The Positive Effects of Growth Hormone-Releasing Peptide-6 on Weight Gain and Fat Mass Accrual Depend on the Insulin/Glucose Status

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Ghrelin and GH secretagogues, including GH-releasing peptide (GHRP)-6, stimulate food intake and adiposity. Because insulin modulates the hypothalamic response to GH secretagogues and acts synergistically with ghrelin on lipogenesis *in vitro*, we analyzed whether insulin plays a role in the metabolic effects of GHRP-6 *in vivo*. Streptozotocin-induced diabetic rats received saline, GHRP-6, insulin, or insulin plus GHRP-6 once daily for 8 wk. Rats receiving saline suffered hyperglycemia, hyperphagia, polydipsia, and weight loss. Insulin, but not GHRP-6, improved these parameters ($P < 0.001$ for all), as well as the diabetes-induced increase in hypothalamic mRNA levels of neuropeptide Y and agouti-related peptide and decrease in proopiomelanocortin. Cocaine amphetamine-related transcript mRNA levels were also reduced in diabetic rats, with GHRP-6 inducing a further decrease ($P < 0.03$) and insulin an increase. Diabetic rats receiving insulin plus GHRP-6 gained more weight and had increased epididymal fat mass and serum leptin levels compared with all other groups ($P < 0.001$). In epididymal adipose tissue, diabetic rats injected with saline had smaller adipocytes ($P < 0.001$), decreased fatty acid synthase (FAS; $P < 0.001$), and glucose transporter-4 ($P < 0.001$) and increased hormone sensitive lipase ($P < 0.001$) and proliferator-activated receptor-γ mRNA levels ($P < 0.01$). Insulin normalized these parameters to control values. GHRP-6 treatment increased FAS and glucose transporter-4 gene expression and potentiated insulin's effect on epididymal fat mass, adipocyte size ($P < 0.001$), FAS ($P < 0.001$), and glucose transporter-4 ($P < 0.05$). In conclusion, GHRP-6 and insulin exert an additive effect on weight gain and visceral fat mass accrual in diabetic rats, indicating that some of GHRP-6's metabolic effects depend on the insulin/glucose status. **(*Endocrinology* 151: 2008–2018, 2010)**

GH secretagogues (GHSs) and ghrelin, the endogenous ligand of the GHS receptor (GHS-R), promote GH secretion (1, 2), regulate food intake and energy homeostasis (3–6), and induce adiposity (7, 8). Recent studies reveal that ghrelin also participates in glucose homeostasis, playing an important role in the regulation of insulin secretion and glucose metabolism (9).

The GHS-R is expressed in peripheral tissues (10, 11) and the central nervous system, with this expression being highest in neurons of the hypothalamic arcuate and ventromedial nuclei (12) where it is involved in the regulation of GH secretion, feeding, and adiposity. Indeed, transgenic rats lacking GHS-R in the arcuate nucleus have decreased body weight, fat mass, and food intake (13). Ghrelin motivates an orexigenic effect by stimulation of neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus (14, 15). However, the induction of body weight gain and increased fat mass by GHSs and ghrelin is due to not only the stimulation of food intake but also to a direct effect on adipose tissue as ghrelin has been

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Abbreviations: AgRP, Agouti-related protein; CART, cocaine amphetamine-related transcript; FAS, fatty acid synthetase; GHRP, GH-releasing peptide; GHS, GH secretagogue; GHS-R, GHS receptor; GLUT, glucose transporter; HRP, horseradish peroxidase; HSL, hormone-sensitive lipase; Ins-R, insulin receptor; NPY, neuropeptide Y; POMC, proopiomelanocortin; PPAR, peroxisome proliferator-activator receptor; STZ, streptozotocin.

shown to stimulate adipogenesis and increase the number of adipocytes *in vitro* by inhibiting apoptosis and activating their proliferation and differentiation (16, 17).

The stimulatory effects of ghrelin and GHSs on food intake and adiposity are influenced by other circulating hormones, among which is insulin (18). The insulin receptor is expressed in hypothalamic neurons (19) and adipocytes (20), indicating a possible interaction of these two hormones in both central and peripheral tissues. Indeed, central insulin administration modulates the activation of hypothalamic neurons by GH-releasing peptide (GHRP)-6 (21). However, how circulating insulin affects the ability of ghrelin or GHSs to modulate the expression of hypothalamic neuropeptides related to food intake has not been reported.

Ghrelin's actions on fat mass may also be influenced by insulin and vice versa. *In vitro* studies demonstrate that ghrelin directly potentiates insulin-stimulated glucose uptake in isolated adipocytes, suggesting a synergic role for ghrelin and insulin in adipocyte regulation of glucose and lipid metabolism (17, 22, 23). However, to date no *in vivo* experiments have been reported evaluating the possible role of insulin in ghrelin or GHS-mediated actions in visceral fat or on overall weight gain and metabolism. Thus, our aim was to determine whether circulating insulin modulates the metabolic effects of GHRP-6, a ghrelin analog that binds to GHS-R1a (1), in the hypothalamus and in epididymal fat. For this purpose, streptozotocin (STZ)-induced diabetic rats, with severely reduced levels of circulating insulin, were treated with saline, GHRP-6, insulin, or insulin+GHRP-6 and different parameters related to food intake and adipose tissue metabolism were analyzed. Because this analog binds specifically to GHS-R1a (1), whereas acylated ghrelin and possibly des-acylated ghrelin may have non-GHS-R1a-mediated effects, these studies were designed to clarify the insulin-dependent actions mediated through this receptor.

Materials and Methods

Animals

All experiments were designed according to the European Union laws for animal care, and the study was approved by the local institutional ethical committee. Adult male Wistar rats from Harlan Iberica S.A. (Barcelona, Spain) were housed two per cage with free access to food and water, under constant conditions of temperature (20–22 C) and light/dark cycles (lights on from 0730 to 1930 h). Before diabetes induction, rats were adapted for 1 wk to the new environment and diet.

The rats, weighing approximately 250 g (~8 wk of age), were injected (ip) with 65 mg/kg streptozotocin (Sigma, Steinheim, Germany). Controls received vehicle. Blood glucose concentrations were measured via tail puncture (Glucocard Memory 2; Menarini Diagnostic, Florence, Italy) to verify the diabetic state (defined as blood glucose levels >300 mg/dl). All rats became diabetic within 1–3 d after streptozotocin injection. Immediately after the onset of diabetes, the rats were randomly divided into treatment groups, such that there was no difference between the groups in the mean time to develop diabetes. The treatments consisted in a daily sc injection of saline (1 ml/kg, n = 11), GHRP-6 (Bachem, Bubendorf, Switzerland; 150 μg/kg · d, n = 11), insulin (Humulin NPH pen, Eli Lilly and Company, Indianapolis, IN, 100 IU/ml, 1–8 U/d, n = 12), or insulin plus GHRP-6 (n = 12). Control rats received saline (n = 12). All rats received their treatments between 1800 and 1900 h, just before the lights were turned off and the animals naturally began their feeding period. After 8 wk of treatment, all rats were killed by decapitation 15 h after the last injection. Trunk blood was collected in cooled tubes, allowed to clot, and then centrifuged. Serum was stored at −80 C until hormone levels were measured. The brains, epididymal fat pads, pancreas, gastrocnemius, and soleus were removed, weighed, and stored at −80 C until processed.

Control of body weight and glycemia levels

Glycemia and body weight were assessed daily before treatment administration. The insulin dose was adjusted, depending on glycemia levels according to the following criteria: no insulin if glycemia was less than 50 mg/dl, 1 U insulin if glycemia was between 50 and 70 mg/dl, 2 U insulin if glycemia was between 70 and 100 mg/dl, 4 U insulin if glycemia was between 100 and 200 mg/dl, 6 U insulin if glycemia was between 200 and 400 mg/dl, 7 U insulin if glycemia was between 400 and 500 mg/dl, and 8 U insulin if glycemia was greater than 500 mg/dl.

Measurement of food intake and water consumption

Food intake and water consumption were measured weekly from diabetes onset until the moment the animals were killed. Constant amounts of food and water (800 g and 800 ml, respectively) were placed in each cage and the remaining amount of chow or water was measured the following day.

RNA preparation and purification

The hypothalami were isolated on ice by using the following boundaries: an anterior cut was made at the level of the optic chiasm, a posterior coronal section anterior to the mammillary bodies, two sagittal cuts parallel to the lateral ventricles, and a dorsal horizontal cut at the level of the anterior commissure. Finally, they were stored at −80 C until processed for RNA extraction. Total RNA was extracted from the hypothalami and epididymal adipose tissue according to the Tri-reagent protocol (24).

Quantitative real-time PCR

Proopiomelanocortin (POMC), NPY, AgRP, cocaine amphetamine-related transcript (CART), insulin receptor (Ins-R), and GHS-R mRNA levels were assessed in hypothalamic samples by quantitative real-time PCR. In adipose tissue, Ins-R, GHS-R, fatty acid synthetase (FAS), hormone-sensitive lipase (HSL), glucose transporter (GLUT)-4, and peroxisome proliferator-activator receptor (PPAR)-γ gene expression was measured.

Briefly, cDNA was synthesized from 1 μg of total RNA isolated from hypothalamic or adipose tissue (high capacity cDNA reverse transcriptase kit; Applied Biosystems, Foster City, CA).

Quantitative real-time PCR was performed by using assay-on-demand kits (Applied Biosystems) for each gene: NPY (Rn 01410145), AgRP (Rn 01431703), POMC (Rn 00595020), CART (Rn 00567382), GHS-R (Rn 00821417), Ins-R (Rn 00567670), FAS (Rn 00569117), HSL (Rn 00563444), PPAR-γ (Rn 00440945), and GLUT-4 (Rn 00562597). TaqMan Universal PCR master mix (Applied Biosystems) was used for amplification according to the manufacturer's protocol in an ABI PRISM 7000 sequence detection system (Applied Biosystems). Values were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. According to the manufacturer's guidelines, the $\Delta\Delta C_T$ method was used to determine relative expression levels. Statistics were performed using $\Delta\Delta C_T$ values (25).

Determination of serum total and acylated ghrelin and IGF-I levels by RIA

Total and acylated ghrelin were measured by RIA following the manufacturer's instructions (Linco Research, St. Charles, MO). The sensitivity of the method was 93 pg/ml and the intraassay variation was 6.4% for both assays.

Serum IGF-I concentrations were measured by a double-antibody RIA. Serum IGF-I binding proteins were removed by acid-ethanol extraction. The IGF-I antiserum (UB2-495) was a gift from Dr. Underwood and Dr. Van Wyk and is distributed by the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases through the National Hormone and Pituitary Program, Gropep Ltd. (Adelaide, Australia). The intraassay coefficient of variation was 8%. All samples were run in duplicate and within the same assay.

Determination of serum leptin, insulin, and adiponectin levels by ELISA

Serum levels of these hormones were measured by ELISA following the manufacturer's instructions (Linco Research). The sensitivity of the method for leptin, insulin, and adiponectin were 0.04, 0.2, and 0.16 ng/ml, respectively. Absorbance in each well was measured by using a Tecan Infinite M200 (Grödig, Austria), and leptin, insulin, and adiponectin serum concentrations were calculated from the standard curve. All samples were run in duplicate and within the same assay for all analyses. The intraassay variation was 2.2% for leptin, 1.9% for insulin, and 1.3% for adiponectin.

Determination of serum C-peptide levels by EIA

Serum C-peptide concentrations were measured by enzyme immunoassay according to the manufacturer's instructions (Yanaihara Institute Inc., Shi, Japan). This assay combines a highly specific antibody to rat C-peptide and the biotin-avidin affinity system. Briefly, the 96-well plate was coated with goat antirabbit IgG and C-peptide standards or samples. Then 50 μl of labeled antigen and antirat C-peptide antibody were added to all wells to allow the competitive immunoreaction. After a 3-h incubation at room temperature, the plate was washed three times and then horseradish peroxidase (HRP)-labeled streptavidin was added to form HRP-labeled streptoavidin-biotinylated rat C-peptide-antibody complexes on the surface of the wells. Finally, HRP activity was determinate by addition of *o*-phenylenediamine dihydrochloride. All samples were run in duplicate. The sensitivity of the method was 1.56 ng/ml and the intraassay variations was 6.1%.

Analysis of epididymal fat adipocytes

Cryostat sections of 10 μm from epididymal fat mass were fixed with 10% formol, stained with hematoxylin and eosin, and visualized by using a light microscope. To examine the size of adipocytes the major axis (maximum diameter) of each adipocyte was measured using Image Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD). At least five cells from three randomly selected fields on each section were analyzed in all rats from each group. Finally, the mean adipocyte diameter was then calculated for each group.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA for multiple comparisons, following by Bonferroni's test. Two-way ANOVA was performed to determine whether there was an interaction between effects of insulin and GHRP-6 in diabetic rats. The values were considered significantly different when $P < 0.05$.

Results

Blood glucose levels

All diabetic rats, regardless of the treatment received, had significantly increased mean blood glucose levels over the 8-wk treatment compared with control rats (Table 1; $P < 0.001$). Insulin treatment significantly decreased blood glucose levels (F: 434.38, $P < 0.0001$) with no effect of GHRP-6 and no interaction between these two factors, such that the mean glycemia of diabetic rats treated with insulin or insulin+GHRP-6 was significantly lower than that of diabetic rats that received either saline or GHRP-6 alone ($P < 0.001$).

Total and weekly body weight gain

Weekly and cumulative body weight gains are shown in Fig. 1, A and B, respectively. Whereas control rats continued to gain weight throughout the 8-wk study, diabetic rats injected with either saline or GHRP-6 lost weight ($P < 0.001$). Although GHRP-6 alone had no effect, there was a significant effect of insulin (F: 437.31, $P < 0.0001$) and an interaction between these two factors (insulin-GHRP-6 F: 4.80, $P < 0.03$) on weight gain in diabetic rats, with insulin-inducing weight gain and GHRP-6 potentiating this effect. The body weight gain of diabetic rats treated with insulin was not statistically different from that of control rats. However, diabetic rats treated with insulin plus GHRP-6 gained significantly more weight than both controls and diabetic rats injected with only insulin ($P < 0.0001$).

TABLE 1. Glycemia, C-peptide, total and acylated ghrelin, leptin, adiponectin, IGF-I, and insulin concentrations (nanograms per milliliter) in control (C) and diabetic (DB) rats

	C + saline	DB + saline	DB + GHRP-6	DB + insulin	DB + insulin and GHRP-6
Glycemia	86 ± 1	543 ± 11[a]	529 ± 16[a]	249 ± 12[a]	198 ± 11[a]
C-peptide (ng/ml)	2.2 ± 1	ND	ND	ND	ND
Insulin (ng/ml)	5 ± 0.9	0.8 ± 0.17[a]	0.61 ± 0.05[a]	16.8 ± 5.8[b]	35.7 ± 11.5[a,b]
Total ghrelin (pg/ml)	1821 ± 219	1775 ± 185	2082 ± 232	1988 ± 341	1797 ± 135
Acylated ghrelin (pg/ml)	291 ± 64	208 ± 33	196 ± 37	227 ± 39	338 ± 9
Leptin (ng/ml)	15 ± 1	0.3 ± 0.01[a]	0.34 ± 0.03[a]	8 ± 2[a,b]	22 ± 1.5[b]
Adiponectin (ng/ml)	33.3 ± 2.8	8.5 ± 2.5[a]	12 ± 1.4[a]	76.5 ± 8[a,b]	78.6 ± 1.7[a,b]
IGF-I (ng/ml)	1075 ± 93	295 ± 57[a]	252 ± 52[a]	1045 ± 138[b]	996 ± 64[b]

Data are represented as mean ± SEM; n = 11–12 rats/group. ND, Not detected.

[a] Significantly different compared to C + saline.

[b] Significantly different compared to DB + saline.

Weights of epididymal fat pads, gastrocnemius, soleus, and pancreas

Epididymal fat pads weighed less in diabetic rats treated with saline or GHRP-6 than in controls and diabetic rats treated with insulin or insulin plus GHRP-6 (Table 2; $P < 0.001$). There was an interaction between insulin and GHRP-6 (F: 5.11, $P < 0.03$) on epididymal fat weight with insulin promoting visceral fat accumulation and GHRP-6 potentiating this effect ($P < 0.001$).

The weights of the gastrocnemius and soleus were also decreased in nontreated diabetic rats compared with controls (Table 2; $P < 0.001$). Insulin, but not GHRP-6, increased the weights of the pancreas (F: 29.16, $P < 0.001$) and the gastrocnemius (F: 84.43, $P < 0.001$) and soleus muscles (F: 3.6, $P < 0.001$), with no interaction between these two treatments.

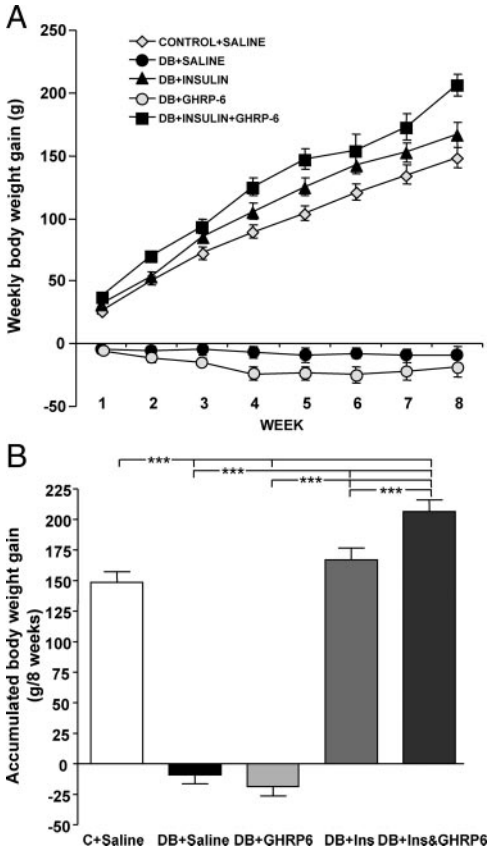

FIG. 1. Weekly (A) and accumulated (B) body weight gain over the 8-wk treatment period. Data are represented as mean ± SEM (n = 11–12 rats/group). DB, Diabetic; Ins, insulin. ***, ANOVA, $P < 0.001$.

Food and water intake

Saline- and GHRP-6-treated diabetic rats presented hyperphagia and polydipsia ($P < 0.001$). Insulin had a significant effect on food intake in diabetic rats (F: 74.96, $P < 0.0001$), with no effect of GHRP-6 or interaction between these two factors, such that food and water consumption of rats treated with insulin, alone or with GHRP-6, was not different from that of control rats (Fig. 2, A and B, respectively).

Circulating C-peptide, total and acylated ghrelin, leptin, adiponectin, insulin, and IGF-I levels

Serum C-peptide levels were undetectable in all diabetic rats, regardless of treatment (Table 1). There was an interaction between insulin and GHRP-6 on serum insulin levels (F; 4.8, $P < 0.04$; Table 1). The concentrations of this hormone were significantly decreased in diabetic rats administered saline or GHRP-6 ($P < 0.001$), whereas the concentrations were not statistically different between insulin-treated rats and controls. However, circulating insulin concentrations were above control levels in the diabetes (DB)+insulin (Ins)+GHRP-6 experimental group ($P < 0.001$) but not statistically different from serum insulin levels of insulin-treated diabetic rats.

Serum total and acylated ghrelin levels were not significantly different between groups (Table 1). Serum leptin levels were significantly decreased in diabetic rats injected with saline ($P < 0.001$). Whereas GHRP-6 had no effect, insulin significantly increased leptin levels in diabetic rats (F: 173.17, $P < 0.0001$), although it did not restore them to control values. There was a significant interaction between insulin and GHRP-6 (insulin-

TABLE 2. Body weight and epididymal fat, gastrocnemius, soleus, and pancreas weights of control (C) and diabetic (DB) rats after treatment with GHRP-6, insulin, or the combination of GHRP-6 and insulin

	C + saline	DB + saline	DB + GHRP-6	DB + insulin	DB + insulin and GHRP-6
Body weight (g)	422 ± 13	249 ± 7[a]	234 ± 9[a]	423 ± 14[b]	460 ± 12[b]
Epididymal fat (mg)	6581 ± 476	273 ± 52[a]	203 ± 30[a]	6410 ± 188[b]	8738 ± 792[a,b,c]
Gastrocnemius (mg)	1990 ± 51	908 ± 53[a]	812 ± 84[a]	1914 ± 52[b]	1996 ± 46[b]
Soleus (mg)	150 ± 5	93 ± 10[a]	94 ± 5[a]	161 ± 6[b]	152 ± 5[b]
Pancreas (mg)	779 ± 39	734 ± 82	714 ± 33	943 ± 23[a,b]	1020 ± 51[a,b]

Data are represented as mean ± SEM; n = 11–12 rats/group.

[a] Significantly different compared to C + saline.

[b] Significantly different compared to DB + saline.

[c] Significantly different compared to DB + insulin.

GHRP-6 F: 39.52, $P < 0.0001$), with the combination of the two treatments inducing a further increase in serum leptin levels (Table 1).

Circulating adiponectin levels were also decreased in diabetic rats treated with saline (Table 1; $P < 0.001$). GHRP-6 treatment of diabetic rats had no effect, whereas insulin treatment significantly increased (F: 259.23, $P < 0.0001$) the levels of this adipokine above control levels, with no interaction between these two factors.

Serum IGF-I levels were significantly reduced in diabetic rats treated with saline compared with controls (Table 1; $P < 0.001$). Insulin treatment inhibited the decrease in circulating IGF-I levels (F: 68.06, $P < 0.0001$), but GHRP-6 had no effect nor was there an interaction between insulin and GHRP-6 on IGF-1 levels.

Pituitary GH content

As previously described (26), GH content was significantly reduced in the pituitary of diabetic rats injected with saline (Fig. 3, $P < 0.001$). Both GHRP-6 (F: 7.06, $P < 0.02$) and insulin (F: 85.34, $P < 0.0001$) alone or in combination increased GH levels in the anterior pituitary ($P < 0.001$), with no interaction between these two treatments.

Hypothalamic NPY, AgRP, POMC, CART, GHS-R, and Ins-R mRNA levels

Hypothalamic NPY (Fig. 4A) and AgRP (Fig. 4B) mRNA levels were increased in diabetic rats treated with

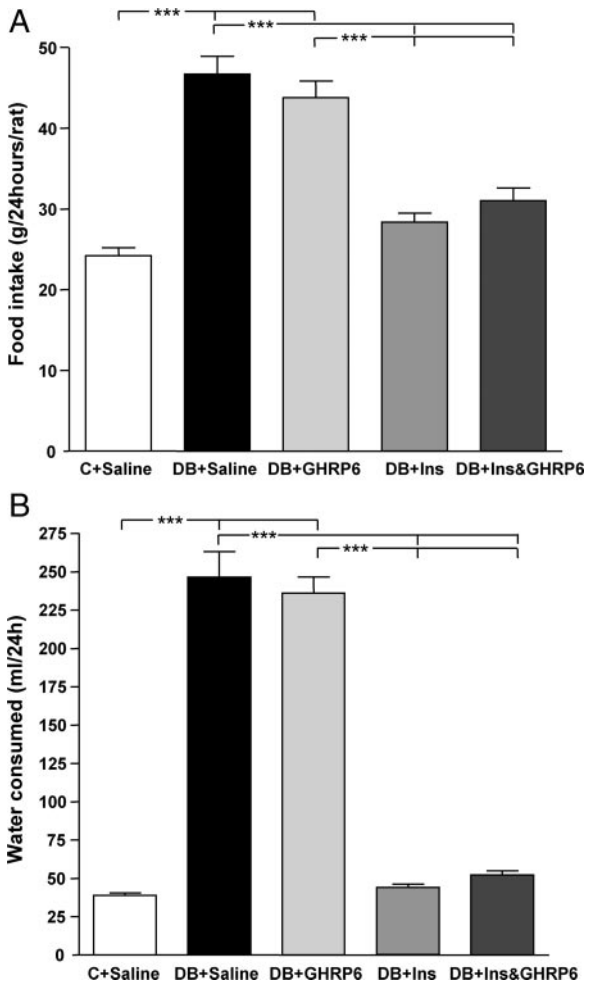

FIG. 2. Twenty-four-hour food (A) and water (B) intake over the 8-wk treatment period. Data are represented as mean ± SEM (n = 11–12 rats/group). C, Control; DB, diabetic; Ins, insulin. ***, ANOVA, $P < 0.001$.

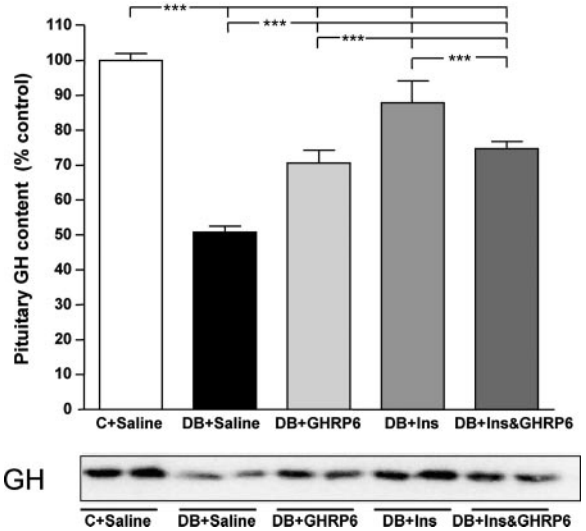

FIG. 3. Pituitary GH content measured by Western blot. Data are represented as mean ± SEM and referred to control values (n = 5–6 rats/group). C, Control; DB, diabetic; Ins, insulin. ***, ANOVA, $P < 0.001$.

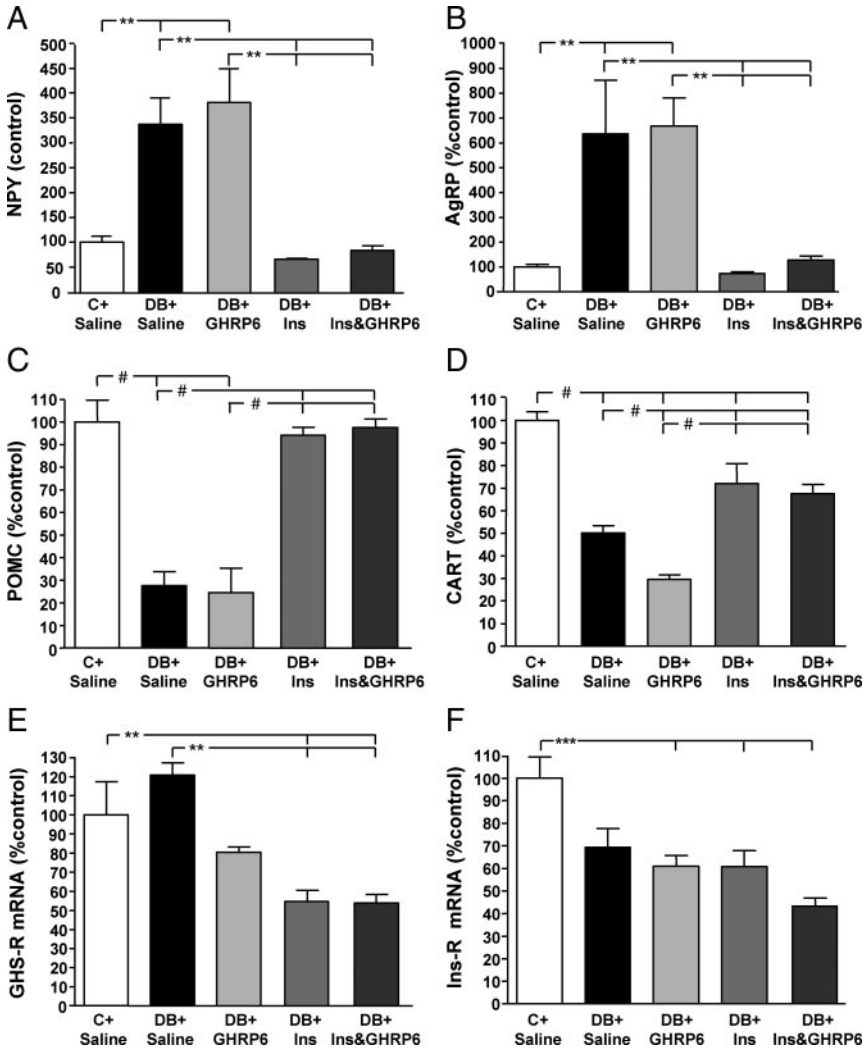

FIG. 4. Relative mRNA levels of NPY (A), AgRP (B), POMC (C), CART (D), GHS-R (E), and Ins-R (F) in the hypothalamus. Data are represented as mean ± SEM and referred to control values (n = 5–6 rats/group). C, Control; DB, diabetic; Ins, insulin. **, ANOVA, $P < 0.01$; #, ANOVA, $P < 0.0001$; ***, ANOVA, $P < 0.001$.

saline compared with control rats ($P < 0.01$ for both neuropeptides). GHRP-6 had no effect, whereas insulin significantly decreased the mRNA levels of both NPY (F: 14.77, $P < 0.002$) and AgRP (F: 13.92, $P < 0.003$), with no interaction between the two treatments.

Both POMC (Fig. 4C, $P < 0.0001$) and CART (Fig. 4D, $P < 0.0001$) mRNA levels were significantly decreased in the hypothalamus of diabetic rats compared with controls. Insulin treatment significantly increased (F: 107.33, $P < 0.0001$) POMC mRNA levels in diabetic rats, whereas GHRP-6 had no effect and there was no interaction between the two treatments. Insulin also significantly increased hypothalamic CART mRNA levels in diabetic rats (F: 13.11, $P < 0.005$). In contrast, GHRP-6 treatment exacerbated the diabetes-induced reduction in CART mRNA levels (F: 6.08, $P < 0.03$).

Hypothalamic GHS-R mRNA levels were not modified in diabetic rats treated with saline or GHRP-6 when compared with controls, whereas insulin-treated diabetic rats had lower expression of this receptor compared with diabetic rats receiving saline ($P < 0.01$; Fig. 4E). Ins-R mRNA levels were down-regulated in diabetic rats treated with saline, although this did not reach statistical significance (Fig. 4F); However, treatment of diabetic rats with GHRP-6, insulin, or insulin+GHRP-6 resulted in a significant reduction in Ins-R compared with control rats ($P < 0.001$), with no interaction between insulin and GHRP-6 treatments.

FAS, HSL, GLUT-4, PPAR-γ, GHS-R, and Ins-R mRNA levels in epididymal fat

Diabetic rats injected with saline had decreased FAS and GLUT-4 ($P < 0.001$ and $P < 0.05$, respectively; Fig. 5, A and B) and increased HSL and PPAR-γ mRNA levels ($P < 0.001$; Fig. 5, C and D) in epididymal fat tissue. Both insulin (F: 47.31, $P < 0.0001$) and GHRP-6 (F: 9.61, $P < 0.005$) increased FAS mRNA levels in epididymal fat, with an additive effect of these two treatments. Likewise, insulin increased GLUT-4 mRNA levels (F: 13.91, $P < 0.01$) as did GHRP-6, and insulin plus GHRP-6 further increased the gene expression of this glucose transporter in epididymal fat (F: 6.73, $P < 0.05$). Insulin treatment significantly decreased HSL (F: 21.21, $P < 0.0005$), with no effect of GHRP-6. Insulin also prevented the diabetic-induced increase in PPAR-γ mRNA levels (F: 31.90, $P < 0.0001$), with no effect of GHRP-6 or interaction between the treatments.

Levels of GHS-R (Fig. 5E) and Ins-R (Fig. 5F) mRNA were increased in epididymal adipose tissue of diabetic rats receiving saline ($P < 0.05$ and $P < 0.01$, respectively). Insulin treatment significantly decreased Ins-R (F: 8.88, $P < 0.02$) and GHS-R mRNA levels (F: 15.34, $P < 0.001$), whereas GHRP-6 had no effect and there was no interaction between the treatments.

Adipocyte size

The mean size of adipocytes from the visceral fat of diabetic rats injected saline (20.6 ± 2 μm) was significantly smaller than that of controls (69.5 ± 3.2 μm) and insulin-treated diabetic rats (63.9 ± 5.8 μm), with insulin treatment reversing this parameter to control levels (Fig. 6;

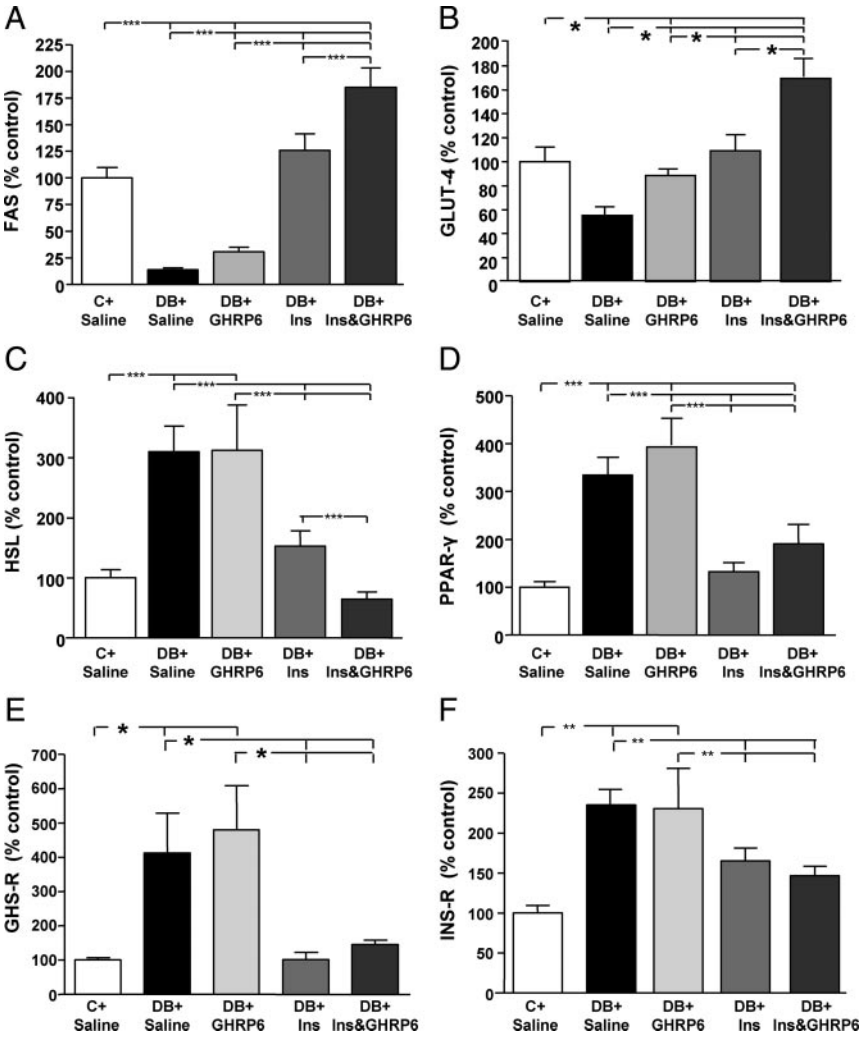

FIG. 5. Relative mRNA levels of FAS (A), GLUT-4 (B), HSL (C), PPAR-γ (D), GHS-R (E), and Ins-R (F) in epididymal fat. Data are represented as mean ± SEM and referred to control values (n = 5–6 rats/group). C, Control; DB, diabetic; Ins, insulin. *, ANOVA, $P < 0.5$; **, ANOVA, $P < 0.01$; ***, ANOVA, $P < 0.001$.

$P < 0.001$). There was an interaction between insulin and GHRP-6 on adipocyte size (F: 12.06; $P < 0.01$) because GHRP-6 administered alone had no effect on adipocyte size (16.8 ± 1.1 μm) but administered with insulin increased the size of adipose cells above that seen in controls (104.9 ± 11.4 μm; $P < 0.001$).

Discussion

The results reported here indicate that some of the metabolic effects of GHRP-6, and hence possibly of ghrelin, on weight gain and fat mass accrual depend on the insulin status. Ghrelin and GHSs are reported to have beneficial effects in some catabolic diseases, including type 1 diabetes, by reducing body weight loss and improving the negative energy balance found in these conditions (27). However, we found that in STZ-induced diabetic rats, daily treatment with GHRP-6 for 8 wk starting at the onset of diabetes did not affect diabetes-induced weight loss or hyperphagia when insulin levels were extremely low and glucose levels high. In contrast, insulin treatment prevented the diabetes-induced increase in food intake and water consumption as well as the decrease in weight gain, even though glucose levels remained above control levels. However, whereas diabetic rats treated with insulin gained approximately the same weight as nondiabetic rats, diabetic rats treated with GHRP-6 plus insulin gained more weight than control rats and those diabetic rats receiving only insulin. This suggests that the positive effect of GH secretagogues on body weight gain (4, 5, 7, 26, 28) may be subjugated by the insulin/glucose status.

Circulating glucose, C-peptide, and insulin levels in GHRP-6-treated rats were not different from diabetic rats receiving saline, suggesting that this secretagogue did not stimulate pancreas regeneration, insulin synthesis, or insulin secretion in diabetic rats, as previously reported (29), and that these rats were indeed insulin deprived. This is in contrast to a previous study (30) in which ghrelin treatment of newborn STZ-diabetic rats stimulated β-cell replication and insulin production, preventing the development of diabetes during adulthood. However, other reports indicate that neither ghrelin nor GHS improves glycemia or insulin production in adult STZ-diabetic rats or mice (26, 31). Thus, the beneficial effects of GHS-R agonists on STZ-induced damage of β-cells may be exerted only during the neonatal period. In support of this, ghrelin has recently been reported to promote pancreas regeneration in newborn pancreatectomized rats (32). It is also possible that ghrelin's protective effect on β-cells is mediated by a GHS-R-independent mechanism (33), and thus, GHRP-6 may not have the same effect.

STZ treatment induced diabetes but had no effect on pancreatic weight as previously reported (34), although this treatment has been shown to induce the specific loss of pancreatic β-cells (34–36). Other cell types, such as α-cells, increase their proliferation after STZ treatment (34), which could explain why no overall pancreatic

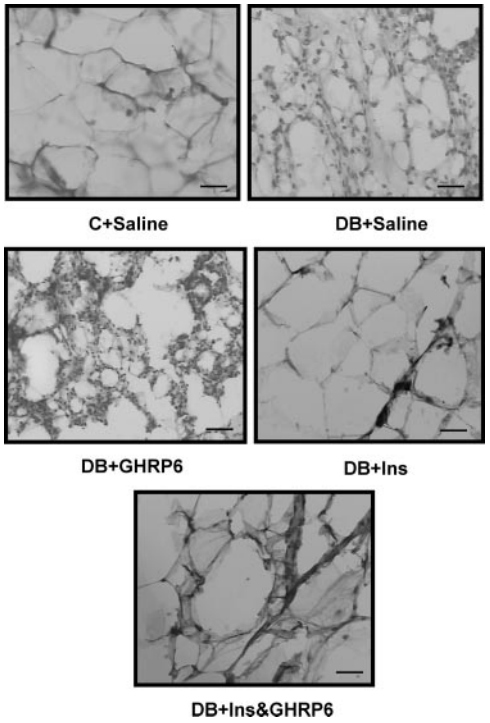

FIG. 6. Photomicrographs of adipocytes from epididymal fat from control (C) rats and diabetic (DB) rats injected saline, GHRP-6, insulin, and insulin+GHRP-6. *Bar*, 20 μm.

weight change was observed. As mentioned above, GHRP-6 had no effect on pancreatic weight, C-peptide, or insulin levels. In contrast, insulin treatment increased the weight of the pancreas in diabetic rats. This hormone has been shown to protect β-cells in diabetes, as well as to stimulate their proliferation (35, 37); however, again no change in C-peptide was observed with insulin treatment, suggesting that at least functional β-cells were not induced by this treatment. If other cell types increased their proliferation or size in response to insulin remains to be determined.

Insulin treatment prevented the diabetes-induced decrease in serum adiponectin and IGF-I levels, as previously reported (38–40). Likewise, as described previously (29, 41), insulin increased leptin levels in diabetic rats, although not to control values. In contrast, GHRP-6 did not modify circulating leptin, adiponectin, total ghrelin, or acylated ghrelin levels, similar to what we observed with iv infusion of GHRP-6 (our unpublished results) or ghrelin to diabetic rats (26). This raises the question as to whether insulin is required for the central and/or peripheral effects of ghrelin and GHSs. However, GHRP-6 increased pituitary GH content in diabetic rats, similar to that reported in normal rats (42) but without modifying circulating IGF-I levels. The lack of effect of GHSs on hepatic and serum IGF-I levels in experimental diabetes is suggested to be due to a decrease in hepatic GH receptor levels that results in GH resistance (31). Although it is possible that the GHRP-6 dose used in these studies was insufficient to modify IGF-I levels, even when insulin had been replaced, a significantly lower dose of this analog (30 μg/kg) was shown to be even more effective in the release of GH in STZ-diabetic rats than in controls (43), indicating that the dose used here was sufficient to stimulate GH secretion. However, it is possible that a larger acute GHRP-6 dose could stimulate a further increase in circulating IGF-I levels.

Diabetic rats are hyperphagic, which is coincident with an increase in the hypothalamic mRNA levels of the orexigenic neuropeptides NPY and AgRP and a decrease in the anorexigenic neuropeptides POMC and CART. Insulin normalized the mRNA levels of all four neuropeptides, as previously described (44, 45). The mRNA levels of POMC, NPY and AgRP were not altered by GHRP-6 administration, in neither the presence or absence of insulin. It is conceivable that GHRP-6 alone had no effect on these neuropeptides because these rats were already hyperphagic and food intake could not be further stimulated. However, after insulin treatment when the rats were no longer hyperphagic, GHRP-6 continued to have no effect on food intake or POMC, NPY, and AgRP mRNA levels. It is possible that insulin and leptin, which remained increased after insulin treatment, counteracted the effect of GHRP-6 because they have opposite actions on food intake and the synthesis of these neuropeptides (46). Indeed, chronic central infusion of insulin or leptin suppresses GHRP-6-induced Fos expression in hypothalamic neurons (21).

In contrast to NPY, AgRP, and POMC, CART mRNA levels were significantly decreased by GHRP-6 in the absence of insulin, with no effect on this neuropeptide in the presence of insulin. Other studies have reported that the normalization of CART does not always follow that of NPY, POMC, and AgRP (47), which may have to do with its role in different physiological functions such as stress or other behaviors (48). Indeed, in the hypothalamus in addition to being coexpressed in POMC neurons, CART is also colocalized with vasopressin and CRH (49). Fasting is reported to reduce CART mRNA levels specifically in the paraventricular nucleus and dorsal medial hypothalamus, with no effect in the arcuate nucleus or lateral hypothalamus, whereas STZ-induced diabetes diminished CART in all areas except in the lateral hypothalamus (49). Thus, whether the additional decline in CART mRNA levels in response to GHRP-6 represents a specific subpopulation of CART expressing cells deserves further investigation.

The inability of GHRP-6 to modulate these neuropeptides in diabetic rats does not appear to be due to decreased sensitivity of the hypothalamus as GHS-R mRNA levels

were similar to control levels in diabetic rats injected with saline or GHRP-6. However, insulin significantly decreased the hypothalamic mRNA levels of GHS-R, which may explain the decreased neuropeptide response to GHRP-6 in insulin-treated rats. In contrast, hypothalamic insulin receptor mRNA levels were decreased in diabetic rats, as reported in other brain areas in this experimental model (50), and neither insulin nor GHRP-6 prevented this decline. No effect of insulin on its receptor levels could be due to the dose administered because diabetes-induced alterations of Ins-R gene expression in central and peripheral tissues are reported to be prevented only with moderate doses of insulin (51).

The increased body weight gain of diabetic rats treated with insulin plus GHRP-6 was the result, at least in part, of increased visceral adipose tissue accrual and this was not secondary to an increase in food intake. Indeed, ghrelin and GHSs are reported to increase visceral fat mass by a mechanism independent of GH secretion and food intake (52). Diabetic rats suffered adipose tissue loss and a dramatic decrease in adipocyte size, as previously reported (53), due to increased lipolysis and decreased fatty acid synthesis as suggested by increased HSL and decreased FAS gene expression in the epididymal fat pads (54, 55). Levels of GLUT-4 mRNA were also decreased in nontreated diabetic rats, as previously reported in both STZ-diabetic animals (56, 57) and STZ-incubated adipocytes (58), whereas PPAR-γ gene expression was increased.

This increased gene expression of PPAR-γ in epididymal fat has previously been reported (59) and could be a compensatory mechanism of adipocytes in an attempt to increase insulin sensitivity. As expected, insulin treatment reverted all of these parameters to control levels (29, 54–56). In contrast, GHRP-6 did not modify adipocyte size or HSL or PPAR-γ mRNA levels, although GHRP-6 in combination with insulin induced a further decline in HSL mRNA levels with no further effect on PPAR-γ. However, GHRP-6 treatment alone significantly increased GLUT-4 and FAS mRNAs in the epididymal fat pads of diabetic rats and treatment with insulin plus GHRP-6 had an additive effect. Indeed, adipocytes were significantly larger in diabetic rats receiving insulin plus GHRP-6 than in control or insulin-treated diabetic rats.

Together, these results suggest increased adipogenesis and lipogenesis as a result of increased glucose uptake by adipocytes, with a synergistic effect between insulin and GHRP-6 on adipose tissue. Our results differ from those of Ott *et al.* (60) in which ghrelin pretreatment of differentiating adipocytes did not alter insulin-induced glucose uptake. However, in recent *in vitro* studies, ghrelin was shown to enhance insulin-stimulated glucose uptake in 3T3-L1 adipocytes (17) and isolated retroperitoneal and epididymal adipocytes (22, 23), supporting our findings. Furthermore, Kim *et al.* (17) reported that in the absence of insulin, ghrelin has no effect on adipocyte deoxyglucose uptake, also suggesting that ghrelin acts synergistically with insulin in visceral adipose tissue.

The effects of GHRP-6 and ghrelin on epididymal adipocytes are most likely mediated through GHS-R1a, which is expressed in this tissue (16), because des-acyl ghrelin, a major circulating nonoctanylated form of ghrelin, has no effect on insulin-stimulated glucose uptake (22). However, other authors have reported that both ghrelin and des-acyl ghrelin facilitate preadipocyte differentiation and enhance cellular lipid content *in vitro*, suggesting a GHS-R1a-independent mechanism (61). The differential effects found in these studies could be due to the different types of adipocytes used because GHS-R1a is reported to be differentially expressed in the different types of visceral fat (22).

The gene expression of GHS-R and Ins-R were up-regulated in visceral fat of diabetic rats treated with saline or GHRP-6. Insulin reversed the up-regulation of GHS-R but did not decrease Ins-R mRNA to control levels in adipose tissue. Because glucose levels were not totally normalized with this insulin regimen, Ins-R gene expression in adipose tissue could be affected by glycemia. Again, this effect could also be due to the insulin dose used because diabetes-induced up-regulation of Ins-R in liver and kidney is reversed only with moderate doses of insulin (51).

In conclusion, our results show that some of GHRP-6's metabolic effects are insulin dependent. Indeed, GHRP-6 administration to STZ-diabetic rats did not increase body weight gain or visceral fat mass accrual but in combination with insulin promoted weight gain and increased visceral adiposity due to increased lipogenesis and decreased lipolysis, with no change in food intake. In contrast, GHRP-6 did not modify the insulin-induced normalization of hypothalamic neuropeptides involved in appetite control but had an independent effect on CART. These results indicate that the interaction of insulin and GHSs are different in different tissues and that the response to GHSs, or possibly ghrelin, may be modulated by the insulin/glucose status. Thus, the studies reported here may help to clarify some of the controversial reports found in the literature pertaining to the interaction of ghrelin or GHSs and insulin on metabolic parameters.

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