Direct antidiabetic effect of leptin through triglyceride depletion of tissues

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ABSTRACT Leptin is currently believed to control body composition largely, if not entirely, via hypothalamic receptors that regulate food intake and thermogenesis. Here we demonstrate direct extraneuronal effects of leptin to deplete fat content of both adipocytes and nonadipocytes to levels far below those of paired controls. In cultured pancreatic islets, leptin lowered triglyceride (TG) content by preventing TG formation from free fatty acids (FFA) and by increasing FFA oxidation. In vivo hyperleptinemia, induced in normal rats by adenoovirus gene transfer, depleted TG content in liver, skeletal muscle, and pancreas without increasing plasma FFA or ketones, suggesting intracellular oxidation. In islets of obese Zucker Diabetic Fatty rats with leptin receptor mutations, leptin had no effect in vivo or in vitro. The TG content was ~20 times normal, and esterification capacity was increased 3- to 4-fold. Thus, in rats with normal leptin receptors but not in Zucker Diabetic Fatty rats, nonadipocytes and adipocytes esterify FFA, store them as TG, and later oxidize them intracellularly via an “indirect pathway” of intracellular fatty acid metabolism controlled by leptin. By maintaining insulin sensitivity and preventing islet lipotoxicity, this activity of leptin may prevent adipogenic diabetes.

Leptin, the peptide hormone that controls body composition, is believed to do so largely, if not entirely, via hypothalamic receptors that regulate food intake and body weight (1–3). However, the various leptin receptor isoforms, including the long isoform OB-Rb, are widely expressed throughout the body (4–6), suggesting that leptin may have important actions on extraneuronal tissues as well. The idea that leptin may directly act on peripheral tissues gains further credence from the extraordinary changes in body fat of normal Wistar rats made chronically hyperleptinemic by infusion of the recombinant adenovirus containing the leptin cDNA (AdCMV–leptin) (7). In these rats, all discernible body fat disappeared within 7 days of this infusion whereas in paired normoleptinemic rats only a modest reduction in body fat took place. The magnitude and rapidity of the lipid depletion raised the possibility of a direct hormone-to-cell action of the leptin, in addition to the central effects that occur via the sympathetic nervous system. The goal of this study was to determine if leptin has important effects on cells outside the central nervous system that are involved in regulation of body weight and composition. Obesity is often associated with pancreatic β-cell dysfunction and diabetes, so we selected the pancreatic islets as the target tissue. Moreover, islets are known to express OB-R (6), and they become dysfunctional when they accumulate triglycerides (TG) in excessive amounts (8).

Leptin lowered triglyceride (TG) content by preventing TG formation from free fatty acids (FFA) and by increasing FFA oxidation. In islet Isolation and Culture. Pancreatic islets were isolated according to the method of Naber et al. (10) with some modifications and were maintained in suspension culture in 60-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO2 and 95% air (11). The culture medium consisted of RPMI 1640 medium supplemented with 8.0 mM glucose, 10% fetal bovine serum, 200 units/ml penicillin, 0.2 mg/ml streptomycin, and 2% BSA, fraction V (Miles) either with or without 1 mM long chain fatty acid mixture (oleate-to-palmitate, 2:1; sodium salt; Sigma).

Oxidation and Esterification of [3H]-Palmitate in Pancreatic Islets. Oxidation and esterification of [3H]-palmitate in isolated pancreatic islets was measured as described for use of [3H]-palmitic acid (8). [3H]-palmitate was removed by precipitating twice with an equal volume of 10% trichloroacetic acid with 2% BSA. Supernatants in a microcentrifuge tube were placed in a scintillation vial containing unlabeled water and incubated at 50°C for 18 h. Tritiated water was measured as described for use of [3H]-glucose (11). Esterification of [3H]-palmitate by islets was determined by measuring [3H]-palmitate uptake by islets.

Materials and Methods

Animals. Male Wistar rats were obtained from Charles River Breeding Laboratories. Obese homozygous (fa/fa) Zucker diabetic fatty (ZDF)-dt rats and lean heterozygous (fa/+) ZDF and wild-type (+/+) littermates were bred in our laboratory from [ZDF/Drt-fa (F10)] rats purchased from R. Peterson (University of Indiana School of Medicine, Indianapolis). Male rats from our colony exhibiting the described phenotype (8, 9) were used.

Islet Isolation and Culture. Pancreatic islets were isolated according to the method of Naber et al. (10) with some modifications and were maintained in suspension culture in 60-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO2 and 95% air (11). The culture medium consisted of RPMI 1640 medium supplemented with 8.0 mM glucose, 10% fetal bovine serum, 200 units/ml penicillin, 0.2 mg/ml streptomycin, and 2% BSA, fraction V (Miles) either with or without 1 mM long chain fatty acid mixture (oleate-to-palmitate, 2:1; sodium salt; Sigma).

Triglyceride and DNA Measurements. After 3 days in culture, islets were washed twice with Hank’s balanced salt buffer and suspended in 50 μl of buffer (2 M NaCl, 2 mM EDTA, 50 mM sodium phosphate, pH 7.4). After sonication, 10 μl of the homogenate was mixed with 10 μl of tert-butyl alcohol and 5 μl of a Triton X-100/methyl alcohol mixture (1:1 vol/vol). For TG measurements of the pancreas, skeletal muscle, and liver, total lipids were extracted from ~100 mg of tissue as described (11) and frozen under liquid nitrogen. TG was extracted with 30 μl of tert-butyl alcohol and 20 μl of a Triton X-104/methyl alcohol mixture (1:1 vol/vol). TG content was measured by the Sigma Triglyceride (GPO-Trinder) kit (8), and DNA content was measured by a method of Hopcroft et al. (12).

Oxidation and Esterification of [3H]-Palmitate in Pancreatic Islets. Oxidation and esterification of [3H]-palmitate by islets were determined as described (13, 14). Groups of 100–200 islets were incubated in duplicate with 1 mM 9,10-[3H]-palmitate for 3 days. Palmitate oxidation was assessed by measuring tritiated water in the medium. Excess [3H]-palmitate was removed by precipitating twice with an equal volume of 10% trichloroacetic acid with 2% BSA. Supernatants in a microcentrifuge tube were placed in a scintillation vial containing unlabeled water and incubated at 50°C for 18 h. Tritiated water was measured as described for use of [3H]-glucose (11). Esterification of [3H]-palmitate by islets was determined by measuring [3H]-palmitate uptake by islets.
similarly treated pACCMVpLpA (15). The resulting plasmid was cotransfected with pJM17 (16) into 293 cells by calcium phosphate/DNA coprecipitation to generate the AdCMV–leptin virus (17). Stocks of AdCMV–leptin were amplified and purified as described (17) and stored at −70°C in PBS with 0.2% BSA and 10% glycerol at 1–3 × 1012 plaque-forming units (pfu)/ml. A virus containing the bacterial β-galactosidase (β-gal) gene under control of the CMV promoter (AdCMV–β-gal) was prepared and used as described (18).

Hyperleptinemic Rat Model. Two milliliters of AdCMV–leptin or AdCMV–β-gal containing a total of 1 × 1012 pfu/ml were infused into Wistar heterozygous lean and homozygous ZDF rats over a 10-minute 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 Immunodiassay, St. Charles, MO). Plasma free fatty acids (FFA) were measured with a colorimetric assay using fatty acyl-CoA synthase, acyl-CoA oxidase, and H2O2-superoxide dismutase (19). Urinary ketone (acetoacetic acid) levels were quantified by a Phillips linked dye reagent (Boehringer Mannheim). The β-hydroxybutyrate (β-OH) level was determined spectrophotometrically (19). Lipid measurements in various tissues and liver, pancreas, and skeletal muscle of hyperleptinemic rats with paired controls and free-feeding control animals infected with AdCMV–β-gal. Adipose tissue could not be compared because it could not be identified in the hyperleptinemic rats. As shown in Table 1, the TG content was reduced profoundly in the hyperleptinemic group in every tissue studied. In pancreas, for example, TG content was 4% of the free-feeding control rats and 20% of paired controls. A similar reduction in TG content was noted in skeletal muscle and liver. Phospholipids were unchanged (data not shown).

In Vivo Effects of Leptin on TG of Tissues. In our previous report demonstrating the absence of fat in hyperleptinemic rats (7), lipid measurements in various tissues were not carried out. To quantify the lipid content of tissues, we repeated the study and compared the content of TG and phospholipids in liver, pancreas, and skeletal muscle of hyperleptinemic rats with paired controls and free-feeding control animals infected with AdCMV–β-gal. Adipose tissue could not be compared because it could not be identified in the hyperleptinemic rats. As shown in Table 1, the TG content was reduced profoundly in the hyperleptinemic group in every tissue studied. In pancreas, for example, TG content was 4% of the free-feeding control rats and 20% of paired controls. A similar reduction in TG content was noted in skeletal muscle and liver. Phospholipids were unchanged (data not shown).
To assess more directly the influence of leptin on TG metabolism, we added 1 mM $[^3H]$palmitate to the culture medium and measured the effects of the hormone on oxidation and esterification of the long chain fatty acid, as described (13). As shown in Fig. 1B, a 42% increase in oxidation occurred in islets from Wistar rats when 20 ng/ml leptin was present, and the incorporation of $[^3H]$palmitate into TG was reduced by 54% (Fig. 1C). These effects on FFA metabolism could account for the reduction of the TG content of islets by leptin.

**Plasma FFA and β-Hydroxybutyrate Levels in Hyperleptinemic Rats.** The foregoing results indicate that leptin can reduce the TG content of islet cells by increasing oxidation and reducing esterification of FFA intracellularly. When TG stores are reduced by starvation or insulin deficiency, the FFA are released and delivered to other tissues. This increased lipolysis is reflected by a rise in plasma FFA levels. β-Oxidation of FFA takes place largely in the liver and results in an increase in plasma and urine ketones (24). To determine if the striking leptin-mediated depletion of adipocyte fat involves the classic pathways of lipolysis and ketogenesis, we measured plasma FFA and β-OH in levels in rats before and 3, 7, and 14 days after they had been made hyperleptinemic by infusion of AdCMV–leptin (leptin levels 13.7 ± 2.1 ng/ml). Paired rats and free-feeding AdCMV–β-gal-infused rats served as controls. As shown in Fig. 2A, FFA levels remained at the prehyperleptinemic baseline levels throughout the 14 days, even at the 3-day postinfusion point before body fat had disappeared. β-OH also failed to rise in the hyperleptinemic rats (Fig. 2B), and daily urine determinations were negative for ketonuria (Fig. 2C). By contrast, plasma FFA, β-OH, and urinary ketones were increased in paired controls (Fig. 2A–C). These results suggest that leptin-induced depletion of TG in white adipocytes involves the same increase in intracellular TG metabolism observed in islets, rather than the export of FFA to other tissues.

**Hyperleptinemic Effects in ZDF Rats.** If, in fact, the lipopenic action of leptin is a direct one mediated by leptin receptors, the effect should not occur in cells in which the leptin receptor is defective. The fa mutation in obese Zucker and ZDF consists of a Glu-269 → Pro mutation in the extracellular domain of all OB-R isoforms (20, 25). These fa/fa rats have a phenotype of leptin resistance with obesity despite hyperleptinemia. We therefore tested the effects of infusing AdCMV–leptin into homozygous ZDF rats. The dramatic effects on food intake, body weight, and fat content observed in normal rats did not occur in ZDF rats (Fig. 3), providing further proof of leptin resistance. However, in homozygous (fa/+) rats, food intake and body weight declined as in Wistar rats.

**In Vitro Effects of Leptin in Islets of ZDF Rats.** We therefore isolated islets from homozygous and heterozygous OB-R-defective (fa/fa) rats and wild-type controls (+/+)(Fig. 4A) to determine if their cells were unresponsive to the in vitro lipopenic action of leptin demonstrated in islets from normal rats. We had reported that the TG content of islets from obese ZDF rats is extraordinarily high (8) and that the capacity of their islets to esterify FFA to TG is greatly increased (14). These abnormalities are consistent with resistance to the leptin effects identified above. We cultured the islets of obese ZDF rats with 20 ng/ml leptin both with and without 1 mM FFA mixture. As shown in Fig. 4B, the islets of obese rats had a TG content of 52 ng/islet, 6.7 times that of wild-type, lean ZDF (+/+) rats. Leptin in the culture medium did not lower the TG content, and it did not reduce the greatly exaggerated increase in TG content that occurred in the presence of 1 mM FFA. By contrast, wild-type lean ZDF rats (+/+) with two normal OB-R alleles exhibited the full response to leptin (Fig. 4B). Heterozygous ZDF rats (fa/+) responded partially; the TG-lowering effect of leptin was only 38% compared with 66% in the wild-type rats; TG accumulation was reduced 57% in heterozygous islets compared with 83% of the wild-type/wild-type ZDF rats (Fig. 4B). Leptin failed to increase $[^3H]$-
palmitate oxidation or reduce its incorporation into TG in islets from homozygous (fa/ fa) ZDF rats (Fig. 4 C and D).

**DISCUSSION**

These results provide new insights into the mechanisms of leptin-induced fat depletion and the pathogenesis of the β-cell lipotoxicity described previously in ZDF rats (8, 9, 14). The results demonstrate that leptin depletes TG in cells with OB-R receptors via a direct mechanism that involves both an increase in FFA oxidation and a decrease in esterification. The ubiquity of the expression of leptin receptor isoforms, including the long isoform OB-Rb (4–6, 23), together with the *in vitro* demonstration that leptin reduces acetyl CoA carboxylase activity (26), is consistent with extraneuronal action. Also consistent with this idea are the disappearance within 7 days of all visible body fat in rats made chronically hyperleptinemic by infusion of AdCMV–leptin (7) and the exhibition by paired controls of only a modest reduction in body fat. Although the *in vitro* effect of leptin on TG content was demonstrated only in islets, the fact that TG content in the liver, pancreas, and skeletal muscle also was drastically reduced in hyperleptinemic rats implies that the same direct lipopenic action of leptin may be operative in many other OB-R-expressing tissues. The results further show that this effect requires the presence of normal leptin receptors and that the effect is attenuated if one OB-R allele is abnormal.

It now seems clear that the extraordinarily high TG content and increased esterification of FFA in islets of ZDF rats reported earlier (8, 14) is a reflection of the absence of leptin-mediated inhibition of lipogenesis from FFA. These findings thus provide the first explanation for diabetes in obese ZDF rats by linking the OB-R mutation to the β-cell lipotoxicity. The islets of these fa/ fa animals are overloaded with fat at the time that hyperglycemia begins (8). Because maneuvers that reduce islet fat content prevent the diabetes in ZDF rats (27), it has been proposed that the TG accumulation in islets plays a causal role in the β-cell dysfunction (6, 9, 19). Thus, the predisposition to diabetes in the homozygous ZDF rats may reflect the fact that their tissues have been completely “unleptinized” throughout their lives and therefore have accumulated high levels of TG. In normal rats, this accumulation is prevented by the action of leptin. It is expected that any therapy that reduces tissue TG will improve β-cell function and reduce insulin resistance.

In hyperleptinemic rats, every tissue that was examined was lipopenic. We speculate that nonadipocytes normally carry a small quantity of TG, perhaps to provide a reservoir of fuel and a degree of independence from adipocytes or to serve as an intracellular second messenger. This TG storage function is regulated by leptin. In the obesity of ZDF rats, the regulatory control by leptin is absent, and these putative intracellular TG reserves soar to levels over 50 times that of hyperleptinemic rats. This results in insulin resistance, β-cell dysfunction, and diabetes.

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