

Inhibitory Effects of Leptin on Pancreatic α -Cell Function

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OBJECTIVE—Leptin released from adipocytes plays a key role in the control of food intake, energy balance, and glucose homeostasis. In addition to its central action, leptin directly affects pancreatic β -cells, inhibiting insulin secretion, and, thus, modulating glucose homeostasis. However, despite the importance of glucagon secretion in glucose homeostasis, the role of leptin in α -cell function has not been studied in detail. In the present study, we have investigated this functional interaction.

RESEARCH DESIGN AND METHODS—The presence of leptin receptors (ObR) was demonstrated by RT-PCR analysis, Western blot, and immunocytochemistry. Electrical activity was analyzed by patch-clamp and Ca^{2+} signals by confocal microscopy. Exocytosis and glucagon secretion were assessed using fluorescence methods and radioimmunoassay, respectively.

RESULTS—The expression of several ObR isoforms (a–e) was detected in glucagon-secreting α TC1-9 cells. ObRb, the main isoform involved in leptin signaling, was identified at the protein level in α TC1-9 cells as well as in mouse and human α -cells. The application of leptin (6.25 nmol/l) hyperpolarized the α -cell membrane potential, suppressing the electrical activity induced by 0.5 mmol/l glucose. Additionally, leptin inhibited Ca^{2+} signaling in α TC1-9 cells and in mouse and human α -cells within intact islets. A similar result occurred with 0.625 nmol/l leptin. These effects were accompanied by a decrease in glucagon secretion from mouse islets and were counteracted by the phosphatidylinositol 3-kinase inhibitor, wortmannin, suggesting the involvement of this pathway in leptin action.

CONCLUSIONS—These results demonstrate that leptin inhibits α -cell function, and, thus, these cells are involved in the adipose-pancreatic communication. *Diabetes* 58:1616–1624, 2009

Among the different hormones released by adipocytes, leptin plays a fundamental role in the control of satiety and body weight by acting on the hypothalamus and, thus, regulating food intake and energy expenditure (1,2). Although several factors modulate the release of leptin from adipocytes, its plasma levels are frequently proportional to body fat mass (1). Its action is mediated by the activation of the leptin receptor (ObR), which is highly expressed in the hypothalamus and cerebellum as well as in other tissues involved in metabolism, such as the endocrine pancreas, the liver, and the adipose tissue (3). The ObR gene produces several splicing variants, yet the long form of the receptor (ObRb) is the main isoform involved in the transduction of intracellular signals (3,4). The activation of ObR induces JAK/STAT (Janus kinase/signal transducer and activator of transcription signaling), which is implicated in transcriptional modulation (4). Additionally, leptin can induce rapid effects on membrane potential and secretion in endocrine cells and neurons by activating the phosphatidylinositol 3-kinase (PI3K) signaling pathway (5–10).

Remarkably, leptin can inhibit insulin expression and secretion in pancreatic β -cells, regulating glucose homeostasis directly through its action on the endocrine pancreas in addition to its central effects (7,11–15). The inhibition of insulin release by leptin is mainly mediated by the hyperpolarization of the membrane potential and a subsequent decrease in Ca^{2+} signaling in both human and rodent β -cells (8–13). These effects result from ATP-sensitive K^+ channel (K_{ATP} channel) opening and involve the PI3K pathway (9–11). Since insulin also stimulates leptin release from adipocytes, there is a bidirectional feedback loop between β -cells and the adipose tissue. It has been proposed that the dysregulation of this adipose-pancreatic communication may play a role in the development of diabetes in obese individuals (14,15).

However, despite the importance of glucagon secretion in the regulation of glycemia, the effect of leptin on α -cells has not been studied in detail. The hyperglycemic hormone glucagon increases blood glucose levels essentially by inducing glucose synthesis and mobilization in/from the liver (16,17). Glucagon secretion is the main line of defense against hypoglycemia and, also, counterbalances the effects of insulin on glucose levels (18). In diabetes, glucagon secretion does not respond adequately to glucose changes, which leads to further problems in the control of glucose levels in diabetic patients besides those difficulties derived from β -cell malfunction (17,18). Leptin has been revealed as an important modulator of glucose homeostasis by directly acting on β -cells. Therefore, given that glucagon is an essential player in the islet function

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TABLE 1
PCR primers for ObR isoforms and 18S

Name	Sense primer (5'-3')	Antisense primer (5'-3')
ObRa	AGGGCTGTATGTCATTGTACCCAT	AGTTTAGGTTTGTTCCTCCATC
ObRb	ACAGTTCTGGCTGTCAATTCCC	AGGAGCTGCTAGAAAGACTG
ObRc	ACAGTTCTGGCTGTCAATTCCC	GAAAGGATGAACAGGCTTGAGAAC
ObRd	AGGGCTGTATGTCATTGTACCCAT	CTTCATGTAAGATATATCCTTTTCC
ObRe	AGGGCTGTATGTCATTGTACCCAT	CCATGAAAAGTACAGTACACATACC
18S	GGGAGGTAGTGACGAAAAATAAC	AATCATGGCCTCAGTTCCGAAA

and the regulation of glycemia, it is important to determine whether leptin can affect α -cell function. Since defects in the adipoinular axis may contribute to diabetes associated with obesity (14,15), a better understanding of the communication between the adipose tissue and the two main endocrine cells involved in glucose homeostasis is essential to design potential therapeutic strategies. Several evidences suggest that leptin could have a role in the α -cell. It has been reported that mouse models with defects in leptin signaling can develop hyperglucagonemia (19–21). Additionally, it was recently shown that induction of hyperleptinemia decreases glucagon levels in diabetic mice with hyperglucagonemia (22). However, the direct regulation of glucagon secretion by leptin has not been described so far.

In the present study, we show a direct action of leptin on the α -cell. The presence of ObR was identified in the glucagon-secreting cell line α TC1-9 and in mouse and human α -cells. We found that leptin hyperpolarizes the membrane potential in α -cells, suppressing electrical activity. Additionally, leptin inhibited Ca^{2+} signaling and glucagon secretion induced by low glucose concentrations. All these findings indicate that the α -cell function can be modulated by the adipose tissue through a leptin signaling pathway.

RESEARCH DESIGN AND METHODS

Islet isolation and cell culture. All protocols were approved by our animal care committee according to national regulations. Swiss albino OF1 mice (8–10 weeks old) were killed by cervical dislocation, and islets were then isolated by collagenase digestion (23,24). Isolated islets were dispersed into single cells by trypsin enzymatic digestion and then cultured overnight at 37°C in RPMI-1640 (Sigma, Madrid, Spain) supplemented with 10% FCS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 5.6 mmol/l D-glucose (23,24). The glucagon-releasing α TC1-9 cell line was purchased from the American Type Cultures Collection (ATCC CRL-2350; passage 5–15). α TC1-9 cells were grown in Dulbecco's Modified Eagle's medium (Invitrogen) containing (in mmol/l) 4 L-glutamine, 16 glucose, 19 NaHCO₃, 10% fetal bovine serum, 15 HEPES, and 0.1 nonessential amino acids. Human islets from healthy donors were obtained from the human islet isolation facility of the Clinic Hospital of Barcelona after approval of the hospital ethics committee. Islets were cultured in RPMI-1640 at 37°C and 5% CO₂. Experiments were performed within 48 h of isolation. Leptin (Calbiochem) was used at 6.25 nmol/l (100 ng/ml) and 0.625 nmol/l (10 ng/ml). Except when indicated, all experiments were carried out at 37°C.

Ca²⁺ signaling measurements by confocal microscopy. For Ca²⁺ experiments, cells were loaded for 1 h at 37°C with the Ca²⁺ probe Fluo-4 (2 μ mol/l) (Invitrogen). Islets were loaded with Fluo-4 (5 μ mol/l) for 1 h at room temperature. Islets or cells were placed on a perfusion chamber mounted on the microscope stage (25). Then, they were perfused at a rate of 1.5 ml/min with a modified Ringer solution containing (in mmol/l) 120 NaCl; 5 KCl; 25 NaHCO₃; 1.1 MgCl₂; and 2.5 CaCl₂, pH = 7.4, gassed with 95% O₂ and 5% CO₂. Ca²⁺ signals were monitored in individual cells using a Zeiss LSM 510 laser confocal microscope equipped with a \times 40 oil immersion objective. The configuration of the system was set to excite the Ca²⁺ probe at 488 nm and collect the emission with a band-pass filter at 505–530 nm from an optical section of 8 μ m. Images were collected at 2-s intervals and treated with a low-pass filter (26). As previously reported, individual cells loaded with Fluo-4 were easily identified at the periphery of the islet where α -cells usually are more abundant (26). Fluorescence records were represented as the percent-

age of $\Delta F/F_0$, where F_0 is the fluorescence signal at the beginning of a record and ΔF is $F - F_0$. Background fluorescence was subtracted from F_0 . The frequency of oscillatory Ca²⁺ signals was calculated over a 5-min period of the Ca²⁺ recording, immediately before and 5 min after the application of the stimulus, as previously reported (23). To analyze frequency, a Ca²⁺ oscillation or spike was defined as a rapid increase in the intracellular Ca²⁺ concentration higher than twice the SD of the background signal at the intervals between spikes (23). Some data were expressed in percentages with respect to the frequency before the stimulus.

Glucagon secretion. Batches of 15 islets were preincubated for 60 min at 37°C in 0.5 ml Krebs-Ringer bicarbonate buffer supplemented with 15 mmol/l HEPES, 0.5% bovine serum albumin, and 5.6 mmol/l glucose, pH = 7.4 (23). Then, the islets were incubated at 37°C for 60 min with Krebs-Ringer bicarbonate buffer supplemented with 0.5 mmol/l glucose and additional reagents, as indicated in the RESULTS. At the end of the incubation, the medium was aspirated and assayed for glucagon using a commercial radioimmunoassay kit (Linco Research).

Analysis of exocytosis by fluorescence imaging. The exocytotic response of α TC1-9 cells was monitored at the single-cell level using the styryl dye FM1-43 (Invitrogen), as previously reported (27–29). The cells were incubated for 15 min with FM1-43 (2 μ mol/l), allowing its incorporation into the cell membrane (27). The dye was maintained continuously throughout the experiment. This cell-impermeable probe is nonfluorescent in an aqueous solution but emits intense fluorescence after partitioning into the plasma membrane. Consequently, the incorporation of secretory granules into the plasma membrane during secretion increases the cell surface, augmenting FM1-43 fluorescence (27–29). The FM1-43 signal was monitored by exciting the cells at 488 nm and obtaining the emission with a 560-nm long-pass filter. The relative change in fluorescence (ΔF) was represented with respect to time for each analyzed cell. The average rate of fluorescence changes ($\Delta F/\text{min}$) was also calculated for each experimental condition. Background signal was subtracted in all cases.

Patch-clamp recordings. As previously reported, α -cells were identified by their size, membrane capacitance (<4 pF), and their characteristic electrical activity in the absence of glucose (30,31). Membrane potential and whole-cell currents were recorded in the perforated patch whole-cell configuration using an Axopatch 200B amplifier (Axon Instruments). Patch pipettes were pulled from borosilicate capillaries using a flaming/brown micropipette puller P-97 (Sutter Instruments) with a resistance of 3–5 M Ω when filled with the pipette solution (in mmol/l): 76 K₂SO₄, 10 KCl, 10 NaCl, 1 MgCl₂, 5 HEPES (pH = 7.35). The extracellular solution consisted of (in mmol/l) 138 NaCl, 5.6 KCl, 2.6 CaCl₂, 1.2MgCl₂, and 5 HEPES (pH = 7.4) and supplemented with glucose as indicated (30,31).

Immunocytochemistry. This protocol was performed as previously reported (23). Briefly, islets or cells were fixed with Bouin's solution for 5 min and then dehydrated for 3 min with 30, 50, and 70% ethanol. Triton X-100 (0.5%) was used for permeabilization. To reduce nonspecific binding, cells were first preincubated with a blocking buffer (5% serum in PBS) for 45 min before applying primary antibodies in a buffer containing 1% serum. Glucagon-containing cells were identified with monoclonal anti-glucagon mouse antibodies (1:200; Sigma, Madrid, Spain). ObR receptors were detected with an anti-ObR goat antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-ObRb rabbit antibody (1:500; Alpha Diagnostic, San Antonio, TX). An anti-ObRb rabbit antibody specific for humans was also used (1:100; Millipore). These antibodies were applied overnight at 4°C. After washing, appropriate combinations of secondary antibodies conjugated with Alexa Fluor dyes (1:500; Invitrogen) were applied for 2 h at room temperature. The omission of the first antibody led to the absence of staining.

RT-PCR. Total RNA was isolated using RNeasy Mini Kit (Quiagen). Extracted RNA was quantified by OD260/280 measurement. Extracted RNA was used to generate cDNA using Expand Reverse Transcriptase (Roche, Mannheim, Germany), and 2 μ l of each RT reaction were used as input for the PCR. Primers are detailed in Table 1. PCR amplification was performed

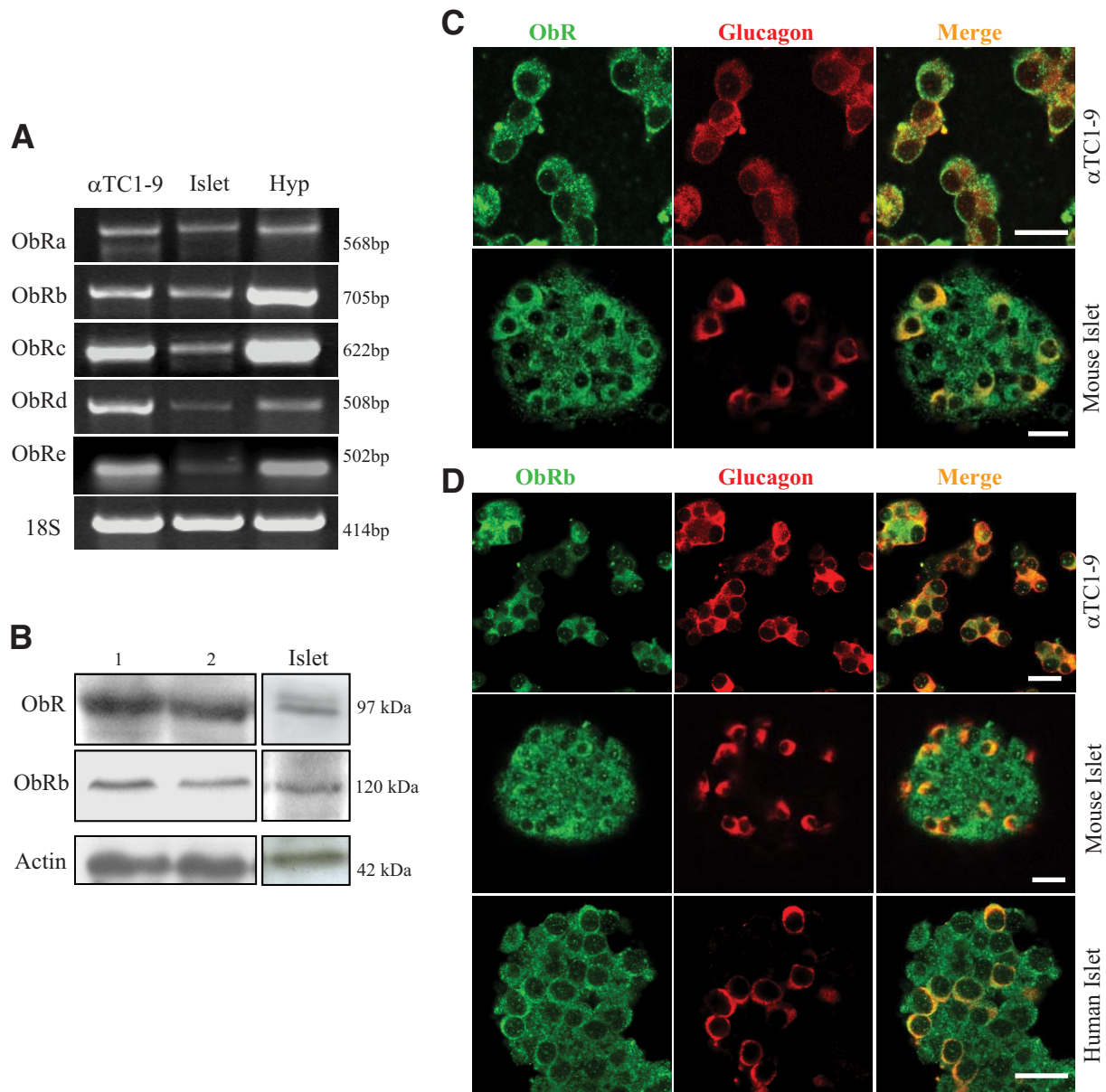


FIG. 1. Expression of leptin receptors in the pancreatic α -cell. **A:** PCR analysis of ObR transcripts shows that multiple isoforms are expressed in α TC1-9 cells. The expression in the mouse hypothalamus (Hyp) and islets is also illustrated. **B:** The presence of ObRb was demonstrated in α TC1-9 cells and in mouse islets by Western blot as well. An antibody that recognizes the extracellular domain of all the isoforms was also tested (ObR). Two examples are shown for the α TC1-9 cells. **C and D:** The spatial localization of ObRb in α TC1-9 cells and in α -cells within mouse and human islets (green) was assayed by immunofluorescence and confocal microscopy. Glucagon staining is shown in red. As in **B**, we also used an antibody that recognizes all the isoforms (ObR). These results are representative of at least three different experiments for each condition. Scale bar: 20 μ m. (A high-quality representation of this figure is available in the online issue.)

using *Taq*-DNA polymerase (Invitrogen, Barcelona, Spain) under the following conditions: 15 s at 94°C, 30 s at 55°C, and 1 min at 72°C. 18S was amplified as an internal control. PCR products were subjected to agarose-gel electrophoresis.

Western blot analysis. Cell pellets were obtained by centrifugation at 1,000 \times 10 min and resuspended in 200 μ l of cell lysis buffer (Cell Signaling Technology, Danvers, MA). Cell extracts were subjected to SDS-PAGE (12% gels). Prestained SDS-PAGE standards were included for molecular mass estimation. The transfer to polyvinylidene fluoride membranes was performed at 125 mA for 90 min in a buffer with 2.5 mmol/l Tris base, 9 mmol/l glycine, and 20% methanol. After membranes were blocked with 2% nonfat dry milk, they were incubated with the above-mentioned anti-ObR (1:100) or anti-ObRb (1:500) antibodies before being incubated with peroxidase-conjugated donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-rabbit (GE Healthcare, Barcelona, Spain) antibodies, respectively. Protein bands were revealed by using the ECL Chemiluminescence Reagents kit (Amersham Biosciences, Barcelona, Spain).

Statistical analysis. Some data are shown as means \pm SE. Student's *t* test or one-way ANOVA were performed, as appropriate, with a level of significance of $P < 0.05$.

RESULTS

Identification of leptin receptors in α -cells. Multiple isoforms have been described for the leptin receptor (ObR) (3,4). Although these variants have a common extracellular domain, the intracellular site varies for each isoform. ObRb is the long full-length isoform, and it is considered the functional receptor in terms of intracellular signaling (4). To investigate the existence of ObR in glucagon-releasing cells, we first performed a PCR analysis in α TC1-9 cells. As shown in Fig. 1A, these cells express

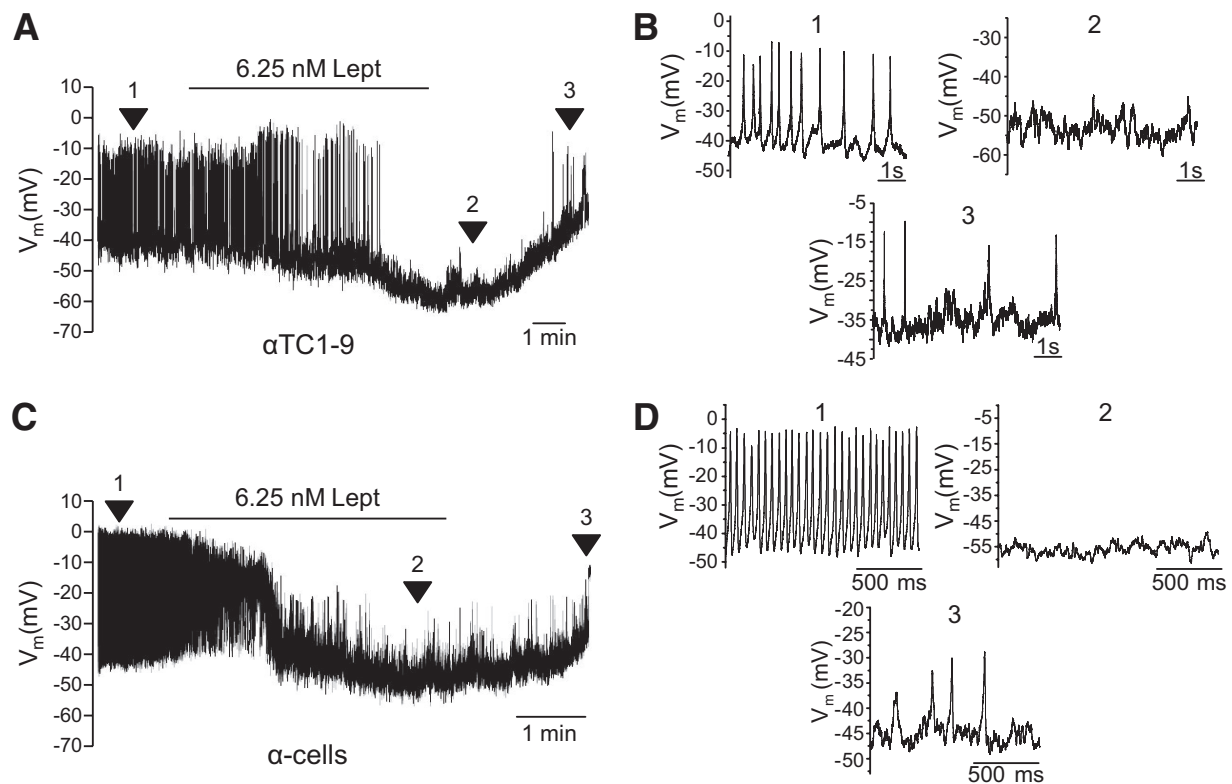


FIG. 2. Leptin induces membrane hyperpolarization and inhibition of electrical activity. **A:** Recording of membrane potential in whole-cell configuration in α TC1-9 cells. With 0.5 mmol/l glucose, characteristic action potentials originated from a membrane potential of -39.9 ± 0.2 mV ($n = 8$). The application of leptin (6.25 nmol/l) induced hyperpolarization (21.1 ± 1.1 mV) and suppression of electrical activity. Removal of leptin allowed for a depolarization and recovery of electrical activity. Lept, leptin. **B:** Expanded records from **A** of different significant instants (indicated by numbers). **C:** With 0.5 mmol/l glucose, an intense electrical activity was recorded in mouse α -cells. Leptin (6.25 nmol/l) hyperpolarized these cells from -37.3 ± 0.6 mV to -60.6 ± 0.4 mV ($n = 3$) and decreased electrical activity. Lept, leptin. **D:** Expanded records from **C** of different significant instants (indicated by numbers).

mRNA for the majority of ObR isoforms (ObRa–e). The presence of these receptors was also studied at the protein level by Western blot in α TC1-9 cells. We initially explored the existence of ObR using an antibody that recognizes the extracellular domain, which is common in all the ObR isoforms (Fig. 1B). Nonetheless, given the importance of ObRb for leptin signaling, its presence in these cells was also confirmed with a specific antibody against this isoform (Fig. 1B). Finally, the spatial localization of ObR and ObRb was studied using these antibodies and confocal microscopy. As shown in Fig. 1C and D, both α TC1-9 cells and α -cells within intact mouse islets contain these receptors. Remarkably, ObRb was also identified in human α -cells. These results indicate that, in addition to β -cells, glucagon-containing cells are also equipped with ObR.

Leptin hyperpolarizes α -cell membrane potential and suppresses electrical activity. In the absence of glucose or at low concentrations, α -cells produce a characteristic, regenerative electrical activity (30–36) that triggers Ca^{2+} signals and glucagon secretion. Thus, to study the functional role of leptin in α -cells, we first recorded their electrical activity using the patch-clamp technique in the whole-cell configuration. In 0.5 mmol/l of glucose, all the analyzed α TC1-9 cells displayed a characteristic electrical activity with action potentials that originated from -39.9 ± 0.2 mV (Fig. 2A). The application of leptin (6.25 nmol/l) hyperpolarized the membrane potential by -21.1 ± 1.1 mV and suppressed electrical activity. Although the effect of leptin persisted in some cells, its removal from the bath allowed for the depolarization of

the membrane potential and the restoration of electrical activity (Fig. 2A and B). Similar effects were found in mouse α -cells, since leptin hyperpolarized the membrane potential from -37.3 ± 0.6 mV to -60.6 ± 0.4 mV, and decreased electrical activity as well (Fig. 2C and D). Thus, leptin is able to induce short-term effects that modulate electrical responses in α -cells, in agreement with findings in other secretory cells (6,8–11).

Regulation of Ca^{2+} signaling by leptin in α -cells. Since glucose-regulated Ca^{2+} signals in α -cells are mainly sustained by their electrical activity (16,17,36), the effect of leptin on membrane potential should affect Ca^{2+} oscillations. It has been reported that among the different islet cell types, α -cells isolated in culture or within mouse islets are unique in displaying Ca^{2+} oscillations at low glucose levels (23,25,37–41). As shown in Fig. 3, α -cells exhibited this characteristic oscillatory Ca^{2+} pattern with 0.5 mmol/l glucose. The application of leptin (6.25 nmol/l) produced either a complete blockage or a decrease in the frequency of the Ca^{2+} oscillations in both α TC1-9 cells and α -cells within intact mouse islets (Fig. 3A–C). The average effect of leptin was a decrease of ~ 60 and $\sim 40\%$ in the Ca^{2+} signaling frequency in α TC1-9 cells and mouse α -cells, respectively (Fig. 3E). A similar response was observed with 3 mmol/l glucose (see supplementary Figure S1 in the online appendix [available at <http://dx.doi.org/10.2337/db08-1787>]). Removal of leptin from the bath allowed for the restoration of Ca^{2+} oscillations (supplementary Figure S2). After long periods of stimulation, this reversibility was not complete during the recording time, indicating that the

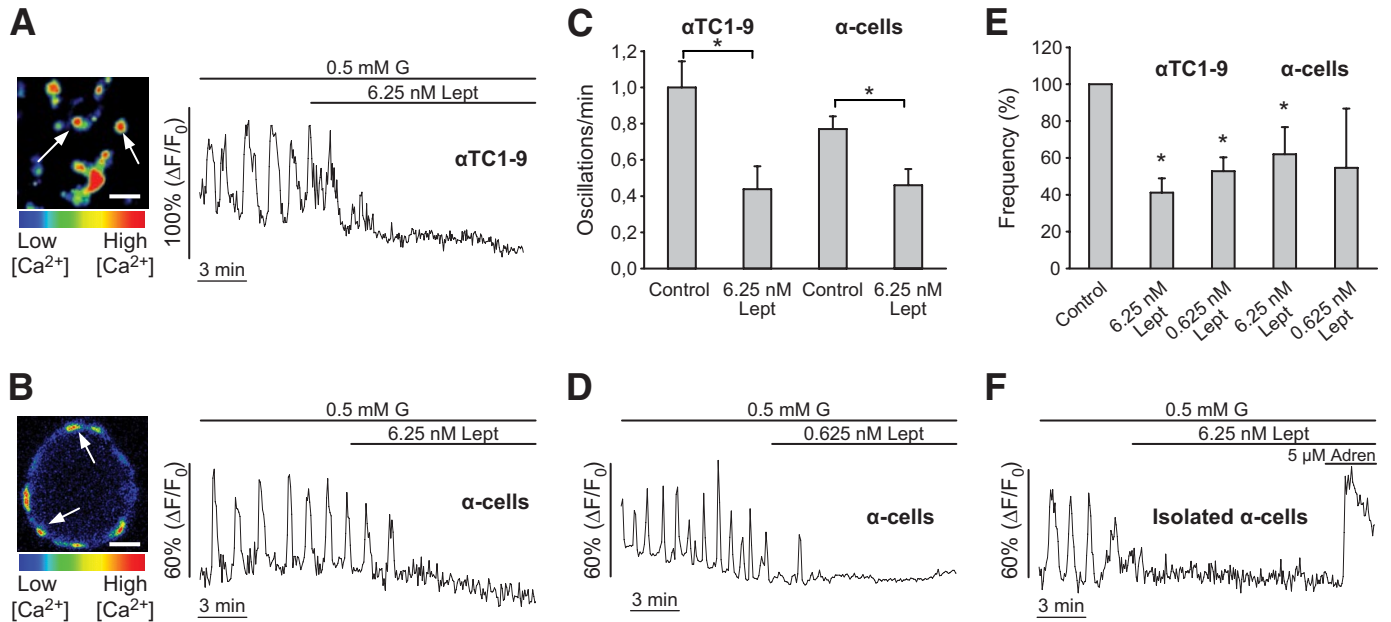


FIG. 3. Leptin inhibits Ca^{2+} signals induced by low glucose concentrations in $\alpha\text{TC1-9}$ cells and mouse α -cells. Leptin (6.25 nmol/l) blocks or reduces the frequency of Ca^{2+} signals induced with 0.5 mmol/l glucose in $\alpha\text{TC1-9}$ cells (A) and α -cells (B) ($n = 13$ and 16, respectively). Images show a culture of $\alpha\text{TC1-9}$ cells (A) and an intact mouse islet (B) loaded with the Ca^{2+} -sensitive probe Fluo-4. Islet images were acquired by confocal microscopy from an optical section close to the equatorial plane. Several individual cells were easily identified at the periphery of the islet (white arrows). C: Average frequency of Ca^{2+} oscillations with 0.5 mmol/l glucose (control) and in the presence of leptin. D: Effect of 0.625 nmol/l leptin in islet α -cells ($n = 20$; $n = 52$ for $\alpha\text{TC1-9}$ cells, not shown). E: Frequency (%) of Ca^{2+} signals after stimuli compared with control conditions. F: Leptin inhibits Ca^{2+} signaling in isolated islet α -cells ($n = 11$). The effect of adrenaline, which is characteristic of this islet cell type (32), is also shown. Data in C and E are shown as means \pm SE. *Statistically significant ($P < 0.05$) compared with control. Adren, adrenaline; G, glucose; Lept, leptin. Scale bar: 20 μm . (A high-quality representation of this figure is available in the online issue.)

leptin effect may be persistent. The addition of 0.625 nmol/l leptin also produced an inhibitory effect in $\alpha\text{TC1-9}$ cells ($\sim 45\%$ decrease) (Fig. 3E). In contrast, although 0.625 nmol/l leptin blocked Ca^{2+} signals in $\sim 57\%$ of α -cells (Fig. 3D), the average effect was not significant because of the heterogeneous response to this concentration (Fig. 3E). To avoid the potential contribution of islet paracrine interactions on the leptin action, some experiments were performed with isolated α -cells (Fig. 3F). In these conditions, leptin produced a similar inhibitory response, proving its direct action on α -cells. The absence of effect on

α -cells from *db/db* mice, which lack functional ObRb, gave further molecular evidence that this receptor is involved in leptin-induced actions (supplementary Figure S3). Finally, the response of α -cells to this hormone was also evaluated in human islets (Fig. 4). Application of leptin at 6.25 and 0.625 nmol/l decreased Ca^{2+} signals by ~ 58 and $\sim 32\%$, respectively. This result indicates that ObR is also functional in human α -cells.

It has been reported that the leptin effect on electrical activity and Ca^{2+} signals is mediated by the PI3K pathway in pancreatic β -cells and neurons (5,6,8–10). To determine

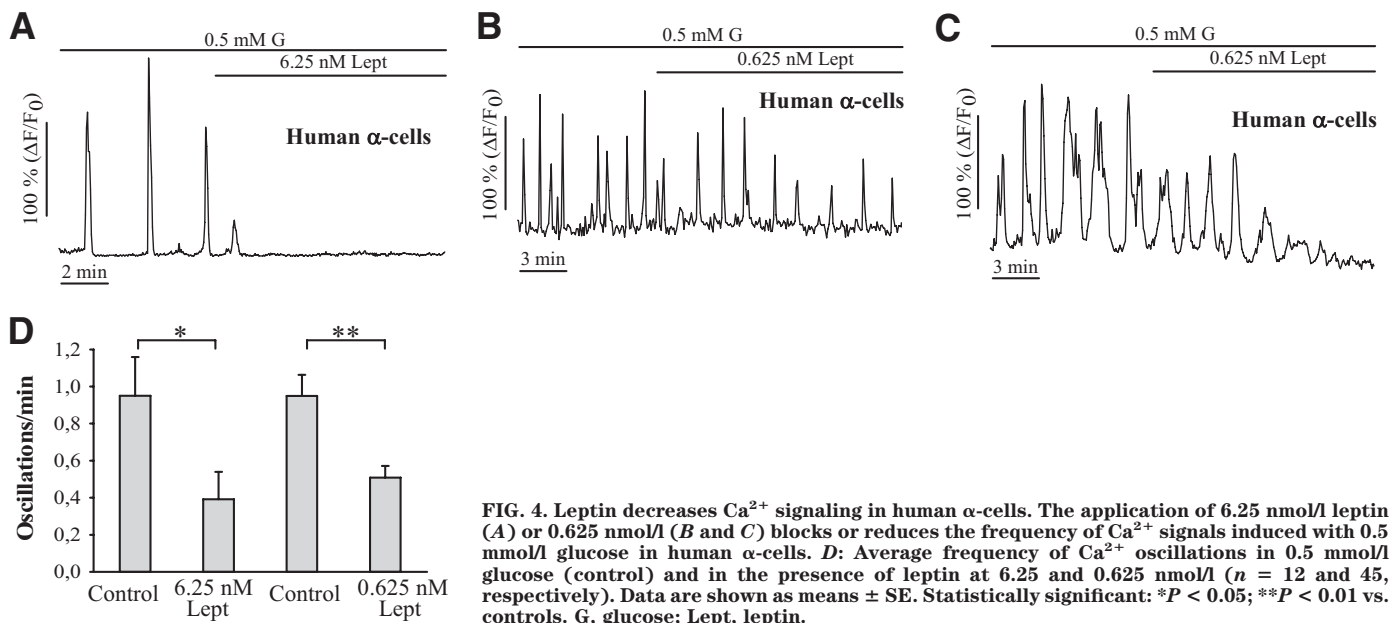


FIG. 4. Leptin decreases Ca^{2+} signaling in human α -cells. The application of 6.25 nmol/l leptin (A) or 0.625 nmol/l (B and C) blocks or reduces the frequency of Ca^{2+} signals induced with 0.5 mmol/l glucose in human α -cells. D: Average frequency of Ca^{2+} oscillations in 0.5 mmol/l glucose (control) and in the presence of leptin at 6.25 and 0.625 nmol/l ($n = 12$ and 45, respectively). Data are shown as means \pm SE. Statistically significant: * $P < 0.05$; ** $P < 0.01$ vs. controls. G, glucose; Lept, leptin.

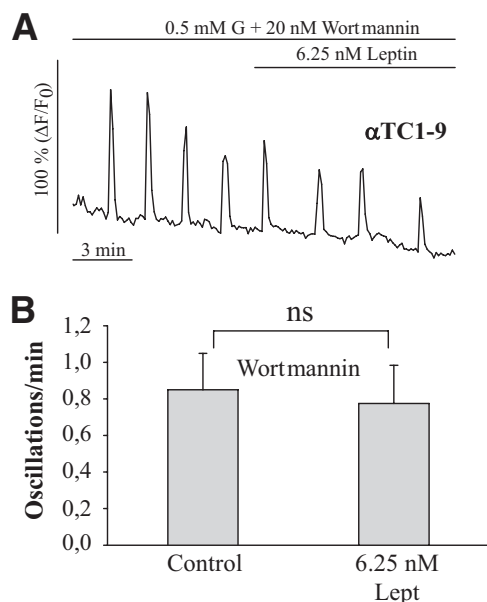


FIG. 5. Leptin does not affect Ca^{2+} signals in the presence of wortmannin. **A:** In the presence of the PI3K inhibitor wortmannin (20 nmol/l), leptin was unable to affect the intracellular Ca^{2+} oscillations induced with 0.5 mmol/l glucose in α TC1-9 cells ($n = 8$). **B:** Average frequency of Ca^{2+} signals with 0.5 mmol/l glucose (control) and leptin, both in the presence of wortmannin. Data in **B** and **C** are shown as means \pm SE. G, glucose; Lept, leptin; ns, nonsignificant.

whether this pathway is also involved in the α -cell, some experiments were performed in the presence of the PI3K inhibitor wortmannin (20 nmol/l) (Fig. 5). In these conditions, leptin failed to inhibit Ca^{2+} signals in α TC1-9 cells (Fig. 5B and C), as shown in other cell types (6,8). Thus, the PI3K pathway may be involved in the leptin effect. The following results further support these observations.

Reduced glucagon secretion in the presence of leptin. Glucagon secretion depends on Ca^{2+} signaling (16,17). Thus, all these changes induced by leptin may be associated with alterations in the secretory pattern. To test this possibility, we analyzed glucagon release from isolated mouse islets using radioimmunoassay (Fig. 6A). Glucagon

secretion with 0.5 mmol/l glucose is almost maximal in mice (42). Incubation with 10 mmol/l glucose reduced glucagon release by $\sim 55\%$, consistent with previous reports (42). In the presence of 6.25 nmol/l leptin, glucagon secretion with 0.5 mmol/l glucose decreased by $\sim 33\%$. The inhibitory effect was not statistically significant with 0.625 nmol/l leptin, which probably may result from the heterogeneous response observed in Ca^{2+} signaling with this concentration (Fig. 3E). In agreement with the previous finding with Ca^{2+} signals (Fig. 5), wortmannin counteracted the blocking action of 6.25 nmol/l leptin on glucagon secretion (Fig. 6A), further indicating the involvement of the PI3K pathway. Finally, to further prove the influence of leptin on the secretory process, we analyzed exocytosis at the single-cell level by monitoring FM1-43 fluorescence in α TC1-9 cells, as previously reported in β -cells (27–29). FM1-43 emits fluorescence when it partitions into the plasma membrane. Thus, the incorporation of secretory granules into the plasma membrane increases the cell surface and, also, the fluorescence of FM1-43 (27–29). As shown in Fig. 6B and C, the fluorescence augmented with 0.5 mmol/l glucose (control), indicating an active secretory response. This increase was significantly reduced in the presence of leptin (6.25 nmol/l). Again, the application of wortmannin counteracted this effect (Fig. 6C). Thus, all these observations indicate that leptin is working as an inhibitory regulator of α -cell function.

DISCUSSION

Glucose homeostasis is mainly regulated by the coordinated action of glucagon and insulin secreted from pancreatic α - and β -cells, respectively (17). These two islet populations respond reciprocally to blood glucose changes. Pancreatic α -cells develop spontaneous electrical activity at low glucose concentrations, leading to Ca^{2+} signals and glucagon secretion, while these processes become inhibited with the elevation of glucose levels (25,26,31,33–35,38). Remarkably, this glucagon secretory response can be impaired in diabetic individuals, aggravating the difficulties in the control of glycemia (17–18). Glucose homeostasis can be further regulated by the

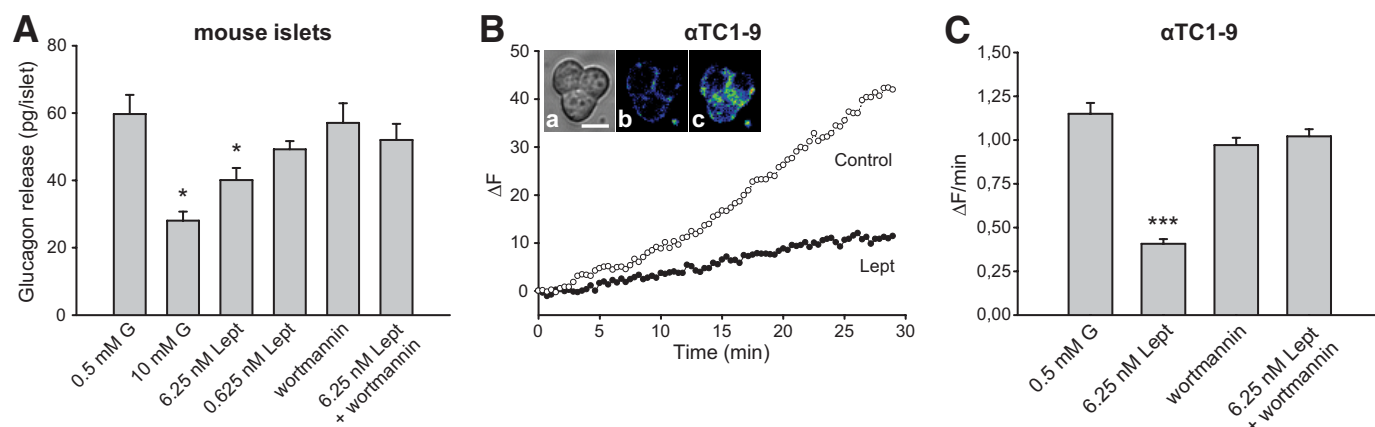


FIG. 6. Effect of leptin on glucagon secretion. **A:** Glucagon secretion from mouse islets with 10 mmol/l glucose or with 0.5 mmol/l glucose plus 0.625 nmol/l or 6.25 nmol/l leptin was compared with the control (0.5 mmol/l glucose). The effect of wortmannin (50 nmol/l) on glucagon release with 0.5 mmol/l glucose in the absence and presence of 6.25 nmol/l leptin is also displayed. Data are shown as means \pm SE ($n = 8$ –13). **B:** Changes in FM1-43 fluorescence (ΔF ; arbitrary units) versus time in α TC1-9 cells in control conditions (0.5 mmol/l glucose) and in the presence of leptin (6.25 nmol/l). *Inset:* images illustrating a group of cells in transmitted light (**A**) and loaded with FM1-43 at the beginning (**B**) and at the end of the record (**C**). Scale bar: 10 μm . **C:** The average rate of fluorescence changes as a function of time ($\Delta F/\text{min}$) indicates that leptin reduces the exocytotic response. This effect was counteracted by wortmannin (20 nmol/l). Data are shown as means \pm SE ($n = 32$ –45 for each condition). * $P < 0.05$; *** $P < 0.001$ vs. control. G, glucose; Lept, leptin. (A high-quality representation of this figure is available in the online issue.)

adipocyte hormone leptin through its inhibitory action on β -cell secretion (7,11–15). It has been proposed that defects in this communication may be implicated in obesity-induced diabetes (12,14,15). The functional role of leptin in pancreatic α -cells and its involvement in the adipoinular communication, however, has not been investigated extensively.

In the present study, we have shown that leptin receptors are present in mouse and human glucagon-containing cells. We demonstrate that leptin produces significant changes in the membrane potential, electrical activity, Ca^{2+} signaling, and glucagon secretion in mouse α -cells as well as glucagon-secreting $\alpha\text{TC1-9}$ cells. This cell line has been previously validated as a good model to study α -cell function (38). Additionally, the blocking action of leptin on Ca^{2+} signaling was also demonstrated in human α -cells. In a previous report (11), the function of leptin was not studied in α -cells because of the lack of ObR expression in hamster glucagonoma INR1G-9 cells and a weak antibody staining in isolated rat α -cells. Although methodological or interspecies differences may account for this discrepancy, our findings on the presence of ObR in α -cells are further sustained by the demonstration of direct leptin actions at the single-cell level.

It has been reported that leptin induces membrane potential changes that can modulate electrical activity in neurons and in β -cells (5,6,8–11,43). In agreement with these findings, we have observed similar effects in the pancreatic α -cell. In contrast to other islet cell populations, α -cells are electrically active in the absence or at low concentrations of glucose (30,33–35). In these conditions, spontaneous action potentials arise from a membrane potential that varies from -60 to -40 mV, depending on experimental conditions (30,33–35). In $\alpha\text{TC1-9}$ cells and mouse α -cells, we observed this characteristic electrical activity with 0.5 mmol/l glucose, where action potentials arose from a membrane potential of about -40 mV. Consistent with the results obtained in other secretory cell types including β -cells (6,8–11,43), leptin hyperpolarized the membrane potential and decreased electrical activity (Fig. 2), which is voltage-dependent (33,34,40). Additionally, this hormone inhibited intracellular Ca^{2+} oscillations and glucagon secretion (Figs. 3, 4, and 6). This effect could be anticipated in α -cells, since their electrical activity triggers Ca^{2+} signals, and exocytosis is Ca^{2+} dependent (16,17). In the case of the pancreatic β -cell, leptin effects on membrane potential are also reflected in Ca^{2+} signals and insulin release (11,12). Like leptin, other hormones such as insulin and somatostatin can inhibit glucagon secretion in α -cells by mechanisms that involve membrane hyperpolarization and suppression of electrical activity, affecting Ca^{2+} signals (38,44,45). It has been proposed that the effects of leptin on β -cell membrane potential are mediated by the opening of K_{ATP} channels (11). A similar process may occur in α -cells, since their membrane potential is controlled by the K_{ATP} channel and its activation causes the hyperpolarization of these cells (40). In any case, further experiments should be performed since other molecular targets may be involved in leptin actions (3,4).

Leptin can activate multiple signaling cascades (4). Among them, PI3K signaling has an important function in leptin short-term effects on membrane potential, Ca^{2+} signals, and secretion in pancreatic β -cells and in neurons (5–10). In agreement with these findings, leptin failed to decrease Ca^{2+} signaling and secretion in α -cells when PI3K was inhibited (Figs. 5 and 6). Therefore, this signal-

ing pathway seems to be involved in the intracellular transduction of leptin short-term effects on the α -cell.

Basal plasma leptin concentrations are in the order of $1\text{--}10$ ng/ml ($\sim 0.0625\text{--}0.625$ nmol/l) during fasting state (1–3,46). These values can significantly increase every day by circadian mechanisms (46). These leptin fluctuations are more marked in women, reaching concentrations of ~ 15 ng/ml in healthy individuals (47). Factors like feeding behavior, sex, age, and pregnancy or elevation of hormones such as insulin, estrogen, or glucocorticoids can notably increase plasma leptin levels in both rodents and humans (1–3). Leptin concentrations can reach $30\text{--}100$ ng/ml ($\sim 1.87\text{--}6.25$ nmol/l) in obesity and are highly elevated in situations such as impaired renal function and inflammatory responses (1–3,46,47). The present study demonstrates a clear inhibitory effect at 6.25 nmol/l leptin. This response was more heterogeneous at 0.625 nmol/l. At this concentration, leptin clearly inhibited Ca^{2+} signals in $\alpha\text{TC1-9}$ and human α -cells (Fig. 3E and Fig. 4), yet the average effect was not found significant in mouse α -cells (Fig. 3E). Nevertheless, 0.625 nmol/l leptin had a blocking action in several of these cells ($\sim 57\%$) (Fig. 3D). Thus, these results indicate that α -cells may be less sensitive to leptin in mice than in humans. Additionally, all these observations suggest that leptin actions on α -cells may be more limited at basal plasma leptin levels. However, leptin effects should be more effective in those physiological situations when this hormone increases above basal values and in pathological situations (1–3), as previously mentioned.

The present findings indicate that leptin can directly regulate α -cell function. This suggests that the adipose tissue may modulate glucose homeostasis not only by inhibiting β -cells and insulin release but α -cells and glucagon secretion as well. Thus, the effect of leptin on islet hormonal responses would be similar to that of somatostatin, which limits both insulin and glucagon release (16,17). Since leptin has been detected in mouse α -cells, this hormone may also play a local autoregulatory effect within the islet (48). Interestingly, hyperglucagonemia has been reported in mouse models that have defects in leptin and ObR, which impairs the signaling by this hormone (19–21). Moreover, a similar suppressive action by leptin has been recently observed in vivo in diabetic mice with hyperglucagonemia and hyperglycemia (22). In these rodents, hyperglucagonemia was reduced by adenoviral-induced hyperleptinemia, which normalized glucose levels. Our results indicate that, although several mechanisms could be involved, this effect may be the result of a direct action on α -cells. It has been demonstrated that leptin regulates hepatic glucose fluxes and, remarkably, that this hormone antagonizes the effects of glucagon in the liver, the main target for α -cell function (49,50). Our present observations suggest that leptin could antagonize glucagon action not only in the liver but also directly in the α -cells. In diabetic individuals, an absolute or relative excess of glucagon along with insulin deficiency can generate an excessive hepatic glucose production (17,18). Since this can be a major problem in these patients, several approaches have been developed to limit glucagon secretion and/or action (17,18). In this regard, the direct inhibitory effect of leptin on the α -cell reported in this study may be explored as a therapeutic strategy for the control of glucagon levels, as has been recently suggested for type 1 diabetes (22). However, the specific management of glucagon levels by leptin would be more complex if β -cells are functional,

since it could also affect insulin release (10,11). Thus, further research will be necessary in this context to develop approaches to discriminate the secretory responses of α - and β -cells to this hormone. Leptin resistance has been reported in obese individuals, and several mechanisms have been proposed to explain this process at the central level (2–4). Nevertheless, although it has been suggested that leptin resistance may occur directly in the β -cell, there is no information about the molecular determinants involved (12,14,15). It has been proposed that β -cell leptin resistance and dysregulation of the adiponinular communication may contribute to obesity-induced diabetes (12,14,15). Thus, it would be interesting to analyze whether α -cells may develop leptin resistance in this context and the role that these cells may be playing in this situation.

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