β-Cell Function in Normal Rats Made Chronically Hyperleptinemic by Adenovirus-Leptin Gene Therapy

Kazunori Koyama, Guoxun Chen, May-Yun Wang, Young Lee, Michio Shimabukuro, Christopher B. Newgard, and Roger H. Unger

Leptin was overexpressed in the liver of normal Wistar rats by infusing recombinant adenovirus containing the cDNA encoding leptin. Plasma leptin levels rose to 12–24 ng/ml (vs. <2 ng/ml in control rats), and food intake and body weight fell. Visible fat disappeared within 7 days. Plasma insulin fell to <50% of normal in association with hypoglycemia, suggesting enhanced insulin sensitivity. Although β-cells appeared histologically normal, the pancreases were unresponsive to perfusion with stimulatory levels of glucose and arginine. Since islet triglyceride content was 0, compared with 14 ng/islet in pair-fed control rats, we coperfused a 2:1 oleate:palmitate mixture (0.5 mmol/l). This restored insulin responses to supranormal levels. When normal islets were cultured with 20 ng/ml of leptin, they too became triglyceride-depleted and failed to respond when perifused with glucose or arginine. Perifusion of fatty acids restored both responses. We conclude that in normal rats, hyperleptinemia for 2 weeks causes reversible β-cell dysfunction by depleting tissue lipids, thereby depriving β-cells of a lipid-derived signal required for the insulin response to other fuels. ***Diabetes*** **46:1276–1280, 1997**

The discovery of leptin, the adipocyte-derived polypeptide that regulates food intake, thermogenesis, and body weight in rodents (1–4), has fueled the hope that the most prevalent of American health problems, obesity, might at last be effectively treated. Since even a modest decrease in body weight and food intake would be expected to reduce the incidence of NIDDM, treatment with leptin or an alternative agent acting via the leptin receptor might prevent this disease.

Recently, however, it was suggested that leptin may cause rather than prevent obesity-related diabetes (5). This speculation, based on a report showing that leptin attenuates insulin-signaling in human hepatoma cells (6), raises the possibility that the endogenous hyperleptinemia of obesity may be a cause of insulin resistance. If so, further elevation of leptin levels resulting from leptin therapy might precipitate diabetes and thus be contraindicated. In this report, we examine the possibility that hyperleptinemia is diabetogenic by inducing chronic hyperleptinemia in normal rats (7) and determining if this causes β-cell dysfunction or cytotoxicity. We find that hyperleptinemia is not diabetogenic in normal rats, but rather causes hypoglycemia and hypoinsulinemia, suggesting increased insulin sensitivity. β-cells become unresponsive to fuel stimuli, such as glucose and arginine, when they are rendered lipopenic by hyperleptinemia, but responsiveness is restored by providing free fatty acids (FFAs). We conclude that leptin-induced lipopenia improves insulin sensitivity so that, despite hypoinsulinemia, glucose levels are subnormal.

RESEARCH DESIGN AND METHODS

Hyperleptinemic rats. To induce hyperleptinemia in normal rats, recombinant adenovirus containing the rat leptin cDNA (AdCMV-leptin) was prepared as previously described (7). Briefly, a *Bam*H1- and *Xba* I-restricted leptin cDNA fragment that included 60 bp of 5′ untranslated region and 76 bp of 3′ untranslated region was ligated to similarly treated pACCMVpLpA (8). The resulting plasmid was cotransfected with pJM17 (9) into 293 cells by calcium phosphate/DNA coprecipitation to generate the AdCMV-leptin virus, using previously described methods (10). Stocks of AdCMV-leptin were amplified and purified, as described (10), and stored at −70°C in phosphate-buffered solution (PBS) with 0.2% bovine serum albumin (BSA) and with 10% glycerol at 3×10^{12} plaque-forming units (pfu)/ml. A virus containing the bacterial β-galactosidase gene under control of the CMV promoter (AdCMV-βGal) was prepared and used as described previously (11).

To deliver the AdCMV-leptin or AdCMV-βGal viruses, polyethylene tubing (PE-50; Becton Dickinson, Rutherford, NJ) was anchored in the left carotid artery of 9-week-old Wistar rats of 250–300 g under sodium pentobarbital anesthesia (50 mg/kg; Abbott, North Chicago, IL) and exteriorized via a subcutaneous tunnel 3 days before virus infusion. Also, 2 ml of AdCMV-leptin or AdCMV-βGAL containing a total of 1×10^{12} pfu was infused into unconscious rats over a 30-min period. Animals were studied in individual metabolic cages, and food intake and body weight were measured daily. Blood samples were collected from the tail vein in capillary tubes coated with EDTA for leptin assay at the indicated intervals, beginning 3 days after adenovirus infusion. Plasma leptin was assayed using the Linco leptin assay kit (Linco Research Immunoassay, St. Charles, MO).

Food intake. Free-feeding rats were on standard rat chow (Tekland F6 8664, Teckland, Madison, WI) ad libitum and had free access to water. Pair-fed rats received exactly the amount of food consumed by the AdCMV-leptin–infused animals.

Immunoblotting for leptin-receptor protein (OB-R). Total protein (about 20 μg per lane) was extracted using X-100 containing Hepes buffer (pH 7.5) and was resolved by 7.5% SDS-PAGE and transferred onto PVDF (polyvinylidene difluoride) membranes. The membranes were then treated with antibody K-20 (Santa Cruz Biotechnology, Santa Cruz, CA), a goat polyclonal antiserum, against an epitope corresponding to the NH_2-terminal, extracellular domain common to all mouse leptin-receptor isoforms. After washing the blot, bound primary antibody was detected by reaction with anti-goat IgG antibody-peroxidase conjugate followed by visualizing with a Super Signal-CL kit (Pierce, Rockford, IL) Although the actual identity of isoforms is not established by this experiment, the molecular size is closest to that of OB-Rb, rather than to the short isoforms. Identical results have been obtained by the manufacturer, Santa Cruz Biotechnology, in SK-N-MC whole-cell lysates.

From the Departments of Internal Medicine and Biochemistry, Gifford Laboratories for Diabetes Research (K.K., G.C., M.-Y.W., Y.L., M.S., C.B.N., R.H.U.), University of Texas Southeastern Medical Center; and the Department of Veterans Affairs Medical Center (R.H.U.), Dallas, Texas.

Address correspondence to Roger H. Unger, MD, Center for Diabetes Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8854.

Received for publication 14 February 1997 and accepted in revised form 8 April 1997.

BSA, bovine serum albumin; FFA, free fatty acid; KRBB, Krebs-Ringer bicarbonate buffer; PBS, phosphate-buffered saline; TG, triglyceride.

Pancreatic morphology. Immunofluorescent staining for insulin and routine hemotoxylin and eosin staining were carried out on sections of pancreas as previously described (12).

Pancreas perfusion. Pancreases were perfused by the method of Grodsky and Fanska (13), as previously described. Insulin was measured by standard radioimmunoassay. Baseline perfusate contained 5.6 mmol/l glucose in Krebs-Ringer bicarbonate buffer (KRBB) containing Dextran T70, 1% BSA, and 5 mmol/l each of sodium pyruvate, sodium glutamate, and sodium fumarate. A 20-min equilibration period preceded each perfusion experiment. In some experiments, 0.5% in 1% BSA was coperfused.

Islet isolation and culture. Pancreatic islets were isolated and maintained in suspension culture in 60-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, as described previously (14). The culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (200 U/ml), streptomycin (0.2 mg/ml), and 2% BSA (fraction V; Miles, Kankakee, IL), either with or without 2 mmol/l long-chain fatty acids (2:1 oleate:palmitate, sodium salt; Sigma, St. Louis, MO). The final glucose concentration was 8.0 mmol/l, which is required for long-term survival of at least 80% of islets.

Perifusion of cultured islets. For perifusion, 50–100 islets were collected under a stereoscopic microscope, washed twice with KRB-Hepes buffer (pH 7.4, 3 mmol/l glucose), and loaded into a 13-mm chamber containing an 8-μm nylon membrane filter (Millipore, Bedford, MA). Islets were perifused with buffer containing 3 or 23 mmol/l glucose at a flow rate of 0.8 ml/min for 15 min each (15). Effluent fractions were collected at 2-min intervals and stored at –20°C until insulin assay. Immunoreactive insulin was determined by radioimmunoassay using charcoal separation.

Plasma measurements. Fasting blood samples were collected (1:00–3:00 P.M.) from the tail vein in capillary tubes coated with EDTA. Plasma was stored at –20°C until the time of leptin assay. Plasma leptin was assayed using the Linco leptin assay kit (Linco Research, St. Charles, MO). Plasma glucose was measured by the glucose oxidase method using the glucose analyzer II (Beckman, Brea, CA). Plasma FFAs were determined with kits (Boehringer Mannheim, Indianapolis, IN). Plasma insulins were assayed by the method described previously (14).

Triglyceride (TG) content of pancreas and islets. Tissues were dissected and placed in liquid nitrogen. About 100 mg of tissue was placed in 4 ml of homogenizing buffer containing 18 mmol/l of Tris-HCl (pH = 7.5), 300 mmol/l of D-mannitol, 5 mmol/l of ethylene glycol-bis-(B-amino ethyl ether) *N,N*-tetra acetic acid (EGTA), and polytron for 10 s. Lipids were extracted by the method of Folch et al. (16). Total TG was assayed by the method of Danno et al. (17).

RESULTS

Leptin-receptor protein in β-cells. A direct action of leptin on β-cells would be possible only if they expressed the leptin receptor. Although leptin-receptor (OB-R) mRNA had previously been detected by reverse-transcription–polymerase chain reaction (RT-PCR) in rat islets and in an insulinoma cell line (16), OB-R protein had not been identified in any tissue. We therefore carried out immunoblotting analyses of both normal rat islets and the rat insulinoma cell line Ins-1 (16) using an antibody against the extracellular domain common to all OB-R isoforms (Fig. 1). Doublet bands were present in both islets and Ins-1 cells with an apparent molecular size of ~124 and 119 kDa. These findings, together with the previous report (16), indicate the presence of OB-R in β-cells. The effect of sustained elevations of plasma leptin on β-cells was then studied.

Hyperleptinemic rat model. To induce chronic hyperleptinemia in Wistar rats, we infused a recombinant adenovirus containing the rat leptin cDNA (AdCMV-leptin). In confirmation of earlier findings (7), leptin mRNA was strongly expressed in the liver of AdCMV-leptin–infused rats. In control rats infused with an adenovirus containing the β-galactosidase gene (AdCMV-βGal), leptin was not ectopically expressed. Plasma levels of immunoreactive leptin rose to 13.7 ± 2.1 ng/ml in the AdCMV-leptin–infused control rats (Fig. 2*A*) (Chen et al. [7] showed that leptin levels averaged ~8 ng/ml). The biological activity of the ectopically secreted leptin was evident from the decline in food intake, the profound reduction in body weight gain, and the disappearance of identifiable fat; these changes were greater than in our previous report (7) (Figs. 2*B* and *C*).

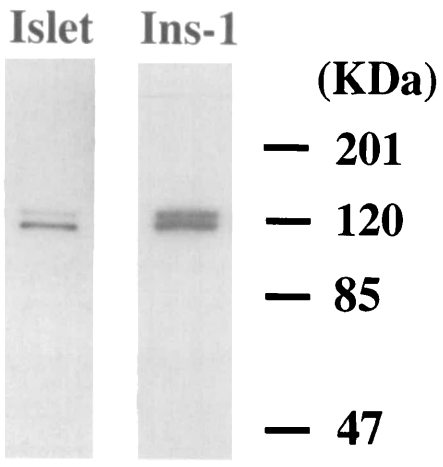

FIG. 1. Immunoblotting analyses showing the presence of leptin receptors in rat islets and Ins-1 cells.

Plasma glucose and insulin levels. Fasting plasma insulin levels were significantly reduced 14 days after the infusion, averaging only 4.7 ± 0.7 μU/ml, compared with 11.1 ± 0.8 and

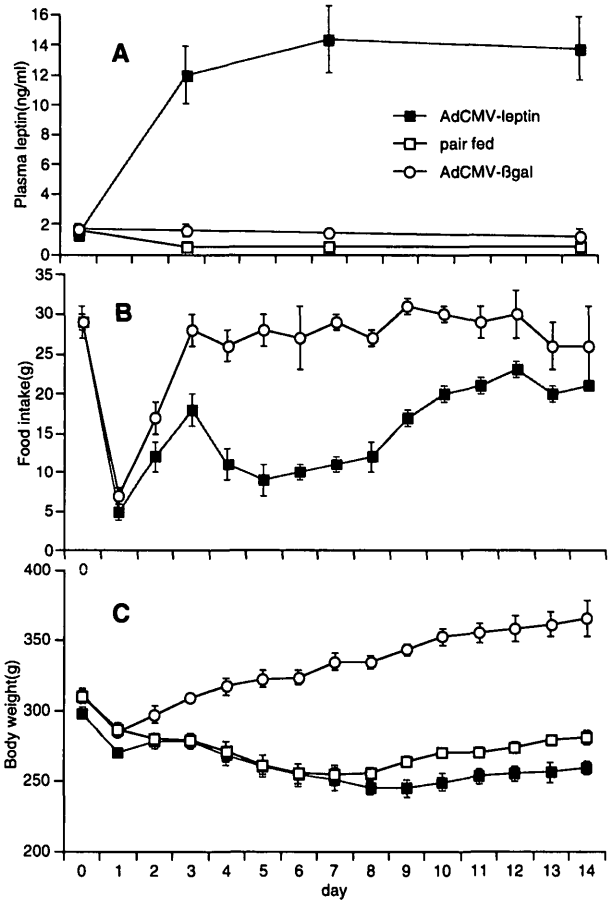

FIG. 2. Plasma leptin levels (*A*), food intake (*B*), and body weight (*C*) in normal male Wistar rats infused with AdCMV-leptin (n = 8), AdCMV-βGal (n = 4), or pair-fed to AdCMV-leptin–infused animals (n = 8).

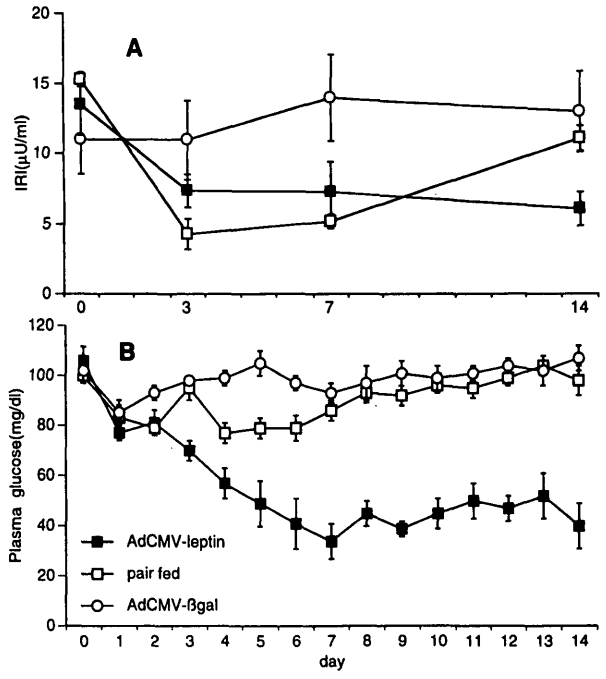

FIG. 3. Plasma insulin (IRI) (*A*) and plasma glucose levels (*B*) in normal male Wistar rats infused with AdCMV-leptin (n = 8) or AdCMV-βGal (n = 4) or pair-fed to AdCMV-leptin–infused animals (n = 8).

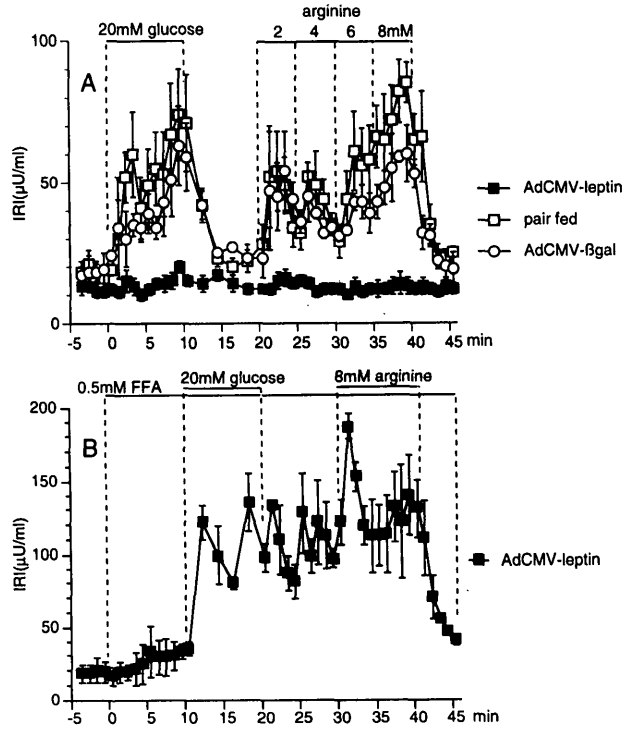

FIG. 4. Perfusion of pancreases isolated from rats infused with AdCMV-leptin (n = 4) or AdCMV-βGal (n = 3) and from intact rats pair-fed to AdCMV-leptin–infused rats (n = 4). *A*: insulin responses to 20 mmol/l glucose and stepwise increments of arginine from 2 to 8 mmol/l, demonstrating β-cell unresponsiveness of AdCMV-leptin–infused rats. *B*: coperfusion of 0.5 mmol/l FFAs in 1% albumin restored the responsiveness of AdCMV. Note the scale differences between *A* and *B*.

13.4 ± 0.5 μU/ml, respectively, in the two control groups (P < 0.01). However, despite this hypoinsulinemia, fasting blood glucose levels fell to subnormal levels throughout the 14 days of observation (Fig. 3). Intraperitoneal glucose tolerance tests also disclosed no evidence of a diabetic abnormality, the glucose levels averaging 130 ± 20 and 81 ± 9 mg/dl at 1 and 2 h after injection of glucose (2.5 g/kg of body weight), respectively. The foregoing findings were consistent with enhanced insulin sensitivity resulting from depletion of tissue lipids.

Studies of β-cell function and morphology of islets in hyperleptinemic rats. Despite the absence of laboratory manifestations of diabetes, it seemed possible that leptin-induced damage to β-cells might have been "masked" by the enhancement of insulin sensitivity resulting from the lipopenia coupled with the reduced food intake. Therefore pancreases were isolated from the hyperleptinemic rats for studies of β-cell function and for morphology. In the hyperleptinemic group, basal insulin secretion at 5.6 mmol/l glucose was slightly below that of pair-fed control rats (P < 0.05), but the striking finding was the complete absence of an insulin response either to 20 mmol/l glucose or to arginine in concentrations of 2, 4, 6, and 8 mmol/l (Fig. 4*A*).

To determine if this abnormality was the result of structural damage to β-cells, hemotoxylin and eosin and insulin immunofluorescence staining of sections of the perfused pancreases was carried out. No obvious structural abnormalities could be identified (not shown).

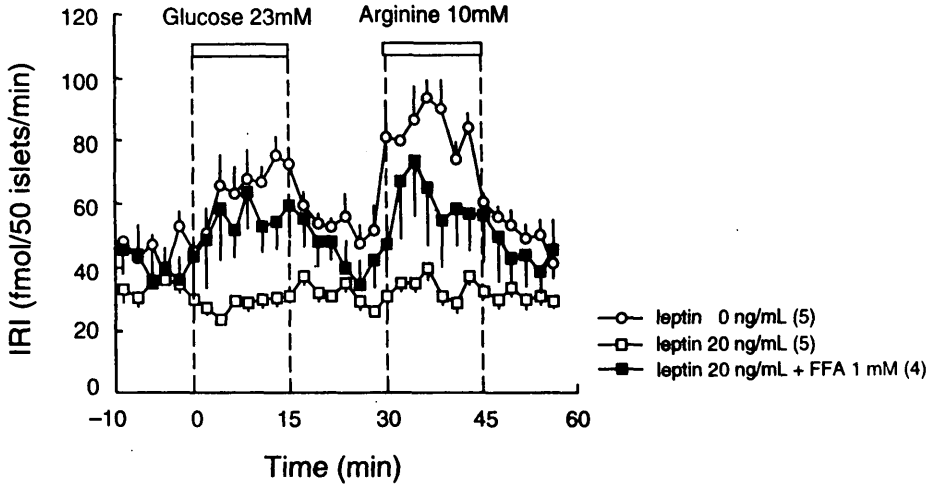

FIG. 5. Perfusion of islets from normal rats cultured for 3 days with 20 mg/ml of leptin (n = 5) or without leptin (n = 5). The leptin-mediated loss of response is partially restored if 0.5 mmol/l FFA is added to the perfusate (n = 4).

TABLE 1
Plasma FFA levels in normal male Wistar rats infused with AdCMV-leptin, AdCMV-βGal, or pair-fed to AdCMV-leptin infused animals

Plasma FFA (mmol/l)	*n*	Day 0	Day 3	Day 7	Day 14
AdCMV-leptin	8	0.71 ± 0.06	0.68 ± 0.09	0.77 ± 0.12	0.69 ± 0.08
Pair-fed	4	0.77 ± 0.10	1.06 ± 0.09	1.02 ± 0.07	0.88 ± 0.08
AdCMV-βGal	3	0.70 ± 0.04	0.54 ± 0.05	0.58 ± 0.01	0.89 ± 0.15

Data are means ± SE. Some of these results were published in different form in Shimabukuro et al. (18).

Evidence for a metabolic cause of leptin-induced β-cell dysfunction. The absence of discernible morphological lesions in islets that were unresponsive to nutrients suggested a metabolic abnormality related to the generalized lipid depletion. We therefore measured the TG content in islets of hyperleptinemic and control rats. In AdCMV-βGal–infused rats, the TG content was 23 ng/islet. In the hyperleptinemic rats, TG was unmeasurable, while in pair-fed control rats it was 14 ng/islets.

Because these results suggested a relationship between the lipopenia and the β-cell dysfunction, we repeated the experiments of Fig. 4*A*, but coperfused 0.5 mmol/l FFA. There was a prompt rise in basal insulin secretion and a vigorous response to both 20 mmol/l glucose and to arginine that exceeded the normal responses (Fig. 4*B*). To determine if the foregoing in vivo effects of hyperleptinemia on β-cell function were the result of direct leptin action on islets, we cultured normal Wistar islets for 3 days in 20 ng/ml of recombinant leptin and then perifused them with or without FFA. As shown in Fig. 5*A*, the insulin response to both glucose and arginine was completely absent in islets cultured in the presence of leptin. The coperifusion of the 1 mmol/l FFA restored these responses (Fig. 5*B*).

DISCUSSION

This study was originally designed to determine if leptin might be diabetogenic as recently suggested (5,6). Normal rats were therefore made hyperleptinemic by adenovirus gene transfer and observed clinically for laboratory evidence of diabetes. All AdCMV-leptin–infused animals exhibited a prompt and sustained reduction in food intake and loss of body weight resulting from disappearance of all visible body fat, confirming our previous report (7). The lipopenia was associated with hypoglycemia as low as 40 mg/dl in association with hypoinsulinemia. By contrast, in intact rats pair-fed to match the food intake of the hyperleptinemic rats, blood glucose levels remained >70 mg/dl, even though their insulin levels were similar to those of the hyperleptinemics. This strongly infers that insulin was much more effective in the hyperleptinemic rats than in the pair-fed control rats, probably reflecting the profound reduction in triglyceride content in target tissues of insulin.

The observation of hypoinsulinemia prompted us to study more carefully the β-cell function of hyperleptinemic rats. In perfused pancreases from these rats, basal insulin secretion at 5.6 mmol/l glucose was subnormal, and the responses to both glucose and arginine were completely absent; by contrast, in pair-fed control rats the glucose and arginine responses were only minimally reduced. The absence of any morphological abnormalities in these islets led us to suspect a metabolic defect, probably related to the lipid depletion. Indeed, triglyceride content in islets of the hyperleptinemic was unmeasurable, whereas in islets of pair-fed control rats it measured 14 ng/islet, ~60% of normal. Given the demonstration by Stein et al. (17) of the essential role of FFAs in the insulin response to glucose, it seemed likely that FFAs would correct the defect. In fact, FFAs restored the response to glucose and to arginine to supranormal levels. The unresponsiveness of β-cells to 20 mmol/l glucose suggests that fuel deprivation was not the cause of the defect, but rather points to a role for FFAs as a signal or messenger required for responses to other fuels. The fact that recombinant leptin produced the same β-cell unresponsiveness in vitro strongly suggests that the leptin-induced effects were the consequence of leptin acting directly on islets rather than of effects mediated via the hypothalamus.

The depletion of both intracellular and extracellular sources of FFAs apparently abolished the normal β-cell responses to nutrients. The teleological role of this loss of β-cell response as a result of lipid depletion may be to protect the fasted organism from protein-induced insulin secretion that might cause hypoglycemia. It also gives vital insulin-independent tissues, such as the central nervous system, an advantage over insulin-dependent tissues in terms of priority of access to glucose during the initial phase of refeeding.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK0270037 and P5OH2598801, a National Institutes of Health/Juvenile Diabetes Foundation Diabetes Interdisciplinary Research Program grant, and Department of Veterans Affairs Institutional Research Support Grant SMI821-109.

We thank Kay McCorkie, Falguni Trieu, and Sharryn Harris for outstanding technical support.

REFERENCES

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432, 1994
2. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F: Effects of the obese gene product on body weight regulation in *ob/ob* mice. *Science* 269:540–543, 1995
3. Considine RV, Considine EL, Williams CJ, Hyde TM, Caro JF: The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the *db/db* mouse and *fa/fa* rat mutations. *Diabetes* 45:992–994, 1996
4. Caro JF, Sinha MK, Kolaczynski JW, Zhang PL, Considine RV: Leptin: the tale of an obesity gene. *Diabetes* 45:1455–1462, 1996
5. Taylor SI, Barr V, Reitman M: Does leptin contribute to diabetes caused by obesity? *Science* 274:1151–1152, 1996
6. Cohen B, Novick D, Rubinstein M: Modulation of insulin activities by leptin. *Science* 274:1185–1188,1996
7. Chen G, Koyama K, Yuan X, Lee Y, Zhou Y, O'Doherty R, Newgard CB, Unger RH: Disappearance of body fat in normal rats induced by adenovirus-mediated leptin gene therapy. *Proc Natl Acad Sci USA* 93:14795–14799, 1996
8. Gomez-Foix AM, Coats WS, Baque S, Alam T, Gerard RD, Newgard CB: Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen metabolism. *J Biol Chem* 267:25129–25134, 1992
9. McCrory WJ, Bautista DS, Graham FL: A simple technique for the rescue of

early region I mutations into infectious human adenovirus type 5. *Virology* 163:614–617, 1988
10. Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB: Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol* 43:161–189, 1994
11. Herz J, Gerard RD: Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci USA* 90:2812–2816, 1993
12. Orci L, Ravazzola M, Baetens D, Inman L, Amherdt M, Peterson RG, Newgard CB, Johnson JH, Unger RH: Evidence that down-regulation of beta-cell glucose transporters in non-insulin-dependent diabetes may be the cause of diabetic hyperglycemia. *Proc Natl Acad Sci USA* 87:9953–9957, 1990
13. Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH: Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships. *Proc Soc Natl Acad Sci USA* 91:10878–10882 1994
14. Milburn JL, Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, Beltran del Rio H, Newgard CB, Johnson JH, Unger RH: Pancreatic β-cells in obesity. *J Biol Chem* 270:1295–1299, 1995
15. Hirose H, Lee YH, Inman LR, Nagasawa Y, Johnson JH, Unger RH: Defective fatty acid-mediated β-cell compensation in Zucker diabetic fatty rats. *J Biol Chem* 271:5633–5637, 1996
16. Kieffer TJ, Heller RS, Habener JF: Leptin receptors expressed on pancreatic beta-cells. *Biochem Biophys Res Commun* 224:522–527, 1996
17. Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S, McGarry JD: Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J Clin Invest* 97:2728–2735, 1996
18. Shimabukuro M, Koyama K, Chen G, Wang M-Y, Trieu F, Lee Y, Newgard CB, Unger RH: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci USA* 94:4637–4641, 1997