acetylcholine were determined by Student's paired t test. A significant (#P < 0.05) phenotype effect on insulin secretion was observed in the presence of acetylcholine at 2 weeks of age.

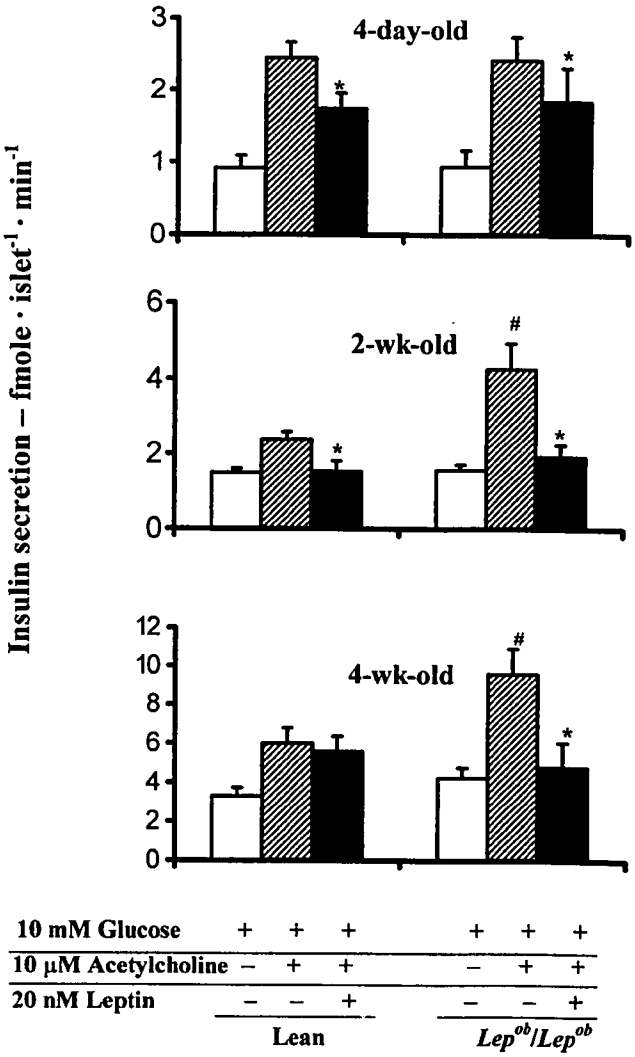

Figure 3. Effects of leptin on acetylcholine potentiation of glucose-induced insulin secretion. Islets from 4-day, and 2- and 4-week-old Lep^{ob}/Lep^{ob} and lean counterparts (n = 11–23, 9–11, and 9–12 mice at 4 days, and 2 and 4 weeks of age, respectively) were incubated in 0.5 mM glucose for 30 min, and then in 10 mM glucose for 30 min, followed by 10 mM glucose + 10 μM acetylcholine ± 20 nM leptin for 30 min. Data represent means ± SE. Significant (P < 0.05) effects of leptin on acetylcholine potentiation of insulin secretion as analyzed by two-way ANOVA in conjunction with LSD adjustment, are indicated by the asterisks. # indicates a significant effect of phenotype on acetylcholine-induced insulin secretion.

Effects of Leptin on Acetylcholine-Potentiated Insulin Secretion. At 4 days of age, acetylcholine-potentiated insulin secretion is equal from islets of lean and Lep^{ob}/Lep^{ob} mice (Fig. 3, upper panel), consistent with observations in 1-week-old mice (Fig. 2). At this age, leptin suppressed acetylcholine-potentiated insulin secretion equally from islets of lean and Lep^{ob}/Lep^{ob} pups (Fig. 3, upper panel). At 2 weeks of age, Lep^{ob}/Lep^{ob} mice begin to hypersecrete insulin in response to acetylcholine (Figs. 2 and 3, middle panel). At this age, islets from both lean and Lep^{ob}/Lep^{ob} mice respond to leptin with lowered acetylcholine-induced insulin secretion (Fig. 3, middle panel). Leptin no longer inhibited *in vitro* acetylcholine-potentiated insulin secretion from islets of young adult (4-week-old) lean mice, but leptin continued to suppress acetylcholine-induced insulin secretion from islets of adult leptin-deficient Lep^{ob}/Lep^{ob} mice (Fig. 3, lower panel).

Discussion

The two major findings from the present study are that hypersecretion of insulin in response to acetylcholine first appears in islets from Lep^{ob}/Lep^{ob} mice between 1 and 2 weeks of age, and that the effectiveness of leptin to constrain acetylcholine-induced insulin secretion persists in islets of Lep^{ob}/Lep^{ob} mice, but moderates in islets from lean mice, between 4 days and 4 weeks of age.

Comparisons of adult lean Lep^{ob}/+ versus +/+ mice indicate that inheritance of a single copy of the mutated Lep^{ob} gene may cause subtle metabolic effects (20). Since one purpose of the present study was to determine when the enhanced insulin secretion response to acetylcholine first appears in Lep^{ob}/Lep^{ob} mice, it was important to determine if inclusion of pups with a single copy of the mutated Lep^{ob} gene would confound this determination. Pups with a single copy of the mutated Lep^{ob} gene were indistinguishable from +/+ pups (Table I and Fig. 1). This enabled us to pool results from +/+ or Lep^{ob}/+ mice in comparison with Lep^{ob}/Lep^{ob} mice.

Acetylcholine potentiates glucose-induced insulin secretion by activating muscarinic receptors to stimulate the phospholipase C-protein kinase C (PLC-PKC) pathway (21). This stimulatory pathway is already present in islets from neonatal mice and it substantially elevates insulin secretion above rates observed in the presence of glucose alone (Figs. 1–3). In 4-day and 1-week-old pups, acetylcholine increased glucose-induced insulin secretion by 169% to 354% (Figs. 1–3). Acetylcholine continued to stimulate insulin secretion from islets of 2- and 4-week-old lean mice, but the percentage increases above glucose-induced insulin were less pronounced (i.e., 81%–103%; Figs. 1–3). In contrast to the age-associated decline in percentage increase in glucose-induced insulin secretion caused by exposure of islets from lean mice to acetylcholine, islets from Lep^{ob}/Lep^{ob} mice continued to respond to acetylcholine with increases in insulin secretion of 186% at 2 weeks of age (Figs. 2 and 3) and 125% at 4 weeks of age (Fig. 3). This failure of islets from Lep^{ob}/Lep^{ob} mice to decrease their stimulatory response to acetylcholine between 1 and 2 weeks of age as much as occurred in islets from lean mice explains why islets from 2-week-old Lep^{ob}/Lep^{ob} mice secrete more insulin in response to acetylcholine than islets from lean mice (Figs. 2 and 3). This implies that during normal development, some constraint of the PLC-PKC pathway in pancreatic islets emerges between 1 and 2 weeks of age to control insulin secretion. As discussed below, the presence of leptin in lean mice appears critically important at this stage of development.

Leptin acutely inhibited acetylcholine-induced insulin secretion from islets of 4-day-old lean and Lep^{ob}/Lep^{ob} pups (Fig. 3). This effect of leptin on islets occurred considerably earlier in development than effects of leptin on food intake or metabolic rate, which are not evident until after 2 weeks of age in these mice (22). It is not clear whether the leptin signal transduction system *per se* matures earlier in islets than in the hypothalamus, or whether other components of these downstream physiological response pathways emerge at differential times during development.

Islets from 2-week-old lean mice continued to respond to acute exposure to leptin with lowered acetylcholine-induced insulin secretion, but this acute effect of leptin on insulin secretion was no longer evident in islets from 4-week-old lean mice (Fig. 3). Islets from 4-week-old leptin-deficient Lep^{ob}/Lep^{ob} mice, however, continued to respond to leptin (Fig. 3). These results suggest that continued exposure of islets to leptin, as occurs *in vivo* in lean mice (22), diminishes the acute effect of leptin on acetylcholine-induced insulin secretion. The chronic *in vivo* exposure of islets in lean mice to leptin may have also constrained the capacity of these islets to increase insulin secretion in response to acetylcholine. These effects of leptin on pancreatic islets, coupled with effects on food intake that emerge after 2 weeks of age (22), help coordinate the regulation of insulin secretion in lean mice.

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