Leptin Administration Normalizes Insulin Secretion from Islets of *Lep^{ob}/Lep^{ob}* Mice by Food Intake-Dependent and -Independent Mechanisms

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Leptin-deficient *Lep^{ob}/Lep^{ob}* mice exhibit elevations in plasma insulin early in development. The present study tested the hypothesis that absence of leptin during neonatal development permanently programs islets from these mice to hypersecrete insulin. Administration of leptin for 8 days to young adult *Lep^{ob}/Lep^{ob}* mice normalized their food intake, plasma insulin concentration, and insulin secretion in response to glucose, acetylcholine, and leptin. Restriction of food intake per se of *Lep^{ob}/Lep^{ob}* mice lowered, but did not normalize, plasma insulin concentrations. Food-restricted *Lep^{ob}/Lep^{ob}* mice continued to hypersecrete insulin in response to glucose, but islets from these mice did not hyperrespond to acetylcholine or respond to leptin as occurs in *ad libitum*-fed *Lep^{ob}/Lep^{ob}* mice. We conclude that neonatal leptin deficiency does not permanently program islets from mice to hypersecrete insulin. The hyperphagia associated with leptin deficiency contributes substantially to the hypersecretion of insulin, but leptin also appears to have more direct effects on regulation of insulin secretion. Exp Biol Med 228:183–187, 2003

Key words: *Lep^{ob}/Lep^{ob}* mice; leptin administration; insulin secretion; acetylcholine; phosphatidylinositol 3-kinase

Leptin-deficient *Lep^{ob}/Lep^{ob}* mice exhibit elevations in plasma insulin early in development (1), before alterations in food intake are evident (2). Pancreatic islets from 1- to 2-week-old *Lep^{ob}/Lep^{ob}* mice do not hypersecrete insulin in response to glucose (3) or activators of the protein kinase A (PKA) signal transduction pathway, such as GLP-1 (4), but specifically hypersecrete insulin in response to activators of the phospholipase C-protein kinase C (PLC-PKC) signal transduction pathway such as acetylcholine and cholecystokinin (5). The addition of leptin to the media suppresses this hypersecretion of insulin in response to acetylcholine (3, 6), possibly via activation of phosphatidylinositol 3-kinase (PI 3-K) (4). These results indicate that leptin functions early in development (i.e., within the first several weeks of age) to regulate a pathway important in the control of plasma insulin concentrations.

The late fetal and early postnatal periods are critical in the development of the pancreas (7). The presence or absence of specific stimuli during this period may have permanent effects on the ability of islets to regulate insulin secretion. For example, a brief exposure to a high-carbohydrate diet during the suckling period causes rat pups to hypersecrete insulin as adults (8–12). Adrenalectomy of young adult *Lep^{ob}/Lep^{ob}* mice normalizes their plasma insulin, but when these mice are fed a high-glucose diet, their hyperinsulinemia reappears (13–15). And, when islets from young adult adrenalectomized *Lep^{ob}/Lep^{ob}* mice are exposed to acetylcholine, the islets still hypersecrete insulin (16). These results raise the possibility that the absence of leptin during neonatal development of *Lep^{ob}/Lep^{ob}* mice permanently programs their islets to hypersecrete insulin when challenged. Although chronic administration of leptin to adult *Lep^{ob}/Lep^{ob}* mice normalize their plasma insulin concentrations (17–20), islets from these mice have not previously been challenged with acetylcholine to determine if the absence of leptin during neonatal development permanently alters their ability to respond normally to acetylcholine.

The present study was undertaken to determine the effects of chronic administration of leptin to *Lep^{ob}/Lep^{ob}* mice on the regulation of insulin secretion from their isolated islets. Islets from these mice were exposed to acetylcholine and leptin. Because leptin lowers food intake, which would

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be expected to lower insulin secretion, a group of Lep^{ob}/Lep^{ob} mice were pair-fed to the leptin-treated Lep^{ob}/Lep^{ob} mice to assess the effects of lowered food intake per se.

Materials and Methods

Animals. Lep^{ob}/Lep^{ob} and lean mice were obtained from our C57BL/6J-$Lep^{ob/+}$ breeding colony. Mice were housed in plastic cages with wood shavings for bedding and were maintained in a room at 25°C with a 12:12-hr light: dark cycle (lights on at 0700 hr). They were fed a nonpurified, commercial diet (Rodent Diet 8640; Harlan Teklad, Madison, WI). Litters were adjusted to six pups per litter within a few days after birth. We attempted to equalize the number of male and female pups within each litter during adjustment. Mice were weaned at 3 weeks of age. Lep^{ob}/Lep^{ob} and lean mice were identified visually and were used at 4 to 5 weeks of age. They were individually housed for 3 days before study and throughout the 8-day experiments. The care and treatment of the mice were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985) and local institutional guidelines.

Leptin Administration. Leptin (PeproTech, Rocky Hill, NJ) was administered to Lep^{ob}/Lep^{ob} mice to lower their food intake to approximate the intake of lean mice. Intraperitoneal injections of leptin (100 μg/d for 4 days, and then 50 μg/d for the last 4 days of the 8-day study) and vehicle (150 μl of 0.01 *M* phosphate-buffered saline [PBS]) were made twice daily (at 0900–0930 hr, and at 1730–1800 hr).

Islet Isolation and Incubation. Pancreatic islets were isolated with collagenase type V (Sigma Chemical, St. Louis, MO) digestion as described previously (3, 21). Isolated islets were selected with the aid of a pipette under a stereoscopic microscope.

Similar-sized islets from single mice (10 islets/dish) were distributed into 35-mm black-bottom petri dishes. Islets were statically incubated at 37°C for 30 min under a 95% O_2/5% CO_2 atmosphere in 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.5 m*M* glucose and 0.1% bovine serum albumin (Amoresco, Solon, OH). Consecutively, islets were incubated in Krebs-Ringer bicarbonate buffer containing 10 m*M* glucose for 30 min, and then in 10 m*M* glucose and 10 μ*M* acetylcholine ± 20 n*M* leptin for 30 min (Experiment 1), or 10 m*M* glucose and 10 μ*M* acetylcholine ± 20 n*M* leptin and ± 20 n*M* wortmannin for 30 min (Experiment 2) as indicated in the figures. Islets secreting more than 2 f*M* insulin islet^{-1} min^{-1} in 0.5 m*M* glucose were considered damaged during isolation. Data from these mice were excluded.

To measure insulin secretion, 0.5 ml of incubation media was collected. Insulin was analyzed by enzyme-linked immunoabsorbant assay (ELISA) (22). Diameters of islets were measured with the aid of a stereoscopic microscope after the incubation.

Experimental Design. *Experiment 1.* Lep^{ob}/Lep^{ob} mice were administered leptin twice daily for 8 days and were fed *ad libitum*. Additional Lep^{ob}/Lep^{ob} mice were fed *ad libitum* or were pair-fed to the leptin-treated Lep^{ob}/Lep^{ob} mice. These control mice were injected twice daily with vehicle. Lean mice ($Lep^{ob/+}$ or $^{+/+}$ mice) were also injected with vehicle and fed *ad libitum*. Food intake and body weight were measured daily at 0830–0900 hr. Pair-fed Lep^{ob}/Lep^{ob} mice were fed one-half of their daily food allotment at 0900 hr and one-half at 1800 hr. All mice were decapitated at ~0930 hr on Day 9, approximately 16 hr after the last leptin or vehicle injection. Blood was collected for insulin assay (22), liver and abdominal fat pad weights were recorded, and pancreatic islets were obtained for measurement of insulin secretion.

Experiment 2. Islets from pair-fed Lep^{ob}/Lep^{ob} mice in Experiment 1 unexpectedly exhibited a minimal insulin secretion response to acetylcholine, and failed to suppress insulin secretion in response to leptin addition to the media. This observation was in sharp contrast to the robust response of islets from *ad libitum*-fed Lep^{ob}/Lep^{ob} mice to acetylcholine stimulation of insulin secretion, and to the suppressive effects of leptin. Additional Lep^{ob}/Lep^{ob} mice were thus pair-fed for an 8-day period, and were sacrificed at ~0930 hr on Day 9 to obtain pancreatic islets for examination of insulin secretion.

Statistical Analysis. Data are presented as means ± SE. Data were analyzed by one-way analysis of variance (ANOVA) in conjunction with LSD adjustment. Differences were considered statistically significant at $P < 0.05$.

Results

As expected (19, 20, 23–25), Lep^{ob}/Lep^{ob} mice were hyperphagic, and leptin administration lowered their food intake (Table I). We attempted to administer a dose of leptin that would lower food intake of the Lep^{ob}/Lep^{ob} mice to approximate food intake of lean mice. Because administration of 100 μg of leptin per day for 4 days lowered food intake of Lep^{ob}/Lep^{ob} mice below intakes of lean mice (2.07 ± 0.30 g vs 3.37 ± 0.28 g on Day 4), the dose of leptin was lowered to 50 μg/day for the last 4 days of the experiment. Food intakes of leptin-injected Lep^{ob}/Lep^{ob} mice and lean mice averaged 2.36 ± 0.16 g and 3.23 ± 0.18 g on Day 8 of the experiment. Body weight changes paralleled food intake, as did fat pad and liver weights. Treatment of Lep^{ob}/Lep^{ob} mice with leptin lowered their islet diameter and plasma insulin concentrations to the same as those in lean mice. Food intake reduction alone was less effective in lowering plasma insulin (Table I), consistent with earlier reports (19, 26, 27).

Islets from adult Lep^{ob}/Lep^{ob} mice, as observed previously (3, 6), were much more responsive to glucose- and acetylcholine-induced insulin secretion than islets from lean mice (Fig. 1). Again, as expected (3, 6), leptin effectively suppressed acetylcholine-induced insulin secretion from is-

Table I. Food Intake, Body and Tissue Weights, and Plasma Insulin in Leptin-Treated Lep^{ob}/Lep^{ob} Mice

	Lep^{ob}/Lep^{ob}			Lean
	Vehicle (2/3)	Leptin (3/3)	Pair fed (2/3)	Vehicle (2/4)
Cumulative food intake (g)	41 ± 2^a	20 ± 1^b	20 ± 2^b	26 ± 1^c
Body weight change (g/8 d)	6.7 ± 0.5^a	-1.0 ± 0.7^b	-1.2 ± 0.8^b	1.1 ± 0.5^c
Fat pad (g)	2.94 ± 0.23^a	1.15 ± 0.11^b	1.60 ± 0.11^c	0.27 ± 0.04^d
Liver weight (g)	2.38 ± 0.15^a	1.14 ± 0.04^b	1.04 ± 0.04^b	1.07 ± 0.06^b
Islet diameter (μm)	144 ± 7^a	113 ± 3^b	129 ± 6^c	115 ± 2^b
Plasma insulin (ng/ml)	79 ± 26^a	1.1 ± 0.4^b	11 ± 5^b	0.5 ± 0.2^b

Note. Values are means ± SEM. Numbers of male/female animals are indicated in parentheses. All mice were injected intraperitoneally twice daily with leptin (100 μg/day for the first 4 days, and then 50 μg/day for the second 4 days) or vehicle for 8 days. Food was provided *ad libitum*, except for Lep^{ob}/Lep^{ob} mice who were pair fed to the leptin-treated mice. Initial body weights were 21.8 ± 0.6 and 17.4 ± 0.6 g for Lep^{ob}/Lop^{ob} and lean mice, respectively. Islet diameter was obtained by measuring 12–20 islets from each mouse. Data were analyzed by one-way ANOVA in conjunction with LSD. Superscripts indicate significant differences among groups ($P < 0.05$).

lets of adult Lep^{ob}/Lep^{ob} mice, but not from islets of lean mice (Fig. 1).

Insulin secretion responses of islets from Lep^{ob}/Lep^{ob} mice treated with leptin for 8 days were similar to response of islets from lean mice (Fig. 1). The lowered food intake of leptin-treated Lep^{ob}/Lep^{ob} mice per se would be expected to lower their insulin secretion. Consequently, a group of Lep^{ob}/Lep^{ob} mice were pair fed. Restriction of food intake per se did not lower glucose-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice, but markedly blunted the stimulating effect of acetylcholine on glucose-induced insulin secretion (Fig. 1). Additionally, leptin failed to suppress acetylcholine-induced insulin secretion from islets of pair-fed Lep^{ob}/Lep^{ob} mice (Fig. 1).

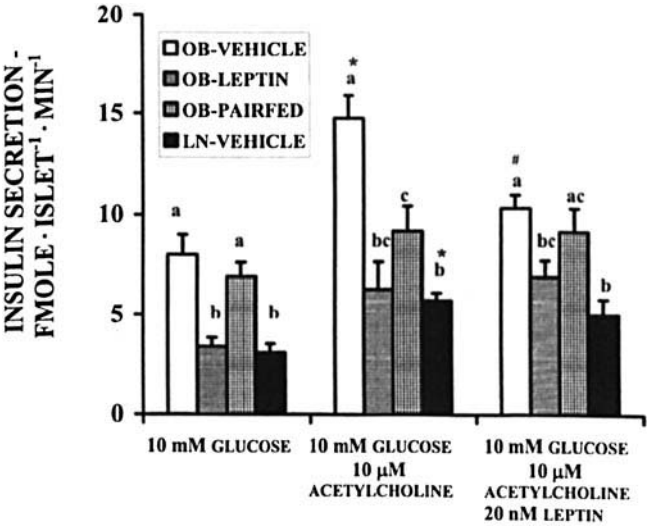

Figure 1. Effect of chronic administration of leptin to Lep^{ob}/Lep^{ob} mice on insulin secretion. Lep^{ob}/Lep^{ob} and lean mice at 4 to 5 weeks of age were treated for 8 days as indicated in the figure. Islets were then isolated (two dishes of 10 islets/dish per mouse) and incubated with 10 m*M* glucose for 30 min followed by 10 m*M* glucose plus 10 μ*M* acetylcholine ± 20 n*M* leptin for 30 min. Data represent means ± SE for five to six mice. Bars with different letters are significantly ($P < 0.05$) different, as determined by one-way ANOVA in conjunction with the LSD *post hoc* test. Comparisons were made within the same incubation media (i.e., glucose, glucose + acetylcholine, or glucose + acetylcholine + leptin). * indicates significantly increased acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} and lean mice treated with vehicle, and # indicates a significant inhibitory effect of leptin on acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice treated with vehicle, as determined by one-way ANOVA in conjunction with the LSD *post hoc* test.

Insulin secretion responses of islets from Lep^{ob}/Lep^{ob} mice treated with leptin for 8 days were similar to response of islets from lean mice (Fig. 1). The lowered food intake of leptin-treated Lep^{ob}/Lep^{ob} mice per se would be expected to lower their insulin secretion. Consequently, a group of Lep^{ob}/Lep^{ob} mice were pair fed. Restriction of food intake per se did not lower glucose-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice, but markedly blunted the stimulating effect of acetylcholine on glucose-induced insulin secretion (Fig. 1). Additionally, leptin failed to suppress acetylcholine-induced insulin secretion from islets of pair-fed Lep^{ob}/Lep^{ob} mice (Fig. 1).

The earliest detectable alteration in islets that occurs in Lep^{ob}/Lep^{ob} mice is an enhanced response to acetylcholine-induced insulin secretion, which is suppressible by addition of leptin to the islets (3). This alteration in insulin secretion occurs by 2 weeks of age (3). We did not expect that food restriction per se would correct this alteration, as occurred in Experiment 1 (Fig. 1). Thus, a second experiment was conducted. Again, restricted food intake per se of Lep^{ob}/Lep^{ob} mice blunted the effectiveness of acetylcholine to stimulate insulin secretion ($P > 0.05$), as well as the ability of leptin to suppress acetylcholine-induced insulin secretion ($P > 0.05$; Fig. 2). Wortmannin, an inhibitor of PI-3 K (28), stimulated acetylcholine-induced insulin secretion from islets of food-restricted Lep^{ob}/Lep^{ob} mice (Fig. 2), in contrast to our earlier observation in *ad libitum*-fed Lep^{ob}/Lep^{ob} mice where wortmannin failed to increase insulin secretion (4). Costimulation of islets from food-restricted Lep^{ob}/Lep^{ob} mice with leptin and wortmannin blocked the wortmannin-induced increase in insulin secretion (Fig. 2).

Discussion

The main finding of this study was that leptin administration to Lep^{ob}/Lep^{ob} mice for 8 days normalized every aspect of insulin secretion measured (i.e., plasma insulin, islet diameter, and insulin secretion responses to glucose, acetylcholine, and leptin). The food intake suppressive effect of leptin accounted for a portion of these change, but Lep^{ob}/Lep^{ob} mice pair fed to the leptin-treated Lep^{ob}/Lep^{ob} mice still exhibited hyperinsulinemia and elevated insulin secretion in response to 10 m*M* glucose.

We had postulated that the absence of leptin during neonatal development might program islets from Lep^{ob}/Lep^{ob} mice to permanently hyperrespond to acetylcholine. Contrary to this expectation, islets from 4- to 5-week-old

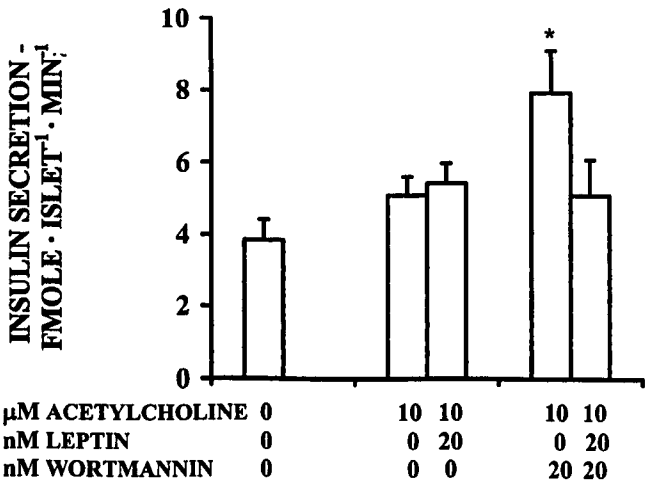

Figure 2. Wortmannin increased acetylcholine-induced insulin secretion from islets of pair-fed *Lep*^{ob}/*Lep*^{ob} mice. Islets from pair-fed *Lep*^{ob}/*Lep*^{ob} mice were isolated (four dishes of 10 islets/dish per mouse) and incubated with 10 m*M* glucose for 30 min. All islets were then exposed to 10 m*M* glucose plus 10 μ*M* acetylcholine ± 20 n*M* leptin and ± 20 n*M* wortmannin for 30 min, as indicated in the figure. Data represent means ± SE for six mice. * indicates a significant ($P < 0.05$) effect of wortmannin on acetylcholine-induced insulin secretion, as determined by one-way ANOVA in conjunction with *post hoc* LSD test.

Lep^{ob}/*Lep*^{ob} mice treated with leptin for only 8 days exhibited a response to acetylcholine similar to that of islets from lean control mice (Fig. 1). A deficiency of leptin during neonatal development does not appear to cause a permanent change in the ability of the islets to respond normally to acetylcholine. Although removal of corticosterone from *Lep*^{ob}/*Lep*^{ob} mice by adrenalectomy also lowered plasma insulin concentration as effectively as did leptin treatment, adrenalectomy failed to correct the hyperresponsiveness of the islets to acetylcholine (16). Glucocorticoids are known to antagonize some actions of leptin (29). Leptin would appear to have distinct effects in regulation of insulin secretion independent of glucocorticoids interactions because the absence of glucocorticoids did not totally mimic effects of leptin on insulin secretion. Additional studies are needed to determine how the concurrent absence of leptin and glucocorticoids enables *Lep*^{ob}/*Lep*^{ob} mice to regulate plasma insulin concentrations within a normal range even though the islets remain hyperresponsive to acetylcholine.

The impact of the leptin-induced reduction in food intake per se in leptin-treated *Lep*^{ob}/*Lep*^{ob} mice was examined by inclusion of *Lep*^{ob}/*Lep*^{ob} mice pair fed to the leptin-treated mice. Food restriction per se attenuated the acetylcholine-induced insulin secretion from islets of these mice, and blocked the ability of leptin to suppress acetylcholine-induced insulin secretion (Figs. 1 and 2). These results suggest that the failure of leptin to inhibit acetylcholine-induced insulin secretion from islets of leptin-treated *Lep*^{ob}/*Lep*^{ob} mice, or from lean mice, is not secondary to "leptin resistance" per se because islets from leptin-deficient, food-restricted *Lep*^{ob}/*Lep*^{ob} mice also failed to decrease acetylcholine-induced insulin secretion in response to leptin. Leptin, secondary to constraint of food intake, appears to assist in the regulation of acetylcholine-induced insulin secretion.

We showed previously that wortmannin, an inhibitor of PI 3-K, increased acetylcholine-induced insulin secretion from islets of lean mice, but not from islets of *ad libitum*-fed *Lep*^{ob}/*Lep*^{ob} mice (4). These findings are consistent with results in Zucker lean and fatty rats (30), and suggest that islets from *Lep*^{ob}/*Lep*^{ob} mice and fatty rats have low PI 3-K activity. Zawalich and Zawalich (30) suggest that PI 3-K inhibits acetylcholine-induced insulin secretion. They attributed low PI 3-K activity in the Zucker fatty rats to insulin resistance. These animals also are leptin resistant, secondary to a mutation in the leptin receptor (31, 32). When islets from pair-fed *Lep*^{ob}/*Lep*^{ob} mice were exposed to wortmannin, acetylcholine-induced insulin secretion increased (Fig. 2). This suggests that PI 3-K is active in islets from food-restricted *Lep*^{ob}/*Lep*^{ob} mice to help constrain acetylcholine-induced insulin secretion.

Leptin is known to activate PI 3-K in islets (33, 34). We hypothesized that leptin via activation of PI 3-K constrains acetylcholine-induced insulin secretion in islets from mice. We showed, in a previous report (4), that preincubation of islets with wortmannin blocks the ability of leptin to inhibit acetylcholine-induced insulin secretion. Islets from *Lep*^{ob}/*Lep*^{ob} mice presumably have low PI 3-K activity because wortmannin does not increase acetylcholine-induced insulin secretion from islets of these mice (4). The addition of leptin to the islets would be expected to activate PI 3-K and this may explain the lowered acetylcholine-induced insulin secretion in the presence of leptin. In contrast, islets from food-restricted *Lep*^{ob}/*Lep*^{ob} mice appear to have high PI 3-K activity because wortmannin increases insulin secretion (Fig. 2). If PI 3-K activity is already high, leptin would be expected to be ineffective *in vitro*, as was observed (Fig. 2). Food restriction of *Lep*^{ob}/*Lep*^{ob} mice lowered plasma insulin and may have reduced insulin resistance. This may have activated PI 3-K in the islets to constrain acetylcholine-induced insulin secretion. Others have reported that food deprivation for 48 hr activated PI 3-K in peripheral tissues of obese neonates (35). It remains to be established how food restriction per se affects PI 3-K activity in islets of mice.

Food restriction per se did not totally normalize insulin secretion of *Lep*^{ob}/*Lep*^{ob} mice (Fig. 1). Leptin lowers insulin mRNA abundance (36, 37) and in some studies is reported to affect ATP-sensitive K^+ and Ca^{2+} channels, which leads to a reduction in glucose-induced insulin secretion (33, 38). It is not clear whether leptin treatment, but not pair feeding, acted via these mechanisms to normalize insulin secretion.

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