OBJECTIVE—Carbohydrate-responsive element-binding protein (ChREBP) is a transcription factor that has been shown to regulate carbohydrate metabolism in the liver and pancreatic β-cells in response to elevated glucose concentrations. Because few genes have been identified so far as bona fide ChREBP-target genes, we have performed a genome-wide analysis of the ChREBP transcriptome in pancreatic β-cells.

RESEARCH DESIGN AND METHODS—Chromatin immunoprecipitation and high-density oligonucleotide tiling arrays (ChIP-chip; Agilent Technologies) using MIN6 pancreatic β-cell precipitation and high-density oligonucleotide tiling arrays were performed together with transcriptional and other analysis using standard techniques.

RESULTS—One of the genes identified by ChIP-chip and linked to glucose sensing and insulin secretion was aryl hydrocarbon receptor nuclear translocator (ARNT)/hypoxia-inducible factor-1β (HIF-1β), a transcription factor implicated in altered gene expression and pancreatic-β-cell dysfunction in type 2 diabetes. We first confirmed that elevated glucose concentrations decreased ARNT/HIF-1β levels in INS-1 (832/13) cells and primary mouse islets. Demonstrating a role for ChREBP in ARNT gene regulation, ChREBP silencing increased ARNT mRNA levels in INS-1 (832/13) cells, and ChREBP overexpression decreased ARNT mRNA in INS-1 (832/13) cells and primary mouse islets. We demonstrated that ChREBP and Max-like protein X (MLX) bind on the ARNT/HIF-1β promoter on the proximal region that also confers the negative glucose responsiveness.

CONCLUSIONS—These results demonstrate that ChREBP acts as a novel repressor of the ARNT/HIF-1β gene and might contribute to β-cell dysfunction induced by glucotoxicity. Diabetes 59:153–160, 2010

Pancreatic β-cell dysfunction or loss is the hallmark of all forms of diabetes (1,2). Transcription factors have proven to be critical for the maintenance of normal β-cell function, and mutations in many of these, including pancreatic duodenum homeobox-1 (PDX1) (3) and the hepatocyte nuclear factors HNF1α (4) and HNF4α (5) lead to monogenic forms of inherited type 2 diabetes (6), whereas polymorphisms in others, notably T-cell factor 7-like 2 (TCF7L2), are associated with more common forms of the disease (7).

Recently, a transcription factor termed aryl hydrocarbon receptor nuclear translocator (ARNT), or hypoxia-inducible factor-1β (HIF-1β), has emerged as a potentially important player in the pathogenesis of pancreatic β-cell dysfunction and type 2 diabetes in humans (8). ARNT/HIF-1β is a member of the basic helix-loop-helix (HLH) Per/AhR/ARNT/Sim (PAS) family of transcription factors and binds DNA as an obligate heterodimer with the oxygen-sensitive HIF-1α, HIF-2α, or aryl hydrocarbon receptor (AhR). When mRNA levels were compared in human pancreatic islets of Langerhans isolated from five type 2 diabetic donors and seven nondiabetic donors by oligonucleotide microarrays and real-time PCR, an 82% reduction in the expression of the ARNT/HIF-1β gene was observed in islets from type 2 diabetic donors. Confirming a role for ARNT/HIF-1β in β-cell function, β-cell-specific ARNT/HIF-1β gene knockout in mice, or ARNT/HIF-1β silencing in MIN6 cells, led to defects in glucose-stimulated insulin secretion (GSIS) and alterations in islet gene expression comparable to those observed in human type 2 islets (8).

Carbohydrate-responsive element-binding protein (ChREBP) (9) (also termed MondoB or Williams-Beuren syndrome critical region gene 14 [WBSCR14]) (10) is a transcription factor that regulates de novo lipogenesis in the liver in response to elevated glucose concentrations (11). It is a member of the basic HLH (bHLH) family and transactivates glucose-responsive genes by binding DNA on carbohydrate response element (ChoRE), as a heterodimer with Max-like protein X (MLX) (12). In addition to its lipogenic role in the liver, we have recently shown (13) that, in clonal pancreatic β-cells, 1) ChREBP mRNA levels are increased in response to elevated glucose concentrations; 2) ChREBP translocates to the nucleus in response to high glucose; 3) ChREBP directly binds to the promoters of lipogenic genes fatty acid synthase (FAS) and L-type pyruvate kinase (L-PK) in a glucose- and calcium-dependent, but insulin-independent, manner in intact cells; and 4) ChREBP silencing by small-interfering RNA (siRNA) improves GSIS (13).

To identify other genes directly regulated by ChREBP in the β-cell, we performed a genome-wide analysis of the ChREBP transcriptome using chromatin immunoprecipitation and high-density oligonucleotide tiling arrays (ChIP-on-chip; Agilent Technologies) in MIN6 cells. ARNT/HIF-1β fell within the statistical cutoff and was considered positive for ChREBP binding. Consequently, we demonstrate that ChREBP directly binds to ARNT/HIF-1β promoter in a glucose-dependent manner in clonal MIN6 and
INS-1 (832/13) cells and negatively regulates ARNT/HIF-1β gene expression in pancreatic islet β-cells.

These results suggest that ChREBP, in addition to being a critical regulator of lipogenic genes in the liver and pancreatic β-cell, may also play a role in the development of β-cell failure and type 2 diabetes through alteration of ARNT/HIF-1β gene expression.

RESEARCH DESIGN AND METHODS

Reagents. The Slcener siRNA construction kit was from Ambion (Huntingdon, U.K.). Primers for siRNA construction and PCR were from MWG Biotech (Milton Keynes, U.K.). Transf-TKO transfection reagent was from Mirus (Madison, WI). The rat insulin radioimmunoassay kit was from Linco (St. Charles, MO). Tissue culture reagents were from Sigma-Aldrich (Dorset, U.K.) or Invitrogen (Paisley, U.K.). Lipofectamine 2000 and STYR Green platinum were from Invitrogen (Paisley, U.K.). Collagenase from C. histolyticum was obtained from Serva Electrophoresis (Heidelberg, Germany). Anti-ChREBP antibody was described by da Silva Xavier et al. (13). Mouse monoclonal anti-hemaglutinin (HA) antibody was provided by Anindiya Roy (Cancer Research U.K.). Other reagents were from Sigma or Fisher.

Plasmids, adenoviruses, and SirNA generation. Plasmid pChREBP was described (13). Plasmid bearing HA-tagged MLX cDNA was provided by Dr H. Plasmids, adenoviruses, and SiRNA generation.

obtained from Serva Electrophoresis (Heidelberg, Germany). Anti-ChREBP 1028[-443T and Spe ARNT-630.LucFF plasmids were introduced using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primer sequences for each mutant are indicated. FGF21 expression was driven by the rat ChREBP promoter from 129 to 5679, which contains 11 mmol/l glucose and then incubated for 16 h at 3 mmol/l glucose and finally for 20 h at either 3 or 17 mmol/l glucose as indicated. Levels of mRNA were determined by quantitative real-time RT-PCR and were normalized with cyclophilin mRNA as described by da Silva Xavier et al. (22). Results are expressed as the fold change over control (null, 3 mmol/l glucose) and presented as the means ± SE.

Insulin secretion and assay. For insulin secretion experiments, islets were incubated for 60 min in a shaking water bath at 37°C in 1 ml Krebs-Ringer bicarbonate medium containing 11.5 mmol/l NaCl, 1.5 mmol/l KCl, 2.0 mmol/l CaCl2, 1.0 mmol/l MgSO4, 0.5 mmol/l NaH2PO4, 10 mmol/l HEPES) supplemented with 0.5 mmol/l glucose. The DNA was hybridized onto microarray slides for up to 40 h at 40°C. The slides were washed, and the data were analyzed by high-resolution scanning and Agilent ChIP Analytics software. To detect significant binding events, a minimum cutoff P value of <0.001 was used.

DNA isolation and quantification real-time PCR. Primary mouse pancreatic islets were treated for 24 h with ChREBP or null adenovirus in culture medium containing 11 mmol/l glucose and then incubated for 16 h at 3 mmol/l glucose and finally for 20 h at either 3 or 17 mmol/l glucose as indicated. Levels of mRNA were determined by quantitative real-time RT-PCR and were normalized with cyclophilin mRNA as described by da Silva Xavier et al. (22). Results are expressed as the fold change over control (null, 3 mmol/l glucose) and presented as the means ± SE.

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FIG. 1. ChREBP binds to ARNT/HIF-1β promoter in vivo in a glucose-dependent manner. A: Sequence of the mouse ARNT/HIF-1β promoter highlighting (bold-italic) those sequences present on the Agilent chip. Underlined is the region that gave the strongest hybridization signal in the ChIP-on-chip assay. The position of the ATG is shown in bold.

B: MIN6 cells were incubated in 3 versus 30 mmol/l glucose for 16 h, as indicated before cross-linking and chromatin immunoprecipitation using our polyclonal anti-ChREBP antibody. The primer sequences used to amplify mouse ARNT/HIF-1β and L-PK promoters and the PCR conditions are given in RESEARCH DESIGN AND METHODS. Data are representative of three independent experiments.
Student's t test for unpaired data, with Bonferroni's correction for multiple tests where appropriate.

RESULTS

ChREBP binds directly and glucose dependently to the ARNT/HIF-1β proximal promoter in living clonal β-cells to regulate its transcription. To identify genomic targets of ChREBP, we used an unbiased "ChIP-on-chip" approach using high-density oligonucleotide arrays (see RESEARCH DESIGN AND METHODS). The immediate 5′ flanking region of the ARNT/HIF-1β gene was one of the promoter elements identified on the array as a site at which binding of ChREBP achieved statistical confidence at $P < 0.1\%$. Given previous reports that ARNT/HIF-1β expression in islets is downregulated in type 2 diabetes in humans and that this factor is required for the normal regulation of insulin secretion (8), we explored the nature and role of the ChREBP-ARNT/HIF-1β promoter interaction in more detail. First, we confirmed, by specific ChIP assay, the binding of ChREBP to a region of the mouse ARNT/HIF-1β promoter that gave the strongest signal on the ChIP-on-chip (Fig. 1A, underlined), which was located between −249 and −530 bp upstream of the ATG (Fig. 1B, upper panel). Our anti-ChREBP antibody also immunoprecipitated a region on the mouse L-PK promoter containing the ChoRE (23,24) and known to bind ChREBP (13,25) (Fig. 1B, lower panel), whereas regions on the ARNT/HIF-1β promoter located either upstream or downstream of −530 to −249 bp area were not pulled down (Fig. 1B, middle panels). Whereas immunoprecipitation of ChREBP from cells incubated at 3 mmol/l glucose, and PCR amplification of this fragment, generated a relatively minor product, a band of more than threefold greater intensity was generated from cells incubated at elevated glucose concentrations (30 mmol/l; Fig. 1B, upper panel). These findings thus demonstrated that ChREBP was bound 1) directly and 2) in a glucose-regulated manner to the ARNT/HIF-1β promoter in living MIN6 β-cells.

To further explore the roles of glucose and ChREBP in controlling ARNT/HIF-1β gene expression, we measured ARNT/HIF-1β mRNA levels in the highly glucose-responsive β-cell line, INS-1 (832/13) (20). Confirming the glucose sensitivity of ARNT/HIF-1β expression, as recently described (26), incubation of INS-1 (832/13) cells at 20 versus 3 mmol/l glucose led to a substantial (40–50%) decrease in the corresponding ARNT/HIF-1β mRNA levels (Fig. 2A). These findings are consistent with the view that ChREBP induction (27) and nuclear accumulation (13) at high glucose levels contribute to the regulation of ARNT/HIF-1β expression in pancreatic β-cells.

FIG. 2. Glucose and ChREBP inhibits ARNT/HIF-1β gene expression in INS-1 (832/13) cells. A and B: INS-1 (832/13) cells were transfected for 16 h with either ChREBP-specific or -scrambled SiRNAs using TransIT-TKO and Opti-mem or (C and D) infected with ChREBP expressing or null adenovirus at an multiplicity of infection of 100 and then incubated in 3 mmol/l glucose RPMI for 16 h followed by either 3 or 20 mmol/l glucose RPMI as indicated for 20 h, before total RNA extraction. ARNT/HIF-1β (A and C) and ChREBP (B and D) mRNA levels were estimated by quantitative real-time RT-PCR. The results are expressed as mean ± SE of three independent experiments. *$P < 0.05$ for the effect of glucose.
glucose concentrations may be involved in the effects of glucose on ARNT/HIF-1β expression. Accordingly, transfection with a siRNA selective for rat ChREBP, which decreased the corresponding mRNA levels by ~50% (Fig. 2B), resulted in a twofold increased in ARNT/HIF-1β mRNA levels at high glucose (Fig. 2A). Conversely, adenoviral ChREBP overexpression (supplementary Fig. 1 and Fig. 2C in the online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db08-0868/DC1) resulted in significant reduction of ARNT/HIF-1β mRNA levels.

To further analyze the effect of ChREBP binding on ARNT/HIF-1β gene transcription, we next generated promoter-reporter luciferase constructs using fragments of the mouse ARNT/HIF-1β promoter from −2,369 to 293 base pairs with respect to the ATG, a region of the promoter highly conserved between species. Because ChREBP has been shown to regulate lipogenic gene expression as a heterodimer with MLX (28), we next examined the effects of ChREBP and/or MLX overexpression on ARNT/HIF-1β reporter activity in HEK293 cells. As shown in Fig. 3A, overexpression of ChREBP resulted in substantial inhibition of ARNT/HIF-1β promoter activity, even without cotransfection with exogenous MLX, whereas MLX overexpression alone activated the ARNT/HIF-1β promoter. However, as verified by qRT-PCR, MLX was expressed in HEK293 cells [although at lower levels than in INS-1 (832/13) cells], whereas ChREBP mRNA was practically undetectable (not shown). To find out whether ChREBP binding to the ARNT/HIF-1β promoter occurs after heterodimerization with MLX in pancreatic β-cells, we performed a ChIP assay in INS-1 (832/13) cells transfected with MLX-HA, using an anti-HA antibody. As shown in Fig. 3B, both L-PK and ARNT/HIF-1β segments of promoter previously pulled down using anti-ChREBP antibody were also immunoprecipitated using anti-HA antibody, whereas the region of ARNT/HIF-1β promoter located between −1,300 and −1,001 bp was not detected. This strongly suggests that ChREBP and MLX form a heterodimeric complex on each promoter.

In an attempt to locate the ChREBP/MLX binding site, truncation and deletion mutants were generated in ARNT/HIF-1β luciferase reporter plasmid by restriction digestion (see RESEARCH DESIGN AND METHODS for details) and cotransfected with either empty pcDNA3 vector or pChREBP into HEK293 cells. As expected, and in accordance with the ChIP-on-chip data and the ARNT/HIF-1β-specific ChIP experiment presented in Fig. 1, the deletion mutant lacking the whole proximal region pulled down by ChIP was no longer repressed by exogenous ChREBP (Fig. 4A). We next mutated the five consensus individual E-boxes present between −512 and −329 bp on the ARNT/HIF-1β promoter. None of these mutants, at least individually, displayed a loss of ChREBP repression (supplementary Fig. 3A in the online appendix). Subsequently, using the NoShift Transcription Factor Assay Kit (Novagen) as an alternative to electrophoretic mobility shift assay, and nuclear extracts from HEK293 cells cotransfected with c-Myc–tagged ChREBP and HA-tagged MLX, we scanned the ARNT/HIF-1β promoter between −519 and −220 bp, using overlapping double-stranded DNA probes (supplementary Fig. 2A). The L-PK ChoRE was used as an internal positive control. Because only the probe located between −441 to −411 bp gave a positive signal (supplementary Fig. 2B), we generated deletion mutants around this region, but none of these mutants lost ChREBP repression (supplementary Fig. 3B), suggesting that ChREBP repression of ARNT/HIF-1β promoter could demand a complex cis element configuration.

Glucose repression of ARNT/HIF-1β gene expression requires the proximal promoter. To investigate the role of ChREBP in mediating the glucose repression of the ARNT/HIF-1β gene, we transected the various ARNT promoter-reporter luciferase constructs in INS-1 (832/13) cells and incubated them in 3 or 20 mmol/l glucose for 24 h. Only the construct lacking the whole of the proximal promoter (−1,028 to −769 ARNT) was no longer repressed by glucose; all the other mutants still showed significant glucose repression (Fig. 4B and data not shown).

ChREBP overexpression inhibits ARNT/HIF-1β expression and regulates glucose-induced insulin secretion from primary mouse islets. To determine whether the observations reported above in clonal β-cells were relevant to the primary β-cell, we infected isolated mouse islets of Langerhans with ChREBP-overexpressing adenovirus. Whereas endogenous ChREBP mRNA levels were
augmented in islets cultured for 20 h at 17 versus 3 mmol/l glucose, as expected, transduction of islets with ChREBP adenovirus caused a >100-fold increase in ChREBP mRNA levels compared with those observed at either 3 or 17 mmol/l glucose in null adenovirus-transduced islets (Fig. 5A). This forced increase in ChREBP expression was associated with a decrease in ARNT/HIF-1α mRNA levels at both low and high (3 and 17 mmol/l) glucose (Fig. 5B).

We have previously demonstrated that ChREBP silencing enhanced basal and glucose-stimulated insulin secretion from MIN6 β-cells (13). Correspondingly, we observed here that adenovirus-mediated ChREBP overexpression in mouse islets inhibited GSIS by ~30% (Fig. 5C).

**DISCUSSION**

The principal aim of the present study was to identify and characterize new target genes that may mediate the effects of ChREBP on β-cell function. To this end, and using an unbiased genome-wide approach, we have identified ARNT/HIF-1α as such a target. Previously shown to be downregulated in type 2 diabetic human islets, and to modulate insulin secretion in the mouse (8), repression of ARNT/HIF-1α by ChREBP represents an important potential mechanism through which the inhibitory effects of high glucose and of ChREBP may be exerted.

**Regulation of ARNT/HIF-1α by ChREBP and glucose.** ARNT/HIF-1α expression has recently been shown (26) to be elevated at low glucose concentrations in pancreatic MIN6 β-cells. Both ARNT/HIF-1α and presenilin-1 expression were induced by low glucose, the latter being a component of the γ-secretase complex involved in the processing of the β-amyloid precursor processing in the brain, which may regulate β-cell survival via the Notch pathway (26). It was suggested in these earlier studies that ARNT/HIF-1α lies downstream of presenilin in a signaling pathway.
play a role that is complementary to that of preseminin-1 to control transcription of the ARNT/HIF-1β gene as glucose concentrations change. However, elevated glucose concentrations were still able to decrease ARNT/HIF-1β mRNA levels in the presence of increased, inhibitory, ChREBP levels (Fig. 3), suggesting that multiple signaling mechanisms may be involved in the regulation of ARNT/HIF-1β transcription by glucose. Interestingly, in a previous report (13), we demonstrated that ChREBP binding to the liver type (l-) pyruvate kinase gene was regulated in part by an increase in intracellular free Ca2+ concentrations. Recent studies by Scott and colleagues (29) suggested that activation by glucose of the l-PK gene in INS-1–derived 832/13 cells involves recruitment of c-Myc, Max, and ChREBP to the promoter and a glucose-stimulated increase in ChREBP transactivation. Whether the inhibitory effects of ChREBP binding at the ARNT/HIF-1β gene promoter are similarly mediated remains to be explored.

ChREBP has previously been shown to regulate transcription of target genes both positively or negatively, depending on the gene and context (10,12). Using ChIP, NoShift assay, and mutational analysis, we located the ChREBP/MLX binding site to the proximal region of ARNT/HIF-1β promoter, a region also mediating the negative glucose effect, but the precise cis element(s) have remained elusive. Although glucose repression of gene expression in yeast is now well understood (30), and involves the AMP-activated protein kinase analog Snf1 (31), our understanding of glucose inhibition of gene expression in mammalian cells is still rudimentary. In primary rat hepatocytes, Ma et al. (28) found 59 genes that were repressed by high glucose, and only eight of these were also increased by dominant-negative MLX. Whether or not ChREBP was the interacting partner of MLX in regulating the expression of these genes and whether ChREBP/MLX was mediating the glucose response needs further study.

**Potential impact of ARNT/HIF-1β regulation on β-cell function.** What may be the physiological consequences for β-cell function or survival of ChREBP-mediated ARNT/HIF-1β repression at high glucose concentrations? The HIF-1 complex is believed to be an important regulator of the response to hypoxic stresses in other cell types (32). Inhibition of ARNT/HIF-1β expression by high glucose may thus contribute to the deleterious effects on β-cell mass or function during sustained hyperglycemia observed in type 2 diabetes. This may further exacerbate the effects of elevated nuclear levels of ChREBP to drive lipogenic gene expression and hence, potentially, contribute to “gluco-lipotoxicity” (1,33) under these conditions. Correspondingly, we show here for the first time that ChREBP overexpression inhibits glucose-stimulated insulin secretion from primary mouse islets by 50%. Therapeutic approaches that may suppress ChREBP function in islets may thus be useful for the treatment of insulin secretory deficiencies in some forms of type 2 diabetes.

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