Hepatocyte Nuclear Factor 4α Regulates the Expression of Pancreatic β-Cell Genes Implicated in Glucose Metabolism and Nutrient-induced Insulin Secretion*

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Mutations in the HNF4α gene are associated with the subtype 1 of maturity-onset diabetes of the young (MODY1), which is characterized by impaired insulin secretory response to glucose in pancreatic β-cells. Hepatocyte nuclear factor 4α (HNF4α) is a transcription factor critical for liver development and hepatocyte-specific gene expression. However, the role of HNF4α in the regulation of pancreatic β-cell gene expression and its correlation with metabolism secretion coupling have not been previously investigated. The tetracycline-inducible system was employed to achieve tightly controlled expression of both wild type (WT) and dominant-negative mutant (DN) of HNF4α in INS-1 cells. The induction of WT-HNF4α resulted in a left shift in glucose-stimulated insulin secretion, whereas DN-HNF4α selectively impaired nutrient-stimulated insulin release. Induction of DN-HNF4α also caused defective mitochondrial function substantiated by reduced [14C]pyruvate oxidation, attenuated substrate-evoked mitochondrial membrane hyperpolarization, and blunted nutrient-generated cellular ATP production. Quantitative evaluation of HNF4α-regulated pancreatic β-cell gene expression revealed altered mRNA levels of insulin, glucose transporter-2, L-pyruvate kinase, aldolase B, 2-oxoglutarate dehydrogenase E1 subunit, and mitochondrial uncoupling protein-2. The patterns of HNF4α-regulated gene expression are strikingly similar to that of its downstream transcription factor HNF1α. Indeed, HNF4α changed the HNF1α mRNA levels and HNF1α promoter luciferase activity through altered HNF4α binding. These results demonstrate the importance of HNF4α in β-cell metabolism-secretion coupling.

The hepatocyte nuclear factor 4α (HNF4α),1 a transcription factor of the nuclear hormone receptor superfamily, is expressed in liver, kidney, gut, and pancreatic islets (1–3). Mutations in the human HNF4α gene lead to maturity onset diabetes of the young subtype 1 (MODY1), which is characterized by autosomal dominant inheritance and impaired glucose-stimulated insulin secretion from pancreatic β-cells (4–6). These MODY1 mutations located in various domains of the HNF4α protein result in defective function of the transcription factor (6). The clinical phenotype of MODY1 patients is indistinguishable from that of MODY3 patients who carry mutations in the HNF1α gene (5, 6). HNF4α acts upstream of HNF1α in a transcriptional cascade that drives liver-specific gene expression and hepatocyte differentiation (7–9). A naturally occurring mutation in the HNF4α-binding site of the HNF1α promoter identified in a MODY3 family (10) suggests that the transcriptional hierarchy could also be involved in pancreatic β-cell gene expression and function.

HNF4α defines the expression of liver-specific genes encoding apolipoproteins, serum factors, cytochrome P-450 isoforms, and proteins involved in the metabolism of glucose, fatty acids, and amino acids (reviewed in Ref. 11). However, clinical characterization of MODY1 subjects reveals that the primary defect is impaired glucose-stimulated insulin secretion from pancreatic β-cells rather than liver dysfunction (5, 12–14). Unfortunately, little is known as to how HNF4α regulates β-cell-restricted gene expression and glucose metabolism and associated insulin secretion. Targeted disruption of the hnf4α gene results in defective gastrulation of mouse embryos due to dysfunction of the visceral endoderm (15). This early embryonic lethality prevents further analysis of the HNF4α function in pancreatic β-cells. The precise role of HNF4α in pancreatic β-cells would best be examined by conditional β-cell-specific deletion of the mouse hnf4α gene. Another alternative is to up- and down-regulate HNF4α function in pancreatic β-cell lines through gene manipulation.

In the present study, the wild type HNF4α (WT-HNF4α) and its dominant-negative mutant (DN-HNF4α) could be induced in INS-1 cells under tight control of the reverse tetracycline-dependent transactivator (16). DN-HNF4α represents the epitope Myc-tagged truncated HNF4α mutant protein lacking the first 111 amino acids (mycΔ111HNF4α) (17). The HNF4α protein consists of an N-terminal ligand-independent transactivation domain (amino acids 1–24), a DNA binding domain containing two zinc fingers (amino acids 51–117), and a large hydrophobic portion (amino acids 163–368) composed of the dimerization, ligand binding, cofactor binding, and ligand-dependent transactivation domain (18, 19). DN-HNF4α therefore suppresses the endogenous WT-HNF4α transcriptional activity by the formation of heterodimers lacking DNA binding capacity (17). We have investigated in a quantitative manner the consequences of altered HNF4α function on β-cell-specific expres-

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‡ The abbreviations used are: HNF4α, hepatocyte nuclear factor 4α; MODY, maturity-onset diabetes of the young; WT, wild type; DN, dominant-negative; PCR, polymerase chain reaction; USF, upstream stimulatory factors; OGDH, 2-oxoglutarate dehydrogenase; L-PK, L-pyruvate kinase; EMSA, electrophoretic mobility shift assay; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Dox, doxycycline.
sion of genes implicated in glucose metabolism and insulin secretion. This allowed us to elucidate the molecular basis and HNF4α target genes responsible for impaired metabolism secretion coupling in β-cells deficient in HNF4α function.

**EXPERIMENTAL PROCEDURES**

**Generation of Stable Cell Lines**—The rat insulinoma INS-1 cell line-derived stable clones were cultured in RPMI 1640 in 11 mM glucose, unless indicated otherwise (20). The establishment of the first step stable clone INS-3r, which expresses the reverse tetracycline-dependent transactivator, was reported previously (21). Plasmids used in the secondary stable transfection were constructed by subcloning the cDNAs encoding the rat WT-HNF4α (a generous gift from Dr. Darnell Jr., New York) and DN-HNF4α into the expression vector PUHD10-3 (a kind gift from Dr. H. Bujard, University Heidelberg, Germany). DN-HNF4α was PCR-amplified from WT-HNF4α using the following primers, ctagagtctggctggtgtaggaggggccag and cagaaattcctgcagatgttctg. The PCR fragment was subcloned into pcDNA3.1myc (Invitrogen, Netherlands) and sequenced. Transfection, clone selection, and screening procedures were described previously (21).

**Immunoblot**—Immunoblotting procedures were performed as described previously using enhanced chemiluminescence (Pierce) for detection (22). Dilutions for antibody against HNF4α (kindly supplied by Dr. F. M. Sladek, University of California, Riverside, CA) and anti-Myc tag (9E10) in myeloma SP2/0 culture medium were 1:6,000 and 1:10.

**Insulin Secretion and Cellular Insulin Content**—Cells in 24-well dishes were cultured in 2.5 mM glucose medium with or without indicated doses of doxycycline for 14 or 48 h. Insulin secretion was measured over a period of 30 min, in Krebs-Ringer/bicarbonate-HEPES buffer (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES, 0.1% bovine serum albumin) containing indicated stimulators. Insulin content was measured over a period of 30 min, in Krebs-Ringer/bicarbonate-HEPES (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES, 0.1% bovine serum albumin) containing indicated stimulators. Insulin content was determined after extraction with acid ethanol following the procedures of Asfari et al. (20). Insulin was detected by radioimmunoassay using rat insulin as standard (22).

**Intracellular ATP**—Cells in 6-well dishes were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 48 h. The production of ATP was measured during 8 min of stimulation in KRBH. ATP assay was performed as reported previously (22).

**14C-Pyruvate Oxidation**—The production of 14CO2 from 14C-pyruvate or [2-14C]pyruvate was measured over 1 h in KRBH containing either 0.05 or 1.0 mM pyruvate as described previously (23, 24).

**Mitochondrial Membrane Potential (Δψm)**—After a 48-h culture period in 2.5 mM glucose medium with or without 500 ng/ml doxycycline, cells were trypsinized (0.025% trypsin, 0.27 mM EDTA), and the cell suspension was maintained for 2 h in a spinner culture with 2.5 mM glucose RPMI 1640 plus 1% newborn calf serum at 37 °C. Mitochondrial membrane potential (Δψm) was measured as described (25). Briefly, after the spinner culture period, cells were loaded with 10 μg/ml rhodamine-123 (Rh-123) for 10 min at 37 °C. After centrifugation, the cells were resuspended and transferred to the fluorometer cuvette at 37 °C with gentle stirring in an LS-50B fluorimeter (PerkinElmer Life Sciences), and fluorescence, excited at 490 nm, was measured at 530 nm.

**Total RNA Isolation and Northern Blotting**—Cells in 10-cm dishes were harvested for nuclear extracts at the indicated times. Nuclear extracts from WT-HNF4α-26 (10 μg/lane) (A) and DN-HNF4α-26 (10 μg/lane) (B) were resolved in 9% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with antibodies against HNF4α (A) and the Myc-tag (B), respectively.

**ATP assay**—Cells in 10-cm dishes were harvested for nuclear extracts at the indicated times. Nuclear extracts from WT-HNF4α-26 (10 μg/lane) (A) and DN-HNF4α-26 (10 μg/lane) (B) were resolved in 9% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with antibodies against HNF4α (A) and the Myc-tag (B), respectively.

**RESULTS**

**HNF4α-regulated β-Cell Gene Expression and Insulin Secretion**

Insulin secretion was quantified as described under “Experimental Procedures” and normalized by cellular DNA content. A, glucose-stimulated insulin secretion in WT-HNF4α-28 cells induced with indicated doses of doxycycline for 14 h. Data represent the means ± S.E. of six independent experiments. Statistical significance between doxycycline-induced and non-induced cells was observed at 2.5 and 6 mM glucose (p < 0.001, unpaired Student's t test). B, glucose-, leucine-, and K+-elicted insulin secretion in DN-HNF4α-26 cells induced with 500 ng/ml doxycycline for 48 h. Insulin secretion was measured during 30 min of incubation with 2.5 mM (Basal) and 24 mM glucose in KRBH, or with 20 mM leucine and 20 mM KCl added in KRBH containing 2.5 mM glucose. Data are the mean ± S.E. of six separate experiments. Statistical significance between doxycycline-induced and non-induced cells was observed at 24 mM glucose- and 20 mM leucine-stimulated conditions (p < 0.001). Insulin content was reduced by 30 ± 8.2% after induction of DN-HNF4α.
DN-HNF4, B. as in with varying doses of doxycycline in a defined period. Similar containing 2.5 mM glucose. Data represent mean ± S.E. performed in triplicate from one of four similar experiments. *p < 0.02. C. [1-14C]pyruvate oxidation was measured with identical conditions in the same preparation of cells as in B. Data represent the mean ± S.E. performed in triplicate from one of three similar experiments.

Fig. 3. Induction of DN-HNF4α impairs cellular ATP production and mitochondrial oxidation. A, cellular ATP levels in DN-HNF4α-26 cells were measured after 8 min of incubation with 2.5 (Basal) and 24 mM glucose in KRBH (or 20 mM leucine and 20 mM KCl added in KRBH containing 2.5 mM glucose. Data represent mean ± S.E. of three independent experiments. Glucose- and leucine-stimulated ATP production was significantly inhibited after treatment with 500 ng/ml doxycycline for 48 h (p < 0.005 and p < 0.001, respectively). B, [2-14C]pyruvate oxidation was measured during 1 h of incubation in KRBH containing 0.05 or 1 mM pyruvate. Data represent the mean ± S.E. performed in triplicate from one of four similar experiments. *p < 0.02. C, [1-14C]pyruvate oxidation was measured with identical conditions in the same preparation of cells as in B. Data represent the mean ± S.E. performed in triplicate from one of three similar experiments.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY—The HNF1α gene promoter luciferase reporter plasmids, WT-HNF1αLuc (wild type) and ΔAHNF1αLuc (HNF4α-binding site deleted), were kindly provided by Dr. N. Miura (Akita University, Japan) (27).

Transient transfection experiments and luciferase reporter enzyme assays were carried out as previously reported (22).

RESULTS

WT-HNF4α or DN-HNF4α Protein Was Induced in INS-1 Cells in a Dose- and Time-dependent Manner—We have obtained 10 and 8 clones positively expressing WT-HNF4α and DN-HNF4α, respectively. The clones designated as WT-HNF4α-28 and DN-HNF4α-26 that displayed highest induction levels of transgene proteins were chosen for the present study. The time course and dose response of doxycycline effect on WT-HNF4α and DN-HNF4α expression are illustrated in Fig. 1, A and B, respectively. WT-HNF4α protein could be induced within a range from 2- to 50-fold above the endogenous protein level (Fig. 1A). Thus, graded overexpression of WT-HNF4α could be achieved by culturing the WT-HNF4α-28 cells with varying doses of doxycycline in a defined period. Similar induction of DN-HNF4α protein was detected in the nuclear extracts from DN-HNF4α-26 cells (Fig. 1B). No leakage of this doxycycline-dependent promoter was observed, since the expression of DN-HNF4α protein was not detectable in non-induced DN-HNF4α-26 cells (Fig. 1B). Therefore, the dominant-negative suppression of HNF4α function in INS-1 cells could be rapidly achieved by culturing the DN-HNF4α-26 cells with a maximum dose of doxycycline (500 ng/ml).

EFFECTS OF WT-HNF4α AND DN-HNF4α ON INSULIN SECRETION—Impaired glucose-stimulated insulin secretion from pancreatic β-cells is the primary defect causing hyperglycemia in MODY1 patients carrying HNF4α mutations. We therefore examined the consequences of induction of WT-HNF4α and DN-HNF4α on insulin secretion in INS-1 cells. The graded overexpression of WT-HNF4α led to a left shift of glucose-stimulated insulin secretion (Fig. 2A). However, the maximal (above 12 mM) glucose-elicited insulin secretion remained unchanged (Fig. 2A).

Glucose generates ATP and other metabolic coupling factors important for insulin secretion through glycolysis and mitochondrial oxidation (28). The physiological insulin secretagogue, leucine, is transported directly into mitochondria to provide substrates for the tricarboxylic acid cycle (28). K+ causes insulin secretion by depolarization of the β-cell membrane, resulting in an increase in cytosolic Ca2+ (28). We therefore examined the insulin secretory responses to these three secretagogues that act at different levels of the signal transduction cascade following induction of DN-HNF4α. As demonstrated in Fig. 2B, DN-HNF4α selectively inhibited glucose- and leucine-stimulated insulin secretion. This could be explained by defective glucose and leucine metabolism.

EFFECTS OF DN-HNF4α ON CELLULAR ATP PRODUCTION AND MITOCHONDRIAL OXIDATION—To investigate whether impaired nutrient-evoked insulin secretion is correlated to defective cellular ATP production, we analyzed the impact of DN-HNF4α expression on the level of ATP generated by glucose and leucine. As shown in Fig. 3A, induction of DN-HNF4α indeed abolished the ATP generation by glucose and leucine. Since the mitochondrial substrate leucine failed to generate ATP after induction of DN-HNF4α, it would seem that HNF4α is required for maintaining normal mitochondrial function.

To test this hypothesis, we examined the consequences of DN-HNF4α induction on mitochondrial oxidation of pyruvate. Pyruvate-derived carbons enter the tricarboxylic acid cycle as either acetyl-CoA, catalyzed by pyruvate dehydrogenase, or oxaloacetate via pyruvate carboxylase. By using pyruvate radiolabeled at either the first or second carbon, the putative defects at various steps in pyruvate metabolism can be assessed. The radiolabeled carbon of [1-14C]pyruvate is lost to CO2 at the pyruvate dehydrogenase step as pyruvate is converted into acetyl-CoA. Alternatively, if pyruvate enters the
tricarboxylic acid cycle via oxaloacetate, the label is lost to CO2 at isocitrate dehydrogenase within one turn of the cycle. Radiolabeled CO2 is generated from 2-14C at either OGDH or isocitrate dehydrogenase when pyruvate enters the tricarboxylic acid cycle as acetyl-CoA. Decreased isocitrate dehydrogenase activity would also be unlikely since impairment at this point of entry of pyruvate into the tricarboxylic acid cycle, suggesting that the defect in mitochondrial metabolism is not at the end product of glycolysis pyruvate (Fig. 4B), indicating mitochondrial dysfunction. Direct activation with methyl succinate of the electron transport chain at complex II resulted in mitochondrial dysfunction. Direct activation with methyl succinate of the electron transport chain at complex II resulted in mitochondrial dysfunction. Direct activation with methyl succinate of the electron transport chain at complex II resulted in mitochondrial dysfunction. Direct activation with methyl succinate of the electron transport chain at complex II resulted in mitochondrial dysfunction. Direct activation with methyl succinate of the electron transport chain at complex II resulted in mitochondrial dysfunction. Direct activation with methyl succinate of the electron transport chain at complex II resulted in mitochondrial dysfunction.
HAS Regulates Pancreatic β-Cell Gene Expression and Insulin Secretion

**DISCUSSION**

It has been demonstrated that HNF4a controls the expression of a large array of liver-specific genes encoding several apolipoproteins, metabolic proteins, and serum factors that are essential for hepatocyte differentiation and liver development (9). HNF4a is also required for HNF1α expression in hepatocytes (7–9). Another study in embryonic stem cell-differentiated embryoid bodies (33) shows that the absence of HNF4a affects the expression of genes encoding GLUT-2, aldolase B, and L-PK, which are involved in glucose transport and glycolysis. However, little is known as to how HNF4a regulates pancreatic β-cell gene expression. The primary cause of the MODY1 phenotype is impaired glucose-stimulated insulin secretion from pancreatic β-cells (5). The present study was therefore designed to investigate the role of HNF4a in the regulation of the expression of β-cell genes implicated in glucose metabolism and associated insulin secretion.

We found that overexpression of WT-HNF4α caused a left shift of glucose-stimulated insulin secretion, whereas dominant-negative suppression of HNF4α selectively blunted the insulin release induced by glucose and leucine but not by K+ depolarization. The diminished nutrient-evoked insulin secretion is associated with reduced ATP production in DN-HNF4α-expressing cells. The physiological insulin secretagogue leucine raises the cytosolic and mitochondrial Ca2+ concentrations through mitochondrial metabolism downstream of glycolysis (28, 34). Therefore, we suggest that loss of HNF4a function leads to defective mitochondrial metabolism and, as a consequence, impaired insulin secretion. The reduced mitochondrial...
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oxidation of [2-14C]pyruvate and the abrogation of mitochondrial membrane hyperpolarization elicited by glucose, pyruvate, and methyl succinate indicate impaired mitochondrial tricarboxylic acid cycle enzyme activity and partial uncoupling of the mitochondrial respiratory chain.

Quantitative Northern blot analysis allows us to identify HNF4α target genes responsible for defective metabolism-secretion coupling. HNF4α indeed regulates the expression of genes encoding GLUT-2, aldolase B, and L-PK in pancreatic β-cells (Fig. 5), as inferred from previous studies in hepatocytes and embryonic stem cell-differentiated embryoid bodies (8, 9). Most importantly, we demonstrate that HNF4α alters the mRNA expression of mitochondrial OGDH E1 subunit and UCP-2 (Fig. 5), which may indeed contribute more significantly to the impaired metabolism-secretion coupling. The phenotype and gene expression patterns in DN-HNF4α-expressing cells are strikingly similar to those of DN-HNF1α-expressing cells (22, 29). This prompted us to investigate whether HNF4α regulates β-cell expression through HNF1α function, as in hepatocytes (9).

We provide unprecedented evidence that HNF4α is required for HNF1α expression in pancreatic β-cells.

This conclusion is based on the use of an artificial dominant-negative hnf4α mutation. The naturally occurring human mutations of HNF4α do not function in a dominant-negative manner (6, 35). It is to be expected that a mutation with such repressive action on the endogenous HNF4α function would cause embryonic lethality, as is the case in the hnf4α knock-out mouse (15). Haploinsufficiency or reduced gene dosage of HNF4α may thus explain the mechanism leading to the MODY1 phenotype (33). The INS-1 cell line expressing DN-HNF4α provides a convenient model to explore the impact of impaired HNF4α function on β-cell gene expression and metabolism-secretion coupling. This goal cannot be achieved by the introduction of one of the human HNF4α mutations into β-cell lines. In fact, the induction of a nonsense mutation HNF4αQ288X to a level similar to DN-HNF4α had no detectable consequences on β-cell gene expression and metabolism-secretion coupling.2

MODY1 patients display secretory defects not only in β-cells but also in the glucagon-secreting α-cells and the pancreatic polypeptide-secreting cells (36, 37). However, this general effect on islet hormone release does not seem due to an effect on the development and differentiation of the endocrine pancreas, since altered HNF4α function did not affect the expression of PDX1 and other transcription factors determining pancreatic phenotype. On the other hand, loss of HNF4α function may cause reduced β-cell insulin content secondary to defective HNF1α function (22, 29).

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2 H. Wang and C. B. Wollheim, unpublished results.
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