The Clock Gene Rev-erbα Regulates Pancreatic β-Cell Function: Modulation by Leptin and High-Fat Diet

Elaine Vieira, Laura Marroquí, Thiago M. Batista, Ernesto Caballero-Garrido, Everardo M. Carneiro, Antonio C. Boschero, Angel Nadal, and Ivan Quesada

Instituto de Bioingeniería (E.V., L.M., E.C.-G., A.N., I.Q.), Universidad Miguel Hernandez, Elche, and Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, Alicante 03202, Spain; and Departamento de Anatomía (T.M.B., E.M.C., A.C.B.), Biología Celular, Fisiología e Biofísica, Instituto de Biología, Universidad Estadual de Campinas, Campinas, and Instituto Nacional de Ciência e Tecnologia de Obesidade e Diabetes, Sao Paulo 13083-970, Brazil

Disturbances of circadian rhythms have been associated with obesity and type 2 diabetes. The nuclear receptor Rev-erbα was suggested to link circadian rhythms and metabolism in peripheral tissues. The aim of the present study was to dissect the role of this clock gene in the pancreatic β-cell function and to analyze whether its expression is modulated by leptin and diet-induced obesity.

To address the function of Rev-erbα, we used small interfering RNA in mouse islet cells and in MIN-6 cells. Cell proliferation was measured by bromodeoxyuridine incorporation, apoptosis by the terminal deoxynucleotidyl transferase dUTP nick end labeling technique, insulin secretion by RIA, and gene expression by RT-PCR. Pancreatic islets were isolated at different zeitgeber times 0, 6, and 12 after 6 wk of high-fat diet treatment, and then gene expression and insulin secretion were determined. Rev-erbα down-regulation by small interfering RNA treatment in islet cells and MIN-6 cells impaired glucose-induced insulin secretion, decreased the expression of key lipogenic genes, and inhibited β-cell proliferation. In vivo and in vitro leptin treatment increased Rev-erbα expression in isolated islets through a MAPK pathway. High-fat diet treatment disrupted the circadian Rev-erbα gene expression profile along with insulin secretion, indicating an important role of this clock gene in β-cell function. These results indicate that the clock gene Rev-erbα plays multiple functions in the pancreatic β-cell. Although the increase in Rev-erbα expression may promote β-cell adaptation in different metabolic situations, its deregulation may lead to altered β-cell function.

(Many organisms have 24-h rhythms in their physiology that are driven by cell circadian pacemakers (1). Complex circuitries of transcriptional/posttranslational regulatory loops allow organisms to coordinate physiological processes in response to environmental changes. Blood pressure and lipid and carbohydrate metabolism as well as the secretion of hormones such as insulin, leptin, and cortisol are few examples of many aspects of mammalian physiology that are subjected to circadian oscillations. In mammals, the central pacemaker is localized in the suprachiasmatic nuclei in the hypothalamus, and it is controlled by transcriptional/translational feedback loops involving a set of clock genes. However, recent studies indicate that, besides the central location in the brain, peripheral molecular clocks exist in several organs, including liver, kidneys, muscle, adipose tissue, and pancreas (2–5), where feeding/fasting are the most dominant time signals for these tissues (6). Recently, disturbances in the regulation of circadian rhythms have been implicated in the development of metabolic disorders such as obesity and type 2 diabetes. Studies in humans showed that alterations in the control of central and peripheral biological clocks by sleep loss can lead to decreased insulin sensitivity and increased risk of obesity and diabetes mellitus (7, 8). Additionally, long-term shift work has been associated with dyslipidemia and increased risk of diabetes and cardio-

Abbreviations: BrdU, Bromodeoxyuridine; HFD, high-fat diet; PI3K, phosphoinositide 3-kinase; NAD(P)H, reduced nicotinamide adenine dinucleotide phosphate; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; ZT, zeitgeber time.
vascular disease (9). These findings indicate the important role of circadian rhythms in the regulation of metabolism.

Rev-erbα (nuclear receptor encoded by NR1D1), one of the key clock genes, was suggested to link circadian rhythms and metabolism (10). Rev-erbα is part of the clock machinery and plays an important role in maintaining proper timing of the circadian system (11). In addition to its role in circadian rhythms, Rev-erbα regulates triacylglyceride and lipoprotein metabolism in the liver (12), adipogenesis (13), and gluconeogenic genes (14, 15) and plays a determinant role in the modulation of vascular inflammatory processes (16). These functions of the Rev-erbα gene were mainly suggested in peripheral tissues. However, its role in the endocrine pancreas remains to be explored. In this study, we demonstrate that Rev-erbα is involved in glucose-induced insulin secretion, proliferation and controls the expression of key lipogenic genes in mouse pancreatic β-cells as well as in mouse insulinoma (MIN-6) cells. Additionally, we demonstrate that leptin regulates the expression of Rev-erbα through a MAPK pathway. We also show that Rev-erbα is one of the main clock genes whose expression is disrupted by high-fat diet (HFD) in pancreatic mouse islets. Thus, Rev-erbα plays an important role in islet function and might be involved in defects of the β-cell function during obesity and type 2 diabetes.

Materials and Methods

Animals and diets

All protocols were approved by our Animal Care Committee according to national regulations. Experiments were performed with 9-wk-old male Swiss albino O.F1 mice that were kept under a 12-h light, 12-h dark cycle (lights on at 0600 h and lights off at 1800 h) with food ad libitum. After weaning, mice were divided into two groups. The first group was fed with chow diet (17% fat), and the second group was fed with HFD (60% fat) for 6 wk. Ten-week-old male ob/ob and db/db mice were purchased from Harlan Laboratories (Indianapolis, IN).

Plasma levels of insulin and leptin

Blood samples were collected for biochemical analysis. Plasma insulin and leptin levels were analyzed by ELISA (Merckodia, Uppsala, Sweden, and Crystal Chem, Downers Grove, IL, respectively).

Glucose tolerance test

A glucose load of 2 g/kg body weight was administered ip on overnight fasted mice. Blood samples were collected at different times for measurements of plasma glucose using a glucose meter (Accu-Check; Roche Diagnostics, Madrid, Spain).

Islet isolation and cell culture

After 6 wk treatment with chow or HFD, mice were killed by cervical dislocation at different zeitgeber times (ZT) 0, 6, and 12 (with ZT0 defined as 0800 h, ZT6 as 1400 h, and ZT12 as 2000 h). Islets were then isolated by collagenase digestion (17) and separated into two groups. One group was collected for gene expression analysis and the other one for insulin secretion measurements. Except when indicated in some experiments using different times of the day, animals in the majority of experiments were killed at 0800 h. There was a time delay of 2 h between the time of killing and the beginning of the experiments. When necessary, single-cell suspensions were obtained from the islets by trypsin enzymatic digestion. Isolated cells or islets were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 5 mM d-glucose. The insulin-releasing MIN-6 cell line was kindly provided by Dr. Jun-Ichi Miyazaki (Osaka University, Osaka, Japan) and used between passages 20 and 30. Cells were grown as previously described (18).
To measure circadian Rev-erba gene expression in vitro, we performed a short treatment with 50% serum (serum shock) to the confluent, serum-starved MIN-6 cells, according to previous studies (19, 20). After 2 h of serum shock, the medium was changed to serum-free DMEM, and RNA was extracted every 6 h during 48 h for gene expression measurements. The experiments were done in serum-free medium to discriminate between oscillations in the cell cycle and intrinsic oscillations (19, 20).

In vivo leptin treatment

Vehicle (PBS) or leptin (500 ng/g body weight) was administered twice daily to mice by ip injection at ZT0 and ZT12 for 5 consecutive days. To discard the possibility that the reduced food intake induced by leptin in the treated mice might induce changes in Rev-erba gene expression, a pair-fed control was also included. In this group, the food intake of vehicle-treated mice was matched to that consumed by the leptin-treated mice on the previous day (21).

Insulin secretion and content

Insulin secretion was measured in MIN-6 cells and isolated islets as previously described (18). Afterward, the islets or cells were lysed with 50 μl lysis buffer (70% ethanol, 0.4% HCl at 30%, 24.6% distilled water) and incubated overnight at 4 C. Samples were centrifuged at 2500 rpm for 5 min, and the supernatant was collected for insulin content analysis. The insulin release and content were measured by RIA and normalized by total protein concentration, which was determined by the Bradford method. Hemin, GSK4112, and SR8278 were purchased from Sigma.

Quantitative real-time PCR

Quantitative PCR assays were performed using the CFX96 Real Time System (Bio-Rad, Hercules, CA). Reactions were carried out in a final volume of 10 μl, containing 200 nm of each primer, 100 nm endogenous control primer, 1 μl cDNA, and IQ Sybr Green Supermix (Bio-Rad). Samples were subjected to the following conditions: 10 min at 95 C; 40 cycles of 10 sec at 95 C, 7 sec at 60 C, and 12 sec at 72 C; and a melting curve of 63–95 C with a slope of 0.1 C/sec. The housekeeping gene rplp0 (ribosomal protein large P0, alias 36B4) was used as the endogenous control for quantification (4). The results were analyzed with CFX Manager version 1.6 (Bio-Rad), and values are expressed as the relative expression with respect to control levels (2−ΔΔCt). Primers sequences are described in Supplemental Table 1 (published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org).

Interference RNA

Small interfering RNA (siRNA) treatment was performed in MIN-6 cells and islet cells dispersed in isolated cells or in small clusters as previously described (21, 22). Cells were transfected overnight with 50 nm siRNA Silencer Predesigned Rev-erba (Ambion, Austin, TX), signal transducer and activator of transcription 3 (STAT3) (Ambion), or 50 nm Silencer labeled negative control no. 2 siRNA (Ambion) in optiMEM I (Invitrogen, Carlsbad, CA) culture medium without antibiotics and 1% Lipofectamine 2000 (Invitrogen). The following Rev-erba siRNA sequences were used (5’–3’): GCAUCGUGUGCAACGUGAAtt (sense) and UCACGUGAAACAACGAUGCaa (antisense). The following STAT3 siRNA sequences were used (5’–3’): GGAU-CUAGAAGCGAAAAGt (sense) and CAUUUUCUGUUC-UAGAUGCag (antisense). After overnight incubation, transfection medium was replaced by DMEM culture medium for 24 h before the start of the experiments.

Western blot analysis

Cell pellets were obtained by centrifuging at 1000 × g for 10 min and resuspended in 50 μl lysis buffer (Cell Signaling Technology, Danvers, MA). Cell extracts were subjected to SDS-PAGE (Mini-ProteanTGX Precast Gel, 4–20% gels; Bio-Rad). Prestained SDS-PAGE standards were included for molecular mass estimation. The transfer to polyvinylidene difluoride membranes was performed at 125 mA for 90 min in a buffer with 2.5 mM Tris base, 9 mM glycine, and 20% methanol. After membranes were blocked with 2% nonfat dry milk, they were incubated with the following antibodies: rabbit polyclonal anti-actin (1:1000; Sigma), anti-Rev-erba (1:500; Abcam, Cambridge, UK), and anti-FAS (1:1000; Cell Signaling Technology). Membranes were incubated with appropriate horseradish peroxidase-conjugated antibodies (Bio-Rad). Protein bands were revealed by using the ECL Western blot substrate (Thermo Fisher Scientific, Madrid, Spain). Intensity of the bands was quantified using Scion Image software (Scion Corp., Frederick, MD).

Cell proliferation

MIN-6 cells were first incubated for 3 h with bromodeoxyuridine (BrdU) and then fixed with 70% ethanol for 30 min at 4 C. Primary cells were incubated for 24 h with BrdU and fixed with Bouin’s solution (HT10132; Sigma, Steinheim,
Germany) for 5 min and permeabilized with 1% Triton X-100.
Both cell types were washed with PBS and treated with HCl (2 N) for 30 min. HCl was neutralized with 1 M borate and then washed with PBS for subsequent blocking with 3% BSA in PBS for 1 h. Cells were incubated overnight with mouse monoclonal anti-BrDU (1:500; Dako, Barcelona, Spain) and anti-insulin (sc-9168; Santa Cruz Biotechnology, Santa Cruz, CA) with 1% BSA and 0.3% goat serum. After that, they were incubated for 2 h at room temperature with goat antimouse polyclonal secondary antibody conjugated with Alexa Fluor 488 (1:500; Molecular Probes, Invitrogen) and goat antirabbit 546 (Invitrogen A11010) with 1% BSA. To counterstain nuclei, cells were incubated with 1 μM ethidium homodimer-1 for 10 min at room temperature (Molecular Probes, Invitrogen).

Cell viability and detection of apoptotic cells
To test viability, cells were incubated with calcein (2 μM) and ethidium homodimer-1 (1 μM) according to the instructions of the viability/cytotoxicity kit (Molecular Probes, Invitrogen). To analyze apoptosis, we used the APO-BrdU terminal deoxynucleotidyl transferase dUTP nick end labeling assay kit (Molecular Probes, Invitrogen).

Reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] measurements
Images was acquired with an extended C4742-95 digital camera (Hamamatsu Photonic, Barcelona Spain), using a dual-filter wheel (Sutter Instrument Co., Novato CA). Data were acquired using ORCA software. MIN-6 cells were excited with 363-nm band-pass filter, whereas emission was filtered at 445 nm. An image was acquired every 60 sec, and 40–50 cells were measured in four different experiments.

Statistical analysis
Data are shown as mean ± SEM. Student’s t-test or one-way or two-way ANOVA was performed as appropriate with a level of significance P < 0.05.

Results
Pancreatic β-cells express Rev-erba
The mRNA expression of Rev-erba and other clock genes was analyzed in mouse skeletal muscle, islets, and MIN-6 cells (Fig. 1, A–C) as well as hypothalamus, where the master clock is located. The mRNA expression levels in islets and MIN-6 were lower compared with hypothalamus, whereas there was a tendency of lower mRNA levels also in skeletal muscle (P = 0.09). Among the clock genes, Rev-erba together with Clock and Cry1 are most expressed in MIN-6-cells compared with Rev-erba mRNA levels. To demonstrate that β-cells exhibit circadian oscillations of clock genes throughout the day, we next performed mRNA measurements every 6 h during a 48-h period in MIN-6 cells after serum shock, as previously described (20). Figure 1, D–F, shows that the housekeeping gene rplp0 (ribosomal protein, large, P0 alias 36B4) did not oscillate during the 48 h. However, Rev-erba mRNA levels oscillated in vitro in MIN-6 cells with a peak of expression at ZT30. The oscillations pattern of Bmal1 had a peak of expression at ZT6 and ZT24, whereas Clock did not show a clear oscillatory pattern with a very small peak of expression at ZT30. The oscillations pattern of Bmal1 had a peak of expression at ZT6 and ZT24, whereas Clock did not show a clear oscillatory pattern with a very small peak of expression at ZT30. The oscillations pattern of Bmal1 had a peak of expression at ZT6 and ZT24, whereas Clock did not show a clear oscillatory pattern with a very small peak of expression at ZT30.
together, these findings indicate that circadian oscillations of Rev-erbα and Bmal1 are an intrinsic property of pancreatic β-cells that occurs independently of the master clock located in the brain.

To dissect the functional role of Rev-erbα, we used siRNA to down-regulate this gene in MIN-6 cells as well as in isolated mouse islet cells. Gene-silencing efficiency in MIN-6 cells and islet cells was about 70–80% compared with cells treated with a scramble siRNA (control siRNA) (Fig. 1, G and H). The decrease in Rev-erbα gene was also confirmed at the protein level as shown in Fig. 1I.

**Rev-erbα plays an important role in the regulation of β-cell function**

Because Rev-erbα was shown to have an important role in adipogenesis and lipid metabolism (13), we measured the expression of key genes involved in lipogenesis. Down-regulation of Rev-erbα led to a decrease in the mRNA levels of sterol regulatory element-binding protein 1c (Srebp-1c) and its target gene fatty acid synthase (FAS) in both MIN-6 and mouse islet cells (Fig. 2, A and B and D and E). Protein levels of FAS were also down-regulated by Rev-erbα siRNA in MIN-6 cells as shown in Fig. 2C. These results demonstrated a potential role of Rev-erbα in the regulation of lipid metabolism in pancreatic β-cells.

Using both islet cell models, we further investigated whether Rev-erbα is involved in insulin secretion. Figure 3, A and D, shows glucose-stimulated insulin secretion from MIN-6 cells and isolated mouse islet cells after Rev-erbα silencing. This protocol did not affect the viability and function of these cells (Supplemental Fig. 1A). In these conditions, basal insulin secretion at 2.8 mM glucose was unchanged in both cell types. However, although glucose-stimulated insulin secretion (16.8 mM glucose) was increased in the presence of the control siRNA (scramble), glucose-stimulated insulin secretion was decreased in both cell models when Rev-erbα was silenced (Fig. 3, A and D). No significant differences were observed in the insulin content and insulin gene expression after Rev-erbα silencing (Fig. 3, B, C, E, and F), except a small decrease of insulin content at 2.8 mM glucose in the islet cells treated with siRev. At present, we do not know the reason for this decrease. To investigate the mechanism underlying the reduced glucose-stimulated insulin secretion, we first studied glucose metabolism by measuring NAD(P)H levels in MIN-6 cells after siRev treatment. Silencing Rev-erbα did not affect the glucose-induced rise in NAD(P)H levels (Supplemental Fig. 1B). However, when we checked the mRNA expression of important genes involved in the exocytotic machinery such as Vamp3, Munch18, SNAP25, and Syntaxin1a, there was a down-regulation of all exocytotic genes in MIN-6 cells after siRev treatment compared with controls (Fig. 3, G–J). Thus, it seems that exocytotic events rather than glucose metabolism would be involved in the decreased insulin secretion in cells treated with siRev.

The activity of REV-ERBα proteins can be regulated by the natural ligand heme (15, 23). Therefore, we next checked the modulation of heme levels on Rev-erbα-dependent gene expression and insulin secretion in MIN-6 cells. Hemin (30 μM) had no effect on Rev-erbα mRNA expression (Fig. 4A) but led to a decrease in ALAS-1, a gene that encodes the δ-aminolevulinate synthase 1, a rate-limiting enzyme in the mammalian heme biosynthetic pathway (Fig. 4B). Hemin also led to a decrease in Bmal1, a Rev-erbα target gene (Fig. 4C). These experiments confirm the activation of Rev-erbα in MIN-6 cells in the presence of heme, as previously shown (23). Hemin stimulated both basal and glucose-stimulated insulin secretion in MIN-6 cells (Fig. 4D). Additionally, the synthetic Rev-erbα agonist GSK41112 also stimulated glucose-induced insulin secretion (Fig. 4E), whereas the synthetic Rev-erbα antagonist SR8278 reduced insulin release (P = 0.05) (Fig. 4F). These results further show that Rev-erbα is implicated in insulin release.

To have a global view of Rev-erbα function in pancreatic β-cells, we next analyzed the effect of its down-regulation on proliferation, cell viability, and apoptosis. As shown in Fig. 5, the decrease in Rev-erbα mRNA levels by siRNA had no effect on cell viability (Fig. 5A) in MIN-6 cells and primary

**FIG. 4.** Activation of Rev-erbα stimulates insulin secretion in pancreatic β-cells. A–C, mRNA levels of Rev-erbα (A) ALAS-1 (B), and Bmal1 (C) in MIN-6 cells treated with 30 μM hemin (n = 4–5). D, Glucose-stimulated insulin secretion in MIN-6 cells stimulated with 30 μM hemin (D), 10 μM Rev-erbα agonist GSK4112 (E), and 10 μM Rev-erbα antagonist SR8278 (F). (n = 4–5). *, P < 0.05; **, P < 0.01. Data are expressed as mean ± SEM.
cells (Supplemental Fig. 1A) and apoptosis in MIN-6 cells (Fig. 5B) compared with the control (Sc), indicating that Rev-erbα was unlikely to be involved in these cell processes. However, when we measured cell proliferation in MIN-6 and primary β-cells, the reduced Rev-erbα expression led to a remarkable decrease in β-cell proliferation (Fig. 5, C and D). These results indicate that this clock gene is involved in β-cell growth.

**Leptin up-regulates Rev-erbα expression through the MAPK pathway**

Previous studies have shown that leptin can affect clock gene expression in the liver (24) and bone (25). Therefore, we hypothesized that leptin might be involved in the modulation of Rev-erbα gene expression in the endocrine pancreas. To test the in vivo leptin effect (Fig. 6A), we chose one time point of the day at ZT0 in this experiment. At this time, Rev-erbα expression had a lower expression level, as shown in Fig. 6. Mice were treated for 5 d with two daily ip injections of leptin (500 ng/g body weight) or with vehicle (PBS) as previously reported (21). A third group (pair-fed) was also included to discard the potential influence of the anorexigenic leptin effect on gene expression. The levels of Rev-erbα expression remained similar between islets from pair-fed and control groups (Fig. 6A). However, Rev-erbα gene expression was significantly increased in isolated islets of mice treated in vivo with leptin (Fig. 6A). To study whether this in vivo effect was due to a direct action of leptin on β-cells, we next performed in vitro experiments in MIN-6 cells and isolated islets from control, ob/ob, and db/db mice. Leptin treatment (6.25 nM) for 24 h increased Rev-erbα gene expression in pancreatic islets (Fig. 6B) and MIN-6 cells (Fig. 6C). Interestingly, in ob/ob mice, which lack the leptin protein due to a mutation in the gene, Rev-erbα gene expression was almost absent, and leptin incubation dramatically increased (4-fold) the Rev-erbα mRNA levels (Fig. 6B). Islets from db/db mice, which lack leptin receptors, showed a tendency to have decreased Rev-erbα mRNA levels compared with control islets, whereas leptin treatment had no effect on Rev-erbα gene expression (Fig. 6B). These results show that leptin modulates Rev-erbα gene expression by direct action on pancreatic β-cells.

To investigate the downstream signaling mechanism by which leptin modulates Rev-erbα gene expression, we analyzed the main leptin-induced signaling cascades in pancreatic β-cells. We first studied the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway using siRNA to knock down STAT3 (26, 27). STAT3 silencing efficiency in MIN-6 cells was about 70–80% (Fig. 6D). As expected, leptin increased Rev-erbα mRNA levels in cells treated with a control siRNA (Fig. 6E). However, STAT3 silencing did not prevent leptin-induced increase of Rev-erbα (Fig. 6E). Using isolated islets from control mice, we further examined the role of the phosphoinositide 3-kinase (PI3K) and MAPK pathways. Inhibition of the PI3K pathway with LY (10 μM) or wortmannin (100 nM) did not prevent leptin-induced increase in Rev-erbα expression (Fig. 6F). In contrast, in the presence of PD 98059 (10 μM), an inhibitor of the MAPK pathway, leptin failed to increase Rev-erbα gene expression (Fig. 6F), indicating that leptin regulation of Rev-erbα occurs through a MAPK pathway.

**HFD disrupts the temporal expression profile of Rev-erbα in pancreatic islets**

Given that several clock genes may be affected in obesity (26, 27), we hypothesized that Rev-erbα expression in
that HFD not only changes the expression profile throughout the day but also decreases the amplitude of the change (~6 vs. 2.5-fold comparing control and HFD conditions). Although we focused on Rev-erba, we also observed that other clock genes exhibited changes in the expression level throughout the day (particularly Clock, Bmal1, Per1, and Per2) and that they were altered by HFD in some cases (Clock and Per1) (Fig. 7, G–L).

Additionally, an interesting finding was that Rev-erba expression levels throughout the day followed a similar pattern as that of changes in glucose-induced insulin secretion in islets from both control and HFD-treated mice (Fig. 7F). In these experiments (Fig. 7, E and F), islets from HFD and control mice were separated into two groups to analyze both Rev-erba expression and insulin release. Basal insulin secretion did not significantly change during the different ZT measured (Fig. 7F), and HFD treatment increased basal insulin release only at ZT12 compared with controls (Fig. 7F). In contrast, glucose-stimulated insulin release in control islets showed a secretion peak at ZT6 (Fig. 7F). If we compare the HFD group with controls, HFD treatment increased glucose-induced insulin release at ZT0 and ZT12 and decreased insulin secretion at ZT6 (Fig. 7F). This pattern in secretion comparing HFD mice and controls is similar to that of Rev-erba expression levels throughout the day between both groups (Fig. 7E). These similar changes in glucose-stimulated insulin secretion and Rev-erba gene expression further indicate that this gene is important for the β-cell secretory function.

**Discussion**

Recently, studies in humans and rodents demonstrated that clock genes are closely related to obesity and diabetes (4, 28–30). For instance, Clock and Bmal1 mutant mice are hyperphagic and obese and develop diabetes mellitus (31, 32). In the present study, we identify the clock gene Rev-erba as a new regulator of glucose-induced insulin secretion, a key gene involved in lipid metabolism and pancreatic β-cell proliferation. Furthermore, we showed that leptin regulates Rev-erba expression in pancreatic islets via a MAPK pathway. The fact that in pathological conditions such as obesity, the expression levels of Rev-erba throughout the day are altered along with insulin secretion further indicate that this clock gene has an important role in β-cell function.

The nuclear receptor Rev-erba integrates signals from the circadian pacemaker and other nuclear receptor sig-
timing of Srebp-1c gene expression for a proper control of lipid metabolism in pancreatic β-cells. Additionally, Rev-erba was shown here to regulate insulin secretion and β-cell proliferation. Indeed, when Rev-erba was down-regulated in both MIN-6 and islet cells by siRNA, glucose-stimulated insulin secretion and β-cell proliferation were decreased. Supporting the role of Rev-erba in the regulation of insulin release, both the natural (hemin) and synthetic Rev-erba agonists GSK4112 (34) stimulated insulin secretion, whereas the Rev-erba antagonist SR8278 (35) suppressed insulin release. Additionally, β-cell proliferation was also down-regulated. Whether Rev-erba directly regulates proliferation or is the result of the decreased insulin secretion is still unknown. These findings are in agreement with the fact that other clock genes such as Bmal1 and Clock modulate insulin secretion and pancreatic islet cell proliferation (31). Interestingly, these previous findings in Bmal1 and Clock mutant mice reported reduced expression levels of Rev-erba in pancreatic islets. Thus, the altered islet function in these animal models may be also, in part, due to reduced islet Rev-erba expression.

Leptin has been shown to modulate Clock and Per2 gene expression in osteoblasts (25) and was recently suggested to be involved in the proper control of Clock, Bmal1, Cry1, Per1, Per2, and Dbp genes in the liver and adipose tissue from ob/ob mice (24). Our results showed for the first time that leptin has also a direct effect on Rev-erba gene expression in the endocrine pancreas, and this effect occurs through a MAPK pathway. It has been previously demonstrated that leptin activates the MAPK pathway in two insulin-secreting cell lines: RINm5f and MIN-6 cells (36, 37). It was also suggested that leptin could control growth and/or differentiation of β-cells via this pathway. Thus, it could be possible that Rev-erba may function downstream of the MAPK cascade to regulate leptin-induced β-cell proliferation during obesity, as has been previously proposed (36, 37). In any case, we showed here that Rev-erba is involved in insulin secretion and proliferation and that leptin regulates Rev-erba expression. However, this does not nec-

FIG. 7. HFD disrupts circadian expression of Rev-erba gene. Mice were fed with normal chow diet and a HFD for 6 wk. A, Body weight (n = 8); B, glucose tolerance test (n = 10); C, plasma insulin (n = 8–10); D, plasma leptin (n = 8–10). E–L, After treatment, pancreatic islets were isolated in different ZT (ZT0, ZT6, and ZT12) and separated into two groups for subsequent measurements of Rev-erba mRNA expression (n = 4–5) (E), insulin secretion (n = 10) (F), Clock mRNA expression (n = 4–5) (G), Bmal1 mRNA expression (n = 4–5) (H), Per1 mRNA expression (n = 4–5) (I), Per2 mRNA expression (n = 4–5) (J), Cry1 mRNA expression (n = 4–5) (K), and Cry2 mRNA expression (n = 4–5) (L). 2.8G, 2.8 mM glucose; 16.8G, 16.8 mM glucose. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. control (chow); and #, P < 0.05; ###, P < 0.01; ####, P < 0.001 for comparison differences between different ZT (ZT0, ZT6, and ZT12) within the control (chow) group. No statistical differences were found in the Rev-erba mRNA levels between ZT within the HFD group. White squares and bars, chow-treated group; black triangles and bars, HFD group. Data are expressed as mean ± SEM.
essarily mean that the effects of leptin on secretion and proliferation are attributed to its Rev-erbα modulation, given that leptin triggers a great variety of signaling pathways and actions in the β-cell (38), and also, it is expected that this clock gene might be under the regulation of several other control points. This situation will be probably more complicated in vivo, where Rev-erbα expression is probably the result of the modulation by several hormones, neurotransmitters, or other molecules.

Our in vivo studies showed that HFD treatment for 6 wk altered the temporal expression pattern of Rev-erbα in pancreatic islets. Given the sensitivity of Rev-erbα to leptin, the present results indicate that this hormone may be one of the obesity-related factors that can alter the expression of Rev-erbα in the endocrine pancreas during obesity. However, we cannot exclude other hormones such as insulin and inflammatory cytokines, which have an important role in obesity, or processes such as leptin and insulin resistance, which are also implicated in the pathophysiology of obesity. Most probably, the altered temporal profile of Rev-erbα in HFD-treated animals is a consequence of several of these obesity-related factors.

Interestingly, glucose-stimulated insulin secretion followed a similar temporal pattern as that of Rev-erbα expression at the time points examined in the islets of both control and HFD-treated mice. This temporal pattern of insulin secretion in mice is in agreement with findings in perfused rat pancreatic islets showing a circadian insulin release with a secretion peak during the day (39). The present ex vivo observations of similar changes in Rev-erbα gene expression and insulin release in both normal and HFD conditions suggest that changes in Rev-erbα levels may be involved in the daily dynamics of insulin secretion. However, additional research should be performed to establish whether leptin-induced Rev-erbα expression is also involved in the circadian pattern of insulin secretion, because the leptin effects on insulin secretion have been shown to be inhibitory (40), stimulatory (41), or without effect (42).

Overall, our results give new insights regarding the role of the clock gene Rev-erbα in the pancreatic β-cell function. It is plausible that during the onset and progression of obesity, the increasing leptin levels may up-regulate Rev-erbα, promoting pancreatic islet adaptations such as β-cell growth. However, prolonged obesity may lead to leptin resistance at the level of the β-cells (27, 38), which may down-regulate Rev-erbα gene expression and, eventually, promote β-cell dysfunction.

Acknowledgments

We thank A. B. Rufete, M. L. Navarro, and M. Giner for their technical assistance and Dr. Atul Deshmukh for the correction of the manuscript.

Address all correspondence and requests for reprints to: E. Vieira. Instituto de Bioingenieria, Universidad Miguel Hernandez, Avenida de la Universidad s/n, 03202 Elche, Spain. E-mail: evieira@umh.es; or I. Quesada. Instituto de Bioingenieria, Universidad Miguel Hernandez, Avenida de la Universidad s/n, 03202 Elche, Spain. E-mail: ivanq@umh.es.

This work was supported by grants from the FP7 Marie Curie Program, Ministerio de Educacion y Ciencia (BFU2010-21773; BFU2008-01492), and Generalitat Valenciana (PROMETEO/2011/080). Centro de Investigacion Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas is an initiative of the Instituto de Salud Carlos III.

Disclosure Summary: The authors have nothing to disclose.

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